December 2014

Novel Protein Secretion and Chitin Utilization Machinery of Flavobacterium Johnsoniae

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NOVEL PROTEIN SECRETION AND CHITIN UTILIZATION MACHINERY OF *FLAVOBACTERIUM JOHNSONIAE*

by

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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

December 2014

ABSTRACT

NOVEL PROTEIN SECRETION AND CHITIN UTILIZATION MACHINERY OF *FLAVOBACTERIUM JOHNSONIAE*

by

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The University of Wisconsin-Milwaukee, 2014
Under the Supervision of Dr. Mark J. McBride

Flavobacterium johnsoniae, a member of phylum *Bacteroidetes*, is a gliding bacterium that digests insoluble chitin. A novel protein secretion system, the Type IX secretion system (T9SS), secretes the motility adhesins SprB and RemA and is also required for chitin utilization. In order to understand *F. johnsoniae* chitin utilization and the role of the T9SS, Fjoh_4555 (*chiA*) was targeted for analysis. Disruption of *chiA* resulted in cells that failed to digest chitin and complementation restored this ability. Antisera raised against ChiA were used to characterize its secretion. ChiA was secreted in soluble form by wild-type cells but remained cell-associated in T9SS mutant strains. Proteins secreted by T9SSs typically have conserved carboxy-terminal domains (CTDs) belonging to the TIGRFAM families, TIGR04131 and TIGR04183. ChiA did not exhibit strong similarity to these sequences but instead had a novel CTD. Deletion of this CTD resulted in accumulation of ChiA inside of cells. Fusion of the ChiA CTD to mCherry resulted in secretion of mCherry into the medium. These results indicate that ChiA is a

soluble extracellular chitinase required for chitin utilization and that it relies on a novel CTD for its secretion by the *F. johnsoniae* T9SS.

Proteins involved in secretion by the T9SS include GldK, GldL, GldM, GldN, SprA, SprE, and SprT. *Porphyromonas gingivalis* has orthologs for each of these T9SS proteins and they are required for secretion of gingipain proteases. *P. gingivalis porU* and *porV* have also been linked to T9SS-mediated secretion and *F. johnsoniae* has orthologs of these. Cells of an *F. johnsoniae porV* deletion mutant failed to secrete ChiA and RemA, but retained the ability to secrete SprB. The *porV* mutant was partially deficient in attachment to glass, apparently because of the absence of RemA and other adhesins on the cell surface. The *porV* mutant also appeared to be deficient in secretion of numerous other proteins that have CTDs associated with targeting to the T9SS. PorU was not required for secretion of ChiA, RemA, or SprB, indicating that it does not play an essential role in the *F. johnsoniae* T9SS.

chiA is located downstream of a cluster of genes likely to be involved in chitin utilization. Deletion of Fjoh_4558 ($cusD_I$) resulted in a partial defect in chitin utilization, and deletion of the region spanning Fjoh_4558 through Fjoh_4562 which includes $cusD_I$, $cusD_{II}$, $cusC_I$ and $cusC_{II}$ resulted in almost complete loss of ability to utilize chitin. The CusC and CusD proteins are similar in sequence to the *Bacteroides thetaiotaomicron* starch utilization system outer membrane proteins SusC and SusD respectively. SusC and SusD are involved in active uptake of starch oligomers across the outer membrane. The *F. johnsoniae* CusC and CusD proteins may perform similar functions, and cooperate with ChiA to allow efficient utilization of insoluble chitin.

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ACKNOWLEDGMENTS

My sincere gratitude and appreciation goes first towards my advisor Dr. Mark J. McBride for his guidance, camaraderie, and mentorship that has led me to successfully complete my doctoral research dissertation. Looking back at my email correspondence with him while applying for doctoral program, his emails were always very enthusiastic about his research work and I am glad that I got the opportunity to share that enthusiasm for research with him in his lab. I could not have asked for a better advisor for my doctoral dissertation. Not only has he advised me during my research but also given me enough independence to grow as a research scientist. The other qualities I admire in him are his patience and humor that not only made some of my horrific and miserably failed experiment days bearable, but also allowed me to be persistent and most importantly enjoy research. Mark, I really cannot thank you enough!

The other person that has played a very important role in my graduate life is Dr. Daad Saffarani. I would like to thank her for the advice and guidance during graduate application process, lab and committee meetings and also for letting me use her lab instruments. I thank my committee members Dr. Charles Wimpee, Dr. Sonia Bardy and Dr. Sergei Kuchin for their inputs and expertise towards my research.

I acknowledge Dr. Ryan Rhodes (who calls himself my adopted American dad) for his mentorship and guidance that has been invaluable in my research. I acknowledge my previous and current lab members. Dr. Yongtao Zhu, Surashree Kulkarni and Joseph

Johnston, I will remember all the crazy and not so crazy scientific discussion. Most of all I will remember the ‘arts and crafts’ done in lab.

I would also like to thank National Science Foundation (NSF MCB-1021721), Research Growth Initiative (RGI), Ruth Walker Grant and University of Wisconsin Milwaukee in acknowledging my hard work and providing financial aid and supporting my research. I would like to acknowledge Ryan and Yongtao for editorial suggestions for the published manuscripts. I also thank Greg Sabat and Greg Barrett-Wilt at the University of Wisconsin Madison Mass Spectroscopy Facility for the LC-MS/MS analyses.

Most importantly, I would like to thank my family and friends for my research accomplishments. Thank you dad: Dr. Suresh D. Kharade, for babysitting me in your clinic where, I initially developed an interest for science. Thank you mom: Sanjivani S. Kharade, you have always inspired me, and taught me to be strong yet humble. I would like to thank both of you for your selflessness and love that has allowed me to attain my doctoral degree. I would also like to thank my loving sisters: Smruti S. Kharade and Samruddhi S. Kharade and my husband: Nishant N. Trivedi and his parents for their unconditional love and support, thus letting me successfully persevere and complete this doctoral dissertation. ‘To transmute dreams into reality one has to have a consistent purpose.’ is something my parents have taught me and this has been my self-motivating adage throughout my life. I would like to thank all of you for allowing me to attain my purpose of achieving and successfully completing my doctoral degree.

Chapter 1. Introduction

Flavobacterium johnsoniae, belonging to the phylum *Bacteroidetes,* is a Gram-negative rod shaped bacterium that digests the insoluble polymer chitin (65). Cells of *F. johnsoniae* crawl over a wide range of surfaces by a process called gliding motility (41). Gliding motility has been loosely defined as cell movement over surfaces without the help of flagella or pili (40). *gld* and *spr* genes are required for gliding motility because cells with mutations in these genes are defective in gliding. *gld* mutant cells are completely non-motile, whereas *spr* mutant cells exhibit reduced but detectable movement (1, 7, 28, 37, 39, 40, 42). *F. johnsoniae* gliding motility relies on cell surface motility adhesins, such as SprB and RemA (46, 62). These adhesins are propelled rapidly along the cell surface. Attachment of the adhesins to a substratum results in cell movement. Mutations in gliding motility genes often result in inability to digest chitin, but the reason for this was not known.

A novel protein secretion system, the type IX secretion system (T9SS), initially known as the Por Secretion System, was recently discovered in *F. johnsoniae* and in the oral pathogen, *Porphyromonas gingivalis* (44, 54, 55). The *F. johnsoniae* T9SS is required for secretion of the motility adhesins SprB and RemA, and thus T9SS mutants are nonmotile. T9SSs are common among members of the *Bacteroidetes* phylum and are important for secretion of extracellular enzymes and virulence factors in addition to secretion of cell surface motility adhesins (35, 44). This thesis explores *F. johnsoniae* chitin utilization and the role of the T9SS in this process.

The type IX secretion system

The T9SS was first identified in *F. johnsoniae* and *P. gingivalis* and later found to be common throughout the phylum *Bacteroidetes* (44, 54, 55) (Figure 1). The *F. johnsoniae* T9SS is required for secretion of the cell surface motility adhesins SprB and RemA (Figure 2). The *F. johnsoniae* T9SS is composed of some of the Gld and Spr proteins. Mutations in *gldK, gldL, gldM, gldN, sprA, sprE*, and *sprT* result in defects in secretion of SprB and RemA (61). This results in inability to glide and the formation of non-spreading colonies (Figure 3). These mutants are also deficient in chitin utilization (Figure 4), suggesting the possibility that a secreted chitinase may rely on the T9SS to exit the cell. Since mutations in the *F. johnsoniae* T9SS genes result in loss of motility and inability to utilize chitin (53), this suggested that *F. johnsoniae* gliding motility and chitin utilization were dependent on each other. Although much progress has recently been made in our understanding of gliding motility, the mechanism of *F. johnsoniae* chitin utilization has not been explored in detail.

Proteins secreted by T9SSs have N-terminal signal peptides, allowing transit across the cytoplasmic membrane via the Sec system, and conserved carboxy-terminal domains (CTDs) that are thought to target the proteins to the T9SS (22, 44, 55, 57, 60, 63). These CTDs typically belong to TIGRFAM families TIGR04131 or TIGR04183. *F. johnsoniae* has fifty-three proteins with these CTD's, including SprB and RemA (35, 54). The exact role of the CTD and its involvement in targeting proteins to the T9SS is not well understood. Identification of the major chitinase responsible for chitin utilization in *F. johnsoniae,* determination of the role of the T9SS in its secretion and the possible

involvement of the major chitinase CTD in this process, are some of the topics addressed in chapter 2.

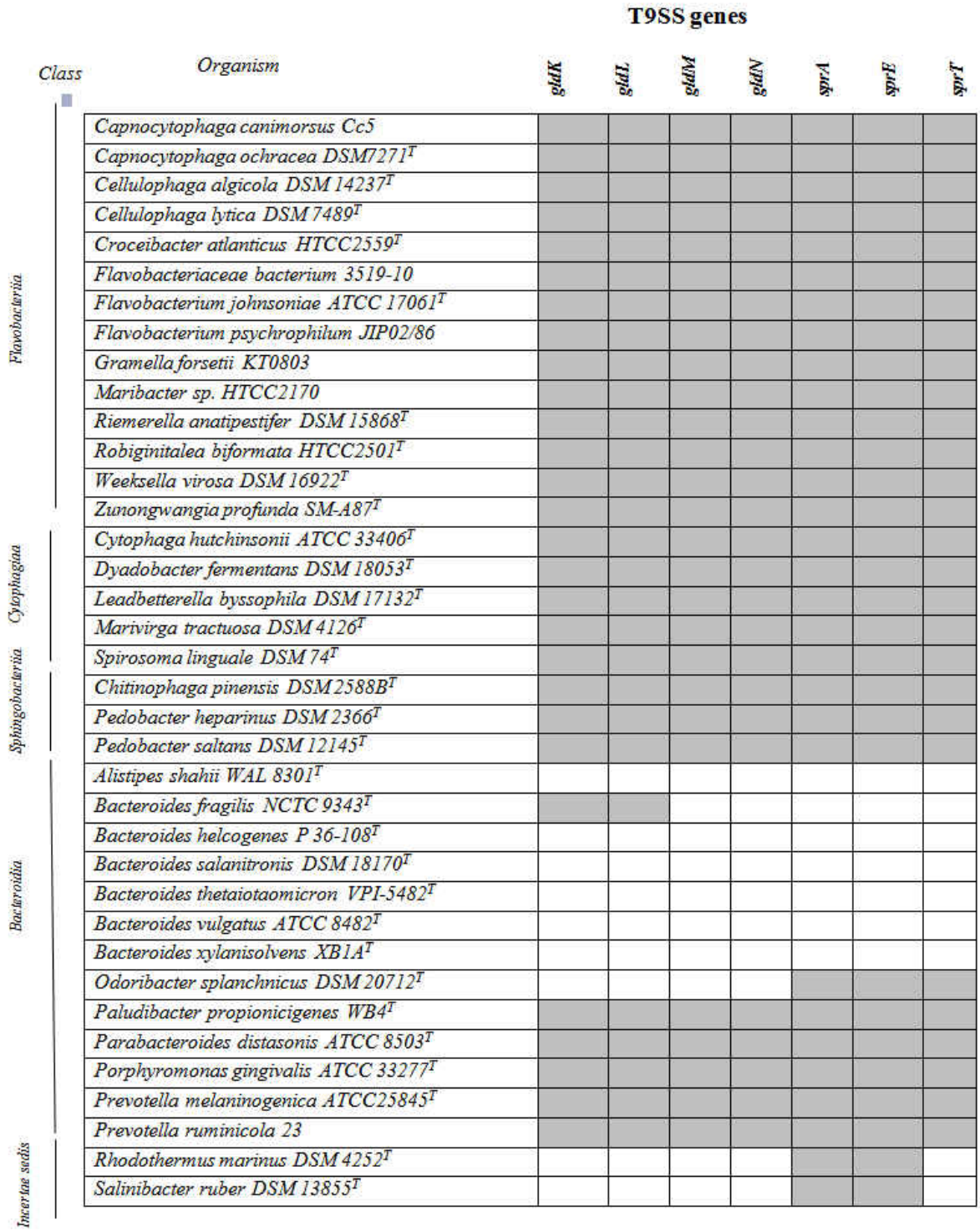

Figure 1. Distribution of T9SS genes among members of the phylum *Bacteroidetes* (44). White squares indicate the absence of an ortholog, whereas a grey square indicates the presence of an ortholog. Modified from (44).

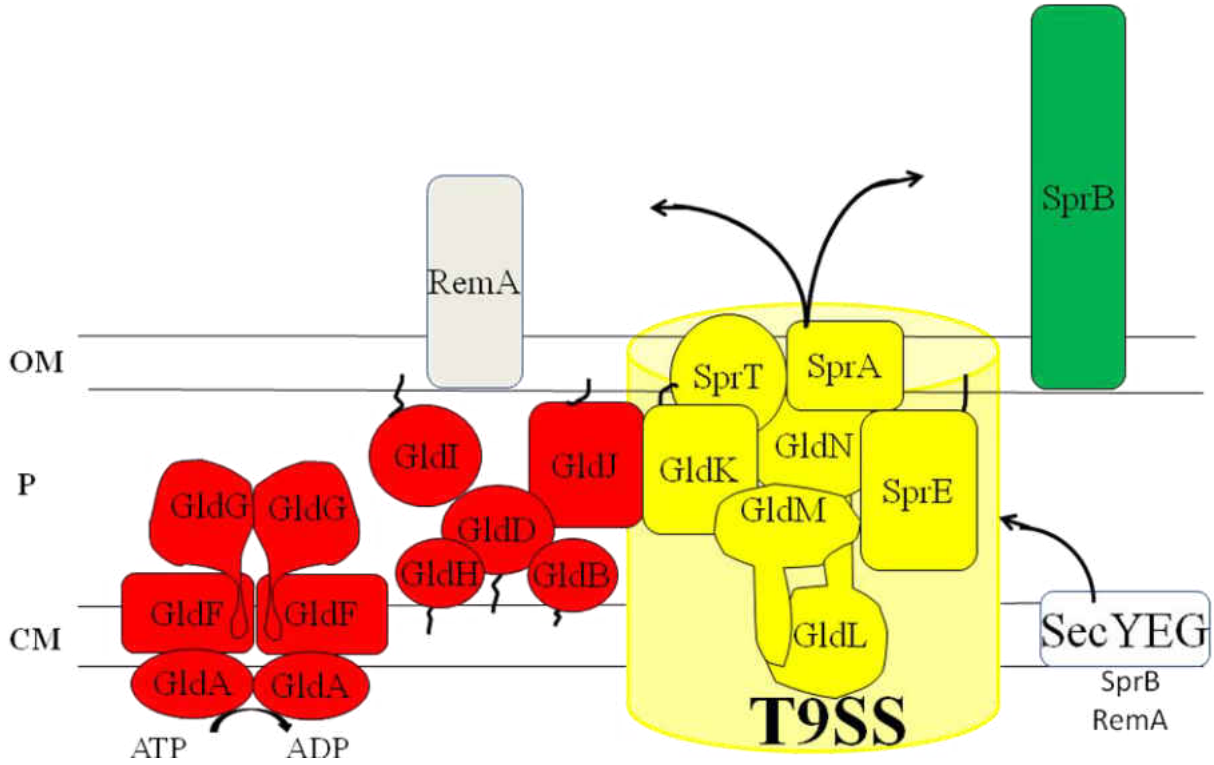

Figure 2. *F. johnsoniae* motility and secretion proteins. T9SS proteins are in yellow. Other proteins related to gliding motility are in red. The T9SS is required for secretion of the motility adhesins SprB and RemA. OM-outermembrane, P-periplasm and CM-cytoplasmic membrane.

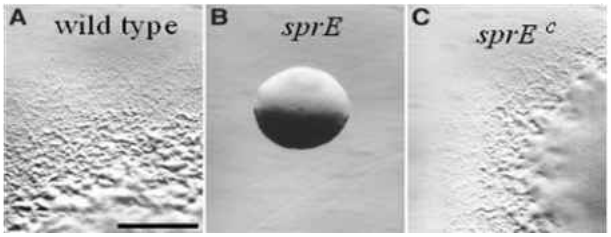

Figure 3. Photomicrographs of *F. johnsoniae* colonies. (A) wild type *F. johnsoniae* FJ1 (B) *sprE* mutant FJ149 C) *sprE* mutant FJ149 complemented with pNap2 carrying *sprE*. Bar in panel A = 0.5 mm applies to all panels. Modified from (53)

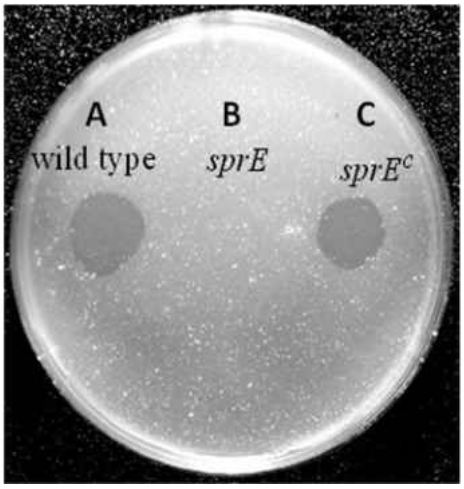

Figure 4. *sprE* is required for chitin utilization. Approximately 10^6 cells of (A) wild type *F. johnsoniae* UW101, (B) *sprE* mutant FJ149 and (C) *sprE* mutant FJ149 complemented with pNap2 carrying *sprE* were spotted on MYA- chitin medium and incubated at 25°Cfor 2 days. Modified from (53).

P. gingivalis is a human periodontal pathogen that secretes gingipain proteases resulting in tissue damage (Figure 5). The *P. gingivalis* T9SS, composed of homologs of the *F. johnsoniae* Spr and Gld proteins, is involved in secretion of gingipain proteases which are its major virulence factors (54). *P. gingivalis* strains with mutations in *porK*, *porL*, *porM*, *porN*, *sov*, *porW*, and *porT* (homologs to *F. johnsoniae gldK, gldL, gldM, gldN, sprA, sprE* and *sprT* respectively) are defective for gingipain secretion (29, 54).

In *P. gingivalis*, two additional proteins, PorU and PorV, are linked to T9SS mediated secretion (12, 22, 54). *P. gingivalis* PorU is thought to function as the peptidase that removes the CTDs from proteins that are secreted via the T9SS (Figure 5) (22). *P. gingivalis* PorV is required for secretion of proteins targeted to the T9SS, including the gingipains RgpA, RgpB and Kgp (Figure 5) (29, 30). PorV has also been suggested to play a role in LPS modification. *P. gingivalis* produces two distinct forms of LPS, one of which carries O-antigen (O-LPS) whereas the other carries an anionic polysaccharide (A-LPS). PorV has also been called LptO, and has been shown to affect *O*-deacylation of lipopolysaccharide (12). PorV directly or indirectly affects the partial deacylation of A-LPS prior to attachment of this lipopolysaccharide to cell-surface proteins secreted by the T9SS. PorV may function as a deacylase, or it may be involved in secretion of a deacylase. *F. johnsoniae* has homologs of *P. gingivalis porU* and *porV* but these were not previously studied. Chapter 3 explores the roles of *F. johnsoniae* PorU and PorV in secretion.

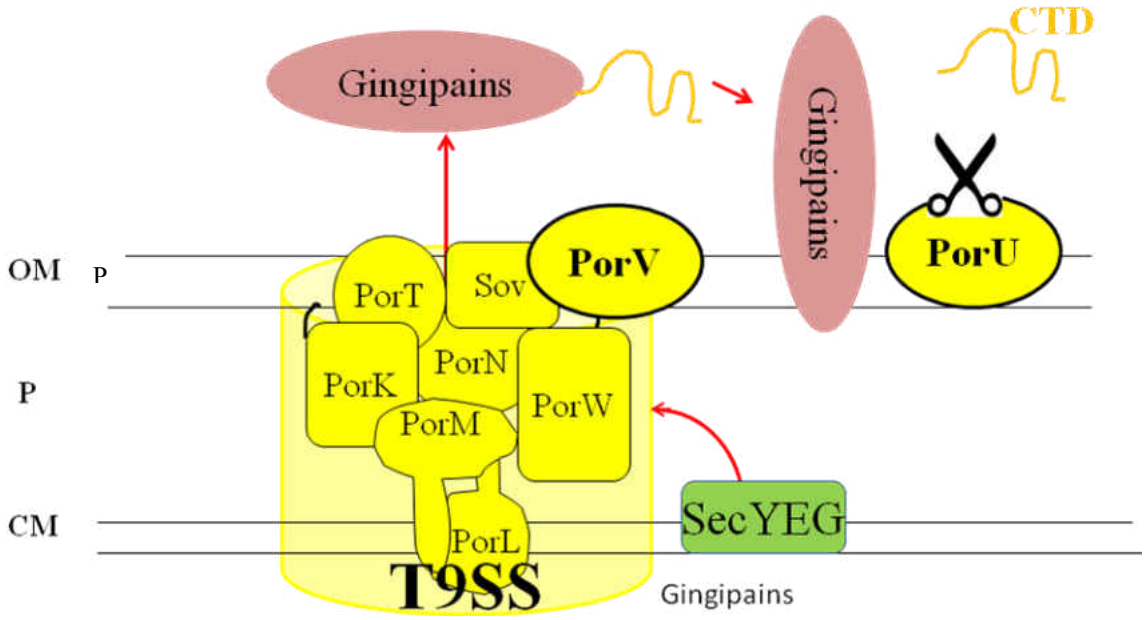

Figure 5. Model of T9SS in *P. gingivalis.* PorK, PorL, PorM, PorN, PorT, PorU, PorV, PorW and Sov form the T9SS required for secretion of its virulence factors, gingipains. Based on the information from (12, 22, 54). PorU appears to cleave CTD of proteins secreted via the T9SS. OM-outer membrane, P-periplasm and CM-cytoplasmic membrane. ✂ indicates cleavage.

T9SSs is not closely related to the well studied type I through type VIII protein secretion systems (T1SS-T8SS) of Gram-negative bacteria (Figure 6) (Table 1) and appears to be confined to the phylum *Bacteroidetes* (11, 54, 69). Type I, type III, type IV and type VI secretion systems are used for transport of proteins across the cytoplasmic and outer membranes in a single step (Figure 6 right side) (11, 18, 26, 37, 44). In contrast, the type II, type V, type VII and type VIII secretion systems function in conjunction with the Sec or Tat protein export pathways (Figure 6 left side). The Sec or Tat protein export pathways transport proteins across the cytoplasmic membrane into the periplasm (48). These proteins are then transported across the outer membrane via the secretion systems. The components of the T9SS are not similar to T1SS-T8SSs of Gram-negative bacteria (4, 9, 10, 16).

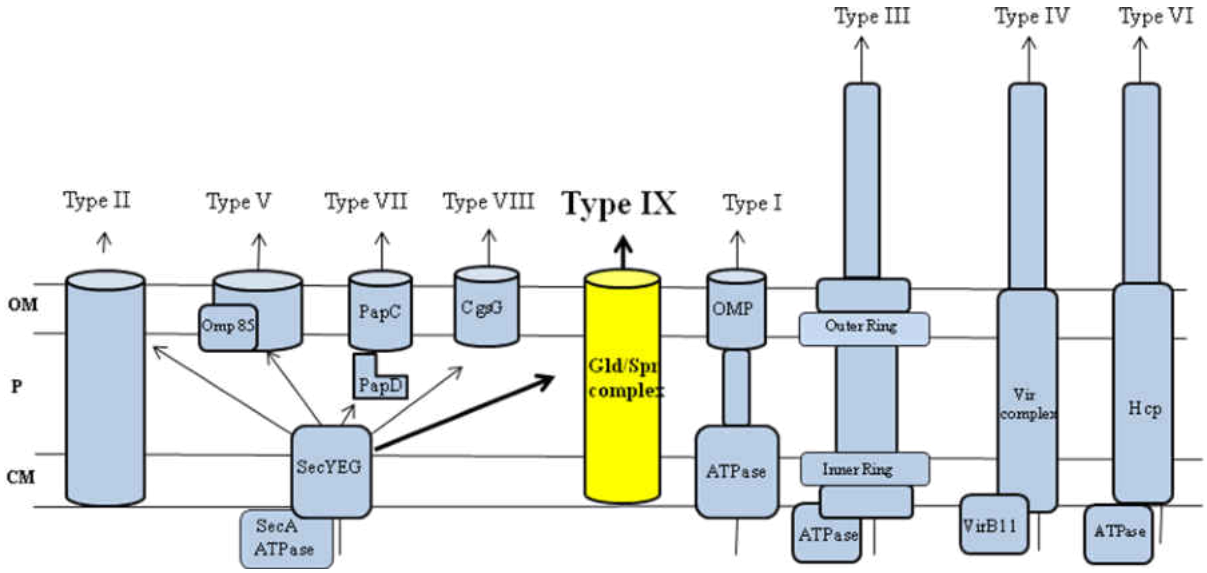

Figure 6. Bacterial protein secretion systems. The TISS, T3SS, T4SS and T6SS transport proteins from the cytoplasm directly to the cell exterior. For the T3SS, T4SS, and T6SS this involves a needle-like structure at the cell surface. The T2SS, T5SS, T7SS, T8SS and T9SSs function in conjunction with the Sec system or the Tat system (not shown). OM-outer membrane, P-periplasm, CM-cytoplasmic membrane. Modified from (69)

Table 1. Bacterial secretion systems

Secretion system	Example	References
Type I	*E. coli* HlyA secretion by HlyB, HlyD, TolC	(10, 20, 27)
Type II	*Klebsiella oxytoca* pullulanase secretion by *GspC, GspD, GspE, GspF, GspG, GspL, GspM*	(48, 49)
Type III	Secretion of flagellin by bacterial flagella Secretion of virulence factors by *Yersinia pestis* Ysc proteins	(2) (14)
Type IV	*Agrobacterium tumefaciens* protein secretion and conjugative transfer of DNA by Vir proteins	(13, 38)
Type V	*E. coli* EspP autotransporter *Bordetella pertussis* two-partner secretion system for filamentous hemagglutinin (FHA, TpsA)	(8) (32, 33)
Type VI	*Vibrio cholerae* VgrG effector protein secretion	(50)
Type VII	*E. coli* chaperone usher pathway	(9, 10, 16)
Type VIII	*E.coli* extracellular nucleation-precipitation (ENP) pathway involved in assembly of cell-surface curli fibers	(4, 10, 16)
Type IX	*Bacteroidetes* specific secretion system found in *F. johnsoniae*, *P. gingivalis*, and many related bacteria	(44, 54)

Chitin and chitinases

Insoluble polysaccharides such as cellulose and chitin are thought to be the most abundant biopolymers on our planet (15). These polymers play important structural roles in plants and insects. Although these polymers are resistant to digestion, many soil and aquatic microorganisms have strategies to attack them. Some of these organisms are being used to convert polysaccharides into bio-fuels such as ethanol (23). Studies of the mechanisms employed by bacteria to digest insoluble polysaccharides therefore have environmental and biotechnological significance (17).

Chitin is a linear insoluble polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) (19). Chitin and its modified forms are employed in wastewater treatment, drug delivery, wound healing and as dietary fiber (15). Enzymatic conversion of chitin has received attention because of prospects in transforming an abundant insoluble component of biomass into useful products such as soluble chito-oligomers. These chito-oligomers have medical, agricultural and industrial applications as antibacterial, antifungal, hypocholesterolemic and food quality enhancing agents (5). Chitin is a major constituent of the shells of crustaceans such as crab and shrimp, the exoskeletons of insects, and the cell walls of yeasts and other fungi (5).

Digestion of polysaccharides is performed by a multitude of bacterial and fungal enzymes termed glycoside hydrolases (GHs) (72). GHs are classified into families based on amino acid sequence similarities, structures, activities, and evolutionary relationships

(72). The most recent and up to date sequence-based classifications are found in the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org).

Chitin can be digested primarily in two ways. The first approach is chitinolytic that involves hydrolysis of the β-glycosidic bond in the chitin chain by chitinases (GH18 and GH19 families) to form chito-oligosaccharides (often chitobiose) (19). These chito-oligosaccharides are further hydrolyzed to GlcNAc monomers by β-*N*-acetylglucosaminidases (typically belong to family GH20) (19). The second approach towards chitin digestion involves deacetylation of chitin by chitin deacetylases (usually belong to the carbohydrate esterase family 4) to form chitosan (a deacylated form of chitin), that is further converted to glucosasmine by chitosanases (often belong to the families GH8, GH46, GH75 and GH80).

Chitinases typically belong to the GH18 and GH19 families (25). Family GH19 (originally identified in plants) exclusively contains chitinases. Chitinases belonging to the family of GH18 are the most widespread and well studied chitinolytic enzymes. The GH18 family in addition to chitinases contains endo-β-*N*-acetylglucosaminidases, and also a subclass of non-hydrolytic proteins including lectins and xylanase inhibitors (19). GH18 chitinases share conserved residues DXXDXDXE at their catalytic region (19, 24). Additonally, some family 18 chitinases may digest chitosan (64) and some chitinases in this family also break down peptidoglycans (6).

Biochemically chitinases can be classified by their mode of action on chitin chains. Endo-chitinases cleave chitin randomly at internal sites to form long oligomers of

GlcNAc. Exo-chitinases, on the other hand, cleave chitin from either the reducing or non-reducing end. Exo-chitinases are typically chitobiosidases that catalyze release of chitobiose from the non-reducing end of chitin. (15). Typically exo-chitinases are processive, repeatedly releasing sugars from the end of the oligomer. A deep substrate binding cleft or tunnel is often observed in exo-acting enzymes allowing this processive mode of action. In contrast, most non-processive endo-acting enzymes have shallow substrate binding clefts (19). Endo-acting and exo-acting enzymes appear to work synergistically to hydrolyse chitin (19, 15).

Many chitinolytic organisms synthesize multiple chitinases (15). Some of these undergo post-translational modifications such as glycosylation and proteolysis (15). Chitinases are isolated and purified from many sources including plants, animals, fungi, bacteria, and viruses (15). Multiple chitinases have been reported in several microorganisms such as *Alteromonas* sp Strain O-7 (47), *Serratia marcescens* (71), *Bacillus circulans* WL-12 (45), *Streptomyces griseus* (31) and *Pyrococcus kodakaraensis* KOD1(67). Orikoshi et. al. reported the synergistic action of four chitinases, *AO7*ChiA, *AO7*ChiB, *AO7*ChiC and *AO7*ChiD, in chitin degradation in the marine bacterium *Alteromonas* sp. Strain O-7. Although the *Alteromonas* chitinases have similar catalytic domains some were more active against powdered insoluble chitin, whereas others were more active against more soluble forms of chitin (47). Synergistic action of the *S. marcescens* chitinases *Sm*ChiA, *Sm*ChiB and *Sm*ChiC1 was demonstrated (66). *Sm*ChiA and *Sm*ChiB are thought to digests chitin chains from opposite ends, whereas *Sm*ChiA acts on the reducing end and, *Sm*ChiB degrades chitin from the non-reducing end (66).

Chitinases are often modular enzymes that consist of catalytic modules and carbohydrate binding modules (CBMs) that bind specifically to chitin (19, 24). Many common bacterial chitin binding domains belong to CBM families 5 and 12 (19). CBMs are thought to bind the substrate and position the enzyme to facilitate digestion (72). In addition they may modulate the activity of the enzyme, attach the enzyme to the bacterial cell surface, and potentially disrupt crystalline portions of the substrate thus improving accessibility to the catalytic domain (72). Recently some CBMs have also been shown to sense the target substrate and regulate transcription of genes associated with polysaccharide digestion (34).

Carbohydrate binding module family 33 (CBM33) until recently was considered to comprise of enzymes that were thought to be chitin binding proteins (19). Due to the observed oxidative activity of the enzymes in the family of 'CBM33', it has been renamed 'auxiliary activity family 10' (AA10). AA10 enzymes are lytic polysaccharide monooxygenases (LPMOs) (70). Some AA10 enzymes oxidize chitin whereas others oxidize cellulose. These enzymes introduce chain breaks in the polysaccharides, generating oxidized chain ends (70). They are thought to convert the crystalline insoluble polysaccharides into forms that can be readily attacked by glycoside hydrolases. The *F. johnsoniae* genome does not encode any AA10 family proteins so it apparently does not rely on this mechanism to attack chitin.

The *F. johnsoniae* major chitinase discussed in chapter 2, Fjoh_4555 (ChiA), has 2 GH18 domains that appear to be involved in chitin degradation. No CBM has been

recognized in *F. johnsoniae* ChiA, although it may have novel chitin binding domains or may interact with other proteins that bind chitin.

F. johnsoniae chitin utilization locus

Bacteria including *F. johnsoniae* employ various strategies to digest polysaccharides (Figure 7). Some bacteria including *Saccharophagus degradans,* secrete enzymes that digest the polysaccharides into small monomers or dimers that are taken up by the cell (Figure 7) (68). In contrast, other bacteria including the cellulolytic clostridia, *Clostridium thermocellum* and *Clostridium cellulovorans,* produce multi-protein complexes of enzymes and polysaccharide binding proteins called cellulosomes that are exposed on the cell surface (21). These interact with the polysaccharides and release soluble monomers and dimers for utilization by the cell (21). Both of the above-discussed strategies result in the polysaccharide being digested primarily outside of the cell. Members of the phylum *Bacteroidetes* have evolved a different strategy for polysaccharide utilization. *Bacteroides thetaiotaomicron*, an anaerobic inhabitant of the human large intestine, is well studied for employing the **S**tarch **U**tilization **S**ystem (Sus) pathway (51). In *B. thetaiotaomicron*, the *sus* locus consists of eight genes *susR, susA, susB, susC, susD, susE, susF* and *susG* (58). Starch binding is achieved by the concerted function of the cell surface proteins SusD, SusE and SusF (52, 58). SusD binds starch and facilitates limited digestion by nearby glycoside hydrolases such as the outer membrane α-amylase SusG resulting in long oligomers (52, 59). These oligomers are actively transported across the outer membrane through a channel-like protein, SusC. SusC is a member of the TonB-dependent receptor family that transports macromolecules through

an energy dependent mechanism. Transport is thought to require a proton motive force and the TonB-ExbBD complex (56). Further digestion of starch oligomers in the periplasm by SusA and SusB results in release of monosaccharides and disaccharides. Transcriptional regulation of the *sus* locus genes is accomplished by the sensor/ regulator SusR (72). *B. thetaiotaomicron* has numerous genes encoding SusC-like and SusD-like proteins allowing it to utilize many polysaccharides. *susC*-like and *susD*-like genes are paired with each other and are usually adjacent to genes encoding the glycoside hydrolases that digest the polysaccharide. Such a gene cluster is called a **P**olysaccharide **U**tilization **L**ocus (**PUL**).

The *Bacteroidetes* SusCD strategy has been demonstrated only for utilization of relatively soluble substrates such as starch. Genome analysis of *F. johnsoniae* suggests that the SusCD paradigm may extend to the digestion of highly insoluble polysaccharides as well, since many genes encoding proteins similar to *B. thetaiotaomicron* SusC and SusD were adjacent to genes predicted to encode glycoside hydrolases that attack insoluble polysaccharides (43). *F. johnsoniae* digests chitin, plant cell wall hemicelluloses such as xylans and mannans and many other polysaccharides. It has 44 *susC*-like genes and 42 *susD*-like genes within its many PULs (3). One of its PULs is predicted to be involved in chitin utilization (Fjoh_4564-Fjoh_4555). Fjoh_4555 encodes ChiA, the major extracellular chitinase discussed in chapter 2. The other genes in this PUL including $cusC_I$ and $cusC_{II}$ (*susC*-like genes of the **c**hitin **u**tilization **s**ystem) and $cusD_I$ and $cusD_{II}$ (*susD*-like genes) appear to play roles in chitin utilization and are discussed in chapter 4.

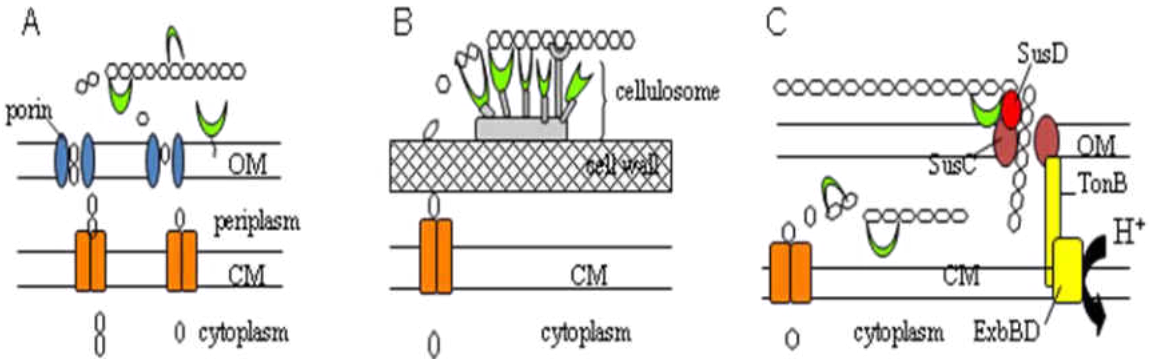

Figure 7. Bacterial strategies for polysaccharide utilization. (A) Extracellular enzyme strategy used by *Saccharophagus degradans.* Extracellular enzymes digest the polymer releasing monomers or dimers that passively diffuse through outer membrane porins and are actively transported across the cytoplasmic membrane. (B) Enzyme complex (cellulosome) strategy used by *Clostridium thermocellum*. Multiple polysaccharide lytic enzymes are displayed on the cell surface as a complex. The enzymes work together to digest polysaccharides to monomers and dimers that are actively transported across the cytoplasmic membrane. (C) *Bacteroidetes* SusCD strategy. Polysaccharides attach to SusD-like protein (red) on cell surface. Nearby cell associated enzymes (green) cut the polysaccharide into long oligomers that are actively transported across the outer membrane through the SusC channel (purple), with the help of TonB-ExbBD (yellow). Further digestion occurs in the periplasm and cytoplasm. OM- outer membrane and CM- cytoplasmic membrane. (Courtesy Mark McBride)

This thesis is focused on the *F. johnsoniae* major extracellular chitinase ChiA, the T9SS protein PorV, and the chitin utilization proteins $CusC_I$, $CusC_{II,}$ $CusD_I$ and $CusD_{II}$. Chapter 2 covers the extracellular chitinase ChiA and its secretion by the T9SS. A slightly modified form of this chapter was published in the Journal of Bacteriology (35). Chapter 3 covers the T9SS protein PorV and its involvement as an accessory protein of the T9SS. A modified form of this chapter was also published in the Journal of Bacteriology (36). The only difference between the papers published in Journal of Bacteriology and the chapters in this thesis are that some of the online supplemental materials associated with the published papers are integrated into the thesis chapters. Chapter 4 is focused on *F. johnsoniae* chitin utilization system proteins $CusC_I$, $CusD_I$, $CusC_{II}$ and $CusD_{II}$. Results that relate to chapter 2 but that were not included in the associated publication are presented in appendix 1 following chapter 4

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Chapter 2. The *Flavobacterium johnsoniae* chitinase ChiA is required for chitin utilization and is secreted by the type IX secretion system.

This chapter is a modified version of a paper published in Journal of Bacteriology (68) that includes some of the online supplemental materials of the published paper integrated into it.

Abstract

Flavobacterium johnsoniae, a member of phylum *Bacteroidetes*, is a gliding bacterium that digests insoluble chitin and many other polysaccharides. A novel protein secretion system, the type IX secretion system (T9SS), is required for gliding motility and for chitin utilization. Five potential chitinases were identified by genome analysis. Fjoh_4555 (ChiA), a 168.9 kDa protein with two glycoside hydrolase family 18 (GH18) domains, was targeted for analysis. Disruption of *chiA* by insertional mutagenesis resulted in cells that failed to digest chitin, and complementation with wild-type *chiA* on a plasmid restored chitin utilization. Antiserum raised against recombinant ChiA was used to detect the protein and to characterize its secretion by *F. johnsoniae*. ChiA was secreted in soluble form by wild-type cells but remained cell-associated in strains carrying mutations in any of the T9SS genes, *gldK, gldL, gldM, gldNO, sprA, sprE* and *sprT*. Western blot and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses suggested that ChiA was proteolytically processed into two GH18 domain-containing proteins. Proteins secreted by T9SSs typically have conserved carboxy-terminal domains (CTDs) belonging to the TIGRFAM protein domain families,

TIGR04131 and TIGR04183. ChiA does not exhibit strong similarity to these sequences and instead has a novel CTD. Deletion of this CTD resulted in accumulation of ChiA inside of cells. Fusion of the ChiA CTD to recombinant mCherry resulted in secretion of mCherry into the medium. The results indicate that ChiA is a soluble extracellular chitinase required for chitin utilization, and that it relies on a novel CTD for secretion by the *F. johnsoniae* T9SS.

Introduction

The gliding bacterium *Flavobacterium johnsoniae* efficiently digests many polysaccharides including insoluble chitin, a homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) (62). Chitin is one of the most abundant biomolecules on earth, and determination of the mechanisms of its digestion has biotechnological and environmental implications (39). Analysis of the *F. johnsoniae* genome revealed ten genes predicted to encode glycohydrolases involved in chitin utilization (37). These included five predicted chitinases to cut the long chitin polymers, and five predicted β-N-acetylglucosaminidases to release N-acetylglucosamine from the soluble chitooligosaccharides. The exact functions of each of these enzymes in chitin utilization are not known.

It has been known for many years that mutations that disrupt gliding motility often result in inability to digest chitin (12). More recently it was recognized that *F. johnsoniae* has a protein secretion system, originally called the Por secretion system and now referred to as the type IX secretion system (T9SS), that is required for both motility

and chitin utilization (38, 53, 55, 59). The components of T9SSs are not closely related to those of the well-studied type I-type VI secretion systems of Gram-negative bacteria (17, 20, 38). They are also unrelated to the components of the chaperone-usher pathway that has recently been called the type VII secretion system (9, 11, 16), the components of the extracellular nucleation-precipitation pathway involved in secretion and assembly of curli amyloid fibers, which has been referred to as the type VIII secretion system (3, 16), and to the mycobacterial ESX (ESAT-6) system (1, 11). The T9SS is required for secretion of the cell surface motility proteins SprB and RemA, and is thus needed for motility. SprB and RemA are adhesins that move rapidly on the cell surface, apparently propelled by the still poorly defined gliding motor (40, 41, 60). Cells with mutations in T9SS genes fail to utilize chitin and lack extracellular chitinase activity (49, 50, 53, 59). One predicted chitinase (Fjoh_4555, which we refer to as ChiA) was identified in the spent culture medium of wild-type cells but not of a T9SS mutant (53). ChiA was thus predicted to be secreted by the T9SS and to have a role in chitin utilization.

T9SSs are common in members of the phylum *Bacteroidetes*, of which *F. johnsoniae* is a member (38). They have been studied not only in *F. johnsoniae*, but also in the nonmotile oral pathogen *Porphyromonas gingivalis*, which uses its T9SS to secrete gingipain protease virulence factors and other proteins (53-55). Proteins secreted by T9SSs have N-terminal signal peptides, allowing transit across the cytoplasmic membrane via the Sec system, and conserved C-terminal domains (CTDs) that are thought to target the proteins to the T9SS (18, 38, 55, 57, 58, 60, 61). These CTDs typically belong to TIGRFAM families TIGR04131 or TIGR04183. *F. johnsoniae* has

fifty-three proteins with these CTD's, including SprB and RemA (59). One predicted chitinase, Fjoh_4175, has a CTD that belongs to TIGR04183 and thus may be secreted by the T9SS. Surprisingly, the secreted chitinase ChiA does not have a recognizable T9SS CTD, so its relationship to the T9SS or to another secretion system required further study.

Here we demonstrate that *chiA* encodes the major extracellular chitinase required for chitin utilization and that ChiA is a soluble enzyme that requires the T9SS for secretion. We also show that the C-terminal 105 amino acids of ChiA are necessary for secretion, and are sufficient to target a foreign protein for secretion by the T9SS.

Materials and Methods

Bacterial and bacteriophage strains, plasmids, and growth conditions. *F. johnsoniae* ATCC 17061 strain UW101 was the wild-type strain used in this study (34, 37). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30^{o}C (36), or in motility medium (MM) at 25^{o}C (32), as previously described. *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C. Strains and plasmids used in this study are listed in Table 2 and primers are listed in Table 3. Antibiotics were used at the following concentrations when needed: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; erythromycin, 100 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 20 μg/ml.

Disruption and complementation of *chiA*. For disruption of *chiA*, a 1299-bp region internal to *F. johnsoniae chiA* was amplified by PCR using primer 937 with engineered BamHI site and primer 938 with engineered SalI site. The fragment was inserted into pLYL03 that had been digested with BamHI and SalI to generate pSAM1.

pSAM1 was introduced into *F. johnsoniae* by conjugation (21, 36) and recombined into the chromosome to yield the *chiA* mutant, CJ1808. The insertion was confirmed by PCR using primer 737 and primer 941.

For complementation of *chiA,* a 4974 bp fragment was amplified using primer 974 (engineered XbaI site) and 975 (engineered BamHI site). This fragment was introduced into complementation vector pCP23, which had been digested with BamHI and XbaI, to generate pSSK05.

Deletion of the *chiA* CTD-encoding region. The previously described strategy to generate unmarked deletions was employed (48) to generate a truncated gene encoding ChiA lacking the C-terminal 106 amino acids. A 2121 bp fragment upstream of the *chiA* CTD-encoding region was amplified using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1378 and 1379. The amplified fragment was digested with BamHI and SalI and cloned into pRR51 that had been digested with the same enzymes, generating pSSK26. A 2033 bp fragment downstream of *chiA* was amplified by PCR using primers 1380 (engineered SalI site) and 1381(engineered SphI site). This fragment was ligated into pSSK26 that had been digested with SalI and SphI, to generate pSSK27. pSSK27 was introduced into the streptomycin-resistant wild type *F. johnsoniae* strain CJ1827 by conjugation. The *chiA* CTD deletion mutant was isolated essentially as previously described (48). The *chiA* CTD deletion mutant, CJ2325, was confirmed by PCR amplification using primers 1391 and 1392 and by sequencing the product.

Deletion of Fjoh_4175. A 2118 bp fragment upstream of Fjoh_4175 was amplified using primers 1229 (engineered SalI site) and 1230 (engineered SphI site). The amplified fragment was digested with SalI and SphI and cloned into pRR51 that had been digested with the same enzymes, generating pSSK32. A 1948 bp fragment downstream of Fjoh_4175 was amplified using primers 1227 (engineered XbaI site) and 1228 (engineered SalI site). This fragment was introduced into pSSK32 that had been digested with XbaI and SphI, to generate pSSK34. pSSK34 was introduced into *F. johnsoniae* CJ1827 by conjugation and the Fjoh_4175 deletion mutant CJ2355 was isolated essentially as previously described (48) and confirmed by PCR amplification and sequencing using primers 1463 and 1464.

Generation of mCherry fusion constructs. A plasmid expressing the N-terminal region of ChiA (encoding the signal peptide) fused to mCherry and to the C-terminal 105 amino acids of ChiA (CTD_{ChiA}) was constructed. A 708 bp region of mCherry was amplified from pME-mCherry using primer 862 (engineered BamHI site) and primer 1266 (engineered XbaI site). This fragment was cloned into the BamHI and XbaI sites of pCP23, generating pSSK30. A 484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region was amplified using primer 1593 (engineered KpnI site) and primer 1516 (engineered BamHI site). The fragment was inserted into KpnI and BamHI digested pSSK30 to generate pSSK51. To introduce the CTD-encoding region, 566-bp was amplified using primer 1600 (engineered XbaI site) and primer 1404 (engineered SphI site). The product was cloned into pSSK51, to generate pSSK52. A plasmid expressing ChiA N-terminal signal peptide fused to

mCherry without CTD_{ChiA} was also constructed as a control. mCherry was amplified using primers 862 and 1443 (engineered XbaI site) and introduced into BamHI and XbaI digested pCP23 generating pSSK45. The ChiA N-terminal signal peptide-encoding region was amplified using primers 1593 and 1516 and was cloned into pSSK45 to generate pSSK54.

Determination of chitinase activity. Chitin utilization on agar was observed as previously described using colloidal chitin prepared from crab shells (34, 47, 49). Chitinase activities in cell-free culture supernatants (spent media), whole cells, and cell extracts were measured as previously described (49) using the synthetic substrates 4-methylumbelliferyl β-D-N,N'-diacetyl-chitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl β-D-N, N', N''-diacetyl-chitotrioside [4-MU-$(GlcNAc)_3$] (Sigma-Aldrich, St. Louis, MO) except that activities were measured for 30 min. Activities in the spent media (secreted chitinase), whole cells, and cell extracts were indicated as pmol 4-methylumbelliferone released during the 30 min per μg total protein in the original cell suspension. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA).

Protein expression and antibody production. An 1833-bp fragment of *chiA* encoding a region spanning the N-terminal glycoside hydrolase domain was amplified by PCR using primers 1066 (engineered BamHI site) and 1067 (engineered SalI site). This fragment was digested with BamHI and SalI and ligated into pET30a that had been digested with the same enzymes, generating pSSK07. pSSK07 was introduced into *E. coli* Rosetta 2(DE3) (Novagen, Madison, WI), which expresses seven rare tRNAs

required for efficient ChiA expression. Cells were grown to mid-log phase at 37°C in LB and expression of recombinant ChiA was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for an additional 4 h. Cells were collected by centrifugation, disrupted using a French press, and inclusion bodies containing recombinant ChiA were isolated by centrifugation at 6,415 × *g* for 30 min. Inclusion bodies were suspended in 20 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, 1 mM EDTA, and 200 μg/ml lysozyme and incubated for 15 min at 25°C. Inclusion bodies were collected by centrifugation at 20,000 × *g* for 15 min and washed twice by suspension in 20 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, 1mM EDTA and 1% Triton X-100 with sonication followed by centrifugation. The inclusion bodies were solubilized in 8 M urea for 1 h at room temperature. Insoluble material was removed by centrifugation at 20,000 × *g* for 30 min, and the soluble material containing ChiA was boiled in SDS-PAGE loading buffer and separated on 7.5% acrylamide gels by SDS-PAGE. Recombinant ChiA was visualized by $CuCl_2$ staining (30), the band was cut from the gel and destained in 0.25 M Tris (pH 9.0), 0.25 M EDTA, and the protein was electroeluted at 60 mA for 5 h into 25 mM Tris, 192 mM glycine, 0.1% SDS (pH 8.8) using a model 422 Electro-Eluter (Bio-Rad). Polyclonal antibodies against recombinant ChiA were produced and affinity purified using the recombinant protein by Proteintech Group, Inc. (Chicago, IL).

Western blot analyses. *F. johnsoniae* cells were grown to mid-log phase in MM 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 filtered using 0.22 μm pore-size

polyvinyllidene difluoride filters (Thermo Fisher Scientific). 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 9 min. Proteins were separated by SDS-PAGE, and western blot analyses were performed as previously described (49) using affinity purified antibody against ChiA (1:5,000 dilution). Equal amounts of each sample based on the starting material were loaded in each lane. For cell extracts this corresponded to 15 μg protein whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 15 μg cell protein before the cells were removed. To determine whether ChiA was associated with membrane vesicles that had been released into the spent medium, the sample was fractionated into soluble and insoluble (membrane) fractions by centrifugation at 352,900 x g for 30 min. For detection of mCherry by Western blot, commercially available antibodies against mCherry (0.5 mg per ml; BioVision Incorporated, Milpitas, CA) were used at dilution of 1:5,000.

Liquid chromatography-tandem mass spectroscopy (LC-MS/MS analysis). *F. johnsoniae* cells were grown to mid-log phase in MM at 25°C with shaking. Cells were pelleted by centrifugation at 22,000 x g for 15 min, and the spent culture medium was filtered (0.22 μm polyvinyllidene difluoride filters) to remove residual cells. The spent medium was concentrated 1000 fold using Pierce concentrators (Thermo Fisher Scientific) and proteins were separated by SDS-PAGE and detected using the BioRad (Hercules, CA) Silver Stain kit.

Enzymatic in-gel digestion and mass spectrometric analysis of the peptides were performed at the University of WI-Madison Mass Spectrometry Facility essentially as previously described (14, 56). Enzymatic digestion and peptide recovery was performed as outlined on the website:

http://www.biotech.wisc.edu/facilities/massspec/protocols/ingelprotocol. Peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent Technologies, Palo Alto, CA) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific) equipped with a nanoelectrospray ion source. Chromatography of peptides prior to mass spectral analysis was accomplished using a C18 reverse phase HPLC trap column (Zorbax 300SB-C18, 5 µM, 5 x 0.3 mm, Agilent Technologies) and a capillary emitter column (in-house packed with MAGIC C18, 3 µM, 150 x 0.075 mm, Michrom Bioresources, Inc.) onto which 8 µl of extracted peptides were loaded. NanoHPLC system-delivered solvents were as follows: A) 0.1% (v/v) formic acid in water, and B) 95% (v/v) acetonitrile, 0.1% (v/v) formic acid. Sample loading was performed at 10 µL/min and peptide elution was performed at 0.20 µL/min using a gradient from 1% (v/v) B to 60% (v/v) B over 60 minutes followed by a gradient from 60% (v/v) B to 100% (v/v) B over 10 minutes. As peptides eluted survey MS scans were acquired in the Orbitrap with a resolving power of 100,000 over the mass range 300 to 2000 m/z. The 5 most intense peptides detected per scan were fragmented and detected in the ion trap. Raw MS/MS data were converted to mgf file format using the Trans Proteomic Pipeline (Seattle Proteome Center, Seattle, WA). The resulting files were used to search against the *F. johnsoniae* protein database

concatenated with a list of common lab contaminants (5,057 protein entries) with cysteine carbamidomethylation as fixed modification and methionine oxidation and asparagine/glutamine deamidation as variable modifications. Peptide mass tolerance was set at 20 ppm and fragment mass tolerance at 0.8 Da. Matrix Science Mascot version 2.2.07 was used as search engine and protein identifications with at least two matched peptides with ion scores of 25 or above were reported.

Table 2. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or description[a]	Source or reference
F. johnsoniae strains		
ATCC 17061 strain UW101	Wild type	(34, 37)
UW102-3	Spontaneous *sprA* mutant	(49, 66)
UW102-57	Spontaneous *gldK* mutant	(6, 12)
UW102-176	Nitrosoguanidine-induced *gldM* mutant	(12, 49)
UW102-344	Spontaneous *gldL* mutant	(49, 66)
CJ1631A	Δ(*gldN gldO*)	(49)
CJ1808	*chiA* disruption mutant; (Em^r)	This study
CJ1827	Strain used for construction of deletion mutants; *rpsL2*; (Sm^r)	(48)
CJ2325	Mutant lacking CTD encoding region of *chiA*; *rpsL2*; (Sm^r)	This study
CJ2355	Fjoh_4175 deletion mutant; *rpsL2*; (Sm^r)	This study
FJ149	*sprE* disruption mutant; (Em^r)	(50)
KDF001	*sprT* disruption mutant; (Em^r)	(53)
Plasmids		
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; Ap^r(Tc^r)	(2)
pET30a	Protein expression vector; (Km^r)	Novagen
pKF002	pCP23 carrying *sprT*; Ap^r (Tc^r)	(53)
pLYL03	Suicide vector used for insertional mutagenesis; Ap^r (Em^r)	(31)
pME-mCherry	Plasmid expressing fluorescent protein mCherry; Km^r	(29)
pNap2	pCP23 carrying *sprE*; Ap^r (Tc^r)	(50)
pRR51	*rpsL*-containing suicide vector for construction of deletions; Ap^r (Em^r)	(48)
pSAM1	1,299 bp fragment of *chiA* in pLYL03 for constructing *chiA* disruption mutant CJ1808; Ap^r (Em^r)	This study
pSN48	pCP23 carrying *sprA*; Ap^r (Tc^r)	(42)
pSSK05	pCP23 carrying *chiA*; Ap^r (Tc^r)	This study
pSSK07	1,833 bp fragment of *chiA* inserted into pET30a; (Km^r)	This study
pSSK26	2,121 bp fragment upstream of *chiA* CTD-encoding region in pRR51; Ap^r (Em^r)	This study
pSSK27	Construct used to delete CTD-encoding region of *chiA*; 2,033 bp region downstream of *chiA* inserted into pSSK26; Ap^r (Em^r)	This study

pSSK30	pCP23 carrying *mcherry*; Ap^r (Tc^r)	This study
pSSK32	2,118 bp region downstream of Fjoh_4175 inserted into pRR51; Ap^r (Em^r)	This study
pSSK34	Construct used to delete Fjoh_4175; 1,948 bp region upstream of Fjoh_4175 inserted into pSSK32; Ap^r (Em^r)	This study
pSSK45	*mcherry* with stop codon amplified with primers 862 and 1443 and cloned into pCP23; Ap^r (Tc^r)	This study
pSSK51	484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region inserted into pSSK30; Ap^r (Tc^r)	This study
pSSK52	566 bp region encoding 105 amino acid CTD_{ChiA} inserted into pSSK51; Ap^r (Tc^r)	This study
pSSK54	484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region inserted into pSSK45; Ap^r (Tc^r)	This study
pTB79	pCP23 carrying *gldN*; Ap^r (Tc^r)	(6)
pTB81a	pCP23 carrying *gldL*; Ap^r (Tc^r)	(6)
pTB94a	pCP23 carrying *gldM*; Ap^r (Tc^r)	(6)
pTB99	pCP23 carrying *gldK*; Ap^r (Tc^r)	(6)

[a]Antibiotic resistance phenotypes are as follows: ampicillin, Ap^r; cefoxitin, Cf^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 3. Primers used in this study

Primers	Sequence and Description
737	5'-AGGCACCCCAGGCTTTACACT-3'; Reverse primer binding downstream of multiple cloning site of pLYL03.
862	5’-GCTAGGGATCCATGGTGAGCAAGGGCGAGG-3’; mCherry forward primer used in construction of pSSK30 and pSSK45; BamHI site underlined.
937	5’-GCTAGGGATCCTGATCCGTCAAGAACTGTTCCGC-3’; Reverse primer used in construction of pSAM1; BamHI site underlined.
938	5’-GCTAGGTCGACAGTCCGGTAGCAAGAGCTGCATTA-3’; Forward primer used in construction of pSAM1; SalI site underlined.
941	5’-TTGCACCTGCAACCGGATTTGTTC-3’; Reverse primer used for confirming and sequencing *chiA* disruption mutant CJ1808; Binds 532 bp upstream of primer 937.
974	5’-GCTAGTCTAGAGGTTCATAATGCGCATCCTTAGGCA-3’; Reverse primer used to amplify *chiA* for construction of complementation plasmid pSSK05; XbaI site underlined.
975	5’-GCTAGGGATCCCTTCCAACCTGCAGTTGAGCGAAA-3’; Forward primer used to amplify *chiA* for construction of complementation plasmid pSSK05; BamHI site underlined.
1066	5’-GCTAGGGATCCAGTCCGGTAGCAAGAGCTGCATTA-3’; Forward primer used to amplify *chiA* for constructing pSSK07; BamHI site underlined.
1067	5’-GCTAGGTCGACTTTTGCACCTGCAACCGGATTTGTTC-3’; Reverse primer used to amplify *chiA* for constructing pSSK07; SalI site underlined.
1227	5’-GCTAGTCTAGATGCAGATCAGTCACCATCGCTTCA-3’; Forward primer used to amplify upstream region of Fjoh_4175 for constructing pSSK34; XbaI site underlined.
1228	5’-GCTAGGTCGACAGAAACAGAACCTCCTCCAAGCGA – 3’; Reverse primer used to amplify upstream region of Fjoh_4175 for constructing pSSK34; SalI site underlined.
1229	5’-GCTAGGTCGACTTCTTCTCGGCAGAAGTTTCGGGA- 3’; Forward primer used to amplify downstream region of Fjoh_4175 for constructing pSSK32; SalI site underlined.
1230	5’-GCTAGGCATGCTCCTAAAGTTGTTGTTCCGTTTGC-3; Reverse primer used to amplify downstream region of Fjoh_4175 for constructing pSSK32; SphI site underlined.
1266	5’-GCTAGTCTAGACTTGTACAGCTCGTCCATGCCG– 3’; Reverse primer to amplify mCherry for constructing pSSK30; XbaI site underlined.
1378	5’- GCTAGGGATCCGCAGTTCCTGCAAATCCAACAGTT- 3’; Forward primer to amplify the upstream region of *chiA* CTD for constructing pSSK26; BamHI site underlined.
1379	5’- GCTAGGTCGACAGATAATTCAGATGAATTACCGCAAGA-3’; Reverse primer to amplify the upstream region of *chiA* CTD for constructing pSSK26; SalI site

	underlined.
1380	5’-GCTAGGTCGACAACTAATAAATGATTGAAAATTTAGAA -3’; Forward primer to amplify the region downstream of *chiA* for constructing pSSK27; SalI site underlined.
1381	5’-GCTAGGCATGCTGAAATTTCCATTAGCCAGC -3’; Reverse primer to amplify the region downstream of *chiA* for constructing pSSK27; SphI site underlined.
1391	5’-TCTGGAAGAACATATACTATGCAGCCA- 3’; Forward primer used to confirm and sequence *chiA* CTD deletion.
1392	5’-TCACCTAATACAATAACTAACCTC-3’; Reverse primer used to confirm and sequence *chiA* CTD deletion.
1404	5’-GCTAGGCATGCTCACCTAATACAATAACTAACCTC-3’; Reverse primer to amplify *chiA* CTD for making construct pSSK52; SphI site underlined.
1443	5’-GCTAGTCTAGATTACTTGTACAGCTCGTCCATGCCG– 3’; Reverse primer to amplify mCherry for constructing pSSK45; XbaI site underlined.
1463	5’-AACAGTATCGATGTTTCGCATTTAG-3’; Used for confirming and sequencing Fjoh_4175 deletion.
1464	5’-GCAAAGAGCGCCAAGTTTAC-3’; Used for confirming and sequencing Fjoh_4175 deletion.
1516	5’-GCTAGGGATCCCACTACTTTTTTCCCGTGGGCTGGCTG -3’; Reverse primer to amplify short N-terminal region of *chiA* to construct pSSK52 and pSSK54; BamHI site underlined.
1593	5’- GCTAGGGTACCTTCCCCGGTAGAGATAGTTATGGCTAT -3’ Forward primer to amplify N-terminal region of *chiA* to make constructs pSSK52,,and pSSK54; Binds 400 bp upstream of *chiA* start codon; KpnI site underlined.
1600	5’GCTAGTCTAGAGCTTATGCAGCTTATTTCGCATCACAA -3’ forward primer to amplify *chiA* CTD region for making construct pSSK52; XbaI site underlined

Results

***chiA* mutant cells are defective in chitin utilization**. Chitinases have catalytic glycoside hydrolase domains belonging to families 18 (GH18) and 19 (GH19) (19). The *F. johnsoniae* genome encodes five predicted chitinases with such domains (37). One of these, Fjoh_4555 which we refer to as ChiA (Figure 8), has previously been implicated in chitin utilization. Cells with a mutation in the T9SS gene *sprT* failed to accumulate ChiA in the extracellular fluid and failed to utilize chitin (53). ChiA has two GH18 domains, each predicted to have chitinolytic activity (Figure 8). We disrupted *chiA* to determine its role in chitin utilization. Cells of the *chiA* mutant CJ1808 failed to utilize chitin (Figure 9A) and the mutant cells were deficient in extracellular chitinase activity (Figure 10). Complementation with pSSK05, which carries *chiA*, restored extracellular chitinase activity and the ability to utilize chitin (Figure 9A, 10). Chitinase activities associated with intact cells and with cell extracts were less affected by disruption of *chiA*, suggesting that the other predicted chitinases may contribute to these cell-associated activities (Figure 10). Extracts prepared from cells carrying pSSK05 exhibited elevated levels of activity against 4-MU-$(GlcNAc)_3$, perhaps indicating that ChiA expressed from the plasmid was not efficiently secreted. Deletion of the T9SS genes *gldN* and *gldO* also resulted in decreased extracellular chitinase as previously reported (49), presumably because of a failure to secrete ChiA. Another predicted chitinase, Fjoh_4175, exhibits sequence similarity to the GH18 chitinase domain near the amino terminus of ChiA (GH18N). The CTD of Fjoh_4175 is similar in sequence to the CTDs of members of TIGRFAM family TIGR04183. These CTDs are thought to target the proteins for

secretion by the T9SS. Given the importance of the T9SS in chitin utilization we examined the role of Fjoh_4175 in this process. The Fjoh_4175 deletion mutant CJ2355 digested and grew on chitin (Figure 9B). Cells of the mutant also had as much extracellular and cell-associated chitinase activities as did wild type cells (Figure 10) indicating that Fjoh_4175 does not play a major role in chitin utilization under the conditions examined.

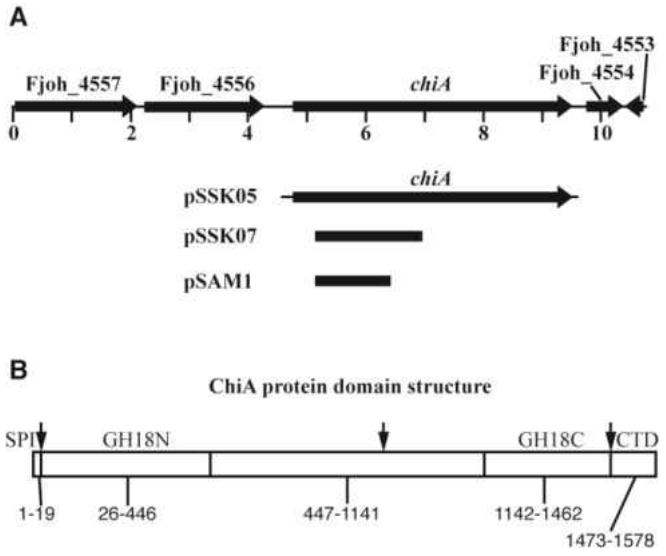


Figure 8. The *chiA* gene and predicted features of the ChiA protein. A) Map of the *chiA* region. Numbers below the map refer to kilobase pairs of sequence. The regions of DNA carried by the plasmids pSSK05 (used for complementation), pSSK07 (used for expression of recombinant ChiA in *E. coli*) and pSAM1 (used for insertional mutagenesis of *chiA*) are indicated beneath the map. B) Predicted features of the ChiA protein. SPI: Type I signal peptide. GH18N and GH18C: glycohydrolase 18 family domains located near the amino and carboxy termini respectively. CTD: C-terminal domain involved in secretion by the type IX secretion system. Arrows denote approximate sites of apparent proteolytic processing, and numbers indicate approximate amino acid ranges for each predicted domain.

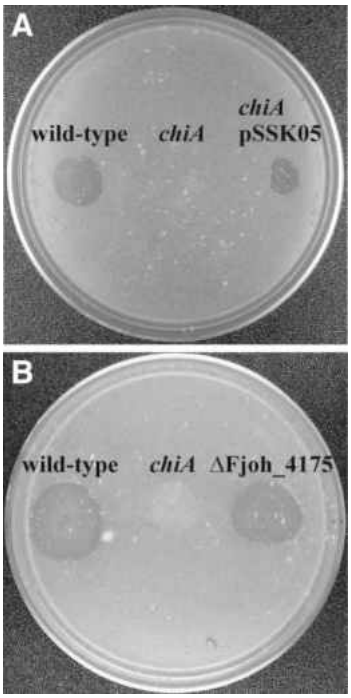


Figure 9. *chiA* is required for chitin utilization. A) Approximately 10^6 cells of wild-type *F. johnsoniae* UW101, *chiA* mutant CJ1808, and CJ1808 with pSSK05 which carries *chiA*, were spotted on MYA-chitin media and incubated at 25°C for 2.5 d. B) Wild type *F. johnsoniae* CJ1827, *chiA* mutant CJ1808, and Fjoh_4175 deletion mutant CJ2355 were spotted on MYA-chitin media and incubated at 25°C for 2.5 d.

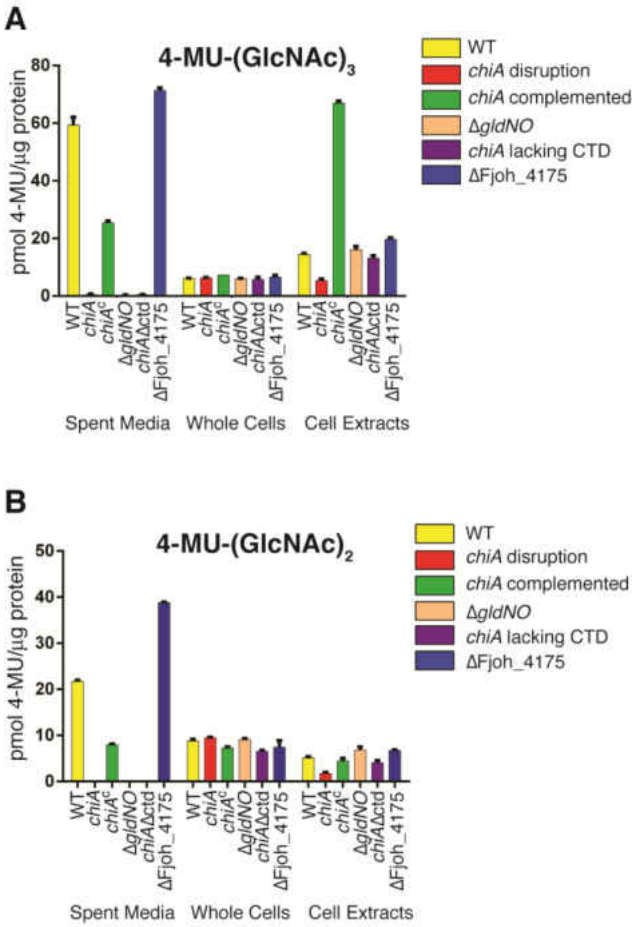


Figure 10. Chitinase activities of wild-type and mutant cells. Chitinase activities of spent media, intact cells, and cell extracts were determined using the synthetic substrates 4-MU-$(GlcNAc)_2$, and 4-MU-$(GlcNAc)_3$. Equal amounts of each sample, based on the protein content of the cell suspension, were incubated with 10 nmol of synthetic substrate for 30 min at 37°C, and the amount of 4-MU released was determined by measuring fluorescence emission at 460 nm following excitation at 360 nm. Yellow, wild type *F. johnsoniae* UW101. Red, *chiA* mutant CJ1808. Green, CJ1808 with pSSK05 which carries *chiA*. Tan, *gldNO* deletion mutant CJ1631A. Purple, CJ2325, which produces ChiA lacking the C-terminal 106 amino acids. Blue, Fjoh_4175 deletion mutant CJ2355.

ChiA is a soluble extracellular protein. A portion of ChiA spanning the N-terminal GH18 domain (GH18N) was overexpressed in *E. coli* and polyclonal antiserum was raised against this fragment. The antiserum was used to detect ChiA in cultures of wild-type *F. johnsoniae*. ChiA was present primarily in the cell-free spent medium, with little if any cell-associated ChiA (Figure 11A). ChiA was detected in spent media from wild-type cells but was absent from spent media of the *chiA* mutant (Figure 11B). Introduction of *chiA* into the mutant on pSSK05 restored production of ChiA. Expression from pSSK05 resulted in large amounts of ChiA, and fragments of ChiA, in the spent medium and in intact cells (Figure 11B). The extra bands observed for the complemented strain may be the result of failure to efficiently secrete the overexpressed protein, perhaps resulting in degradation. To determine whether ChiA in the spent medium from wild type cells was present in soluble form or was associated with membrane vesicles or cell debris, particulate material was pelleted by ultracentrifugation twice at 352,900 x g for 30 min. ChiA was found in the soluble fraction (Figure 11C), indicating that ChiA is a soluble secreted protein.

ChiA is predicted to be 166 kDa in size after removal of its N-terminal signal peptide. We refer to the cell-associated 166 kDa protein as proChiA (Figure 11B). In contrast, the secreted ChiA detected with our antiserum migrated with an apparent molecular mass of approximately 92 kDa (Figure 11) suggesting that the protein was proteolytically processed. The antiserum used to detect ChiA was raised against the region spanning the N-terminal GH18 domain, and thus did not efficiently detect other regions of ChiA released during processing.

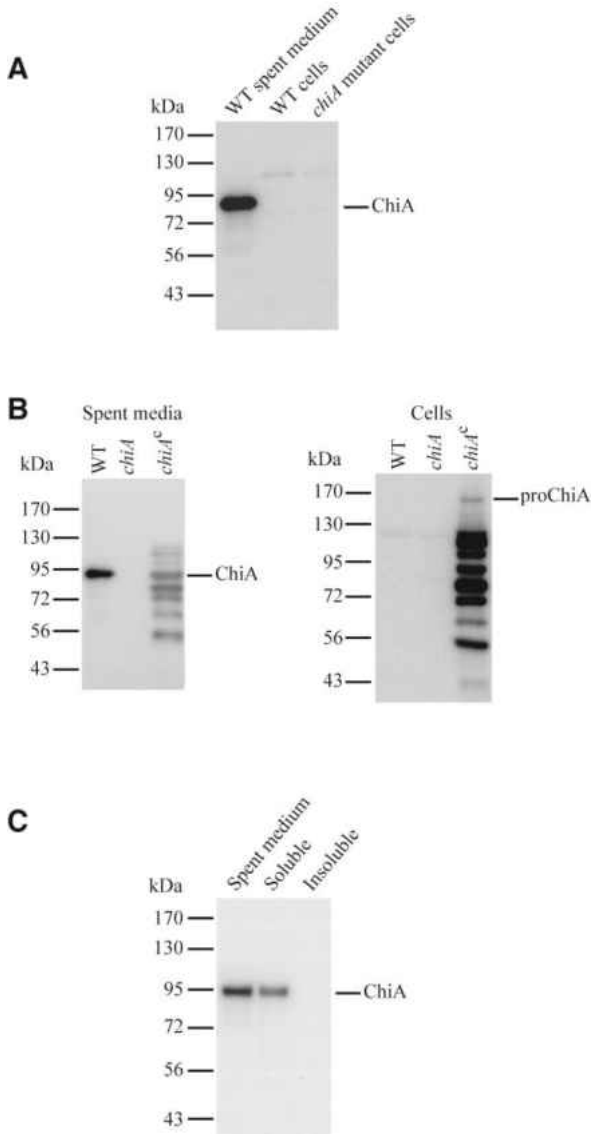


Figure 11. ChiA is a soluble extracellular protein. Panels A and B: Spent medium and whole cells of wild-type (WT), *chiA* mutant (*chiA*), and *chiA* mutant complemented with pSSK05 (*chiA*^{c}) were examined for ChiA by SDS-PAGE followed by Western blot analysis. Panel C: Spent medium from a culture of wild-type *F. johnsoniae* UW101 was examined for ChiA before (Spent medium) and after fractionation into Soluble and Insoluble (membrane) fractions by centrifugation at 352,900 x g for 30 min. For each panel, equal amounts of each sample based on the starting material were loaded in each lane. For cells this corresponded to 15 μg protein, whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 15 μg cell protein before the cells were removed.

Cell-free spent media from wild-type, *chiA* mutant, and complemented cells were also examined by SDS-PAGE followed by silver staining to identify the prominent bands. Proteins of approximately 92 kDa and 65 kDa were observed in spent media from wild type and complemented cells, but not in spent medium of the *chiA* mutant (Figure 5). The 92 kDa and 65 kDa proteins secreted by cells of the *chiA* mutant CJ1808 complemented with pSSK05 which expresses ChiA were identified by LC-MS/MS (Figure 13 and 14). The two bands corresponded to the two GH18 domains of ChiA with flanking sequences, suggesting that proteolytic processing released these in soluble form. The 92 kDa band ($ChiA_{GH18N}$) corresponded to a fragment containing the amino-proximal GH18 fragment and adjacent regions, and the 65 kDa band ($ChiA_{GH18C}$) corresponded to a fragment containing primarily the carboxy-proximal GH18 and adjacent regions. LC-MS/MS analysis of the 65 kDa band also revealed two peptides corresponding to regions of the protein closer to the amino terminus. These were apparently low abundance proteins in the band (Figure 14) and may have corresponded to breakdown products of the 92 kDa protein. Such breakdown products of approximately 65 kDa were expected, because they were also observed by Western blot using antiserum against the amino terminal portion of ChiA (Figure 11B). LC-MS/MS analysis of the 65 kDa and 92 kDa proteins failed to detect the amino terminal signal peptide and the C-terminal 91 amino acid region.

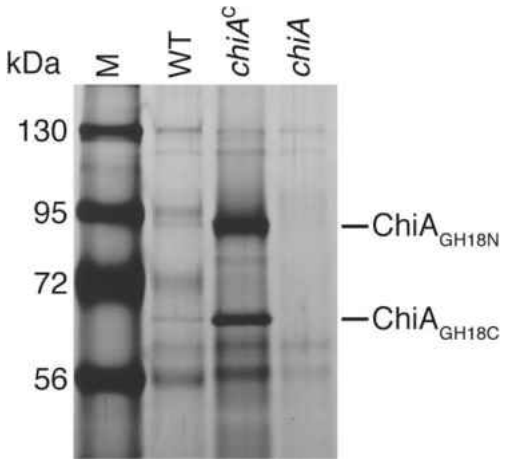


Figure 12. Analysis of secreted ChiA protein by SDS-PAGE. Equal amounts of cell-free spent media from cultures of wild-type (WT), *chiA* mutant CJ1808 (*chiA*), and CJ1808 complemented with pSSK05 which carries *chiA* (*chiA*^{c}) were separated by SDS-PAGE. Proteins were detected by silver staining. $ChiA_{GH18N}$ and $ChiA_{GH18C}$ refer to amino proximal and carboxy proximal GH18-containing fragments respectively of ChiA as determined by LC-MS/MS analysis of material from the 92 kDa and 65 kDa bands (Figure 13 and 14). M= molecular weight markers.

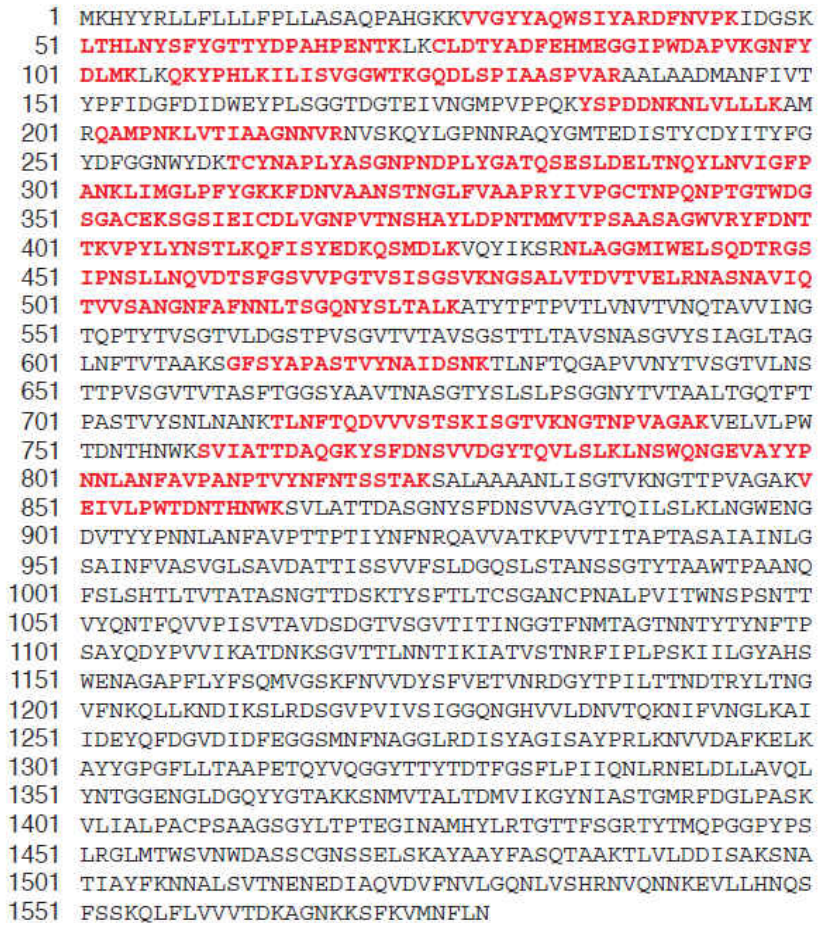


Figure 13. The approximately 92 kDa secreted form of ChiA corresponds to the amino-proximal portion of the full length ChiA. Cell-free spent medium from the *chiA* mutant CJ1808 complemented with pSSK05 which carries *chiA* was separated by SDS-PAGE and proteins were detected by silver staining.The approximately 92 kDa band was cut from the gel shown in Figure 12 and subjected to LC-MS/MS analysis. The primary amino acid sequence of ChiA is shown, with the regions detected by LC-MS/MS highlighted in red. 131 spectral matches were detected to ChiA. All of these fell between amino acids 27 and 864, which corresponds to the amino-proximal region of the protein after removal of the signal peptide.

Figure 14. The approximately 65 kDa secreted form of ChiA appears to correspond to the carboxy-proximal portion of full length ChiA. Cell-free spent medium from the *chiA* mutant CJ1808 complemented with pSSK05 which carries *chiA* was separated by SDS-PAGE and proteins were detected by silver staining. The approximately 65 kDa band was cut from the gel shown in figure 12 and subjected to LC-MS/MS analysis. The primary amino acid sequence of ChiA is shown, with the regions detected by LC-MS/MS highlighted. Regions in red correspond to sequences for which multiple (2 to 7) spectral matches were obtained, and regions in orange correspond to sequences for which single spectral matches were obtained, and which were thus apparently less abundant. In total, 79 spectral matches were detected to ChiA. 77 of these fell between amino acids 850 and 1487, which corresponds to the carboxy-proximal region of the protein immediately upstream of the C-terminal region involved in targeting to the type IX secretion system. The 2 spectral matches to sequences at positions 315 to 332 and 449 to 478 probably correspond to breakdown products of the 92 kDa amino-proximal portion of ChiA. No spectral matches were detected to the carboxy-terminal 91 amino acids suggesting that this region may have been removed by proteolysis during secretion.

The T9SS is required for secretion of ChiA. Mutations in the *F. johnsoniae* T9SS genes *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, and *sprT* result in defects in chitin utilization (49, 50, 53, 59). The effect of such mutations on secretion of ChiA was examined. ChiA accumulated in the spent culture medium of wild type cells, but not of cells of the T9SS mutants (Figure 15A). Complementation of the T9SS mutants with plasmids carrying the appropriate T9SS genes restored secretion of ChiA into the culture medium. Cells were also examined for ChiA. Wild type cells accumulated little if any ChiA protein, whereas cells of the T9SS mutants accumulated some proChiA (Figure 15B). The amount of proChiA that accumulated in cells of the T9SS mutants was less than the amount of processed ChiA found in the culture fluid of wild-type cells. We do not know the reason for this, but likely explanations could include decreased expression of ChiA or degradation of the improperly localized ChiA in the T9SS mutants.

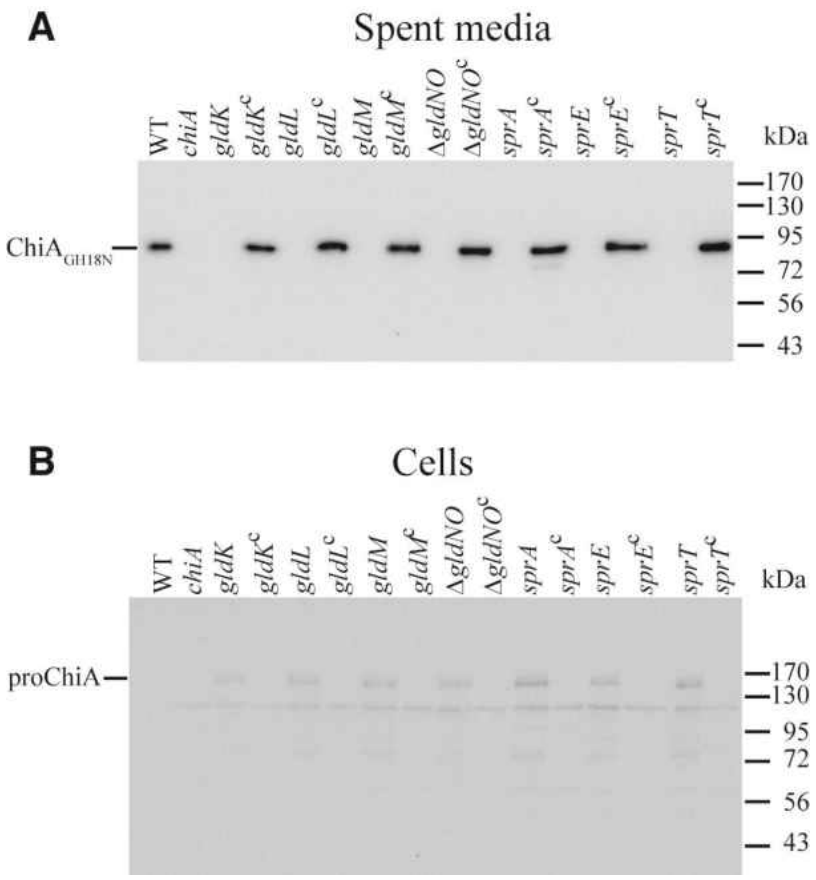


Figure 15. Mutations in T9SS genes disrupt secretion of ChiA. Cell-free spent media (Panel A) and cells (Panel B) were examined for ChiA by SDS-PAGE followed by Western blot analysis using antiserum against recombinant ChiA. WT: wild-type *F. johnsoniae* UW101. *chiA*: *chiA* mutant CJ1808. *gldK*: *gldK* mutant UW102-57. *gldK*^{c}: UW102-57 complemented with pTB99 which carries *gldK*. *gldL*: *gldL* mutant UW102-344. *gldL*^{c}: UW102-344 complemented with pTB81a which carries *gldL*. *gldM*: *gldM* mutant UW102-176. *gldM*^{c}: UW102-176 complemented with pTB94a which carries *gldM*. Δ*gldNO*: *gldNO* deletion mutant CJ1631A. Δ*gldNO*^{c}: CJ1631A complemented with pTB79 which carries *gldN*. *sprA*: *sprA* mutant UW102-3. *sprA*^{c}: UW102-3 complemented with pSN48 which carries *sprA*. *sprE*: *sprE* mutant FJ149. *sprE*^{c}: FJ149 complemented with pNap2 which carries *sprE*. *sprT*: *sprT* mutant KDF001. *sprT*^{c}: KDF001 complemented with pKF002 which carries *sprT*. Samples loaded in panel B corresponded to 15 μg protein per lane, and samples loaded in panel A corresponded to the volume of spent medium that contained 15 μg cell protein before the cells were removed.

The C-terminal region of ChiA is necessary and sufficient for secretion. *F. johnsoniae* proteins known to be secreted by the T9SS have conserved CTDs that belong to TIGRFAM families TIGR04131 (such as SprB) and TIGR04183 (such as RemA). ChiA does not exhibit strong similarity to members of these TIGRFAM families (Figure 18 and 19). However, ChiA has a C-terminal region of unknown function that might perform a similar role, and this region does exhibit limited similarity to the CTDs of TIGR04183 members (Figure 19). A mutant, CJ2325, which expresses ChiA lacking the C-terminal 106 amino acids was constructed. Cells of CJ2325 failed to utilize chitin and failed to accumulate ChiA extracellularly (Figure 10 and 16). Instead, the mutant cells accumulated cell-associated proChiA, suggesting a role for the C-terminal region in secretion.

To determine if the ChiA CTD is sufficient for secretion we constructed a plasmid that expressed the foreign protein mCherry sandwiched between the ChiA signal peptide (at the amino terminus) and a 105 amino acid region encompassing the ChiA CTD (at the carboxy terminus). Expression of mCherry with the 105 amino acid C-terminal region of ChiA resulted in accumulation of mCherry in the spent medium, whereas expression of mCherry without the ChiA C-terminal region did not (Figure 17). Cells of a strain lacking the T9SS genes *gldN* and *gldO* failed to secrete mCherry-CTD_{ChiA}. Together these results suggest that the CTD of ChiA targets proteins for secretion by the T9SS.

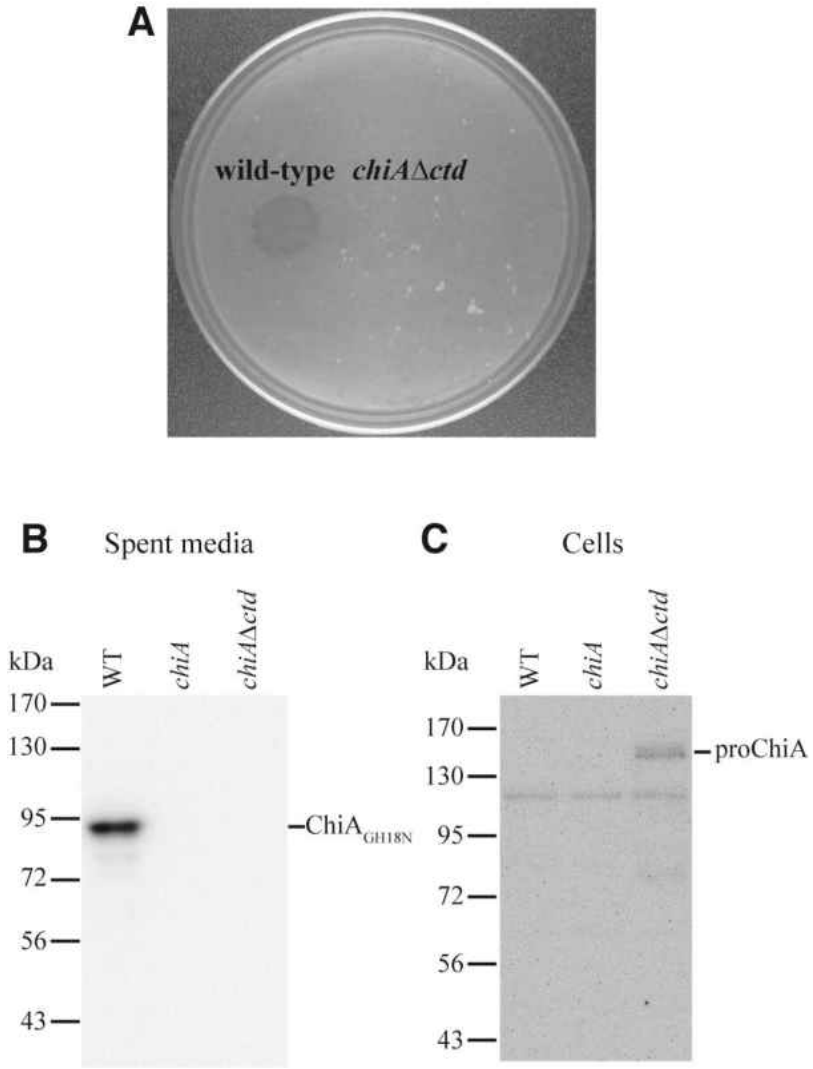


Figure 16. The C-terminal region of ChiA is required for chitin utilization. Panel A) Deletion of the region of *chiA* encoding the CTD results in defects in chitin utilization. Approximately 10^6 cells of wild-type *F. johnsoniae* CJ1827 and of the *chiA*Δ*ctd* mutant CJ2325 were spotted on MYA-chitin medium and incubated at 25°C for 2.5 d. Panels B and C) The C-terminal region of ChiA is required for secretion. Cell-free spent media (B) and cells (C) were examined for ChiA by SDS-PAGE followed by Western blot analysis using antiserum against recombinant ChiA. WT: wild-type *F. johnsoniae*. *chiA*: *chiA* disruption mutant CJ1808. *chiA*Δ*ctd*: *chiA* mutant CJ2325 which encodes ChiA lacking its CTD. Samples loaded in Panel C corresponded to 15 μg protein per lane, and samples loaded in Panel B corresponded to the volume of spent medium that contained 15 μg cell protein before the cells were removed.

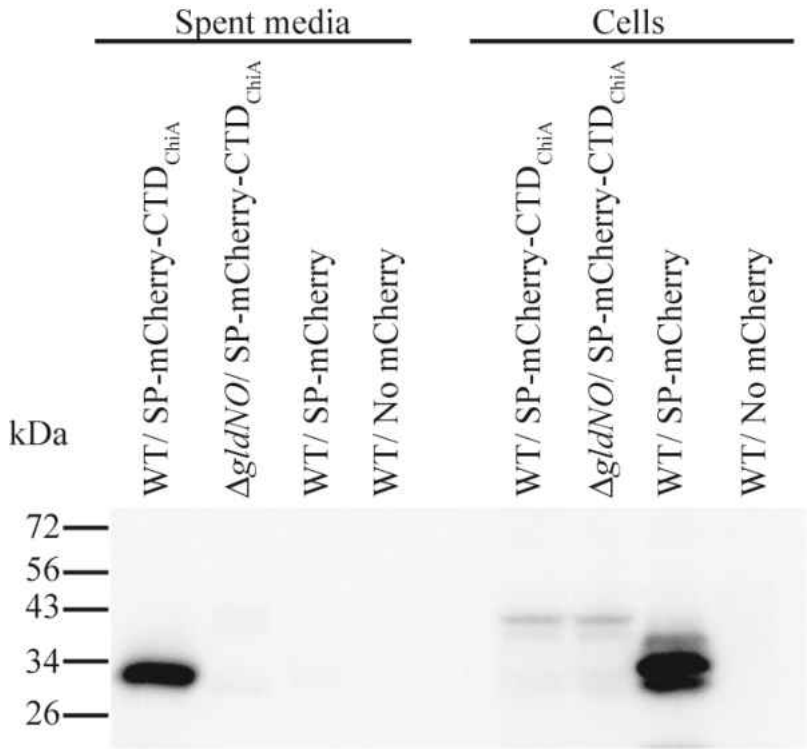


Figure 17. ChiA CTD is sufficient for secretion of the heterologous protein mCherry. Cell-free spent media and whole cells were analyzed for cultures of wild-type (WT) cells carrying pSSK54 that expresses mCherry with the N-terminal signal peptide from ChiA (SP-mCherry), or carrying pSSK52 that expresses mCherry with the N-terminal signal peptide from ChiA and the 105 amino acid C-terminal region of ChiA (SP-mCherry-CTD_{ChiA}). Cells and spent media from cultures of the T9SS mutant CJ1631A (Δ*gldNO*) carrying pSSK52 were also analyzed. 'No mCherry' indicates spent media or cells from *F. johnsoniae* carrying the control vector pCP23. Cell samples corresponded to 15 μg protein per lane and samples from spent media corresponded to the volume of spent media that contained 15 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE and mCherry was detected using antiserum against mCherry.

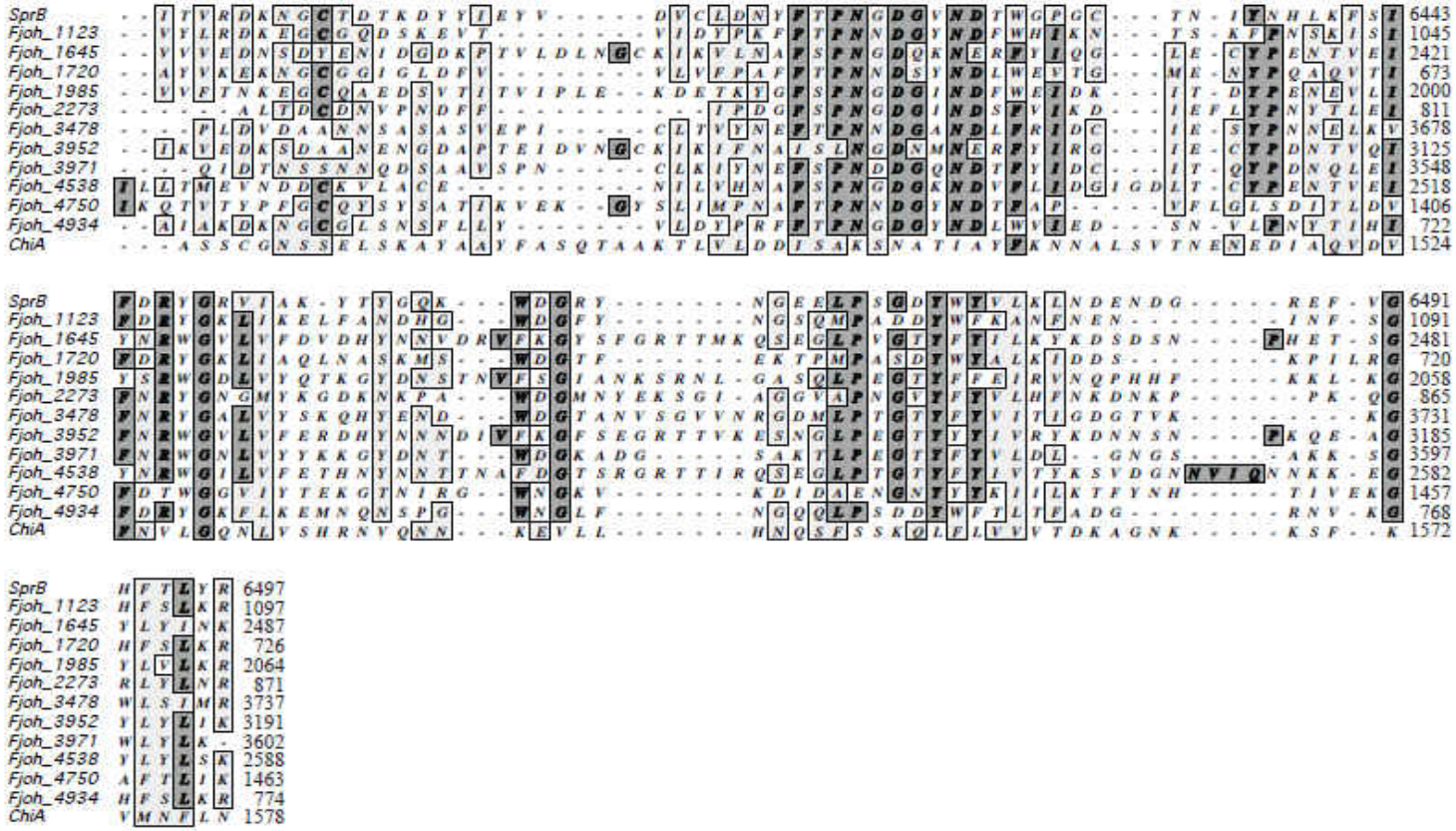
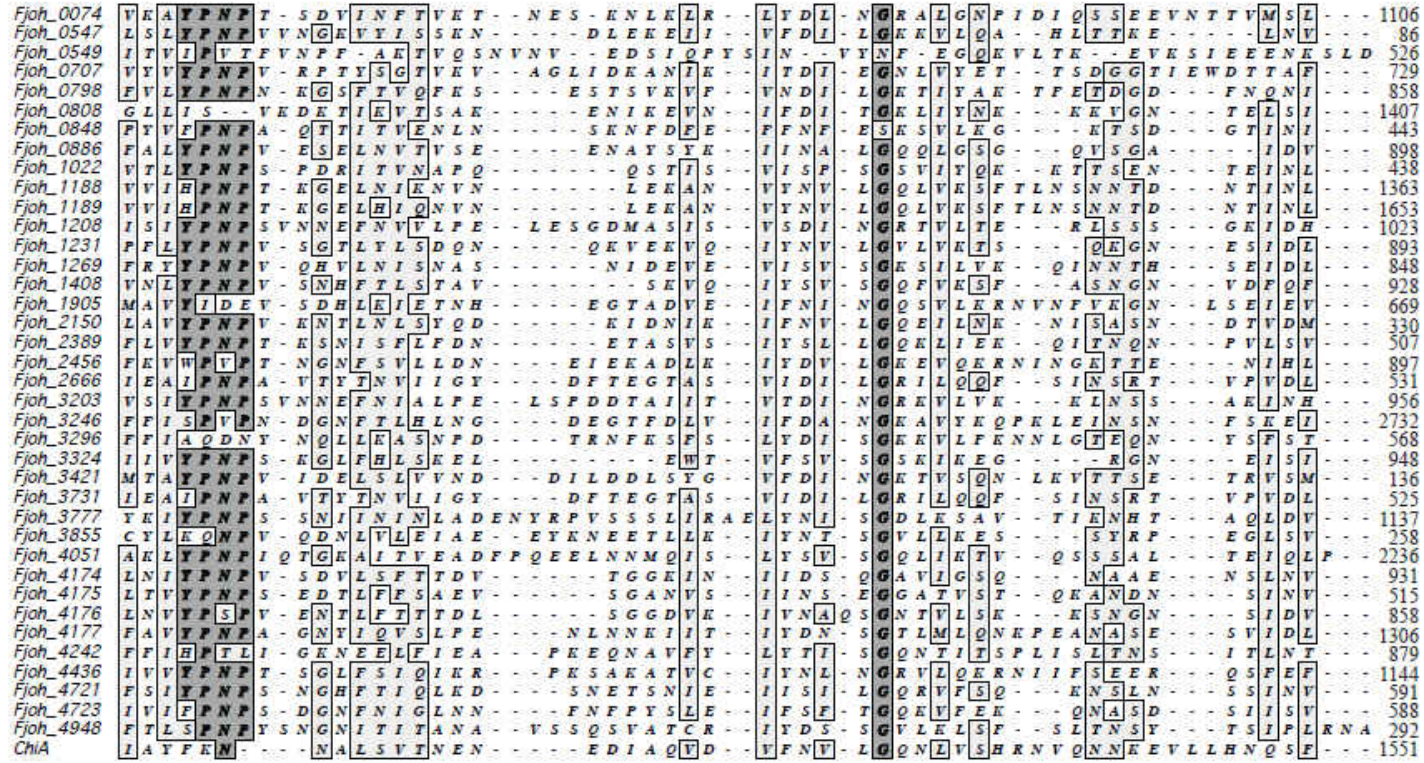


Figure 18. Alignment of the C-terminal domain (CTD) of ChiA with the CTDs of *F. johnsoniae* TIGR04131 family members using MUSCLE. Dark shading indicates identical amino acids and light shading indicates similar amino acids. TIGR04131 is described as 'gliding motility-associated C-terminal domain' on the TIGRFAM website (http://www.jcvi.org/cgi-bin/tigrfams/HmmReportPage.cgi?acc=TIGR04131). TIGR04131 family members were identified by searching the *F. johnsoniae* genome using the IMG v 4.0 Function Profile Tool. As shown, the ChiA CTD has little if any similarity to the CTDs of TIGR04131 family members.

Alignment of the CTD of ChiA with the CTDs of *F. johnsoniae* TIGR04183 family members



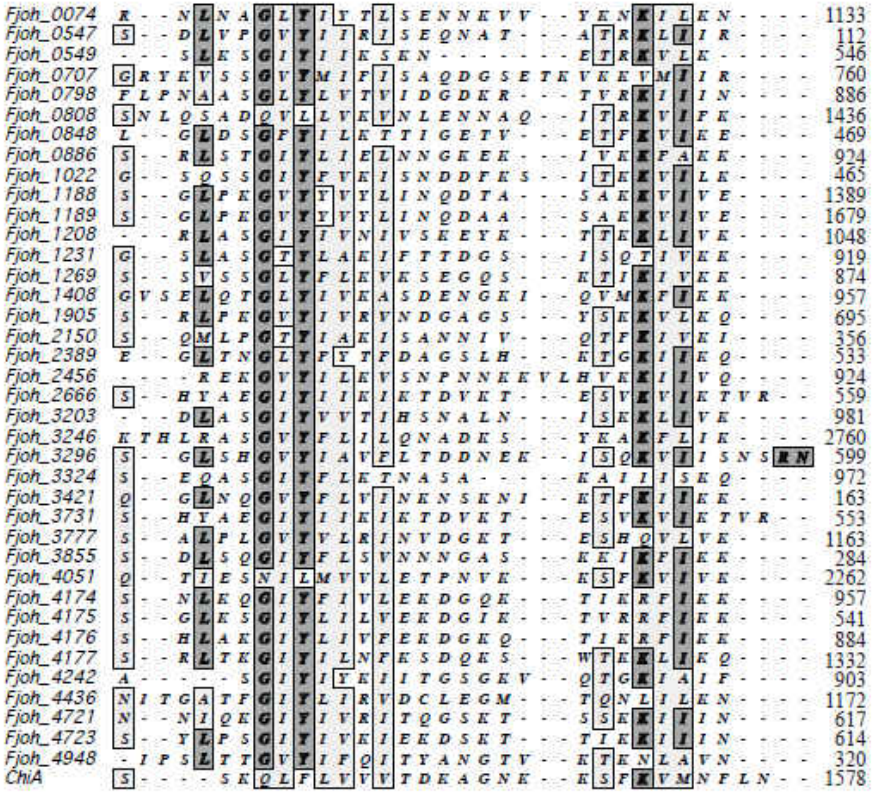


Figure 19. Alignment of the C-terminal domain (CTD) of ChiA with the CTDs of *F. johnsoniae* TIGR04183 family members using MUSCLE. Dark shading indicates identical amino acids and light shading indicates similar amino acids. TIGR04183 is described as 'Por secretion system C-terminal sorting domain' on the TIGRFAM website (http://www.jcvi.org/cgi-bin/tigrfams/HmmReportPage.cgi?acc=TIGR04183). TIGR04183 family members were identified by searching the *F. johnsoniae* genome using the IMG v 4.0 Function Profile Tool. Fjoh_2336, Fjoh_2338, Fjoh_2339, and Fjoh_3296 were eliminated because of poor matches to the consensus. As shown, the ChiA CTD has limited similarity to the CTDs of TIGR04183 family members.

Cells of the *chiA* mutant exhibit wild-type gliding motility. Many mutants of *F. johnsoniae* that have defects in chitin utilization have been studied, and each of these also had defects in gliding motility (7, 34, 35, 49, 50, 53, 59). The connection between chitin utilization and gliding motility was unclear until it was recognized that assembly of the gliding motility apparatus and secretion of ChiA relied on the same T9SS. Unlike the T9SS mutants, cells of the *chiA* mutant CJ1808 formed spreading colonies on agar (Figure 20), and cells moved rapidly over surfaces similar to wild-type cells, demonstrating that the ability to utilize chitin was not required for gliding motility.

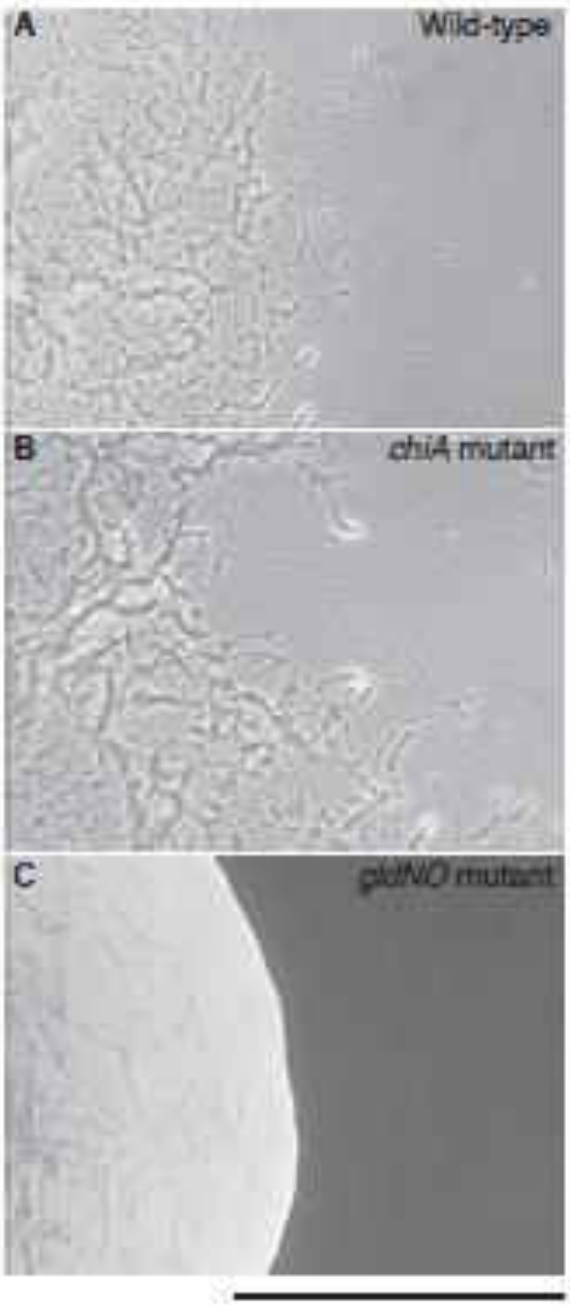


Figure 20. Disruption of *chiA* does not affect gliding motility. Colonies were grown for 42 h at 25°C on PY2 agar medium. Photomicrographs were taken with a Photometrics CoolSNAP$_{cf}^{2}$ camera mounted on an Olympus IMT-2 phase-contrast microscope. (A) Wild-type *F. johnsoniae* UW101. (B) *chiA* mutant CJ1808. (C) *gldNO* deletion mutant CJ1631A. Bar indicates 0.5 mm and applies to all panels.

Discussion

F. johnsoniae rapidly digests insoluble chitin, and its genome encodes five predicted chitinases that may have roles in this process (37). Here we demonstrate that one of these, ChiA, is essential for chitin utilization. ChiA is a soluble extracellular enzyme. Disruption of *chiA* eliminates soluble extracellular chitinase activity and results in inability of cells to digest insoluble chitin. Cell associated chitinase activities were still present, presumably contributed by some of the remaining four predicted chitinases. Some of these may reside on the cell surface whereas others may be periplasmic, allowing the digestion of oligomers of chitin that have been imported across the outer membrane. At least one of the predicted chitinases, Fjoh_4175, was not essential for chitin digestion under the conditions employed. Deletion of Fjoh_4175 failed to decrease the levels of secreted or cell-associated chitinase activities. Fjoh_4175 may be a minor chitinase, may not be expressed under the conditions of our experiments, or may not assist in digestion of the form of chitin (insoluble colloidal chitin prepared from crab shells) used in this study.

ChiA appears to be secreted by the T9SS. Mutations in any of the T9SS genes resulted in failure to secrete soluble ChiA, and accumulation of unprocessed proChiA inside of cells. Sequence analysis did not predict that ChiA would be secreted by the T9SS. Most other proteins known to be secreted by T9SSs have CTDs that belong to TIGRFAM families TIGR04131 (which includes SprB) and TIGR04183 (which includes RemA), but ChiA was not recognized by algorithms used to detect members of these families. ChiA does however have a region C-terminal to the predicted catalytic domains

that appears to perform a similar function. Deletion of this region resulted in failure to secrete ChiA, and attachment of this region to a foreign protein, mCherry, resulted in secretion in soluble form. BlastP analysis of the C-terminal 106 amino acid sequence against the non-redundant protein sequences in Genbank identified only three proteins that exhibit significant similarity to the *F. johnsonaie* ChiA CTD. Each of these are predicted chitinases from *Flavobacterium* species, and each are similar to ChiA not only over the CTD, but also over the entire protein. The ChiA CTD thus does not seem to represent a large family of previously unrecognized T9SS CTDs. The results reported here, and those previously published (18, 44, 54, 57, 58, 61, 63, 67), indicate that CTDs are involved in secretion by the T9SS but that there is considerable variation in the CTD sequences. Some features are apparently common to all, including the presence of multiple positively charged residues near the carboxy terminus. The sequence variations suggest that the structures of the CTDs may be more important than the exact sequences in targeting proteins to the T9SS.

The T9SS probably secretes many proteins besides those involved in motility and chitin utilization. *F. johnsoniae* is predicted to encode 53 proteins that have CTDs that belong to TIGRFAM families TIGR04131 and TIGR04183, which are thought to target proteins for secretion by the T9SS (59). This list includes nine predicted glycoside hydrolases, one polysaccharide lyase, and four peptidases in addition to proteins such as SprB and RemA that were previously known to be secreted by this system. Mutations that disrupt the T9SS are thus likely to have pleiotropic effects in addition to the known defects in motility and chitin utilization.

Proteins secreted by T9SSs often localize to the outer surface of the outer membrane. The *F. johnsoniae* motility proteins SprB and RemA, and the *P. gingivalis* gingipains and adhesins are examples of such proteins (13, 57-60, 63, 67). Some of these surface associated proteins have been shown to be modified by attachment of a glycolipid that may anchor them to the cell surface (13). This modification has been proposed as a general property of proteins secreted by T9SSs (63). Our results with ChiA indicate that it is secreted in soluble form by the T9SS, suggesting that this type of modification is not a requirement for secretion by the system. Many members of the phylum *Bacteroidetes* have dozens or even hundreds of genes predicted to encode CTD-containing proteins secreted by T9SSs (38, 63). It is perhaps not surprising that among this large number of proteins some are cell-surface associated and others are soluble extracellular proteins.

ChiA may undergo multiple processing events during or after secretion from the cell. ChiA has a predicted cleavable N-terminal signal peptide that is thought to target it to the Sec system for transit across the cytoplasmic membrane. Mutations in *secDF* result in decreased digestion of chitin (43), which is consistent with the involvement of the Sec system in export of ChiA. T9SS mediated secretion across the outer membrane may involve cleavage of the CTD from ChiA. This C-terminal region was not detected by LC-MS/MS analysis of secreted ChiA, suggesting that it may have been removed from the major secreted products. Evidence of removal of T9SS CTDs by proteolytic processing during secretion was recently reported for proteins of *P. gingivalis*, *Tannerella forsythia*, *Parabacteroides distasonis*, *Prevotella intermedia*, and *Cytophaga hutchinsonii* (18, 55, 58, 63, 67). PG0026, also referred to as PorU, was required for removal of the CTDs

from secreted proteins of *P. gingivalis* (18). *F. johnsoniae* has an ortholog of PorU that may perform a similar function. In addition to removal of the amino- and carboxy-terminal regions, *F. johnsoniae* ChiA may have had another processing event involving proteolysis between the two GH18 domains, resulting in two major soluble products each predicted to have chitinase activity. We do not know whether this processing event is important for the functioning of ChiA or whether it is the result of nonspecific digestion by one of the many proteases produced by *F. johnsoniae* (37). $ChiA_{GH18N}$ is similar in sequence to *Bacillus circulans* ChiA1 (65), and $ChiA_{GH18C}$ is similar in sequence to *B. circulans* ChiD (64) (see Fig. S6 and Fig. S7 in the supplemental material of (68)). The two GH18 domains of *F. johnsoniae* ChiA exhibit little similarity to each other, but each has the signature active site sequence (DXXDXDXE) that is characteristic of GH18 chitinases (19). *B. circulans chiA1* and *chiD* are adjacent on the genome, and the protein products presumably work together to digest chitin (64). Additional experiments are needed to determine the exact functions of *F. johnsoniae* $ChiA_{GH18N}$ and $ChiA_{GH18C}$ and the synergy, if any, that they exhibit.

In addition to their catalytic domains, many bacterial chitinases have carbohydrate-binding modules (CBMs) belonging to families 5 or 12 (19). Examination of proteins encoded by the *F. johnsoniae* genome revealed the complete absence of such domains, as presented in the Carbohydrate Active enZYmes (CAZY) database (http://www.cazy.org/) (5, 10). ChiA itself does not harbor a recognizable CBM of any family. ChiA may have novel CBMs or may rely on its catalytic domains to interact with chitin.

ChiA is required for *F. johnsoniae* chitin digestion, but further experiments are needed to determine if the four other predicted chitinases (37) have roles in this process. Synergistic interactions between multiple chitinases may be needed to efficiently digest crystalline chitin in nature. Such synergy has been demonstrated for the chitinases of other bacteria (8). Variations in organization of the polymer strands in the α-, β-, and γ-forms of crystalline chitin, variation in the degree of acetylation, and variations regarding the components complexed with chitin (proteins, polysaccharides, inorganic materials) (4, 19) may mean that no single enzyme or set of enzymes is ideally suited to efficiently digest all forms of chitin. Additional experiments will be needed to determine the entire complement of chitinolytic enzymes that allows optimal digestion and utilization of different forms of chitin by *F. johnsoniae* cells.

Chitin is one of the most abundant biopolymers produced on earth and is a common component of organisms in soil, freshwater and marine environments (22, 26, 39, 46). Bacteria of the phylum *Bacteroidetes* are important and sometimes dominant members of the chitinolytic communities in these environments (25). Members of the phylum *Bacteroidetes* are known to use novel strategies to utilize polysaccharides (51), and an improved understanding of the mechanisms used by *F. johnsoniae* and related bacteria to digest chitin may enhance our understanding of the turnover of this important biopolymer in nature. Such studies may also have more targeted practical value. For example, *F. johnsoniae* and closely related bacteria are common in the rhizosphere (23, 24, 27, 28, 33, 45), and have been linked to enhanced disease resistance of plants (28, 52). Chitinases released by these bacteria may contribute to this resistance because of

their activities against fungal or insect pests. The chitin modifying enzymes may also be useful for the production of chitooligosaccharides and other pharmaceutical products (15).

The results presented in this paper identify the major extracellular chitinase, ChiA, of *F. johnsoniae* and characterize its secretion by the T9SS. The motility adhesins SprB and RemA are also known to be secreted by the *F. johnsoniae* T9SS. Unlike SprB and RemA, ChiA is not attached to the cell surface after secretion, but instead is released in soluble form. Further study is needed to determine what features of the proteins result in anchoring on the cell surface or release in soluble form. The results of such studies could have broad implications. Analysis of the *F. johnsoniae* genome suggests that many proteins are secreted by the T9SS, and these are likely to undergo similar CTD recognition and processing events. Moreover, T9SSs appear to be common in the large and diverse phylum *Bacteroidetes* (38, 63), and an understanding of the events occurring during secretion of cell-surface and extracellular proteins of these bacteria will likely be of both practical and fundamental significance.

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Chapter 3. *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system.

This chapter is a modified version of a paper published in Journal of Bacteriology (47) that includes some of the online supplemental materials of the published paper integrated into it.

Abstract

Flavobacterium johnsoniae exhibits gliding motility and digests many polysaccharides, including chitin. A novel protein secretion system, the type IX secretion system (T9SS), is required for gliding and chitin utilization. The T9SS secretes the cell-surface motility adhesins SprB and RemA, and the chitinase ChiA. Proteins involved in secretion by the T9SS include GldK, GldL, GldM, GldN, SprA, SprE, and SprT. *Porphyromonas gingivalis* has orthologs for each of these that are required for secretion of gingipain protease virulence factors by its T9SS. *P. gingivalis porU* and *porV* have also been linked to T9SS-mediated secretion and *F. johnsoniae* has orthologs of these. Mutations in *F. johnsoniae porU* and *porV* were constructed to determine if they function in secretion. Cells of a *porV* deletion mutant were deficient in chitin utilization and failed to secrete ChiA. They were also deficient in secretion of the motility adhesin RemA, but retained the ability to secrete SprB. SprB is involved in gliding motility and is needed for formation of spreading colonies on agar, and the *porV* mutant exhibited gliding motility and formed spreading colonies. However the *porV* mutant was partially deficient in attachment to glass, apparently because of the absence of RemA and other adhesins on the cell surface. The *porV* mutant also appeared to be deficient in secretion of numerous

other proteins that have carboxy-terminal domains associated with targeting to the T9SS. PorU was not required for secretion of ChiA, RemA, or SprB, indicating that it does not play an essential role in the *F. johnsoniae* T9SS.

Introduction

Cells of *Flavobacterium johnsoniae,* and of many members of the phylum *Bacteroidetes*, crawl rapidly over surfaces by a process known as gliding motility (18). *F. johnsoniae* gliding involves the rapid movement of the adhesins SprB and RemA along the cell surface (23, 24, 41). These proteins are secreted across the outer membrane by a novel protein secretion system originally called the Por secretion system, and more recently referred to as the type IX secretion system (T9SS) (35, 40). Motility proteins are not the only cargo for the *F. johnsoniae* T9SS. It is also required for secretion of the soluble extracellular chitinase ChiA (15), and 51 other *F. johnsonaie* proteins are predicted to use this secretion system (40). Many proteins secreted by T9SSs are very large. ChiA, RemA, and SprB, for example, are 166, 152, and 669 kDa respectively (15, 24, 41). The mechanism that allows efficient secretion of such large proteins by the T9SS is not known. T9SSs are found in many members of the phylum *Bacteroidetes*, and they are apparently limited to this phylum (22, 42). The proteins required for T9SS-mediated secretion are not similar in sequence to proteins of other bacterial secretion systems (4, 22, 35). Although the T9SS was only recently discovered, it has already been associated with motility (31), virulence (35), chitin and cellulose digestion (15, 46), and colonization of and protection of plants from pathogens (16).

T9SSs were originally identified in *F. johnsoniae* and in the oral pathogen *Porphyromonas gingivalis* (31, 35). *P. gingivalis* secretes virulence factors such as gingipain proteases and adhesins using its T9SS. Proteins secreted by T9SSs have cleavable N-terminal signal peptides and are apparently exported across the cytoplasmic membrane via the Sec system (37, 42). They also have conserved carboxy-terminal domains (CTDs) of approximately 60 to 100 amino acids that target them for secretion across the outer membrane by the T9SS (15, 26, 37, 40, 42). The CTDs appear to be proteolytically cleaved during or after secretion across the outer membrane (8, 42). The CTDs are necessary and sufficient for secretion by the T9SS. *P. gingivalis* HBP35 and *F. johnsoniae* ChiA lacking their CTDs are not secreted, and heterologous fusion proteins carrying the HBP35 and ChiA CTDs are efficiently secreted (15, 38). Many T9SS CTDs of *F. johnsoniae* and *P. gingivalis* belong to TIGRFAM protein domain family TIGR04183 (22, 37, 40). There appears to be considerable diversity in T9SS CTDs, and not all fall within the boundaries of TIGR04183. *F. johnsoniae* SprB, for example, requires the T9SS for secretion but its carboxy-terminal region exhibits no similarity to TIGR04183 family members, but rather belongs to the unrelated domain family TIGR04131. Eleven other *F. johnsoniae* proteins have TIGR04131-type CTDs, as do numerous proteins from other species belonging to the phylum *Bacteroidetes* that have T9SSs. We have speculated that these TIGR04131-type CTDs target proteins for secretion by the T9SS (15, 22), but with the exception of SprB, T9SS-mediated secretion of these proteins has not been experimentally demonstrated in any organism.

Proteins required for secretion by the *F. johnsoniae* T9SS include GldK, GldL, GldM, and GldN or its paralog GldO (31, 40). SprA, SprE, and SprT also have important roles in T9SS-mediated secretion and cells with mutations in the genes encoding these proteins are severely but incompletely deficient in secretion (32, 35, 40). The *P. gingivalis* T9SS has orthologs for GldK, GldL, GldM, GldN, SprA, SprE, and SprT, and these are required for secretion (33, 35, 36). *P. gingivalis* PorP is also required for secretion. Unlike *P. gingivalis*, *F. johnsoniae* has multiple genes that exhibit similarity to *porP*. One of these, *sprF*, is required for secretion of SprB but is not needed for secretion of other proteins by its T9SS (29). The *F. johnsoniae* genome is predicted to encode ten PorP-like proteins in addition to SprF, and each of these may facilitate secretion of specific cargo proteins.

Five additional *P. gingivalis* proteins PorQ, PorU, PorV, PorX, and PorY are linked to T9SS function (7, 8, 12, 35). Mutations in *P. gingivalis porQ*, *porX* and *porY* result in partial defects in T9SS-mediated secretion. The function of PorQ is not known, but PorX and PorY are thought to form a two-component regulatory system that controls expression of the T9SS genes (35). The related *F. johnsoniae* proteins do not appear to play similar roles since deletion of the *F. johnsoniae* orthologs of *porQ*, *porX*, and *porY* has no effect on secretion of SprB, RemA or ChiA (39). The functions of *F. johnsoniae* PorU and PorV in secretion have not previously been studied. *P. gingivalis* PorU is thought to function as the peptidase that removes the CTDs of secreted proteins (8). *P. gingivalis* PorV is required for secretion of proteins targeted to the T9SS, including the gingipain proteases RgpA, RgpB and Kgp (11, 12). PorV, which has also been called

LptO, is required for the partial *O*-deacylation of lipopolysaccharide (7). PorV may have deacylation activity or it may be required for secretion of a deacylase. *F. johnsoniae* has orthologs of *porU* and *porV* but their functions have not been determined. In this study we constructed and examined *F. johnsoniae* mutants to determine the roles of PorU and PorV in secretion. Deletion of *porU* had little effect on secretion indicating that it was not essential for *F. johnsoniae* T9SS function. In contrast, PorV was required for the secretion of many but not all proteins targeted to the T9SS. Deletion of *porV* eliminated secretion of RemA and ChiA but had no effect on secretion of SprB.

Materials and Methods

Bacterial and bacteriophage strains, plasmids, and growth conditions. *F. johnsoniae* ATCC 17061^T strain UW101 was the wild-type strain used in this study (5, 19, 21). The streptomycin resistant *rpsL* mutant of UW101 (CJ1827) was used to construct deletion mutants (30). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30^{o}C (20). To observe colony spreading cells were grown on PY2 agar at 25^{o}C (1), and to observe motility of individual cells they were grown in motility medium (MM) at 25^{o}C (17). *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C (34). Strains and plasmids used in this study are listed in Table 4 and primers are listed in Table 5. Antibiotics were used at the following concentrations when needed: ampicillin, 100 μg/ml; cefoxitin, 100 μg/ml; erythromycin, 100 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 20 μg/ml.

Construction and complementation of *porV* and *porU* mutants. Unmarked deletions were generated as previously described (30). To delete *porV*, a 2,442 bp fragment upstream of *porV* was amplified using Phusion DNA polymerase (New England Biolabs, Ipswich, MA), and primers 1203 (engineered BamHI site) and 1204 (engineered SalI site). The amplified fragment was digested with BamHI and SalI and cloned into pRR51 that had been digested by the same enzymes, generating pSSK20. A 2,271 bp fragment downstream of *porV* was amplified using primers 1201 (engineered SalI site) and 1202 (engineered SphI site). This fragment was introduced into pSSK20 that was digested with SalI and SphI, to generate pSSK22. pSSK22 was introduced into the *F. johnsoniae* strain CJ1827 by triparental conjugation. Colonies containing the plasmid integrated into the chromosome were obtained by selecting for erythromycin resistance, and *porV* deletion mutants that had lost the integrated plasmid were obtained by selecting for streptomycin resistance and confirmed by PCR essentially as previously described (30).

porU deletion mutants were constructed in a similar manner. A 2,282 bp fragment upstream of *porU* was amplified using primers 1207 (engineered BamHI site) and 1208 (engineered SalI site). The amplified fragment was digested with BamHI and SalI and cloned into pRR51 that had been digested with the same enzymes, generating pSSK21. A 2,305 bp fragment downstream of *porU* was amplified using primers 1205 (engineered SalI site) and 1206 (engineered SphI site). This fragment was ligated with pSSK21 that had been digested with SalI and SphI, generating pSSK23. pSSK23 was used to construct the *porU* deletion strain as described above. A strain with a polar insertion mutation in

porU (CJ1818) was also constructed. For this purpose a 1050-bp fragment internal to *porU* was amplified using primers 948 (engineered BamHI site) and 949 (engineered SalI site). This fragment was cloned into pLYL03 which had been cut with BamHI and SalI to generate pSSK01. pSSK01 was introduced into wild-type *F. johnsoniae* UW101 by conjugation, and selection for erythromycin resistance resulted in integration of the plasmid into the genome and disruption of *porU*. The insertion was confirmed by PCR using primers 737 and 948.

For complementation of the *porV* mutant, a 1,516 bp region spanning *porV* was amplified using primers 972 (engineered SphI site) and 973 (engineered KpnI site) and introduced into pCP29, to generate pSSK03. Similarly, for complementation of *porU* mutants, a 4,309 bp region spanning *porU* was amplified using primers 988 (engineered XbaI site) and 989 (engineered BamHI site) and introduced into pCP23, to generate pSSK04.

Determination of chitinase activity. Chitin utilization on agar was observed as previously described using colloidal chitin prepared from crab shells (19, 28, 31). Chitinase activities in cell-free culture fluid (spent media), intact cells, and cell extracts were measured as previously described (31) using the synthetic substrates 4-methylumbelliferyl β-D-N,N'-diacetyl-chitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl β-D-N, N', N''-diacetyl-chitotrioside [4-MU-$(GlcNAc)_3$] (Sigma-Aldrich, St. Louis, MO) except that activities were measured for 30 min. Activities in the spent media (secreted chitinase), intact cells, and cell extracts were indicated as pmol 4-methylumbelliferone released during the 30 min per μg total protein in the original cell

suspension. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA).

Western blot analyses. *F. johnsoniae* cells were grown to late-exponential phase in MM at 25°C with shaking. Cells were pelleted by centrifugation at 22,000 x g for 15 min, and the spent culture fluid was filtered using 0.22 μm pore-size polyvinylidene difluoride filters (Thermo Fisher Scientific). For whole-cell samples, the cells were suspended in the original culture volume of phosphate buffered saline (PBS) 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 9 min. Proteins were separated by SDS-PAGE, and Western blot analyses were performed as previously described (31) using affinity purified antibody against ChiA (1:5,000 dilution). Equal amounts of each sample based on the starting material were loaded in each lane. For cell extracts this corresponded to 15 μg protein whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 15 μg cell protein before the cells were removed. For detection of mCherry by Western blot, commercially available antibodies against mCherry (0.5 mg per ml; BioVision Incorporated, Milpitas, CA) were used at dilution of 1:5,000.

Myc-tagged RemA was detected as previously described (41). *F. johnsoniae* cells were grown to late-exponential phase in CYE at 25°C with shaking. Whole cells and spent culture fluid were prepared for SDS-PAGE and Western blots were performed as described above for ChiA, except that antisera against the *c-myc* epitope (1:10,000 dilution; AbCam, Cambridge, MA) were used.

Analysis for secretion of cell-surface SprB and Myc-tagged RemA. Secretion of SprB was examined essentially as previously described (32, 40). Briefly, cells were grown overnight in MM without shaking at 25°C. Purified anti-SprB (1 μl of a 1:10 dilution of a 300-mg/liter stock), 0.5- μm-diameter protein G-coated polystyrene spheres (1 μl of a 0.1% stock preparation; Spherotech, Inc., Libertyville, IL), and bovine serum albumin (1 μl of a 1% solution) were added to 7 μl of cells (approximately 5 x 10^8 cells per ml) in MM. The cells were introduced into a tunnel slide (41) and examined by phase contrast microscopy at 25°C. Samples were examined 2 min after spotting, and images were recorded for 30 s to determine the percentage of cells that had anti-SprB-coated spheres attached to them. Surface-localized Myc-tagged RemA was detected similarly, except that antisera against the Myc tag (EQKLISEEDL; AbCam) was used.

Cell aggregation studies. The effect of RemA on aggregation was determined as previously described (41). Cultures (10 ml) were grown overnight in test tubes at 25°C in EC medium (5) with appropriate antibiotics on a platform shaker set at 120 rpm. Cultures were examined for turbidity and for accumulation of cell aggregates at the bottom of the tubes.

Measurement of bacteriophage sensitivity. *F. johnsoniae* bacteriophages used in this study were ϕCj1, ϕCj13, ϕCj23, ϕCj28, ϕCj29, ϕCj42, ϕCj48 and ϕCj54 (5, 27, 43). Sensitivity to phages was determined as previously described (31). Briefly, 3 μl of phage lysate (approximately 10^9 PFU/ml) was spotted onto lawns of cells in CYE overlay agar. The plates were incubated for 24 h at 25°C. A quantitative assay was also used to measure sensitivity to bacteriophages. This involved serial dilution of phage lysates in 10

mM Tris-8 mM $MgSO_4$ (TM buffer, pH 7.5) and determination of the number of plaque forming units. Wild-type *F. johnsoniae* cells were cultured overnight in CYE at 30°C. 100 µl of phage dilutions were added to 200 µl of cells to allow adsorption. Four ml of overlay agar at 42°C was added and the samples were briefly mixed and poured onto CYE agar plates. After solidification of the overlay agar the plates were incubated for 24 h at 25°C and plaques were counted.

Microscopic observations of cell attachment. Wild-type and mutant cells of *F. johnsoniae* were examined for attachment to glass as previously described (40). Cells were grown overnight in MM without shaking at 25°C and harvested at an OD_{600} of 0.18. Cells (2.5 µl) were added to a Petroff-Hausser counting chamber, covered with a glass coverslip, and allowed to incubate for 2 min at 25°C. The number of cells attached to 9 randomly selected 0.03 mm^2 regions of the glass coverslip was determined.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Cells of *F. johnsoniae* wild type (CJ1827), Δ*porU* mutant CJ2116, Δ*porV* mutant CJ2130 and Δ(*gldN gldO*) mutant CJ2090 (referred to hereafter as Δ*gldNO*) and cells of complemented mutants were grown in MM at 25°C with shaking until cells reached an OD_{600} of 0.7 (late exponential phase of growth). Cells were pelleted by centrifugation at 22,000 x g for 15 min and the spent culture medium was filtered (0.22 µm polyvinylidene difluoride filters) to remove residual cells. Membrane vesicles and insoluble cell debris were removed from the cell-free spent medium by centrifugation at 100,000 x g for 1 h. This process was repeated once to ensure complete removal of insoluble material, the cell-free spent medium was concentrated 1000 fold using Amicon concentrators

(Millipore, Darmstadt, Germany), and proteins were separated by SDS-PAGE and detected using the BioRad (Hercules, CA) silver stain kit. Enzymatic in-gel digestion was performed at the University of WI-Madison Mass Spectrometry Facility as outlined on the website (https://www.biotech.wisc.edu/services/massspec).

Peptides were analyzed by nano LC-MS/MS using the Agilent 1100 nanoflow system (Agilent, Palo Alto, CA) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Bremen, Germany) equipped with an EASY-Spray™ electrospray source. Chromatography of peptides prior to mass spectral analysis was accomplished using a capillary emitter column (PepMap C18, 3 µM, 100Å, 150 x 0.075 mm, Thermo Fisher Scientific) onto which extracted peptides were automatically loaded. NanoHPLC system delivered solvents were as follows: (A) 0.1% (v/v) formic acid in water, and (B) 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid. Sample loading was performed at 0.60 µL/min and peptide elution directly into the nano-electrospray was performed at 0.3 µl/min using a gradient from 0% (v/v) B to 40% (v/v) B over 20 min followed by a gradient from 40% (v/v) B to 100% (v/v) B over 5 min. As peptides eluted from the HPLC-column/electrospray source survey MS scans were acquired in the Orbitrap with a resolution of 100,000. The 5 most intense peptides per scan were fragmented and detected in the ion trap over the mass range 300 to 2000 m/z. Redundancy was limited by dynamic exclusion. Raw MS/MS data were converted to mgf file format and used to search against a *F. johnsoniae* protein database (5,507 protein entries) concatenated with a list of common lab contaminants. Peptide mass tolerance was set at 20 ppm and fragment mass at 0.8 Da. Scaffold version 4.3.2 (Proteome

Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Peptide Prophet algorithm (13) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (25).

Analysis of starch utilization. Wild-type and mutant cells of *F. johnsoniae* were examined for starch hydrolysis using a plate assay. Cells were streaked on CYE agar supplemented with 0.25% starch and incubated overnight at 25°C. Starch was detected by flooding the agar with a solution of 1% KI and 1% iodine.

Sequence analyses. Sequences were analyzed with MacVector software (Cary, NC) and comparisons to database sequences were made using the BLAST algorithm (2). Predictions regarding cellular localization were made using PSORTb (45) TMpredict (9), and CELLO (44).

Table 4. Strains and plasmids used in this study.

Strain or plasmid	Genotype and/ or description	Source or reference
F. johnsoniae strains		
UW101 (ATCC 17061)	Wild type	(19, 21)
CJ1631A	Δ(*gldN gldO*) in *F. johnsoniae* UW101	(31)
CJ1808	*chiA* disruption mutant; (Em^r)	(15)
CJ1818	*porU* disruption mutant; (Em^r)	This study
CJ1827	*rpsL2*; (Sm^r) "wild-type" strain for construction of all deletion mutants except CJ1631A	(30)
CJ1922	*rpsL2* Δ *sprB*; (Sm^r)	(30)
CJ1984	*rpsL2* Δ *remA*; (Sm^r)	(41)
CJ1985	*rpsL2* Δ *sprB* Δ *remA*; (Sm^r)	(41)
CJ2082	*rpsL2* Δ *Fjoh_0288*; (Sm^r)	This study
CJ2083	*rpsL2 remA*::*myc*-tag-1; (Sm^r)	(41)
CJ2089	*rpsL2* Δ (*gldN gldO*) *remA*::*myc*-tag-1; (Sm^r)	(41)
CJ2090	*rpsL2* Δ (*gldN gldO*); (Sm^r)	(41)
CJ2116	*rpsL2* Δ *porU*; (Sm^r)	This study
CJ2130	*rpsL2* Δ *porV*; (Sm^r)	This study
CJ2323	*rpsL2* Δ *porV remA*::*myc*-tag-1; (Sm^r)	This study
CJ2445	*rpsL2* Δ *porV* Δ *sprB*; (Sm^r)	This study
CJ2446	*rpsL2* Δ *porV* Δ *Fjoh_0288*; (Sm^r)	This study
Plasmids		
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; (Ap^r (Tc^r)	(1)
pCP29	*E. coli-F. johnsoniae* shuttle plasmid; (Ap^r (Cf^r Em^r)	(14)
pRR51	*rpsL* containing suicide vector; (Ap^r (Em^r)	(30)
pRR39	pCP23 carrying *remA*; (Ap^r (Tc^r)	(41)
pSSK01	1050-bp fragment of *porU* in pLYL03 for constructing *porU* disruption mutant CJ1818*;* (Ap^r (Em^r)	This study
pSSK03	1,516-bp SphI-KpnI fragment spanning *porV* amplified with primer 972 and 973 and inserted into pCP29; (Ap^r (Cf^r Em^r)	This study
pSSK04	4,309-bp XbaI-BamHI fragment spanning *porU* amplified with primer 988 and 989 and inserted into pCP23; (Ap^r (Tc^r)	This study
pSSK14	2332-bp XbaI-SalI fragment downstream of	This study

	Fjoh_0288 amplified with primers 1104 and 1105 and inserted into pSSK16; Ap^r (Em^r)	
pSSK16	2,301-bp BamHI-XbaI fragment upstream of *Fjoh_0288* amplified with primers 1102 and 1103 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK20	2,442-bp BamHI-SalI region upstream of *porV* amplified with primers 1203 and 1204 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK21	2,282-bp BamHI-SalI region upstream of *porU* amplified with primers 1207 and 1208 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK22	2,271-bp SalI-SphI region downstream of *porV* amplified with primers 1201 and 1202 and inserted into pSSK20; Ap^r (Em^r)	This study
pSSK23	2,305-bp SalI-SphI region downstream of *porU* amplified with primers 1205 and 1206 and inserted into pSSK21; Ap^r (Em^r)	This study
pTB79	pCP23 carrying *gldN*; Ap^r (Tc^r)	(3)

[a]Antibiotic resistance phenotypes are as follows: ampicillin, Ap^r; cefoxitin, Cf^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 5. Primers used in this study

Primers	Sequence and Description
737	5'-AGGCACCCCAGGCTTTACACT-3'; Reverse primer binding downstream of multiple cloning site of pLYL03.
948	5’- GCTAGGGATCCTCTTGCAGGCTCAACTACCGGAAT – 3’; Forward primer to construct pSSK01; BamHI site underlined
949	5’- GCTAGGTCGACCTTTCGCTTGCCAAACCGTCTTCA – 3’; Reverse primer to construct pSSK01; SalI site underlined
972	5’ - GCTAGGCATGC TACGTGGGACGGAAGGGATGATTT – 3’; Forward primer used for constructing pSSK03; SphI site underlined
973	5’ - GCTAGGGTACCCCTGAGAAAGCTCATTGATGGTGTCG – 3’; Reverse primer to construct pSSK03; KpnI site underlined
988	5’ - GCTAGTCTAGAGCCGTGCCCAACATCAATACCATT – 3’; Forward primer used for constructing pSSK04; XbaI site underlined
989	5’ - GCTAGGGATCCAGTTGCAACACCCTGGTCTCCTAA – 3’; Reverse primer to construct pSSK04; BamHI site underlined
1102	5’ - GCTAGGGATCCGGCAAAACTTCGAACTTCGTA – 3’; Forward primer to amplify upstream region of *Fjoh_0288* to construct pSSK16; BamHI site underlined
1103	5’ - GCTAGTCTAGAGGCATCGACACCAATATTCAT – 3’; Reverse primer to amplify upstream region of *Fjoh_0288* to construct pSSK16; XbaI site underlined
1104	5’ - GCTAGTCTAGAGCTTTGTACTCAAATATTTTTTCGATA – 3’; Forward primer to amplify downstream region of *Fjoh_0288* to construct pSSK14; XbaI site underlined
1105	5’ - GCTAGGTCGACCAGTTTTATCGCCTGAGCTTC – 3’; Reverse primer to amplify downstream region of *Fjoh_0288* to construct pSSK14; SalI site underlined
1201	5’- GCTAGGTCGACTACCACGAAAGCCCTATGAAAGGA - 3’; Forward primer to amplify downstream region of *porV* to construct pSSK22; SalI site underlined
1202	5’- GCTAGGCATGCCTCCTTCAGCTACTGTATCACCAAC-3’; Reverse primer to amplify downstream region of *porV* to construct pSSK22; SphI site underlined
1203	5’ - GCTAGGGATCCTGCCATTGATGCGTCTGACTAC – 3’; Forward primer to amplify upstream region of *porV* to construct pSSK20; BamHI site underlined
1204	5’- GCTAGGTCGACAGGGCGCTCAATATCTTGGGCTTT - 3’; Reverse primer to amplify upstream region of *porV* to construct pSSK20; SalI site underlined
1205	5’- GCTAGGTCGACGATTTTGGCGACCGGATTGGAAAAGGA - 3’; Forward primer to amplify downstream region of *porU* to construct pSSK23;

	SalI site underlined
1206	5’- GCTAGGCATGCCCATACCAATTGGCTGAACGTGGT – 3; Reverse primer to amplify downstream region of *porU* to construct pSSK23; SphI site underlined
1207	5’ - GCTAGGGATCCCGACAGTTCCTGCAGTGTTTCTAAGC-3’; Forward primer to amplify upstream region of *porU* to construct pSSK21; BamHI site underlined
1208	5’- GCTAGGTCGACAAAGTATGCGATCAGGGCTTGTTTCAT - 3’; Reverse primer to amplify upstream region of *porU* to construct pSSK21; SalI site underlined

Results

F. johnsoniae porU* and *porV. Analysis of the *F. johnsoniae* genome revealed orthologs of the *P. gingivalis* T9SS genes *porU* and *porV*. *F. johnsoniae* PorU exhibits 26% identity to *P. gingivalis* PorU over 1170 amino acids, and *F. johnsoniae* PorV exhibits 44% identity to *P. gingivalis* PorV over 385 amino acids. *F. johnsoniae* PorU and PorV have predicted N-terminal signal peptides, and both proteins are predicted by PSORTb and CELLO analyses to reside in the outer membrane. *F. johnsoniae porU* and *porV* are located adjacent to each other on the genome (Figure 21) as are *P. gingivalis porU* and *porV* (8, 12, 35). A putative promoter (TTG-N18-TANNTTTG) which matches the *Bacteroidetes* housekeeping promoter consensus (6), lies between *porU* and *porV*, and several possible promoter sequences lie upstream of *porU*, suggesting that these genes may be transcribed separately. A predicted terminator begins 25 bp downstream of the *porV* stop codon (AATCCAAATTTCTGCATTTTAGAAATTTGGATTTTTTTT).

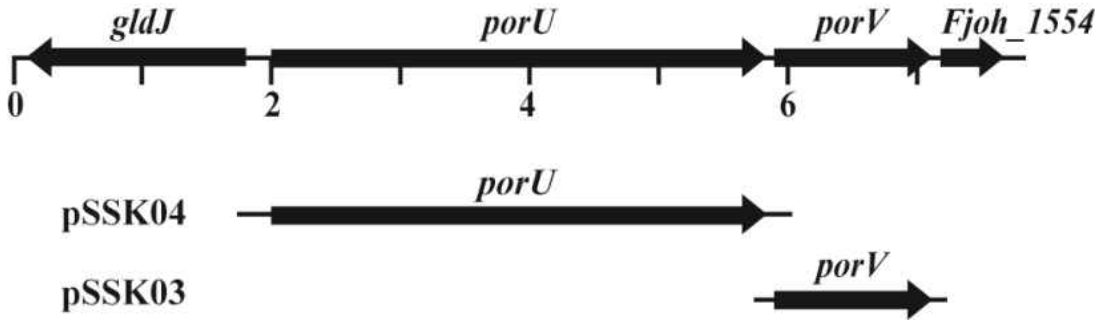

Figure 21. Map of the region spanning *porU* and *porV*. Numbers below the map refer to kilobase pairs of sequence. The regions of DNA carried by complementation plasmids used in this study are indicated beneath the map.

***porV* mutant cells are defective for chitin utilization and for secretion of the chitinase ChiA**. Cells of *F. johnsoniae* T9SS mutants fail to secrete the soluble extracellular chitinase ChiA and are thus defective in chitin utilization (15, 31, 32, 35, 40). The *F. johnsoniae porU* and *porV* genes were deleted to determine whether they have roles in T9SS function. Deletion of *porV* resulted in loss of ability of cells to digest chitin (Figure 22A). Complementation with pSSK03, which carries *porV*, restored this ability. In contrast deletion of *porU* had little effect on chitin digestion. Similarly, insertional disruption of *porU* using the suicide vector pLYL03 also had little effect on chitin utilization. pLYL03 insertions result in polar mutations that prevent expression of downstream genes of an operon (10). The ability of the *porU* disruption mutant to digest chitin (Figure 22A) supports the suggestion made above that *porU* and *porV* are transcribed separately. Chitinase activity was also examined in cell-free spent culture fluid, intact cells, and cell extracts, using a quantitative assay. Chitinase activity was detected in the cell-free spent culture fluid from wild-type cells and from cells of the Δ*porU* mutant, but was not detected in spent culture fluid from cells of the Δ*porV* mutant (Figure 22B), suggesting that PorV was required for secretion of the major soluble extracellular chitinase ChiA. Cell-associated chitinase levels (intact cells and cell extracts) were similar for wild-type and mutant cells. Genome analysis predicted the existence of four chitinases in addition to ChiA that may contribute these activities (21).

Western blot analyses were used to examine the presence of ChiA protein in cells and in the spent culture fluid of wild-type and mutant strains. Wild-type cells secreted ChiA into the culture fluid with little if any ChiA detected in cell extracts (Figure 23). In

contrast cells of the *porV* mutant failed to secrete ChiA and instead accumulated small amounts of the larger proChiA inside of cells. Complementation of the *porV* mutant with pSSK03 resulted in secretion of ChiA into the culture medium and failure to accumulate proChiA inside of cells, similar to wild-type cells. The effect of deletion of *porV* on ChiA secretion was nearly identical to that observed for deletion of the region spanning the T9SS genes *gldN* and *gldO* (Figure. 23). Deletion of other T9SS genes also resulted in failure to secrete ChiA (15). In contrast to the results with the *porV* mutant, cells of the *porU* deletion mutant behaved similar to wild-type cells. The *porU* mutant strain secreted ChiA and failed to accumulate it inside of cells. Secreted ChiA from wild-type and *porU* mutant cells migrated at the same size, suggesting that in *F. johnsoniae* PorU is not required for secretion or processing of ChiA.

Proteins secreted by T9SSs typically have conserved CTDs involved in this process (26, 40, 42). The C-terminal 105 amino acids of ChiA are necessary and sufficient for secretion by the T9SS since mCherry fused to the CTD of ChiA is efficiently secreted by wild-type cells but not by cells of T9SS mutants (15). Secretion of mCherry-CTD_{ChiA} by wild-type cells and by *porU* and *porV* mutant cells was examined. Wild-type cells and cells of the *porU* deletion mutant secreted mCherry-CTD_{ChiA} (Figure 24). The secreted protein corresponded to the size of mCherry, suggesting that the protein was processed, perhaps removing the CTD, during or after secretion. In contrast, cells of the Δ*porV* mutant and of the Δ*gldNO* mutant failed to secrete mCherry-CTD_{ChiA} and instead accumulated a small amount of a protein corresponding in size to mCherry-

CTD_{ChiA} in the cells. The results indicate that PorV is required for secretion of proteins carrying CTD_{ChiA}.

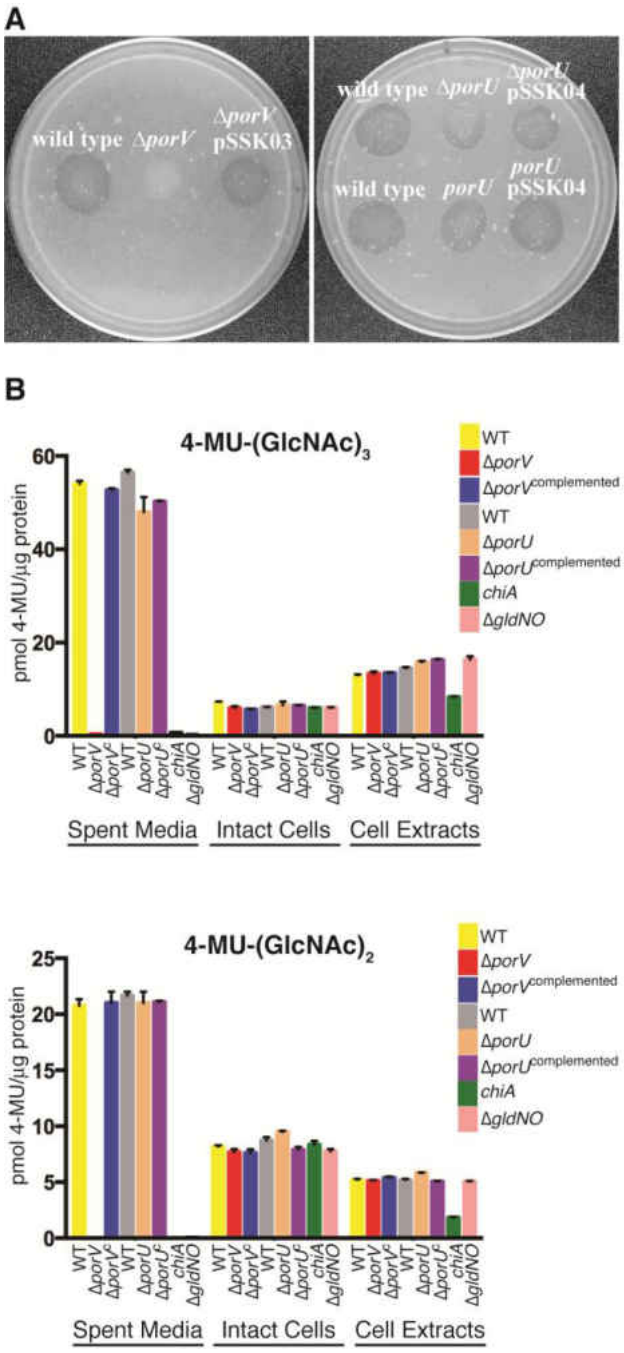

Figure 22. *porV* is required for chitin utilization. A) Chitin digestion on agar media. Approximately 10^6 cells of wild-type and mutant strains of *F. johnsoniae* were spotted on MYA-

chitin media (19) and incubated at 25°C for 2.5 d. Left panel, left to right: wild-type *F. johnsoniae* CJ1827; *porV* deletion mutant CJ2130; CJ2130 complemented with pSSK03 which carries *porV*. Right panel, top row left to right: wild-type *F. johnsoniae* CJ1827; *porU* deletion mutant CJ2116; CJ2116 with pSSK04 which carries *porU*. Right panel, bottom row left to right: wild-type *F. johnsoniae* UW101; *porU* disruption mutant CJ1818; CJ1818 with pSSK04 which carries *porU*. B) Chitinase activities of wild-type and mutant cells. Chitinase activities of spent media, intact cells, and cell extracts were determined using the synthetic substrates 4-MU-$(GlcNAc)_3$, and 4-MU-$(GlcNAc)_2$. Equal amounts of each sample, based on the protein content of the cell suspension, were incubated with 10 nmol of synthetic substrate for 30 min at 37°C, and the amount of 4-MU released was determined by measuring fluorescence emission at 460 nm following excitation at 360 nm. Yellow, wild-type *F. johnsoniae* UW101 carrying control vector pCP29. Red, *porV* deletion mutant CJ2130 carrying pCP29. Blue, CJ2130 complemented with pSSK03 which carries wild-type *porV*. Grey, wild-type *F. johnsoniae* UW101 carrying control vector pCP23. Tan, *porU* deletion mutant CJ2116 carrying pCP23. Purple, CJ2116 complemented with pSSK04 which carries wild-type *porU*. Green, *chiA* mutant CJ1808. Pink, *gldNO* deletion mutant CJ1631A.

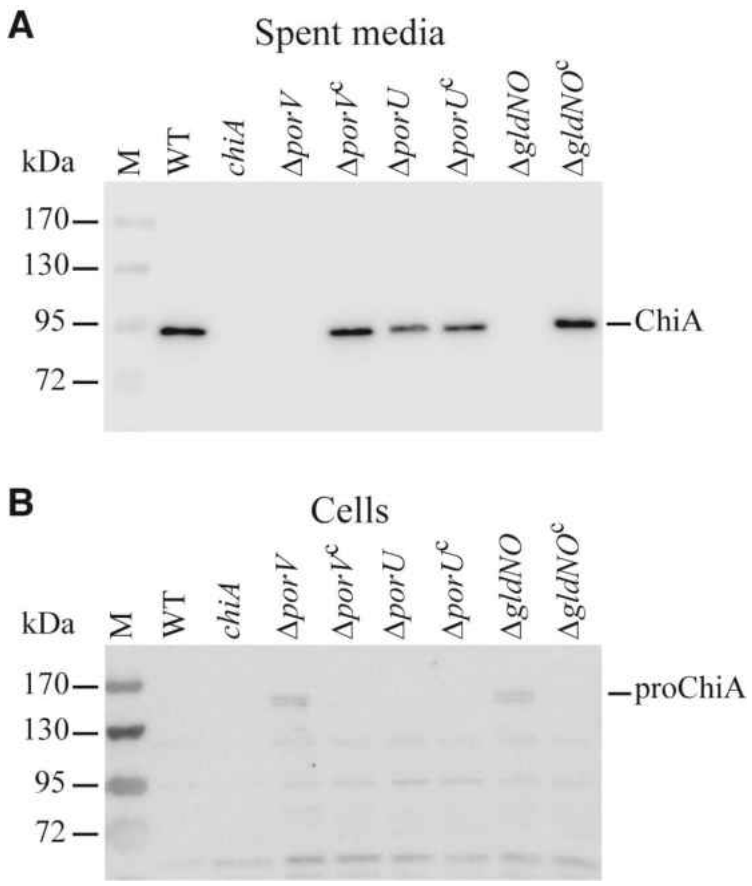

Figure 23. PorV is required for secretion of the soluble extracellular chitinase ChiA. Cell-free spent media (A) and cells (B) were examined for ChiA by SDS-PAGE followed by Western blot analysis using antiserum against recombinant ChiA. M, molecular weight markers. WT, wild-type *F. johnsoniae* CJ1827. *chiA*, *chiA* mutant CJ1808. Δ*porV*, *porV* deletion mutant CJ2130. Δ*porV*^{c}, CJ2130 complemented with pSSK03 which carries *porV*. Δ*porU*, *porU* deletion mutant CJ2116. Δ*porU*^{c}, CJ2116 complemented with pSSK04 which carries *porU*. Δ*gldNO*, *gldNO* deletion mutant CJ1631A. Δ*gldNO*^{c}, CJ1631A complemented with pTB79, which carries *gldN*. Samples loaded in panel B corresponded to 15 μg protein per lane, and samples loaded in panel A corresponded to the volume of spent medium that contained 15 μg cell protein before the cells were removed.

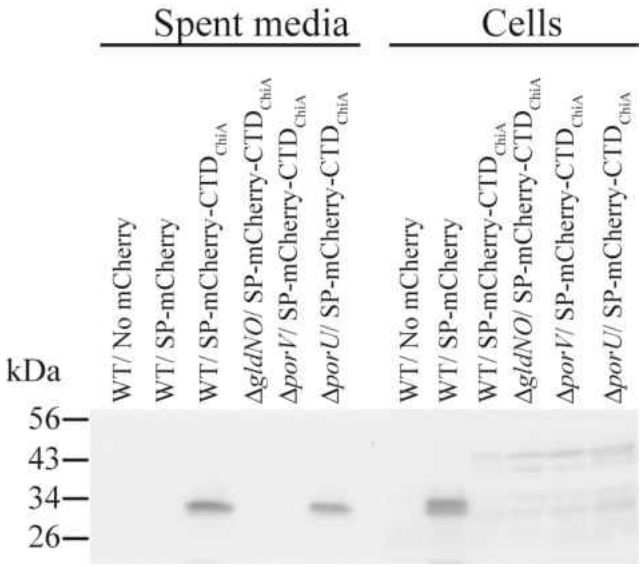

Figure 24. PorV is required for secretion of the heterologous fusion protein mCherry-CTD_{ChiA}. Cell-free spent media and whole cells were analyzed for cultures of wild-type (WT) cells carrying pSSK54 that expresses mCherry with the N-terminal signal peptide from ChiA (SP-mCherry), or carrying pSSK52 that expresses mCherry with the N-terminal signal peptide from ChiA and the 105 amino acid C-terminal region of ChiA (SP-mCherry-CTD_{ChiA}). Cells and spent media from cultures of the T9SS mutant CJ1631A (Δ*gldNO*), the *porV* deletion mutant CJ2130 (Δ*porV*), and the *porU* deletion mutant CJ2116 (Δ*porU*) each carrying pSSK52 were also analyzed. "No mCherry" indicates spent media or cells from *F. johnsoniae* carrying the control vector pCP23. Cell samples corresponded to 15 μg protein per lane and samples from spent media corresponded to the volume of spent media that contained 15 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE and mCherry was detected using antiserum against mCherry.

***porV* mutant cells fail to secrete RemA**. The cell-surface motility adhesin RemA is secreted by the T9SS (41). The ability of cells of the *porV* deletion mutant to secrete a Myc-tagged version of RemA was examined using antisera against the c-*myc* peptide. Wild-type and Δ*porV* mutant cells produced Myc-tagged RemA (Figure 25, right hand side), but whereas wild-type cells localized RemA on the cell surface, cells of the Δ*porV* mutant failed to do this (Table 6). Wild-type cells also secreted substantial amounts of RemA into the culture fluid (Figure. 25, left hand side) whereas cells of the *porV* mutant did not. Soluble secreted RemA from wild-type cells was present as fragments of 100 and 130 kDa, significantly smaller than cell-associated RemA. We do not know if these soluble fragments of RemA are functional, but they serve as additional evidence that PorV is required for secretion of RemA.

RemA is a cell surface galactose/rhamnose-binding lectin, and cells expressing this protein aggregate to form multi-cell clumps (41). This phenomenon is most easily observed when RemA is moderately overexpressed (approximately 10 fold) from plasmid. Wild-type and *porV* mutant cells expressing RemA were examined for the formation of large cell aggregates. Wild-type cells aggregated extensively, rapidly falling to the bottom of the culture fluid, whereas cells of the *porV* mutant remained dispersed (Figure 26), further indicating that *porV* mutant cells fail to secrete RemA to the cell surface.

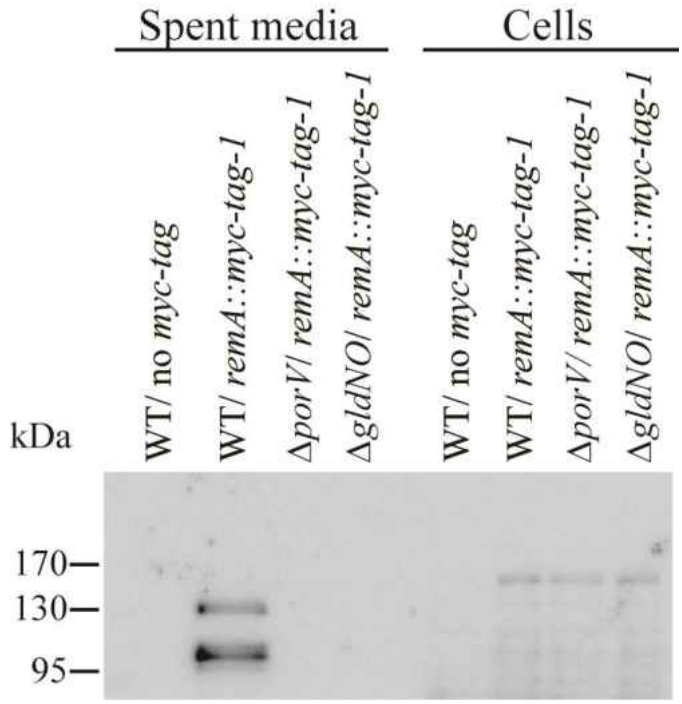

Figure 25. Deletion of *porV* disrupts secretion of RemA. Immunodetection of Myc-tagged RemA in spent media or in cells of wild-type or mutant *F. johnsoniae* strains. Cell-free spent media and whole cells were analyzed for cultures of wild type *F. johnsoniae* CJ1827 (WT, no *myc*-tag), CJ2083 (WT, *remA*::*myc*-tag-1), CJ2323 (Δ*porV remA*::myc-tag-1), and CJ2089 (Δ*gldNO*, *remA*::myc-tag-1). Cell samples corresponded to 20 μg protein per lane and samples from spent media corresponded to the volume of spent media that contained 20 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE and Myc-tagged RemA was detected using antiserum against the Myc-tag peptide.

Table 6. Deletion of *porV* disrupts secretion of Myc-tagged RemA.

Strain	Description	Antibody added	Avg (SD) % of cells with spheres attached[a]
CJ1827	Wild type, no *myc*-tag	Anti-Myc	0.0 (0.0)
CJ2083	Wild type, *remA*::*myc*-tag-1	No antibody	0.0 (0.0)
CJ2083	Wild type, *remA*::*myc*-tag-1	Anti-Myc	44.6 (3.3)
CJ2323	Δ*porV, remA*::*myc*-tag-1	Anti-Myc	0.0 (0.0)
CJ2323/pSSK03	Δ*porV, remA*::*myc*-tag-1, complemented with pSSK03 carrying *porV*	Anti-Myc	40.6 (3.0)

[a] Purified anti-Myc-tag 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 2 min at 25°C, and examined using a phase-contrast microscope. Images were recorded for 30 s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time. The numbers in parentheses are standard deviations calculated from three measurements.

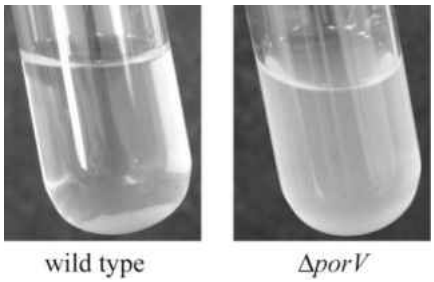

Figure 26. Effect of *porV* on RemA-mediated cell aggregation. Cells of CJ1827 (wild type) and CJ2130 (Δ*porV*) were incubated in EC medium for 16 h at 25°C. Both strains carried *remA*-expressing plasmid pRR39.

PorU and PorV are not required for secretion of the major motility adhesin SprB, or for gliding motility. The T9SS is required for secretion of the motility adhesin SprB to the cell-surface (31, 32, 35, 40). Antibodies against SprB were used to determine if SprB was present on the surface of Δ*porU* and Δ*porV* mutant cells. Latex spheres coated with antibodies against SprB readily attached to wild-type, Δ*porU*, and Δ*porV* mutant cells (Table 7). In contrast, they failed to attach to cells of the T9SS mutant CJ2090 (Δ*gldNO*), or to cells of the *sprB* deletion mutant CJ1922. These results indicate that although PorV is required for secretion of RemA and ChiA it is not required for secretion of SprB.

The presence of SprB on the cell surface is required for efficient cell movement over agar resulting in the formation of spreading colonies. Cells of *sprB* mutants, and cells of T9SS mutants that disrupt secretion of SprB, form nonspreading colonies (24, 31, 32, 35, 40). In contrast, cells of *porU* and *porV* mutants formed spreading colonies (Figure 27), consistent with the ability of these mutants to express and secrete SprB. As shown above *porV* mutant cells were deficient in secretion of the motility adhesin RemA. However, as previously reported (41), and as confirmed in this study (Figure 27), deletion of *remA* had no effect on movement of cells over agar. Although not required for movement over agar, RemA is thought to facilitate attachment to and movement over other types of surfaces (such as glass) that are coated with rhamnose- or galactose-containing polysaccharides produced by the cells (41).

Table 7. PorV and PorU are not required for localization of SprB to the cell surface.

Strain	Description	Antibody added	Avg (SD) % of cells with spheres attached[a]
CJ1827	Wild type	No antibody	0.0 (0.0)
CJ1827	Wild type	Anti-SprB	44.5 (3.54)
CJ1827/pCP29	Wild type with empty vector pCP29	Anti-SprB	43.5 (3.05)
CJ1827/pCP23	Wild type with empty vector pCP23	Anti-SprB	39.5 (3.48)
CJ1922	Δ*sprB*	Anti-SprB	0.0 (0.0)
CJ2090	Δ(*gldN gldO*)	Anti-SprB	0.0 (0.0)
CJ2130/pCP29	Δ*porV* with empty vector pCP29	Anti-SprB	30.4 (2.9)
CJ2130/pSSK03	Δ*porV* complemented with pSSK03 carrying *porV*	Anti-SprB	37.5 (1.88)
CJ2116/pCP23	Δ*porU* with empty vector pCP23	Anti-SprB	37.0 (2.17)
CJ2116/pSSK04	Δ*porU* complemented with pSSK04 carrying *porU*	Anti-SprB	35.8 (2.51)

[a] Purified anti-SprB 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 2 min at 25°C, and examined using a phase-contrast microscope. Images were recorded for 30 s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time. The numbers in parentheses are standard deviations calculated from three measurements.

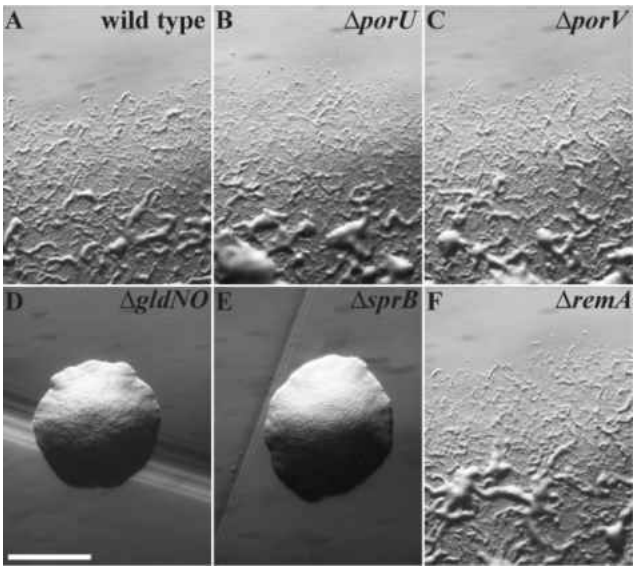

Figure 27. Photomicrographs of *F. johnsoniae* colonies. Colonies grown from single cells were incubated at 25°C on PY2 agar for 44 h. Photomicrographs were taken with a Photometrics Cool-SNAP$_{cf}^{2}$ camera mounted on an Olympus IMT-2 phase-contrast microscope. (A) Wild type *F. johnsoniae* CJ1827. (B) *porU* deletion mutant CJ2116. (C) *porV* deletion mutant CJ2130. (D) *gldNO* deletion mutant CJ2090. (E) *sprB* deletion mutant CJ1922. (F) *remA* deletion mutant CJ1984. Bar in panel D indicates 1 mm and applies to all panels.

***porV* mutant cells are resistant to some *F. johnsoniae* phages**. Cells with mutations in genes essential for T9SS function exhibit resistance to all known bacteriophages that infect *F. johnsoniae* (31, 32, 40). For example, cells of the Δ*gldNO* mutant CJ2090 were resistant to all bacteriophages tested (Figure 28I). This is thought to be the result of inability to secrete cell surface proteins that function as phage receptors, such as SprB, RemA, and other motility adhesins (24, 40, 41). *porU* mutants remained sensitive to all phages (Figure 28B), consistent with the findings presented above that

PorU is not required for secretion by the *F. johnsoniae* T9SS. In contrast, the *porV* deletion mutant CJ2130 was resistant to at least two of the eight phages tested, ϕCj48 and ϕCj54 (Figure 28C and see Table 8). Complementation with pSSK03, which carries *porV*, restored sensitivity to these phages. Previous results indicated that SprB is a likely receptor for phages ϕCj1, ϕCj13, and ϕCj23, and one of several receptors for ϕCj29 (24, 40) (and see Figure 28F). Sensitivity of the *porV* mutant to phages ϕCj1, ϕCj13, ϕCj23, and ϕCj29 supports the suggestion made above that PorV is not required for secretion of SprB. PorV is required for the secretion of RemA, as shown above, and it is also likely to be involved in the secretion of other cell surface proteins. This is illustrated by the sensitivity of the Δ*remA* mutant to phages ϕCj48 and ϕCj54 compared to the complete resistance of the Δ*porV* mutant to these phages (see table 8). Comparison of the phage resistances of the Δ*sprB* and Δ*porV* mutants with the Δ*sprB* Δ*porV* double mutant suggests that some phages may use multiple cell-surface receptors. Cells of the Δ*sprB* or Δ*porV* mutants were susceptible to ϕCj29 and ϕCj42 whereas cells of the double mutant (Δ*sprB* Δ*porV)* were completely resistant to both phages (Figure 28 compare panels C, F and H, and see Table 8). Phages ϕCj29 and ϕCj42 may use either SprB, or cell surface proteins secreted by PorV, as receptors.

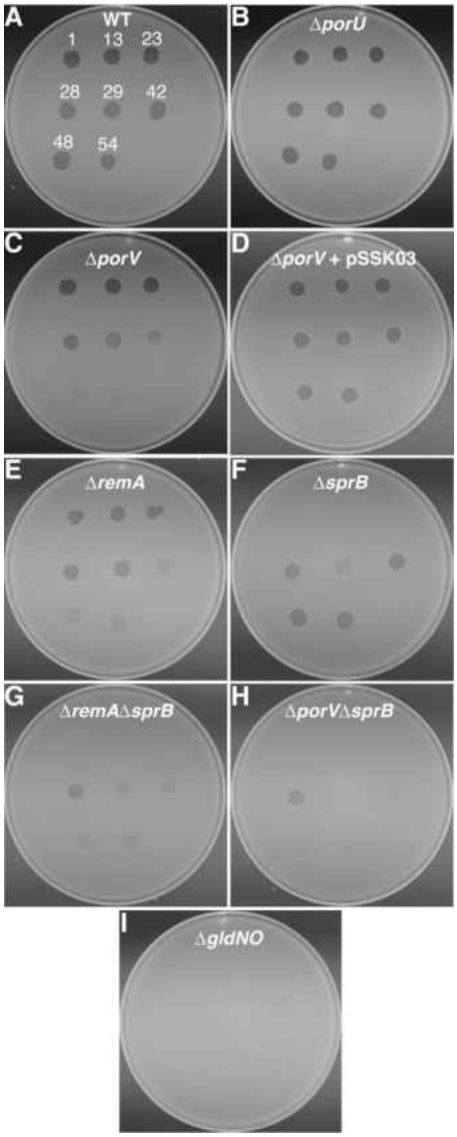

Figure 28. Susceptibility of wild-type and mutant cells to bacteriophages. Bacteriophages (3 µl of lysates containing approximately 10^9 PFU/ml) were spotted onto lawns of cells in CYE overlay agar. The plates were incubated at 25°C for 24 h to observe lysis. Bacteriophages were spotted in the following order from left to right, as also indicated by the numbers in panel A: top row, ϕCj1, ϕCj13, and ϕCj23; middle row ϕCj28, ϕCj29, and ϕCj42; bottom row, ϕCj48 and ϕCj54. (A) Wild-type *F. johnsoniae* CJ1827. (B) CJ2116 (Δ*porU*). (C) CJ2130 (Δ*porV*). (D) CJ2130 complemented with pSSK03 which carries *porV*. (E) CJ1984 (Δ*remA*). (F) CJ1922 (Δ*sprB*). (G) CJ1985 (Δ*remA* Δ*sprB*). (H) CJ2445 (Δ*porV* Δ*sprB*). (I) CJ2090 (Δ*gldNO*).

Table 8. Bacteriophage sensitivity of *F. johnsoniae* wild-type and mutant strains[a].

Phage / Host Strain	ϕ1 pfu/ml	ϕ13 pfu/ml	ϕ23 pfu/ml	ϕ28 pfu/ml	ϕ29 pfu/ml	ϕ42 pfu/ml	ϕ48 pfu/ml	ϕ54 pfu/ml
WT (CJ1827)	1.5×10^8	1.6×10^8	1.5×10^8	1.7×10^9	1.5×10^9	1.7×10^9	1.7×10^9	1.7×10^9
Δ*porU*	1.5×10^8	1.5×10^8	1.5×10^8	1.7×10^9	1.4×10^9	1.6×10^9	1.6×10^9	1.7×10^9
Δ*porV*	1.4×10^8	1.4×10^8	1.5×10^8	1.7×10^9	1.1×10^9	1.3×10^9	< 10	< 10
Δ*porV* + pSSK03	1.5×10^8	1.5×10^8	1.5×10^8	1.7×10^9	1.4×10^9	1.6×10^9	1.6×10^9	1.7×10^9
Δ*remA*	1.5×10^8	1.5×10^8	1.5×10^8	1.7×10^9	1.5×10^9	1.5×10^9	1.5×10^9	1.6×10^9
Δ*sprB*	< 10	1.2×10^3	< 10	1.7×10^9	1.0×10^9	1.7×10^9	1.6×10^9	1.7×10^9
Δ*remA* Δ*sprB*	< 10	1.1×10^3	< 10	1.7×10^9	1.0×10^9	1.4×10^9	1.5×10^9	1.6×10^9
Δ*porV* Δ*sprB*	< 10	< 10	< 10	1.7×10^9	< 10	< 10	< 10	< 10
Δ*gldNO*	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

[a] Each phage stock was serially diluted, mixed with cells of *F. johnsoniae*, plated on CYE agar in CYE overlay agar, incubated 24 h at 25°C, and the number of plaque forming units (pfu) per ml of original phage stock was determined on each bacterial strain. '<10' indicates that no plaques were observed even when 0.1 ml of undiluted phage stock was tested. 'WT' indicates the streptomycin resistant *rpsl2* strain CJ1827, which is wild-type for secretion and motility and was used to construct deletion mutants. Δ*porU*, CJ2116. Δ*porV*, CJ2130. Δ*remA*, CJ1984. Δ*sprB*, CJ1922. Δ*remA* Δ*sprB*, CJ1985. Δ*porV* Δ*sprB*, CJ2445. Δ*gldNO*, CJ2090. pSSK03 carries *porV* and was used to complement the Δ*porV* mutant.

Cells of Δ*porV* mutant are defective in attachment to glass. Wild-type cells attached efficiently to glass whereas cells of Δ*gldNO* mutant CJ2090 were completely deficient in attachment (Table 9). This supports the previous suggestion that the T9SS secretes cell-surface adhesins (40). The motility adhesins SprB and RemA are secreted by the T9SS and their absence on the surface of T9SS mutants accounts for some of the defect in attachment. Cells of the *sprB* deletion mutant CJ1922 attached well to glass and cells of the *remA* deletion mutant CJ1984 appear to have had a slight deficiency in attachment, but cells of the double mutant CJ1985 (Δ*remA* Δ*sprB*) exhibited a substantial defect in attachment (Table 9). An even greater defect in attachment was observed for cells of the Δ*porV* mutant CJ2130, suggesting that PorV is required for secretion of other adhesins in addition to RemA. The few Δ*porV* mutant cells that attached to glass exhibited gliding motility, consistent with the results described above that indicated that PorV was not required for gliding motility. CJ2445 (Δ*porV* Δ*sprB*) was almost entirely deficient in attachment to glass, suggesting that PorV is required for secretion of most of the glass-binding adhesins other than SprB. The results suggest that RemA, SprB, and other adhesins secreted by the T9SS are responsible for attachment to glass. Some of these adhesins may function as phage receptors, helping to explain the phage resistance of *porV* mutant cells (Figure 28). Cells of the *porU* mutant CJ2116 attached to glass almost as well as wild-type cells, indicating that PorU is not required for secretion of the *F. johnsoniae* adhesins.

Table 9. Deletion of *porV* results in decreased attachment of cells to glass.

Strain	Description	Avg (SD) no. of cells attached to 0.03-mm^2 region of glass coverslip[a]
CJ1827	Wild type	40.5 (2.2)
CJ1827/pCP29	Wild type with empty vector pCP29	38.7 (6.7)
CJ1827/pCP23	Wild type with empty vector pCP23	32.4 (6.7)
CJ2090	Δ(*gldN gldO*)	0.0 (0.0)
CJ1984/pCP29	Δ*remA* with empty vector pCP29	32.3 (6.5)
CJ1922/pCP29	Δ*sprB* with empty vector pCP29	41.4 (9.0)
CJ1985/pCP29	Δ*remA* Δ*sprB* with empty vector pCP29	17.8 (5.5)
CJ2130/pCP29	Δ*porV* with empty vector pCP29	7.0 (1.6)
CJ2130/pSSK03	Δ*porV* complemented with pSSK03 carrying *porV*	31.3 (2.7)
CJ2445/pCP29	Δ*porV* Δ*sprB* with empty vector pCP29	0.1 (0.3)
CJ2116/pCP23	Δ*porU* with empty vector pCP23	31.5 (2.7)
CJ2116/pSSK04	Δ*porU* complemented with pSSK04 carrying *porU*	30.3 (2.7)

[a] Approximately 10^6 cells in 2.5 µl of MM medium were introduced into a Petroff-Hausser counting chamber and incubated for 2 min at 25°C. Samples were observed using an Olympus BH-2 phase-contrast microscope, and cells attached to a 0.03-mm^2 region of the cover glass were counted. Numbers in parentheses are standard deviations calculated from 9 measurements.

***porV* mutant cells appear to be defective for secretion of at least thirty-two additional proteins.** Spent culture fluid of wild-type, Δ*gldNO*, Δ*porV*, and Δ*porU* mutant cells, and of complemented cells, were examined for the presence of secreted proteins by SDS-PAGE. Several prominent bands between 60 and 240 kDa that were present in the cell-free spent culture fluids from wild-type and Δ*porU* mutant cells were absent or decreased in intensity in culture fluid of Δ*gldNO*, and Δ*porV* mutant cells, and

were restored to near wild-type levels in the complemented mutants (Figure 29). LC-MS/MS analysis of one of these bands, at approximately 90 kDa, demonstrated that it corresponded to ChiA (Figure 30), which as mentioned above is secreted by the T9SS.

The regions of the gels in Figure 30 between 60 and 240 kDa were excised and analyzed by LC-MS/MS (Table 10), and see TableS3 in the supplemental material of (47)). Proteins present in the culture fluid from wild-type cells, but absent or greatly reduced in culture fluid from the Δ*gldNO* mutant, included eighteen proteins with TIGR04183 CTDs (including RemA), six proteins with TIGR04131 CTDs, and nine proteins that lacked obvious conserved CTDs. Twenty-six of the proteins mentioned above were also absent or greatly reduced in the cell-free culture fluid of the Δ*porV* mutant. The other seven proteins (Fjoh_0601, Fjoh_0602, Fjoh_0604, Fjoh_0606, Fjoh_1123, Fjoh_3952, Fjoh_4934) apparently do not require PorV for secretion by the T9SS. All of the proteins listed in Table 10 that have TIGR04183-type CTDs required PorV for efficient secretion. Proteins in cell-free culture fluid of Δ*porU* mutant cells were similar to those in culture fluid of wild-type cells (Figure 29), although a few proteins were apparently reduced in intensity or absent in the culture fluid from the Δ*porU* mutant (Table 10). These results indicate that PorU is not required for *F. johnsoniae* T9SS-mediated secretion, but it may enhance the secretion of some proteins.

In addition to the proteins that appeared to be secreted by the T9SS mentioned above many other proteins were also identified in the cell-free culture fluid (see Table S3 in the supplemental material of (47)). Analysis of the *F. johnsonaie* genome suggested the presence of a type II secretion system (22) which may account for the secretion of

some of these proteins. Some proteins were present in much higher amounts in the culture fluid of T9SS mutants than in the culture fluid of wild-type cells (see Table S3 in the supplemental material (47). These may be cellular (non-secreted) proteins that were released because of cell surface defects of the T9SS mutants. Consistent with this, many of these proteins were predicted to localize to the cytoplasm, periplasm, or outer membrane, where they presumably reside in wild-type cells.

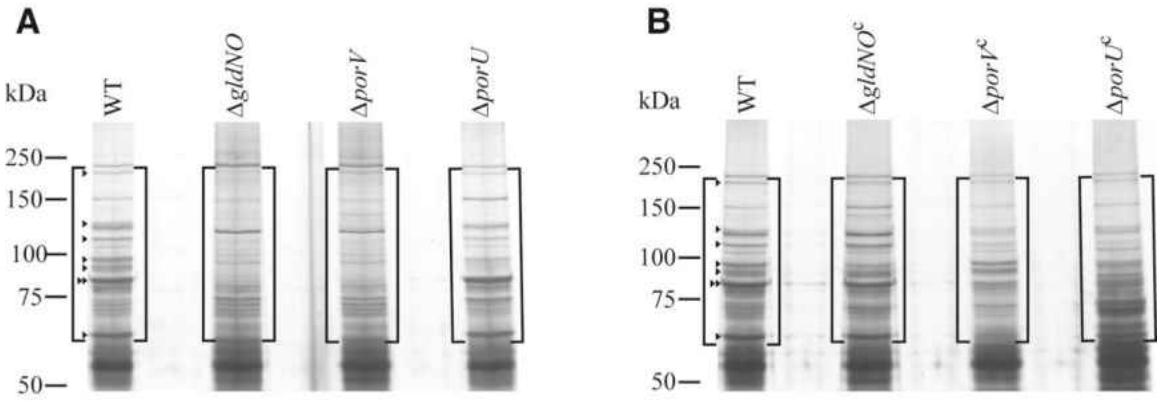

Figure 29. Soluble extracellular proteins of wild-type and mutant cells. Cells of *F. johnsoniae* wild type (CJ1827), Δ*gldNO* mutant (CJ2090), Δ*porV* mutant (CJ2130), Δ*porU* mutant (CJ2116), CJ2090 complemented with pTB79 which carries *gldN*, CJ2130 complemented with pSSK03 which carries *porV*, and CJ2116 complemented with pSSK04 which carries *porU*, were grown in MM at 25°C with shaking until cells reached an OD_{600} of 0.7. Equal amounts of cell-free spent media of wild-type and mutant cells were separated by SDS-PAGE and proteins were detected by silver staining. Arrowheads indicate bands present in the culture fluid of wild-type cells that were absent or reduced in intensity in the culture fluid of the Δ*gldNO* mutant cells. The double arrowhead corresponds to the N-terminal fragment of ChiA (Figure 30). The boxed regions were subjected to LC-MS/MS analysis (Table 10) and see Table S3 in the supplemental material (47)).

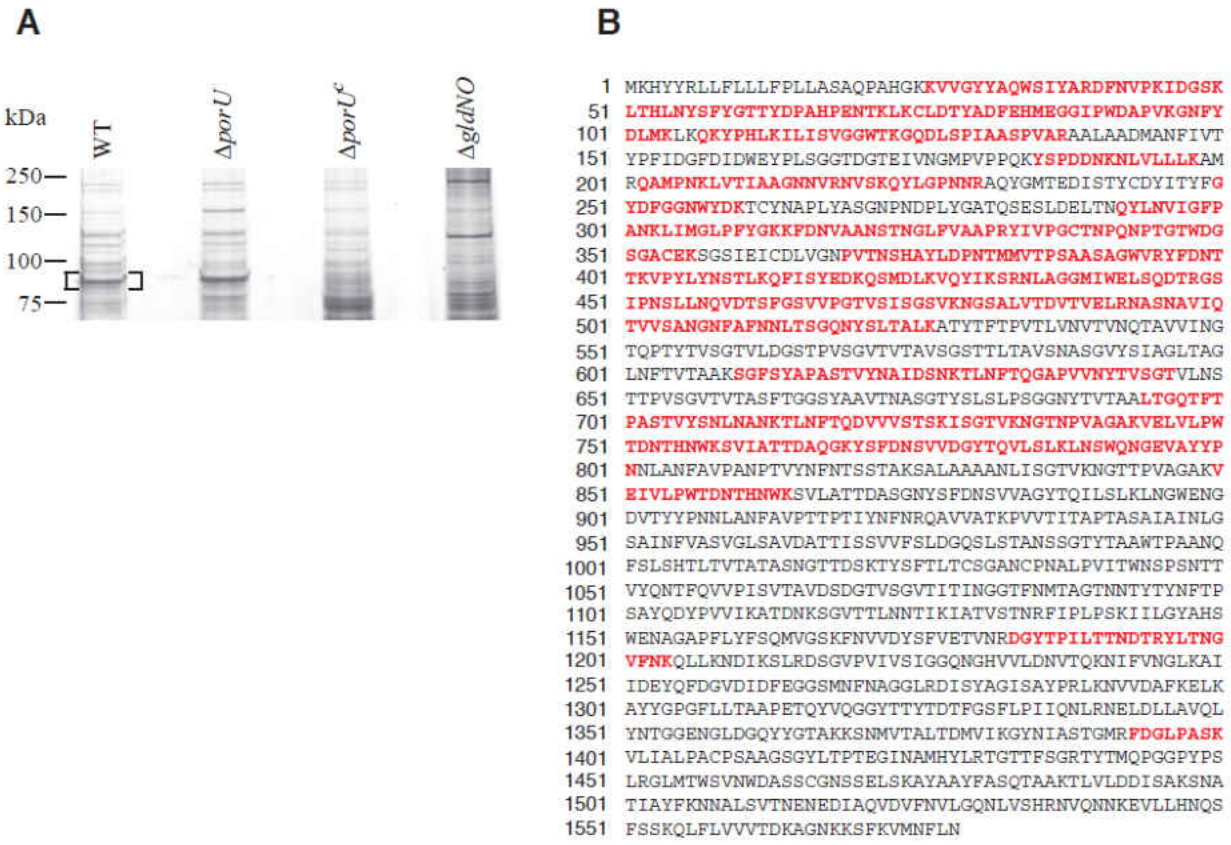

Figure 30. The approximately 90 kDa protein present in culture fluid of wild-type cells but not in culture fluid of the Δ*gldNO* mutant corresponds to the N-terminal portion of ChiA. A) Cell-free spent medium from wild-type cells (WT) and from cells of the Δ*porU* mutant, Δ*porU* mutant complemented with pSSK04, and Δ*gldNO* mutant were separated by SDS-PAGE and proteins were detected by silver staining. The approximately 90 kDa band that was present in the cell-free culture fluid of wild-type cells (bracketed) but was not present in culture fluid of the Δ*gldNO* mutant was cut from the gel and subjected to LC-MS/MS analysis. B) The primary amino acid sequence of ChiA is shown, with the regions detected by LC-MS/MS highlighted in red. 469 spectral matches were detected to ChiA. All of these except for 3 fell between amino acids 26 and 864, which corresponds to the amino-proximal region of the protein after removal of the signal peptide. ChiA has previously been shown to be processed into N-terminal and C-terminal fragments, each of which has a chitinase catalytic domain (15).

Table 10. Candidate proteins secreted by the T9SS identified by LC-MS/MS analysis of cell-free culture fluid[a].

Locus tag/Protein name	Mol mass[b] (kDa)	Predicted localization[c]	CTD[d]	Predicted protein function[e]	Wild type	Δ*gldNO*	Δ*gldNO* + pTB79	Δ*porV*	Δ*porV* + pSSK03	Δ*porU*	Δ*porU* + pSSK04
Fjoh_0074	123.1	OM, E	TIGR04183	Nuclease/phosphatase	42	3	108	7	84	95	129
Fjoh_0601	208.2	OM			115	0	84	71	78	118	148
Fjoh_0602	279.3	OM			68	0	38	57	59	65	86
Fjoh_0604	144.2	E			47	0	39	42	44	40	45
Fjoh_0606	409.5	OM			163	0	172	77	169	176	198
Fjoh_0808/ RemA	154.0	E	TIGR04183	Motility adhesin	38	0	47	0	56	37	67
Fjoh_0886	99.1	E	TIGR04183	Peptidase	12	0	19	0	21	14	18
Fjoh_1022	51.1	E	TIGR04183	Licheninase	6	0	6	0	6	1	8
Fjoh_1123	121.9	E, OM	TIGR04131		34	0	10	27	3	37	32
Fjoh_1188	152.7	E, OM	TIGR04183		49	0	104	0	42	52	75
Fjoh_1189	181.4	E	TIGR04183	Lectin	74	0	112	1	69	112	79
Fjoh_1208	112.5	E	TIGR04183	α amylase	45	0	66	6	91	58	126
Fjoh_1231	97.8	E	TIGR04183	Pectate lyase	9	0	13	0	6	35	31
Fjoh_1269	94.3	E, OM	TIGR04183		27	4	43	3	40	34	56
Fjoh_1408[f]	106.0	E	TIGR04183	α amylase	2	0	4	0	2	0	6
Fjoh_1645[f]	258.1	E	TIGR04131		2	0	6	0	1	2	3
Fjoh_2150	39.0	E, OM	TIGR04183		6	0	6	0	7	3	3
Fjoh_2273	93.3	E	TIGR04131		4	0	5	1	5	5	1
Fjoh_2389[f]	57.7	E, OM	TIGR04183	Peptidase	2	0	7	0	12	0	6
Fjoh_2667	129.7	OM			28	0	7	0	3	5	33
Fjoh_2687	155.8	E			26	1	26	7	35	26	43

Fjoh_3108	30.9	OM, E, P			7	0	10	0	8	0	10
Fjoh_3246	299.4	OM, E	TIGR04183		12	0	77	0	6	23	41
Fjoh_3324	105.3	E	TIGR04183	Carbohydrate-binding	16	1	40	5	20	49	59
Fjoh_3729	195.1	OM			46	0	32	0	52	23	79
Fjoh_3777	128.1	OM, E	TIGR04183	Deacylase	10	0	25	0	9	10	34
Fjoh_3952	330.6	E	TIGR04131		22	0	11	12	16	11	17
Fjoh_4174	102.5	E	TIGR04183	Carbohydrate-binding	40	5	40	6	62	55	36
Fjoh_4176	95.4	E	TIGR04183	Carbohydrate-binding	48	3	65	7	63	108	76
Fjoh_4177	144.9	E	TIGR04183	Glycoside hydrolase	22	0	35	0	34	67	60
Fjoh_4750	158.1	E	TIGR04131		13	0	3	3	3	3	10
Fjoh_4819	112.5	C, OM, P		Glycoside hydrolase	34	0	5	0	1	12	9
Fjoh_4934	84.8	E	TIGR04131		11	1	7	19	4	17	17

[a] Proteins in cell-free culture fluid from wild type *F. johnsoniae* CJ1827, Δ*gldNO* mutant CJ2090, Δ*porV* mutant CJ2130, Δ*porU* mutant CJ2116, Δ*gldNO* complemented with pTB79, Δ*porV* complemented with pSSK03, and Δ*porU* complemented with pSSK04 were separated by SDS-PAGE, silver stained, and the regions shown in Figure 29 spanning approximately 60 to 240 kDa were cut from the gel and analyzed by LC-MS/MS. Total/unweighted spectrum counts corresponding to total number of spectra associated to a single protein and indicative of relative abundance of that protein are indicated for each of the seven strains analyzed. For the complete data set including proteins that were apparently secreted by other secretion systems or were released by cell lysis see Table S3 in the supplemental material of (47).

[b] Mol mass, molecular mass as calculated for full-length protein before removal of signal peptide. [c] Protein localization as predicted by CELLO v 2.5 subcellular localization predictor (44). OM-outer membrane, E-extracellular, P-periplasmic and C-cytoplasmic.

[d] CTD-type identified by BLASTP analysis.

[e] Predicted protein functions as listed on the Integrated Microbial Genomes website (https://img.jgi.doe.gov), except for RemA (41).

[f] The small number of spectra identified from wild type cells for these proteins made the prediction of secretion by the T9SS less certain. These proteins were included because no spectra were observed from Δ*gldNO* or Δ*porV* mutant cells and because complementation of the Δ*gldNO* mutant resulted in 4 to 7 spectral hits for each protein.

The T9SS and PorV are required for efficient starch utilization. The predicted α-amylases Fjoh_1208 and Fjoh_1408 were detected in cell-free culture fluid of wild-type cells but not in culture fluid of Δ*gldNO* mutant cells (Table 10). Examination of wild-type and mutant cells confirmed that the T9SS has a role in starch utilization (Figure 31). The Δ*gldNO* and Δ*porV* mutant cells were partially deficient in digestion of starch. The small amount of residual starch digestion detected with these mutants may be the result of additional amylases released by other secretion systems, or may indicate that a small amount of Fjoh_1208 or Fjoh_1408 was released from the mutant cells.

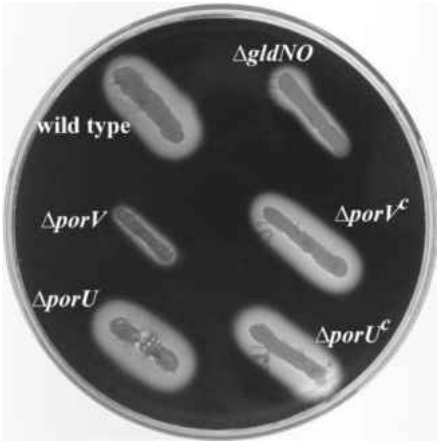

Figure 31. Starch digestion by wild-type and mutant cells. Cells were streaked on CYE agar containing 0.25% starch and incubated overnight at 25°C. The agar was flooded with a solution of 1% KI and 1% iodine to detect starch. Clearing zones around the cells indicate starch digestion. Wild type: *F. johnsoniae* CJ1827. Δ*gldNO*: *gldNO* deletion mutant CJ2090. Δ*porV*: *porV* deletion mutant CJ2130. Δ*porV*^{c}: CJ2130 complemented with pSSK03 which carries *porV*. Δ*porU*: *porU* deletion mutant CJ2116. Δ*porU*^{c}: CJ2116 complemented with pSSK04 which carries *porU*.

Fjoh_0288, which exhibits limited sequence similarity to PorV, does not appear to be required for T9SS function. Examination of the *F. johnsoniae* genome revealed one gene, *Fjoh_0288*, encoding a protein that exhibits 30% amino acid identity with PorV over a 135 amino acid region. *Fjoh_0288* was deleted in wild-type cells and in cells of the Δ*porV* mutant. Cells of CJ2082 (Δ*Fjoh_0288*) and of CJ2446 (Δ*Fjoh_0288* Δ*porV*) spread on agar as well as wild-type cells (data not shown) suggesting that SprB was secreted to the cell surface. Deletion of *Fjoh_0288* also had no effect on secretion of ChiA, and no effect on sensitivity to the eight phages used in this study (data not shown). These results suggest that Fjoh_0288 is not involved in T9SS-mediated secretion.

Discussion

T9SSs, discovered in *P. gingivalis* and *F. johnsoniae*, are common among members of the phylum *Bacteroidetes* (22, 35, 42). Seven proteins (GldK, GldL, GldM, GldN, SprA, SprE, SprT) are important for T9SS function in *F. johnsoniae*, and orthologs of these are required for secretion in *P. gingivalis* (31-33, 35, 36, 40). *P. gingivalis* PorP is also required for secretion (35), whereas in *F. johnsoniae* the function of PorP appears to be split between multiple PorP-like proteins, such as SprF which is required specifically for secretion of SprB (29). Two additional *P. gingivalis* proteins (PorU and PorV) are involved in gingipain secretion (7, 8, 12, 35). In this study we examined the potential roles of *F. johnsoniae* PorU and PorV in secretion.

F. johnsoniae PorU was not required for secretion of proteins by the T9SS. Three proteins, RemA, ChiA, and SprB, known to be secreted by the T9SS (15, 31, 40, 41) were each secreted in functional form by cells of a *porU* deletion mutant. SDS-PAGE

followed by LC-MS/MS analysis of proteins secreted by wild-type and mutant cells revealed that whereas cells of the *gldNO* deletion mutant appeared to be defective for the secretion of at least 33 proteins, proteins secreted by cells of the *porU* deletion mutant were similar to those secreted by wild-type cells. PorU is thought to function as the peptidase that removes the CTDs of *P. gingivalis* proteins during or after secretion (8). Our results suggest that *F. johnsoniae* either does not require CTD processing for secretion or it has other proteases that render PorU unnecessary. No paralogs of *porU* were detected in the genome, but *F. johnsoniae* produces many peptidases (21).

F. johnsoniae PorV was required for secretion of many but not all proteins that are targeted to the T9SS. Secretion of RemA and ChiA required PorV, whereas secretion of SprB did not. Defects in phage sensitivity and in attachment of cells to glass were also associated with deletion of *porV*, and suggested that PorV is required for the secretion of additional cell-surface adhesins besides RemA. Analysis of spent culture fluid of wild-type and mutant cells revealed 26 proteins that appear to require *porV* for efficient secretion. These proteins were also absent in culture fluid of the Δ*gldNO* mutant, suggesting that they are secreted from wild-type cells by the T9SS. Eighteen of these proteins had CTDs that belong to protein domain family TIGR04183, suggesting that proteins with TIGR04183-type CTDs might require PorV for secretion. Some proteins with TIGR04131-type CTDs, and some proteins that lacked recognizable T9SS CTDs also appeared to require PorV for secretion, whereas others did not since they were found in the cell-free culture fluid of both wild-type and *porV* mutant cells.

T9SS-mediated secretion of proteins with TIGR04183-type CTDs has been documented for many proteins of diverse members of the phylum *Bacteroidetes* (22, 37,

40-42). In contrast, the role of TIGR04131-type CTDs in targeting proteins for secretion is less well established. *F. johnsoniae* SprB is the only example of a TIGR04131 family member that has been demonstrated to be secreted by the T9SS (29, 31, 35, 40). The observation of six additional *F. johnsoniae* proteins with TIGR04131-type CTDs that appear to be secreted by the T9SS supports the suggestion that TIGR04131 CTDs target proteins to the T9SS. The *F. johnsoniae* genome is predicted to encode 12 proteins with TIGR04131-type CTDs, and we hypothesize that each of these are secreted by the T9SS. Proteins that have TIGR04131-type CTDs are also common among the many members of the phylum *Bacteroidetes* that have T9SSs. Proteins in addition to those with TIGR04183-type CTDs and TIGR04131-type CTDs are also secreted by T9SSs. For example *F. johnsonaie* ChiA is secreted by the T9SS but its CTD, which is necessary and sufficient for secretion, does not closely resemble members of either TIGR04183 or TIGR04131 (15). Here we identified nine additional proteins (Fjoh_0601, Fjoh_0602, Fjoh_0604, Fjoh_0606, Fjoh_2667, Fjoh_2687, Fjoh_3108, Fjoh_3729, and Fjoh_4819) that were apparently secreted by the T9SS but that did not exhibit similarity to members of either TIGR04183 or TIGR04131. There is considerable sequence diversity among T9SS CTDs (37, 42) and these nine proteins may have novel T9SS-targeting domains that have thus far escaped detection.

The results reported here identify thirty-five proteins (including ChiA, SprB and RemA) that appear to be secreted by the *F. johnsoniae* T9SS. This is probably only a partial list of proteins secreted by this system. Fifty-four *F. johnsoniae* proteins were previously predicted to be secreted by the T9SS based on the presence of CTDs belonging to TIGR04183 and TIGR04131 (40). In addition, the identification of proteins

that lack obvious T9SS-targeting CTDs but that are apparently secreted by the T9SS suggests that additional proteins may be secreted by this system. Further study is needed to determine the diversity of T9SS-targeting sequences and to reveal the mechanism of T9SS-mediated protein secretion in *F. johnsoniae* and in other members of the phylum *Bacteroidetes*.

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Chapter 4. Characterization of the *F. johnsoniae* chitin utilization locus.

Abstract

Chitin and cellulose are thought to be the most abundant polysaccharides on earth and therefore have the potential to be used as sources of energy. Bacteria employ various strategies to digest polysaccharides. Some bacteria secrete extracellular enzymes that hydrolyze the polymers to monomers and dimers, while others rely on their cell-surface enzyme complexes to perform the same task. A member of the phylum *Bacteroidetes, Bacteroides thetaiotaomicron,* uses a different strategy. Its cell surface **s**tarch **u**tilization **s**ystem proteins, SusC and SusD bind long starch oligomers and transport them across the outer membrane where they are digested further. The related bacterium *Flavobacterium johnsoniae* digests many polysaccharides including chitin. *F. johnsoniae* has 44 *susC*-like genes and 42 *susD*-like genes within its many **p**olysaccharide **u**tilization **l**oci (PULs). One PUL predicted to be involved in chitin utilization was investigated. Single and multiple deletion mutants were constructed to determine the roles of the two *susC-like* genes ($cusC_I$ and $cusC_{II}$) and the two *susD-like* genes ($cusD_I$, and $cusD_{II}$) in chitin utilization. Cells of a $cusD_I$ deletion mutant and of a double mutant lacking both $cusD_I$, and $cusD_{II}$ exhibited reduced growth on chitin. Individual deletions of $cusC_I$, $cusC_{II}$ and $cusD_{II}$ did not result in obvious defects in chitin utilization. *chiA,* which encodes the extracellular chitinase required for chitin utilization, is also part of the chitin PUL, as is another predicted chitinase, Fjoh_4560. Cells deleted for Fjoh_4560 grew on chitin as well as the wild-type. Deletion of the region spanning $cusD_I$ (Fjoh_4558) through $cusC_{II}$ (Fjoh_4562) resulted in complete loss of ability to grow on chitin. These results suggest that *F. johnsoniae* employs SusC-like and SusD-like proteins to utilize the insoluble

polymer chitin. ChiA may act synergistically with these proteins to efficiently utilize chitin.

Introduction

Cellulose, hemicelluloses and chitin are thought to be the most abundant insoluble polysaccharides in the environment and are rich sources of energy that can be converted into liquid biofuels (3, 11, 12). Chitin is a major constituent of shells of crustaceans such as crabs and shrimps, the exoskeletons of insects, and the cell walls of yeasts and other fungi (4). Chitin is a linear insoluble polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and is resistant to digestion (6). Chitin and its modified forms have applications in wastewater treatment and drug delivery systems. Moreover modified forms of chitin are also employed as food quality enhancing agents, antibacterial agents, antifungal agents and as dietary fiber supplements (3, 4). Dietary polysaccharides affect human health, since gut microbes that digest them supply energy to the human body, impact the host immune system, and affect the ability of pathogens to colonize the intestinal tract. The human distal gut is inhabited by trillions of microbes. Bacteria in the gut digest and ferment resistant glycans to fatty acids that are easily absorbed (9, 16). This microbial process contributes up to 10% of daily caloric intake (9). Several bacterial phyla including *Bacteroidetes* and *Firmicutes*, are capable of metabolizing a variety of polysaccharides (24). Members of the phylum *Bacteroidetes* often dominate the large intestine. Several *Bacteroidetes* have become model organisms to study the molecular mechanisms underlying polysaccharide digestion, in part because of the genetic tools available for their manipulation (16, 23).

Bacteria employ various strategies to digest polysaccharides some include secretion of extracellular enzymes that hydrolyze the polymers to monosaccharides and disaccharides, while other strategies rely on their cell-surface enzyme complexes to perform the same task. Members of the phylum *Bacteroidetes* employ a different strategy to utilize these polymers (2). This involves cell surface enzymes that partially hydrolyze the polysaccharides into long oligomers. These oligomers apparently bind to a cell surface glycan binding protein SusD, and are taken up actively by a TonB dependent porin, SusC. The oligomers are further digested in the periplasm. *Bacteroides thetaiotaomicron* a starch utilizing anaerobic bacterium belonging to the phylum *Bacteroidetes,* employs this Sus strategy for polymer digestion (20). *B. thetaiotaomicron* enzymes involved in starch utilization are cell associated (25). Some of these are located on the cell surface and others are internal (periplasmic and cytoplasmic) (26). *B. thetaiotaomicron* has numerous genes encoding SusC-like and SusD-like proteins allowing it to utilize many soluble polysaccharides. *susC*-like and *susD*-like genes are paired with each other and are usually adjacent to genes encoding glycohydrolases involved in digesting polysaccharides. These gene clusters are called **P**olysaccharide **U**tilization **L**oci (**PULs**) (19).

Flavobacterium johnsoniae belongs to the phylum *Bacteroidetes* and digests chitin and many other polysaccharides (19). Genome analysis of *F. johnsoniae* suggests that SusC-like and SusD-like proteins may be involved not only in the utilization of relatively soluble polysaccharides such as starch, but also in the utilization of highly insoluble crystalline polysaccharides such as chitin (5). Many genes encoding proteins similar to *B. thetaiotaomicron* SusC and SusD were revealed in the *F. johnsoniae*

genome (19). It has 44 *susC*-like genes and 42 *susD*-like genes within its many PULs (2). One of its PULs is predicted to be involved in chitin utilization (Fjoh_4564-Fjoh_4555) (Figure 32). Fjoh_4555 encodes ChiA, the major extracellular chitinase. The other genes in this PUL including $cusC_I$ and $cusC_{II}$ (*susC*-like genes) and $cusD_I$ and $cusD_{II}$ (*susD*-like genes) were predicted to play roles in chitin utilization.

Materials and Methods

Bacterial and bacteriophage strains, plasmids, and growth conditions. *F. johnsoniae* ATCC 17061 strains UW101 was the wild-type strains used in this study. The streptomycin resistant *rpsL* mutant of UW101 (CJ1827) was used to construct deletion mutants (21). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30^{o}C (18) or Stanier media supplemented with chitin for analyzing growth on chitin. *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C. Strains and plasmids used in this study are listed in Table 11 and primers are listed in Table 12. Antibiotics were used at the following concentrations when needed: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; erythromycin, 100 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 20 μg/ml.

Construction of deletion mutants and complementation.

The previously described strategy was employed (21) to generate single gene deletions in the predicted chitin PUL. A 1718 bp fragment upstream of $cusD_I$ was amplified by PCR using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1166 and 1167. The amplified product was digested with XbaI and SalI and

cloned into pRR51 that had been digested with the same enzymes, generating pSSK13. A 1725 bp fragment downstream of *cusD$_I$* was amplified by PCR using primers 1168 (engineered SalI site) and 1169 (engineered SphI site). This product was ligated into pSSK13 that had been digested with SalI and SphI, to generate pSSK18. pSSK18 was introduced into *F. johnsoniae* strain CJ1827 by conjugation and integration into the genome by recombination was selected using erythromycin. Subsequent selection with streptomycin allowed isolation of the Δ*cusD$_I$,* mutant (CJ2121) that had lost the integrated plasmid (21). The Δ*cusD$_I$* mutant was confirmed by PCR amplification using primers primer 1444 and 1445 and by sequencing the product.

Strains with deletions in *cusD$_{II}$* (CJ2018), *cusC$_I$* (CJ2086), *cusC$_{II}$* (CJ2340), and Fjoh_4560 (CJ2350), were constructed in the same way using the primers and plasmids listed in Table 11 and Table 12. The Δ*cusD$_I$* Δ*cusD$_{II}$* double mutant, CJ2156, was generated by starting with the Δ*cusD$_I$* mutant CJ2121 and deleting *cusD$_{II}$* as described above.

To delete a region spanning *cusD$_I$* to *cusC$_{II}$* a 1766 bp region downstream of *cusC$_{II}$* was amplified by PCR using primers 1252 (engineered SalI site) and 1253 (engineered SphI site). This product was digested with SalI and SphI and ligated into pSSK13 that had been digested with the same enzymes to generate pSSK39. pSSK39 was introduced into *F. johnsoniae* strain CJ1827 by conjugation and streptomycin selection was used to obtain the Δ(*cusD$_I$*-*cusC$_{II}$*) mutant CJ2349. The deletion was confirmed by PCR amplification using primers 1465 and 1466 and sequencing.

To complement the *cusD$_I$* mutant, a 1937 bp region spanning *cusD$_I$* was amplified with primers 1514 (engineered SalI site) and 1515 (engineered SphI site). This product was digested with SalI and SphI and ligated into pCP23 that had been digested with the same enzymes to generate pSSK47.

To express recombinant CusD$_I$ in *E.coli* for biochemical characterization, a 1518 bp region of *cusD$_I$* was amplified with primers 1561 (engineered NheI site) and 1562 (engineered XhoI site). This fragment was digested with NheI and XhoI and ligated into pET28a that had been digested with the same enzymes to generate pSSK49. Similarly a 1461 bp region of *cusD$_{II}$* was amplified using primers 1563 (engineered NheI site) and 1564 (engineered XhoI site). This product was digested with NheI and XhoI and ligated into pET28a that had been digested with the same enzyme to generate pSSK50. pSSK49 and pSSK50 were introduced into *E.coli* Rosetta to allow production of recombinant CusD$_I$ and CusD$_{II}$

Qualitative determination of growth on chitin. Cells of the wild type and mutant strains were inoculated in minimal Stanier broth supplemented with colloidal chitin prepared from crab shells (13, 17, 22). The cells were incubated at 25°C for 48h, and examined for growth of cells and solubilization of chitin.

Table 11. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or description[a]	Source or reference
Strains		
UW 101 (ATCC 17061)	Wild type	(17)
CJ1808	*chiA* disruption mutant; (Em^r)	(13)
CJ1827	*rpsL2*; (Sm^r) "wild type' strain for construction of deletion mutants	(21)
CJ2018	*rpsL2* Δ *cusD*$_{II}$; (Sm^r)	This study
CJ2086	*rpsL2* Δ *cusC*$_I$; (Sm^r)	This study
CJ2121	*rpsL2* Δ *cusD*$_I$; (Sm^r)	This study
CJ2156	*rpsL2* Δ *cusD*$_I$ *cusD*$_{II}$; (Sm^r)	
CJ2340	*rpsL2* Δ *cusC*$_{II}$; (Sm^r)	This study
CJ2349	*rpsL2* Δ *cusD*$_I$ - *cusC*$_{II}$; (Sm^r)	This study
CJ2350	*rpsL2* Δ *Fjoh_4560*; (Sm^r)	This study
Plasmids		
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; Ap^r (Tc^r)	(1)
pRR51	*rpsL* containing suicide vector; Ap^r (Em^r)	(21)
pET28a	pET28a [replaced thrombin site to rTEV (cleavable N-terminal His tag) site in Rosetta2(DE3) (Km^rCm^r)	Nicole Koropatkin
pSSK08	1,827-bp BamHI-SalI region downstream of *cusD*$_{II}$ amplified with primers 1055 and 1056 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK09	Construct used for deletion *cusD*$_{II}$: 1,328-bp SalI-SphI region upstream of *cusD*$_{II}$ amplified with primers 1057 and 1058 and inserted into pSSK08; Ap^r (Em^r)	This study
pSSK13	1,718-bp XbaI-SalI region downstream of *cusD*$_I$ amplified with primers 1166 and 1167 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK15	1,504-bp XbaI-SalI region downstream of *cusC*$_I$ amplified with primers 1170 and 1052 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK17	Construct used for deletion *cusC*$_I$:1,827-bp SalI-SphI region upstream of *cusC*$_I$ amplified with primers 1053 and 1054 and inserted into pSSK15; Ap^r (Em^r)	This study
pSSK18	Construct used for deletion *cusD*$_I$:1,725-bp SalI-SphI region upstream of *cusD*$_I$ amplified with primers 1168 and 1169 and inserted into pSSK13; Ap^r (Em^r)	This study
pSSK29	1,899-bp XbaI-SalI region downstream of *cusC*$_{II}$ amplified with primers 1250 and 1251 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK33	Construct used for deletion *cusC*$_{II}$:1766,-bp SalI-SphI region	This study

	upstream of *cusC$_{II}$* amplified with primers 1252 and 1253 and inserted into pSSK29; Ap^r (Em^r)	
pSSK38	1,510-bp BamHI-SalI region upstream of *Fjoh_4560* amplified with primers 1423 and 1424 and inserted into pRR51; Ap^r (Em^r)	This study
pSSK39	Construct used for deletion (*cusD$_I$ to cusC$_{II}$*)1,766-bp SalI-SphI region upstream of *cusC$_{II}$* amplified with primers 1252 and 1253 and inserted into pSSK13; Ap^r (Em^r)	This study
pSSK40	Construct used for deletion Fjoh_4560:1,560-bp SalI-SphI region downstream of *Fjoh_4560* amplified with primers 1425 and 1426 and inserted into pSSK39; Ap^r (Em^r)	This study
pSSK47	1,937-bp SalI-SphI fragment spanning *cusD$_I$* amplified with primer 1514 and 1515 and inserted into pCP23; Ap^r (Tc^r)	This study
pSSK49	1,518-bp NheI-XhoI fragment spanning *cusD$_I$* amplified with primer 1561 and 1562 and inserted into pET28a; Ap^r (Tc^r)	This study
pSSK50	1,461-bp NheI-XhoI fragment spanning *cusD$_{II}$* amplified with primer 1563 and 1564 and inserted into pET28a; 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 12. Primers used in this study

Primers	Sequence and Description
1052	5’ - GCTAGGTCGACACAGGTGATGCAAGAAATGCAGGC – 3’, reverse primer to amplify downstream region of Fjoh_4559 used for constructing pSSK15; SalI site underlined
1053	5’ - GCTAGGTCGACTTTTACCTGTGCAAGCGAAACCTG – 3’, forward primer to amplify upstream region of Fjoh_4559 used for constructing pSSK17; SalI site underlined
1054	5’ - GCTAGGCATGCGCTCCTGCAAGTCAGGCAAGTATT – 3’, reverse primer to amplify upstream region of Fjoh_4559 used to construct pSSK17, SphI site underlined
1055	5’ - GCTAGGGATCCTTTTACCTGTGCAAGCGAAACCTG – 3’, forward primer to amplify downstream region of Fjoh_4561 to construct pSSK08, BamHI site underlined
1056	5’ - GCTAGGTCGACGCTCCTGCAAGTCAGGCAAGTATT – 3’, reverse primer to amplify downstream region of Fjoh_4561 to construct pSSK08, SalI site underlined
1057	5’ - GCTAGGTCGACTGTAAGCTGACCTGCAGGATTTGG – 3’, forward primer to amplify upstream region of Fjoh_4561 to construct pSSK09, SalI site underlined
1058	5’ - GCTAGGCATGCAATGCACCGGGAGCTTACAAGAAC – 3’, reverse primer to amplify upstream region of Fjoh_4561 to construct pSSK09, SphI site underlined
1166	5' GCTAGTCTAGATACAATTTCGATATCCTCCTGCCC 3', forward primer to amplify downstream region of Fjoh_4558 to construct pSSK13, XbaI site underlined
1167	5 GCTAGGTCGACGGAGTTTCTAAATTGGGCGGACCA ' 3', reverse primer to amplify downstream region of Fjoh_4558 to construct pSSK13, SalI site underlined
1168	5’- GCTAGGTCGACGGCGAGTAACAAAGTACAAATAGTTGCTTT - 3’, forward primer to amplify upstream region of *Fjoh_4558* to construct pSSK18, SalI site underlined
1169	5’- GCTAGGCATGCTGGTTGTCGATTGCTTCTAGATACAGTTAT -3’, reverse primer to amplify upstream region of *Fjoh_4558* to construct pSSK18, SphI site underlined
1170	5’ - GCTAGTCTAGACTGAGCAGTACCGCCCATATTCCA – 3’, forward primer to amplify downstream region of *Fjoh_4559* to construct pSSK15, XbaI site underlined
1250	5’- GCTAGGTCGACGTAGCAAATGGAGTTGTTAATACAGGA - 3’, reverse primer to amplify downstream region of *Fjoh_4562* to construct pSSK29, SalI site underlined
1251	5’- GCTAGTCTAGAAGTTTACATCCCACCAAACCTTACCAG- 3’, forward primer to amplify downstream region of *Fjoh_4562* to construct pSSK29, XbaI site underlined
1252	5’- GCTAGGCATGCCTGCTGCAAATTCTCTAAAAGC – 3, reverse primer to amplify upstream region of *Fjoh_4562* to construct pSSK33, SphI site underlined
1253	5’ - GCTAGGTCGACAGCAGATGCCTGAATCGTATACATACC -3’, forward primer to amplify upstream region of *Fjoh_4562* to construct pSSK33, SalI site underlined
1423	5’- GCTAGGGATCCTTTACTCAATTATGTATGTCTGGAGAC - 3’, forward primer to amplify upstream region of *Fjoh_4560* to construct pSSK38, BamHI site underlined
1424	5’- GCTAGGTCGACAAGGATGCCTAATAAGGCTTTATTTTT - 3’, reverse primer to amplify upstream region of *Fjoh_4560* to construct pSSK38, SalI site

	underlined
1425	5’- GCTAGGTCGACTACAAGACTTCAGGCATGTGCGGT - 3’, forward primer to amplify downstream region of *Fjoh_4560* to construct pSSK40, SalI site underlined
1426	5’- GCTAGGTCGACAAGGATGCCTAATAAGGCTTTATTTTT - 3’, reverse primer to amplify downstream region of *Fjoh_4560* to construct pSSK40, SphI site underlined
1444	5’- TTG ATA TTT ATG GTT TAC CTT CTA CCA-3’ used for sequencing Fjoh_4558
1445	5’- ATT CGG AGC ATT TAC ATC CCA CCA AAC -3’ used for sequencing Fjoh_4558
1465	5’- ATCCTAACGATGAGATGCAGAAA-3’ used for sequencing Fjoh_4560 and checking deletion of Fjoh_4558 to Fjoh_4562
1466	5’- CAATCAATAATCTGCTGCTCGAAA- 3’ used for sequencing Fjoh_4560 and checking deletion of Fjoh_4558 to Fjoh_4562
1467	5’- ACCAGTTGGAGTTGCCATATAA-3’ used for sequencing Fjoh_4559
1468	5’- GGATAACGATTTCGTGCAACATAA-3’ used for sequencing Fjoh_4562
1469	5’- CTGATAATATCCGTCTCCAGACATAC-3’used for sequencing Fjoh_4562
1470	5’- GCGCGAAATCTATTGACATTCAG-3’ used for sequencing Fjoh_4561
1471	5’- CAAAGCTGTCGGCAGATAAGTA-3’ used for sequencing Fjoh_4561
1512	5’ – GCTAGGGTACCGGAACTGGCTCAGGATTCTT – 3’, Forward primer to amplify Fjoh_4559. KpnI site underlined.
1513	5’ – GCTAGGTCGACCGGCGAGTAACAAAGTACAAATAG – 3’, Reverse primer to amplify Fjoh_4559. SalI site underlined.
1514	5’-GCTAGGTCGACCTACACAACTGTTGGAGGAAGA-3’, Forward primer to amplify Fjoh_4558. SalI site underlined.
1516	5’-GCTAGGCATGCCTGCTTGTACCATTTGCTAACC-3’, Reverse primer to amplify Fjoh_4558. SphI site underlined.
1561	5’- GCTAGGCTAGCACAGATAATTTTGAAGACATTAATACT-3’, forward primer to amplify Fjoh_4558. NheI site underlined
1562	5’- GCTAGCTCGAGTTAGAAATTCGGAGCATTTACATCCCA-3’, reverse primer to amplify Fjoh_4558. XhoI site underlined
1563	5’- GCTAGGCTAGCACAGAAAATTTTGACGAACTGATAAAG-3’, forward primer to amplify Fjoh_4561. NheI site underlined
1564	5’- GCTAGCTCGAGTTAGTTTACATCCCACCAAACCTTACC-3’, forward primer to amplify Fjoh_4561. XhoI site underlined

Results

***F. johnsoniae* has a PUL that appears to be involved in chitin utilization.** The *F. johnsoniae* genome was analyzed for PULs containing *susC*-like and *susD*-like genes (19). One PUL (Fjoh_4564 to Fjoh_4555) was predicted to be involved in chitin utilization (Figure 32). It includes Fjoh_4555 that encodes the major extracellular chitinase ChiA required for chitin utilization as discussed in chapter 2. It also includes genes encoding other hydrolytic enzymes, SusC-like proteins, SusD-like proteins and regulatory proteins. As typical in other organisms in the phylum *Bacteroidetes,* the *susC*-like genes are located upstream of *susD*-like genes, and are situated near genes encoding hydrolytic enzymes. In addition to *chiA* (Fjoh_4555) the PUL includes Fjoh_4560 that is predicted to encode another chitinase. The predicted chitin PUL also includes genes predicted to encode a β-*N*-acetylglucosaminidase (Fjoh_4556) and a glucosamine-6-phosphate deaminase (Fjoh_4557). These two enzymes are predicted to be involved in later steps of chitin utilization.

Fjoh_4558 and Fjoh_4561 are predicted to encode SusD-like proteins. They exhibit similarity to members of the SusD-like family pfam12741 (7, 8). Fjoh_4559 and Fjoh_4562 are predicted to encode SusC-like proteins and these proteins exhibit similarity to members of the SusC-like TIGRFAM families TIGR04056 and TIGR04057. These SusC-like and SusD -like proteins were predicted by PSORTb and CELLO to localize to the outer membrane. The *F. johnsoniae,* SusD-like proteins $CusD_I$ and $CusD_{II}$ exhibit 27% sequence identity over 561 amino acids with each other, whereas the SusC-

like proteins $CusC_I$ and $CusC_{II}$ exhibit 33% sequence identity over 1096 amino acids with each other.

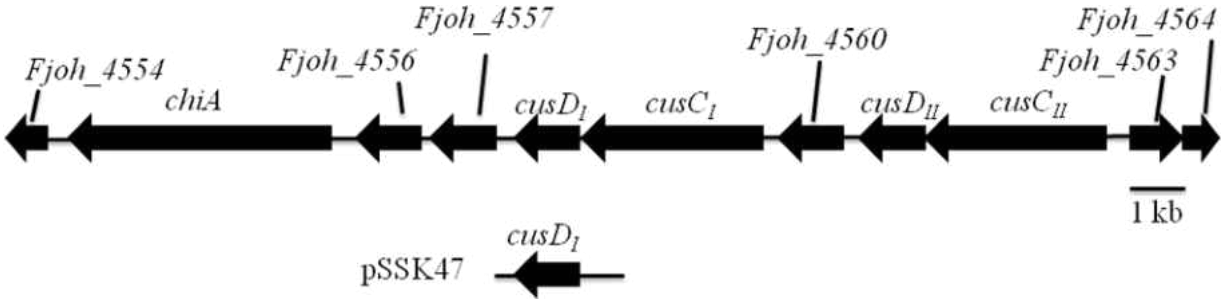

Figure 32. Map of region spanning *susC*-like genes (*cusC$_I$, cusC$_{II,}$*) and *susD*-like genes (*cusD$_I$, cusD$_{II,}$*). Bar indicates one kilobase pair sequences.

The SusC-like and SusD-like proteins appear to function in chitin utilization. Single and multiple deletion mutants were constructed to determine the roles of *F. johnsoniae susC-like* and *susD-like* genes in the putative chitin PUL. The ability to utilize chitin was tested in Stanier broth supplemented with colloidal chitin. Cells of CJ2121 (Δ*cusD$_I$*) and CJ2156 (Δ*cusD$_I$* Δ*cusD$_{II}$*) were partially defective in chitin utilization (Figure 33). In contrast cells with single deletions in CJ2086 (*cusC$_I$*), CJ2340 (*cusC$_{II}$*) and CJ2018 (*cusD$_{II}$*) appeared to utilize chitin as well as the wild type cells. Cells of the *chiA* mutant were completely defective in chitin utilization as reported in chapter 2. In contrast, cells of CJ2350 (ΔFjoh_4560) predicted to encode another chitinase appeared to grow on chitin as well as the wild-type (Figure 33). A strain lacking all of the *susC-like* and *susD-like* genes of the chitin PUL was also generated. Cells of this Δ(*cusD$_I$* to *cusC$_{II}$*) mutant CJ2349, appeared to be completely deficient in growth on chitin (Figure 33). This strain also lacked Fjoh_4560 which as shown above appears not to be essential for chitin

utilization. The cells of Δ(*cusD*$_I$ to *cusC*$_{II}$) mutant appeared not to grow at all in Stanier broth supplemented with chitin, as compared to the small amount of growth exhibited by the (Δ*cusD*$_I$ Δ*cusD*$_{II}$) mutant cells. This suggests that the SusD-like and SusC-like proteins may both have roles in chitin utilization.

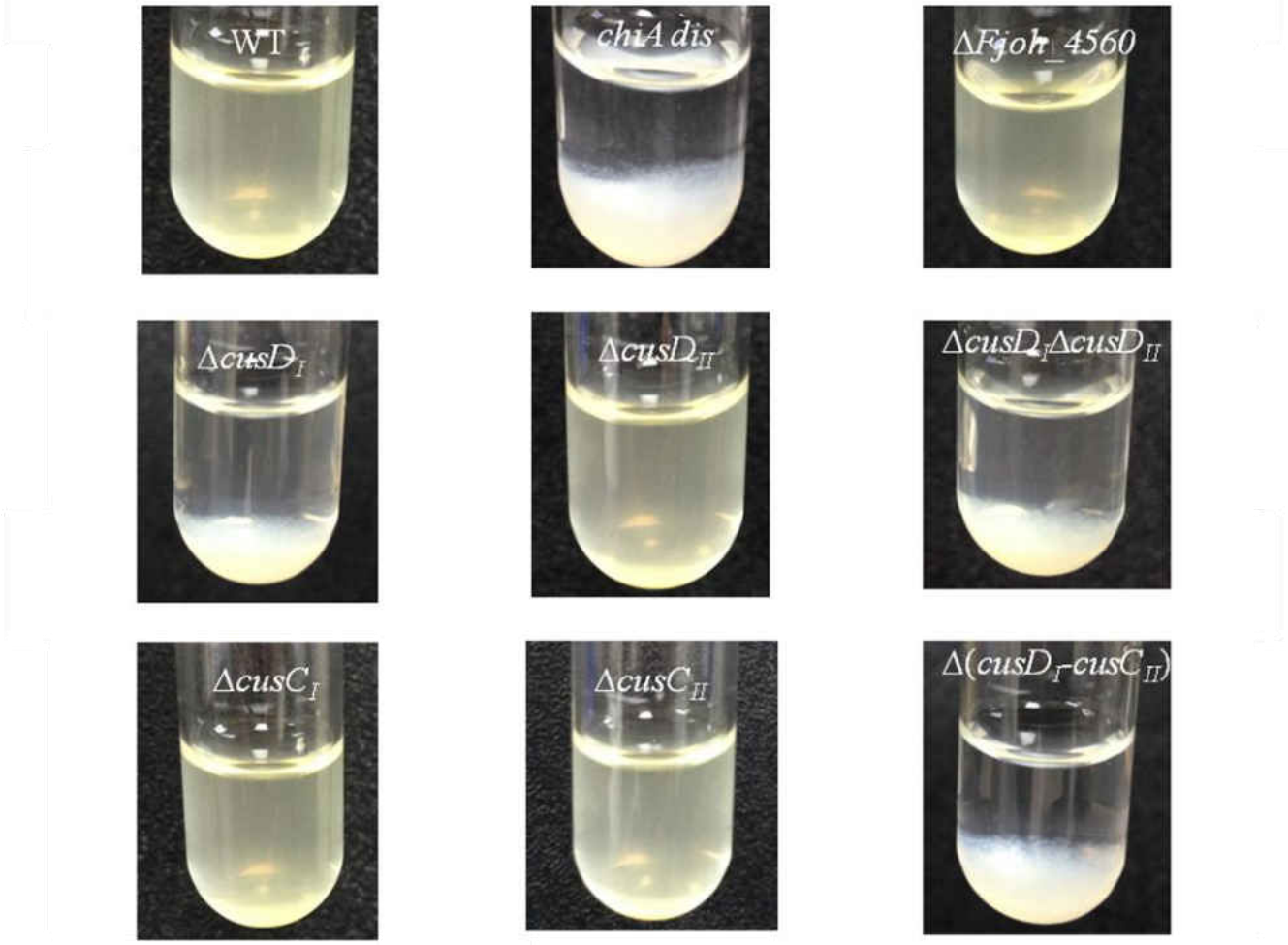

Figure 33. SusC-like and SusD-like proteins appear to function in chitin utilization. Cells of wild type, *chiA* mutant CJ1808, ΔFjoh_4560 mutant CJ2350, Δ*cusD*$_I$ mutant CJ2121, Δ*cusD*$_{II}$ mutant CJ2018, Δ*cusD*$_I$Δ*cusD*$_{II}$ mutant CJ2156, Δ*cusC*$_I$ mutant CJ2086, Δ*cusC*$_{II}$ mutant CJ2340 and Δ(*cusD*$_I$ to *cusC*$_{II}$) mutant CJ2349 were inoculated into Stanier media supplemented with chitin (white) and incubated at 25°C for 48h. Tubes with the Δ*cusD*$_I$ mutant and Δ*cusD*$_I$ Δ*cusD*$_{II}$ mutant appear to exhibit minimal turbidity in the broth (indicating bacterial cells) and chitin (white) settled at the bottom. In the tubes of *chiA* mutant, Δ*cusD*$_I$ mutant, Δ*cusD*$_I$Δ*cusD*$_{II}$ mutant, and Δ(*cusD*$_I$ to *cusC*$_{II}$) mutant chitin (white) can be seen at the bottom of the tubes.

Discussion

F. johnsoniae, a member of the phylum *Bacteroidetes,* digests the insoluble polymer chitin (13, 19). Other members of the phylum *Bacteroidetes* are thought to employ outer membrane SusC-like and SusD-like proteins in polymer utilization (14, 15, 19, 26). Analysis of *B. thetaiotaomicron,* starch utilization resulted in the Sus paradigm for polysaccharide utilization by members of the phylum *Bacteroidetes* (16). SusD has been shown to bind starch and its oligomers on the cell surface and SusC is thought to actively transport the starch oligomers across the outer membrane. The oligomers are further digested in the periplasm (16, 26). The results presented in this chapter elucidate the roles of *F. johnsoniae* SusC-like and SusD-like proteins in chitin utilization. Cells of Δ*cusD*$_I$ and Δ*cusD*$_I$ Δ*cusD*$_{II}$ mutants were defective in chitin utilization. Cells with individual mutations in *cusC*$_I$, *cusC*$_{II}$ and *cusD*$_{II}$ appear to utilize chitin as well as the wild-type cells. However, when the region spanning *cusD*$_I$ to *cusC*$_{II}$ was deleted the ability to utilize chitin appeared to be completely lost. This suggests redundancy between some of the genes in this region. Quantitative analysis to compare the growth observed in the cells of Δ*cusD*$_I$ and Δ*cusD*$_I$ Δ*cusD*$_{II}$ mutants may further elucidate redundant roles in chitin utilization between CusD$_I$, and CusD$_{II}$. Additionally, construction of a double deletion mutant lacking both *cusC*$_I$, and *cusC*$_{II}$ is needed to specifically determine whether CusC$_I$ and CusC$_{II}$ have redundant roles in chitin utilization. Complementation of the Δ(*cusD*$_I$-*cusC*$_{II}$) mutant with individual genes or combinations of these genes could also help to determine the roles of individual proteins in chitin utilization.

In *B. thetaiotaomicron* a regulatory gene that controls expression of the *sus* genes is included in the PUL (26). Genes (Fjoh_4563, Fjoh_4564) predicted to encode the components of a two-component signal transduction system are located immediately upstream of the *F. johnsoniae* chitin PUL. Further study will be needed to determine whether the proteins encoded by these genes regulate expression of the chitin PUL. Moreover the genes *susA* and *susB* the *B. thetaiotaomicron* in starch PUL encode hydrolytic enzymes that are thought to further hydrolyse starch oligomers internally (periplasmic). Fjoh_4556 and Fjoh_4560 are predicted to encode hydrolytic enzymes that might perform the function of further hydrolyzing the internalized chito-oligomers.

The Sus paradigm apparently plays a role in *F. johnsoniae* chitin utilization. Based on the observed results a model for chitin utilization in *F. johnsoniae* is presented (Figure 34). In this model the extracellular chitinase ChiA (discussed in chapter 2) partially digests chitin to produce chito-oligomers that bind to $CusD_I$ and $CusD_{II}$. The bound oligomers may be actively internalized by the $CusC_I$ and $CusC_{II}$ porins. The oligomers could be hydrolyzed further in the periplasm by hydrolytic enzymes predicted to be encoded by Fjoh_4556 and Fjoh_4557. A key feature of Sus-like systems is the coordinated action of several gene products involved in substrate binding, uptake and degradation (16). *F. johnsoniae* $CusC_I$, $CusC_{II}$, $CusD_I$, and $CusD_{II}$ may work synergistically with ChiA to allow efficient utilization of the insoluble polymer chitin.

Insoluble polymers such as chitin and cellulose are thought to be the most abundant polymers on earth (3, 11, 12). These polymers play structural roles in plants, invertebrates and fungi, and they are resistant to digestion. Flavobacteria and related

bacteria are abundant in many environments and presumably contribute to turnover of these polysaccharides. Knowledge of the mechanisms used to utilize insoluble polysaccharides will improve our understanding of carbon turnover in nature. Polysaccharide digesting intestinal bacteria are also beneficial to human health. Understanding the molecular mechanisms underlying insoluble polymer digestion may enable the manipulation of intestinal polysaccharide digesting bacteria to maintain human health. Improved understanding of mechanisms for digestion of insoluble polysaccharides may also allow development of strategies to efficiently convert biomass to biofuels (10). In summary, an improved understanding of the mechanisms involved in the utilization of chitin and other insoluble polysaccharides could have positive biotechnological, economical and human health implications.

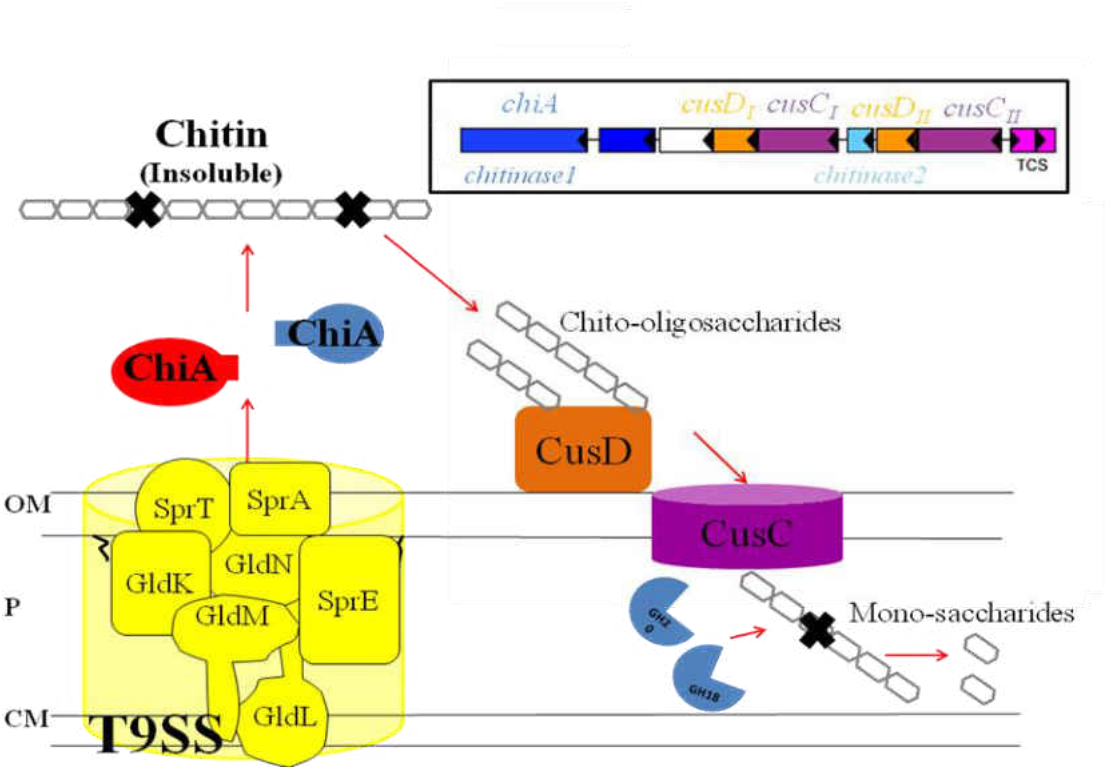

Figure 34. Model for *F. johnsoniae* chitin utilization. Extracellular ChiA (Red and blue) cut chitin into long oligomers. Chitin oligomers bind to SusD-like proteins $CusD_I$ and $CusD_{II}$ (orange) on cell surface and are actively transported (TonB dependent proton pump not shown) across the outer membrane through the SusC like porins $CusC_I$ and $CusC_{II}$ (purple). Other enzymes (light blue) digest the oligomers in the periplasm or cytoplasm. OM-outer membrane, P-periplasm and CM-cytoplasmic membrane. **X**- hydrolysis. The inset shows the chitin utilization locus. TCS-genes encoding the components of the the two component signal transduction system. Modified from (19).

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Chapter 5. Summary

Flavobacterium johnsoniae, a member of phylum *Bacteroidetes*, is a gliding bacterium that digests chitin. A novel protein secretion system Type IX secretion system (T9SS) secretes motility adhesins, SprB and RemA. Based on the results presented in this thesis, a model for protein secretion and chitin utilization machinery in *F. johnsoniae* is hypothesized (Figure 35). ChiA, discussed in chapter 2, is a major extracellular soluble chitinase required for chitin utilization. The novel carboxy terminal domain (CTD) of ChiA appears to target ChiA and foreign proteins to the T9SS. Chapter 3 focuses on PorV, an accessory protein of the T9SS required for the secretion of RemA, ChiA and some other proteins secreted via the T9SS. PorV appears to be required for the secretion of proteins with conserved (TIGRFAM 04183) and novel CTDs. In chapter 4, a polysaccharide utilization locus likely to be involved in chitin utilization is presented. SusC-like (TonB dependent porin) proteins and SusD-like (glycan binding) proteins appear to function in chitin utilization. A model has been hypothesized, ChiA secreted via the T9SS cuts the chitin into long oligomers or chito-oligosaccharides. These oligomers appear to bind SusD-like protein CusD. The oligomers are internalized via the SusC-like protein CusC. Chitoligomers are further hydrolysed by hydrolytic enzymes internally. ChiA appears to work with CusC and CusD synergistically to efficiently utilize chitin.

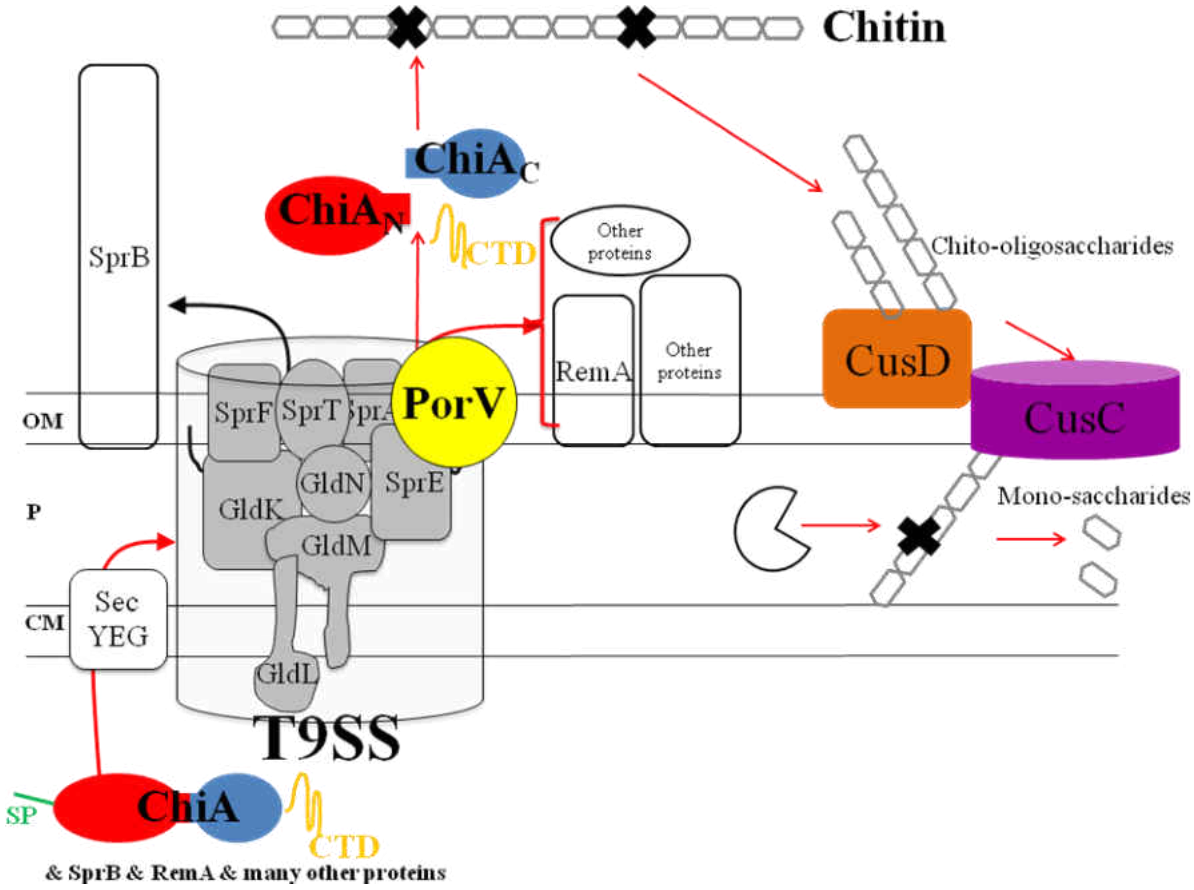

Figure 35. Model of *F. johnsoniae* secretion and chitin utilization machinery based on the results presented in this thesis. In addition to the secretion of motility adhesin SprB and RemA, the major extracellular chitinase ChiA (Red and blue) is secreted by the T9SS. PorV (yellow) is an accessory protein of the T9SS required for the secretion of RemA, ChiA and some other proteins that are secreted via the T9SS. ChiA appears to perform limited hydrolysis of chitin into chito-oligosaccharides, that bind to SusD-like proteins CusD (orange). The oligosaccharides are internalized via the SusC like porins CusC (purple). Chitooligomers are further hydrolyzed internally by hydrolytic enzymes. ChiA appears to work with CusC and CusD protein to allow efficient utilization of chitin. OM-outer membrane, P-periplasm and CM-cytoplasmic membrane. **X**- hydrolysis.

Appendix 1. Analysis of the carboxy terminal domain (CTD) of ChiA required for secretion by the T9SS.

As discussed in chapter 2 the novel carboxy-terminal domain (CTD) of ChiA is necessary and sufficient for secretion by the T9SS (2). Proteins secreted by T9SSs typically have conserved CTDs belonging to the TIGRFAM families, TIGR04131 or TIGR04183 (2). The ChiA CTD does not belong to either of these families but instead is novel. Removal of the C-terminal 106 amino acids of ChiA resulted in accumulation of ChiA inside of cells. Fusion of the C-terminal 105 amino acids of ChiA to recombinant mCherry resulted in secretion of mCherry into the media. To further ascertain the minimal size required for secretion of proteins by the T9SS, the C-terminal 80 amino acids of ChiA was fused to recombinant mCherry and analyzed.

A plasmid expressing the N-terminal region of ChiA (encoding the signal peptide) fused to mCherry and to the C-terminal 80 amino acids of ChiA ($CTD_{ChiA80aa}$) was constructed (Table 13 and 14). A 708 bp region of mCherry was amplified from pME-mCherry using primer 862 (engineered BamHI site) and primer 1266 (engineered XbaI site). This product was digested with BamHI and XbaI and cloned into pCP23 that had been digested with the same enzymes, generating pSSK30. A 484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region was amplified using primer 1593 (engineered KpnI site) and primer 1516 (engineered BamHI site). The product was inserted into KpnI and BamHI digested pSSK30 to generate pSSK51. To introduce the CTD-encoding region, a 491 bp region was amplified using primer 1599 (engineered XbaI site) and primer 1404 (engineered SphI site). The product

was cloned into pSSK51, to generate pSSK53. pSSK53 was conjugated into wild type and Δ*gldNO* mutant cells to generate CJ2434 and CJ2435 respectively.

Secretion of mCherry was examined in wild type and Δ*gldNO* mutant cells expressing mCherry fused to C-terminal 105 and 80 amino acid regions of ChiA. mCherry fused to the C-terminal 105 amino acids of ChiA was efficiently secreted from wild type cells, but was not secreted from cells of the T9SS (Δ*gldNO*) mutant. When the C-terminal 80 amino acids of ChiA were fused to mCherry the protein was also seen in the cell free culture fluid of cells of the wild-type, but at a much lower level. This secretion required the T9SS because no mCherry was seen in the culture fluid of cells of the Δ*gldNO* mutant expressing this protein. When mCherry was expressed without the ChiA CTD it did not accumulate in the spent medium; rather, mCherry accumulated in the cells (Figure 36). The results suggest that the 80 C-terminal amino acids region is sufficient to allow some secretion by the T9SS but the inclusion of the an additional 25 amino acids appear to result in more efficient secretion. Additional studied are needed to define the minimal ChiA CTD region and further identify the features that result in optimal secretion by the T9SS. Since the ChiA CTD is novel, similar studies will also be needed to be performed with other CTDs belonging to TIGRFAM families, TIGR04183 (such as RemA) and TIGR04131 (such as SprB) to gain insights into general features required for secretion.

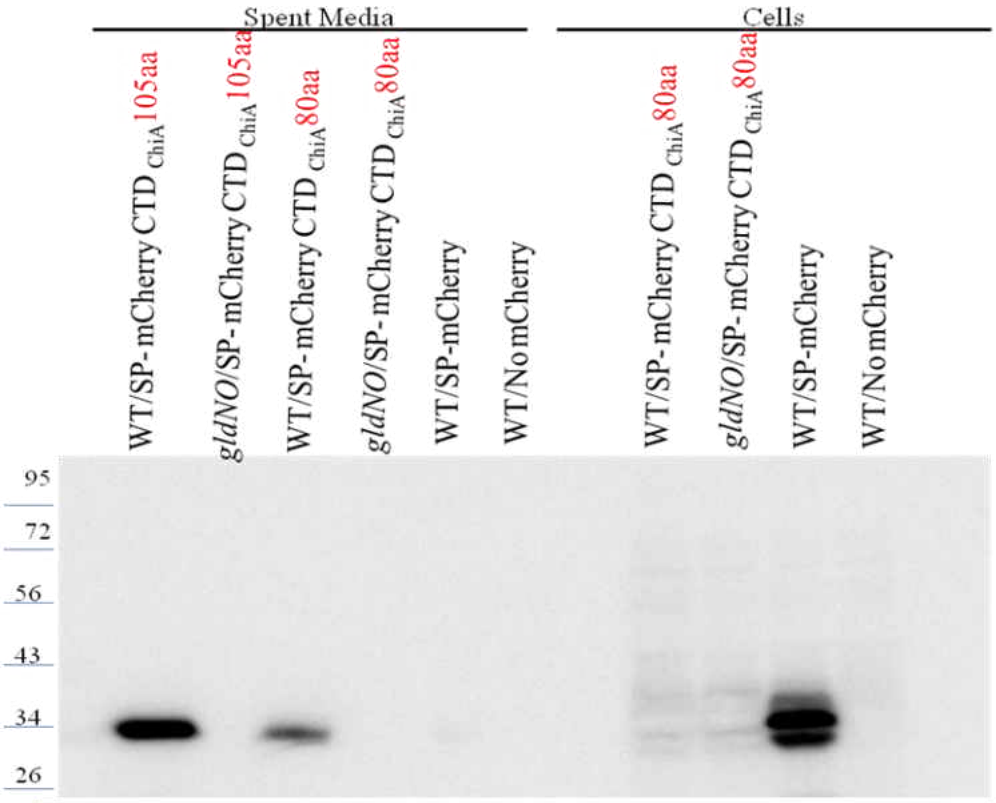

Figure 36. 80 amino acid of ChiA CTD is sufficient for secretion of the heterologous protein mCherry. Cell-free spent media and whole cells were analyzed for cultures of wild-type (WT) cells carrying pSSK54 that expresses mCherry with the N-terminal signal peptide from ChiA (SP-mCherry), or carrying pSSK52 that expresses mCherry with the N-terminal signal peptide from ChiA and the 105 amino acid C-terminal region of ChiA (SP-mCherry-CTD_{ChiA}) or carrying pSSK53 that expresses mCherry with N-terminal signal peptide from ChiA and the 80 amino acid C-terminal region of ChiA. Cells and spent media from cultures of the T9SS mutant CJ1631A (Δ*gldNO*) carrying pSSK52 and pSSK53 were also analyzed. 'No mCherry' indicates spent media or cells from *F. johnsoniae* carrying the control vector pCP23. Cell samples corresponded to 15 µg protein per lane and samples from spent media corresponded to the volume of spent media that contained 15 µg cell protein before the cells were removed. Samples were separated by SDS-PAGE and mCherry was detected using antiserum against mCherry.

Table 13. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or description	Source or reference
F. johnsoniae strains		
ATCC 17061 strain UW101	Wild type	(4, 5)
CJ1631A	Δ(*gldN gldO*)	(7)
CJ1827	Strain used for construction of deletion mutants; *rpsL2*; (Sm^r)	(6)
Plasmids		
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; $Ap^r(Tc^r)$	(1)
pME-mCherry	Plasmid expressing fluorescent protein mCherry; Km^r	(3)
pSSK45	*mcherry* with stop codon amplified with primers 862 and 1443 and cloned into pCP23; Ap^r (Tc^r)	(2)
pSSK51	484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region inserted into pSSK30; Ap^r (Tc^r)	(2)
pSSK52	566 bp region encoding 105 amino acid CTD_{ChiA} inserted into pSSK51; Ap^r (Tc^r)	(2)
pSSK53	491 bp region encoding 80 amino acid CTD_{ChiA} inserted into pSSK51; Ap^r (Tc^r)	This study
pSSK54	484 bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide encoding region inserted into pSSK45; Ap^r (Tc^r)	(2)

Table 14. Primers used in this study

Primers	Sequence and Description
1404	5'-GCTAGGCATGCTCACCTAATACAATAACTAACCTC-3'; Reverse primer to amplify *chiA* CTD for making construct pSSK52; SphI site underlined.
1516	5'-GCTAGGGATCCCACTACTTTTTTCCCGTGGGCTGGCTG -3'; Reverse primer to amplify short N-terminal region of *chiA* to construct pSSK52 and pSSK54; BamHI site underlined.
1593	5'- GCTAGGGTACCTTCCCCGGTAGAGATAGTTATGGCTAT -3' Forward primer to amplify N-terminal region of *chiA* to make constructs pSSK52,,and pSSK54; Binds 400 bp upstream of *chiA* start codon; KpnI site underlined.
1599	5' GCTAG TCTAGA GCAACGATAGCTTATTTTAAAAACAAT -3' forward primer to amplify *chiA* CTD (80 aa) region for making construct pSSK53; XbaI site underlined
1600	5'GCTAGTCTAGAGCTTATGCAGCTTATTTCGCATCACAA -3' forward primer to amplify *chiA* CTD (105 aa) region for making construct pSSK52; XbaI site underlined

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Appendix 2. Protocol for making 2% w/v colloidal chitin slurry

1. Suspend 20 g Chitin in 400 ml HCl (~32%).
2. Stir the mixture for 20-30 min until black colloidal solution appears.
3. Pour into 5 L of ice-cold deionized water. The solution will turn white.
4. Continue stirring for 10 min.
5. Centrifuge at 8,000 rpm for 5 min and remove supernatant.
6. Resuspend the pellet in deionized water.
7. Repeat steps 5-6 for 5 times. A pH value of about 4 will be attained.
8. Adjust the pH to 7 with NaOH.
9. Dilute the volume 1 L (final concentration 2% (w/v))

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