

A holin and an endopeptidase are essential for chitinolytic protein secretion in *Serratia marcescens*

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Pathogenic bacteria adapt to their environment and manipulate the biochemistry of hosts by secretion of effector molecules. *Serratia marcescens* is an opportunistic pathogen associated with healthcare-acquired infections and is a prolific secretor of proteins, including three chitinases (ChiA, ChiB, and ChiC) and a chitin binding protein (Cbp21). In this work, genetic, biochemical, and proteomic approaches identified genes that were required for secretion of all three chitinases and Cbp21. A genetic screen identified a holin-like protein (ChiW)

and a putative L-alanyl-D-glutamate endopeptidase (ChiX), and subsequent biochemical analyses established that both were required for nonlytic secretion of the entire chitinolytic machinery, with chitinase secretion being blocked at a late stage in the mutants. In addition, live-cell imaging experiments demonstrated bimodal and coordinated expression of *chiX* and *chiA* and revealed that cells expressing *chiA* remained viable. It is proposed that ChiW and ChiX operate in tandem as components of a protein secretion system used by gram-negative bacteria.

Introduction

Targeting of proteins to their sites of physiological function is an essential process in all cellular systems. Secretion of specific proteins completely out of the cell is a key process that bacteria use to adapt to and modulate their immediate environment (Trost et al., 2005). Six protein targeting and secretion systems have been identified in gram-negative bacteria, named type 1–type 6 (Economou et al., 2006). In general, protein secretion can occur either in a single step, in which substrates are secreted directly from cytoplasm to the cell exterior, or by a two-step process, in which substrates are first targeted to the periplasm and are then translocated across the outer membrane in a secondary transport event.

Serratia marcescens is an opportunistic pathogen thought to be a significant cause of healthcare-acquired infections (Mahlen, 2011). This bacterium is a prolific secretor of proteins, many of which include virulence factors such as phospholipases, proteases, and nucleases (Hines et al., 1988). In addition, *S. marcescens* secretes three extracellular chitinases (ChiA, ChiB, and ChiC) and one extracellular chitin binding protein

(Cbp21). This chitinolytic machinery allows *S. marcescens* to use chitin as a carbon and nitrogen source for growth (Hejazi and Falkiner, 1997). Moreover, chitinases from bacterial pathogens, together with host chitinase-like proteins, are thought to have increasingly important roles in infection (Tran et al., 2011; Frederiksen et al., 2013), including in the inflammation process (Lee et al., 2011), where the bacterial chitinases and chitin binding proteins are believed to facilitate the initial attachment of bacteria onto host cells (Tran et al., 2011; Frederiksen et al., 2013).

The mechanism of secretion of the components of the *S. marcescens* chitinolytic machinery has not been reported. The *S. marcescens* ChiA and Cbp21 proteins are both predicted to be synthesized as precursors with N-terminal Sec signal peptides (Brurberg et al., 1996; Vaaje-Kolstad et al., 2005a), and as a result, it may have been assumed that they were externalized by a type 2 secretion system, in a similar fashion to the chitinase from *Escherichia coli* (Francetic et al., 2000). Interestingly, the primary sequences of the other chitinases (ChiB and ChiC)

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Abbreviations used in this paper: DIC, differential interference contrast; LB, Luria Bertani; Tat, twin-arginine translocation.

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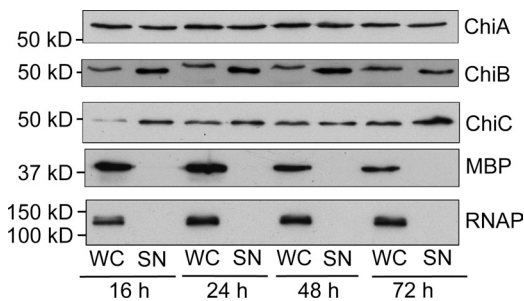


Figure 1. *S. marcescens* chitinases are extracellular proteins. *S. marcescens* Db10 was grown aerobically in rich medium. At the time points indicated, an aliquot was taken and separated into culture supernatant (SN) and whole cells (WC) by centrifugation. Samples were analyzed for the presence of ChiA, ChiB, and ChiC by Western immunoblotting. The locations of a periplasmic control protein (maltose binding protein [MBP]) and a cytoplasmic control protein (RNA polymerase [RNAP]) are shown.

contain no canonical signal peptides or other obvious targeting signals (Brurberg et al., 1995; Suzuki et al., 1999), suggesting that these proteins may be secreted by an alternative route. Indeed, the homologous ChiC protein from *Pseudomonas aeruginosa* was previously implied to be secreted by an unknown mechanism, distinct from the then-characterized systems in that bacterium (Folders et al., 2001).

In this work, the molecular basis of chitinase secretion by *S. marcescens* was studied. A genetic screen initially identified a gene predicted to encode a holin-like integral membrane protein that was shown to be required for secretion of the entire chitinolytic machinery. The holin-like protein is encoded in an apparent four-cistron operon—*chiWXYZ*—that shares similarity with bacteriophage lysis cassettes. The *chiX* gene encodes a putative L-alanyl-D-glutamate endopeptidase that is also essential for chitinase secretion. The phenotype of a Δ *chiW* strain was rescued by expression of a modified version ChiX routed to the periplasm via the twin-arginine translocation (Tat) pathway, suggesting that ChiX normally operates in the periplasm and is targeted there by ChiW. Live-cell imaging experiments revealed bimodal and coordinated expression for *chiX* and *chiA* and demonstrated that cells expressing chitinase remained viable. Together, *chiW* and *chiX* form part of a genetic locus dedicated to chitin metabolism, and it is proposed that they represent components of a protein secretion system used by gram-negative bacteria.

Results

Chitinases are secreted proteins

Initially, the subcellular localization of the *S. marcescens* chitinases was clarified (Fig. 1). *S. marcescens* was cultured aerobically in liquid medium before whole cells and cell-free supernatants were analyzed for the presence of the native chitinases by Western immunoblotting (Fig. 1). ChiA, ChiB, and ChiC were clearly detectable in the culture supernatant over the duration of the experiment (Fig. 1). In contrast, neither the cytoplasmic control protein RNA polymerase nor the normally periplasmic maltose binding protein could be detected in the extracellular milieu, even after an extended 72 h of growth (Fig. 1). It must be concluded that all three chitinases are deliberately secreted from the bacterial cell.

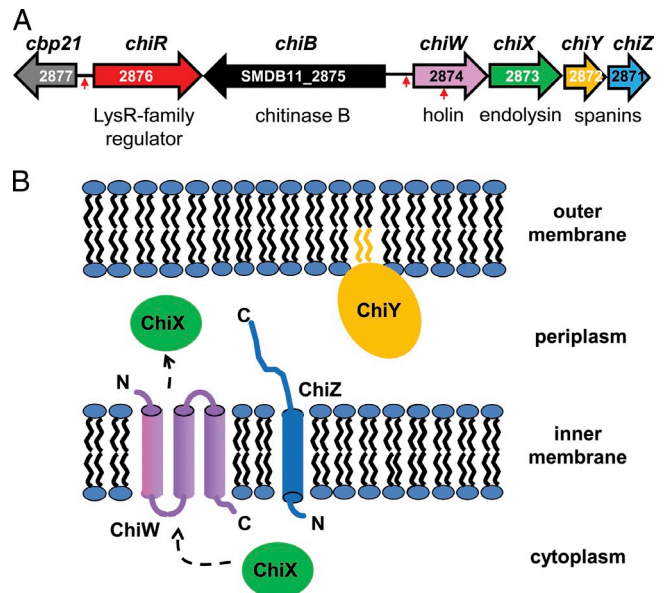


Figure 2. The genetics of chitinase secretion in *S. marcescens*. (A) A cartoon representing the organization of the *S. marcescens* Db10/11 chromosome around region 3,031,956–3,036,024 bp. The red arrows indicate the positions of three transposon insertion sites found to disrupt ChiC secretion. One was located 129 bp upstream of *chiR* (SMDB11_2876), one was 23 bp upstream of *chiW* (SMDB11_2874), and one was at position 165 within *chiW*. (B) Cartoon depicting the predicted subcellular localizations and topologies of the *chiWXYZ* gene products. ChiW is a member of the phage holin-3 family and is predicted to span the membrane three times with an N terminus-out, C terminus-in topology. The predicted endopeptidase ChiX does not have a classical signal peptide and is hypothesized to reach the periplasm via the ChiW holin. The ChiY protein is synthesized with a classical N-terminal type II signal peptide, which is typical of outer membrane lipoproteins. The ChiZ protein is predicted to have a single N-terminal transmembrane domain and span the membrane with an N terminus-in, C terminus-out topology. N, N terminus; C, C terminus.

Identification of a locus required for ChiC secretion

Arguably the most well-studied secretion system is the general secretory pathway or type 2 secretion system (Korotkov et al., 2012). Inspection of the *S. marcescens* Db10/Db11 genome sequence (Iguchi et al., 2014) did not reveal any gene products that would encode typical components of a type 2 secretion system. Furthermore, chitinases ChiB and ChiC display no obvious targeting Sec-type sequences. Thus, to select for mutant strains defective in chitinase secretion, a genetic screen was designed. The initial focus was placed on ChiC and an *S. marcescens* mutant, JchiC (Δ *chiA* and Δ *chiB*), was constructed that would produce only ChiC. Random transposon mutagenesis was used to screen the JchiC (Δ *chiA* and Δ *chiB*) strain for mutants that could no longer degrade extracellular chitin. Eight transposon mutant strains devoid of chitinase activity were isolated (Fig. S1). To differentiate mutants defective in *chiC* expression from those defective in ChiC secretion, the transposon mutants were analyzed for the presence of ChiC in cellular and secreted/supernatant fractions by Western immunoblotting (Fig. S1). This approach revealed that three of the original transposon insertion mutants retained the ability to produce ChiC to normal levels but were apparently completely blocked in their ability to secrete the enzyme into the culture supernatant (Fig. S1).

The locations of the transposon insertions in the three secretion-defective mutant strains were mapped with reference to the genome sequence of *S. marcescens* Db11 (Iguchi et al., 2014), which is a spontaneous streptomycin-resistant derivative of the Db10 strain used in this study. Two of the transposon insertions were mapped to different locations near the same gene (*SMDB11_2874*) and the third was located near *SMDB11_2876*, which had been previously identified as *chiR* (Fig. 2 A; Suzuki et al., 2001). The *chiR* gene encodes a transcriptional regulator of the LysR family thought to be important for expression of chitinase activity (Suzuki et al., 2001); however, *SMDB11_2874* had not been previously studied. Further inspection of the *S. marcescens* Db11 genome sequence revealed that *chiR* and *SMDB11_2874* are located adjacent to *chiB* on the *S. marcescens* chromosome (Fig. 2 A). The *SMDB11_2874* gene is therefore part of a genetic locus dedicated to chitin metabolism and was named *chiW* (Fig. 2). The *chiW* gene encodes a predicted inner membrane protein of the phage holin-3 family. The archetypal member of this protein family is the bacteriophage λ holin S (Gründling et al., 2000). The *chiW* gene is located in an apparent four-cistron operon with a gene encoding a predicted metal-dependent L-alanyl-D-glutamate endopeptidase and two genes encoding homologues of bacteriophage spanins (Fig. 2), similar to a λ lysis cassette (Summer et al., 2007), and the operon was named *chiWXYZ* (Fig. 2).

Proteomics reveals all chitinolytic proteins require ChiW for secretion

Next, it was decided to take a whole systems approach to understand the involvement of ChiW in general protein secretion. First, a new deletion strain (JJH08p) was made that carried an unmarked complete deletion of *chiW*. Total protein extracts of culture supernatant (secretomes) were then prepared from the *S. marcescens* Db10 parental strain and the JJH08p *chiW* mutant. Label-free quantitative proteomics was then used to profile changes in protein abundance in the corresponding secretomes. Four biological replicates of secretome digests of both strains were analyzed by high-resolution mass spectrometry in a mass spectrometer (Orbitrap Velos Pro; Thermo Fisher Scientific), and data were processed through MaxQuant (Cox and Mann, 2008). Using strict filtering, 497 proteins (<1% false discovery rate) were identified, of which 351 showed good reproducibility and were quantified in at least two of the four replicates for each strain.

Although the relative levels of almost all proteins identified by this method remained unaffected by the *chiW* deletion, a subset of 10 proteins showed, with high confidence ($P < 0.01$), a decrease in abundance in the secretome of the mutant compared with the parent strain (Table 1). Strikingly, this group of 10 proteins included not only ChiC but also ChiA, ChiB, and Cbp21 (Table 1). In addition, these four chitinolytic proteins were by far the most abundant of the 10 whose extracellular levels were found to change in this experiment (Fig. 3, Table 1, and Fig. S3). Similarly, only a small number of mostly extracellular proteins, predominantly fimbrial subunits, were found to increase in abundance in the secretome of the *chiW* mutant (Table S1).

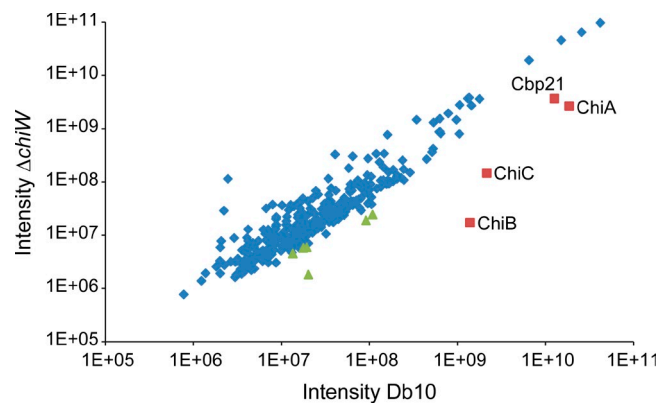


Figure 3. **Intensity scatter plot of proteome results.** Label-free intensities of secretome proteins (mean of four biological replicates) of the Δ *chiW* (JJH08p) strain versus the secretome of the Db10 parental strain. Highly abundant proteins that were reduced significantly ($P < 0.01$) in the Δ *chiW* secretome are labeled red with the locations of the other, lower abundance, proteins shown in green (see Table 1). The three chitinases, ChiA, ChiB, and ChiC, as well as the chitin-binding protein, Cbp21, are the by far the most abundant of the proteins affected by the Δ *chiW* mutation.

Genetic dissection of the *chiWXYZ* locus

To understand the roles of all the members of the *chiWXYZ* locus, a bank of four in-frame deletion strains was constructed, and Western immunoblotting was then used to assess the localization of the three chitinases (Fig. 4 A). In line with the proteomics data (Fig. 3 and Table 1), deletion of the *chiW* gene deleteriously affected the extracellular levels of all three *S. marcescens* chitinases (Fig. 4 A). In addition to *chiW*, it was revealed that ChiX, a putative metal-dependent L-alanyl-D-glutamate endopeptidase, was also essential for secretion of the three known chitinases (Fig. 4 A). However, neither of the genes encoding the spanin-like proteins (*chiY* and *chiZ*) appeared individually essential for chitinase secretion (Fig. 4 A).

Both the Δ *chiW* and the Δ *chiX* mutant phenotypes could be complemented in trans (Fig. 4, B and C). The intact *S. marcescens* *chiW* and *chiX* genes were amplified by PCR and cloned under the control of the constitutive *tat* promoter from *E. coli* in plasmid pUNI-PROM (Jack et al., 2004). Transformation of the either the Δ *chiW* (Fig. 4 B) or Δ *chiX* mutant (Fig. 4 C) strain with the empty vector did not restore secretion of ChiC. However, plasmid-driven production of the holin-like protein in Δ *chiW* (Fig. 4 B) or the endopeptidase in Δ *chiX* (Fig. 4 C) rescued secretion of the ChiC chitinase.

Evidence that ChiW and ChiX operate in tandem to facilitate chitinase secretion

Although sequence analysis suggests ChiX could be an L-alanyl-D-glutamate endopeptidase, which would require a periplasmic localization to access peptidoglycan substrate, this protein is not synthesized with an obvious signal peptide. One hypothesis, consistent with the similarity of the *chiWXYZ* system to phage lysis cassettes, is that the role of the ChiW holin could be to allow ChiX access to the periplasm. To test this hypothesis, an experiment was designed in which ChiX was targeted to the periplasm via an alternative route, thus bypassing any requirement for ChiW (Fig. 5 A).

Table 1. Extracellular proteins secreted in a ChiW-dependent manner identified by label-free mass spectrometry

Identifier	Name	Sec signal peptide	Description	Intensity (relative intensity ^a)	Relative abundance Db10/ Δ chiW	P-value	Unique peptides	Sequence coverage
SMDB11_2875	ChiB	No	Chitinase B	8.5×10^9 (92)	80.7	1.32×10^{-8}	11	32
SMDB11_0468	ChiC	No	Chitinase C	1.4×10^{10} (152)	14.7	3.08×10^{-5}	21	54
SMDB11_3897	–	No	Pirin	1.3×10^8 (1.4)	11.2	3.08×10^{-4}	5	26
SMDB11_4243	ChiA	Yes	Chitinase A	1.2×10^{11} (1,304)	7.0	1.42×10^{-7}	33	69
SMDB11_1933	TrpE	No	Anthranilate synthase	5.5×10^8 (6)	4.8	2.26×10^{-5}	12	26
SMDB11_4522	MdhA	No	Malate dehydrogenase	7.4×10^8 (8)	3.4	1.94×10^{-5}	9	36
SMDB11_2877	Cbp21	Yes	Chitin binding protein	6.7×10^{10} (728)	3.4	0.0054	10	73
SMDB11_2870	MaeB	No	NADP-dependent malic enzyme	1.3×10^8 (1.4)	3.3	0.00032	11	18
SMDB11_2390	–	No	Putative heme oxygenase	1.1×10^8 (1.2)	3.1	0.0044	4	25
SMDB11_3147	ProS	No	Prolyl-tRNA synthetase	9.2×10^7 (1)	3.0	6.33×10^{-5}	9	23

Proteins identified as present in the secretome of the *S. marcescens* parental strain (Db10) at an abundance greater than 3x higher than observed in the secretome of a strain (JH08p) lacking the *chiW* gene ($P < 0.01$) are shown. Four biological replicates of each strain were analyzed. The most abundant proteins in this list are highlighted in bold. Minus signs indicate that these gene products have no name.

^aRelative intensity is calculated in relation to the intensity of least abundant protein in the list (prolyl-tRNA synthetase). Additional mass spectrometry data showing the proteins in the secretome of the Δ chiW strain, which are increased in abundance relative to the secretome of the Db10 parental strain, are shown in Table S1.

A set of plasmids was prepared, based on the L-arabinose-inducible pBAD18 system (Guzman et al., 1995), which allowed controlled production of ChiX, or ChiX fused to the Tat signal peptide of *S. marcescens* SufI (SMDB11_3496), in the *S. marcescens* host. The bacterial Tat pathway is a general protein export system for the transport of folded proteins across the cytoplasmic membrane and was chosen here because ChiX has a predicted metal-binding motif, as have most natural Tat substrates (Palmer and Berks, 2012). A Δ chiW strain, blocked for chitinase secretion, was transformed with the inducible plasmids (Fig. 5 B). Induction of *chiX* alone could not restore chitinase secretion to a Δ chiW strain (Fig. 5 B). Very interestingly, however, production of the spSufI::ChiX fusion was able to rescue secretion of ChiC in the Δ chiW strain (Fig. 5 B). It can be concluded from this that periplasmically located ChiX is required for chitinase secretion and that ChiW may normally play some role in targeting ChiX to this compartment.

Evidence for a two-step pathway for chitinase secretion

It is clear that genetic inactivation of ChiW and ChiX impaired chitinase secretion from the cell; however, it was of interest to determine the subcellular location of mislocalized enzymes in the mutant strains. To address this, the periplasmic, cytoplasmic, and total membrane fractions of the parental strain and the Δ chiW mutant were prepared, separated by SDS-PAGE, and analyzed by Western immunoblotting (Fig. 4 D). Probing with periplasmic and cytoplasmic control antisera established that the fractionation protocol had prepared samples of the Δ chiW strain periplasm that were free from cytoplasmic contamination (Fig. 4 D). Further inspection revealed that both ChiA and ChiC were located in the periplasm in the secretion-defective Δ chiW strain (Fig. 4 D). Similarly, fractionation of the Δ chiX

strain revealed that ChiA and ChiC secretion was blocked at an outer membrane transport step because both proteins could be detected in the periplasm in the mutant background (Fig. 4 D). Taken altogether, these data suggest strongly that export of ChiA and ChiC to the periplasm is not adversely affected by either the *chiW* or *chiX* mutations but that secretion is completely blocked at the final outer membrane translocation stage. Surprisingly, ChiB appeared to behave differently in this experiment, where a periplasmic intermediate for this isoenzyme was not readily detectable in the mutant strains.

Evidence for bimodal and coordinated expression of *chiA* and *chiX*

Although the biochemical (Fig. 1, Fig. 4, and Fig. 5) and proteomic (Table 1, Table S1, Fig. 3, and Fig. S3) analyses described here showed negligible contamination of the extracellular milieu with periplasmic or cytoplasmic proteins, the obvious similarities between *chiWXYZ* and bacteriophage lysis cassettes means it is reasonable to question whether chitinase secretion by *S. marcescens* is associated with a cell lysis event. To address this directly, live single cell imaging experiments were designed.

To visualize expression of the *chiWXYZ* operon at the single cell level, a fluorescent reporter strain was constructed in which the *chiX* gene on the *S. marcescens* chromosome was replaced by the gene encoding the RFP mKate2 (Shcherbo et al., 2007). The JH09 (Δ chiX::mKate) strain was grown for 16 h at 30°C in rich media before analysis by fluorescence microscopy (Fig. 6). The data clearly demonstrate bimodal *chiX* expression in the isogenic *S. marcescens* population, as both *chiX* ON cells (TRITC positive) and *chiX* OFF cells (TRITC negative) were apparent (Fig. 6). Calculations indicated that 0.95% of the cells per field of view ($n > 4,500$ cells) were TRITC (and thus *chiX*) positive (Fig. S4).

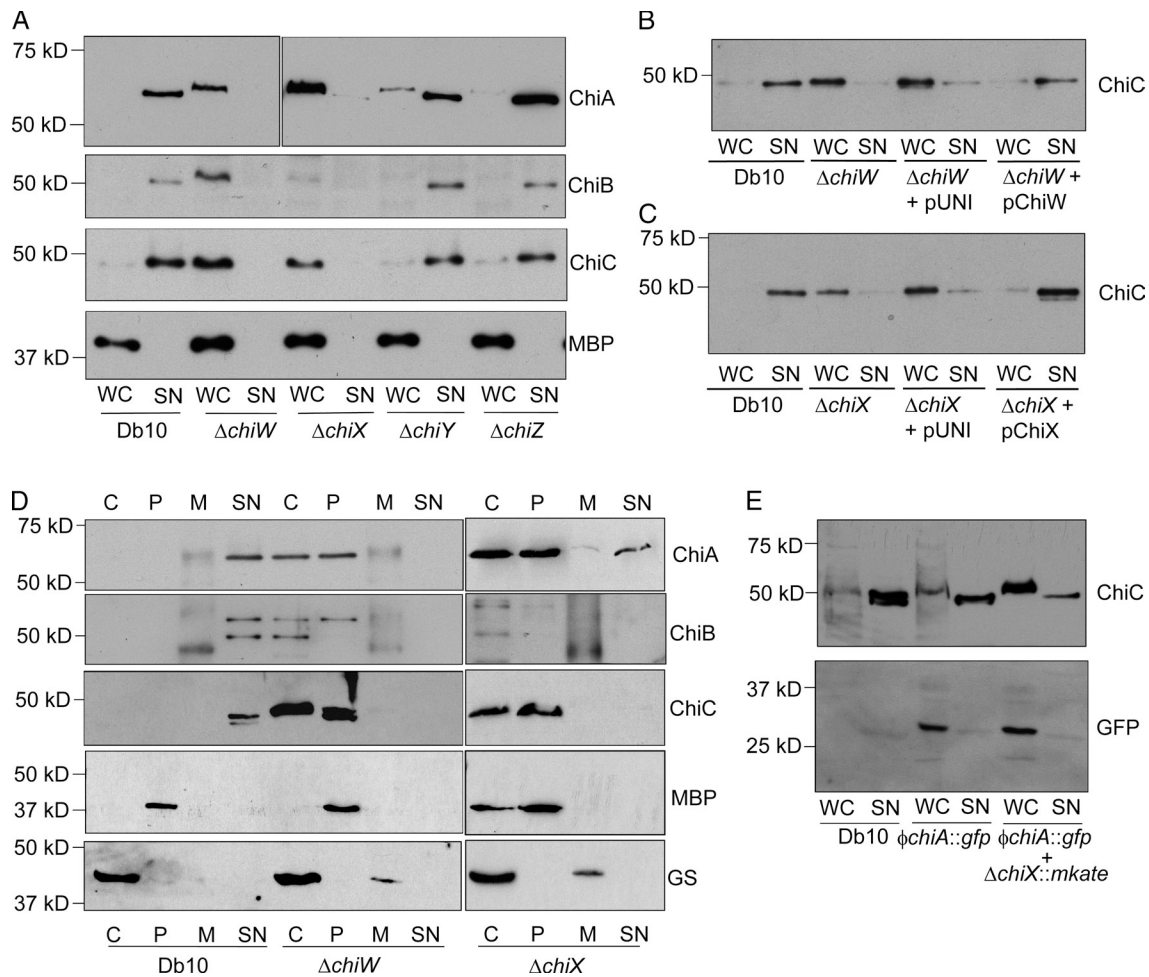


Figure 4. *S. marcescens* chitinases are secreted in *chiW*- and *chiX*-dependent manner. (A) *S. marcescens* (Db10) together with JJH04w ($\Delta chiW$), JJH05x ($\Delta chiX$), JJH06y ($\Delta chiY$), and JJH07z ($\Delta chiZ$) were analyzed by Western immunoblotting using the specific antisera indicated. (B) Complementation of the $\Delta chiW$ strain. The JJH04w strain was transformed with pUNI-PROM or pUNI-ChiW (pChiW), and the localization of ChiC was analyzed. (C) Complementation of the $\Delta chiX$ strain. The JJH05x strain was transformed with pUNI-PROM or pUNI-ChiX (pChiX), and the localization of ChiC was analyzed. (D) *S. marcescens* (Db10) together with the JJH04w ($\Delta chiW$) mutant and the JJH05x ($\Delta chiX$) mutant were fractionated into cytoplasm (C), periplasm (P), total membranes (M), and culture supernatant (SN). Proteins were analyzed by Western immunoblotting using the antisera indicated. Controls were the periplasmic maltose binding protein (MBP) and the cytoplasmic glutamine synthetase (GS) enzyme. In all cases, the band marked by the single asterisk is the ChiA protein, which the polyclonal ChiB antiserum can also detect. (E) The FTG005 strain ($\phi chiA::gfp$) and the FTG006 strain ($\phi chiA::gfp$ and $\Delta chiX::mkate$) were grown in rich media before the localization of ChiC and GFP was analyzed by Western immunoblotting. WC, whole cell.

To visualize expression of a secreted substrate, expression of the chitinase gene *chiA* was monitored at the single-cell level using a transcriptional reporter strain. A modified version of *S. marcescens* strain was constructed, FTG005, which contained a promoterless *gfp* gene (encoding GFP) positioned 23 base pairs downstream of the *chiA* termination codon on the bacterial chromosome. The FTG005 ($\phi chiA-gfp$) strain was grown for 16 h at 30°C and before analysis by fluorescence microscopy (Fig. 6). The data clearly demonstrate bimodal *chiA* expression in the isogenic *S. marcescens* population, as both *chiA* ON cells (FITC positive) and *chiA* OFF cells (FITC negative) were apparent (Fig. 6). Calculations indicated that 0.76% of the cells per field of view ($n > 7,500$ cells) were FITC (and thus *chiA*) positive (Fig. S4).

Next, a dual-fusion strain was constructed that would report both *chiA* (GFP) and *chiX* (mKate) expression simultaneously. Analysis of the FTG006 ($\phi chiA-gfp$ and $\Delta chiX::mkate$)

strain established that bimodal expression of both *chiX* and *chiA* was evident from this experiment (Fig. 7). Most interestingly, however, the microscopy also revealed that expression from the distinct *chiX* and *chiA* operons was frequently coordinated within a single cell (Fig. 7).

Expression of *chiA* does not predispose cells to lysis

Having established that expression of *chiX* and *chiA* is tightly coordinated, it was now possible to use GFP-linked expression of *chiA* to monitor the viability of cells within the subpopulation known to be secreting chitinases. This is because the FTG005 strain produces GFP linked to *chiA* expression but also possesses an intact copy of active *chiX*, which is essential for the extracellular release of chitinases.

First, a biochemical approach was taken. Cells harboring the *chiA::gfp* fusion were analyzed for chitinase secretion activity

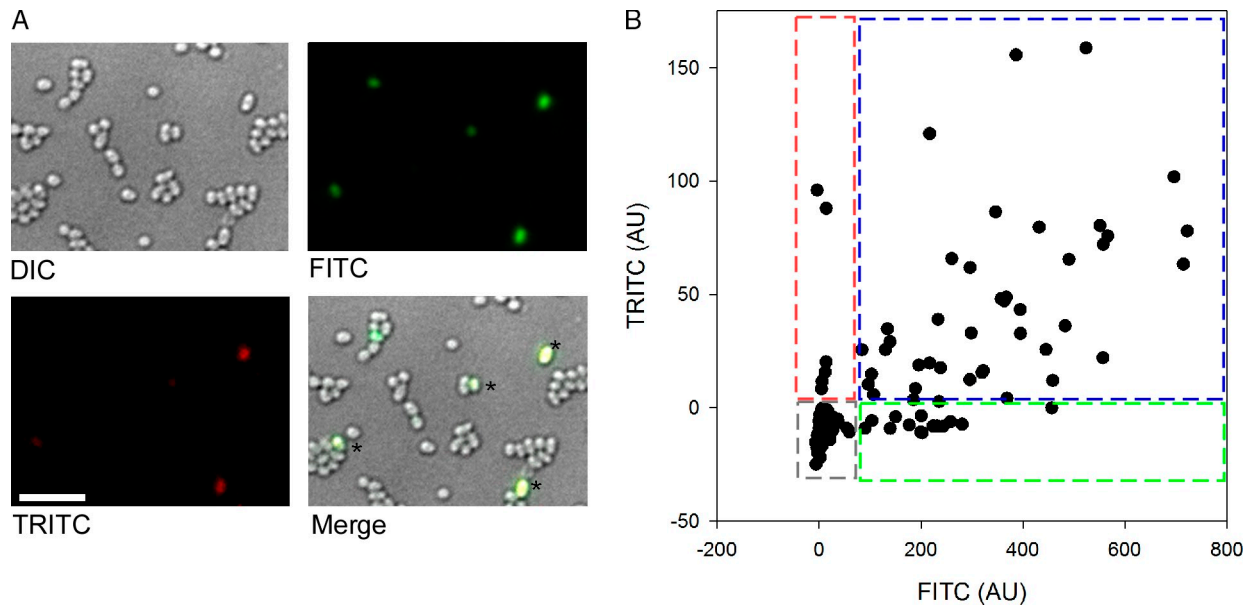


Figure 7. **Coordinated expression of *chiA* and *chiX*.** Representative data for *chiA* and *chiX* expression as followed via GFP and mKate fluorescence, respectively, for strain FTG006 ($\Delta chiX::mkate$ and $\phi chiA::gfp$). (A) Representative still frames of the FTG006 strain. Cells were grown for 16 h in rich media, and images were taken using a 100 \times , 1.4 NA lens (Olympus) and a camera (CoolSNAP HQ). Asterisks highlight cells that exhibit both red and green fluorescence and so appear yellow upon merging. Bar, 10 μ m. (B) The background level for both channels was calculated as the mean value of *S. marcescens* Db10 parent strain grown under the same conditions, plus two times the standard deviation. This value was subtracted from the fluorescence values obtained for strain FTG006. TRITC values in arbitrary units (AU) are plotted on the y axis, and FITC (arbitrary units) is plotted in the x axis. 500 cells are represented, and the boxed areas on the chart highlight cells with TRITC only (red), FITC only (green), and TRITC with FITC (blue) expression. The cell population analyzed was biased toward analysis of the fluorescent cells to allow the ability of coexpression to be viewed.

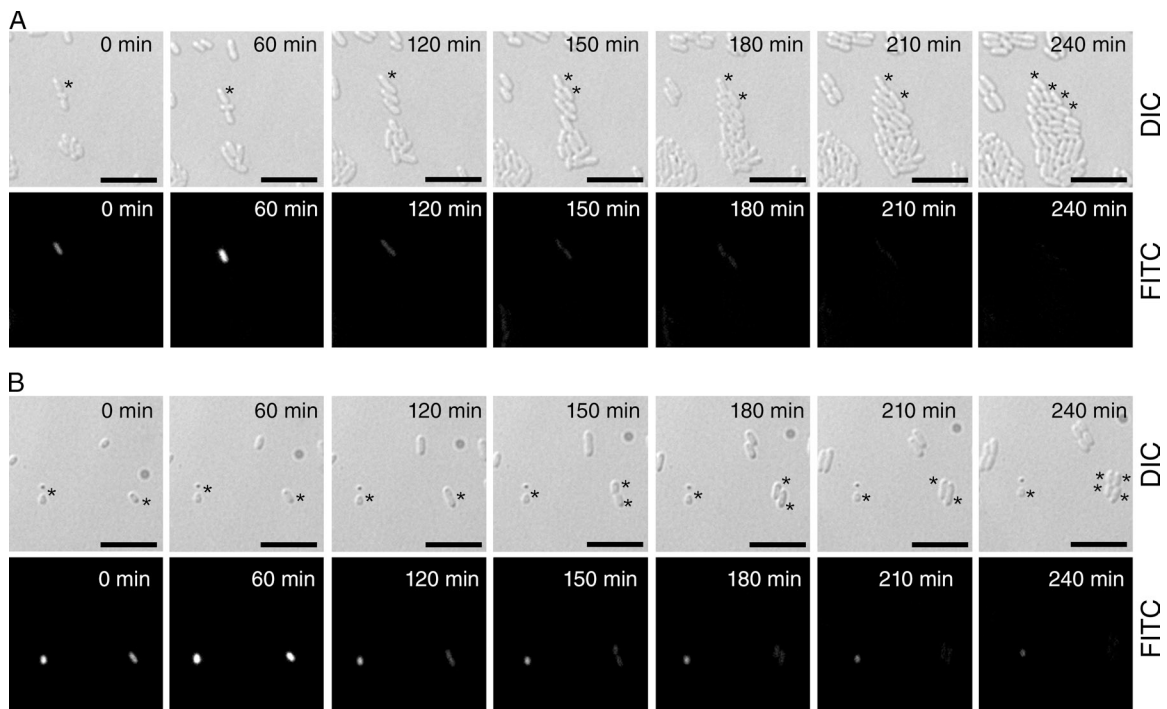


Figure 8. **Expression of *chiA* gene is not concurrent with a lysis event.** Representative still frames (separate DIC and FITC channels) taken from time-lapse videos for strain FTG005 ($\phi chiA::gfp$) demonstrating the two fates of GFP-positive cells upon time-lapse imaging. The *S. marcescens* FTG005 ($\phi chiA::gfp$) strain was grown for 18 h in minimal media before being diluted and applied to a microscope slide. (A) FITC-positive cells divide and propagate. The asterisks highlight a single cell growing and dividing, twice, into daughter cells. (B) A subpopulation of FITC-positive cells remains fluorescent but does not divide even over an extended period of time. Progression from left to right is with respect to time, as noted on the micrograph (minutes). The left-hand asterisks highlight a quiescent cell that does not divide. The right-hand asterisks highlight a single cell growing and dividing, twice, into daughter cells. Bars, 10 μ m. Videos of these images (merged) are available as [Videos 1](#) and [2](#).

oxidoreductase system (Goemans et al., 2014), reinforcing the need for periplasmic intermediates during the secretion of these enzymes.

The *S. marcescens* ChiB and ChiC proteins do not display obvious Sec signal peptides. Proteomic analysis in this work established that the ChiB protein was not proteolytically processed during secretion. However, ChiB does contain a disulfide bond (Vaaje-Kolstad et al., 2004), which implies that a periplasmic intermediate must be a feature of its biosynthesis. Surprisingly, the ChiB protein was not detected in the periplasm of the *chiW* and *chiX* strains. It seems unlikely that ChiB is exported to the periplasm via ChiW because no periplasmic intermediate was obvious even in the Δ *chiX* strain that has an intact *chiW* gene (Fig. 4 D). Instead, it seems more likely that ChiB is rapidly degraded when mistargeted to the periplasm. The secreted version of *S. marcescens* ChiC was also found to be intact in the proteomic experiments and therefore not processed during secretion. However, previous work on *P. aeruginosa* ChiC suggested that secretion of this enzyme was accompanied by an unusual processing at the N terminus (Folders et al., 2001). Indeed, in some instances, the electrophoretic mobility of *S. marcescens* ChiC appeared different in whole cell and supernatant fractions (Fig. 4 D), and this can also be observed in some ChiA (Fig. 4 A) and ChiB (Fig. 1 and Fig. 4 A) samples. The basis of these electrophoretic shifts will require further investigation because the proteomic analysis of the secreted version of these enzymes did not reveal any processing events. The mode of initial export of ChiC to the periplasm remains to be established; however, it is clear that ChiW is not involved in this step (Fig. 4 D). It is possible that ChiC is a Sec-dependent protein but that it lacks an obvious signal peptide, perhaps analogous to SodA from *Rhizobium leguminosarum* (Krehenbrink et al., 2011).

The role of a holin in protein secretion

ChiW is a holin-like protein encoded within a putative four-gene operon that is itself located within a wider genetic locus dedicated to chitin utilization (Fig. 2). The ChiW protein shares sequence identity with bona fide phage holin proteins and is predicted to be an inner membrane protein with three transmembrane domains. A canonical phage or prophage holin would be expected to be involved in catalyzing cell lysis. Thus, because the evidence presented in this work suggests that cell lysis is not the major facilitator of chitinase secretion, ChiW is termed a holin-like protein in this study.

ChiW shares 32% overall sequence identity, and 46% similarity, with the canonical λ S holin. ChiW is clearly a member of the holin family (Reddy and Saier, 2013) but shows a high degree of divergence from phage sequences. Indeed, it is worth noting that of 52 potential holin families, there are 12 holin families predicted to be of proteobacterial origin, which includes ChiW, that have not been extensively characterized (Reddy and Saier, 2013). One key difference is that, unlike most holins encoded by phage or prophage, the *S. marcescens* ChiW protein has a single translation start (Fig. S2). The λ S holin, for example, has two translation initiation codons that result in active λ S105 and anti-holin λ S107 forms (White et al., 2010). ChiW therefore probably has no anti-holin activity

associated with it. Indeed, this itself may point to a different modus operandi compared with the canonical λ S holin.

If the *S. marcescens* ChiW holin-like protein were to behave in the same way as the λ S holin, the possibility would be raised that the chitinases may not be actively secreted but instead released into the extracellular milieu via an altruistic cell lysis event induced by ChiW allowing ChiX, and possibly other hydrolytic enzymes, access to the periplasm. However, the evidence presented in this work allows an argument to be made against a crude cell lysis model. Neither Western analysis, nor the sensitive and wider ranging label-free proteomics experiments, could identify a lysis event associated with the chitinase-positive *S. marcescens* parent strain. Here, 497 different proteins were identified in the extracellular milieu of both parental and *chiW* strains. These included many previously described secreted proteins, as well as a large number of proteins known or predicted to be cytoplasmic or membrane-bound proteins, but that can be detected and identified even at very low levels here using this very sensitive technique. For a ChiWX-triggered general lysis model to be correct, it would be expected that all 497 proteins would be equally decreased in the mutant strain secretome compared with the parent strain, and so after normalization of the data, no significant differences would be expected to be observed. In reality, 10 proteins behaved very differently from the remaining 487 and are clearly reduced in the secretome of the holin-deficient strain (Table 1), with only four of those (the chitinolytic enzymes) being normally very highly abundant in the parental strain secretome (Fig. 3). Furthermore, live-cell imaging techniques revealed that *chiA* was coexpressed with the *chiWXYZ* operon in a subpopulation of cells (Fig. 7) and that cells coexpressing *chiA* and *gfp* did not release GFP into the growth media as would be expected in a lysis event (Fig. 4 D) but were instead able to grow and divide (Fig. 8). This provides strong evidence that, although bimodal, chitinase expression is not concomitant with an altruistic lysis event. Taken altogether, these data clearly point to a specific role in chitinase secretion for the ChiW holin-like protein.

Holins have been implicated in protein secretion in some other systems, but not without controversy. The *S. marcescens* NucE protein (SMDB11_0177) was suggested to be a holin required for secretion of the Sec-dependent NucA nuclease (Berkmen et al., 1997). However, subsequent deletion of *nucE* was found to have no effect on nuclease secretion (Strych et al., 1999). Similarly, one recent study suggested a holin-like protein (TcdE) was dedicated to the nonlytic toxin secretion by *Clostridium difficile* (Govind and Dupuy, 2012), whereas alternative research concluded that *tcdE* was not involved in toxin secretion (Olling et al., 2012).

The role of an endolysin in chitinase secretion

The ChiX protein is predicted to be a 15-kD soluble protein that has been classified in some databases as a D-alanine-D-alanine carboxypeptidase of the VanY family but actually shares very little sequence identity with genuine VanY-like proteins. Instead, ChiX is closely related to the *Bacillus subtilis* CwlK protein, which has been characterized as an L-alanyl-D-glutamate endopeptidase (Fukushima et al., 2007). Unlike CwlK, ChiX contains no obvious targeting

signals, but its coexpression with a gene encoding a holin-like protein (ChiW) suggested that it could be targeted to the periplasm via its partner holin. Experimental evidence presented in this work supports that model because a $\Delta chiW$ mutant phenotype can be complemented by a Tat-targeted ChiX protein (Fig. 5).

The important role of the *chiX* gene in chitinase secretion in *S. marcescens* is clear; however, the precise biochemical function of ChiX protein remains to be determined. Although bacteriophage-encoded endolysins are often closely linked with cell lysis events when they operate in partnership with holins, evidence is now emerging of endolysins being involved in the specific nonlytic secretion of proteins. For example, recently an *N*-acetyl- β -D-muramidase (TtsA) from *Salmonella enterica* serovar Typhi, similar to phage endolysins, was found to be involved in typhoid toxin secretion across the bacterial outer membrane (Hodak and Galán, 2013). It is possible that ChiX is similarly involved in remodeling the peptidoglycan to facilitate protein secretion. Indeed, many apparently homologous enzymes have been identified encoded within bacterial genomes that may have such specialized roles, because it is believed that the periplasmic peptidoglycan layer presents a permeability barrier to all but the smallest of proteins (Scheurwater et al., 2008). Endogenous endolysins may be required to remodel the peptidoglycan to allow, for example, cell division to proceed or for large transmembrane protein complexes, such as secretion systems, to be assembled (Koraimann, 2003; Scheurwater et al., 2008).

A proposed scheme for chitinase secretion

Collectively, the multidisciplinary approaches used in this study allow an initial model to be proposed for chitinase secretion by *S. marcescens*. A small subpopulation of cells coexpress chitinase genes together with *chiWXYZ*. The chitinases produced are most likely first targeted to the periplasm by the Sec pathway, where ChiA and Cbp21 are further proteolytically processed and ChiA, ChiB, and Cbp21 have disulfide bonds correctly inserted. ChiW is a holin-like protein that meanwhile integrates into the inner membrane and, by an as-yet-unexplored mechanism for this system, allows the periplasmic targeting of ChiX, which is predicted to be a metal-dependent L-alanyl-D-glutamate endopeptidase. The role of ChiX is most likely to remodel the peptidoglycan by removing cross-links between the glycan strands, and this activity seems critical for the correct functioning of the final outer membrane secretion step.

The role for a bacterial holin-like protein and a peptidoglycan hydrolase in protein transport across the outer membrane is an intriguing one. It is very likely that these proteins will have to operate in partnership with other, as-yet-unidentified, outer membrane-associated proteins. In addition, ChiA, ChiB, ChiC, and Cbp21 may contain a common signal that allows specific secretion of this subset of proteins above all others.

Materials and methods

Mutant and plasmid construction, transposon mutagenesis, and genetic screening

Deletion strains of *S. marcescens* were constructed by allelic exchange using the suicide vector pKNG101 (Kaniga et al., 1991), and they are listed in

Table 2. *S. marcescens* strains used and constructed in this work

Strain	Relevant genotype	Reference
Db10	Nonpigmented wild strain	Flyg et al., 1980
JJH01	As Db10 $\Delta chiA$ (SMDB11_4243)	This study
JJH02	As Db10 $\Delta chiB$ (SMDB11_2875)	This study
JJH03	As Db10 $\Delta chiC$ (SMDB11_0468)	This study
JchiA	As Db10 $\Delta chiB$, $\Delta chiC$	This study
JchiB	As Db10 $\Delta chiA$, $\Delta chiC$	This study
JchiC	As Db10 $\Delta chiA$, $\Delta chiB$	This study
Nochi	As Db10 $\Delta chiA$, $\Delta chiB$, $\Delta chiC$	This study
JJH04w	As Db10 $\Delta chiW$ (SMDB11_2874)	This study
JJH05x	As Db10 $\Delta chiX$ (SMDB11_2873)	This study
JJH06y	As Db10 $\Delta chiY$ (SMDB11_2872)	This study
JJH07z	As Db10 $\Delta chiZ$ (SMDB11_2871)	This study
JJH08p	As Db10 $\Delta chiW$, <i>chiX</i>	This study
FTG005	As Db10 $\phi chiA::gfp$	This study
JJH09	As Db10 $\Delta chiX::mkate$	This study
FTG006	As Db10 $\phi chiA::gfp$, $\Delta chiX::mkate$	This study

Table 2. All in-frame deletions were constructed to preserve surrounding regulatory elements; however, note that strain JJH08p is devoid of *chiW* and also lacks the initiation codon of *chiX* and therefore exhibits a $\Delta chiW$ and *chiX* genotype. *S. marcescens* strains carrying fluorescent transcriptional markers were constructed using pKNG101. For strain JJH09 ($\Delta chiX::mkate$), a DNA fragment containing a ribosome binding site and the *mkate* gene (Shcherbo et al., 2007) was amplified from a plasmid supplied by T. Norman (Harvard University, Cambridge, MA) and inserted into a $\Delta chiX$ allele prepared in pKNG101. For strain FTG005 ($\phi chiA::gfp$), a 1,000-bp fragment of DNA covering the 3' end of *chiA* was amplified by PCR and cloned into pKNG101. Then, an EcoRV restriction site 35 bp downstream of the *chiA* termination codon was used to insert *gfp-mut2* amplified from pNW704 (Marlow et al., 2014) to give an $\phi chiA::gfp$ that was then transferred to the chromosome of *S. marcescens* Db10.

Transposon mutagenesis of the JchiC (*chiC*⁺, $\Delta chiA$, and $\Delta chiB$) strain was performed using *E. coli* donor strain SM10 λ pir (pUTmini-Tn5^{Sm/Sp}) as outlined previously (de Lorenzo et al., 1990). JchiC colonies that contained Tn5 were patched onto Luria Bertani (LB) agar plates supplemented with 2% (wt/vol) colloidal chitin prepared in house. Transposon insertion sites were mapped using single primer-specific PCR in which genomic DNA was digested with XhoI and PstI and then cloned into pBluescript followed by a round of PCR with vector- and Tn5-specific primers. PCR products were sequenced with Tn5 primers, and the positions of the Tn5 insertions were located by analysis of the *S. marcescens* Db11 genome at the Wellcome Trust Sanger Institute, for which Db10 is the direct parent.

For complementation analysis, the intact *chiW* and *chiX* genes were amplified by PCR and cloned into the vector pUNI-PROM (Amp^r) that allows constitutive gene expression (Jack et al., 2004). *S. marcescens* was transformed by electroporation, and transformants were isolated on solid LB media supplemented with 200 μ g/ml carbenicillin. For protein secretion analysis, the transformants were grown aerobically in liquid LB medium supplemented with 50 μ g/ml carbenicillin at 30°C.

For the holin bypass experiments, the *chiX* gene was amplified by PCR and cloned as an engineered XbaI-SphI fragment into pBAD18 (Kan^r) that allows regulated expression with L-arabinose (Guzman et al., 1995) to give plasmid pBAD-ChiX. To attach a Tat signal peptide onto the N terminus of ChiX, the pBAD18 derivative pSC144 (English et al., 2012) was modified such that a SacI-XbaI fragment encoding an OmpA signal peptide was replaced by a PCR product encoding the initial 30 amino acids of *S. marcescens* SufI (SMDB11_3496) to give plasmid pSC143. Next, *chiX* (minus its initiation codon) was amplified and cloned as an XbaI-SphI fragment, resulting in pBAD-Tat-ChiX encoding an spSufI::ChiX fusion protein.

Protein production and secretion analysis

SMDB11_0468 (ChiC), SMDB11_2875 (ChiB), and SMDB11_4243 (ChiA) were overproduced in *E. coli* as C-terminally His-tagged proteins. Purified proteins were used to raise rabbit polyclonal antisera (Eurogentec). For Western immunoblotting, samples were separated by SDS-PAGE

electroblotted onto polyvinylidene difluoride sheets (EMD Millipore). The in-house α -ChiA, α -ChiB, and α -ChiC sera were raised in rabbits against purified, recombinant, hexa-His-tagged *S. marcescens* chitinases produced in *E. coli* and were each used at a 1:20,000 dilution. The anti-glutamine synthetase antiserum was raised in rabbits against purified, recombinant, *E. coli* glutamine synthetase as previously described (Javelle et al., 2004) and was used at 1:10,000 dilution. Commercially available antibodies purchased for this work were the α -MBP (*E. coli* maltose binding protein) mouse monoclonal antibody (New England Biolabs, Inc.), which was used at a 1:20,000 dilution, and the α -RNAP (*E. coli* RNA polymerase) mouse monoclonal antibody (NeoClone), which was used at a 1:40,000 dilution. Immunodetection was performed using a HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad Laboratories) used at 1:20,000 dilution together with a chemiluminescent substrate (EMD Millipore). For small scale protein secretion assays, strains were grown in 5-ml volumes at 30°C. The OD₆₀₀ for each sample was normalized before whole cell and supernatant fractions were collected by centrifugation. Culture supernatants were subjected to ultrafiltration through a 0.2- μ m device (EMD Millipore).

Proteomic sample preparation and mass spectrometry

S. marcescens Db10 and JH08p (Δ *chiW* and *chiX*) secreted protein samples were prepared for label-free mass spectrometry by growth in 50 ml minimal media at 30°C for 16 h. 2.6 ml of a 6.1-N trichloroacetic acid solution (~100% wt/vol) was added to 40 ml of culture supernatant, and proteins were precipitated overnight at 4°C. The protein precipitates were then washed four times in 1 ml of 80% acetone (-20°C) before being dried at room temperature for 45 min. Samples were resuspended in 500 μ l of 20 mM Tris-HCl, pH 8.0, 4% (wt/vol) SDS, and 1 mM Tris(2-carboxyethyl)phosphine and alkylated by addition of 5 mM iodoacetamide for 20 min in the dark at room temperature. After quenching with 5 mM DTT, samples were treated using the Filter-Aided Sample Prep protocol (Manza et al., 2005; Wisniewski et al., 2009) and digested using trypsin (sequencing grade; Promega). Approximately 0.5 μ g of each digest was injected in an interleaved manner onto a 2-cm \times 100- μ m trap column and separated on a 15-cm \times 75- μ m reversed-phase column (PepMap C18; Thermo Fisher Scientific) on a Rapid Separation LC (UltiMate 3000; Dionex). Peptides were eluted by a linear 2-h gradient of 95% A/5–35% B (A: 0.1% [wt/vol] formic acid; B: 80% [vol/vol] acetonitrile and 0.08% [wt/vol] formic acid) at 300 nl/min into a LTQ Orbitrap Velos Pro. Data were acquired using a data-dependent top 20 method, dynamically choosing the most abundant precursor ions from the survey scan (350–1,650 Th, 60,000 resolution, and automatic gain control target value of 10⁶). Precursors above the threshold of 500 counts were isolated within a 2 Th window and fragmented by collision-induced dissociation in the LTQ Velos using a normalized collision energy of 35% and an activation time of 10 ms. Dynamic exclusion was defined by a list size of 500 features and exclusion duration of 60 s. Lock mass was used and set to 445.120025 for ions of polydimethylcyclsiloxane (Ritorto et al., 2013).

Label-free quantitation was performed using MaxQuant v1.3.0.5 (Cox and Mann, 2008). Mass spectrometric runs of four biological replicates of Db10 and JH08p were searched against a combined database of *S. marcescens* containing 4,720 sequences and a list of common contaminants in proteomics experiments using the following settings: enzyme trypsin/P, allowing for two missed cleavages, fixed modifications were set to carbamidomethyl, and variable modifications were set to acetyl (protein N terminus) and oxidation. Mass spectrometry/mass spectrometry tolerance was set to 0.5 D, and precursor tolerance was set to 6 ppm. Peptide and protein false discovery rate was set to 0.01, minimal peptide length was 7, and one unique peptide was required. Requantify and retention time alignment (2 min) were enabled.

Subcellular fractionation

Preparation of cytoplasmic, total membrane, periplasmic, and secreted (culture supernatant) fractions was as follows. Initially, 5-ml cultures were grown overnight in LB medium. The cells were harvested by centrifugation and then suspended in 1 ml of 30 mM Tris-HCl, pH 7.8, 40% (wt/vol) glucose, and 2 mM EDTA. Cell suspensions were incubated at 30°C for 10 min before being harvested by centrifugation and immediately resuspended in 1 ml ice-cold distilled water. After incubation on ice for 10 min, the resultant spheroplasts were harvested by centrifugation, and the supernatant was retained as the periplasmic fraction. Spheroplasts were suspended in 50 mM Tris-HCl, pH 7.8, and 0.2 M Na₂CO₃ and lysed by sonication. Total membranes were recovered by ultracentrifugation and resuspended in 50 mM Tris-HCl, pH 7.8, by homogenization.

Live-cell imaging and image processing

For static images, cells were grown in LB aerobically overnight at 30°C with shaking at 220 rpm. Next, 5 ml of fresh LB was inoculated with 25 μ l of the starter culture and grown for 16 h at 30°C with shaking at 220 rpm. Cells were harvested by centrifugation, washed in 5 ml PBS, and suspended in 10 ml PBS. Each microscope slide was prepared as follows. A 125 μ l Gene Frame (AB-0578; ABgene House) was attached to a standard microscope slide (super premium; VWR International). The Gene Frame was next filled with molten minimal media (0.2% wt/vol glucose) solidified with 1% agarose and covered firmly with a standard microscope slide, to flatten the agarose surface. When the agarose had sufficiently cooled and solidified, the upper slide was carefully removed. After inoculation, the cell suspension was allowed to dry, after which the Gene Frame was sealed with a number 1.5 coverslip (22 \times 22 mm; VWR International). Images were acquired using a wide-field microscope (DeltaVision Core; Applied Precision) mounted on an inverted stand (IX71; Olympus) with a 100 \times , 1.4 NA lens (Olympus) and electron-multiplying charge-coupled device camera (Cascade2 512; Photometrics). Datasets (512 \times 512 pixels with 13 z sections spaced by 0.2 μ m) were acquired with DIC and fluorescence optics. DIC images were acquired with an light-emitting diode transmitted light source (Applied Precision) at 32% intensity and exposure times between 25 and 50 ms. Fluorescence from mKate2 was imaged using a 100-W mercury lamp and a TRITC filter set (excitation of 555/28 nm; emission of 617/73 nm) with an exposure time of 300 ms. Fluorescence from GFP was imaged using a 100-W mercury lamp and an FITC filter set (excitation of 490/20 nm; emission of 528/38 nm) with an exposure time of 200 ms.

For time-lapse microscopy, cells were grown in an identical manner except they were subcultured into minimal media (0.2% wt/vol glucose) and grown aerobically at 30°C with shaking at 220 rpm for 18 h before being diluted to OD₆₀₀ of 0.01 in minimal media. Agarose microscope slides were prepared, exactly as described for collecting static images, and incubated at 30°C in a temperature-controlled environmental chamber (Weather Station; Applied Precision) to allow the cells to equilibrate on the agarose pads for 3 h. Time-lapse imaging of microcolony development and gene expression was performed using a wide-field microscope (DeltaVision Core) mounted on an inverted stand (IX71) with a 60 \times , 1.4 NA lens (Olympus) and a camera (CoolSNAP HQ; Photometrics) with DIC and fluorescence optics. For each experiment, 12 independent fields, each containing one or two cells, were manually identified, and their xyz positions were stored in the microscope-control software (softWoRx; Applied Precision). Datasets (512 \times 512 pixels with 2 \times 2 binning and 12 z sections spaced by 1 μ m) were acquired every 15 min for \leq 12 h. GFP was imaged using a 100-W mercury lamp and a FITC filter set (excitation of 490/20 nm; emission of 528/38 nm) with an exposure time of 50 ms. DIC images were acquired with an light-emitting diode transmitted light source (Applied Precision) at 32% intensity and exposure times between 25 and 50 ms. Postacquisition datasets were rendered and analyzed using OMERO software (Open Microscopy Environment). During analysis, the threshold used to define activation of the transcriptional reporter was set as a fluorescence intensity value greater than two standard deviations above the mean background fluorescence of the wild-type strain Db10.

Online supplemental material

Fig. S1 shows that directed and transposon mutagenesis identifies genes required for in vivo chitinolytic activity by *S. marcescens*. Fig. S2 shows the basic bioinformatics analysis of the ChiW holin-like protein. Fig. S3 shows the additional analysis of the proteomics data, demonstrating the reproducibility of the experiment. Fig. S4 shows the fluorescence data collected and used to calculate the different levels of expression of *chiA* and *chiX* within a population of cells. Table S1 shows analysis of the label-free quantitative proteomic data to reveal secreted proteins that are increased in abundance in the secretome of the Δ *chiW* strain as compared with the parental strain. Video 1 shows time-lapse microscopy of an *S. marcescens* strain expressing GFP transcriptionally coupled to *chiA*. Video 2 shows the same *S. marcescens* strain expressing GFP under time-lapse microscopy, but this time, it demonstrates a small subset of fluorescent cells that are quiescent in that they do not divide over the timescale of the experiment. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201404127/DC1>. Additional data are available in the JCB DataViewer at <http://dx.doi.org/10.1083/jcb.201404127.dv>.

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