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

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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 a chitin-binding protein (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.

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

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

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.
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 chitolytic 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 ΔchiW 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.
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 (*SMB11_2874*) and the third was located near *SMB11_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, *SMB11_2874* had not been previously studied. Further inspection of the *S. marcescens* Db11 genome sequence revealed that chiR and *SMB11_2874* are located adjacent to chiB on the *S. marcescens* chromosome (Fig. 2 A). The *SMB11_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 ∆chiW (JJH08p) strain versus the secretome of the Db10 parental strain. Highly abundant proteins that were reduced significantly (P < 0.01) in the ∆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 ∆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 ΔchiW and the ΔchiX 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 ΔchiW (Fig. 4 B) or ΔchiX 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 ∆chiW (Fig. 4 B) or the endopeptidase in ∆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).
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 ΔchiW 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 periplasmic and cytoplasmic control antisera established that both ChiA and ChiC secretion was blocked at an abundance greater than 3× higher than observed in the secretome of the Db10 parental strain (Fig. 4 D). Further inspection revealed that both ChiA and ChiC were located in the periplasm in the secretion-defective ΔchiW strain (Fig. 4 D). Similarly, fractionation of the ΔchiX 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 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 JJH09 (ΔchiX::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).
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 (ΔchiA-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 (ΔchiA-gfp and ΔchiX::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 chiA and chiX 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.
were grown for 18 h in minimal media and were then diluted and monitored at 15-min intervals under the differential interference contrast (DIC; light microscopy) and FITC (green fluorescence) channels while growing on agarose slides. Growth was followed over a 4-h period and showed unequivocally that fluorescent FTG005 (ΔchiA-gfp) cells were capable of dividing (twice during this time course) and differentiating into a nonfluorescent cell population (Fig. 8 and Videos 1 and 2). From a total of 77 fluorescent cells observed, 55 were noted to undergo cell division (~71%). Furthermore, none of the remaining 22 cells (~29%) exhibited an obvious lysis event but appeared to adopt a quiescent (nondividing) state and were either subsumed within the developing microcolony (not depicted) or remained dormant (Fig. 8 and Video 2). It can be concluded that these behaviors are incompatible with chiA expression, representing a terminal differentiation state that automatically results in lysis.

Discussion

This work has identified three genes—chiW (SMDB11_2874), chiX (SMDB11_2873), and chiR (SMDB11_2876)—that are essential for chitinase secretion by S. marcescens. All three are part of a locus that includes chiB and cbp21 and so is dedicated to chitin metabolism. There are no adjacent genes encoding phage tail proteins or transposases, which suggests that these genes are of proteobacterial origin and not associated with a prophage. The chiR gene was originally identified by a genetic screen designed to identify strains with reduced extracellular chitinase activity (Suzuki et al., 2001), whereas chiW and chiX have not been previously implicated in chitinase secretion.

Evidence presented in this work points to a two-step chitinase secretion process in which the chitinolytic machinery is first targeted to the periplasm and then transported across the outer membrane. Western analysis placed both ChiA and ChiC in the periplasm of the ΔchiW and the ΔchiX strains (Fig. 4 D). In this case, coordinately expressed GFP was found in the whole cells by Western immunoblotting only, rather than in the culture supernatant, whereas chitinase was secreted (Fig. 4 E).

Next, the fate of the FTG005 (ΔchiA-gfp) population expressing chiA and gfp was followed using time-lapse microscopy (Fig. 8 and Videos 1 and 2). FTG005 (ΔchiA-gfp) cells and the localization of coexpressed GFP (Fig. 4 E). In this case, coordinately expressed GFP was found in the whole cells by Western immunoblotting only, rather than in the culture supernatant, whereas chitinase was secreted (Fig. 4 E).

Figure 6. The expression of the chiX and chiA genes exhibit a bimodal distribution. [A] Representative still frames of the JJH09 [ΔchiX::mKate] strain. The ΔchiX::mKate cells were chosen to represent the range of expression values observed. (B) Representative still frames of the chiA::gfp strain (FTG005). In each case, cells were grown for 16 h in rich media, and images were taken using a 100×, 1.4 NA lens (Olympus) and a camera (CoolSNAP HQ). Bars, 10 µm.
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 (ΔchiX::mkate and 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×, 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 µm. (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. TRICT 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 TRICT only (red), FITC only (green), and TRICT 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 (ΔchiA::gfp) demonstrating the two fates of GFP-positive cells upon time-lapse imaging. The S. marcescens FTG005 (ΔchiA::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 µm. 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 ΔchiX 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 holin S.

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 (SMDDB11_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-ala-d-ala 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 CwiK protein, which has been characterized as an t-alamyl-d-glutamate endopeptidase (Fukushima et al., 2007). Unlike CwiK, 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 ΔchiW 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 bio-chemical 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 *chiWXZ*. 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.

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

| Strain (Genotype) | Relevant genotype |
|------------------|-------------------|
| Db10             | Nonpigmented wild strain |
| JJH01            | As Db10 ΔchiA [SMDB11_4243] |
| JJH02            | As Db10 ΔchiB [SMDB11_2875] |
| JJH03            | As Db10 ΔchiC [SMDB11_4068] |
| JchiA            | As ΔchiA, ΔchiC |
| JchiB            | As ΔchiA, ΔchiB |
| JchiC            | As ΔchiA, ΔchiC |
| Nochi            | As ΔchiA, ΔchiB, ΔchiC |
| JJH04w           | As ΔchiW [SMDB11_2874] |
| JJH05x           | As ΔchiX [SMDB11_2873] |
| JJH06y           | As ΔchiY [SMDB11_2872] |
| JJH07z           | As ΔchiZ [SMDB11_2871] |
| JJH08p           | As ΔchiW, ΔchiX |
| FG005            | As ΔchiA::gfp |
| JJH09            | As ΔchiX::mkate |
| FG006            | As ΔchiA::gfp, ΔchiX::mkate |

*Strains used and constructed in this work.*
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 chinatinase produced in E. coli and were each used at a 1:20,000 dilution. The anti–γ-glutamylase antiserum was raised in rabbits against purified, recombinant, E. coli glutamyltranspeptidase as previously described (Bjellqvist et al., 2001) and was used at 1:10,000 dilution. Commercially available antibodies purchased for this work were the α-rabbit IgG (goat anti–mouse monoclonal antibody (New England Biolabs, Inc.).) was used at a 1:20,000 dilution, and the α-RNA pol (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 to detect goat anti–mouse IgG. The slides were then mounted with half-strength VECTASHIELD (Vector Laboratories, CA) at a 1:20,000 dilution, and protein false discovery rate was set to 0.01, minimal peptide length of 7, and dynamic range of 1.5. Data were acquired with a wide-field microscope (DeltaVision Core; Applied Precision) mounted on an inverted stand (IX71; Olympus) with a 100x, 1.4 NA lens (Olympus) and electron-multiplying charge-coupled device camera (Cascade2 510; Photometrics). Datasets (512 × 512 pixels with 13 z sections spaced by 0.2 µm) were acquired with DI and fluorescence optics. DIC images were acquired with an oil-immersion 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 FITC filter set (excitation of 555/28 nm; emission of 517/73 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 OD<sub>600</sub> of 0.01 in minimal media. Agarose microsphere 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 60x, 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 x-y positions were stored in the microscope-control software (softWoRx; Applied Precision). Datasets (512 × 512 pixels with 2 × 2 binning and 12 z sections spaced by 1 µm) were acquired every 15 min for ≤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 5 and 20 ms.

Online supplemental material

Fig. S1 shows directed and transposon mutagenesis identifies genes required for in vivo chitinolytic activity by S. marcescens. Fig. S2 shows the basic bioinformatics 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 AchiW 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/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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