A holin/peptidoglycan hydrolase-dependent protein secretion system

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Abstract
Gram-negative bacteria have evolved numerous pathways to secrete proteins across their complex cell envelopes. Here, we describe a protein secretion system that uses a holin membrane protein in tandem with a cell wall-editing enzyme to mediate the secretion of substrate proteins from the periplasm to the cell exterior. The identity of the cell wall-editing enzymes involved was found to vary across biological systems. For instance, the chitinase secretion pathway of Serratia marcescens uses an endopeptidase to facilitate secretion, whereas the secretion of Typhoid toxin in Salmonella enterica serovar Typhi relies on a muramidase. Various families of holins are also predicted to be involved. Genomic analysis indicates that this pathway is conserved and implicated in the secretion of hydrolytic enzymes and toxins for a range of bacteria. The pairing of holins from different families with various types of peptidoglycan hydrolases suggests that this secretion pathway evolved multiple times. We suggest that the complementary bodies of evidence presented is sufficient to propose that the pathway be named the Type 10 Secretion System (TXSS).

Keywords
chitinase, holin, peptidoglycan hydrolase, protein secretion, toxin, type X secretion system
Bacteria secrete proteins to the extracellular environment to allow them to successfully compete for space and resources. The Sec and Tat machineries in the cytoplasmic membrane serve as general transloca- 
ted to secrete proteins in Gram-positive bacteria, and to export proteins to the periplasm in Gram-negative bacteria (Collinson et al., 2015, Palmer and Stansfeld, 2020). Specialized secretion systems are required by Gram-negative bacteria to export proteins across the outer membrane. Eight Gram-negative protein secretion pathways (named Type I–VI, Type VIII, and Type IX) have previously been described (Costa et al., 2015; Lauber et al., 2018; Gorasia et al., 2020). Some are one-step secretion systems, recognizing their substrates in the cytoplasm and transporting them simultaneously across both bacterial membranes, whereas others rely on the Sec and/or Tat pathways to first translocate substrates to the periplasm (so-called two-step mechanisms). Here we describe the Type X (10) Secretion System, a two-step pathway that relies on a holin and peptidoglycan hydrolase pair for secretion across the outer membrane.

1.1 | Chitinase secretion by Serratia marcescens DB10 as a paradigm pathway dependent on a holin and a peptidoglycan hydrolase

Serratia are a genus of ubiquitous Gram-negative bacteria found in water and soil, and some species have been identified as opportunistic pathogens of humans and insects (Hejazi and Falkiner, 1997, Flyg et al., 1980). Serratia produce a vast number of extracellular enzymes and as a result several different protein secretion systems can be identified encoded across the large number of sequenced Serratia genomes. Major common components of the extracellular proteome of Serratia species are the chitinase enzymes that allow the organism to grow with chitin as the sole carbon and nitrogen source (Vaaje-Kolstad et al., 2013). Most strains encode at least four chitinases (ChiA, ChiB, ChiC, and Cbp21), although some contain more (Purushotham et al., 2012a; Purushotham et al., 2012b), that collectively degrade chitin to chitobi- 
ose, which is taken up into the periplasm assisted by the outer mem-
brane chitoporin, ChiP (Takanoa et al., 2014). This disaccharide may then be further degraded to N-acetyl glucosamine (GlcNAc) monosaccharides by the action of a periplasmic chitobiase, or directly imported into the cytoplasm by a phosphotransferase system for further break-
down (Uchiyama et al., 2003; Vaaje-Kolstad et al., 2013).

Serratia marcescens strains DB10 and 2170 are two of the most well understood biological systems in regard to chitinase secretion and chitino-lytic activity. Where studied in other biological systems, chitinase secretion in Gram-negative bacteria has been observed as a two-step process, relying on the Type II Secretion System for translocation across the outer membrane (Connell et al., 1998; Francetic et al., 2000; DebRoy et al., 2006). However, it is clear that S. marcescens DB10 in particular, and other S. marcescens genomes, lacks any genes encoding the Type II Secretion System. The situa-
tion in S. marcescens DB10 is doubly curious when it is considered that two of the secreted chitinases, ChiA and Cbp21 (more correctly a copper-dependent lytic polysaccharide monooxygenase, LPMO), are synthesized as obvious precursors with N-terminal Sec signal peptides for export across the inner membrane (Brurbation et al., 1994; Suzuki et al., 1998), but neither the ChiB nor the ChiC se-
creted chitinases contain any Sec-type N-terminal signal peptides (Brurbation et al., 1995; Suzuki et al., 1999). By analogy with ChiC from Pseudomonas aeruginosa (Folders et al., 2001), the S. marcescens ChiC protein could potentially undergo unusual processing of a small num-
ber of N-terminal residues during secretion, however the biochemi-

cal evidence for this is to the contrary (Hamilton et al., 2014).

To experimentally determine the secretion pathway taken by ChiC in S. marcescens DB10, Hamilton et al. (2014) undertook trans-


are important biochemical and structural differences. Mutational analysis of the *S. marcescens* chiWXYZ cluster indicates that only chiW and chiX are essential for chitinase secretion, with the spanin homologs ChiY and ChiZ being dispensable, at least under laboratory growth conditions (Hamilton et al., 2014). The *S. marcescens* DB10 ChiW protein comprises 108 amino acids and shares ~32% overall sequence identity with the lambda holin. However, unlike phage holin genes in this class, chiW has a single translational start site and, therefore, has...
no canonical anti-holin associated with it. This suggests that ChiW activity may be regulated differently to the holins of lytic phages. Indeed, the only known function of ChiW is to mediate the specific transport of ChiX to the periplasm, and the essentiality of ChiW for secretion can by completely bypassed if ChiX is routed to the periplasm through the Tat pathway using an engineered signal peptide (Hamilton et al., 2014). Furthermore, the ChiX protein itself is a zinc-dependent peptidoglycan hydrolase with L-Ala D-Glu endopeptidase activity, with this cell wall-editing activity being absolutely critical for chitinase secretion (Owen et al., 2018). This class of enzyme bears no relation to the lambda phage "R" endolysin, which is a transglycosylase with a different target in the peptidoglycan matrix (Bienkowska-Szewczyk and Taylor, 1980).

The similarity of ChiWXYZ with the lambda phage lysis machinery raised the possibility that these proteins-mediated chitinase release through cell lysis, and that such an altruistic process in a minority of cells would benefit the remaining population. However, a number of lines of evidence argue against this. For example, comparative analysis of the extracellular proteomes of a wild-type and isogenic chiW mutant strain using a sensitive mass spectrometry technique identifies only a handful of proteins that decrease significantly in abundance in the absence of ChiW, and these are predominantly extracellular components of chitinolytic machinery (Hamilton et al., 2014; Costa et al., 2019). The S. marcescens DB10 chitinase secretion machinery is co-ordinately regulated with the chitinase enzymes through the action of a LysR-family transcriptional regulator, ChiR (Suzuki et al., 2001; Suzuki et al., 2016; Yan et al., 2017; Costa et al., 2019). LysR-type regulators are generally activated though interaction with a small metabolite, although at present the co-activator of ChiR is not known. Expression of the chitinase genes and the chitinase secretion operon is bimodal—thus in laboratory conditions only a small sub-population of cells (<10%) express the chitinase genes and chiWXYZ, however this can be increased to ~60% of cells without lysis if ChiR is overproduced (Hamilton et al., 2014; Costa et al., 2019). Furthermore, when gfp was transcriptionally coupled with chiA to ensure it was co-ordinately expressed in the population of cells that were actively producing and secreting chitinases, the GFP protein was detected only in the cellular fraction despite an abundance of ChiA in the culture supernatant (Hamilton et al., 2014). Finally, single cell microscopy analysis of S. marcescens actively expressing ChiA showed that the majority of cells underwent cell division events and were not predisposed to lysis (Hamilton et al., 2014). Taken altogether it is clear that a phage lysis cassette-like system has been co-opted to function as a chitinase secretion system in S. marcescens. Since cell lysis is apparently not a general feature of the chitinase secretion process we use the general term peptidoglycan hydrolase rather than endolysin to describe the cell wall-editing component of this protein secretion system.

### 1.2 A holin/ L-Ala D-Glu endopeptidase pairing is conserved across Gram-negative bacteria

To determine whether the system identified in S. marcescens DB10 could be regarded as a general secretion pathway, we undertook a search across bacterial genomes using the sequence of the S. marcescens ChiX L-Ala D-Glu endopeptidase, followed by the analysis of gene neighborhood conservation (Saha et al., 2020). As seen in Figure 3a and S1, the chiR-chiB-chiWXYZ organization is well conserved across Serratia species, as is the presence of maeb, encoding a predicted NADP-dependent oxaloacetate-decarboxylating malate dehydrogenase, downstream of chiZ. Maeb contains no obvious signal peptide but was one of the few proteins other than chitinases that was decreased in abundance in the secretome of an S. marcescens chiW mutant strain (Hamilton et al., 2014), suggesting that Maeb may also be a substrate of the ChiWXYZ secretion system. Interestingly, in some Serratia species, for example S. fonticola, chiB is absent from this locus but maeb remains conserved, potentially supporting the notion that Maeb is a secreted substrate (Figure 3a).

Some strains of Yersinia enterocolitica also encode chiWXYZ, but here the nearby chitinase gene is also transcribed in the same orientation as the secretion machinery genes rather than divergently as seen in Serratia strains (Figures 3b and S1).

In this analysis we also observed ChiX endopeptidase-encoding homologs in additional genomic contexts. In particular, chik is frequently located adjacent to a holin-encoding gene from a different holin family to the S. marcescens ChiW holin. While S. marcescens ChiW is from the holin 3-1 family (pfam05106), chik was more often found directly downstream from a gene encoding a holin of the 3-3 family (pfam16083; see, Reddy and Saier (2013) for a discussion of different holin families). Some of these pairings are clearly within pro-phage genomic islands (Figure S1), which we would consider to be likely involved in cell lysis rather than protein secretion, but in at least two instances the holin3-3/ChiX pairings do not cluster with other pro-phage genes and are probably of bacterial origin. One highly conserved gene organization is seen across strains of Enterobacter and Citrobacter (Figure 4). Here, the holin/endopeptidase pair is encoded at a locus that also has tandem copies of genes encoding predicted bifunctional metallophosphatase/5'-nucleotidases, each having N-terminal Sec signal peptides. Also within this gene cluster is the ttx gene, which encodes a nucleoside-specific outer membrane channel (Ye and van den Berg, 2004). The co-location of a gene encoding an outer membrane transporter to uptake the product of 5'-nucleotidases strongly suggests that the enzymes are secreted, and would be prime candidates as substrates of the holin/endopeptidase secretion pathway. A LysR-family regulator is also encoded in a convergent orientation at the end of the gene cluster that could potentially co-ordinately regulate the secretion system and its substrates. No spanin-encoding genes could be found at these loci.

The second commonly occurring genomic context is found in Enterobacteriales such as Yersinia and Proteus. Here, a holin3-3/ChiX/spanin cassette is genetically embedded within a locus encoding a tripartite Tc insecticidal toxin (Figures 5 and S1). These toxins are large complex assemblies that are released from bacterial cells and perforate target cell membranes to deliver a toxic enzyme (an auto- proteolysis product from the Tcc subunit (Roderer and Raunser, 2019). At present, almost nothing is known about how these large toxins are secreted (McQuade and Stock, 2018). Interestingly, a
chitinase (LPMO) of the Cbp21 family is also frequently encoded at the same locus, and genes for a pair of LysR-family regulators are found adjacent to the gene cluster (Figures 5 and S1). It has been shown that these two regulators control toxin induction in *Yersinia enterocolitica* strain W22703 at low temperatures (Starke et al., 2013). More recently, it was reported that the holin/endopeptidase/spanin genes in the cluster are transcriptionally coupled, and that expression of these genes is exceptionally low (Springer et al., 2018).

Deliberate overproduction of the *Y. enterocolitica* W22703 holin and endopeptidase in *E. coli* was shown to result in cell lysis (Springer et al., 2018), which has also been observed when ChiW/ChiX are highly overproduced (Owen, 2016). However, in the native context *S. marcescens* DB10 chiW and chiX expression is carefully regulated (Hamilton et al., 2014), presumably to prevent the uncontrolled accumulation of these proteins to levels that could induce lysis, and this also appears the case for the *Y. enterocolitica* system (Springer et al., 2018). This underscores the importance of studying the function of these proteins produced at native levels under physiological conditions wherever possible.

### 1.3 A holin/endopeptidase pair may mediate chitinase secretion in other Enterobacteriaceae

During our analyses it was noted that a gene encoding a 3-3 family holin was frequently linked not with a cell wall endopeptidase (such as...
as ChiW) but instead with a cell wall muramidase of the ZliS superfamily (Figures 6 and S2). These enzymes cleave the glycan strands of peptidoglycan more akin to the lambda phage endolysin rather than hydrolyzing the peptide cross-bridges as observed for *S. marcescens* ChiX. Interestingly, the ZliS superfamily is named after the *Zymomonas mobilis* ZliS protein that has been reported to mediate secretion of sucrose-hydrolyzing enzymes (Kondo et al., 1994, Oda et al., 1994).

In strains of *Cronobacter*, *Klebsiella*, and *Citrobacter*, among others, the holin and muramidase genes are usually (although not always) convergently arranged. Within these gene clusters there is always a gene...
for a LysR-family transcriptional regulator, along with genes for one or more chitinases. No spanin-encoding genes are present within the clusters. These observations strongly suggest that a secretion pathway for extracellular chitinases pairing together a type of holin with a type of peptidoglycan hydrolase has evolved more than once.

1.4 Typhoid toxin secretion—a further example of the holin/peptidoglycan hydrolase secretion pathway?

Salmonella enterica serovar Typhi (S. Typhi) produces typhoid toxin, which is encoded on a genomic island. This is an unusual AB-type toxin, which has two distinct toxic activities: a DNase activity from the CdtB subunit, and an ADP-ribosyltransferase activity from PltA toxin, which has two distinct toxic activities: a DNase activity from which is encoded on a genomic island. This is an unusual AB-type Typhi) produces typhoid toxin, serovar Typhi (Salmonella enterica secretion pathway?

We analyzed bacterial genomes to examine the distribution of genes encoding TtsA homologs and found that they are also associated with toxin clusters in other bacteria. Interestingly, we noticed that in Raoultella planticola, and several strains of E. coli, not one but two holin-encoding genes (a 3-7 family holin/pfam05449 and an 8 family holin/pfam16931) preceded ttsA (Figures 7 and S2). This finding strongly suggests that toxin secretion is dependent on a holin/muramidase combination, and that a holin encoded elsewhere on the genome may substitute for the "missing" holin on the typhoid toxin island in S. Typhi. The S. Typhi genome encodes several holins, any one of which could play this role. Furthermore, as part of this analysis we saw that in the same strains of E. coli, and also in Cronobacter sakazakii, the two subunits of heat-labile enterotoxin IIA are encoded adjacent to this holin/holin/muramidase cluster (Figures 7 and S2). A similar arrangement of these toxin genes in some strains of E. coli had previously been noted by Hodak and Galan (2013). The secretion of heat-labile enterotoxin has been studied in an E. coli strain and was found to be dependent on the Type II Secretion System (Tauschek et al., 2002). However, in the particular strains identified here we were unable to detect any genes encoding this secretion pathway, and we, therefore, propose that the heat-labile enterotoxin IIA could be secreted via a holin/muramidase-dependent pathway in these bacteria.

Taken together, it would appear that holin/peptidoglycan hydrolase-dependent secretion systems are widespread in Gram-negative bacteria, have potentially evolved multiple times, and are involved with the secretion of toxins and extracellular enzymes. It is proposed here that the pathway can be termed the Type X (10) Secretion System (T10SS or TXSS).

**FIGURE 7** Holin and muramidase genes are found in toxin gene clusters in some strains of *Citrobacter, Cronobacter sakazakii*, and *Escherichia coli*. NCBI RefSeq protein accessions and sequences were downloaded from the NCBI Conserved Domain Database (Marchler-Bauer et al., 2017) based on presence of the domain “COG3926: ZliS”. The initial 2,740 accessions were reduced to a non-redundant set of 833 accessions using USEARCH with a sequence identity limit of 90% (Edgar, 2010). These were subsequently used as input for FlagS.py (Saha et al., 2020 and http://130.239.193.227/html/webFlagS.html). Genes depicted in white are pseudogenes and those in gray are RNA-encoding. Genes highlighted by the red asterisk correspond to known or predicted secreted substrates. The full output of this FlagS analysis can be found in Fig S2. Manual searching indicated that the genetic organization shown here for a single strain of *E. coli* and of *Citrobacter* was conserved across several strains. The second holin gene in *Raoultella planticola* appears to be frameshifted (shown in white), currently only one accession is currently available for this strain (NZ_JHQH01000003.1). Note that the three subunits of typhoid toxin are annotated on the figure as Pertussis-like toxin subunit ArtA (PltA), Subtilase cytotoxin subunit B (PltB), and Cytotoxic distending toxin subunit B family protein (CdtB).
1.5 | How does a holin and peptidoglycan hydrolase mediate protein secretion?

At present, the complete mechanism by which the holin (an inner membrane protein) and peptidoglycan hydrolase (a periplasmic protein) mediate secretion of proteins across the outer membrane of Gram-negative bacteria is not clear. Much of the work in this area has come from studies on the function of TtsA in *S. Typhi*. It was shown that TtsA is a muramidase that specifically recognizes L-D cross-linked peptidoglycan. In *S. Typhi*, and the closely related *E. coli*, the majority of cross-bridges in peptidoglycan are between D-Ala at position 4 of the stem peptide to meso-diaminopimelic acid (m-DAP) at position 3 of a neighboring peptide, and are DD-cross-links. However, a few percent of the peptidoglycan is cross-linked between two m-DAP residues, known as LD-cross-links (Glauner et al., 1988; Geiger et al., 2018). TtsA specifically recognizes the sugar backbone containing LD-cross-links, which are enriched at the poles of *S. Typhi* through the action of a conserved LD-transpeptidase. Both typhoid toxin and TtsA localize to the cell poles, and the action of TtsA serves to transport the toxin from the cis side of the periplasm across the peptidoglycan to the trans side adjacent to the outer membrane (Geiger et al., 2018; Geiger et al., 2020; Figure 8a). How typhoid toxin then crosses the outer membrane to be released into the extracellular space is not known, but may involve outer membrane disrupting agents produced by the human host, for example, bile salts or antimicrobial peptides (Geiger et al., 2018).

In contrast, it is likely that the mechanism of chitinase secretion mediated by ChiWXYZ is slightly different from that described for typhoid toxin. *S. marcescens* ChiX cleaves within the peptidoglycan cross-bridges rather than the peptidoglycan backbone like TtsA. In Enterobacteriaceae, peptidoglycan is anchored firmly to the outer membrane through the highly abundant Lpp protein (also known as the Braun lipoprotein). At least a third of the cellular Lpp is covalently attached to the peptidoglycan through the formation of a bond between its C-terminal lysine residue and the carboxyl group of m-DAP (Inouye et al., 1972; Glauner et al., 1988, Asmar and Collet, 2018). This serves to stabilize the outer membrane, and ablation of this covalent interaction yields outer membrane defects, including blebbing (Sanders and Pavelka, 2013, Asmar et al., 2017). *S. marcescens* ChiX cleaves between L-Ala and D-Glu at positions 1 and 2 in the stem peptide and would, therefore, not only remove the cross-bridges that hold the glycan strands together, but ultimately disconnect Lpp from the peptidoglycan (Owen et al., 2018). A reduction in the level of cross-linking along with uncoupling the peptidoglycan from the outer membrane would be anticipated to stimulate the formation of outer membrane vesicles (OMVs) (Toyofuku et al., 2019).

One potential mechanism for ChiWXYZ-dependent protein secretion is shown in Figure 8b. In the model ChiW mediates export of ChiX across the inner membrane where it locally cleaves the peptidoglycan cross-bridges. The chitinase enzymes accumulate in the periplasm in the vicinity of active ChiX—this could be achieved for...
example if all of these proteins were found at the poles of the cell as observed for TtsA and periplasmic typhoid toxin (Geiger et al., 2018). Ultimately, the enzymes would be released in OMVs. According to the model, the spanins may serve to delimit the region of the periplasm where this process occurs through interaction with the holin. Alternatively, their interaction across the periplasm may help to anchor the outer membrane, preventing lysis.

1.6 Future perspectives for the Type X (10) Secretion System

It is highly likely that proteins originating from phage lysis cassettes have been adapted, probably multiple times, to facilitate protein secretion across the outer membrane of Gram-negative bacteria. However many questions still remain. For the ChiWXYZ chitinase secretion system, what is the assembly state of the active ChiW holin? How does it specifically recognize and transport ChiX to the periplasm? What is the role of the spanin proteins ChiY and ChiZ, and do they interact with the holin during secretion? Are the chitinase enzymes initially secreted in OMVs and if so how are they released to interact with their substrate? For the TtsA-dependent secretion pathway, how does TtsA reach the periplasm? And are there other variations on this secretion pathway that have co-opted alternative phage lysis mechanisms? Finally, how many other protein secretion systems have yet to be described? The recent report of a TonB-dependent protein secretion pathway in Myxococcus xanthus (Gomez-Santos et al., 2019) takes us to Type XI, however it is likely that there are further fundamental mechanisms that mediate protein secretion which remain to be discovered.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

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