DOCTOR OF PHILOSOPHY

Secretion of the chitinolytic machinery in Serratia marcescens

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Declaration

I declare that I am the author of this thesis and that all of the appropriate references have been consulted. The work presented here-in has been performed by me, and any collaborations are stated explicitly in the text.

Jaeger Hamilton

September 2013

Conferences and Presentations

The 9th International Hydrogenase Conference, July 2010, Uppsala Sweden. Poster Presentation 'GlcNAc as a substrate in Biohydrogen Technology'. Jaeger Hamilton, Lisa Bowman, Jennifer McDowall and Frank Sargent.

SGM Autumn Conference, September 2011, University of York, UK. Poster Presentation 'Chitinase secretion in *Serratia marcescens*: investigating the membrane biology of an important human pathogen'. Jaeger Hamilton, Sarah Coulthurst and Frank Sargent.

Gordon Research Conference 'Bacterial Cell Surfaces', June 2012, Mount Snow Resort, Vermont USA. Poster presentation 'Discovery of a holin essential for chitinase secretion in *Serratia marcescens*'. Jaeger Hamilton, Manman Guo, Matthias Trost, Tracy Palmer, Sarah Coulthurst and Frank Sargent.

Abstract

There are six known secretion systems in Gram negative bacteria, referred to as Type 1 to Type 6 respectively, which are dedicated to moving substrate across the outer membrane. Secretion systems are broadly separated into those that move their substrate across the cell envelope in a single translocation event (one-step systems), and those that are dependent on the Sec or Tat machineries for export to the periplasm (two-step systems). *Serratia marcescens* is an important opportunistic human pathogen and has gathered a lot of interest due to its repertoire of secreted proteins. These include the haem-scavenging protein HasA, which is secreted by a Type 1 secretion system, and the cytotoxic haemolysin ShlA, which is secreted as part of a two-partner Type 5 secretion system. *Serratia marcescens* also encodes a Type 6 secretion system, which is known to translocate at least six effector molecules directly into other bacterial target cells.

Serratia marcescens is a model organism in terms of its ability to degrade the quite intractable polymer chitin, for which it produces three chitinase enzymes ChiA, ChiB, ChiC and a chitin-binding protein Cbp21, which hydrolyse the β-1,4 link in the chitin chain and promote binding of chitinase to the chitin substrate respectively. These chitinolytic enzymes are utilised by *S. marcescens* for both basic physiology and also in pathogenesis. In this work, genetic, biochemical and proteomic approaches identified, for the first time, genes that are essential for the secretion of all three chitinases as well as Cbp21. A genetic screen identified genes encoding a holin-like membrane protein (ChiW) and a putative L-alanyl-D-glutamate endopeptidase (ChiX). Subsequent quantitative proteomics experiments and biochemical analyses established that ChiW and ChiX were required for secretion of the entire chitinolytic machinery. Chitinase secretion was observed to be blocked at a late stage in the mutant strains as normally secreted enzymes were found to accumulate in the periplasm, thus implicating ChiW and ChiX in a novel outer membrane protein translocation process. It is proposed that the bacterial genome-encoded holin-like protein and endopeptidase identified represent a putative secretion system utilised by Gram-negative bacteria. In addition to this, genes encoding the chitinolytic machinery and the putative secretion apparatus were shown to be bimodally regulated and co-ordinately expressed.

Common abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BLAST	Basic alignment search tool
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	(sodium) diaminoethanetetra acetate
GFP	Green fluorescent protein
GlcNac	N-acetylglucosamine
Hcl	Hydrochloric acid
IPTG	Isopropyl-β-thiogalactoside
kDa	Kilo Dalton
l	Litre
LB	Luria Broth
M	Molar
Mw	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl) aminomethane
µ	micro

Antibiotic abbreviations

Amp – ampicillin

Kan – kanamycin

Strep – Streptomycin

Tet - Tetracycline

Amino acid abbreviations

Alanine	**A**	**Ala**		
Arginine	**R**	**Arg**		
Asparagine	**N**	**Asn**		
Aspartate	**D**	**Asp**		
Cysteine	**C**	**Cys**		
Glutamate	**E**	**Glu**		
Glutamine	**Q**	**Gln**		
Glycine	**G**	**Gly**		
Histidine	**H**	**His**		
Isoleucine	**I**	**Ile**		
Leucine	**L**	**Leu**		
Lysine	**K**	**Lys**		
Methionine	**M**	**Met**		
Phenylalanine	**F**	**Phe**		
Proline	**P**	**Pro**		
Selenocysteine	**U**	**SeCys**		
Serine	**S**	**Ser**		
Threonine	**T**	**Thr**		
Tryptophan	**W**	**Trp**		
Tyrosine	**Y**	**Tyr**		
Valine	**V**	**Val**	**Any amino acid**	**X**

1 Introduction

1.1 At the interface: the cell envelope of Gram negative bacteria

In order to survive and flourish cellular life has to maintain the integrity of the cell as a relatively enclosed site for basic biochemical and metabolic reactions, and this form is conserved by a semi-permeable lipid membrane. Many core features of the biosphere (such as the oxygen we breathe) is determined by the way bacteria interact with and actively change their environment: one key process by which bacteria adapt to their environment is *via* the transport of effector molecules, whereby proteins, or peptides or nucleic acids are actively transported to different cellular, or extracellular, locations. Gram negative bacteria are characterised by their distinctive cell envelope, which is ~20-40 nm in diameter and consists of an asymmetric membrane bilayer: there is an inner cytoplasmic membrane composed of phospholipid, and an outer membrane that also consists of phospholipid, but with bound outward facing lipopolysaccharide (LPS) (FIGURE 1.1). There are several forms of phospholipid found in Gram negative cell membranes, these are phosphatidylethanolamine (PE), which together with phosphatidylglycerol (PG) constitute most of the lipids found in the inner membrane, as well as phosphatidyl serine and cardiolipin (Silhavy *et al.*, 2010). Situated between the two membranes is the periplasm, which contains a loose network of peptidoglycan. Peptidoglycan consists of an interlocking mesh of β-1,4 linked GlcNAc-MurNAc (N-acetylglucosamine and N-acetylmuramic acid) sugar residues, which are further crosslinked by pentapeptide chains, this forms the basic cell wall structure and gives the bacterial cell a durable form (FIGURE 1.1).

In addition to providing structural stability the cell envelope is also studded with integral membrane proteins and different sugar molecules that provide each bacterial strain with important distinguishing characteristics. One such key feature is the presence of a crystalline lattice of proteins that forms the S-layer, consisting of oligosaccharides and glycoproteins (Sara and Sleytr, 2000), as well as long appendages such as fimbriae that facilitate adhesion to surfaces (Connell *et al.*, 1996). The outer and inner membranes both contain integral membrane proteins, but these differ significantly in their structure: the inner cytoplasmic membrane contains proteins that exhibit α-helical structure consisting of hydrophobic non-polar transmembrane domains, whereas the outer membrane proteins usually consist of monomeric, dimeric and trimeric β-barrels (Berven *et al.*, 2004). These integral membrane proteins usually serve to mediate nutrient intake and ion transport. Gram negative bacteria also contain outer membrane lipoproteins, including Lpp, which interacts with the peptidoglycan cell wall enhancing the overall integrity of the cell envelope (Chang *et al.*, 2012).

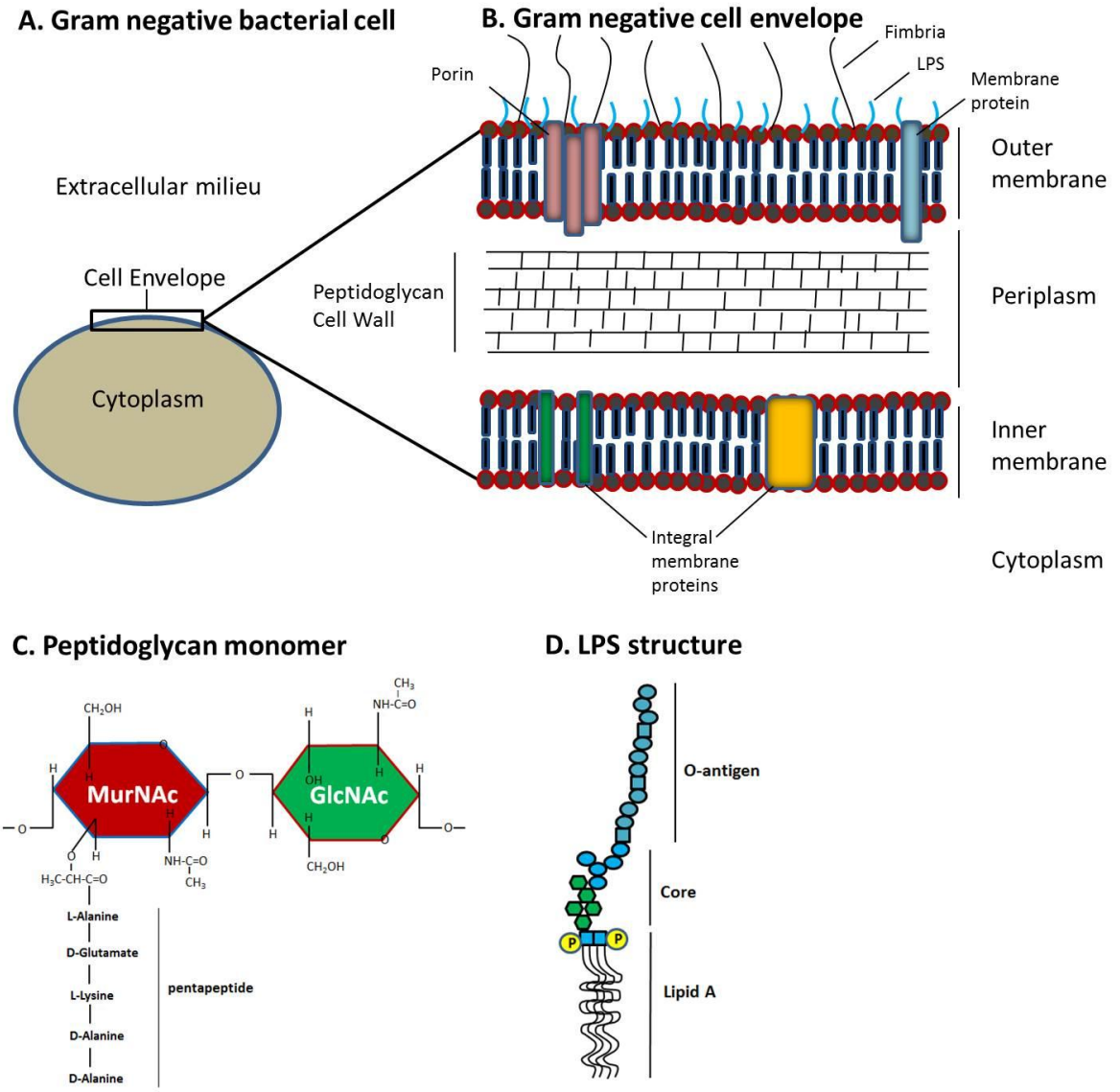


Figure 1.1 The Gram negative cell envelope consists of an asymmetric membrane bilayer. (A) Basic cartoon representation of a bacterial cell. (B) A 'close up' of the Gram negative cell envelope. The inner membrane is composed of a phospholipid bilayer (shown as dark grey/ red circles) and the outer membrane consists of phospholipid with an embedded, outward facing layer of lipopolysaccharide (LPS, represented as blue lines). Between these two membranes is the periplasmic space, which contains the cell wall composed of peptidoglycan (shown as cross linked black lines). (C) Peptidoglycan is composed of β-1,4 linked chains of amino sugars N-acetylglucosamine (GlcNAc or NAG) and N-acetylmuramic acid (MurNAc or NAM), these chains are connected by pentapeptide bonds coming off the MurNAc molecule, connecting to the amino group of the GlcNAc molecule causing the interlocking residues to stack in layers to form peptidoglycan. (D) Lipopolysaccharide (LPS) is embedded in the Gram negative outer membrane, facing outward, and consists of O-antigen, core residues and lipid A tail. O-antigen triggers an innate immune response in animals.

The secreted proteome is an important distinguishing feature of any given bacterium since effector molecules, whether targeted to another cell or to the extracellular environment, are the means by which bacteria facilitate change in their environment. In terms of protein effector molecules

produced by Gram negative bacteria, a key distinction is whether proteins are targeted to the periplasm, defined as protein *export*, or whether they are targeted to the extracellular environment, which requires a dedicated machinery to facilitate movement across the entire cell envelope, which is defined as protein *secretion*.

1.2 Protein Export *via* the Sec Pathway

In bacteria most extracytoplasmic proteins are targeted to the Sec machinery for export. Targeting to the Sec pathway involves the synthesis of a precursor polypeptide containing an N-terminal signal sequence, which itself is essential for translocation of the passenger protein across the inner membrane (von Heijne, 1990). N-terminal signal peptides can vary in size between 5-30 residues, but always consist of three domains: a positively charged N-region (1-5 residues), a central hydrophobic region (7-15 residues), and a polar region at the carboxy terminal end (the C-region, usually 3-7 residues), which is recognised and cleaved by a signal peptidase after translocation (von Heijne, 1990).

The Sec apparatus consists of the inner membrane heterotrimeric translocase SecYEG, the ATPase SecA, the ancillary proteins SecDF and the chaperone SecB. The Sec precursor is captured at the ribosome by the SecB chaperone and transported to the SecA motor ATPase (Lycklama and Driessen, 2012). Currently the most refined model of the Sec mechanism is the 'reciprocating piston model' (Kusters and Driessen, 2011). In this model, the unfolded SecB-bound precursor is delivered to a SecA dimer, which subsequently binds to SecYEG (Mao *et al.*, 2009). The binding of the preprotein to SecA initiates a conformational change in SecA, which causes SecB to dissociate from SecA, and also triggers the ATPase activity, whereupon it inserts a loop like structure containing the N-terminal signal motif and the 'early mature domain' of the preprotein into the SecYEG channel (Engelman and Steitz, 1981; Joly and Wickner, 1993; Lycklama and Driessen, 2012). Additional binding of ATP to SecA pushes the substrate through the SecYEG channel in further sequential translocation steps (Economou and Wickner, 1994). In addition to SecA, the SecDF complex facilitates a proton-motive-force-assisted translocation of the substrate through the channel from the periplasmic side of the membrane – hence SecA provides a pushing force from the cytoplasm, and SecDF a pulling force from the periplasm. Upon arrival in the periplasm the exported protein folds into its mature functional state (Lycklama and Driessen, 2012).

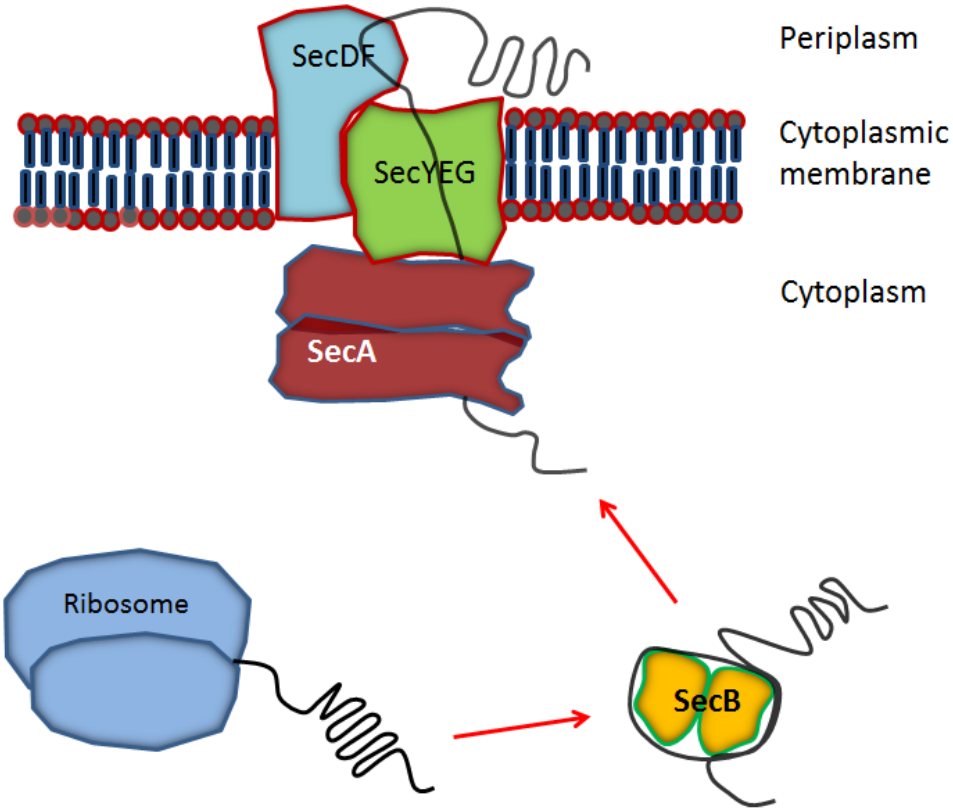


Figure 1.2 Schematic representation of the general secretion (Sec) pathway in Gram negative bacteria. After elongation the chaperone SecB (yellow) binds the unfolded preprotein and transports it to dimeric SecA (maroon), which subsequently binds to the SecYEG channel complex (green). The binding of ATP to SecA initiates a conformational change in SecA, at which point SecB dissociates and the preprotein is inserted into the SecYEG pore and proceeds in an ATP-driven stepwise manner aided by the ancillary SecDF complex (marine blue), which pulls the preprotein through from the periplasmic milieu. During this process the Sec signal peptide is recognised and cleaved by a signal peptidase. Once released to the periplasm the protein undergoes folding. Adapted from (Lycklama and Driessen, 2012).

1.2.1 Protein folding and modification in the periplasm

For most Sec-exported proteins there is a careful process of quality control to ensure that extracytoplasmic proteins are correctly folded; these substrates are folded and assembled in the periplasm – a process that requires the action of various proteases, folding catalysts and chaperones (Miot and Betton, 2004). Two key folding catalysts are protein disulphide isomerases, Dsb proteins, and the peptidyl-prolyl isomerases, PPIase, and these proteins facilitate disulphide bond formation and isomerisation of peptidyl bonds respectively.

The generation of disulphide bridges for the stabilisation of secreted proteins requires an oxidised environment, and therefore presents bacteria with a unique challenge: the bacterial cytoplasm is kept in a reduced state, hence disulphide bond formation in this compartment is not normally possible, and for this reason bacteria will frequently target such proteins to the periplasm, which provides a more suitable (oxidising) environment for effective protein folding (de Marco, 2009).

Disulphide bond formation is essential for the proper assembly of most Sec-exported proteins, and is mediated by Dsb oxidoreductase electron transfer pathways (Kadokura *et al.*, 2003). DsbA and DsbB introduce disulphide bonds into proteins, whereas DsbC and DsbD mediate a disulphide-isomerizing reductive pathway (Sato and Inaba, 2012). The process involves membrane-bound DsbB oxidation of DsbA, which in turn catalyses the oxidation of adjacent cysteines in the folding protein. The resultant electrons are relayed to the quinone pool (Bader *et al.*, 1999; Miot and Betton, 2004). Any production of non-native disulphide bonds made between incorrect pairs of cysteine residues in the folded protein are subsequently rearranged by periplasmic thiol isomerases, DsbC and DsbG (Miot and Betton, 2004).

Such examples serve to illustrate the importance of protein folding and assembly in the appropriate physiological environment, and that the primary amino acid sequence of each protein contains sufficient information to ensure it is processed and targeted correctly.

1.3 Protein export *via* the Tat Pathway

An important mechanism dedicated to the export of proteins across the inner membrane is the twin-arginine translocation pathway, or Tat system. The Tat system is distinctive for its capacity to translocate fully folded proteins across the cytoplasmic membrane: for this the Tat substrates contain a distinctive and highly conserved twin-arginine amino acid motif within their N-terminal signal peptides, defined as SRRXFLK, that distinguishes it from Sec-targeted proteins (Berks *et al.*, 2000). In addition to the consecutive arginine residues, the hydrophobicity of the Tat signal peptide is considerably less than Sec signal sequences and the C-region of Tat signals contains more basic residues than is found in Sec signal peptides (Cristobal *et al.*, 1999). Another remarkable feature of the Tat system is that it can transport proteins that do not possess any recognisable signal sequence: this is achieved by a hitch-hiking mechanism, whereby one protein forms a complex with another bearing a signal peptide in order to co-translocate to the periplasm (Rodrigue *et al.*, 1999).

In bacteria, the number of substrates exported *via* the Tat pathway varies dramatically between different organisms. For example, some Gram positive Firmicutes, such as *Bacillus subtilis* and *Staphylococcus aureus* possess few Tat substrates (Dilks *et al.*, 2003; Palmer and Berks, 2012), whereas other Gram positives such as *Streptomyces coelicolor* export up to one-sixth of its total secretome *via* the Tat apparatus (Widdick *et al.*, 2006). Gram negative enteric bacteria, such as *Escherichia coli* and *Salmonella* sp., encode approximately 20-30 Tat-targeted substrates in their genomes (Berks *et al.*, 2000).

At present, three reasons have been established as to why some proteins need to be exported in a fully folded conformation: the insertion of complex cofactors; the transport of hetero-oligomeric complexes; or the need to avoid competing metal ions (Palmer and Berks, 2012). In the case of cofactor containing enzymes, many cofactors are labile, such as iron-sulphur clusters, so must be assembled within a stable protein scaffold prior to export (Berks *et al.*, 2003). A good example of a protein that requires folding in the cytoplasm to avoid incorrect metal ions being inserted in to the active site is provided by the Mn^{2+}-containing MncA protein, produced by certain *Synechocystis* sp., which must first bind manganese in the absence of competing copper or zinc ions that are abundant in the periplasm (Tottey *et al.*, 2008).

The Tat system consists of three components, TatA, TatB and TatC, which together form a complex that mediates the passage of substrates through the TatA component (FIGURE 1.3). The best characterised Tat system is in *E. coli*, where Tat export is initiated by the substrate signal peptide binding to the TatBC complex – TatC contains a binding site that recognises the twin-arginine motif

(Cline and Mori, 2001). Once bound, TatBC undergoes a conformational change, which triggers TatA to polymerise and associate with TatBC (Mori and Cline, 2002). TatA has been shown to form ~4 subunit oligomers in the absence of TatBC whereas it forms a 25 subunit complex in the presence of TatBC (Leake *et al.*, 2008), which provides further evidence that TatABC together form the translocation system (FIGURE 1.3). Some models suggest that the Tat substrate is in contact with the whole TatABC complex during transport (Mori and Cline, 2002), whereas others suggest that TatBC passes the substrate on to TatA for transport (Alami *et al.*, 2003). After formation of TatABC and transport of substrate to the periplasm, the Tat signal peptide is cleaved by a signal peptidase (Luke *et al.*, 2009) and the complex disassembles (Mori and Cline, 2002) (FIGURE 1.3)..

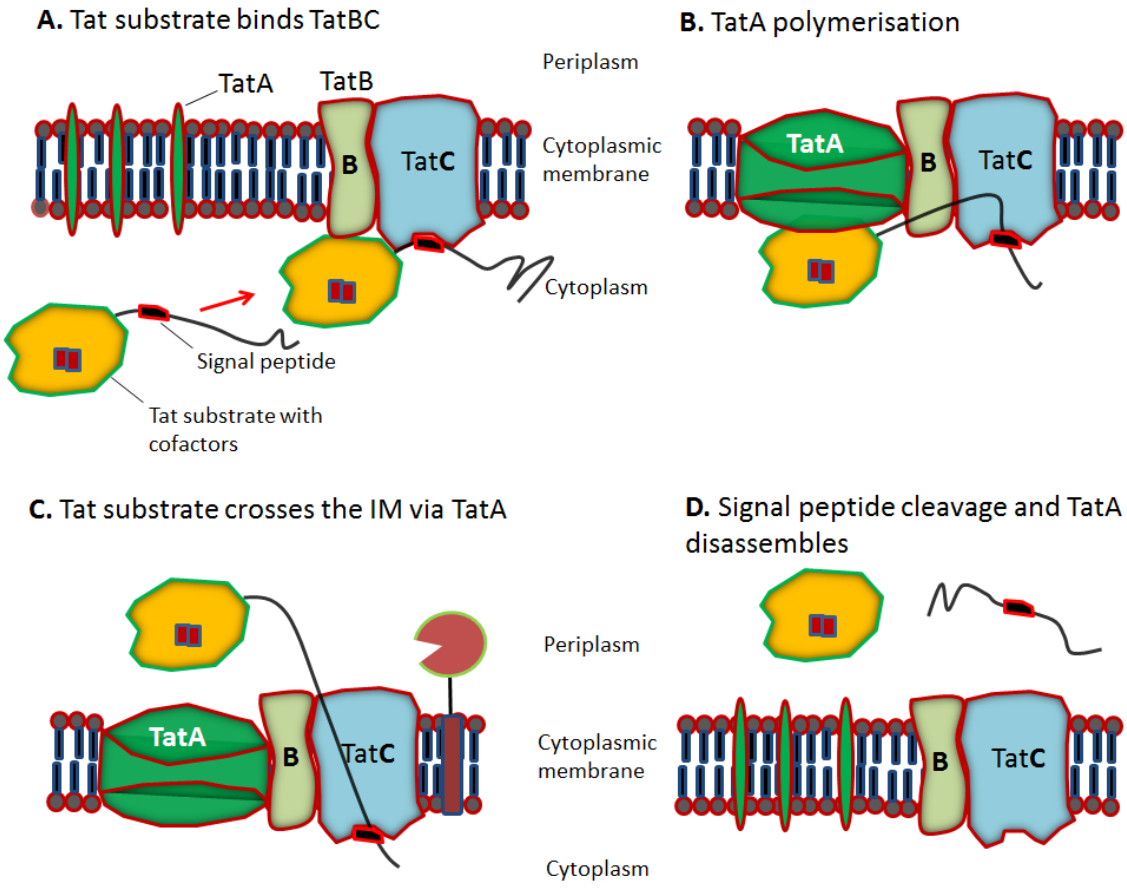


Figure 1.3 Schematic representation of the Tat translocation cycle in *E. coli*. (A) The Tat substrate is folded in the cytoplasm ensuring correct assembly and cofactor/ion insertion, and is targeted to the TatBC complex. TatC specifically recognises the twin-arginine (RR) motif of the Tat substrate. At this stage TatA is present in the membrane as dispersed protomers. TatB and TatC are composed of multiple copies of TatB and C, but are represented here as single proteins for clarity. (B) Binding of the Tat substrate causes TatBC to undergo a conformational change that, in turn, causes TatA to polymerise and associate with TatBC to form the translocation complex TatABC and the substrate is in contact with each of the three components. (C) The Tat substrate is transported across the IM through the TatA component. (D) The signal sequence is cleaved by a signal peptidase. Adapted from (Palmer and Berks, 2012).

1.4 Protein Secretion by Gram Negative Bacteria

In order for effector molecules to be externalised across the cell envelope to the extracellular environment, where they carry out their functions, most substrates will require active secretion. In this work, a secretion system in Gram negative bacteria is defined as any mechanism that is dedicated to the selective movement of a substrate across the outer membrane that can also maintain the integrity of the envelope without non-specific leakage of cellular or periplasmic contents. In Gram negative bacteria six different secretion systems have been identified: these are broadly defined as systems that involve a 'one-step' or a 'two-step' translocation event. One step machineries can act in conjunction with inner membrane components to form a contiguous channel that spans the bilayer and moves substrate across the envelope in a single translocation event. Conversely the two-step systems moves substrate across the inner and outer membranes in two separate events and these systems are dependent on the Sec or Tat pathways for export to the periplasm, before the dedicated secretion apparatus moves the substrate across the outer membrane.

1.4.1 Type 1 Secretion

The Type 1 secretion system (T1SS) is ubiquitous in bacteria and secretes an eclectic set of proteins including adenylate cyclases, pore forming hemolysins (such as HlyA), lipases, proteases and the extracellular haem scavenging protein HasA (Delepelaire, 2004). The Type 1 secretion system consists of a tripartite structure that forms a contiguous channel across the cell envelope: this structure forms transiently upon binding of the substrate to the inner membrane ABC (ATP binding cassette) transporter, after which the substrate passes through a periplasmic adaptor protein (membrane fusion protein MFP), and finally an outer membrane channel of the TolC family (Delepelaire, 2004), which is exemplified by the *E. coli* HlyA Type 1 system composed of HlyB, HlyD and TolC components. One general characteristic of Type 1 substrates is the presence of an essential non-cleavable C-terminal signal sequence, which unlike the Sec-signal peptide lacks any consensus motif and consists of an alpha-helical secondary structure that interacts with the ABC protein at the cytoplasmic membrane (Zhang *et al.*, 1995).

The Type 1 secretion components together form a tunnel-like structure that translocates the substrate directly from the cytoplasm to the extracellular environment, as exemplified by the HlyA system in *E. coli* (Holland *et al.*, 2005). The inner membrane ABC transporter generates the driving

force for protein translocation from ATP hydrolysis, whereas the membrane fusion protein serves to join the ABC protein to the periplasmic domain of the outer membrane TolC pore-forming protein (Holland *et al.*, 2005). In terms of substrate selection, the recognition event between the Type 1 substrate and the ABC component is highly exclusive; interestingly the MFP component is also thought to act in substrate recognition, mediated by its N-terminal cytoplasmic domain, since deletion of this was shown to abolish HlyA secretion completely (Pimenta *et al.*, 1999; Kanonenberg *et al.*, 2013). By contrast the outer membrane TolC protein exhibits considerable promiscuity: in addition to facilitating the movement of Type 1 substrate (such as HlyA) across the outer membrane, TolC has also been shown to mediate the passage of a number of other substrates by interacting with different inner membrane proteins, which is thought to aid in the extrusion of cytotoxic compounds and drug resistance (Kanonenberg *et al.*, 2013). The crucial point is that Type 1 secretion involves the conjunction of all three ABC/ MFP/ TolC components, and therefore that these systems do not exist in constant association, static in the cell envelope; ABC/MFP are colocalised, but their association with TolC to form the functional Type 1 complex is triggered by the ABC/MFP substrate recognition event (Balakrishnan *et al.*, 2001).

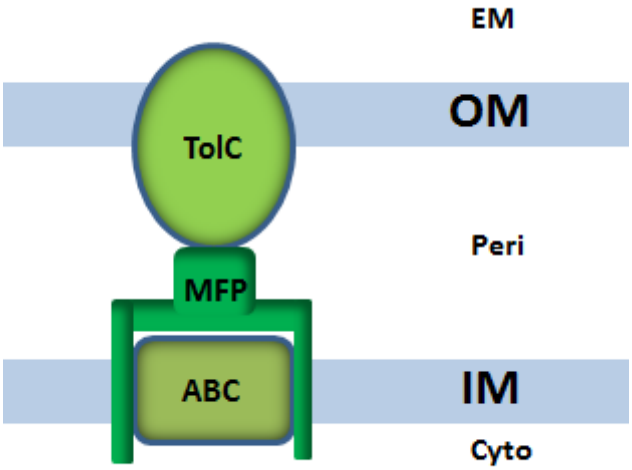


Figure 1.4 The Type1 secretion system exhibits a tripartite structure consisting of ABC/ MFP/ TolC proteins. The complex association of ABC/MFP with TolC is known to be triggered by the ABC/MFP substrate recognition, upon which the substrate is moved from the cytoplasm to the extracellular environment in a one step process. EM, extracellular milieu; OM, outer membrane; Peri, periplasm; IM, inner membrane; Cyto, cytoplasm. Adapted from (Kanonenberg *et al.*, 2013).

1.4.2 Type 2 Secretion

The Type 2 secretion system (T2SS), or general secretory pathway (GSP), involves a two-step process whereby the substrate is exported to the periplasm *via* the Sec or Tat machineries (Voulhoux *et al.*, 2001; de Keyzer *et al.*, 2003); after cleavage of the signal peptide by the leader peptidase, the periplasmic mature exoprotein is pushed through the outer membrane "secretin" component (GspD) by the formation of a pseudopilus structure comprised of a helical assembly of pseudopilins (GspG-K), which is hypothesised to push the substrate through in a piston fashion, driven by a cytoplasmic traffic ATPase, GspE (FIGURE 1.5) (Filloux, 2004). The Type 2 system, or main terminal branch of the GSP, consists of 12-15 different proteins named GspA-O and GspS, which are usually encoded within a single operon (Korotkov *et al.*, 2012). The Type 2 system is homologous to archael flagella, the transformation system of Gram positive bacteria, and to the type IV pilus system: indeed, in *Pseudomonas aeruginosa* both Type 2 pseudopilins and type IV pilins are generated by the same prepilin peptidase, GspO/ PilD (Hahn, 1997; Filloux, 2004; Korotkov *et al.*, 2012).

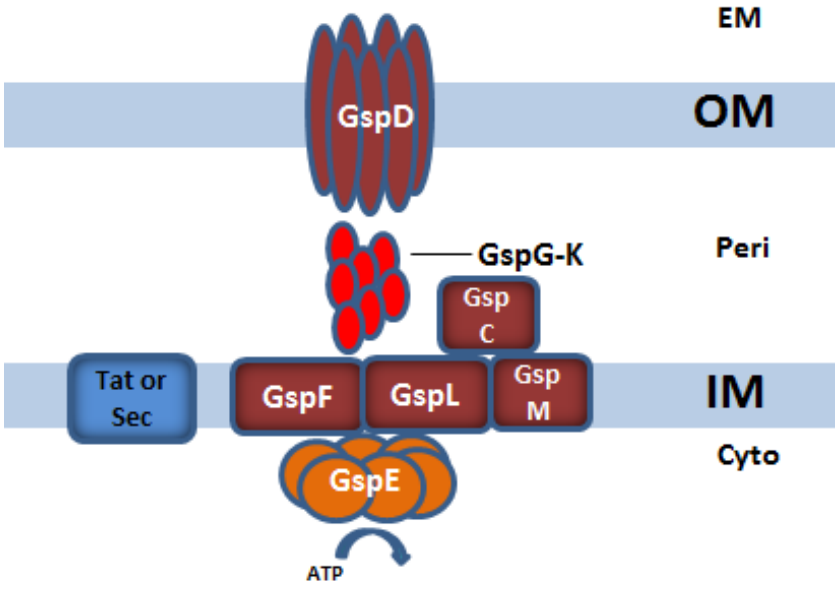


Figure 1.5 The two-step Type 2 secretion system of Gram negative bacteria facilitates movement of substrate across the outer membrane. T2SS consists of 12-15 core proteins: core features are cytoplasmic ATPase GspE, pseudopilus GspG-K, which is hypothesised to push substrate out through the outer membrane secretin (GspD) component in a piston fashion. T2SS substrates are dependent on Sec or Tat for export to the periplasm. Adapted from (Korotkov *et al.*, 2012).

Type 2 secretion is very common in Gram negative bacteria. It is present in both pathogenic bacteria, such as *Vibrio cholerae*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella spp*., *Legionella pneumophila* and *Yersinia enterocolitica*, as well as non-pathogenic organisms such as *Shewanella oneidensis* (Korotkov *et al.*, 2012). Each of these organisms may contain more than one copy of T2SS, and use these to secrete a diverse array of substrates, such as enterotoxin and cholera toxin in enterotoxic *E. coli* and *V. cholera* respectively (Mudrak and Kuehn, 2010), or more benignly, to secrete cell surface exposed lipoproteins in *S. oneidensis* in order to reduce solid metal oxides (Shi *et al.*, 2008).

1.4.3 Type 3 Secretion

The Type 3 'injectisome' (T3SS) was discovered in *Yersinia pestis* and was the first secretion system to demonstrate active translocation of effector molecules from the bacterial cytoplasm directly into the host cell, as opposed to being secreted into the external medium (Cornelis and Van Gijsegem, 2000). The Type 3 system forms a needle/ syringe like structure that bears homology to the bacterial flagella basal body (Saier, 2004). Type 3 systems are widely dispersed amongst Gram negative bacteria (e.g it is encoded by Pathogenicity Islands in the human pathogen *Salmonella* and also by plant pathogens / symbionts such *Rhizobium leguminosarum* (Cornelis, 2006).

Pathogenic *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* encode a T3SS as part of a 70 kb plasmid pCD1 (Hu *et al.*, 1998). In the absence of the T3SS, *Y. pestis* is rendered completely avirulent, even when introduced into the bloodstream (Viboud and Bliska, 2005). The effector molecules of the *Yersinia* T3SS are collectively referred to as Yops, many of which have specific chaperones (such as SycD, SycE, SycH) that mediate effector delivery to their target site (Wattiau *et al.*, 1994; Shao, 2008), and these effectors have been shown to target proinflammatory signalling pathways as well as the host cytoskeleton (Pan *et al.*, 2009). The injectisome is a remarkably complex secretion system: besides the ~20 conserved core features, there are an additional 39 predicted other proteins that facilitate regulation, secretion and translocation of the various core components (Pan *et al.*, 2009). Key features of the injectisome complex include the ATPase YscN (Blaylock *et al.*, 2006), which provides the energy source to drive the Yop effector molecules through the hollow needle channel composed of YscF proteins (Edqvist *et al.*, 2007). The translocon consists of YopB and YopD proteins, in addition to LcrV at the tip of the needle, which together form a pore in the host cell membrane (FIGURE 1.6) (Pan *et al.*, 2009).

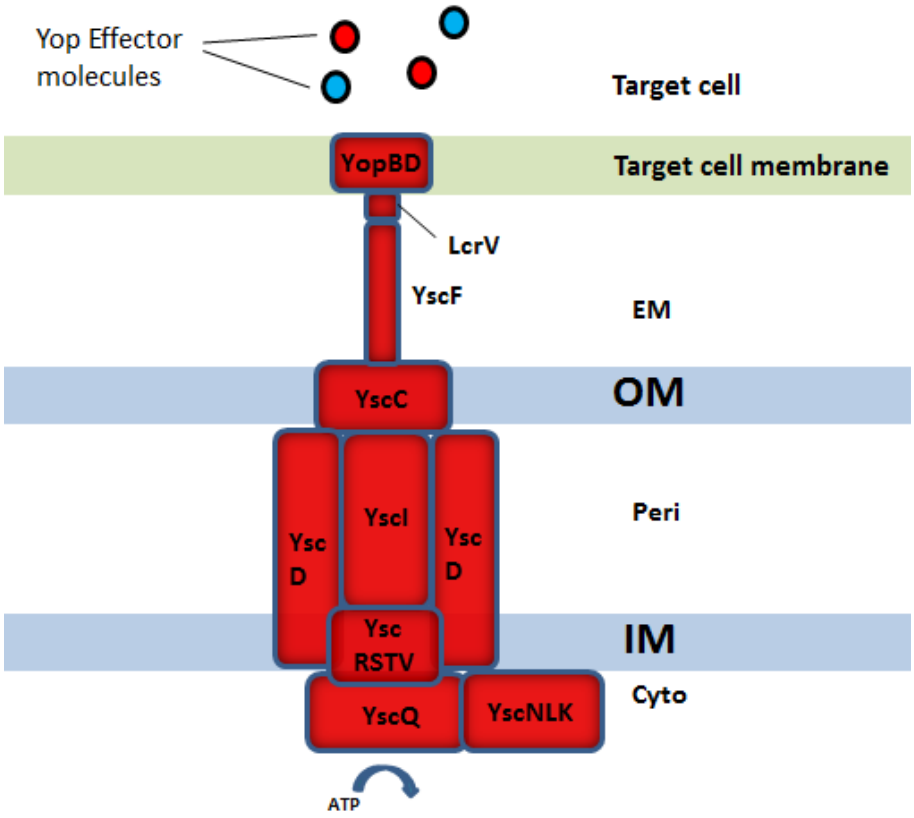


Figure 1.6 The Type 3 injectisome delivers effector molecules directly from the bacterial cytoplasm into target cells. Cartoon representation of the *Yersinia pestis* T3SS, which is essential for virulence, and delivers Yop effector molecules through the hollow YscF channel, across the host cell membrane. Energy for the translocation of effector molecules is derived from YscN ATP hydrolysis. Adapted from (Dewoody *et al.*, 2013).

1.4.4 Type 4 Secretion

The Type 4 system (T4SS) bears homology to the bacterial conjugation system and translocates effector molecules directly in to host cells in one step (Juhas *et al.*, 2008). The Type 4 system is exemplified by the plant pathogen *Agrobacterium tumefaciens* Vir machinery, which translocates T-DNA and nucleoproteins encoded by its Ti plasmid, directly into its plant host, which thereby induces the formation of crown galls (Christie, 2004). Not all Type 4 systems follow this pattern however: the pertussis toxin is Sec-dependent and is a notable exception to the T4 one-step dogma (Cascales and Christie, 2003). The human gastric pathogen *Helicobacter pylori* translocates CagA virulence factors *via* the Type 4 system in to gut epithelial cells (Backert and Selbach, 2008), which is in part responsible for the formation of peptic ulcers.

In *A. tumefaciens* the T-DNA effector is encoded on the Ti-plasmid, but this plasmid also encodes a separate region, the virulence *vir* region that encodes the membrane spanning Type 4 apparatus

(Aguilar *et al.,* 2010). In addition to the secretion of single stranded T-DNA, there are several nucleoproteins, VirD2, VirE2, VirE3 and VirF that bear a positively charged C-terminal transport signal, and are transferred into the plant host by this Type 4 system (Vergunst *et al.*, 2005). The *A. tumefaciens* T4SS consists of a double–chambered channel, composed of VirB7, VirB9 and VirB10, which spans the entire cell envelope to form the core of the Type 4 machinery (Fronzes *et al.*, 2009). The pilus is composed of VirB2 major component (Lai and Kado, 1998), which forms the delivery channel, and VirB5 minor component, which localises at the tip (Aly and Baron, 2007; Aguilar *et al.,* 2010). Assembly of the Type 4 machinery is facilitated by the muramidase VirB1, which in addition to creating a furrow in the peptidoglycan for Type 4 assembly (Zahrl *et al.*, 2005), is also secreted to the cell surface and facilitates pilus formation (Zupan *et al.*, 2007). Several features localised at the inner membrane, including VirB4, VirB11 and VirD4, of the *A. tumefaciens* T4SS are thought to provide ATP driven power to move substrate through the system (Atmakuri *et al.*, 2004).

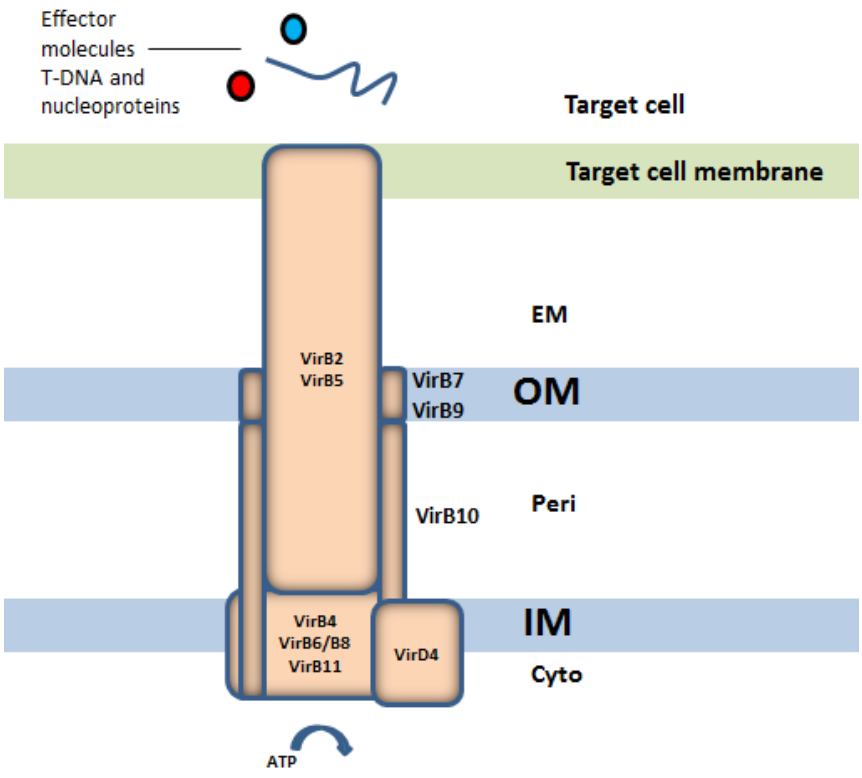


Figure 1.7 The Type 4 secretion system resembles the bacterial conjugation machinery. *A. tumefaciens* uses a T4SS to deliver oncogenic T-DNA and nucleoprotein effector molecules directly into plant host cells, which induces crown gall formation. Adapted from (Cascales and Christie, 2003).

1.4.5 Type 5 Secretion

Type 5 secretion is a two-step mechanism whereby substrates are dependent on the Sec machinery for export to the periplasm, after which they cross the outer membrane facilitated by a β-barrel; transport across the outer membrane takes place in the absence of either ATP hydrolysis or a proton gradient, for this reason Type 5 secretion is also referred to as the 'autotransporter' system (Leo *et al.*, 2012). Currently there are five recognised subclasses of Type 5 secretion systems, which are defined by their outer trans-membrane domain, denoted Va, Vb, Vc, Vd and Ve respectively (Leo *et al.*, 2012). An interesting feature of autotransporter substrates is their relative lack of cysteine residues, since these substrates require folding in the extracellular environment, not in the periplasm; indeed the presence of disulphide bonds is known to have an inhibitory effect on Type 5 secretion (Jose *et al.*, 1996).

The 'classical' autotransporter Va proteins form a β-barrel at their C-terminus which acts as a channel for the N-terminal passenger domain, released by proteolytic cleavage (Henderson *et al.*, 2004). This subclass of Type 5 proteins include some important virulence factors, most notably the adhesin Pertactin produced by *Bordetella pertussis* (Inatsuka *et al.*, 2010), the adhesin AIDA-I produced by *E. coli* (Wells *et al.*, 2010), and another adhesin, IgA, produced by *Neisseria meningitidis* (Virji, 2009). Type Va substrates encode an extended Sec signal peptide, which keeps the substrate polypeptide tethered to the translocon during a quality control process involving Skp, FkpA, SurA, and DegP, which ensure there is no premature folding before outer membrane insertion (Ruiz-Perez *et al.*, 2009). The signal sequence is cleaved once C-terminal POTRA domain is recognised by BamA, which inserts the β-barrel domain into the outer membrane (Knowles *et al.*, 2009). The linker region of the passenger domain then forms a hairpin in the β-barrel pore, which is subsequently pulled through the pore and released to the extracellular milieu – the energy for this process is thought to derive from the secreted protein being folded sequentially as it leaves the outer membrane (Leo *et al.*, 2012). Finally the linker region undergoes cleavage, which in many cases is autocatalytic, releasing the passenger domain to the environment, and the remaining α-helical linker plugs the β-barrel pore (FIGURE 1.8) (Leo *et al.*, 2012).

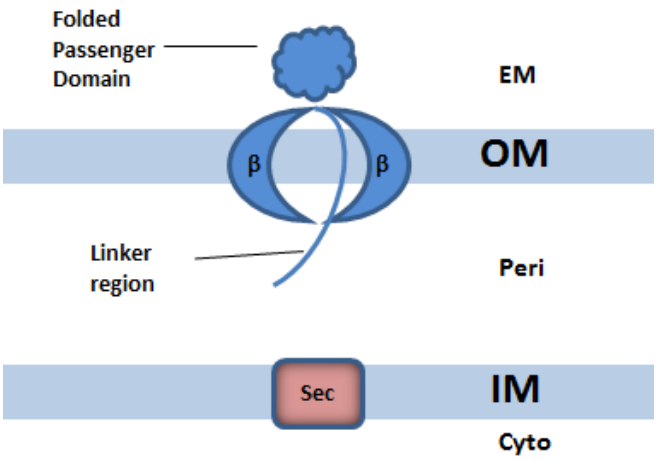


Figure 1.8 The classical Va autotransporter consists of an N-terminal passenger domain and a C-terminal β-barrel domain. After export via the Sec translocon, the POTRA domain at the C-terminus is recognised by BamA, which inserts the β-barrel into the OM, the passenger domain is then fed through sequentially and folds outside the cell, eventually the N-terminal linker region is cleaved, releasing the passenger domain and plugging the remaining β-barrel pore. Adapted from (Henderson *et al.*, 2004).

In contrast to the classical Va autotransporters, the two-partner Vb system contains a separate pore channel (TpsB) and passenger domain (TpsA), and these are usually organised into operons where the pore channel encoding gene precedes the passenger encoding gene (Henderson *et al.*, 2004). The TpsA and TpsB polypeptides are threaded through the Sec translocon separately: TpsB forms a β-barrel in the outer membrane, which contains two periplasmic POTRA domains that recognise the N-terminal ~300 residue two-partner secretion TPS domain of the passenger protein (Delattre *et al.*, 2011). This subclass of Type 5 substrates include heavy weight adhesins, HMW1 and HMW2, produced by *Haemophilus influenza* (St Geme and Yeo, 2009) and haemagglutinin FHA produced by *B. pertussis* (Guedin *et al.*, 2000).

In contrast to the Va and Vb Type 5 systems, the Vc autotransporters are not cleaved after their passage across the outer membrane, and instead remain cell-associated, covalently attached to the membrane anchor, facing outward as far as 250 nm (Linke *et al.*, 2006) – unsurprisingly, this subclass consist of some important adhesins, such as YadA of *Yersinia entercolitica*, which is known to bind collagen of its target host cells (Hoiczyk *et al.*, 2000). The Vc Type 5 system externalises three polypeptide chains, as opposed to one, and for this reason is often referred to as 'trimeric autotransport' (Linke *et al.*, 2006). Substrates of the Vc system, like Va and Vb, also encode extended signal peptides and are kept in an unfolded 'translocation-competent' conformation until they are secreted across the outer membrane, and are also dependent on the Bam complex for insertion of

the membrane anchor region into the outer membrane (Linke *et al.*, 2006). The recently described Vd Type 5 'fused two-partner system' is exemplified by the lipolytic patatin-like protein PlpD of *Pseudomonas aeruginosa* (Salacha *et al.*, 2010): what distinguishes this subclass of Type 5 system is that the passenger domain is joined to the β-barrel domain via a POTRA domain, which has led to the proposition that this system resembles a fusion of the Vb two-partner system, but with quite different passenger substrates (Salacha *et al.*, 2010). The Vd systems are also dependent on BamA for insertion into the outer membrane and also for initiation of transport (Ieva *et al.*, 2011). The most recently discovered Ve subclass are very similar to the classical Va mode of Type 5 secretion, except that the C- and N-terminus of the passenger domain is reversed for these substrates, whereby the α-helical hairpin loop first enters the β-barrel and draws the passenger polypeptide through from its N-terminus, as opposed to the C-terminus in Va secretion (Leo *et al.*, 2012). Ve Type 5 substrates include some important virulence factors such as Intimin and Invasin produced by *E. coli* and enteropathogenic *Yersinia spp*. respectively (Leo *et al.*, 2012).

1.4.6 Type 6 Secretion

Type 6 secretion systems (T6SS) are composed of 13 core components that together form a membrane spanning needle-like structure that resembles an inverted bacteriophage tail-spike and, like the Type 3 system, delivers virulence effector molecules into target cells (Silverman *et al.*, 2012). Interestingly, Type 6 systems have been shown to target both eukaryotic and bacterial host cells, and furthermore different Type 6 systems within a given strain can be used for different purposes and exhibit quite different patterns of regulation (Jani and Cotter, 2010).

The current model of Type 6 secretion involves a core ATPase motor, TssH in the standard nomenclature. The TssD proteins form a hexameric ring that is hypothesized to form a conduit that surrounds, and is capped by, the TssI proteins, and these are capable of puncturing the outer membrane of the target cell to deliver effector molecules (Silverman *et al.*, 2012). The proteins TssB and TssC form a sheath around TssD and TssI at the inner membrane that forms a baseplate structure for the Type 6 apparatus (Silverman *et al.*, 2012). It is thought that close contact with neighbouring cells causes the assembly of the Type 6 apparatus (Basler *et al.*, 2013) and triggers the VipAB sheath to contract, pushing the Hcp/ VgrG needle out of the cell (Basler *et al.*, 2012). The first substrates shown to be secreted to the extracellular milieu by the Type 6 system were Hcp (TssD) and VgrG (TssI) (Pukatzki *et al.*, 2009).

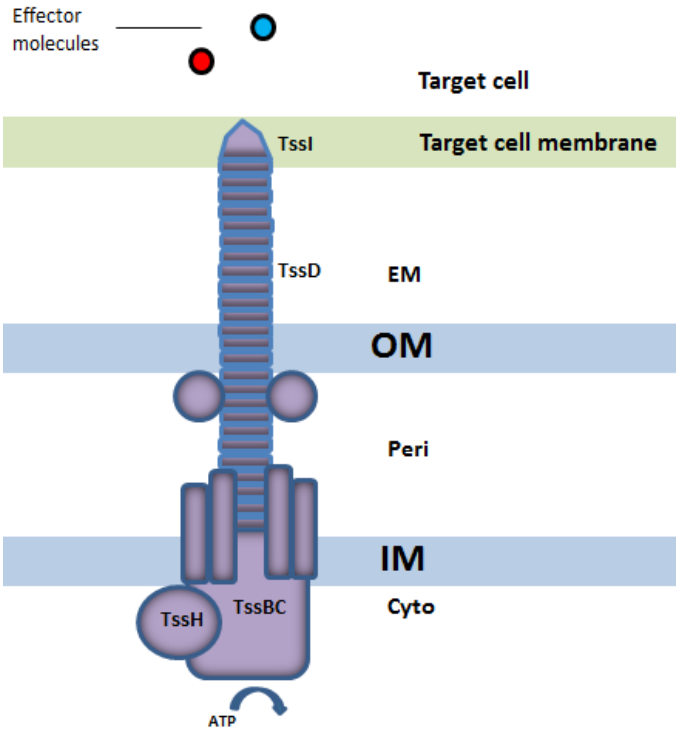


Figure 1.9 The Type 6 secretion system resembles an inverted bacteriophage tail-spike and delivers effector molecules directly into target cells. Key features of T6SS include TssH ATPase motor, the TssD channel capped by TssI, and the base plate structure, which is sheathed by TssB and TssC. Adapted from (Silverman *et al.*, 2012).

In *Pseudomonas aeruginosa* there are three separate Type 6 secretion systems (H1-H3 T6SS) that have been described, as well as three different protein effectors Tse1-3 that have been shown to be virulence factors targeted to other bacteria (Hood *et al.*, 2010). *P. aeruginosa* is the third most common nosocomial pathogen, and can be lethal in chronic respiratory infections such as cystic fibrosis (CF) (Hachani *et al.*, 2011): it is thought that possessing multiple Type 6 systems aids the survival of *P. aeruginosa* within polymicrobial environments, such as in biofilms and/or within the CF lung (Southey-Pillig *et al.*, 2005). Recent work (Sana *et al.*, 2012) is beginning to distinguish between the different *P. aeruginosa* H1-H3 Type 6 systems and their separate functions: for example the H2-T6SS, in contrast to the antibacterial activity of H1-T6SS, was shown to enhance the uptake of strain PAO1 in HeLa cells and lung epithelial cells, and was also shown to be essential for infection in a worm host, suggesting the H2-T6SS targets eukaryotic cells and may have an important role in establishing infection in humans. Little is known about the function of the *P. aeruginosa* H3-T6SS at present, but there is sufficient evidence that the H1-H3 Type 6 systems play diverse roles that enable *P. aeruginosa* to flourish in different environments (Sana *et al.*, 2012).

In *P. aeruginosa*, the best characterised system is the H1-T6SS, which consists of IcmF (TssM), DotU (TssL) and Lip (TssJ), which form the cell envelope platform where it is thought that the C-terminal peptidoglycan binding domain of DotU functions as an anchor (Lossi *et al.*, 2013). The VgrG (TssI) proteins forms the puncturing device reminiscent of the T4 bacteriophage tail spike, which sits at the tip of a conduit/ needle composed mostly of hexameric Hcp (TssD) protein (Pukatzki *et al.*, 2007; Lossi *et al.*, 2013), and the baseplate consists of HsiF1 (TssE) and likely HsiB1 (TssB), HsiC1 (TssC), HsiG1 (TssF) and HsiH1 (TssG) – recent work (Lossi *et al.*, 2013) has established that HsiB1 and HsiC1 predicted baseplate components could be co-purified and that HsiC1 exhibited tubule and cog-wheel structures similar to the phage tail sheath proteins, as previously exemplified by *Vibrio cholerae* Type 6 system (Basler *et al.*, 2012), where these components where shown to exhibit a dynamic process of assembly, disassembly, reassembly at the inner membrane.

1.5 The secreted proteome of *Serratia marcescens*

1.5.1 Introducing *Serratia marcescens*: a prolific secretor of proteins and an opportunistic human pathogen

Serratia marcescens is a Gram-negative γ-Proteobacterium, and is a member of the large family Enterobactericeae. *Serratia* is a rod shaped flagellate bacillus and is, in part, characterised by its production of the pigment prodigiosin, which is a secondary metabolite with antibacterial properties produced especially under phosphate limiting conditions (Williams, 1973). The most striking thing about prodigiosin is the distinctive red quality it gives some *Serratia* strains, indeed the word prodigiosin derives from the Latin word 'prodigiosus' meaning miraculous, wonderful, divine and for this reason is sometimes referred to as the "miracle bacillus". *Serratia* was first discovered by the Italian pharmacist Bartolomeo Bizio in 1819 due to its bloody discolouring of polenta. Prior to this, *Serratia* is thought to have made at least one star appearance in history related to its red pigmentation. In Italy in the middle ages there were some reported miracles during the Catholic Eucharist, or Holy Communion, which is a celebration that involves taking bread and wine as symbolic of the body and blood of Christ. There were several reported miracles of holy bread seeping with 'blood', which has subsequently been attributed to *Serratia*. It was in 1264 that the Pope Urban started the feast of Corpus Christi to celebrate this 'miracle at Bolsena', and was later depicted in a fresco by Raphael on the walls of the Vatican.

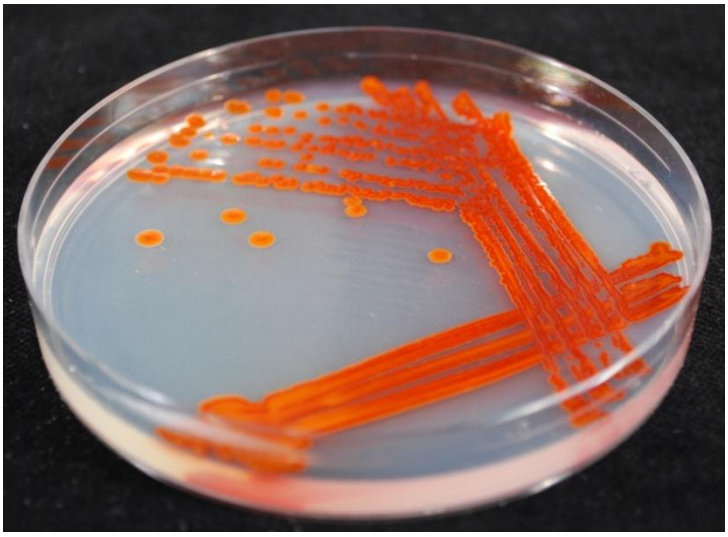


Figure 1.10 *Serratia marcescens* strain 274 produces the brilliant red pigment prodigiosin.

Serratia is a widely dispersed saprophyte in nature: it has been isolated from soil and water environments, from plant and animal hosts and is present in most households growing on tile grout, soap or fatty deposits (Hejazi and Falkiner, 1997). Interestingly *Serratia* was regarded as harmless for many years, from the early to mid-1900s it was used in 'handshake experiments' to demonstrate the importance of handwashing, and in 1951-1952 the U.S army performed 'operation sea spray' where they filled balloons with *Serratia* and burst them over San Fransisco in order to assess the wind dispersal of biological weapons; the subsequent increase in urinary tract infections and pneumonia contributed to *Serratia* being classified as an opportunistic pathogen in the 1960s. Once thought to be a pathogen only in severely debilitated individuals, *Serratia* has been implicated in a plethora of infections including meningitis, wound infections, respiratory infection, septicaemia, ocular infections and as a causative agent of endocarditis both in hospitals and in the community (Mills and Drew, 1976; Cox, 1985; Gouin *et al.*, 1993; Korner *et al.*, 1994; Mahlen, 2011). Indeed, *Serratia* is estimated to be responsible for ~1.4% of hospital acquired infections in the US (Hejazi and Falkiner, 1997). It is especially prevalent in neonatal units where it typically presents itself with meningitis-like symptoms, or cerebral abscess, particularly in infants that have undergone head surgery or those who have suffered a prior sepsis. Indeed, as recently as March 2011, *S. marcescens* was identified as responsible for the infection of eight infants, resulting in one death, in the neonatal unit at Glasgow's Princess Royal Maternity (PRM) hospital. There are multidrug resistant strains of *Serratia*: treatment usually consists of an antibiotic course of fluoroquinolones, amino-glycosides or beta-lactam agents. *Serratia* is known to have innate resistance to ampicillin, macrolides and first generation cephalosporins, as well as reported increasing resistance to gentamicin and tobramycin (O'Dell, 1976).

Although, due to its red pigmentation, *Serratia* was once used to track infections in human patients, today it is still used as a biomarker for pathogenicity in *Drosophila* and in nematode infection models. The genome of *S. marcescens* Db11 - a spontaneous streptomycin resistant derivative of the Db10 strain used in this study - has been made available by the Sanger Institute and was shown to contain 5.12 Mb with 59.51% G+C content. *Serratia* is renowned for the quantity and diversity of its secreted proteome, which includes virulence factors such as phospholipases, the extracellular protein HasA, several proteases, a nuclease, a lipase, the surfactant 'Serrawettin' and several chitinases; it is this eclectic repertoire of secreted proteins that enables *Serratia* to flourish in such a broad range of environmental niches (Hejazi and Falkiner, 1997).

1.5.2 Type 1 secretion in *S. marcescens*

In *S. marcescens* the haem scavenging hemophore HasA is secreted by an archetypal Type 1 system consisting of an inner membrane ABC transporter (HasD), a periplasmic adaptor (HasE) and an outer membrane TolC protein (HasF) (Letoffe *et al.*, 1994; Binet and Wandersman, 1996). The working model of Type 1 secretion proposes an essential C-terminal signal peptide that interacts with the ABC transporter. However, recent work (Masi and Wandersman, 2010) has demonstrated that HasA requires additional linear regions called 'anchoring sites' to mediate HasA secretion. This work has also shown that synthesis and secretion of HasA is closely coupled and that the C-terminal region of HasA actively promotes HasD ATP hydrolysis leading to disassembly of the complex (FIGURE 1.11) (Masi and Wandersman, 2010). In previous work it was also established that the C-terminus of HasA, although essential for secretion, is not essential for targeting to the Type 1 ABC component, since HasA lacking 14 C-terminal amino acid residues was shown to be capable of interacting with the ABC protein, but was jammed at the TolC component (Cescau *et al.*, 2007). It has been proposed that this model of Type 1 mediated secretion adds another level of complexity to the N-terminal signal sequence paradigm, and suggests that the overall secretion process might be more protracted than first imagined, involving certain 'checkpoints' corresponding to separate motif-recognition events (Masi and Wandersman, 2010).

In addition to the HasDEF Type 1 system in *S. marcescens*, there is another Type 1 system, the Lip system, which consists of the ABC protein LipB, the MFP component LipC, and the OMP LipD (Akatsuka *et al.*, 1997). *S. marcescens* uses the Lip Type 1 system to secrete the lipase LipA and the metalloprotease PrtA (Akatsuka *et al.*, 1997). The Lip ABC transporter (LipB) and the MFP (LipC) components exhibit 45% and 53% sequence identity to HasD and HasE in *S. marcescens* respectively (Akatsuka *et al.*, 1995; Akatsuka *et al.*, 1997).

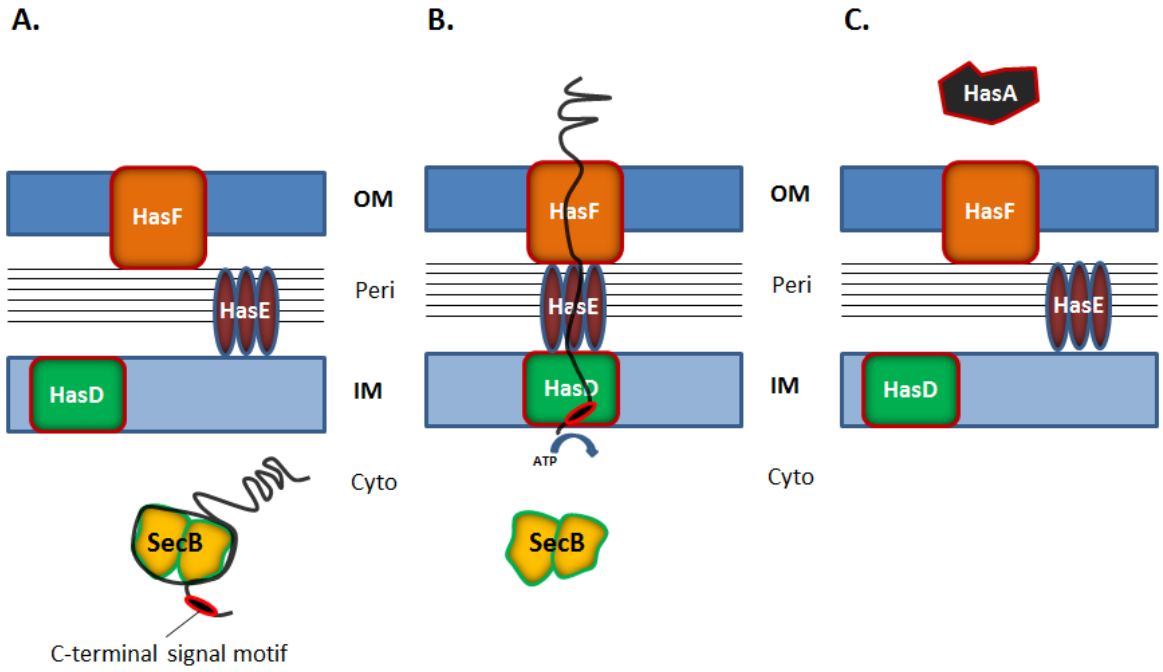


Figure 1.11 Schematic of HasA secretion by Type 1 system HasDEF in *S. marcescens*. (A) Unfolded HasA polypeptide encoding Type 1 C-terminal motif is targeted to the HasD ABC component located in the inner membrane (IM) by the SecB chaperone. (B) HasDEF translocase forms a channel spanning the cell envelope: HasA interaction with HasD induces hydrolysis of ATP that drives HasA through the channel. Linear regions at the C-terminal end of HasA are required for a step-wise recognition process while passing across the channel. (C) The HasA hemophore folds in the extracellular environment and the Type 1 system disassembles. Cyto, cytoplasm; IM, inner membrane; Peri, periplasm (peptidoglycan cell wall shown as black lines); OM, outer membrane. Adapted from (Masi and Wandersman, 2010).

1.5.3 Type 2 secretion in *S. marcescens*

At present there is no published work investigating Type 2 secretion in *S. marcescens*. Analysis of the Db11 genome (Wellcome Trust Sanger Institute, UK) by searching for homologues of several genes (e.g. *gspD*, *gspE* and *gspG*) encoding core Type 2 components in *E. coli* revealed that *S. marcescens* Db10 does not encode a GspD secretin. Indeed, the closest homologue to this is the PilQ protein (SMA3852), a type IV assembly protein. Other gene products similar to Type 2 components are encoded by SMA0091, SMA0092 and SMA0093. Gene SMA0091 encodes a protein, HofC, which has 45% overall sequence identity with the type IV pilin subunit HofC from *E. coli*. SMA0092 encodes HofB, which shares 52% overall sequence identity with the PulE/GspE ATPase from *E. coli*. SMA0093 encodes PppD, a prepilin peptidase dependent protein, which is a predicted major pseudopillus subunit in *S. marcescens* strain WW4 (Kuo *et al.*, 2013), but also has similarity to major prepilin

peptidase dependent protein D (60% overall sequence identity) from *E. coli*. Overall, although the *Serratia marcescens* Db10/Db11 genome encodes a handful of proteins that resemble a T2SS, there are no large and highly organised gene clusters or operons that might encode a complete Type 2 secretion system.

1.5.4 Type 3 secretion in *S. marcescens*

At present there is no published work investigating Type 3 secretion in *S. marcescens*. Analysis of the Db11 genome (Wellcome Trust Sanger Institute, UK) for genes that bear a similarity to core Type 3 components encoded by *Yersinia enterocolitica yopB* (encoding YopB major translocon subunit), *lcrV* (needle tip), *yscN* (ATPase), did not reveal anything that might obviously encode a T3SS. Unsurprisingly, *Y. enterocolitica yscN* gene does have similarity to *S. marcescens* SMA2198 *fliI* encoding a flagellum-specific ATP synthase.

1.5.5 Type 4 secretion in *S. marcescens*

There is currently no published work investigating Type 4 secretion in *S. marcescens*. Searching the Db11 genome (Wellcome Trust Sanger Institute, UK) for genes that have similarity to genes (*virD4*, *virB2* and *virB4*) encoding core components of the *Agrobacterium tumefaciens* Type 4 secretion machinery did not reveal anything resembling Type 4 secretion components. However, we did identify SMA3852, encoding PilQ, which is a putative Type 4 fimbrial component in *S. marcescens* strain WW4 (Kuo *et al.*, 2013), and has similarity to some putative Type 4 secretin components encoded by various *Serratia spp*.. *S. marcescens* PilQ does not have any clear sequence similarities to Type 4 secretins produced by either *Helicobacter spp*. or *Agrobacterium spp.*; it does, however, exhibit a 25% overall sequence identity with a type IV pilus protein from *Agrobacterium* H13-3. Nearby to SMA3852 on the chromosome there is SMA3855, which encodes a putative fimbrial assembly protein, as well as SMA3856, which encodes PilM, a putative type IV pilus assembly protein. Based on this sequence analysis, *S. marcescens* Db10 does not appear to encode anything resembling a complete *bona fide* Type 4 secretion apparatus.

1.5.6 Type 5 Secretion in *S. marcescens*

The hemolysin ShlA is a cytotoxin secreted by *S. marcescens* and is known to interact with ShlB as part of a two-partner (Vb) Sec-dependent Type 5 secretion system (Walker *et al.*, 2004; Hodak and Jacob-Dubuisson, 2007). The ShlA cytotoxin (encoded by the *shlA* gene) causes ATP depletion and potassium efflux in epithelial cells and fibroblasts (Hertle *et al.*, 1999; Walker *et al.*, 2004), and has been shown to contain two conserved regions (68-ANPNL and 109-NPNGIS) that are important for activity, since replacement of N-69 and N-111 residues by isoleucine has been shown to abolish haemolytic activity (Schonherr *et al.*, 1993; Walker *et al.*, 2004). ShlA is known to be secreted across the outer membrane by the ShlB protein in the presence of the cofactor phosphatidylethanolamine: in addition to actively secreting ShlA, ShlB also converts ShlA into an active hemolysin (FIGURE 1.12) (Schiebel *et al.*, 1989; Walker *et al.*, 2004; Shimuta *et al.*, 2009).

In addition to ShlA, the phospholipase PhlA is another virulence factor produced by *S. marcescens* that exhibits haemolytic and cytotoxic activity (PhlA haemolytic activity is dependent on the presence of phospholipids, which it cleaves to form lysophospholipids resulting in haemolysis and cell death) (Shimuta *et al.*, 2009). PhlA is hypothesised to be secreted by a two-partner Type 5 system since a PhlA homologue in the insect pathogen *Photorhabdus luminescens* has been shown to be secreted by the two-partner system (Brillard *et al.*, 2002). However, this remains to be proven experimentally.

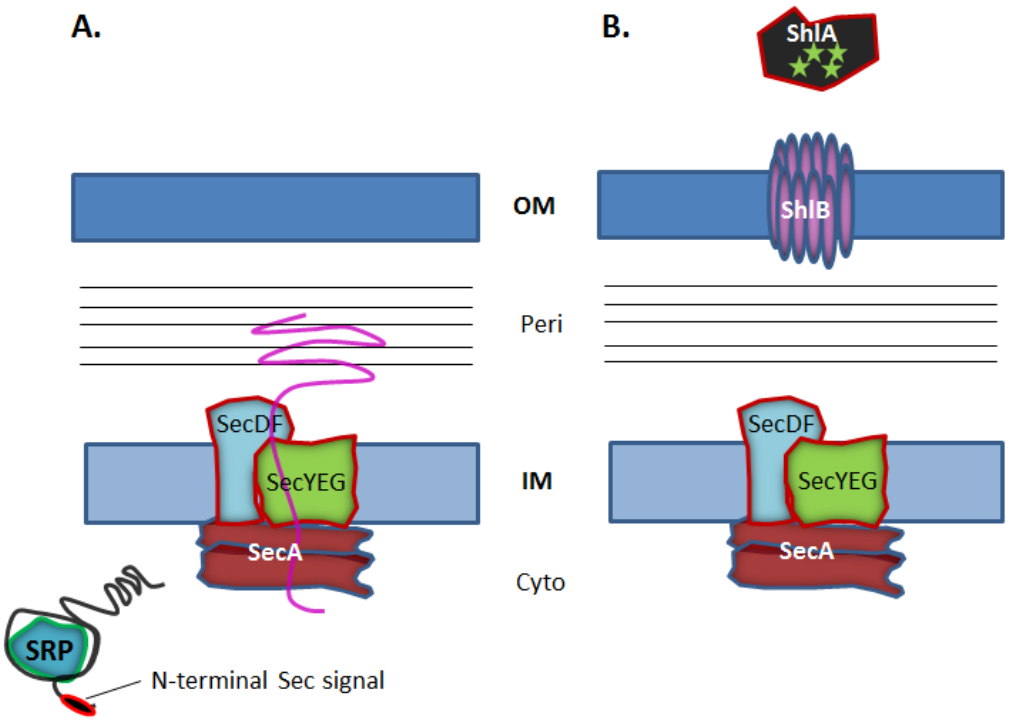


Figure 1.12 Cartoon representation of Type 5 two-partner secretion of cytotoxic hemolysin ShlA in *S. marcescens*. (A) Both partners, ShlA and ShlB, are Sec dependent for export to the periplasm. Interestingly, the preproteins are not targeted to Sec by the SecB chaperone, but by SRP signal recognition particle. (B) Once in the periplasm ShlB forms the β-barrel outer membrane component that mediates transport of ShlA virulence factor to the extracellular environment. ShlB not only facilitates transport but converts ShlA into an active hemolysin by inserting four phosphatidylethanolamine cofactors (green stars). Cyto, cytoplasm; IM, inner membrane; Peri, periplasm (peptidoglycan cell wall shown as black lines); OM, outer membrane. Adapted from (Walker *et al.*, 2004).

1.5.7 Type 6 Secretion in *S. marcescens*

In recent years the research group led by Dr Sarah Coulthurst, University of Dundee, has identified a functional Type 6 secretion system in *Serratia marcescens* that was shown to predominantly target other bacteria and help *S. marcescens* Db10 to flourish within a polymicrobial niche (Murdoch *et al.*, 2011). *S. marcescens* exhibited strong antibacterial killing activity against *Pseudomonas fluorescens* and the opportunistic pathogen *Enterobacter cloacae*, whereas it did not play a role in virulence of non-mammalian hosts such as the nematode *Caenorhabditis elegans* and wax moth larva *Galleria mellonella* (Murdoch *et al.*, 2011). This same work also showed that Hcp was secreted to the extracellular milieu in a Type 6 dependent manner, and that the Type 6 apparatus was constitutively expressed from a gene cluster encoding 13 core components that together was postulated to form the main features of a Type 6 secretion system (FIGURE 1.13) (Murdoch *et al.*, 2011).

The first substrates shown to be secreted to the extracellular milieu by the Type 6 system were Hcp and VgrG (Pukatzki *et al.*, 2009), however these represent structural components of the Type 6 needle and are not regarded as genuine effector proteins. Recent work identified two *bona fide* virulence effectors, Tae4 homologs Ssp1 and Ssp2, which are targeted to other bacterial cells where they act as amidases that hydrolyse the cell wall, and *S. marcescens* protects itself in turn by producing immunity proteins, Rap1a and Rap2a, specific to each of these effectors (English *et al.*, 2012). In more recent work, further label-free quantitation (LFQ) mass spectrometry experiments identified an additional four novel Type 6 anti-bacterial virulence factors secreted by *S. marcescens*, for which another three specific immunity proteins were also discovered (Fritsch *et al.*, 2013). This work also identified that the assembly of the Type 6 machinery is regulated by a post-translational phosphorylation cascade involving homologues of *P. aeruginosa* PpkA, PppA and Fha Type 6 components (Fritsch *et al.*, 2013). In *S. marcescens*, FhA phosphorylation by PpkA was shown to be crucial in activating the Type 6 machinery, which can be subsequently reversed by PppA phosphatase activity; interestingly this form of activation was not cell-to-cell contact dependent, as was proposed to be the case in *Vibrio cholerae* (Basler *et al.*, 2012; Fritsch *et al.*, 2013).

In *S. marcescens* the membrane associated components include IcmH (TssL in the standard Type 6 nomenclature), IcmF (TssM) and Lip (TssJ); the phage tail-like components include the VgrG (TssI) and Hcp (TssD) proteins, Hcp forms hexameric rings that stack on top of one another in a tube-like structure that is thought to be capped by a VgrG trimer (Fritsch *et al.*, 2013) – it has been proposed to form a conduit delivering effector proteins to target cells. The proteins VipA (TssB) and VipB (TssC) are hypothesised to form a sheath around the VgrG/ Hcp needle, which is also very similar to T4 phage tail architecture (Bonemann *et al.*, 2009). It is thought that close contact with neighbouring cells causes the assembly of the Type 6 apparatus (Basler *et al.*, 2013) and triggers the VipAB sheath to contract, pushing the Hcp/ VgrG needle out of the cell (Basler *et al.*, 2012). The current model of Type 6 secretion involves a core ATPase motor, ClpV (TssH), which is also essential for the assembly of the VipAB sheath *in vivo*, and in *S. marcescens* ClpV is known to be recruited to the Type 6 apparatus upon activation by PpkA, which phosphorylates the Fha (TagH) component (FIGURE 1.13) (Fritsch *et al.*, 2013).

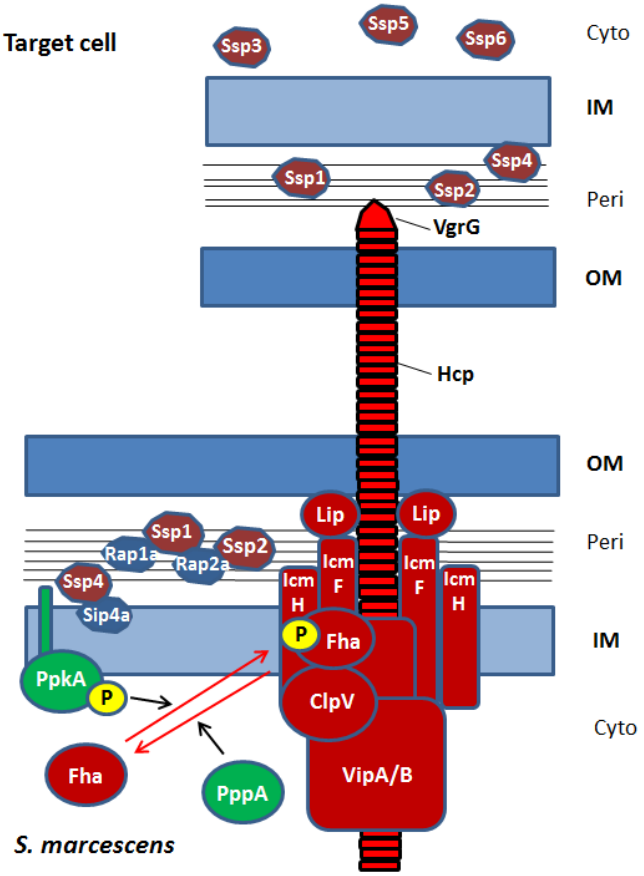


Figure 1.13 Model of *S. marcescens* Type 6 secretion system. *S. marcescens* assembly of Type 6 secretion apparatus is activated by PpkA phosphorylation of Fha, which induces the VipAB sheath to form a baseplate complex with VgrG/Hcp, whereupon the ClpV ATPase is recruited to the complex and initiates transport of effectors. The Hcp and VgrG components form a conduit to transport virulence effectors into the target host cell. Some effectors, such as Ssp1/Ssp2 hydrolyse peptidoglycan, whereas others attack the inner membrane (Ssp4) or are cytotoxic (effectors Ssp3, Ssp5 and Ssp6). For each of these virulence factors a cognate immunity protein has been identified (Rap1-2a and Sip3-5a), except for effector protein Ssp6. Cyto, cytoplasm; IM, inner membrane; Peri, periplasm (peptidoglycan cell wall shown as black lines); OM, outer membrane. Adapted from (Coulthurst, 2013; Fritsch *et al.*, 2013).

1.6 Chitin and chitinolytic activities

1.6.1 Chitin and chitinase enzymes

Chitin is a polymer of (1,4)-*N*-acetyl-D-glucosamine (GlcNAc), itself an important signalling molecule in mammalian cells. Chitin is a key structural component in fungal cell walls, the nematode eggshell and pharyngeal lining, insect and crustacean exoskeletons, and is the second most abundant polysaccharide on Earth after cellulose (there is an estimated 10 gigatons of chitin recycled in the biosphere each year). Chitin exhibits a similar structure to cellulose: the 2-hydroxyl group in cellulose is replaced with an N-acetyl group in chitin and the resultant hydrogen bonding between bordering polymers accounts for the harder structure and greater stability of chitin. The absence of the acetyl group produces the chitin-derivative chitosan. In its unmodified form chitin exists as a translucent pliable "leather-like" material: in arthropod exoskeletons, however, it is embedded within a calcium carbonate matrix which forms a much harder, rigid substance. The chemical structures of cellulose, chitin, chitosan and GlcNAc are given in FIGURE 1.14 below.

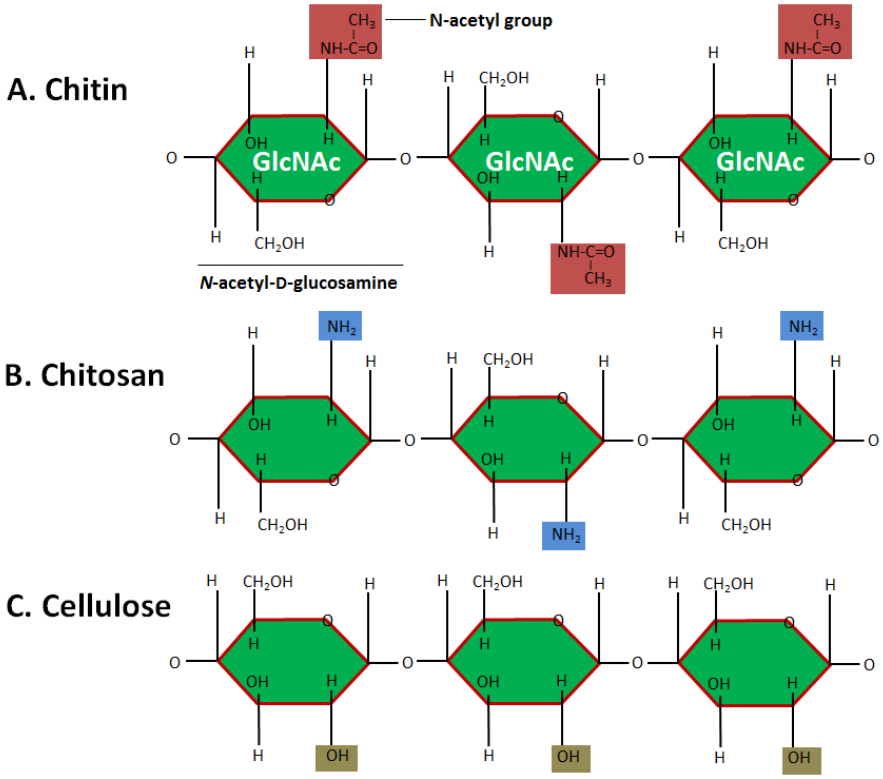


Figure 1.14 Chemical structure of chitin, chitosan and cellulose. (A) Chitin is a polymer of β-1,4 linked monosaccharide *N*-acetyl-D-glucosamine (GlcNAc). (B) Chitosan is chitin lacking most of the acetyl groups. (C) Cellulose is very similar to chitin: chitin has an acetylamine group where cellulose has a hydroxyl group; this quality gives the chitin polymer matrix more stability.

Many organisms produce chitinase enzymes that are part of a repertoire of complex carbohydrate degrading enzymes that hydrolyse the β-1,4 glycosyl bond in the chitin chain. They are frequently classified as 'exo' or 'endo' chitinases depending on whether they cleave randomly within the chitin polymer (endo), or at the end of the polymer (exo) to leave a GlcNAc oligomer or dimer (chitobiose). If the resulting chitobiose is to be used as an energy source, it can be subsequently taken into the cell and cleaved by a chitobiase to leave GlcNAc monomers (Brurberg *et al.*, 2000). The GlcNAc molecule can be further broken down by a deacetylase and a deaminase (in some Gram negative bacteria such as *E. coli* and *S. marcescens* these are encoded by *nagA* and *nagB* genes, respectively) to render glucose (carbon source) and ammonia (N source) available. A general schema of chitin metabolism in enteric bacteria is given in FIGURE 1.15 below.

Due to the ubiquity of chitin in the biosphere, chitinase enzymes are utilised not just by bacteria but also by plants, fungi, viruses, animals and even humans. It is very common for plants to up-regulate the expression of chitinase-encoding genes in response to a fungal or insect infection. For example, the fescue grass *Festuca arundinacea* was shown to dramatically enhance the expression of chitinase FaChit1 as a stress response to fungal attack, whereas mechanical wounding did not greatly affect chitinase expression levels (Wang *et al.*, 2009). Fungi commonly produce their own chitinase enzymes that facilitate the turnover of chitin at the growing apical tip in hyphae, for example the germination and hyphal growth of *Aspergillus nidulans* is inhibited in the absence of the *chiA* chitinase-encoding gene (Takaya *et al.*, 1998). Insect viruses commonly express chitinases to facilitate terminal release of viral progeny from the infected host, such as the granulovirus PiraGV-K of *Pieris rapae* (cabbage butterfly), which expresses a chitinase that mediates virulence in host larvae, and is thought to play an important role in managing the populations of this important crop pest (Oh *et al.*, 2013). In addition to expressing their own chitinases, viruses are also of particular interest for heterologous expression of chitinases as biocontrol agents: for example a baculovirus isolated from the moth *Autographa californica* expressing a foreign chitinase, AcMNPV-CHT1, expressed in *Spodoptera frugiperda* (The Fall Armyworm) cells, was shown to mediate enhanced killing of *Haemaphysalis longicornis* ticks, and could potentially alleviate the incidents of Lyme disease and spotted fever (Assenga *et al.*, 2006).

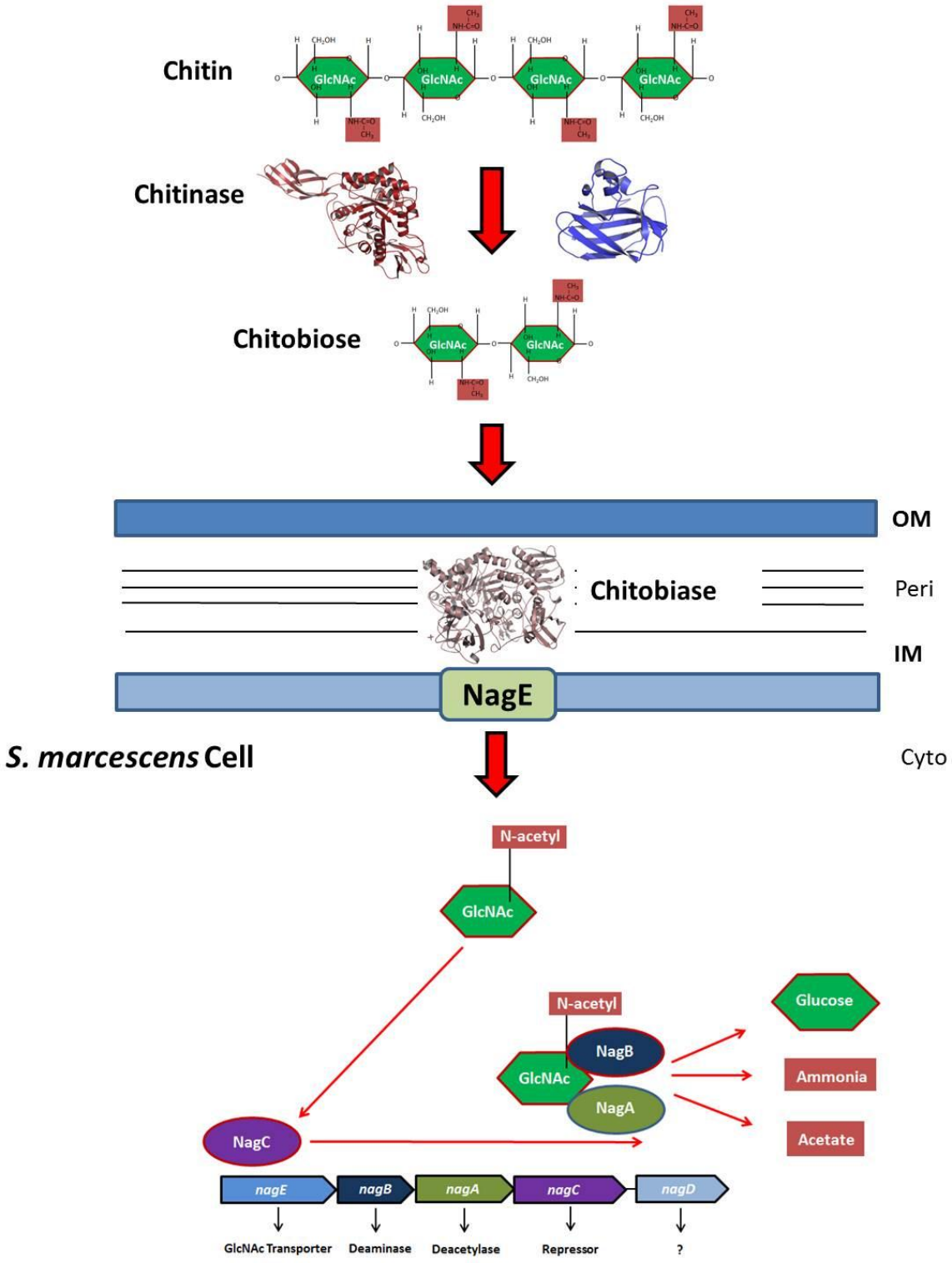


Figure 1.15 General schema of chitin metabolism in *Serratia marcescens*. Chitin is a polymer of N-acetyl-β-D-glucosamine (GlcNAc) which is hydrolysed to chitobiose by chitinases (such as ChiA, ChiB, ChiC in *Serratia*), which is cleaved again by a periplasmic chitobiase to form the monosaccharide component, GlcNAc. In *S. marcescens*, binding of the chitinase to the chitin substrate is promoted by chitin binding protein CBP21. GlcNAc is transported into the cell via NagE whereupon it binds to the NagC repressor of the NagBACD operon and enables constitutive expression: *nagA* and *nagB* encode a deacetylase and a deaminase respectively, and these protein products cleave GlcNAc to produce glucose, ammonia and an acetate group that can provide a carbon and nitrogen source for the cell.

1.6.2 Chitinases and chitin-binding proteins as virulence factors

There are currently two major families of bacterial chitinases, these are classified as families 18 and 19 of the glycoside hydrolases depending on the amino acid sequence of their catalytic domains (Suzuki *et al.*, 1999). Family 18 chitinases include bacterial, fungal, animal and plant chitinases, whereas family 19 contain just plant chitinases and chitinase C from *Streptomyces griseus* (Suzuki *et al.*, 1999). These two families do not exhibit any amino acid sequence similarity and are thought to have emerged independently from separate ancestors (Suzuki *et al.*, 1999). Chitinases have a crucial role in recycling environmental chitin. However, in addition to this there is increased focus on studying them as virulence factors. This section aims to give a brief introduction to the various uses of bacterial chitinolytic activities in relation to the production of chitinase-like molecules in humans, and the conditions in which these molecules mediate important host-microbe interactions.

One of the most interesting features to have emerged from this field is a greater appreciation of the diversity of chitinolytic substrates. Although mammals do not produce chitin, it is thought that many bacterial pathogens, in particular opportunistic human pathogens exploit 'chitinous' receptors, such as mucin, on the epithelial cells of the immunocompromised host (Tran *et al.*, 2011). In these cases it is thought that bacterial chitinases are used to promote adhesion and penetration of target cells, even though these targets do not contain 'true' chitin. For example *Pseudomonas aeruginosa* has been shown to upregulate chitinase expression in response to immunocompromised patients, despite being unable to utilize chitin as a carbon source in itself (Tran *et al.*, 2011).

The Gram positive soil bacterium *Listeria monocytogenes* possesses two chitinase encoding genes *chiA* and *chiB*, as well as *cbp* encoding chitin binding protein (chitin-binding proteins promote the binding of chitinase to its chitin substrate), and is also an opportunistic human pathogen responsible for the food-poisoning condition listeriosis, which typically manifests with meningitis and gastroenteritis symptoms (Vazquez-Boland *et al.*, 2001; Leisner *et al.*, 2008). In a mouse infection model an *L. monocytogenes* strain devoid of *chiA*, *chiB* or *cbp* genes was shown to be significantly impaired in its ability to be translocated to the liver and spleen (important sites for colonisation) (Chaudhuri *et al.*, 2010). In addition to this, the level of *chiA* expression was shown to be enhanced in murine macrophages (Chatterjee *et al.*, 2006), and ChiA has been identified to actively suppress the host innate immune response by decreasing the expression of nitric oxide synthase, which provides the first concrete evidence of a bacterial chitinase actively suppressing host immune defences *in vivo* (Chaudhuri *et al.*, 2013). Since the mouse model does not encode chitin, it is proposed that *Listeria monocytogenes* chitinases are not only capable of hydrolysing chitin, but are also able to bind glycoproteins or carbohydrate moieties in host cells (Chaudhuri *et al.*, 2010).

The Gram negative bacterium *Pseudomonas aeruginosa* is present in the soil, aquatic environments, and is also normally a human commensal present on the skin, as well as being an opportunistic human pathogen. As mentioned above, *P. aeruginosa* is the primary agent of pulmonary infection in cyctic fibrosis (CF) patients, and is unable to utilize chitin as a sole carbon source (Stover *et al.*, 2000; Folders *et al.*, 2001). The chitin binding protein encoding gene was found to be up-regulated in a *P. aeruginosa* strain isolated from a CF patient, suggesting this chitinolytic protein might help establish infection of the CF lung (Salunkhe *et al.*, 2005). Furthermore, expression of the chitinase encoding gene *chiC* was shown to be enhanced when *P. aeruginosa* was grown in culture with samples that mimicked sputum from the lung of CF patients. It was hypothesised that mucin was the key factor that initiated *chiC* expression (Fung *et al.*, 2010). In addition to this, it has also been shown elsewhere that both *chiC* and *cbp* transcript levels were elevated in a CF-associated strain of *P. aeruginosa* (Manos *et al.*, 2009).

Besides thinking about the chitinolytic activity of the microbial pathogen, another important consideration is the chitinolytic activity, and the chitinous molecules, produced by the mammalian host. Although human beings do not produce chitin, we do express two chitinases, acidic mammalian chitinase AMCase and chitotriosidase CHIT1, as well as the chitinase-like enzyme YKL-40 (or CHI3L1), which have been isolated from gut and blood plasma and are thought to aid in defence against pathogens. Despite the role of AMCase in innate immunity, enhanced levels of AMCase have also been shown to play a role in T helper-2 inflammation leading to asthma (Zhu *et al.*, 2004). In addition to mammals encoding chitinases, or chitinase-like enzymes, it is thought that mammalian chitinase expression is enhanced in response to the presence of bacterial chitinases. For example, there is now evidence that early exposure to fungal systemic infection in neonates causes a spike in the presence of chitotriosidase (Labadaridis *et al.*, 2005). Interestingly, the expression of CHIT1 was also shown to be heightened in the early immune response of neonates to bacterial infection, suggesting it is not the presence of chitin that elicits such a response, but an alternative substrate such as a glycoprotein, that sufficiently resembles chitin (Labadaridis *et al.*, 2005). It is clear from these examples that expression of chitinolytic, chitin-binding and chitinous molecules form a dense network of host-microbial interactions that is complex and multifaceted.

1.7 The Chitinolytic machinery of *S. marcescens*

1.7.1 *S. marcescens* is a model organism in terms of chitin-degradation

Serratia is renowned for its secreted proteome and in particular for its ability to degrade chitin: it is a model chitin-degrading organism due to its repertoire of chitinolytic exoproteins (Brurberg *et al.*, 1996). The *S. marcescens* Db10 genome encodes three extracellular chitinases (ChiA, ChiB, and ChiC) and one extracellular chitin binding protein (Cbp21). *S. marcescens* also encodes a periplasmic 95kDa chitobiase (Chb), which cleaves $(GlcNAc)_2$ to produce monosaccharide GlcNAc. Chb belongs to family 20 of the glycosyl hydrolases and consists of four domains. The catalytic domain contains a TIM-barrel fold like the family 18 chitinases, however the active site is different. The function of the remaining three domains is unknown (Brurberg *et al.*, 2000). In addition to the chitobiase, *S. marcescens* also encodes the *nagEBACD* operon, which degrades cytoplasmic GlcNAc to glucose, ammonia and acetate, and hence *S. marcescens* is able to fully utilise chitin as a carbon and nitrogen source. The LysR-type transcriptional regulator ChiR is also thought to be essential for the expression of each of the chitinase encoding genes and *cbp21* (Suzuki *et al.*, 2001). The genomic organisation of each of these chitinolytic components is illustrated in FIGURE 1.16.

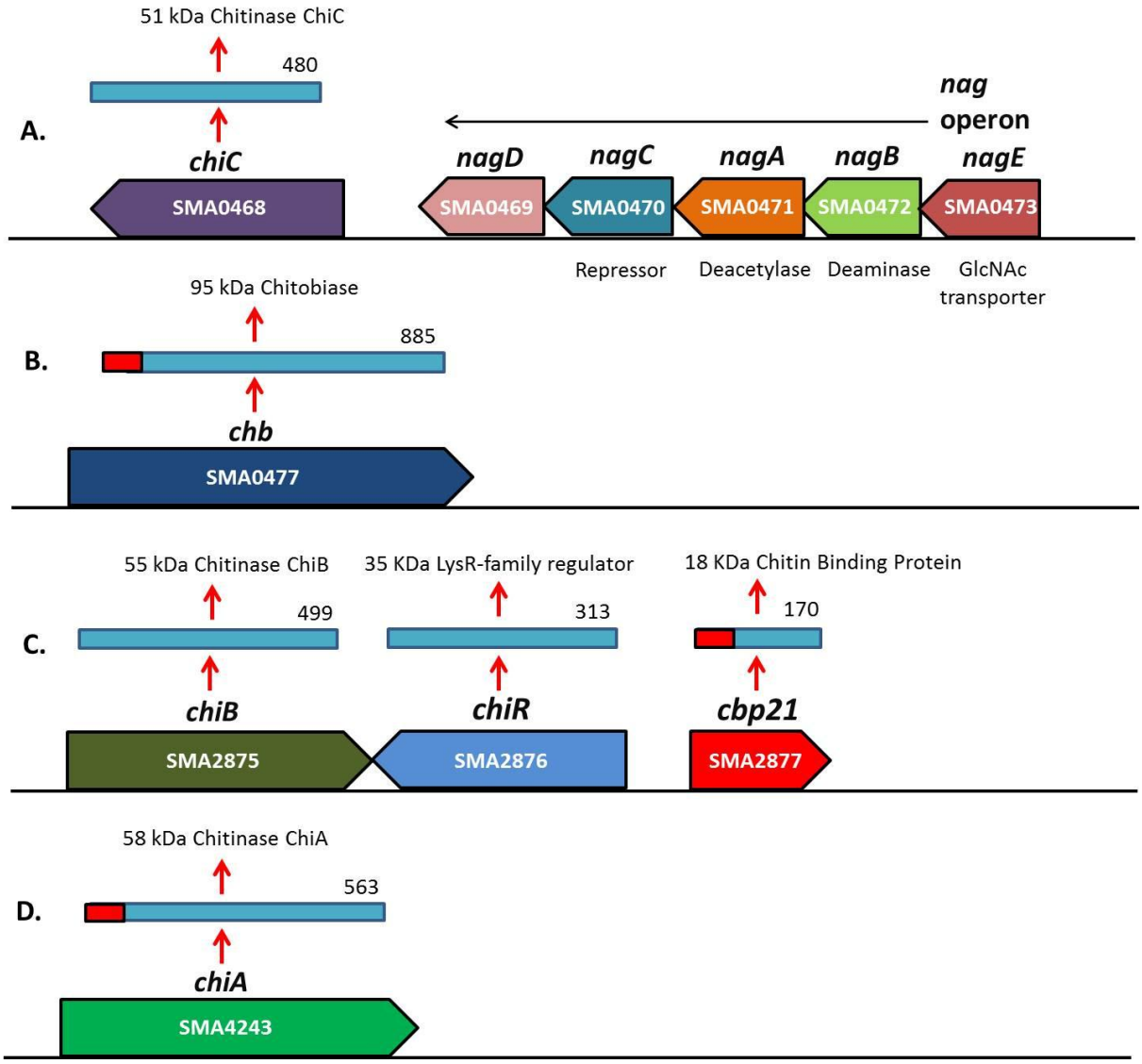


Figure 1.16 Genetic material required for chitin utilisation in *Serratia marcescens* Db10. (A) Genomic loci of chitinase encoding gene *chiC* and the *nag* operon encoding GlcNAc uptake and utilization proteins. (B) Chitobiase encoding gene *chb.* (C) Chitinase encoding gene *chiB*, gene *chiR* encoding a LysR-type transcriptional regulator, and *cbp21* gene encoding chitin binding protein. (D) Genomic loci of chitinase encoding *chiA*. ChiA, Chb and Cbp21 contain N-terminal Sec signal peptide for export to the periplasm (red tags). The SMA number corresponds to the specific gene. The number of residues and the mass of the mature protein is given.

1.7.2 *S. marcescens* ChiA

ChiA is synthesised as a 563-residue precursor with a canonical Sec-type N-terminal signal peptide for targeting the enzyme to the periplasm, whereupon it is cleaved and the resulting 540 residues has a molecular mass 58.5 kDa (FIGURE 1.17) (Brurberg *et al.*, 2000). ChiA contains five cysteine residues and there are two predicted disulphide bonds (115-218, 120-195) (Ferre and Clote, 2006). The structure of *S. marcescens* ChiA was the first crystal structure of a bacterial chitinase, and revealed many of the core features of family 18 glycoside hydrolases, in particular the presence of a TIM-barrel fold, very common for protein active sites, and consists (usually) of eight parallel α-helices and eight parallel β-strands (Perrakis *et al.*, 1994). Like the other chitinase enzymes produced by *S. marcescens*, ChiA encodes SXGG and DXXDXDXE sequence motifs that are characteristic of family 18 glycosyl hydrolases (Brurberg *et al.*, 2000). In addition to this, the structure of ChiA revealed two key residues, E144 and E315, that are responsible for the nucleophilic attack that cleaves the β-1,4 link in the chitin chain (Perrakis *et al.*, 1994; van Aalten *et al.*, 2001). ChiA contains a deep substrate binding groove, and contains three domains that are thought to interact with the chitin substrate, these are termed the ChiN, FnIII and ChBD domains. The ChBD domain is thought to be the 'true' chitin binding domain and it is proposed that the ChiN and FnIII domains are involved in guiding the substrate through the active site cleft (Perrakis *et al.*, 1994; Watanabe *et al.*, 1994; Perrakis *et al.*, 1997; Brurberg *et al.*, 2000).

According to the carbohydrate chemistry, hydrolysis of the β-1,4 glycosidic bond by family 18 chitinases does not follow a 'classical' mechanism and is thought to degrade chitin in a substrate-assisted manner (Brameld *et al.*, 1998). This has led some authors to propose that family 18 chitinases have an absolute preference for chitin substrate bearing an N-acetyl group (Honda *et al.*, 2000), although this strict substrate selection would conflict with the current hypothesis that family 18 chitinases can be routinely used to bind 'chitinous' glycoproteins such as mucin (Frederiksen *et al.*, 2013). In all family 18 chitinases the signature DXXDXDXE motif facilitates hydrolysis of the chitin substrate, all of the aspartate residues, and especially the final glutamate, are key residues in mediating catalysis (Tsujibo *et al.*, 1993; Watanabe *et al.*, 1994; Brurberg *et al.*, 1996). *S. marcescens* ChiA exhibits a strong exo-chitinase character and ChiA-cleavage produces $(GlcNAc)_2$ (Brurberg *et al.*, 2000).

A.

MRKFNKPLLALLIGSTLCSA**AQAAA**PGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKNAADVSVSWNLWNGDAGTTAKI
LLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGK
VVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK
GVTAWDDPYKGNFGQLMALKQAHPDLKILP**SIGG**WTLSDPFFFMGDKVKRDRFVGSVKEFLQTWKF**FDGVDIDWE**FP
GGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGRKYELTSAISAGKDKIDKVAYNVAQNSMDQIFLMSYDFYGAF
DLKNLGHQTALNAPAWKPDTAYTTVNGVNALLTQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWE
NGIVDYRQIASQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDADNGDILNSM
NASLGNSAGVQ

B.

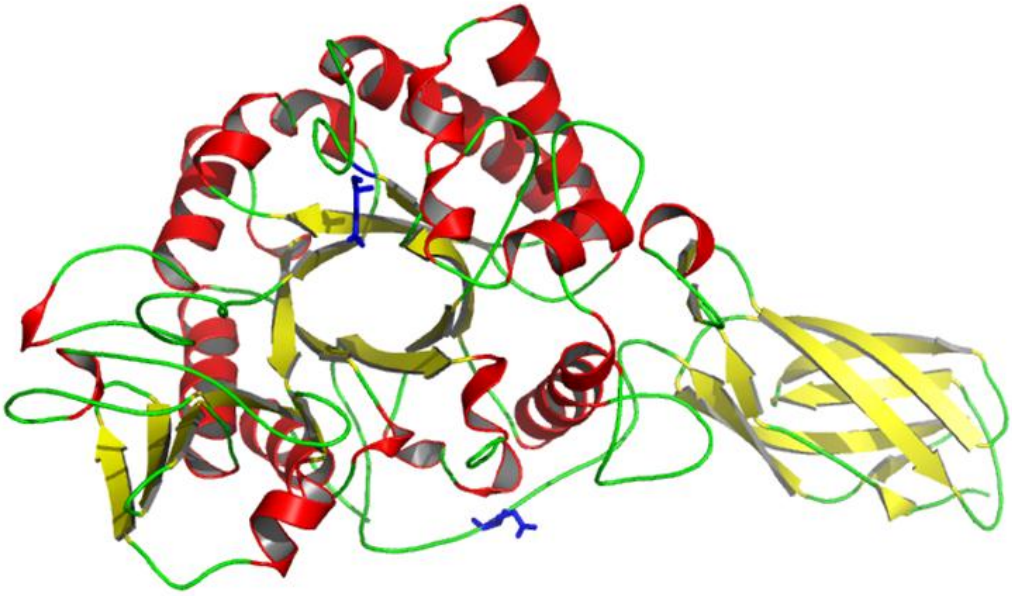


Figure 1.17 ChiA is a family 18 glycosyl hydrolase. Structure of chitinase ChiA from *Serratia marcescens* (Perrakis *et al.*, 1994)**.** (A) Full length amino acid sequence of ChiA is shown and consists of 563 amino acid residues and has a molecular mass of 61,019 Da. ChiA contains an N-terminal signal peptide for targeting to the periplasm (shown in red, the cleavage site in bold), and also bears the signature SXGG/ DXXDXDXE motif of family 18 glycoside hydrolases (shown in green, bold underlined). (B) The structure of *S. marcescens* ChiA. The catalytic E144 and E315 residues are shown as blue sticks.

1.7.3 *S. marcescens* ChiB

ChiB of *S. marcescens* contains the signature SXGG and DXXDXDXE sequence motifs typical of family 18 glycosyl hydrolases, and the crystal structure revealed that the catalytic domain possesses a TIM-barrel fold, which is also characteristic of chitinolytic members of this family (FIGURE 1.18). ChiB exhibits a distinctive tunnel-shaped active site that mediates its exochitinase activity (van Aalten *et al.*, 2001). Mature ChiB contains 498 residues with a mass of 55.4 kDa and is moved to the extracellular environment apparently without proteolytic processing, despite not containing a recognisable N-terminal signal peptide (Brurberg *et al.*, 1995), but ChiB does contain four cysteine residues, with two predicted disulphide bonds (60-328, 127-331), suggesting it is targeted to the periplasm (Ferre and Clote, 2006). In addition to this, early studies on *S. marcescens* ChiB showed that, although it was clearly detectable in the extracellular environment, it was mostly localised in the periplasm (Brurberg *et al.*, 1995), although this conflicted with other studies concluding that ChiB is above all a secreted (extracellular) enzyme (Jones *et al.*, 1986; Watanabe *et al.*, 1997).

The structure of ChiB contains a deep substrate binding groove and exhibits a strong exo-chitinase character. ChiB cleavage produces $(GlcNAc)_3$ and also exhibits a very low level of endochitinase activity (Brurberg *et al.*, 1996). A key feature of ChiB mediated degradation is that ChiB degrades chitin from the non-reducing end, as opposed to the reducing end for ChiA (Brurberg *et al.*, 2000).

The crystal structure of ChiB revealed that it mediates catalysis *via* a substrate-assisted mechanism, with the acidic E144 and E315 being the key catalytic residues, and also that E144 is integral to a 'roof' structure described for the catalytic domain (van Aalten *et al.*, 2001). The ChiB structure also identified some novel features of the catalytic mechanism. Studies of $(GlcNAc)_5$-bound ChiB proposed that following catalysis the 'roof' of ChiB re-opens, and that during catalysis the TIM-barrel catalytic domain undergoes a conformational change involving conserved polar residues resulting in the reaction intermediate being displaced and then stabilised (van Aalten *et al.*, 2001).

A.

MSTRKAVIGYYFIPTNQINNYTETDTSVVPFPVSNITPAKAKQLTHINFSFLDINSNLECAWDPATNDAKARDVVNRLTALKA
HNPSLRIMF**SIGG**WYYSNDLGVSHANYVNAVKTPASRTKFAQSCVRIMKDYG**FDGVDIDWE**YPQAAEVDGFIAALQEIRT
LLNQQTVTDGRQALPYQLTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKVTNHQAALFGDAAGPTFYNAL
REANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGSNGGQYSSHSTPGEDPYPNT
DYWLVGCEECVRDKDPRIASYRQLEQMLLGNYGYQRLWNDKTKTPYLYHAQKGLFVTYDDAESFKYKAKYIKQQQLGGV
MFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLRYTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQT
KWGYITSAPGSDSAWLKVGRVA

B.

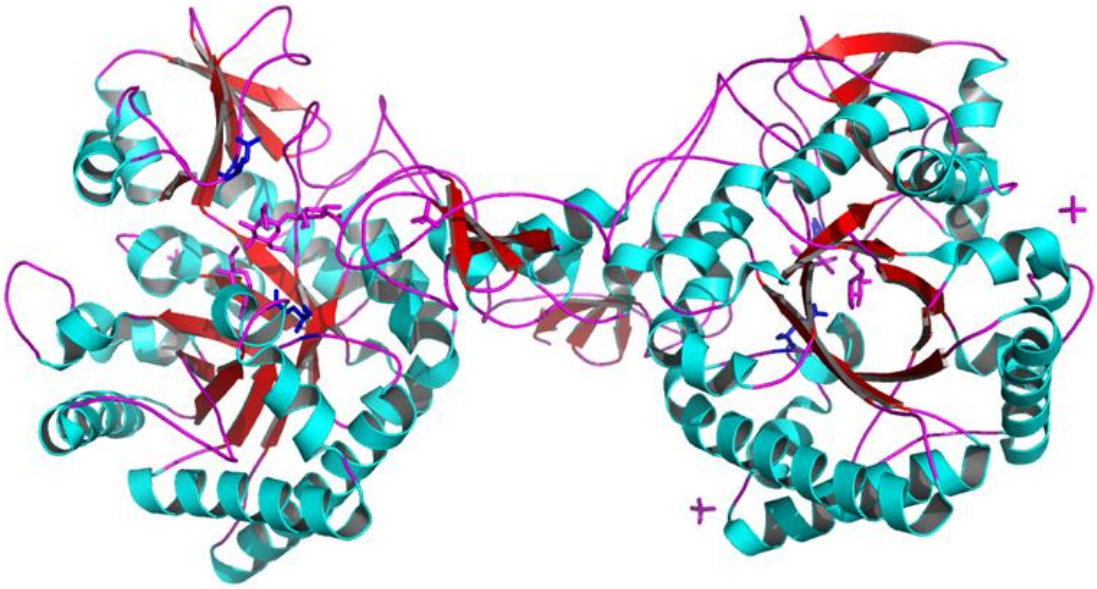


Figure 1.18 ChiB is a family 18 glycosyl hydrolase. Structure of chitinase ChiB from *S. marcescens* (van Aalten *et al.*, 2001)**.** (A) Full length amino acid sequence of ChiB is shown and consists of 499 amino acid residues and has a molecular mass of 55,541 Da. ChiB contains the signature SXGG/ DXXDXDXE motif of family 18 glycoside hydrolases (shown in green, bold underlined). (B) The structure of dimeric *S. marcescens* ChiB. The catalytic E144 and E315 residues are shown as blue sticks.

1.7.4 *S. marcescens* endochitinase ChiC

The full length crystal structure of *S. marcescens* ChiC is yet to be solved. Sequence analysis suggests that ChiC bears the signature SXGG/ DXXDXDXE motif characteristic of family 18 glycoside hydrolases (FIGURE 1.19), and is a predicted endochitinase (Brurberg *et al.*, 2000). ChiC contains only 1 cysteine, so does not contain disulphides. ChiC does not encode any recognisable N-terminal signal peptide, however there has been reports suggesting that ChiC in *S. marcescens* undergoes non-specific processing of the N-terminal region resulting in ChiC variants lacking 8-12 N-terminal residues, which suggests that ChiC is involved in some sort of processing/ recognition event indicative of export (Suzuki *et al.*, 1999). In addition to this, expression of the *chiC* gene in *E. coli* gives rise to two variants, or fragments, of ChiC: there is ChiC1 consisting of 479 residues with a mass 51.6 kDa, whereas ChiC2 is the C-terminally truncated version of ChiC1 consisting of 325 residues (Gal *et al.*, 1998; Suzuki *et al.*, 1999). ChiC is regarded as an exoprotein that is moved deliberately to the extracellular milieu (Fuchs *et al.*, 1986; Watanabe *et al.*, 1997).

Based on amino acid sequence, the predicted structure of ChiC suggests that it consists of a catalytic domain and two putative chitin-binding domains, and is also predicted to have endochitinase activity (Suzuki *et al.*, 1999). It is predicted that an $\alpha+\beta$ domain, that forms the wall of the substrate binding groove in ChiA (residues 295-373) and ChiB, is absent in ChiC, which would account for a much more open substrate binding groove, and hence its endochitinase activity (Brurberg *et al.*, 2000). More recent work examining the function of *S. marcescens* chitinases concludes that ChiC is a 'non-processive' endochitinase. When ChiC was incubated with $(GlcNAc)_6$ oligomers ChiC initially produced $(GlcNAc)_4$ and $(GlcNAc)_2$ (Horn *et al.*, 2006).

The structure of the catalytic site of *S. marcescens* ChiC has recently been resolved (Payne *et al.*, 2012), and this revealed that ChiC has a novel calcium-binding site, and the catalytic glutamic acid is located at E141. Both the partial ChiC structure (Payne *et al.*, 2012) and the Phyre^2 predicted structure (Kelley and Sternberg, 2009) of *S. marcescens* ChiC suggests that it exhibits the greatest similarity to the endochitinase *Ll*Chi18A produced by the Gram positive *Lactococcus lactis* (PDB entry 3IAN).

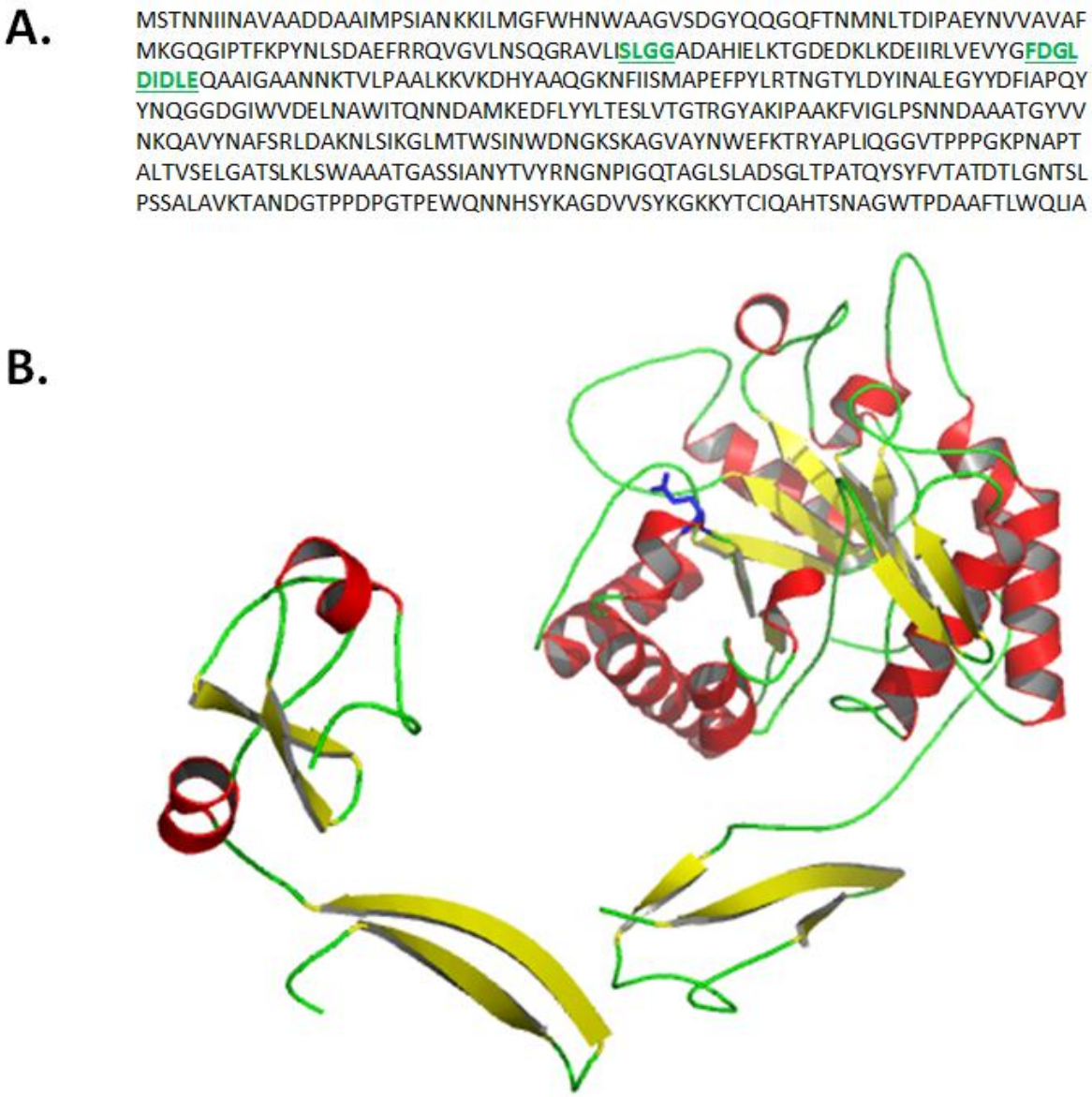


Figure 1.19 ChiC is a family 18 glycosyl hydrolase. (A) Full length amino acid sequence of ChiC is shown and consists of 480 amino acid residues and has a molecular mass of 51,808 Da. ChiC contains the signature SXGG/ DXXDXDXE motif of family 18 glycoside hydrolases (shown in green, bold underlined). (B) Phyre[2] predicted structure of ChiC (Kelley and Sternberg, 2009). The catalytic E141 residue is shown as a blue stick.

FIGURE 1.20 provides a sequence alignment of *S. marcescens* ChiA, ChiB and ChiC. At the level of sequence identity, the three chitinase isoenzymes do not exhibit much similarity: ChiA exhibits 28% sequence identity and 41% similarity to ChiB with 85% sequence coverage, and 36% sequence identity to ChiC with just 25% coverage. ChiB exhibits a small region (22% coverage) bearing similarity to ChiC, with 48% sequence identity and 69% similarity to ChiC. ChiA has a very similar substrate binding groove to ChiB, however ChiA exhibits less of a 'tunnel' shape due to the absence of a loop

that serves as a ‘roof’ in ChiB; in addition to this ChiA has more of an open groove, which means that chitin has greater access to the active site and also that ChiA has some additional low-level endochitinase activity (more akin to ChiC), this structure affords ChiA a certain catalytic flexibility, unlike the other chitinases it can produce GlcNAc monomers, in addition to dimers (Brurberg *et al.*, 2000).

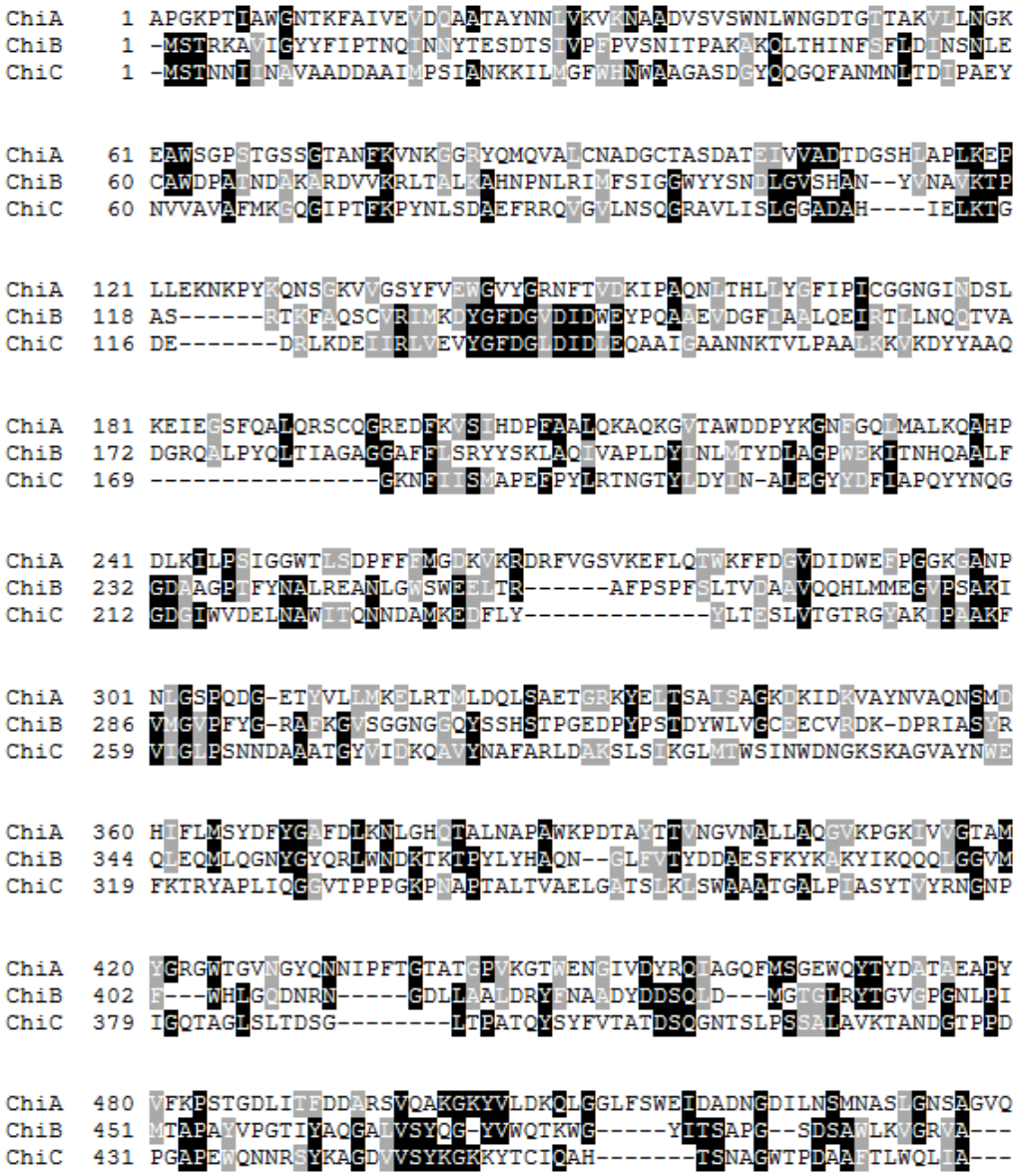


Figure 1.20 Amino acid sequence alignment of chitinases ChiA, ChiB and ChiC from *Serratia marcescens*.

1.7.5 *S. marcescens* chitin binding protein Cbp21

S. marcescens produces an 18 kDa (mature) chitin binding protein CBP21, which is classified as belonging to family 33 CBMs (carbohydrate binding module) (Fuchs *et al.*, 1986). The 197 amino acid precursor contains a Sec-type N-terminal signal peptide for targeting to the periplasm (FIGURE 1.21) (Watanabe *et al.*, 1997). The mature chitin binding protein exhibits general similarity to cellulose binding domains of cellulases, and is known to promote binding of the chitinase to the chitin substrate (Brurberg *et al.*, 2000). An *in vitro* study investigating the role of Cbp21 in *S. marcescens* chitin degradation concluded that Cbp21 strongly promotes ChiA and ChiC-mediated cleavage, and is essential for ChiB-mediated degradation, by binding chitin substrate through polar interactions and inducing structural changes of the chitin substrate that enhances accessibility to chitinase (Vaaje-Kolstad *et al.*, 2005). This work was carried out *in vitro* by incubating chitin with Cbp21 and chitinase and analysing the degradation products using liquid chromatography (Vaaje-Kolstad *et al.*, 2005), it does not establish whether Cbp21 is essential for the chitinolytic activity of *Serratia marcescens in vivo* i.e by making targeted deletion of the *cbp21* gene. Cbp21 has two predicted disulphide bonds (41-145, 49-162) (Ferre and Clote, 2006).

Figure 1.21 Chitin binding protein Cbp21. Structure taken from (Vaaje-Kolstad *et al.*, 2005). (A) Cbp21 contains an N-terminal Sec signal peptide, shown in red, cleavage site in red bold. The crystal structure identified key hydrophilic surface exposed residues that facilitate substrate binding (Y54, E55, E60, H114, D182), these are shown in blue bold. (B) The structure of *S. marcescens* Cbp21 consists of a fibronectin type III fold forming two β-sheet sandwich (Vaaje-Kolstad *et al.*, 2005). The conserved polar residues are shown as blue sticks.

1.8 The problem situation – a hypothesis to be tested

Serratia marcescens is an important human pathogen and is a model organism in terms of chitin degradation. The bacterium produces at least four extracellular proteins that are required for the use of chitin as a carbon and nitrogen source. Considering the fact that *S. marcescens* is renowned for its elaborate secreted proteome, it is surprising that very little is known about the secretion mechanism responsible for exporting the various chitinases outside the cell. Since the ChiA and Cbp21 precursors contain Sec-type signal peptides it may have been assumed that such proteins would be externalised *via* a Type 2 secretion system. Indeed, the marine pathogen *Vibrio cholerae* is known to secrete a chitinase *via* a Type 2 system (Sikora *et al.*, 2011), as does *E. coli* (Francetic *et al.*, 2000). However, our analysis of the *S. marcescens* Db10 genome was unable to identify any genes encoding typical Type 2 components. Since both ChiA and Cbp21 contain disulphide bridges, it makes sense to target these proteins first to the periplasm and then out to the extracellular milieu in a two-step process. However, to deepen the mystery further, ChiB also has a disulphide bond but in this case does not bear a Sec-signal peptide. Moreover, ChiC has neither disulphides nor a signal peptide, which begs the question how these enzymes are targeted to the extracellular environment.

Other than the Type 1-6 systems, are there other routes to protein export and secretion used by bacteria? Non-classical secretion in bacteria can be defined as protein secretion in the absence of any recognisable signal motif. There are some fascinating examples of non-classical secretion provided by Gram positive bacteria. For example, the human pathogen *Mycobacterium tuberculosis* produces the secreted substrate superoxide dismutase SodA, which it is able to secrete in the absence of any recognisable signal motif (Harth and Horwitz, 1999). For this the pathogen uses a variant of SecA known as SecA2 (Braunstein *et al.*, 2001). There are additional reported cases of SecA2 mediated secretion in other Gram positives, such as *Listeria monocytogenes*, which also utilises SecA2 in virulence to mediate smooth-rough phenotypic variation (Lenz and Portnoy, 2002), or *Staphylococcus aureus*, which uses the accessory secretory factors SecA2 and SecY2 to export the glycoprotein GspB (Siboo *et al.*, 2008). Interestingly, recent work investigating the nature of SodA dismutase in the Gram negative *Rhizobium leguminosarum* identified it as being targeted to the Sec apparatus for export, despite not bearing a recognisable Sec signal peptide (Krehenbrink *et al.*, 2011).

It is both interesting and highly relevant to this study that non-classical secretion of chitinase has been identified. Work done on chitinase ChiC in *P. aeruginosa*, which exhibits sequence similarity to *S. marcescens* ChiC (FIGURE 1.22), was first to address the question of non-classically secreted

chitinase (Folders *et al.*, 2001). This research identified some unusual characteristics about *Pseudomonas* ChiC with regard to its status as an exoprotein: it lacks a typical N-terminal signal sequence, and yet the first 11 residues of the N-terminus were apparently cleaved off in the secreted form (FIGURE 1.22) (Folders *et al.*, 2001). These authors also showed that expression of *Pseudomonas* ChiC is regulated by quorum sensing and that it is secreted gradually over a four day time course, and this behaviour was not attributable to cell lysis (Folders *et al.*, 2001).

Thus, chitinase secretion may follow an unusual route in a wide range of pathogenic bacteria. It is tempting to speculate that, in addition to the six known protein secretion systems, a seventh may be waiting to be discovered.

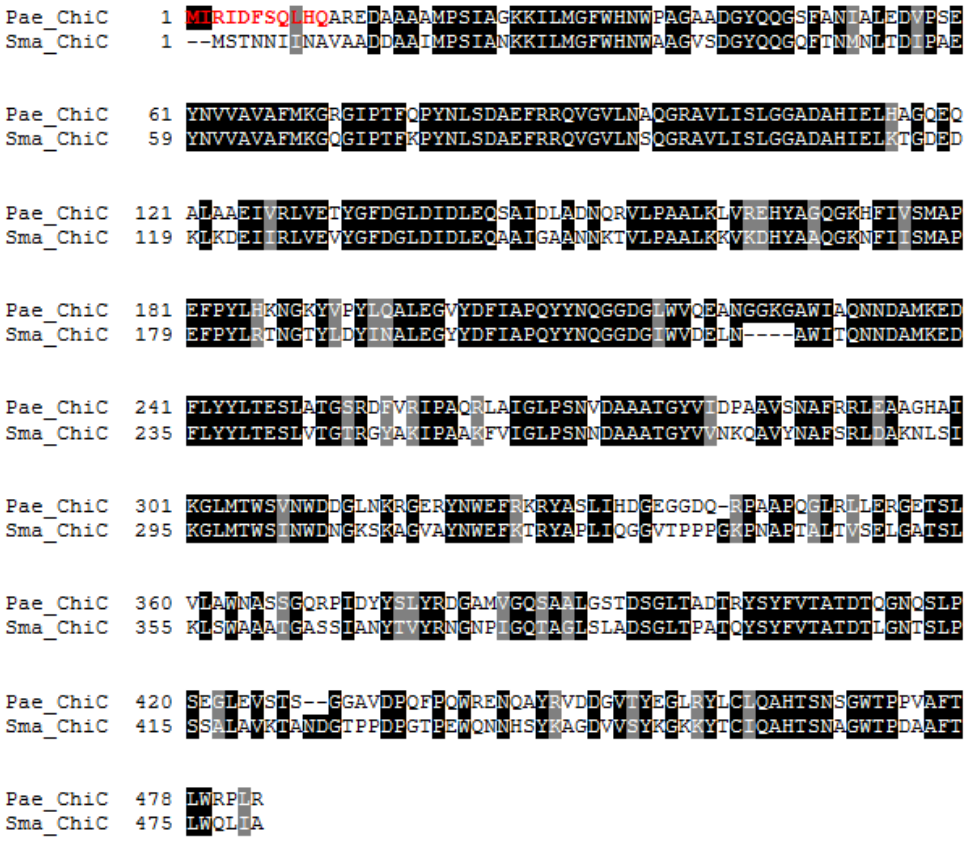


Figure 1.22 An alignment of ChiC sequences from *Serratia marcescens* Db10 (Sma) and *Pseudomonas aeruginosa* (Pae) ((Folders *et al.*, 2001) shows considerable sequence conservation. To the sequence of ChiC from *S. marcescens*, ChiC from *P. aeruginosa* has 65% identity and 76% similarity. Previous work (Folders *et al.*, 2001) has shown that *P. aeruginosa* ChiC is processed, the first 11 residues are cleaved (shown in red). By comparison, ChiA from *Vibrio harveyi* has 31% identity and 44% similarity to *S. marcescens* ChiC, and ChiD from *B. circulans* has 29% identity and 44% similarity to *S. marcescens* ChiC. Sequence alignments were performed with Clustal Omega (2), and displayed using the freely-available on-line tool Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

1.9 Aims

The overall aim of this project was to address the core hypothesis that there could be a secretion system dedicated to the transport of chitinases across the cell envelope in *S. marcescens*. We will take molecular genetic, proteomic, and biochemical approaches in order to challenge this hypothesis. This research has the following specific objectives:

1. To identify new genes involved in chitin utilisation.
2. To understand the roles of any new genes in chitinase export and secretion.
3. To form a new hypothesis on the mechanism of chitinase secretion.

2 Identification of genes required for chitinolytic phenotype

2.1 Introduction

One key process by which bacteria adapt to their environment is *via* the secretion of effector molecules, whereby proteins, or peptides or nucleic acids are actively translocated from inside the cell across the cell envelope into the environment. *Serratia marcescens* is renowned for the quantity and diversity of its secreted proteome, which includes a metalloprotease PrtA, a phospholipase PhlA, a haem-scavenging hemophore HasA and the cytotoxic hemolysin ShlA (Akatsuka *et al.*, 1997; Hejazi and Falkiner, 1997; Hertle *et al.*, 1999; Shimuta *et al.*, 2009). *Serratia marcescens* is also remarkable for its ability to degrade the quite intractable polymer chitin, for which it utilizes three chitinases ChiA, ChiB, ChiC and a chitin-binding protein Cbp21. ChiA and Cbp21 both contain signal peptides targeting them to the Sec-apparatus (Table 2.1) for export: this was enough to infer that these were secreted substrates, and since the *S. marcescens* Db10 genome does not encode a Type 2 secretion system, we postulated that *S. marcescens* could encode an unknown mechanism dedicated to moving these enzymes across the cell envelope. Moreover, ChiB and ChiC do not contain any recognisable signal peptides, which makes the question as to how these enzymes - of considerable size (ChiC is 50 kDa) - are released outside of the bacterial cell even more pertinent.

2.2 Aims

The aim of this Chapter was to begin to address the mechanism responsible for the extracellular presence of the *S. marcescens* chitinolytic system. We aimed to establish whether chitinases are deliberately moved to the extracellular environment by *Serratia* and if, therefore, we could regard these as true secreted proteins. We aimed to take a molecular-genetic approach by performing a classical transposon mutagenic screen to establish what genes may be responsible for the extracellular presence of chitinases in *S. marcescens*.

2.3 Results

2.3.1 Tools for investigating chitinase biology in *Serratia*: a bank of deletion mutants

In order to understand the different roles of each component in the *S. marcescens* chitinolytic system, it was necessary to produce a bank of deletion mutants that would enable us to study each of the chitin-degrading components in isolation. This would establish the core components of the system, identify any hitherto unknown chitinolytic proteins, and serve as the basis for a genetic screen to search for a secretion system. To do this, we constructed deletion alleles for *chiA*, *chiB* and *chiC* in the pKNG101 suicide vector: a system that facilitates the targeted deletion of genes on the *S. marcescens* Db10 chromosome by homologous recombination (Kaniga *et al.*, 1991).

Initially, we constructed three strains each lacking a single chitinase encoding gene: JJH01 (Δ*chiA*), JJH02 (Δ*chiB*), and JJH03 (Δ*chiC*). In each case the entire gene was removed, with only the start and stop codons remaining. Next, the same constructs were used to produce three double mutants that would express only one chitinase activity: JchiA (*chiA*$^{+}$, Δ*chiB*, Δ*chiC*), JchiB (*chiB*$^{+}$, Δ*chiA*, Δ*chiC*) and JchiC (*chiC*$^{+}$, *ΔchiA*, *ΔchiB*). Finally, a strain lacking all three of the known chitinase encoding genes was also constructed, Nochi (Δ*chiA*, Δ*chiB*, Δ*chiC*).

2.3.2 Tools for investigating chitinase biology in *Serratia*: antisera against the isoenzymes

In order to understand the molecular basis of chitinase secretion in *S. marcescens* it was anticipated that Western immunoblot analysis would be a powerful tool in assessing the subcellular localisation and post-translational modifications of the enzymes. Thus, with the aim of generating polyclonal antibodies for each isoenzyme, we cloned the *chiA* gene into pQE70, and cloned separately the *chiB* and *chiC* genes into the pQE60 overproduction vector (Qiagen). Upon transformation of the *E. coli* M15 [pRep4] host strain, each of the chitinases were observed to be overproduced by induction with 2 mM IPTG (final concentration) (FIGURE 2.1). In addition, the engineered C-terminal hexa-histidine tags were found to be intact for each isoenzyme following immunobloting with an anti-pentaHis monoclonal antibody (FIGURE 2.1). Small scale solubility tests were carried out and, under the conditions tested, the recombinant ChiC^{His} was the only isoenzyme present in the soluble fraction (FIGURE 2.1).

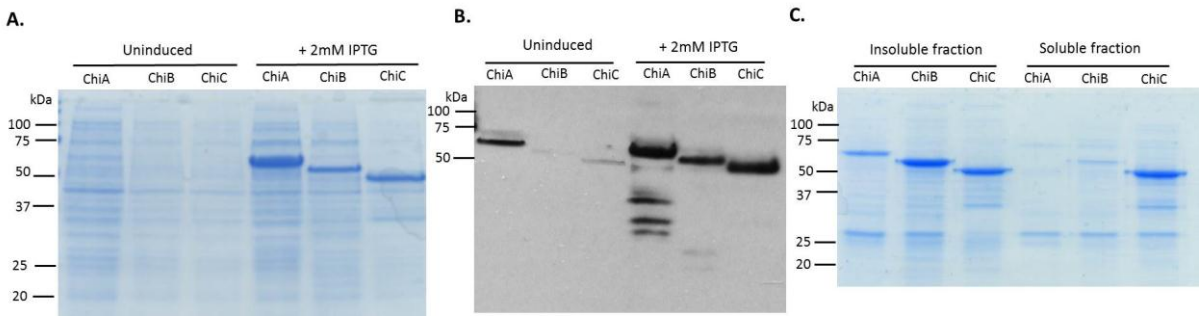


Figure 2.1 Overproduction of the *S. marcescens* chitinases in *E. coli*. (A) Each of the *S. marcescens* chitinase encoding genes *chiA*, *chiB*, *chiC* were overexpressed from pQE60/ pQE70 vector induced with 2mm IPTG in LB medium at 37 °C for 3 hours. Whole cell fractions were loaded onto a 14 % (w/v) acrylamide gel and stained with Coomassie brilliant blue (B) Western immunoblotting of whole cell fractions using a penta-His mouse monoclonal antibody shows each of the C-terminal hexa-Histidine tags were present. (C) Following IPTG induction, cells were harvested, washed, and sonicated. Insoluble material was removed by centrifugation and compared to the remaining soluble protein by SDS-PAGE.

Since ChiC was the only chitinase to appear in the soluble fraction, and because ChiC is one of the least well characterised members of the *S. marcescens* chitinolytic system, we purified ChiC^{His} by immobilised metal affinity chromatography (IMAC) and size exclusion chromatography (FIGURE 2.2). The resultant protein was concentrated to 0.74 mg/ml and identified as *S. marcescens* ChiC by tryptic peptide mass fingerprinting, before a fraction of this was used to generate a rabbit anti-ChiC polyclonal antiserum (Eurogentec). ChiC was shown to be present in both the cellular fraction and in the extracellular medium in *S. marcescens* Db10 and for the strain JchiC (*chiC*$^{+}$, Δ*chiB,* Δ*chiC*), but not for any of the mutants lacking the *chiC* gene (FIGURE 2.2).

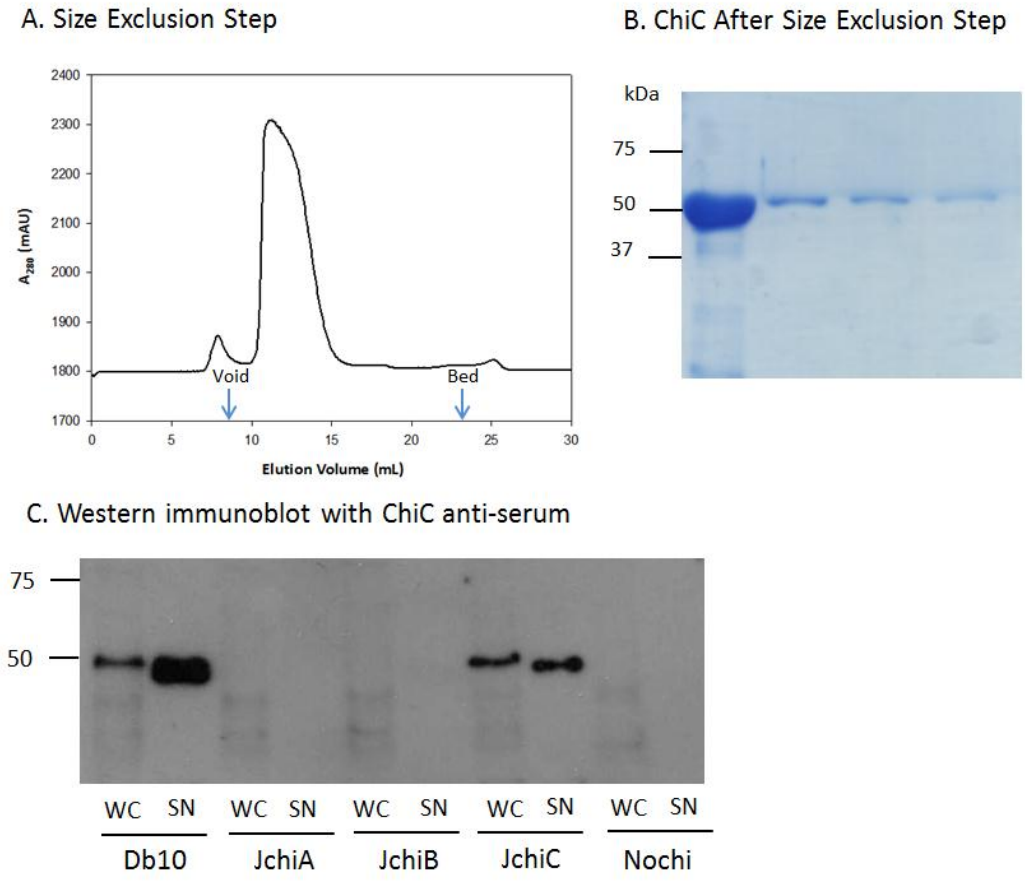


Figure 2.2 Generation of ChiC polyclonal anti-sera. First, ChiC^{His} was overproduced and purified by Ni-IMAC. The protein was then pooled and concentrated in 10 kDa MWCO device and loaded onto SuperdexTM 75 10/30 size exclusion column. (A) Elution profile of ChiC^{His} at A_{280} by SEC. Fractions corresponding to the peak shown around elution volume 10-16mL were pooled. (B) SDS-PAGE analysis of the SEC fractions revealed one major protein at the correct molecular mass for ChiC^{His} (C) Western immunoblotting of whole cell (WC) and supernatant (SN) fractions with ChiC polyclonal antisera. The in-house α-ChiC sera was used at a 1:20,000 dilution, while the secondary antibody was horseradish peroxidase-conjugated (BioRad) and used at 1:10,000 dilution. Westerns were developed using enhanced chemoluminescence (Millipore).

Next, we wanted to generate ChiB antisera. Since ChiB was insoluble, we overproduced ChiB, but then resuspended the cell lysate pellet in 5M urea buffer before refolding and purification *via* an IMAC step. The now soluble ChiB was then concentrated using a 10,000 MWCO device and subjected to SEC (FIGURE 2.3). The resultant protein was then pooled and concentrated to 1.51 mg/ml, and was confirmed to be *S. marcescens* ChiB by tryptic peptide mass fingerprinting, a sample of which was used to generate polyclonal ChiB antisera (Eurogentec). ChiB was detectable in the supernatant in 'wild type' strain *S. marcescens* Db10 and in strain JchiB (*chiB*$^{+}$, Δ*chiA*, Δ*chiC*). Although the band corresponding to ChiB is prominent, the polyclonal antiserum is not immunologically distinct: it is able to detect ChiA and at least one other non-specific band in a triple chitinase mutant Nochi (Δ*chiA*, Δ*chiB*, Δ*chiC*) (FIGURE 2.3).

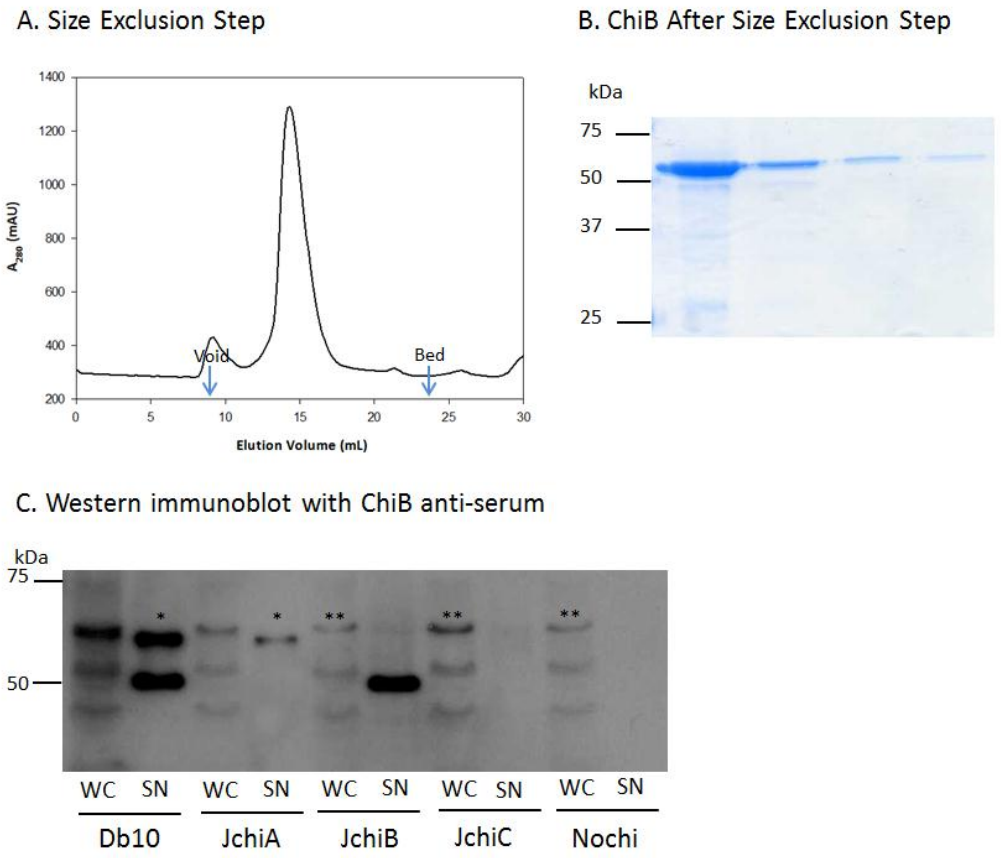


Figure 2.3 Generation of ChiB polyclonal anti-sera. ChiB^{His} was overproduced; the low-speed pellet was resuspended in 5M Urea, and purified by Ni-IMAC. The protein was then pooled and concentrated in 10 kDa MWCO device and loaded onto Superdex^{TM} 75 10/30 size exclusion column. (A) Elution profile of ChiB^{His} at A_{280} by SEC. Fractions corresponding to the peak shown around elution volume 12-18mL were pooled. (B) SDS-PAGE analysis of the SEC fractions showed one protein of the correct molecular mass for ChiB^{His}. (C) Western immunoblotting of whole cell (WC) and supernatant (SN) fractions with ChiB polyclonal antibody detected ChiB is present in the extracellular medium of *S. marcescens* Db10 and JchiB mutant encoding just *chiB*. The ChiB antiserum can recognise the ChiA protein (*) and at least one unidentified protein (**). The in-house α-ChiB sera was used at a 1:20,000 dilution, while the secondary antibody was horseradish peroxidase-conjugated (BioRad) and used at 1:10,000 dilution. Westerns were developed using enhanced chemoluminescence (Millipore).

Finally, we also needed to generate ChiA antisera for Western blot analysis. The cell lysate pellet (containing overproduced ChiA in inclusion bodies) was denatured in 5M urea before renaturation and purification by IMAC. The fractions corresponding to the imidazole elution were pooled and dialysed. A 58 kDa band was identified as ChiA by tryptic peptide mass fingerprinting. The aggregation and degradation bands apparent around ChiA by SDS-PAGE (FIGURE 2.4) were revealed also to be ChiA by western immunoblotting against the His-tag (FIGURE 2.4).The concentration of the protein after dialysis was 0.57 mg/ml and a fraction of this was used to produce polyclonal ChiA antisera (Eurogentec). Western immunoblotting against ChiA with the resultant antibody shows ChiA is present both in the cell (Db10) and also in the extracellular supernatant, for Db10 and JchiA (*chiA*$^{+}$, Δ*chiB*, Δ*chiC*), and shows that it is immunologically distinct, since it does not appear in strains devoid of the *chiA* gene.

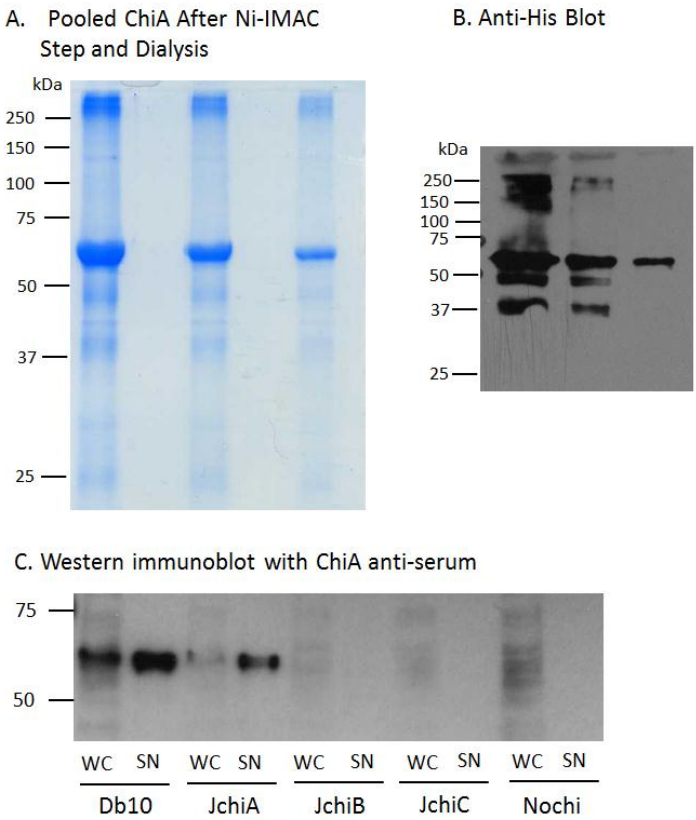


Figure 2.4 Generation of ChiA polyclonal anti-sera. ChiA^{His} was overproduced in *E. coli*, the low speed pellet was resuspended in 5M urea and gently stirred for 5 days at room temperature, and then purified by Ni-IMAC. The fractions corresponding to the peak from the IMAC step were then pooled and dialysed. (A) The pooled sample was loaded onto an acrylamide gel and separated by SDS-PAGE, which revealed one major protein at the correct molecular mass for ChiA^{His} and its identity was confirmed by tryptic peptide mass fingerprinting. We loaded three different volumes (10 µl, 5 µl and 2.5 µl) of the same 0.57 mg/ml sample in order to determine the most prominent band/s. (B) Western immunoblotting of the same sample revealed aggregates surrounding ChiA^{His} were also ChiA. (C) The ChiA antibody shows ChiA is present in both the cellular fraction and the extracellular medium of *S. marcescens* Db10 and JchiA mutant encoding just *chiA*.

2.3.3 Dissecting the chitin-degrading phenotype of *Serratia marcescens*

To test for secreted chitinase activity, each of the new deletion strains were grown on LB-agar plates supplemented with 2% (w/v) colloidal chitin for 48 hours at 30 °C (FIGURE 2.5). Chitinolytic activity was not detectable in the triple mutant Nochi strain (FIGURE 2.5), and appears compromised in other strains containing one or two chitinases (FIGURE 2.5). This important initial data show that a strain lacking all known chitinases is unable to degrade colloidal chitin in this plate test, while strains that express only one of the three chitinases retains detectable chitinase activity. This served as the basis of a robust genetic screen to identify the secretion systems for each of the chitinase isoenzymes.

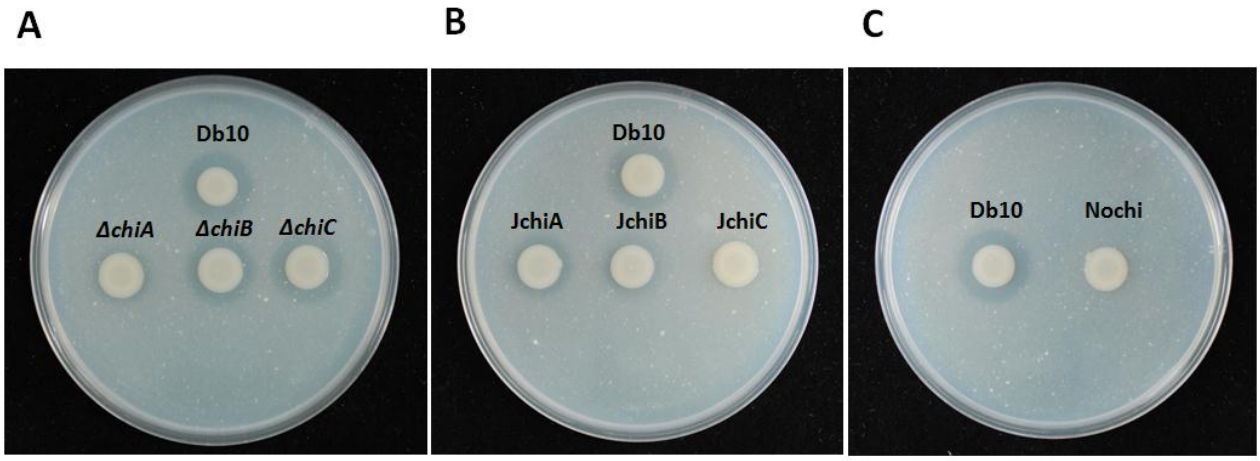


Figure 2.5 The chitin-degrading phenotype is compromised in *S. marcescens* mutant strains. Chitinolytic activity is compromised in strains lacking one or two chitinase encoding genes, and lost completely in strain (Nochi) devoid of any chitinase encoding genes. The parent *S. marcescens* Db10 and chitinase mutants were first grown in liquid cultures for 16 hours and then spotted on LB-agar containing 2% (w/v) colloidal chitin and incubated aerobically at 30 °C for 48 hours. Chitinase activity is visualised as zones of clearing. (A) Single chitinase mutants. (B) Double chitinase mutants. (C) A strain (Nochi) devoid of the known chitinase-encoding genes. Parental strain *S. marcescens* Db10 is included in each image for comparison.

In order to quantify the extracellular chitinolytic activity of the mutant strains we performed an enzymatic assay. The supernatants from liquid cultures were grown in the presence of 2% (w/v) colloidal chitin for 16 hours and were isolated and incubated with chitin azure for 48 hours. Chitinolytic activity was then measured as an increase in A_{560} caused by release of a blue dye from the chitin azure substrate (FIGURE 2.6). The activity recorded suggests that ChiA is the dominant chitinase activity expressed by *S. marsescens* Db10 and that ChiB may be a minor player in the utilisation of chitin under these experimental conditions (FIGURE 2.6).

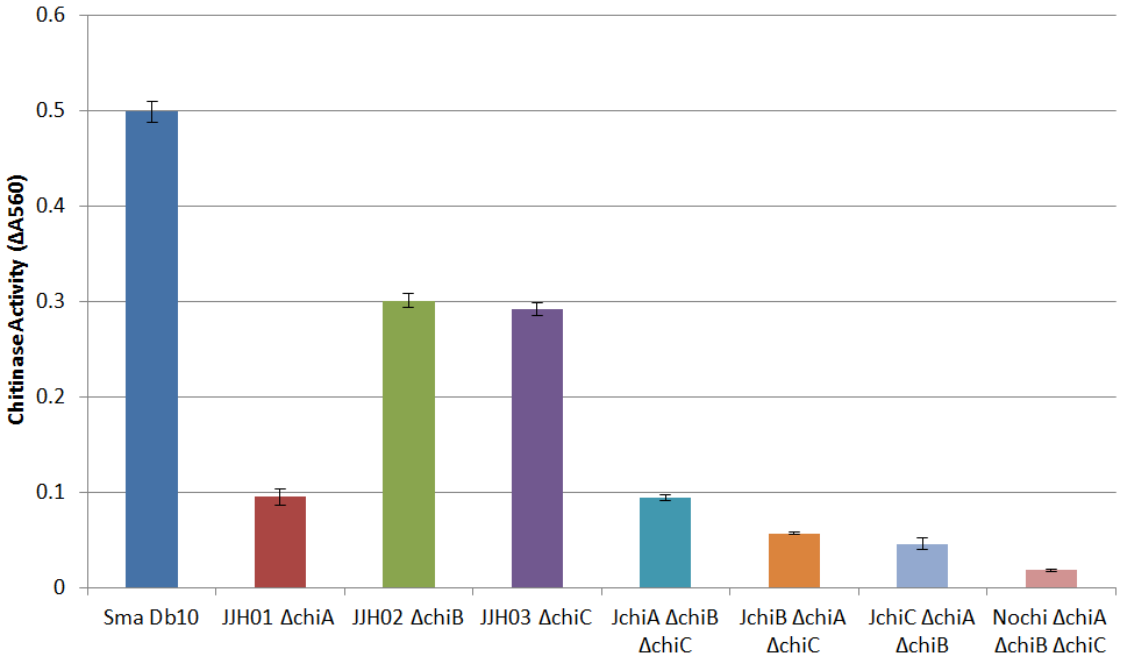


Figure 2.6 Chitinase secretion assay showing chitinolytic activity is compromised in each of the mutant strains. The spent culture supernatants from the *S. marcescens* parental strain ('*Sma* Db10') and a selection of the chitinase mutants grown for 16 hours overnight were concentrated and assayed for 48 hours at 30°C to measure chitinase activity with 1% chitin azure in 100 mM succinate pH 6.0. Error bars represent SEM, *n* = 3.

2.3.4 Deletion of the *cbp21* gene encoding chitin-binding protein does not appear to affect the chitin degrading phenotype

On the basis of previous *in vitro* work (Vaaje-Kolstad *et al.*, 2005), which suggested that Cbp21 is essential for chitin degradation by *S. marcescens*, we decided to make a targeted deletion of *cbp21* in order to assess whether the phenotype appears compromised *in vivo*. The *SMA2877 cbp21* gene was deleted from both parental strain *S. marcescens* Db10, to assess whether the whole chitinolytic phenotype was altered, but also JchiB (*chiB*$^{+}$, Δ*chiA*, Δ*chiC*) since previous work had suggested that Cbp21 was most essential for promoting binding of ChiB to its substrate (Vaaje-Kolstad *et al.*, 2005). Strains were grown for 16 hours and spotted onto solid 2% w/v colloidal chitin rich media and grown at 30^{o}C for 48 hours. Deletion of *SMA2877 cbp21* appears to have no obvious effect on the *in vivo* chitinolytic phenotype of *S. marcescens* under these experimental conditions, in either *S. marcescens* Db10 or JchiB backgrounds (FIGURE 2.7).

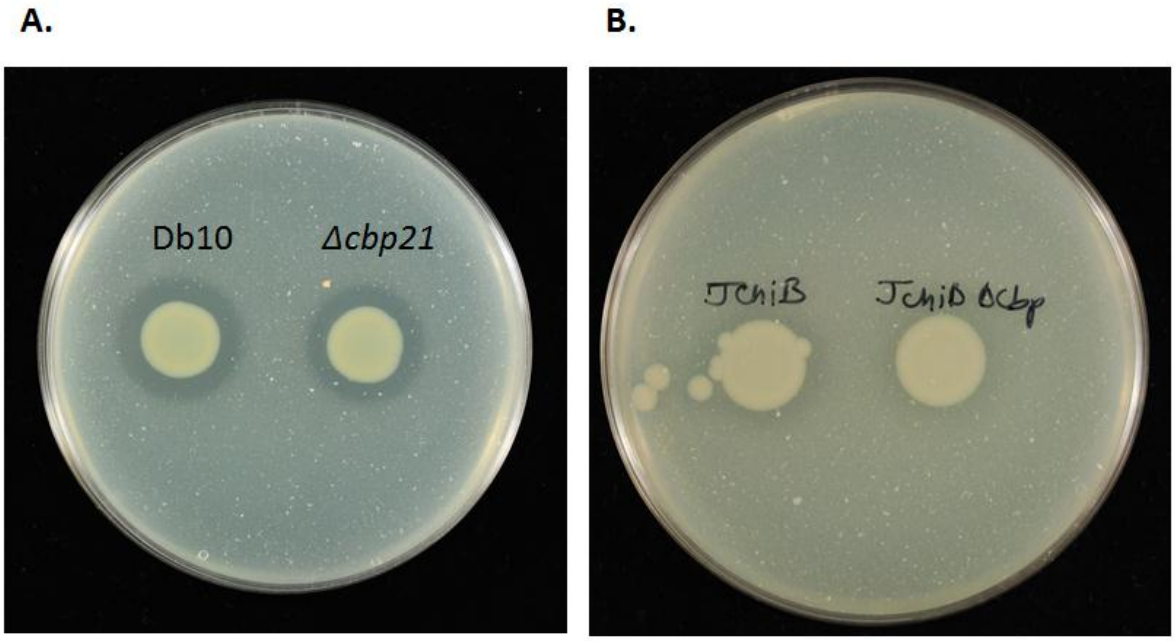


Figure 2.7 Deletion of gene *SMA2877* encoding chitin-binding protein Cbp21 appears to have no effect on the *in vivo* chitinolytic phenotype of *S. marcescens*. Targeted deletion of *SMA2877* from parental strains Db10 and JchiB (*chiB*$^{+}$, Δ*chiA*, Δ*chiC*) were made. Strains were grown for 16 hours at 30^{o}C in rich media and then spotted onto solid 2% w/v colloidal chitin rich media and grown at 30^{o}C for 48 hours.

2.3.5 Chitinases are predominantly localised in the extracellular environment

To assess whether each of the *S. marcescens* chitinolytic proteins were truly secreted enzymes, it was important to assess the relative levels of intra- against extracellular presence of chitinase. To test this, a biochemical assessment of the localisation of each *S. marcescens* chitinase was undertaken: *S. marcescens* was cultured aerobically at 30 ^{o}C in liquid rich medium, over the course of three days before whole cell and cell free supernatants were separated and the respective presence of native chitinase was compared using Western immunoblotting (FIGURE 2.8). ChiA, ChiB and ChiC were clearly detectable in the culture supernatant over the duration of the experiment, whereas neither the cytoplasmic control protein RNA polymerase, nor the periplasmic maltose binding-protein, could be detected in the extracellular milieu even after an extended 72 hours of growth (FIGURE 2.8). This strongly suggested that all three chitinases are deliberately externalised from the bacterial cell.

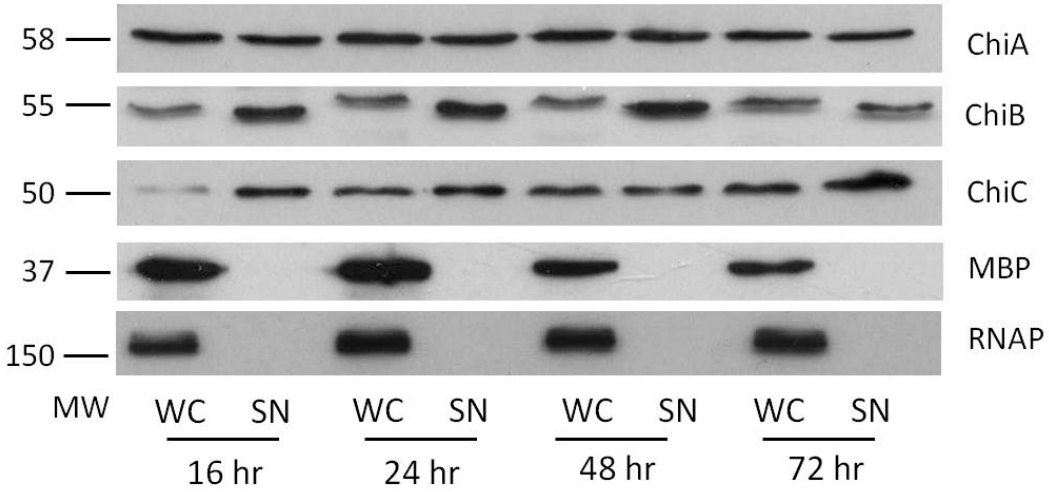


Figure 2.8 *S. marcescens* chitinases are located in the extracellular environment. *S. marcescens* was grown aerobically at 30^{o}C in rich medium. At the time points indicated an aliquot was taken and separated into culture supernatant 'SN' and whole cells 'WC' by centrifugation. The fractions were analysed for the presence of ChiA, ChiB, ChiC by Western immunoblotting using polyclonal antibodies. The locations of the periplasmic control protein (maltose binding-protein, MBP) and a cytoplasmic control protein (RNA polymerase, RNAP) were also determined using commercially-available antibodies.

2.3.6 A transposon mutagenic screen to search for a chitinase secretion system

The gross phenotypes of the chitinase mutants on colloidal chitin plates (FIGURE 2.9) were used as the basis of genetic screens to identify the secretion pathways used by each enzyme. Strains JchiA *(chiA*$^{+}$, Δ*chiB*, Δ*chiC*), JchiB (*chiB*$^{+}$, Δ*chiA*, Δ*chiC*), JchiC (*chiC*$^{+}$, Δ*chiA*, Δ*chiB*) where separately mated with an *E. coli* donor strain harbouring a mini-Tn5 plasmid containing a transposable element (*E. coli* SM10 λpir pUTmini-Tn5Sm/Sp). *S. marcescens* colonies containing a transposon insertion were selected on minimal media plates supplemented with streptomycin, and each mutant was patched onto 2% (w/v) colloidal chitin plates to screen for candidates that were defective for chitinolytic activity. For the JchiA strain (expressing *chiA* and *cbp21* only) 2,400 transposon insertion mutants were screened; for the JchiB strain (expressing *chiB* and *cbp21* only) 3,800 transposon insertion mutants were screened; and for JchiC (expressing *chiC* and *cbp21* only) 2,750 transposon insertion mutants were screened. Mutant candidates apparently exhibiting a loss of the chitinolytic halo were re-struck onto fresh colloidal chitin plates. We identified 14 JchiA candidates that had lost *in vivo* chitinase activity; five candidates for the JchiB strain; and nine candidates for the JchiC experiment (FIGURE 2.9).

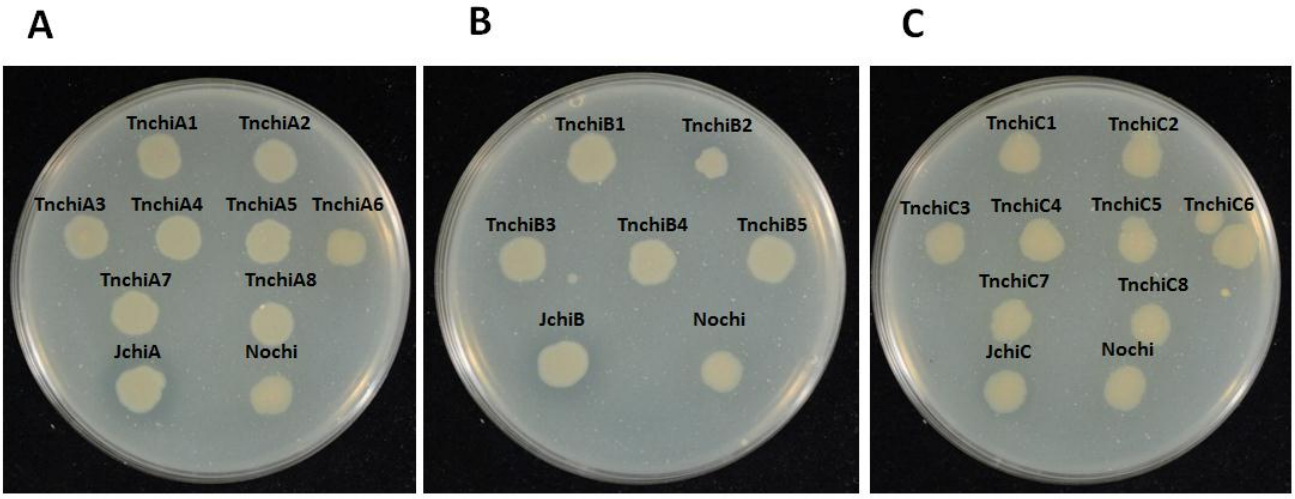


Figure 2.9 Transposon screen to identify genes encoding a possible chitinase secretion system and responsible for *in vivo* chitinolytic phenotype. Transposon mutagenesis candidates harbouring a single chitinase encoding gene exhibiting a loss of chitinolytic activity were grown at 30^{o}C for 48 hours on LB media supplemented with 2% (w/v) colloidal chitin. (A) There are currently 14 JchiA candidates (8 are shown) (B) There are 5 JchiB candidates. (C) There are 9 JchiC candidates (8 are shown). Parental strains (JchiA, JchiB or JchiC) exhibiting a chitinolytic halo, as well as the triple mutant (Nochi) were included for comparison.

2.3.7 Mapping Tn5 insertions in the JchiA strain

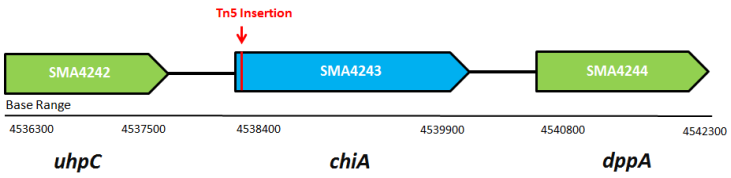
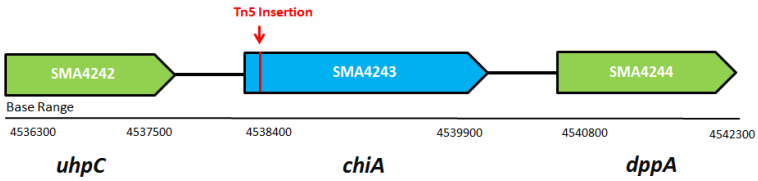
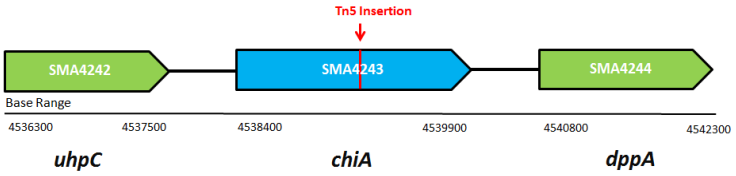
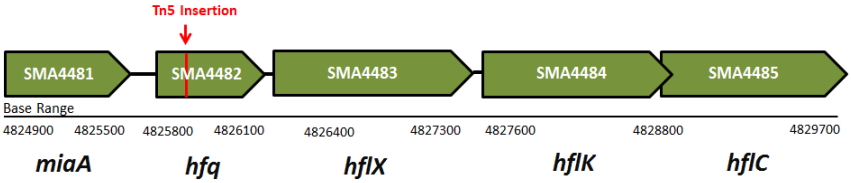
The locations of the transposon insertions in the JchiA (*chiA*$^{+}$) strain that exhibited a loss of chitin-degrading ability were mapped (Table 2.1), using a single primer specific PCR, with reference to the genome sequence of *S. marcescens* Db11 (Wellcome Trust Sanger Institute, UK).

TnchiA Candidate	**Disrupted ORF**	**Putative Product/ Function**
TnchiA3	*SMA4243 chiA*	Chitinase ChiA
TnchiA4	*SMA4243 chiA*	Chitinase ChiA
TnchiA5	*SMA4243 chiA*	Chitinase ChiA
TnchiA6	*SMA4482 hfq*	Host Factor I Protein mRNA Binding Regulator Hfq
TnchiA7	*SMA4243 chiA*	Chitinase ChiA
TnchiA8	*SMA4243 chiA*	Chitinase ChiA
TnchiA9	*SMA4243 chiA*	Chitinase ChiA
TnchiA11	*SMA4243 chiA*	Chitinase ChiA
TnchiA13	*SMA4243 chiA*	Chitinase ChiA
TnchiA14	*SMA4579 speF*	Ornithine Decarboxylase SpeF

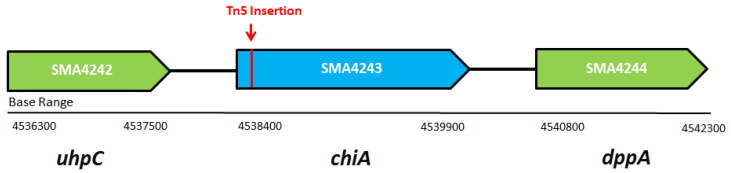
Table 2.1 Genetic loci of remaining JchiA Tn5 candidates exhibiting loss of chitin-degrading phenotype. Of the ten 'TnchiA' candidates that were mapped, all but two contained a transposable element in the chitinase encoding gene *chiA*. Disruption of *SMA4482 hfq* encoding a global mRNA-binding regulator was shown to be responsible for loss of phenotype in one candidate 'TnchiA6', as was disruption of *SMA4579 speF*, encoding an Ornithine Decarboxylase in a separate candidate 'TnchiA14'.

Of the fourteen mutant candidates that were defective on chitin rich media, ten were successfully mapped, eight of which were shown to reside in *SMA4243*, the chitinase encoding gene *chiA* (Table 2.1). Of the remaining two candidates, one transposon was located in *SMA4579 speF*, encoding an ornithine decarboxylase, for which there is no published work investigating the role of this in relation

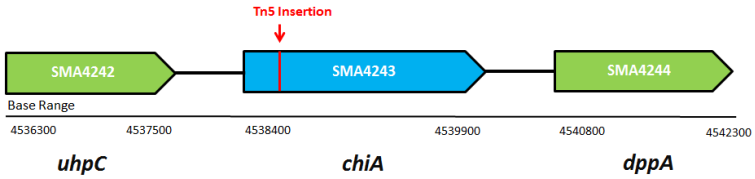
to chitin metabolism. The other Tn5 insertion was mapped to *SMA4482 hfq*, which encodes a global mRNA binding transcriptional regulator. At present there is no published work linking Hfq to regulation of the chitinolytic machinery in *Serratia marcescens*: here we have shown that Hfq is likely to be essential for the chitin-degrading phenotype produced by ChiA. Cartoon representations of each of the Tn5 insertions are given in FIGURE 2.10.

TnchiA Candidate	**Genomic Location *S. marcescens***	**Cartoon Representation of Tn5 Insertion and Surrounding Genomic Loci**
TnchiA3	Base pair location 4538485-4538486	
TnchiA4	Base pair location 4538497-4538498	
TnchiA5	Base pair location 4539373-4539374	
TnchiA6	Base pair location 4825944-4825945	

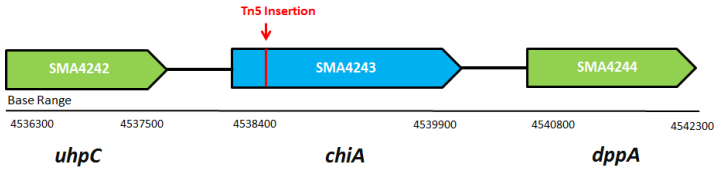
TnchiA7 Base pair location 4538538-4538539



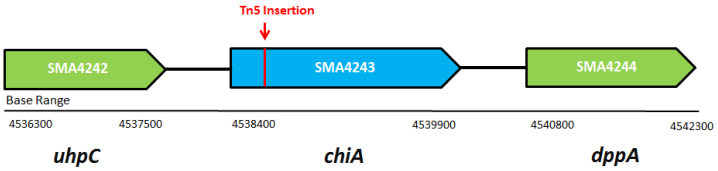
TnchiA8 Base pair location 4538717-4538718



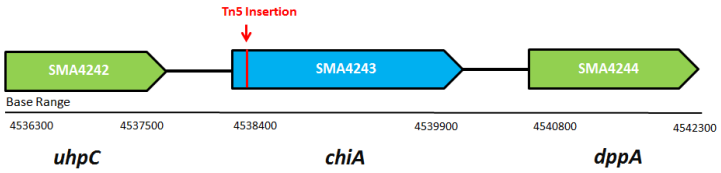
TnchiA9 Base pair location 4538683-4538684



TnchiA11 Base pair location 4538692-4538693



TnchiA13 Base pair location 4538492-4538493



TnchiA14 Base pair location 4924106-4924107

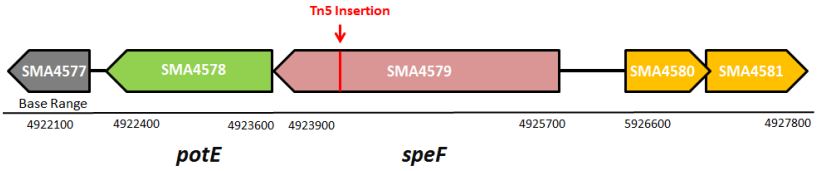


Figure 2.10 Cartoon representation of the ten JchiA candidate ('TnchiA') Tn5 insertions that exhibited a loss of chitin-degrading phenotype, including nearby genomic ORF's. Eight of the ten candidates contained a transposon insertion in *chiA* chitinase encoding gene itself, all except one of these were located toward the 5' end of *chiA*. In one of the candidates the Tn5 was located in *SMA4482*, which encodes Hfq, or host factor-I protein (HF-I), a global mRNA-binding regulator. In a separate candidate, TnchiA14, the Tn5 was mapped to *SMA4579 speF*, encoding an ornithine decarboxylase.

Western immunoblotting of the 'TnchiA' candidates revealed that none of these strains, mapped or otherwise, were able to produce ChiA, suggesting all of the genes disrupted were directly involved in *chiA* production, rather than transport, if not embedded in *chiA* itself (FIGURE 2.11).

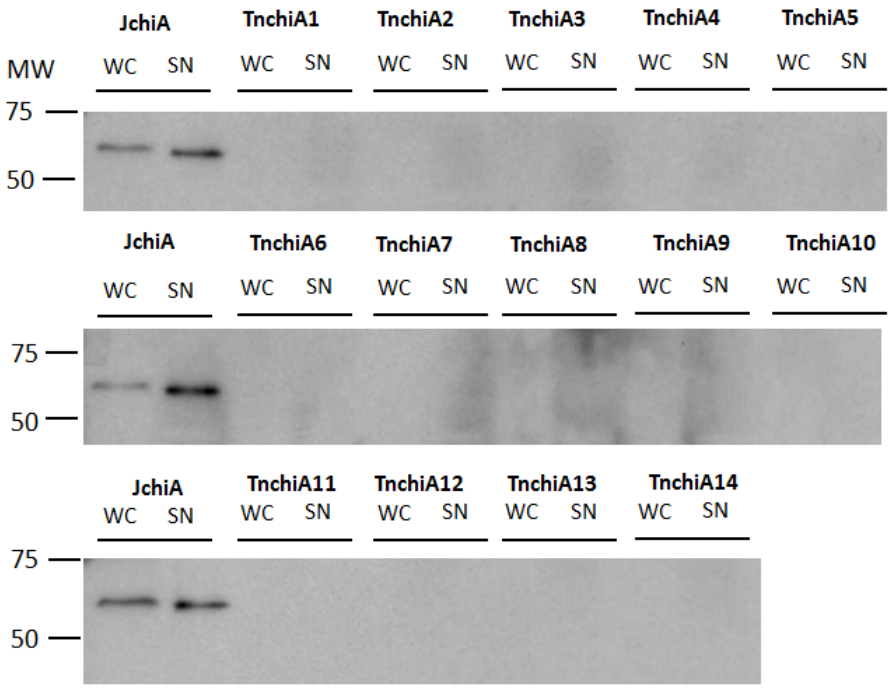


Figure 2.11 ChiA was not detectable in Tn5-containing JchiA candidates. Western immunoblotting to detect the presence of ChiA: strains were grown for 16 hours and separated into whole cell (WC) and supernatant (SN) fractions and blotted against using polyclonal ChiA anti-sera. ChiA was not detectable in any of the Tn5-containing strains. The JchiA parental strain is included for comparison.

2.3.8 Mapping Tn5 insertions in the JchiB strain

Two of the five 'TnchiB' candidates were successfully mapped with respect to the published *S. marcescens* Db11 genome (Wellcome Trust Sanger Institute, UK). One Tn5 was mapped to *chiB*, the other was mapped to *chiR*, encoding a LysR type transcriptional regulator and known to be an essential player in the *S. marcescens* chitinolytic phenotype (Table 2.2) (Suzuki *et al.*, 2001).

Candidate	Disrupted ORF	Putative Product/ Function
TnchiB3	*SMA2875 chiB*	Chitinase ChiB
TnchiB5	*SMA2876 chiR*	LysR type Regulator of Chitinase transport ChiR

Table 2.2 Genetic loci of JchiB Tn5 candidates exhibiting a loss of chitin-degrading phenotype. Of the two 'TnchiB' candidates that were mapped, one Tn5 was located in *SMA2875 chiB* chitinase encoding gene, and the other was located in *SMA2876 chiR* regulator.

A cartoon of each insertion shows a Tn5 was isolated well within the respective ORFs of chitinase encoding *chiB*, and the regulator encoding *chiR* gene for each candidate (FIGURE 2.12).

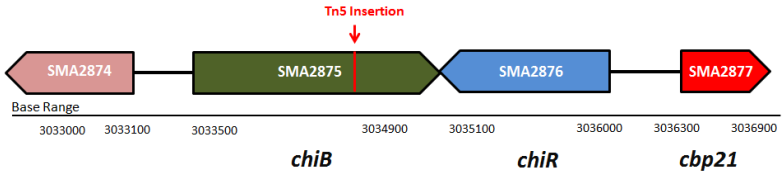
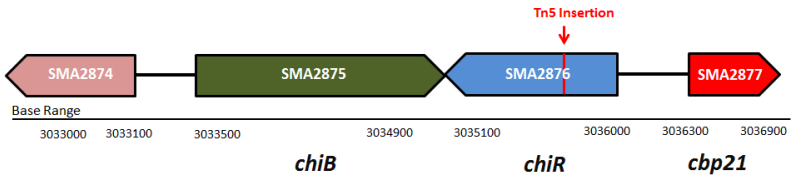
TnchiB Candidate	Genomic Location *S. marcescens*	Cartoon Representation of Tn5 Insertion and Surrounding Genomic Loci
TnchiB3	Base pair location 3034536-3034537	
TnchiB5	Base pair location 3035608-3035609	

Figure 2.12 Cartoon representation of Tn5 insertions for candidates TnchiB3 and TnchiB5. Tn5 insertions were mapped well within the respective *SMA2875 chiB* and *SMA2876 chiR* ORF's.

Western immunoblotting each of the 'TnchiB' candidates, however, revealed that strain 'TnchiB2' was able to move ChiB to the extracellular milieu and yet exhibited a loss of *in vivo* chitinolytic activity when grown on colloidal chitin rich media (FIGURE 2.13). This candidate is yet to be successfully mapped.

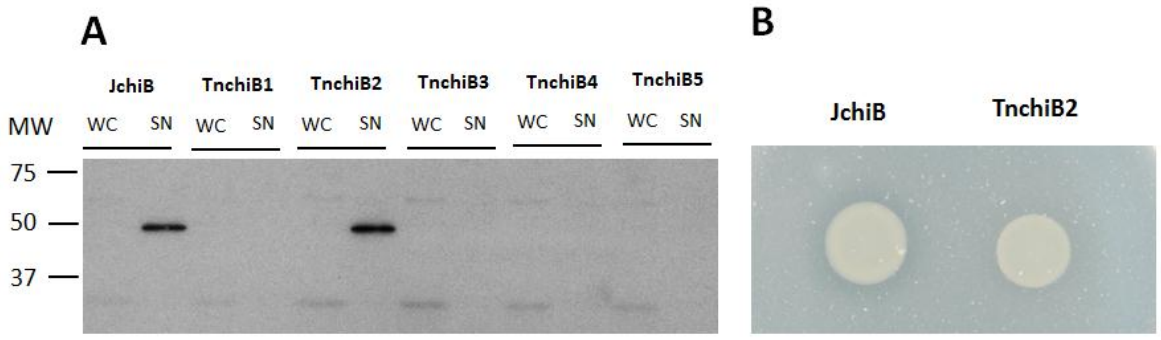


Figure 2.13 Strain TnchiB2 secretes ChiB to the supernatant but exhibits a loss of chitinolytic activity. (A) Strains were grown for 16 hours in liquid rich media before separation into whole cell (WC) and supernatant fractions (SN) and the presence of ChiB was determined using polyclonal ChiB-antisera by Western immunoblotting. (B) Strain TnchiB2 exhibited a loss of chitinolytic activity *in vivo*. This was shown by growing parental JchiB strain and TnchiB2 for sixteen hours in liquid rich media and then spotting on 2% w/v colloidal chitin rich media and grown aerobically at 30°C for two days.

2.3.9 Mapping Tn5 insertions in the JchiC strain

The JchiC (*chiC*$^{+}$, *ΔchiA*, *ΔchiB*) strain remained chitinase-positive on the colloidal chitin solid media and would only produce ChiC. The random transposon mutagenic screen identified nine mutant isolates that could no longer degrade extracellular chitin. To differentiate between mutants defective in *chiC* expression, from those defective in ChiC secretion, the transposon mutants were grown in liquid culture and separated into whole cell/ intracellular and supernatant/ secretion fractions which were compared using Western immunoblotting (FIGURE 2.14). This approach identified three candidates that had lost *in vivo* chitin-degrading phenotype: these candidates were producing ChiC to normal levels but were blocked in their ability to move ChiC to the extracellular supernatant (FIGURE 2.14).

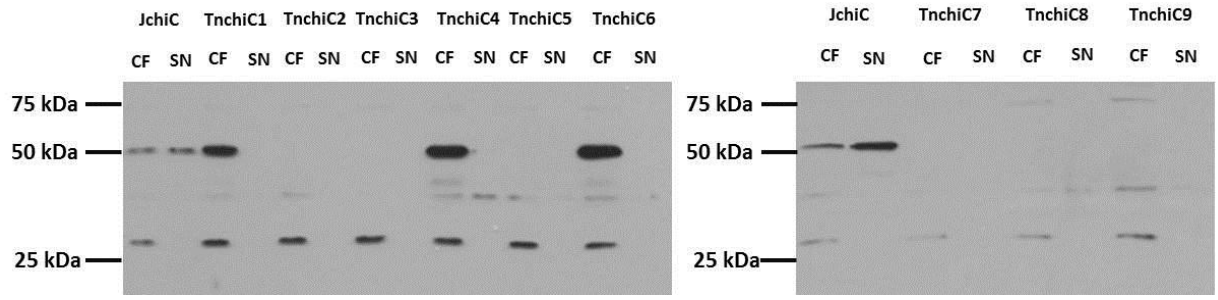


Figure 2.14 Detection of three JchiC strains producing native ChiC that was not detectable in the culture supernatant. Western immunoblot with polyclonal anti-ChiC serum with whole cell and supernatant fractions of JchiC transposon mutagenesis candidates 1-9 grown for 16 hours at 30 ^{o}C.

The locations of the transposon insertions in the three secretion-defective mutant strains were mapped, using single primer specific PCR, with reference to the genome sequence of *S. marcescens* Db11 (Wellcome Trust Sanger Institute, UK), which is a spontaneous streptomycin-resistant derivative of the *S. marcescens* Db10 strain used in this study. Two of the transposon insertions were mapped to different locations in the same gene (*SMA2874*) and the third was located in *SMA2876*, which had been previously identified as *chiR* (TABLE 2.3) (Suzuki *et al.*, 2001).

Candidate	Disrupted ORF	Putative Product/ Function
TnchiC1	*SMA2876 chiR*	LysR type Regulator of Chitinase transport ChiR
TnchiC4	*SMA2874*	Phage holin-like protein
TnchiC6	*SMA2874*	Phage holin-like protein

Table 2.3 Genetic location of Tn5 insertion in JchiC candidates responsible for loss of chitinolytic phenotype.

The *chiR* gene encodes a transcriptional regulator of the LysR family already known to have an important role in the chitin-degrading phenotype (Suzuki *et al.*, 2001), however *SMA2874* had not been previously studied. Further inspection of the *S. marcescens* genome sequence revealed that *chiR* and *SMA2874* are located adjacent to *chiB* and *cbp21* on the *S. marcescens* chromosome. The relative positions of each Tn5 insertion are represented in cartoon format in (FIGURE 2.15). The TnchiC1 transposon was mapped 129 base pairs upstream of the 5' initiation codon of *chiR*, and 276 base pairs upstream of the 5' start of *cbp21* ORF.

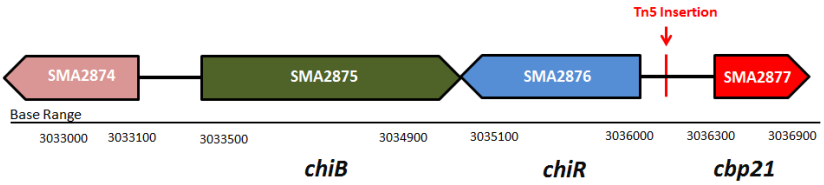
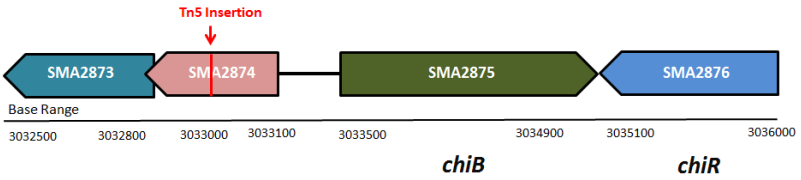
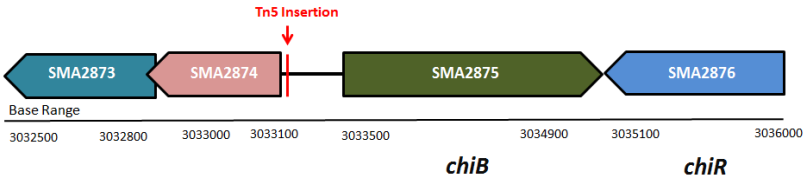
TnchiC Candidate	Genomic Location *S. marcescens*	Cartoon Representation of Tn5 Insertion and Surrounding Genomic Loci
TnchiC1	Base pair location 3036153-3036154	
TnchiC4	Base pair location 3033033-3033034	
TnchiC6	Base Pair Location 3033227-3033228	

Figure 2.15 Cartoon representations of the three JchiC candidate ('TnchiC') Tn5 insertions that were blocked in their ability to move ChiC to the extracellular environment. One shows insertion in *chiR*, the other two in *SMA2874*, and including nearby genomic organisation. For candidate TnchiC1, the Tn5 was located 129 base pairs upstream of the *chiR* initiation codon. The Tn5 insertion of candidate TnchiC4 was mapped clearly within the *SMA2874* ORF, and the insertion for candidate TnchiC6 was located 23 bases upstream of the 5' initiation codon of *SMA2874*.

2.4 Discussion

The work presented here investigated the role of each component responsible for the *S. marcescens* chitinolytic phenotype. Each of the single chitinase producing strains were shown to be individually chitinase positive when grown on chitin-rich media, whereas *cbp21* encoding chitin binding protein, Cbp21, was not essential for the chitinolytic phenotype *in vivo*.

In this Chapter a transposon mutagenic screen was used to attempt to identify a secretion system responsible for moving chitinase across the cell envelope. We have uncovered new evidence that genes *SMA4482 hfq*, *SMA4579 speF*, *SMA2876 chiR*, and *SMA2874* may have crucial roles in the *S. marcescens* chitinolytic phenotype. Of these four genes, *SMA4579* (*speF*) and *SMA2874*, have never been previously associated with chitin degradation, or chitinase secretion.

Candidates from the transposon screen with JchiC parental strain bearing a Tn5 in *chiR* or *SMA2874* were shown to produce intracellular ChiC that was clearly blocked in its capacity to move to the extracellular environment, suggesting both of these genes encode proteins involved in transport of ChiC, as opposed to transcription or translation of *chiC*. *SMA4579* (*hfq*) encodes a global mRNA binding regulator, Hfq, which plays an important role in the chitinolytic phenotype of the Gram positive bacterium *Listeria monocytogenes* (Nielsen *et al.*, 2011). Furthermore, *chiR* encodes the LysR regulator ChiR, which is already known to have an essential role in the *S. marcescens* chitinolytic phenotype (Suzuki *et al.*, 2001).

2.4.1 The potential role of Hfq regulator in the *S. marcescens* chitinolytic phenotype

Although the *SMA4482 hfq* gene was only hit in our screen for genes involved in ChiA secretion, rather than ChiB and ChiC, it has subsequently been shown that deletion of *SMA4482 hfq* results in a complete loss of chitin-degrading activity in *Serratia marcescens* (Sarah Murdoch and Marilia Costa, University of Dundee). From this we can infer that Hfq may be essential for the expression of all chitinase enzymes produced by *S. marcescens*.

The crystal structure of *E. coli* Hfq has been solved (Beich-Frandsen *et al.*, 2011), and to date the only published work related to Hfq regulation of *chiA* was done in the Gram positive bacterium *Listeria monocytogenes* (Nielsen *et al.,* 2011). We therefore made an alignment of an Hfq sequence from each of these organisms (FIGURE 2.16), which showed considerable sequence conservation between all three. The *S. marcescens* Hfq protein has 88% overall sequence identity, and 91% overall similarity, with *E. coli* Hfq, and 46% overall sequence identity, and 70% overall similarity, with *L. monocytogenes* Hfq. It may be worth noting that in β- and γ-proteobacteria, Hfq has an extended C-terminal domain that is thought to have a role in the binding of longer mRNAs, but is unnecessary in the binding of small RNAs (Vecerek *et al.*, 2008). Hfq is known to contain an evolutionarily conserved core sequence (residues 7-66), from this it is inferred there has been selective pressure to conserve the sequence, and the function, of Hfq (Beich-Frandsen *et al.*, 2011).

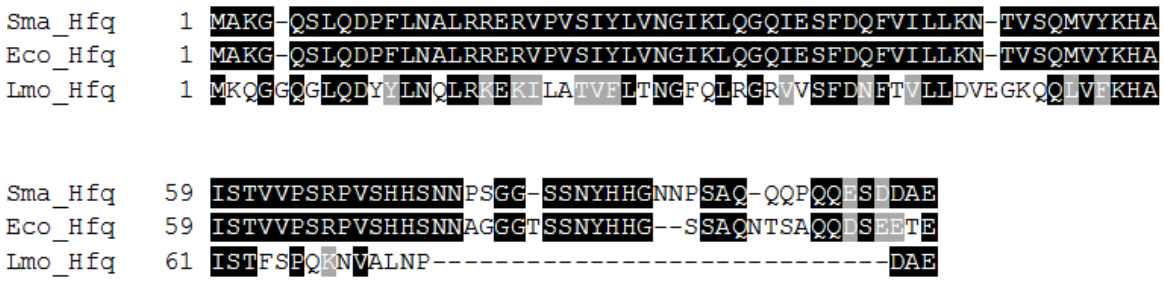


Figure 2.16 An alignment of Hfq sequences from *Serratia marcescens* Db10 (Sma), *Escherichia coli* ER2566 (Eco) and the Gram positive bacterium *Listeria monocytogenes* EGD-e (Lmo). Sequence alignments were performed with Clustal Omega (2), and displayed using the freely-available on-line tool Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Hfq is involved in riboregulation, a mechanism of stress response involving regulation by small trans-encoded RNAs (sRNAs) that bind to mRNAs at the ribosome binding site and preclude ribosome binding, leaving the mRNA subject to rapid degradation (Papenfort and Vogel, 2009). Many sRNAs are functionally dependent on Hfq for stabilization and for its role as a chaperone in mediating the interaction between the sRNA and its target mRNA. Deep sequencing of *Salmonella enterica* serovar Typhimurium has shown that Hfq is involved in regulating the expression of 20% of its genes (Moller *et al.*, 2002; Sittka *et al.*, 2008). Although most work investigating the role of Hfq in facilitating sRNA-mediated riboregulation has been done in Gram negative bacteria, Hfq itself in fact belongs to the Sm- and Sm-like family of proteins (Moller *et al.*, 2002), but it is commonly classified as an 'RNA-chaperone' (Moll *et al.*, 2003). The Sm-like family of bacterial, eukaryotic and archael proteins are comprised of a diverse, and conserved, group of RNA-binding chaperone proteins that operate by assisting in the proper spatial configuration of RNA molecules, and they also commonly act as helpers in RNA-RNA and RNA-protein interactions (Murina and Nikulin, 2011).

E. coli Hfq consists of six protomers that form a hexameric doughnut-like structure (Beich-Frandsen *et al.*, 2011). This work has shown that the presence of the variable C-terminus, as well as the hexameric conformation, have little effect on the Sm-core, which is highly conserved between distantly related phyla (Beich-Frandsen *et al.*, 2011). The *E. coli* Hfq structure exhibits polar and hydrophobic interactions between three C-termini, which stabilizes the Hfq hexamers in hexagonal layers, and these layers pack against each other to form a double layer (FIGURE 2.17) (Beich-Frandsen *et al.*, 2011).

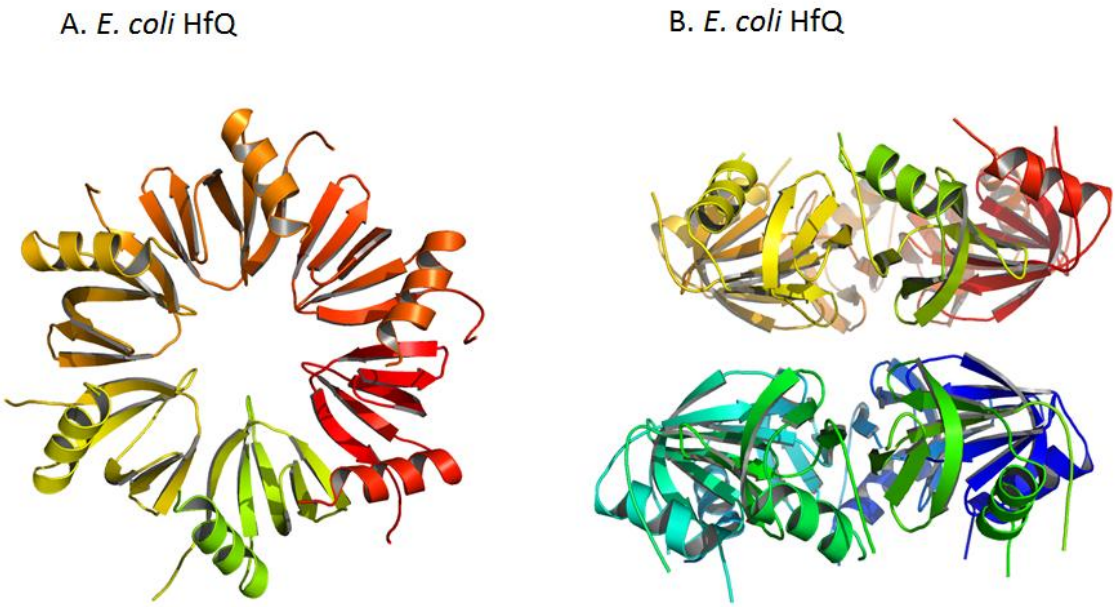


Figure 2.17 *E. coli* Hfq forms a hexameric doughnut structure (Beich-Frandsen *et al.*, 2011). Each *E. coli* Hfq hexameric molecule (A) packs to form a hexagonal 'honey-comb' pattern. The hexameric layers then stack to form a staggered double layer (B) with a ~30 Angstroms void between them.

To our present knowledge, the only work to have investigated connections between Hfq and chitin metabolism has been done in the Gram positive *Listeria monocytogenes* (Nielsen *et al.*, 2011). This work showed that Hfq acts as a post-transcriptional regulator of *chiA*, it promotes base pairing of the LhrA sRNA to the *chiA* mRNA transcript and hence acts to down regulate the expression of *chiA* (Nielsen *et al*., 2011). Some recent work carried out in the Division of Molecular Microbiology, University of Dundee, by Sarah Murdoch and Sarah Coulthurst (unpublished observations) revealed that a FLAG-tagged Hfq of *S. marcescens* was bound to a small RNA corresponding to a sequence located at the 3' end of *chiA*. The possibility that the 3' end of *chiA* encodes a possible sRNA involved in regulation of *chiA* expression is currently under investigation. This is a tantalizing discovery in the light of recent work (Chao *et al.*, 2012) that has identified an sRNA, DapZ, which is encoded in the 3'-UTR of the *dapB* gene and acts as a translational repressor. This work also discovered another seven genes in *Salmonella typhimurium* encoding 3'-UTR harbouring sRNAs that are transcribed from a gene-internal promoter (Chao *et al.*, 2012). The authors conclude that the 3' mRNA regions potentially provide a rich source of sRNAs that function as Hfq targets and plays an important role in Hfq networks (Chao *et al.*, 2012). The transposon screen carried out in this study did not disrupt the 3'-end of *chiA*, so it is unlikely that the resulting loss of chitinolytic phenotype is attributable to disruption of 3' encoded sRNA regulation.

Since targeted deletion of the *hfq* gene results in a complete loss of chitinolytic phenotype in *S. marcescens*, it is likely that Hfq plays a stimulatory role in the regulation of chitin metabolism. Whether this is mediated by a single sRNA, as most Hfq-associated sRNAs are known to regulate multiple targets, is an open question.

For future work, there is a publicly available tool *TargetRNA* (http://cs.wellesley.edu/~btjaden/TargetRNA2/) that will predict mRNA targets for a given sRNA (Tjaden *et al.*, 2006), and it will be interesting to see whether this supports any new findings in our *hfq* / *chiA* research. The next task would be to generate a clean deletion of the sRNA encoding locus and to compare global transcript levels using microarray analysis, and also to validate any change in RNA products of interest using either northern blotting or quantitative RT-PCR – this should provide hard evidence that the sRNA of interest plays a role in regulation of chitinolytic, and possibly unknown, components.

2.4.2 The putative role of ornithine decarboxylase in the chitinolytic phenotype

From the transposon mutagenic screen with JchiA parental strain we mapped one Tn5 to gene *SMA4579 speF* encoding a putative ornithine decarboxylase (ODC). This protein belongs to members of the ornithine decarboxylase family, their general structure is a dodecamer consisting of six homodimers, and they serve to decarboxylate ornithine to form putrescine (John, 1995). The amino acid sequence of *S. marcescens* SpeF exhibits considerable similarity, 72% sequence identity and 84% positive identity, to the *E. coli* ornithine decarboxylase SpeC. In *E. coli*, SpeC is known to have an important role in polyamine biosynthesis: it converts L-ornithine to putrescine, which then undergoes condensation with decarboxylated *S*-adenosylmethionine to produce spermidine, which is carried out by a spermidine synthase SpeE (Xie *et al.*, 1989). Regulation of polyamine pools has been shown to be important for growth in both bacteria and mammals (Davis *et al.*, 1992). To our current knowledge, an ornithine decarboxylase has never been investigated in relation to either membrane biology or chitin metabolism. It was therefore a surprising result. Since it is very likely that SpeF, as yet uncharacterised in *S. marcescens*, plays an important role in regulating polyamine pools, it is unlikely to be involved directly in facilitating chitinase transport. Furthermore Western blot analysis revealed that the presence of ChiA is lost altogether in the *speF*-Tn5 (TnchiA14) strain (FIGURE 2.11), which suggests it is more likely that spermidine/ polyamine pools play a role in transcription or translation of *chiA*, as opposed to transport of ChiA.

Polyamines, such as putrescine, are known to play important roles in gene regulation due to their positive-charge, DNA/RNA-binding potential: for example, in *E. coli* 90% of spermidine is bound to RNA, and of the total cellular putrescine available 9.3% is bound to DNA, 48% to RNA and 39% is free (Miyamoto *et al.*, 1993; Wortham *et al.*, 2007). In addition to this, polyamines are also thought to be involved in stabilising DNA bending, an important feature for many regulatory proteins, such as the LysR-type transcriptional regulators (see discussion of ChiR regulation below) (Lindemose *et al.*, 2005; Pastre *et al.*, 2006). There is evidence that polyamines, including putrescine, alleviate DNA-bending by preferential binding to adenine tracts in double stranded DNA, and by sequence specific interactions with gene promoter regions (Lindemose *et al.*, 2005). The DNA-interacting properties of polyamines has led some researches to coin the term ‘polyamine modulon’ to denote this form of regulation (Yoshida *et al.*, 2004).

On the *S. marcescens* Db11 genome, *speF* lies adjacent to *SMA4578 potE* encoding a putrescine-ornithine antiporter, a predicted 46 kDa membrane protein involved in putrescine transport. In *E. coli* there are two ABC transporters, encoded by *potABCD* and *potFGHI*, involved in uptake of spermidine

and putrescine respectively (Igarashi *et al.*, 2001): in addition to this *E. coli* also encodes a putrescine/ ornithine antiporter, *potE*, that also forms part of an operon in which it lies adjacent to the gene encoding an ornithine decarboxylase, *speF*, and PotE has been shown to excrete putrescine in a 1:1 exchange in response to the presence of ornithine (Kashiwagi *et al.*, 1997)

In order to take this work further, a clean Δ*speF* strain would need to be constructed, and its effect on the secretion of each chitinase determined. The best way to approach this would be to perform a chemical complementation using the Δ*speF* strain, the *S. marcescens* Db10 parental control strain, and Δ*speF* encoding *speF* on a plasmid: these strains would then be treated with exogenous application of putrescine, applied in a gradient (e.g. 1 µM-10 mM) and the levels of chitinase production, as a function of putrescine addition, would be assessed by Western immunoblotting (Patel *et al.*, 2006). This will establish whether or not the presence of putrescine plays an important role in regulating chitinase gene expression. It may also be worthwhile isolating mRNA from the different putrescine-treated samples, from there it will be possible to do qPCR to assess whether the levels of chitinolytic gene expression, including *chiR* and *SMA2874*, are affected by the presence of polyamines such as putrescine.

2.4.3 The role of ChiR and the LysR family

In this work, we have identified two separate chitinase secretion mutants – one from the JchiB and one from the JchiC screen - bearing Tn5 insertions in *SMA2876* (*chiR*). Moreover, it was very interesting that the TnchiC1 mutant was able to produce intracellular ChiC, but appeared blocked in its ability to move ChiC to the extracellular milieu. The Tn5 insertion for mutant TnchiB5 was mapped well within the *chiR* ORF, whereas the TnchiC1 transposon was mapped 129 base pairs upstream of the 5' initiation codon of *chiR*, 276 base pairs upstream of the divergent *cbp21* initiation codon, and was therefore in an intergenic region. Previous work has implicated the intergenic region between *chiR* and *cbp21* to be an important binding site for ChiR (Suzuki *et al.*, 2001).

ChiR is a LysR-type transcriptional regulator (LTTR). The LysR family are ubiquitous transcriptional regulators in bacteria and with 800 members identified they comprise the largest family of DNA binding proteins in prokaryotes (Maddocks and Oyston, 2008). In previous work, it was established that Tn5 disruption, and targeted deletion, of *chiR* in *S. marcescens* results in a complete loss of the extracellular presence of each chitin-degrading character (ChiA, ChiB, ChiC and Cbp21) (Suzuki *et al.*, 2001). This important work identified *chiR* as essential in the *S. marcescens* chitinolytic phenotype, it identified ChiR as being an LTTR, and also demonstrated that it binds the *chiR-cbp21* intergenic region (Suzuki *et al.*, 2001). However, this paper made the assumption that if the chitinolytic phenotype is lost in a *chiR*-Tn5 mutant, this infers that ChiR is essential for the transcription of the entire chitinolytic set (even though ChiR was shown not to bind *chiA*, *chiB*, or *chiC* promoter regions in the same work). Suzuki and co-workers (2001) demonstrate that the extracellular presence of the chitinolytic proteins is lost in the culture supernatant of the *chiR*-Tn5 strain, but do not test (or have no way of testing) whether intracellular levels are affected. Testing whether any chitinolytic proteins are present within the cell would have enabled them to determine whether ChiR is involved directly in transcription of the chitinolytic genes or if it could be regulating something else, for example a transport or secretion system.

Originally, the LysR family were thought to be a small group of transcriptional activators that positively regulated expression of a divergently transcribed gene, and exhibited negative autoregulation. This classical model of LysR regulation (based on LysR regulation of *lysA*, encoding an enzyme that decarboxylates diaminopimelate to produce lysine in *E. coli* (Stragier *et al.*, 1983)) has since been redefined. LysR regulators are the most abundant family of transcriptional regulators in bacteria, they both activate and repress transcription of a very diverse set of genes involved in virulence, metabolism, quorum sensing, cell division and motility (Maddocks and Oyston, 2008). The LysR family exhibit a conserved structure, characterised by an N-terminal helix-turn-helix (HTH) motif, and a C-terminal co-inducer-binding domain. The LTTR N-terminal 'winged'-HTH motif has

three helical domains, the second and third interact with DNA and the third domain inserts into the major groove of the DNA helix (Brennan and Matthews, 1989); in addition to this the winged-HTH motif also possesses a single anti-parallel β-sheet hairpin between the second and third helix (Maddocks and Oyston, 2008). Unlike the N-terminal HTH motif, the C-terminus of the LysR family exhibits very little conservation in terms of amino acid sequence. This region is characterised by the presence of two subdomains, RD1 and RD2, and the co-inducer binds to the cleft produced by the two cross over regions where these interact (Stec *et al.*, 2006).

An important feature of LTTR regulation is DNA bending: there are two key sites upstream of the LTTR target gene, referred to as the RBS (regulatory binding site, which can span -35 to +20 bp of the transcriptional start site) and the ABS (activation binding site, which spans -40 to -20 bp) (Porrua *et al.*, 2007); another important feature is the 'LTTR Box' present at the RBS, it consists of the sequence T-N_{11}-A, and is generally palindromic – LTTRs differ in their affinity for the LTTR Box depending on whether they are co-inducer bound, which subsequently affects DNA bending and interaction with RNA polymerase (Parsek *et al.*, 1994). The LTTR is known to act as a tetramer: the RBS site is usually bound by the apo-form of an LTTR dimer, whereas the ABS site is only usually occupied, by a separate dimer, once the LTTR is bound by the co-inducer (Tropel and van der Meer, 2004). It is thought that the two LTTR dimers located at the RBS and ABS sites come into contact to form a tetrameric structure as a result of DNA bending, which facilitates the formation of a higher-order complex involving RNA polymerase, leading to transcription - irrespective of whether the LTTR is acting as an activator or repressor (Maddocks and Oyston, 2008). A good example of LTTR regulation mediated by DNA bending is provided by OccR, which is encoded on the *Agrobacterium tumefaciens* Ti plasmid: it positively regulates genes involved in octopine (a nutrient released from crown gall tumours) catabolism, and exhibits negative autoregulation (Akakura and Winans, 2002). When the octopine coinducer is bound to OccR, it causes a shift from a high angle bend, OccR occupying base pairs 280-228 upstream of target gene initiation codon *occQ*, to a low angle bend with OccR occupying a shorter region of 280-238 bp upstream (Akakura and Winans, 2002). Transcriptional activation is dependent on the DNA bending caused by the multimeric OccR octopine-bound complex (Akakura and Winans, 2002) (FIGURE 2.18).

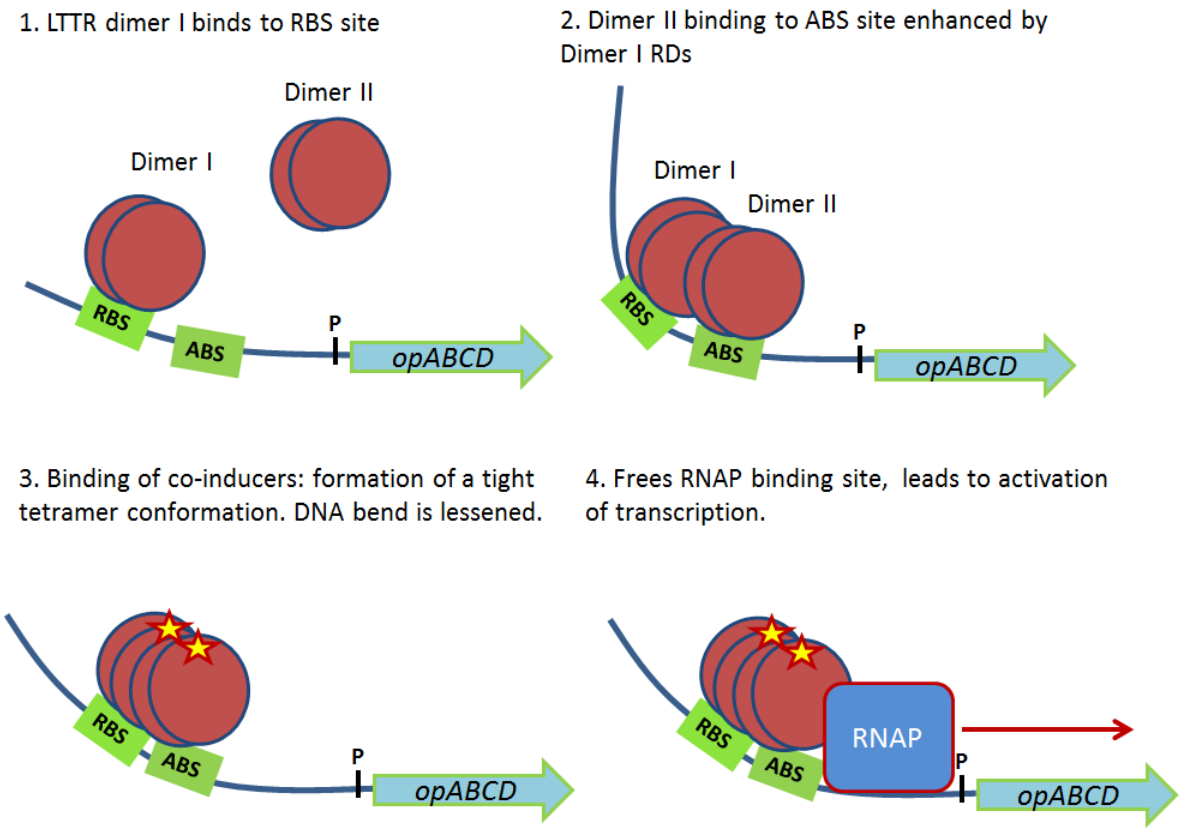


Figure 2.18 General mechanism of LTTR regulation involves DNA bending. A schematic representation of LTTR dimers binding separately (the putative 'step-binding' model) to the RBS and ABS to form a tightly associated tetramer with bound co-inducer. (1) Shows LTTR Dimer I binding to RBS site with high affinity. (2) Shows binding of Dimer II, which is enhanced by RDs from Dimer I, which form a loosely associated 'dimer of dimers' causing the DNA to bend. (3) The coinducer bound dimer of dimers undergoes a conformational change to produce a tightly bound tetramer, causing the DNA bend to lessen, which subsequently frees the RNAP promoter region. (4) RNAP binds to the promoter, forms a ternary complex, and initiates transcription. Adapted from (Maddocks and Oyston, 2008) and (Zhou *et al.*, 2010).

One important factor that accounts for the ubiquity of LTTRs is the diversity of roles they can employ, both positive and negative autoregulation, in addition to acting as either transcriptional activator or repressor of target genes. One example of LTTR positive autoregulation is provided by YtxR in *Yersinia enterocolitica*: expression of *ytxR* from a non-native promoter was shown to increase expression of *ytxA* (*ytxAB* together encode an ADP-ribosylating toxin) 35-fold, and was shown to induce expression from its own promoter 100-fold (Axler-Diperte *et al.*, 2006). In contrast to YtxR, relatively early work on another LTTR, IlvY from *Salmonella typhimurium*, revealed this LTTR is a negative regulator of *ilvY* expression, hence it is a negative autoregulator, although it acts as a transcriptional activator of its divergently transcribed target gene *ilvC* (encoding IlvC, an acetohydroxy-acid isomeroreductase, which is involved in the synthesis of L-valine and L-isoleucine) (Biel and Umbarger, 1981). The substrates of IlvC, α-acetolactate and α-acetohydroxybutyrate, are both co-inducers of IlvY, which in turn are necessary for transcriptional activation of *ilvC* – this type of

regulation coupled to a feedback loop is considered paradigmatic of LTTR regulation. Perhaps the best example of an LTTR acting as a repressor is provided by CcpC regulation of *citB* (encoding aconitase) and *citZ* (encoding citrate synthase) in *Bacillus subtilis*. Negative regulation by CcpC plays an important role in the Krebs cycle: it has been shown that mutations at the -27 position upstream of *citB* reduces DNA bending, and has a similar effect to the addition of citrate (the co-inducer), which provides greater access to RNA polymerase, leading to derepression (Kim *et al.*, 2003). However, in contrast to all of these LTTRs, another LysR regulator in *Bacillus subtilis*, GltC, is able to act as both a transcriptional activator and repressor (Picossi *et al.*, 2007). GltC regulates the *gltAB* operon, which encodes glutamate synthase, an essential enzyme of nitrogen metabolism. Work done by Picossi et al., (2007) identified three regions ('dyad symmetry elements') called Box I, Box II and Box III located in the intergenic region of *gltA* and the divergently transcribed *gltC*. In order to activate transcription, GltC requires the co-inducer α-ketoglutarate (a substrate of glutamate synthase): in the absence of inducer, GltC only binds to the Box I region and only marginally activates transcription of *gltAB* (Picossi *et al.*, 2007). However, in the presence of glutamate (the product of glutamate synthase), glutamate-bound GltC was shown to bind to Box I and Box III, leading to repression of *gltAB* transcription (Picossi *et al.*, 2007). But in the presence of α-ketoglutarate, GltC was shown to bind to Box I and Box II (which overlaps slightly with the -35 region of the *gltA* promoter), and to stabilize binding of RNA polymerase to the *gltA* promoter in order to activate transcription of *gltAB* (Picossi *et al.*, 2007). This mechanism is thought to be essential for expression of *gltAB* and provides a complex example of LTTR regulation that exhibits both activator and repressor characteristics.

Full length crystal structures of LTTRs are sparse because of their insolubility, and also because of the flexibility of the winged HTH-motif (Maddocks and Oyston, 2008). At present, five full length LTTR crystal structures have been resolved: the first to be resolved was CysB from *Klebsiella aerogenes* (Verschueren *et al.*, 2001), then CbnR from *Ralstonia eutropha* (Muraoka *et al.*, 2003), DntR from *Burkholderia* sp. strain DNT (Smirnova *et al.*, 2004), CrgA from *Neisseria meningitidis* (Sainsbury *et al.*, 2009) and most recently ArgP from *Mycobacterium tuberculosis* (Zhou *et al.*, 2010). As mentioned above, the C-terminal hinge region/ co-inducer binding cleft is a conserved feature of all LTTR structures, although the C-terminal region of different LTTRs exhibits very little sequence similarity. The full length structure of CbnR is regarded as the archetypal LTTR: CbnR is a homo-tetramer, a dimer of dimers, and each dimer – held together by an anti-parrallel helix-helix interaction - consists of two subunits in different conformations, one short-form and one extended-form subunit, which gives the tetramer an overall ellipsoid shape (Muraoka *et al.*, 2003). The DNA-binding domain (DBD residues 1-58) of CbnR forms a V-shaped bottom that interacts with the two DNA binding sites (RBS and ABS) spanning a 60bp stretch of the *cbnA* promoter (the *cbnABCD* operon encodes enzymes

involved in the degradation of chlorocatechol, *cbnR* is divergently transcribed) (Muraoka *et al.*, 2003). The regulatory domain (residues 88-294) and the DBD are held together by a flexible linker region (residues 59-87) (Muraoka *et al.*, 2003). In addition to this, recent structural work with ArgP (an LTTR regulator of outward arginine transport) has confirmed many of the details first described in the CbnR structure (Zhou *et al.*, 2010): like CbnR, ArgP forms a homodimer, each subunit consists of two domains, a regulatory domain (RD) and a DNA binding domain (DBD), and two distinct subunit conformations were observed, closed or open (Zhou *et al.*, 2010) (FIGURE 2.19). Based on this most recent structure, these authors propose a 'step-binding' model for LTTR regulation (FIGURE 2.18): the first dimer binds to the RBS site (higher affinity), which facilitates binding of a second dimer to the ABS site (lower affinity) to form a dimer of dimers in loose conformation covering the RNAP binding sites; subsequent binding of effectors (co-inducers) to the dimer of dimers is hypothesised to cause conformational changes in both RD and DBD leading to formation of a tight tetramer conformation that frees the RNAP binding site, and enables formation of a ternary complex leading to transcription (Zhou *et al.*, 2010).

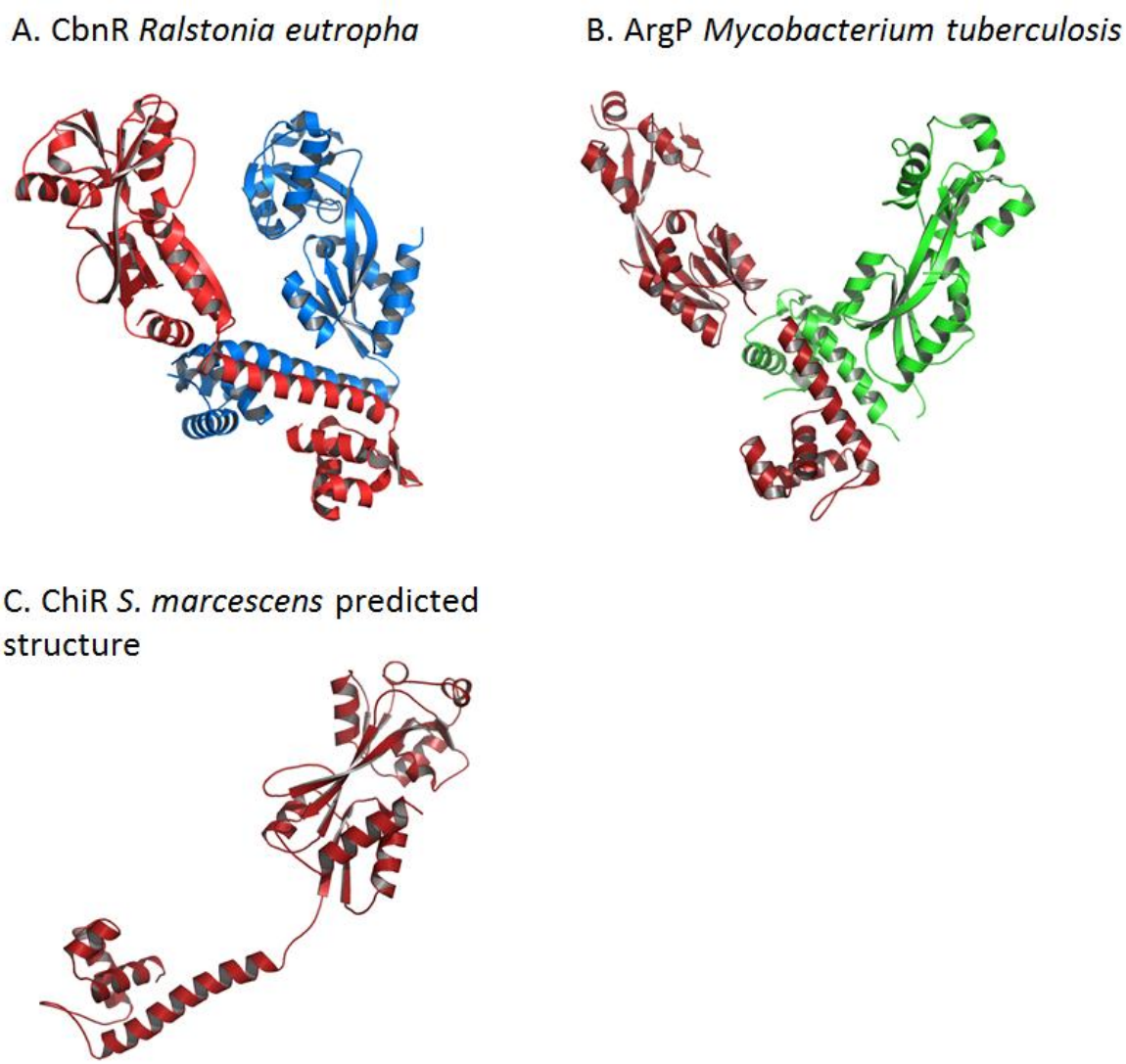


Figure 2.19 LTTR crystal structures. (A) The structure of CbnR from *Ralstonia eutropha* (Muraoka *et al.*, 2003) is paradigmatic of LysR family structure. The main body of the tetramer forms a V-shape, with the DNA binding domains located at the bottom of the V, and this is suited to interact with the full 60 bp length of the promoter region (Muraoka *et al.*, 2003). The target DNA is bent along the V-shaped bottom of the CbnR tetramer, and relaxes when CbnR is bound by its co-inducer (Muraoka *et al.*, 2003). (B) The structure of ArgP from *Mycobacterium tuberculosis* exhibits the same LTTR core features first elucidated in the CbnR structure (Zhou *et al.*, 2010): ArgP forms a homodimer and each subunit contains two domains, a DNA-binding domain (DBD) and a regulatory domain (RD), and these exhibit two distinct conformations, closed or open, depending on whether it is bound by its co-inducer. (C) Predicted structure of *S. marcescens* ChiR (Kelley and Sternberg, 2009). In terms of predicted structure (Kelley and Sternberg, 2009), *S. marcescens* ChiR exhibits similarity to BenM from *Acinetobacter sp.* and to CrgA from *Neisseria meningitidis*, both LysR family regulators.

In this work, we have isolated a *chiR*-Tn5 candidate that is blocked in its ability to move ChiC to the extracellular supernatant. Based on this result, and since it has already been shown that ChiR does not bind to the promoter regions of *chiA*, *chiB* or *chiC*, we hypothesise that ChiR is involved in regulating the transcription of components involved in the secretion of chitinase, as opposed to the chitinase genes directly. However, we cannot rule out the possibility that ChiR might regulate transcription of ChiB, since we were unable to detect the intracellular presence of ChiB in mutant

TnchiB5 (*chiR*-Tn5) (FIGURE 2.13), and also because *chiR* is divergently transcribed from *chiB*, which is typical of LTTR target genes. However, since the intracellular presence of each chitinase is lost in a clean Δ*hfq* strain, it seems more likely that Hfq mediates transcriptional regulation of the chitinase genes. In order to test this we will need to generate a clean targeted deletion of the *chiR* gene, and confirm that each of the chitinases are blocked in terms of movement to the culture supernatant by Western immunoblotting. From there the first crucial task will be to identify the small molecule coinducer of ChiR. The work of Picossi *et al*., (2007) identified the coinducer of GltC as α-ketoglutarate, and since ornithine is also known to be an important molecule in the same metabolic pathways in *B. subtilis* (the Krebs cycle and the Roc pathway), this work potentially outlines a good approach. To begin with we will engineer both N-terminal and C-terminal His_6-tagged ChiR (including a TEVP cleavage site) on the *S. marcescens* chromosome, and ensure that these retain a chitinase positive phenotype by Western blot analysis and also on chitin rich media. If the candidate shows a positive phenotype, indicating that the $ChiR^{His}$ strain is still active, we can then overexpress and purify the physiologically active $ChiR^{His}$ in *E. coli*, which will provide the basis for an *in vitro* transcription assay in the presence of different potential coinducers. The transcription assay will involve adding purified $ChiR^{His}$ to ~300 bp regions encoding the upstream promoter regions of *chiR* (autoregulator) and also *chiB* (likely target gene) and *chiC* (control) genes in the presence of *E. coli* RNA polymerase (*S. marcescens* RNAP does not appear to be available), and these mixtures will be incubated separately with different metabolites (possible co-activators): initially it would be best to start with the following candidates, NAD, NADH, NADP, NADPH, ornithine, putrescine, spermidine, NH_4Cl, glutamate, glutamine, α-ketoglutarate, glucose and GlcNAc at their respective physiological concentrations. Including α-32P labelled dNTPS in the reaction will enable the transcription reactions (incubated at 37°C for 20 mins) to be visualised on sequencing gels, and we can check the transcripts correspond to the target genes using internal primers for sequencing. If transcription levels appear elevated in the presence of a particular small molecule, we will then repeat the experiment with that particular potential coactivator in a dose-dependent manner to assess its overall sensitivity to the molecule.

Identifying the ChiR coinducer in this way will enable us to further characterise the regulatory role of ChiR. A microarray experiment would identify the expression of genes that are affected by a *chiR* deletion (in comparison to *S. marcescens* Db10), the important thing in this case would be to identify whether the target genes encode anything that could facilitate secretion of chitinase (such as *SMA2874*). Further gel mobility shift assays, or transcription assays will clarify whether ChiR binds to the promoter regions of candidate target genes directly.

2.4.4 The role of the SMA2874 holin-like protein in chitinase secretion

Two independent transposon mutants were identified in a holin-encoding gene (*SMA2874*) as being responsible for the chitin-degrading ability of ChiC in *S. marcescens*. This is the first time a holin has been implicated as being important in chitin degradation by *S. marcescens*. The *SMA2874* gene was discovered to be nestled within a genomic region dedicated to chitin metabolism including genes encoding chitinase B, the LysR regulator *chiR*, and chitin-binding protein Cbp21. This genomic organisation sparked further in-depth investigation of the role of *SMA2874* in chitinase secretion in Chapter 3.

2.5 Conclusions

Since, ideally, we should have screened 10,000 candidates for each of the three strains - JchiA, JchiB, JchiC - the transposon mutagenic screen conducted here was not exhaustive and we cannot rule out the possibility that there may remain important genes that are not yet identified. Nevertheless, the genetic screens performed in this Chapter identified four genes with potentially important roles in chitinase expression and/or secretion - *SMA4482* (*hfq*), *SMA4579* (*speF*), *SMA2876* (*chiR*) and *SMA2874.* Of these, only *chiR* was identified from screens of two separate strains (once in a screen for ChiB secretion and again in a screen for ChiC secretion). The *SMA2874* gene encoding the holin-like protein was isolated twice with two independent insertions identified in a screen against JchiC. The four independent isolations of mutations in *chiR* and *SMA2874* give confidence that these genes have roles in chitin degredation by *S. marcescens*.

3 A holin and an endolysin are essential for secretion of the chitinolytic machinery

3.1 Introduction

The transposon mutagenesis carried out in Chapter 2 identified a gene (*SMA2874*) that was apparently required for the externalization of ChiC by *S. marcescens* Db10. The *SMA2874* gene product comprises 108 amino acid residues and has a predicted molecular mass of 11,787 Da. BLAST analyses suggest that *SMA2874* is a member of the phage holin-3 family that includes the archetypal holin protein encoded by the λ bacteriophage. The *SMA2874* protein shares 35% overall sequence identity, and 50% overall similarity, with the canonical lambda S105 holin (FIGURE 3.1) (White *et al.*, 2010). The number of transmembrane helices predicted by TMHMM gives three helices, 20-39, 48-70, and 74-93 with an N-out, C-in topology (FIGURE 3.1). In addition to canonical λ-S, ChiW is also similar to holins produced by the Enterobacterial phages, especially to HK97 (33% sequence identity, 49% similarity) (Juhala *et al.*, 2000), and Sf6 (35% sequence identity, 50% similarity) (FIGURE 3.1) (Casjens *et al.*, 2004).

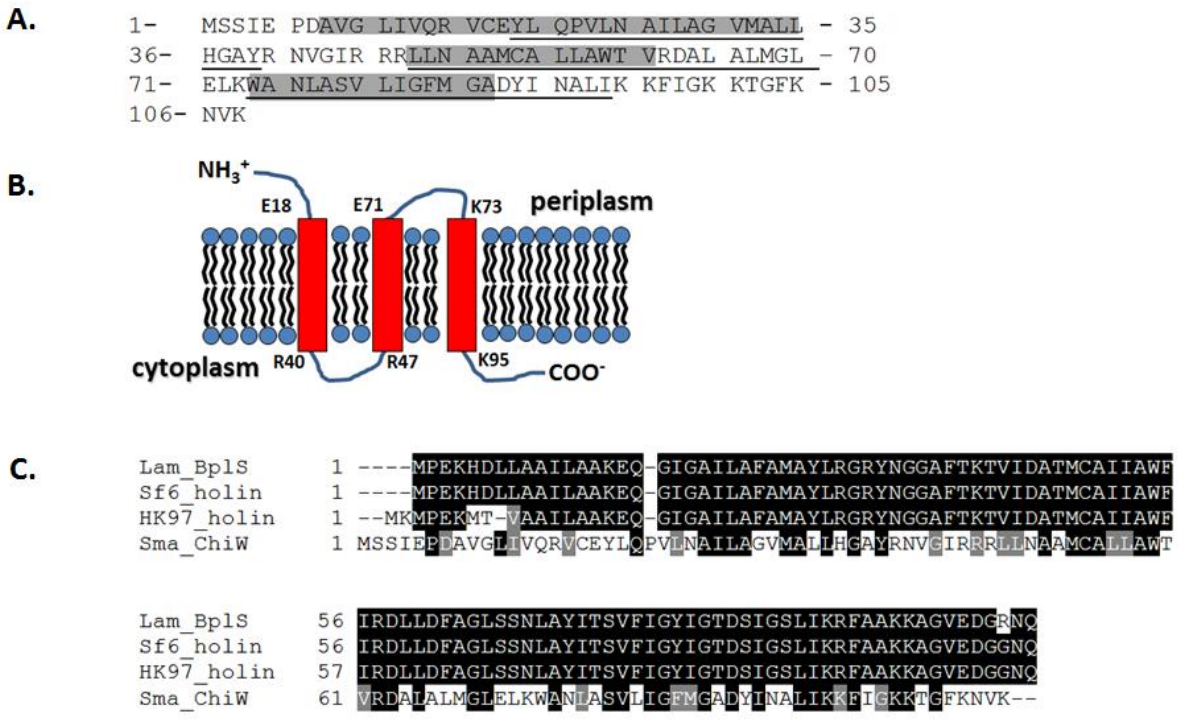


Figure 3.1 *SMA2874* is a holin-like protein with three predicted transmembrane helices. (A) The primary sequence of *SMA2874*. Predicted transmembrane domains are underlined, and transmembrane domains inferred from the phage lambda S holin protein are shaded (White *et al.*, 2010). (B) Putative inner membrane localisation and topology of *SMA2874*. The number of transmembrane helices predicted by TMHMM prediction gives three helices (20-39, 48-70, and 74-93 with N-out, C-in). (C) An alignment of *S. marcescens* ChiW with holins produced by bacteriophage: the canonical lambda S105 holin ('Lam-BplS') (White *et al.*, 2010), a holin produced by the Enterobacteria phages HK97 ('HK97_holin') (Juhala *et al.*, 2000) and Sf6 ('Sf6_holin') (Casjens *et al.*, 2004). Sequence alignments were performed with Clustal Omega (2), and displayed using the freely-available on-line tool Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

SMA2874 has not been previously studied in relation to chitin metabolism. The *SMA2874* gene is located in an apparent four-cistron operon with a gene encoding a predicted L-alanyl-D-glutamate endopeptidase (*SMA2873*) and two genes encoding homologues of bacteriophage spanins (*SMA2872*, *SMA2871*) (FIGURE 3.2). Intriguingly, *SMA2874* is located adjacent to three genes already known to be involved in chitin metabolism, namely *chiB*, *chiR* and *cbp21*. The role of *SMA2874* in chitinase secretion therefore deserved further investigation.

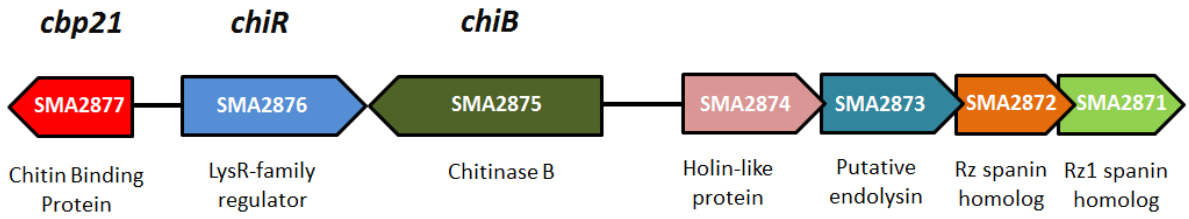


Figure 3.2 A cartoon representation of the genetic organisation of the *S. marcescens* chromosome around region 3,032,110 – 3,037,022. *SMA2874* encodes a bacteriophage holin-like protein and forms part of an apparent four-cistron operon encoding a predicted endopeptidase (*SMA2873*) and two genes (*SMA2872*, *SMA2871*) encoding proteins that resemble bacteriophage spanins. *SMA2874* also lies adjacent to genes that encode known chitinolytic proteins, such chitin binding protein encoding *cbp21*, LysR-type regulator encoding *chiR* and chitinase encoding *chiB*.

3.2 Aims

The aim of this Chapter was to explore the role of the *SMA2874* phage holin-like protein in chitinase secretion and chitin degradation. The initial aim was to construct an in-frame, unmarked, isogenic deletion mutant in the *SMA2874* gene and then take a combination of genetic, biochemical and proteomic approaches to understand its role in chitinase secretion. A second aim was to examine the roles of adjacent genes at the *SMA2874* locus in chitinase secretion.

3.3 Results

3.3.1 The phage holin-like protein encoded by *SMA2874* is essential for secretion of the entire chitinolytic machinery in *S. marcescens* Db10

Tn5 transposon disruption of *SMA2874*, encoding a phage holin-like protein, apparently results in a loss of ChiC associated chitinolytic phenotype in *S. marcescens* (Chapter 2). In order to determine whether the phage holin-like protein is in fact responsible for the extracellular presence of ChiC we constructed an unmarked deletion strain (JJH08p) in *SMA2874*. Initial Western immunoblotting analysis demonstrated that the new deletion in the gene encoding the phage holin-like protein phenocopied both of the Tn5 insertion mutants isolated in Chapter 2 (FIGURE 2.15). The ChiC protein is produced to a similar level as the parent strain, but remains associated with the whole cells and is not detected in the culture supernatant (FIGURE 3.3).

Next, the role of *SMA2874* in secretion of ChiA and ChiB was examined. Very interestingly, Western immunoblotting revealed that both ChiA and ChiB were mislocalised in the JJH08p (Δ*SMA2874*) strain (FIGURE 3.3). This data gives initial, but strong, evidence that the phage holin-like protein has a pleiotropic role in mediating translocation of each chitinase across the cell envelope in *S. marcescens*.

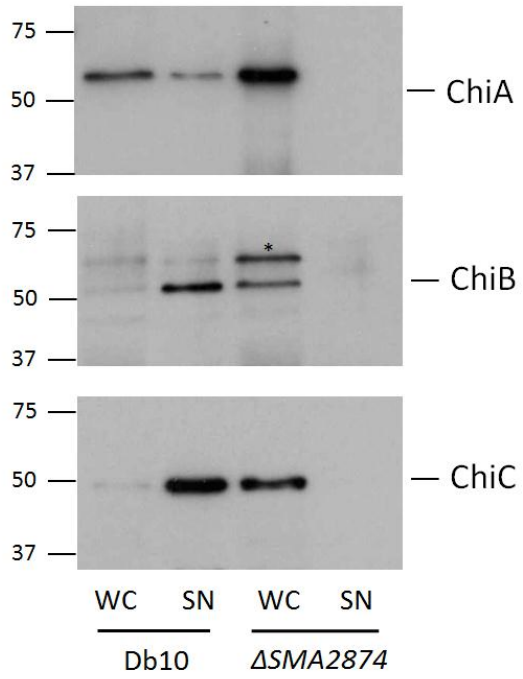


Figure 3.3 A phage holin-like protein facilitates the movement of ChiA, ChiB and ChiC to the extracellular environment. The *S. marcescens* parent strain Db10, and strain JJH08p (Δ*SMA2874*) were grown aerobically in rich media for 16 hours at 30°C before aliquots of whole cells (WC) and culture supernatants (SN) were taken and analysed for the presence of ChiA, ChiB and ChiC by Western immunoblotting. The band marked by the single asterisk (*) is the ChiA protein, which the polyclonal ChiB antiserum can also detect.

Next, it was important to take a whole systems approach to understand the involvement of the phage holin-like protein in protein secretion in general. Thus a new collaboration was initiated with Dr Matthias Trost of the Protein Phosphorylation and Ubiquitination Unit, University of Dundee, who is an expert in label-free quantitative proteomics. The broad aim was to compare the total extracellular secretome of the *S. marcescens* Db10 and with that of the JJH08p (Δ*SMA2874*) mutant, and obtain quantitative data on the relative amounts of each protein in each extract. To do this, total protein extracts of culture supernatants ('secretomes') were prepared. The strains were grown in minimal media supplemented with glucose for 16 hours at 30°C, and the proteins present in the supernatant were concentrated by TCA precipitation. Label-free quantitative mass spectrometry was then used to profile changes in protein abundance in the corresponding secretomes. Four biological replicates of secretome digests of both strains were analysed by high-resolution mass spectrometry in an Orbitrap Velos Pro mass spectrometer and data processed through Maxquant (Cox and Mann, 2008). Using strict filtering, 497 proteins (<1% FDR) 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 *SMA2874* deletion, a subset of ten proteins (shown as green and red points in FIGURE 3.4) showed with high

confidence ($p<0.01$) a significant increase in abundance in the secretome of the parent strain compared to the mutant (Table 3.1). Strikingly, the most abundant proteins by far in this small group of ten proteins were the entire known chitinolytic machinery produced by *S. marcescens* (ChiA, ChiB, ChiC and Cbp21) (FIGURE 3.4).

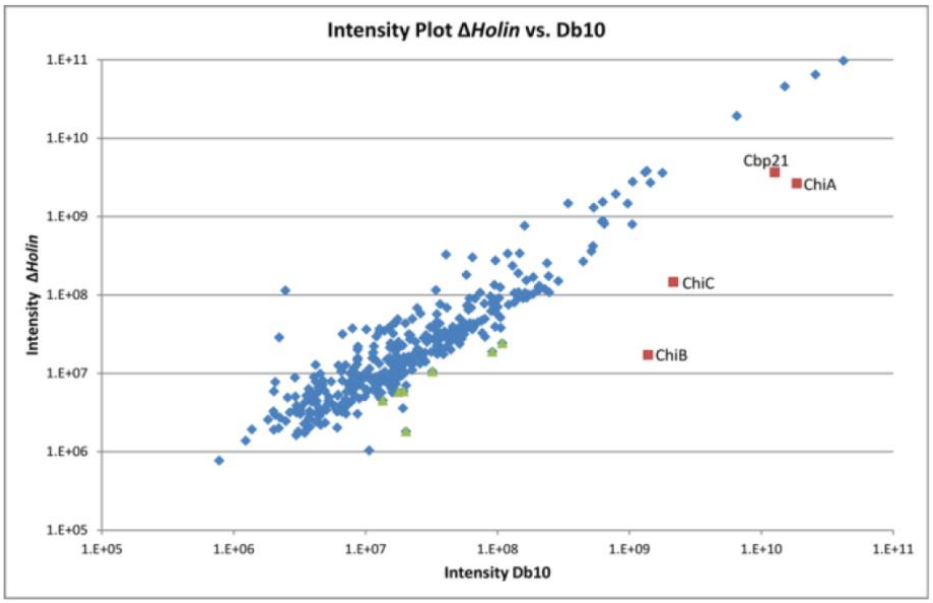


Figure 3.4 Intensity scatter plot of secretome data shows the extracellular presence of each of the four main chitinolytic proteins is dramatically affected by a *SMA2874* deletion. Label-free intensities of secretome proteins (average of four biological replicates) of the Δ*SMA2874* ('ΔHolin') strain *versus* the secretome of the Db10 parental strain. Highly abundant proteins that were reduced significantly ($p<0.01$) in the Δ*SMA2874* secretome are labelled red and, with the locations of the other, lower abundant, proteins shown in green (see Table 3.1). The three chitinases, ChiA, ChiB and ChiC, as well as the chitin-binding protein, Cbp21, are by far the most abundant of the proteins affected by the Δ*SMA2874* mutation.

In total there were ten proteins whose extracellular levels were significantly affected by the *SMA2874* deletion. In addition to the four chitinolytic proteins, the removal of the *SMA2874* gene deleteriously affected the extracellular levels of six other proteins, including those as diverse as a pirin (encoded by *SMA3897*), an anthranilate synthase TrpE (*SMA1933*), a malate dehydrogenase MdhA (*SMA4522*), a NADP-dependent malic enzyme MaeB (*SMA2870*), a putative haem oxygenase (*SMA2390*) and a proly-tRNA synthetase ProS (*SMA3147*) (TABLE 3.1). It should be noted, however, that the relative abundance of these proteins in the secretome was very low when compared to the chitinases (TABLE 3.1).

Identifier	Name	Sec signal peptide	Description	Intensity (relative intensity*)	Relative abundance Db10/ *ΔSMA2874*	p-value	Unique peptides	Sequence coverage (%)
SMA2875	**ChiB**	**No**	**Chitinase B**	**8.5E+09 (92)**	**80.7**	**1.32E-08**	**11**	**32**
SMA0468	**ChiC**	**No**	**Chitinase C**	**1.4E+10 (152)**	**14.7**	**3.08E-05**	**21**	**54**
SMA3897		No	Pirin	1.3E+08 (1.4)	11.2	3.08E-04	5	26
SMA4243	**ChiA**	**Yes**	**Chitinase A**	**1.2E+11 (1304)**	**7.0**	**1.42E-07**	**33**	**69**
SMA1933	TrpE	No	Anthranilate synthase	5.5E+08 (6)	4.8	2.26E-05	12	26
SMA4522	MdhA	No	Malate de-hydrogenase	7.4E+08 (8)	3.4	1.94E-05	9	36
SMA2877	**Cbp21**	**Yes**	**Chitin binding protein**	**6.7E+10 (728)**	**3.4**	**0.0054**	**10**	**73**
SMA2870	MaeB	No	NADP-dependent malic enzyme	1.3E+08 (1.4)	3.3	0.00032	11	18
SMA2390		No	Putative haem oxygenase	1.1E+08 (1.2)	3.1	0.0044	4	25
SMA3147	ProS	No	Prolyl-tRNA synthetase	9.2E+07 (1)	3.0	6.33E-05	9	23

Table 3.1 Extracellular proteins secreted in a holin-dependent manner identified by label-free mass spectrometry. Proteins identified as present in the secretome of the *S. marcescens* parental strain (Db10) at an abundance > 3 × higher than observed in the secretome of a strain (JJH08p) lacking the gene encoding the phage holin-like protein ($p<0.01$) are shown. Four biological replicates of each strain were analysed. * relative intensity is calculated in relation to the intensity of the least abundant protein in the list (prolyl-tRNA synthetase).

Each of the proteins from the label free MS show a tight distribution: the extracellular presence of the ten proteins identified (TABLE 3.1) are also clearly reduced in the Δ*SMA2874* deletion strain when presented on a volcano intensity plot (FIGURE 3.5). Of the 497 proteins identified by label free Mass Spec, 351 (71%) exhibited a high level reproducibility (FIGURE 3.5).

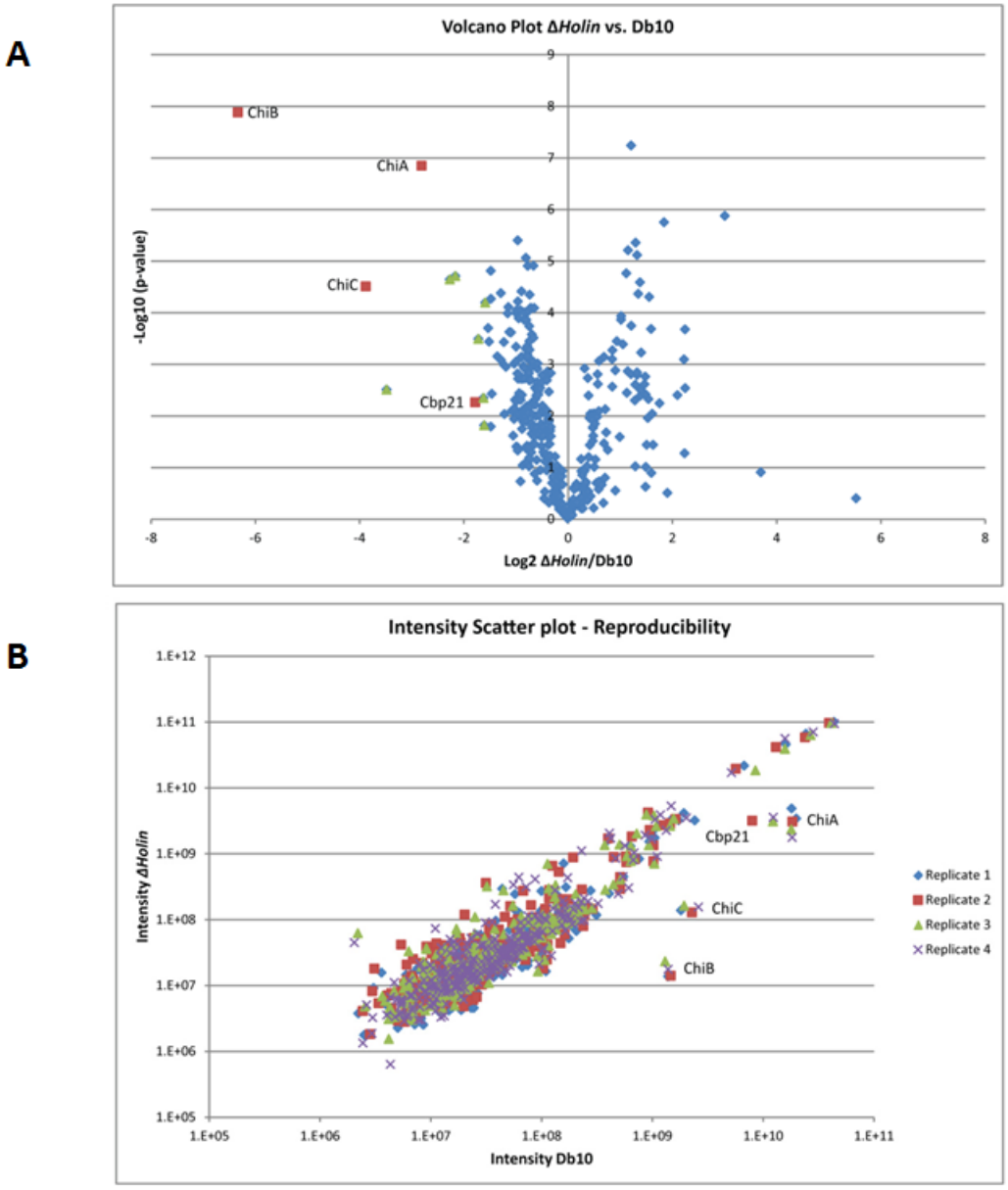


Figure 3.5 Proteomics quality control. (A) Volcano plot of the proteomics experiment shows an expected tight distribution for the majority of proteins with the few significantly reduced proteins in the JJH08p strain ('Δ*Holin*') mutant coloured green and red. (B) An intensity scatter plot of all four biological replicates shows the high reproducibility of the changing chitinases and the chitin-binding protein Cbp21.

In addition, in-gel analysis of the same *S. marcescens* Db10 and JJH08p (Δ*SMA2874*) secretome samples used in the quantitative proteomics experiments clearly showed ChiA and Cbp21 were missing in the JJH08p (Δ*SMA2874*) secretome (FIGURE 3.6). Minor Coomassie-stained bands correseponding to ChiB and ChiC were also missing in the JJH08p (Δ*SMA2874*) extract, while flagellin (FliC) and a Type V-dependent secreted serine protease (SSP) acted as internal controls whose extracellular levels remained completely unaffected (FIGURE 3.6).

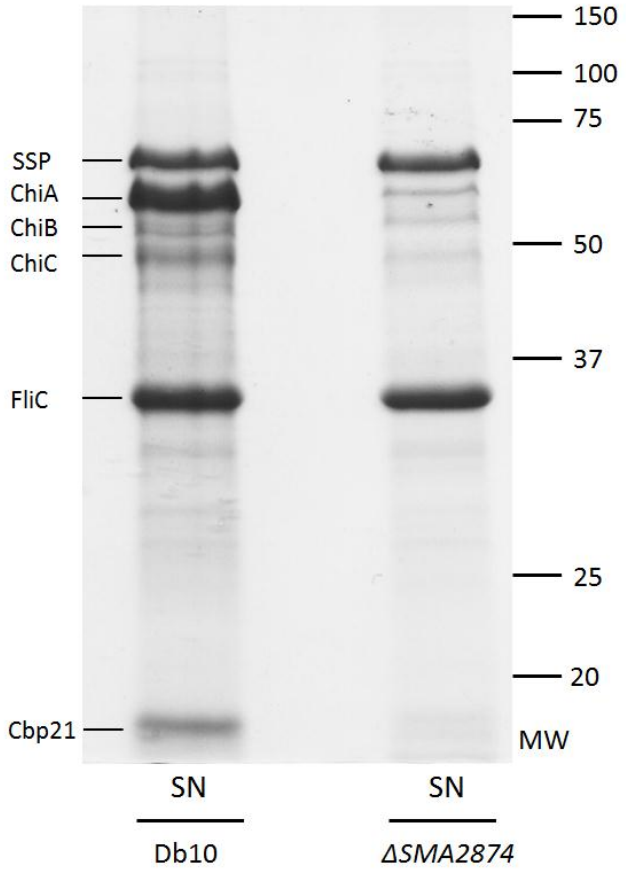


Figure 3.6 In-gel comparison of secretion profiles reveals ChiA, ChiB, ChiC and Cbp21 are missing from Δ*SMA2874* secretome. The relative levels of ChiA, ChiB, ChiC and CBP21 are diminished in the profile of the Δ*SMA2874* strain in comparison to the secretion profile of Db10 parental strain. Strains were grown aerobically in minimal media supplemented with glucose before culture supernatants were separated and their proteinaceous content analysed by SDS-PAGE and Coomassie staining. Tryptic peptide mass fingerprinting was used to identify each indicated protein: SSP (*SMA1670*), Type V-secreted serine protease; FliC, flagellin; ChiA, ChiB, ChiC and Cbp21.

Further analysis of the protein bands in Figure 3.6 by tryptic peptide mass fingerprinting revealed excellent peptide coverage for each chitinolytic enzyme (FIGURE 3.7). Indeed, the quality of the tryptic peptide mass fingerprinting data allows some conclusions to be drawn regarding post-translational processing of the proteins. The N-terminal Sec signal peptides of both ChiA and Cbp21 are clearly missing, confirming that these proteins must be targeted to the periplasm by the Sec pathway (FIGURE 3.7). More interestingly, this mass spec data provides the first definitive evidence that neither ChiB nor ChiC are proteolytically processed during the secretion event (FIGURE 3.7).

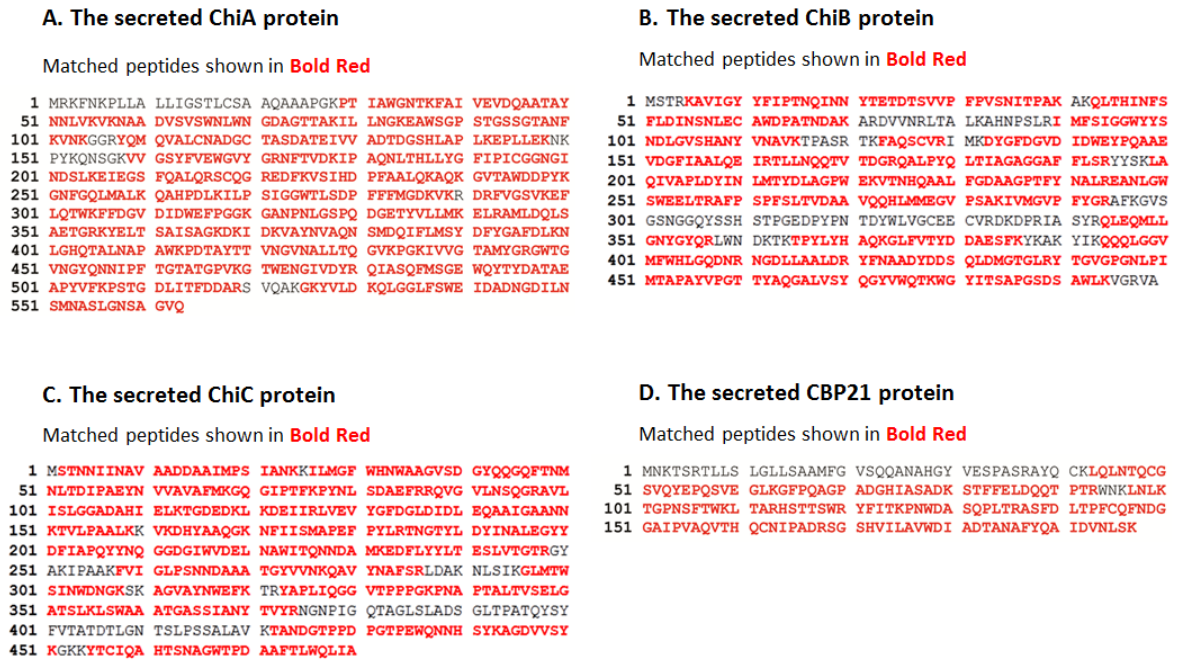


Figure 3.7 Tryptic Peptide Mass Fingerprinting of the secreted chitinolytic set of *S. marcescens*. (A) Analysis of excised gel pieces around ChiA (Fig. 3.6) identified the ChiA protein (score 3247) and 92% sequence coverage showed clearly that the N-terminal Sec signal peptide was not present. (B) Analysis of excised gel pieces around ChiB (Fig. 3.6) identified the ChiB protein (score 3851), 78% overall sequence coverage revealed that neither the N- nor C-terminus of the polypeptide was proteolytically processed during secretion. (C) Analysis of excised gel pieces around ChiC identified peptides corresponding to ChiC (score 5336). 84% overall sequence coverage revealed that, although the initiator methionine was not present, neither the N- nor C-terminus of the polypeptide was proteolytically processed during secretion. (D) Analysis of excised gel pieces around CBP21 identified the Cbp21 protein (score 914) and showed that its N-terminal Sec signal peptide was not present.

In addition to the substrates identified as having a reduced presence in the Δ*SMA2874* secretome, the label free mass spectrometry also identified nine proteins that underwent a >3-times increased abundance in the Δ*SMA2874* extracellular fraction. These included Type-1 fimbrins FimA (encoded by *SMA3915*), FimI (*SMA1250*), and SafA (*SMA1052*); 50S ribosomal proteins RplU (*SMA4526*) and RplI (*SMA4500*); predicted fimbrial-like adhesion proteins, PmfE (*SMA3920*) and another encoded by *SMA0789*; a flagellar assembly protein FlgD (*SMA2217*); and a component of the Type 6 secretion system Hcp (*SMA2263*) (TABLE 3.2).

Identifier	Name	Sec signal peptide	Description	Intensity (relative intensity*)	Relative abundance Δ*SMA2874* / Db10	p-value	Unique peptides	Sequence coverage (%)
SMA3915	FimA	Yes	Type-1 fimbrin	4.1E+07	8.0	1.33E-06	6	60
SMA1250	FimI	Yes	Type-1 fimbrin	1.6E+08	4.7	0.0028	3	34
SMA4526	RplU	No	50S ribosomal protein L21	6.7E+06	4.7	0.0002	3	26
SMA0789	-	Yes	Predicted fimbrial-like adhesion protein	6.5E+07	4.7	0.0008	4	35
SMA1052	SafA	Yes	Mannose sensitive Type-1 fimbrin	3.4E+08	4.3	0.0039	8	70
SMA3920	PmfE	Yes	Fimbrial adhesion	1.0E+07	3.6	1.77E-06	7	27
SMA2217	FlgD	No	Flagellar assembly protein	3.4E+07	3.4	0.0057	4	32
SMA4500	RplI	No	50S ribosomal protein L9	4.2E+06	3.1	0.0091	4	30
SMA2263	Hcp	No	Type 6 secreted protein	1.5E+10	3.0	0.0002	19	92

Table 3.2 Extracellular proteins of increased abundance in Δ*SMA2874* strain identified by label-free mass spectrometry. Proteins identified as present in the secretome of a holin-deficient mutant (JJH08p) at an abundance >3X higher than observed in the secretome of the parent strain Db10 ($p<0.01$) are shown. Four biological replicates of each strain were analysed.

3.3.2 Genetic dissection of the *chiWXYZ* locus

We have now gathered compelling genetic and biochemical evidence that the phage holin-like protein encoded by the *SMA2874* gene plays a central role in the secretion of the entire chitinolytic machinery in *S. marcescens*. In view of this we now propose to name the *SMA2874* gene as *chiW*. In every sequenced strain of *Serratia* the *chiW* gene is found in a locus that includes *chiB*, *chiR* and *cbp21,* together with three other genes. We hypothesise that, since the downstream ORFs overlap considerably, that *chiW* forms part of an apparent four-cistron operon, here named *chiWXYZ* (FIGURE 3.8).

```
atgtccagcatagagcctgacgctgttggcttaatagtccagcgagtatgtgaatatctt
 M  S  S  I  E  P  D  A  V  G  L  I  V  Q  R  V  C  E  Y  L
 chiW

cagccggtattgaacgccattctggccggcgtgatggcgctattgcacggtgcctatcgc
 Q  P  V  L  N  A  I  L  A  G  V  M  A  L  L  H  G  A  Y  R

aacgtgggtattcggcgccgtttattgaatgcggcgatgtgcgcgttattggcctggacg
 N  V  G  I  R  R  R  L  L  N  A  A  M  C  A  L  L  A  W  T

gtgcgcgatgcgctggcgttgatgggcctggaattaaagtgggcgaatttggccagcgta
 V  R  D  A  L  A  L  M  G  L  E  L  K  W  A  N  L  A  S  V

ctgattggttttatgggggcggattacatcaacgccttaattaaaaaattcatcggcaaa
 L  I  G  F  M  G  A  D  Y  I  N  A  L  I  K  K  F  I  G  K

aagacggggtttaaaaatgttaaatgacatcgaagagattcgctttactgcccgcagcga
 K  T  G  F  K  N  V  K  *
                        M  L  N  D  I  E  E  I  R  F  T  A  R  S  E
                        chiX
```

agaaaacctgcgcggcgtgcacccggatctggtgcgcgttattcgtctggcgctgcgtta

E N L R G V H P D L V R V I R L A L R Y

ttccctggtgccgttttccgtcagcgaggggctgcgcagtatggcgcgccagcgggaaat

S L V P F S V S E G L R S M A R Q R E M

ggtgcgcgccggcagcagccaaacgctgcgcagccgccatctgaccggtcacgcggtgga

V R A G S S Q T L R S R H L T G H A V D

tgtggtggcgatgccggcgggtgtggtctcctgggagtgggattactacgcgcagattgc

V V A M P A G V V S W E W D Y Y A Q I A

ggtggcggtgcggcgcgcggcgcgtgaatgcggcatcatcgtcgaatggggcggcgaatg

V A V R R A A R E C G I I V E W G G E W

gaaaaccctcaaggatggtccgcacttccagctgacgttccgggactacccggcatgagc

K T L K D G P H F Q L T F R D Y P A *

M S

chiY

ggttggctgcaaaaactgatgcagggcgggttgctgctgctgttgctggcggctatctgc

G W L Q K L M Q G G L L L L L L A A I C

ctcggcggctacagctcgctgctgtcgcaccagttggcgtctgcacggcaacaggcggca

L G G Y S S L L S H Q L A S A R Q Q A A

gagttacagaaaagtctggcgcagcaggcggggctgatcgccaccctgcagactcaggat

E L Q K S L A Q Q A G L I A T L Q T Q D

gcgcaaaatcgtgcgctgatggcggcgcagcagcggcaggaacagcagctacgccaacaa

A Q N R A L M A A Q Q R Q E Q Q L R Q Q

cacgaggcttatcagaggaaataccgtgaagcgattaaaaacgatccctgcgccgctcag

H E A Y Q R K Y R E A I K N D P C A A Q

M K R L K T I P A P L S

Figure 3.8 The *chiWXYZ* operon and its protein products. The coding sequence of the *chiWXYZ* operon. The DNA sequence shown is the complementary strand reading base pairs 3,033,204 – 3,031,956 from the Wellcome Trust Sanger Centre database entry for the *S. marcescens* Db11 genome. Note that each of the genes in the operon overlap considerably, and also that translation of the spanin homologue *chiZ* is predicted to initiate at a GUG codon, encoding formyl-Methionine.

Next, it was important to determine whether any other component of the *chiWXYZ* operon, besides *chiW*, is potentially important in facilitating chitinase secretion. In order to establish whether each of the individual members of the *chiWXYZ* locus is essential for chitinase secretion, a new bank of four in-frame deletion strains was constructed, and the localisation of each chitinase was assessed using Western immunoblotting (FIGURE 3.9). In addition to *chiW*, it was revealed that ChiX, a putative L-alanyl-D-glutamate endopeptidase, was also essential for secretion of the three known chitinases (FIGURE 3.9). However, the genes encoding the spanin-like proteins, *chiY* and *chiZ*, were shown not to be individually essential for chitinase secretion (FIGURE 3.9).

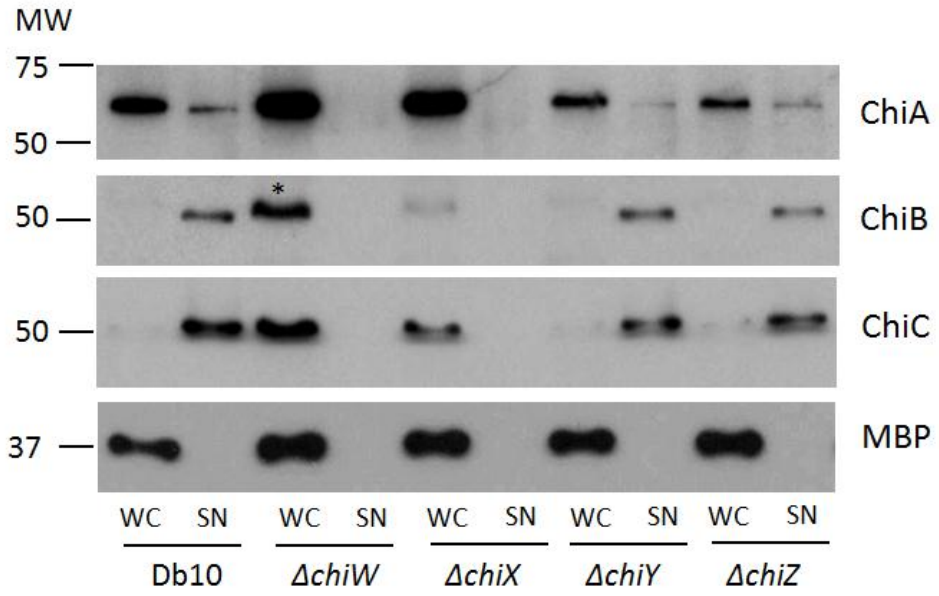


Figure 3.9 The ChiW holin and ChiX endopeptidase are both essential for secretion of each chitinase. The *S. marcescens* parental strain ('Db10') together with JJH04w (Δ*chiW*), JJH05x (Δ*chiX*), JJH06y (Δ*chiY*) and JJH07z (Δ*chiZ*) were all grown aerobically in rich media and separated into whole cell ('WC') and culture supernatant ('SN') fractions. Proteins were separated by SDS-PAGE and analysed by Western immunoblotting using the specific antisera indicated. MBP 'maltose binding protein'. ChiA detected by the ChiB antisera is indicated (*).

3.3.3 A two-step pathway for chitinase secretion

A secretion system is defined as a mechanism that facilitates the movement of a particular substrate across the cell envelope, while also maintaining the membrane integrity. Each chitinolytic component of this system has been shown to be dependent on the presence of ChiW and ChiX for movement to the extracellular environment. Since two of these components, ChiA and Cbp21, contain Sec type N-terminal signal peptides, we hypothesised that the putative secretion apparatus is dedicated to facilitating the movement of the chitinolytic enzymes across the outer membrane - which would classify it as a two-step secretion system. In order to test this we prepared periplasmic, cytoplasmic, extracellular and total membrane fractions from *S. marcescens* Db10 parental and Δ*chiW* strains, grown aerobically at 30°C for 16 hours. The periplasm was prepared using an osmotic shock method and the cytoplasmic and membrane fractions were separated using ultracentrifugation at 80,000 rpm. The fractions were separated by SDS-PAGE and analysed by Western immunoblotting (FIGURE 3.10). Probing with periplasmic and cytoplasmic control antisera established that the fractionation protocol had prepared samples of the *S. marcescens* Db10 and

Δ*chiW* periplasm free from cytoplasmic contamination (FIGURE 3.10). Further inspection revealed that both ChiA and ChiC were located in the periplasm in the secretion-defective strain (FIGURE 3.10). Similarly, fractionation of the Δ*chiX* strain also revealed that ChiA and ChiC secretion was blocked at the outer membrane transport step, since both proteins could be detected in the periplasm in the mutant background (FIGURE 3.10). Taken altogether, these data suggest strongly that export of ChiA and ChiC to the periplasm is not adversely affected by 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.

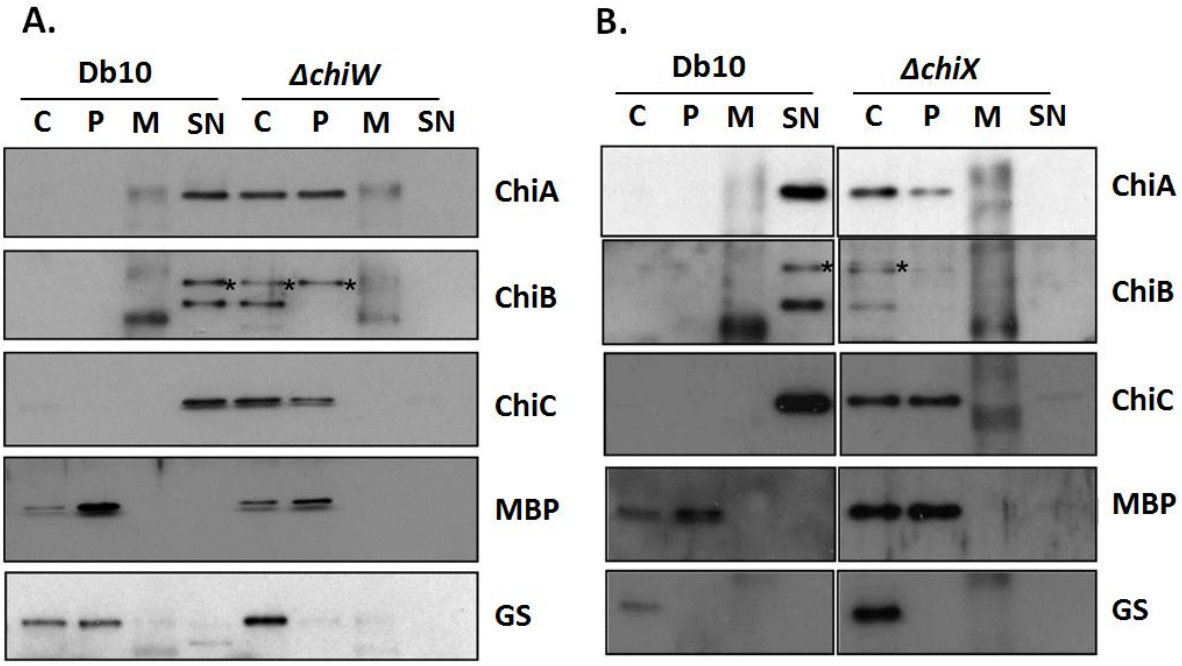


Figure 3.10 The ChiW holin and ChiX endopeptidase facilitate movement of ChiA and ChiC across the outer membrane. In strains lacking the *chiW* holin encoding gene (A), and the *chiX* endopeptidase encoding gene (B), movement of chitinase ChiA and ChiC is blocked across the outer membrane. In both strains, ChiA and ChiC are located in the periplasm, suggesting ChiW and ChiX facilitate their passage across the outer membrane, as part of a two-step secretion mechanism. The *S. marcescens* parental strain (Db10) together with the JJH04w (*ΔchiW*) mutant (panel A) and the JJH05x (*ΔchiX*) mutant (panel B) were grown aerobically in rich media, harvested, and fractionated into cytoplasm ('C'), periplasm ('P'), total membranes ('M') and culture supernatant ('SN'). Proteins were separated by SDS-PAGE and analysed by Western immunoblotting using the antisera indicated. The band marked by the single asterisk (*) is the ChiA protein, which the polyclonal ChiB antiserum can also detect. Control blots were against the periplasmic maltose binding protein (MBP) and the cytoplasmic glutamine synthetase (GS) enzyme.

3.3.4 Regulation of chitinase secretion

A stretch of DNA sequence 203 base pairs upstream of the *chiW* translation start was analysed by a bacterial transcriptional promoter scanning program (softberry.com), which predicted that *chiW* may have a single transcription initiation site (FIGURE 3.11).

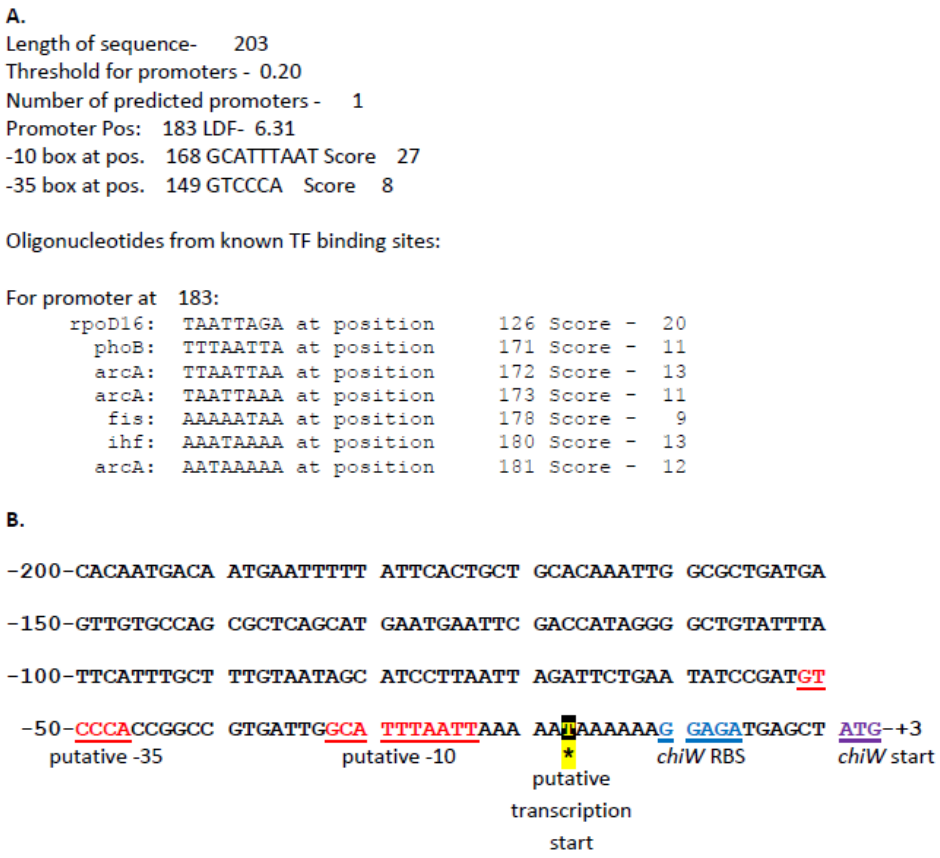


Figure 3.11 Analysis of *chiW* regulatory region. (A) Output from the sequence analysis and predicted locations of regulator binding-sites. (B) The sequence as analysed. The position of the *chiW* translation start codon is highlighted in purple, and the sequence given is numbered relative to this position. The putative ribosome binding site for *chiW* is shown in blue. The predicted transcription start site is highlighted in yellow and by the asterisk, with the predicted -10 and -35 sigma (70) factor binding sites red underlined.

We were interested in whether ChiR regulates expression of the *chiWXYZ* operon since it has been shown that ChiR has an essential role in the *S. marcescens* chitinolytic phenotype (Suzuki *et al.*, 2001). Moreover, we had observed in previous work (FIGURE 3.12) that a Tn5 disruption of ChiR (in strain TnchiC1) did not affect the intracellular expression of ChiC, but did prevent the movement of ChiC to the extracellular milieu. So the question became if ChiR was not involved in the transcription

of chitinase genes *per se*, could it be alternatively involved in regulating the expression of the transport system? One way to test this is to look at transcript levels in a basic RT-PCR experiment. Cultures were grown for 6 hours before total RNA was isolated and cDNA was generated and used as a template in a PCR to assess the relative transcript levels of *chiWX* and *chiC*. What is especially clear is that transcript levels of *chiWX* are dramatically reduced in a strain harbouring a Tn5 in *chiR*, TnchiB5, and we also see a less-dramatic reduction of transcript levels in candidate TnchiC1 (FIGURE 3.12 A). A strain with a Tn5 in *chiW* (TnchiC4) was used as a control, showing no transcript as expected (FIGURE 3.12 A). We also hypothesised that ChiR would not regulate the chitinase encoding genes directly, since ChiR has been shown not to bind the promoter regions of *chiA*, *chiB* or *chiC* (Suzuki *et al.*, 2001). In this case we did not see a strong reduction in *chiC* transcript levels in the absence of *chiR* (FIGURE 3.12 B, bottom panel).

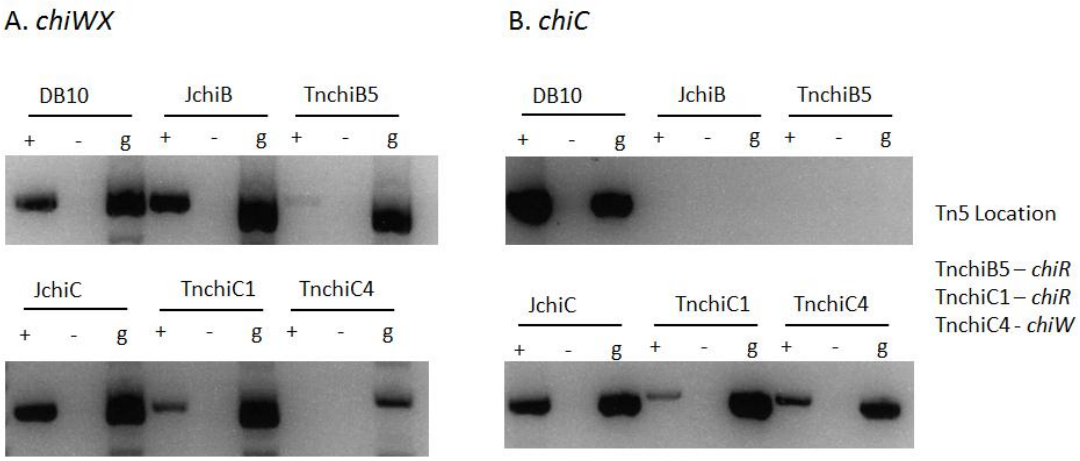


Figure 3.12 Trancript levels of *chiWX* are reduced in strains harbouring a Tn5 in *chiR*. Strains were grown for 6 hours at 30°C, total RNA was isolated, from which cDNA templates were made and used as the template for PCR with primers specific for either *chiWX*, or *chiC*. PCR with reverse transcriptase (+), PCR without reverse transcriptase to control for DNA 'carry over' (-), and gDNA positive control (g).

3.4 Discussion

3.4.1 ChiW and ChiX are essential for chitinase secretion in *S. marcescens*

Work in this Chapter has identified two genes – *chiW* (*SMA2874*) and *chiX* (*SMA2873*) – that are essential for chitinase secretion by *S. marcescens*. ChiW is a holin-like protein encoded within a putative four gene operon, *chiWXYZ*, that is itself located within a wider genetic locus dedicated to chitin degradation, which includes *chiB* (*SMA2875*), *cbp21* (*SMA2877*) and *chiR*. The *chiX* gene overlaps *chiW*, and encodes a predicted L-alanyl-D-glutamate endopeptidase. Both *chiW* and *chiX* appear to facilitate secretion of chitinase across the outer membrane, since deletion of these genes results in the stalling of ChiA and ChiC in the periplasm (FIGURE 3.10). Of the genes encoded within the *chiWXYZ* operon, *chiW* and *chiX* were essential for mediating chitinase secretion, whereas *chiY* and *chiZ* (encoding putative spanins) were not essential since targeted deletion of these did not appear to have an obvious effect on chitinase secretion.

Bioinformatic analysis of ChiW reveals that it exhibits amino acid similarity to prophage-encoded holins located within the sequenced genomes of Gram negative enteric bacteria such as *Klebsiella pneumonia* and *Xenorhabdus bovienii*, and to holins produced by Enterobacterial phages, such as HK97 and Sf6, and also to canonical lambda S105 holin. At present there is no published work examining the role of phage holin–related proteins in mediating protein secretion in *S. marcescens*, and, until now, work attempting to draw solid evidence for holin-mediated protein secretion in other biological systems has proved inconclusive. Although there is currently no published work providing a plausible mechanism of ChiW-mediated chitinase secretion, it is worth noting that, of fifty two holin families in total, there are twelve holin families predicted to be of proteobacterial origin (Reddy and Saier, 2013) – none of these have been extensively characterised.

3.4.2 The role of holins in the bacteriophage lytic cycle

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 (FIGURE 3.1). At present, there are two general forms of bacteriophage holin: canonical holin, such as S105, and pinholins, such as $S^{21}68$ produced by lambdoid phage 21 (Park *et al.*, 2007; Pang *et al.*, 2009). There are also two general forms of peptidoglycan-degrading endolysin: canonical endolysins such as λ-R, and signal anchor release (SAR) endolysins, such as R21 of phage 21 and Lyz of phage P1 (Berry *et al.*, 2012). As part of the phage lytic cycle, canonical holins are known to accumulate until, at an allele specific time point, they form an oligomeric pore in the inner membrane. The pore is often of considerable size: λ-S105 forms one single pore per host cell, with an average pore diameter of 340 nm (but these pores can be as large as 1 μm), and positions itself randomly in the host membrane (Dewey *et al.*, 2010). The holin pore permits the passage of the fully folded endolysin (canonical R protein), which degrades the cell wall and, in conjunction with spanins that join the inner and outer membrane, results in catastrophic cell lysis (Wang *et al.*, 2000; Berry *et al.*, 2008). Another key feature of the canonical bacteriophage lytic cycle is the production of the S107 antiholin, which functions as a negative regulator of the S105 holin (Young, 2002). The S gene consists of 107 codons, the mRNA transcript contains a stem-loop structure that partitions two translation initiation codons designated Met1 and Met3, resulting in translation two separate products: S107 and S105 (Wang *et al.*, 2000). Indeed, a key distinguishing feature of holins is that they encode two translation initiation codons and hence the anti-holin/holin are considered together as integral features (Barenboim *et al.*, 1999). The S107 anti-holin is completely inactive and does not form a pore. Instead, it binds to the S105 holin and so maintains it too in an inactive form. It is thought that anti-holins therefore serve a role in 'fine-tuning' the lysis event: for example, in lambdoid phages the anti-holin serves to slightly delay lysis (Barenboim *et al.*, 1999); whereas in other systems, such as P1, the absence of the antiholin can result in premature cell lysis before the phage virion assembly has taken place, and hence for these systems anti-holin function is essential (Walker and Walker, 1980; Young, 2002).

In contrast to the canonical mechanism exhibited by λ-S105 and λ-R, the alternative mechanism involves the accumulation of pinholins that, upon allele specific 'triggering', form heptameric foci, or rafts, in the inner membrane and generate pores that are just 2 nm in diameter and so are too small to accommodate the passage of folded proteins (Park *et al.*, 2007; Pang *et al.*, 2009). The pinholin triggering event depolarises the inner membrane and induces the periplasmic, Sec-dependent, SAR endolysin to fold at which point it degrades the peptidoglycan and induces lysis (Pang *et al.*, 2013).

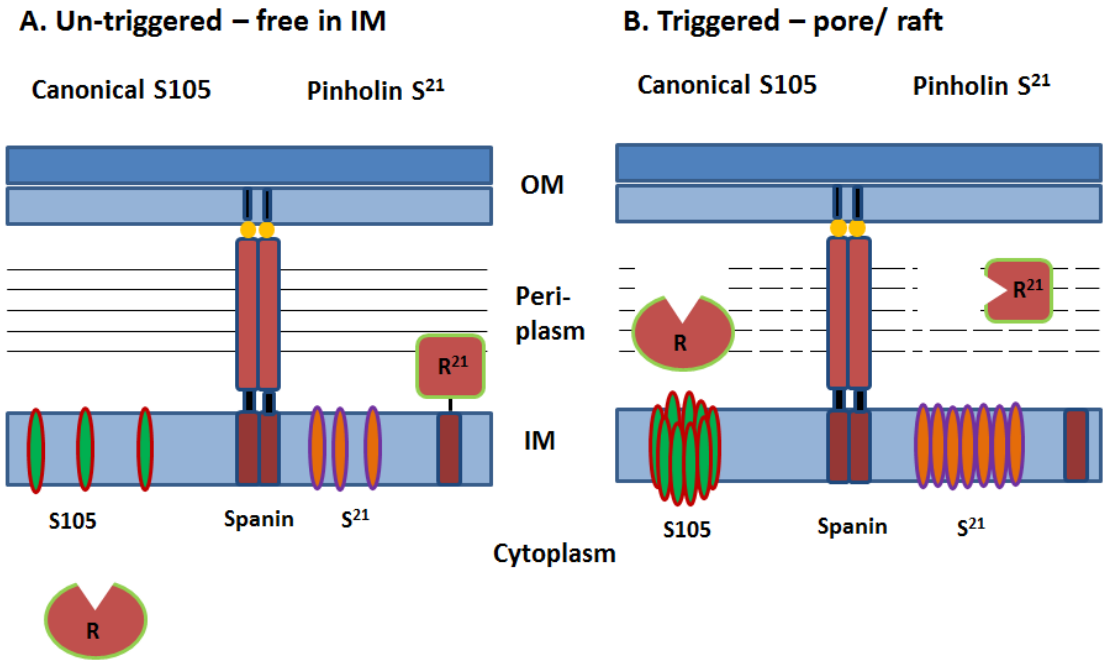


Figure 3.13 Two general phage lysis mechanisms exemplified by canonical λ-S105/R and phage 21 S^{21}/R^{21}. Figure adapted from (Berry *et al.*, 2012). Both canonical holin (S105) and pinholin (S^{21}) accumulate harmlessly in the IM, until an allele specific triggering permits the endolysin R/R^{21} to attack the peptidoglycan. Once triggered, the canonical holin forms an oligomeric pore that permits the endolysin to pass through, whereas the pinholin forms heptameric foci, or rafts, containing 2nm pores too small to accommodate folded proteins. The pinholin raft disrupts the energy gradient of the IM and releases the Sec-dependent (previously membrane tethered) endolysin, which refolds into its muralytic conformation and degrades the peptidoglycan. The spanins have an essential role in disrupting the OM. IM 'inner membrane', OM 'outer membrane'.

If ChiW were to function like a canonical phage holin, it would be involved in catalysing cell lysis. However, we have already identified several features of this system that are incompatible with this idea. First, various cytoplasmic protein controls have been used in these experiments, such as MBP, RNAP and GS, that were not detected in the extracellular supernatant. In addition, the ChiW system appears to be highly *selective*: of the 497 proteins identified in the *S. marcescens* Db10 secretome, only a subset of ten proteins, including the chitinolytic machinery, were shown to be significantly diminished in a *chiW* deletion strain (TABLE 3.1 AND FIGURE 3.4). There are also some notable differences between the phage S105 encoding gene and *chiW*. For example, the lambda S holin has two translation initiation codons that result in active lambda S105 and anti-holin lambda S107 forms. However, unlike most holins encoded by phage or prophage, the *S. marcescens* ChiW protein has a single translation start (FIGURE 3.11) and therefore probably has no 'anti-holin' activity associated with it. Indeed, we propose that this may point to a different mechanism for ChiW, involved in mediating protein secretion, compared to cell lysis induced by canonical lambda holin S.

3.4.3 The roles of prophage and their holins in protein externalisation

The term 'bacteriophage' (a composite of 'bacteria', and the Greek *phagein* 'devour') denotes viruses that inject their genetic material and replicate within bacteria: their presence is vast and, according to some estimates, they account for 90% of phages discussed in published research (Krupovic *et al.*, 2011). A distinguishing feature of some bacteriophages, such as phage λ, is their capacity to integrate their DNA onto the chromosome of their respective host, and to subsequently lie dormant until environmental signals trigger the lytic cycle and release of phage progeny (Krupovic *et al.*, 2011). This quiescent or temperate stage of the phage life cycle, where the phage coexists with the host, is referred to as a 'prophage'. Not all prophages are integrated onto the bacterial chromosome, some, such as *E. coli* phage P1 exist as extrachromosomal circular plasmids, or even, in the case of N15, as a linear plasmid (Krupovic *et al.*, 2011). The integration of novel genetic components by phage is considered a major driving force in evolution and this is sometimes, together with transposon and plasmid-mediated evolution, collectively referred to as the 'mobilome' (Canchaya *et al.*, 2004). Indeed, prophage genes, or remnants of prophage genes, have been identified in most bacterial genomes, and can in some cases account for as much as 10-20% of the genetic material (Casjens, 2003).

Unsurprisingly, there are examples in the literature of prophage encoded holin/ endolysin-mediated release of proteins. For example the spirochete *Borrelia burgdorferi*, which is the causative agent of Lyme disease, has been shown to encode a prophage holin/ endolysin system BlyA/BlyB that mediates release of the SheA protein (Damman *et al.*, 2000). Similarly, the enteric *Xenorhabdus nematophila* genome contains a 5.7 kbp prophage locus that includes a holin encoding gene that causes a SheA mediated haemolytic phenotype when expressed in *E. coli*, the functional holin was also shown to complement a λ-S mutant in mediating host cell lysis (Brillard *et al.*, 2003). There are other examples of prophage encoded holin-mediated lysis, such as the prophage NgoΦ1 element of *Neisseria gonorrhoeae*, which encodes a holin that performs the same lytic function as λ-S holin when expressed in *E. coli* (Piekarowicz *et al.*, 2007).

Bioinformatic analysis of the lambdoid prophage DLP12 identified a putative holin encoding gene *essD* and endolysin *ybcS*, that was shown to function as a two-component lysis system akin to phages p21 and P1: like a canonical holin, *essD* was shown to encode two initiation sites that produced both holin and antiholin (Srividhya and Krishnaswamy, 2007). The endolysin, encoded by *ybcS*, was a SAR endolysin and exhibited lytic activity when expressed in *E. coli* and *Salmonella typhi* (Srividhya and

Krishnaswamy, 2007). Similar prophage encoded holin/ endolysin activities have also been described in *Listeria monocytogenes* (Zink *et al.*, 1995). In the case of prophage DLP12 encoded holin, the important thing to note is that it encodes two initiation codons for holin and anti-holin activities (Srividhya and Krishnaswamy, 2007), and that (to our knowledge) all of the prophage encoded holins described in the literature have mediated lysis, none appear to be involved in facilitating protein secretion.

Based on its amino acid sequence, the *chiW* holin exhibits some similarity to prophage-encoded holins in *Klebsiella pneumoniae* (Fouts *et al.*, 2008) with 34% identity and 50% similarity (FIGURE 3.1), and *Xenorhabdus bovienii* (Chaston *et al.*, 2011) with 32% identity and 47% similarity (FIGURE 3.14). However, in contrast to *S. marcescens* ChiW, both of these prophage-encoded holins are regarded as homologues of λ-S in the literature. They also both harbour two initiation codons for holin/ anti-holin proteins, characteristic of canonical holin function (Fouts *et al.*, 2008; Chaston *et al.*, 2011). Both *Klebsiella pneumoniae* and *Xenorhabdus bovienii* produce at least one chitinase (belonging to family 18 glycosyl hydrolases) – the *X. bovienii* chitinase exhibits 32% sequence identity to *S. marcescens* ChiA.

It is important to note that the *S. marcescens chiWXYZ* operon is not framed within a prophage in the bacterial chromosome. There are no other phage-related genes of any kind located adjacent or near the *chiWXYZ* operon. Instead, ChiW is an example of a holin encoded by a bacterial genome.

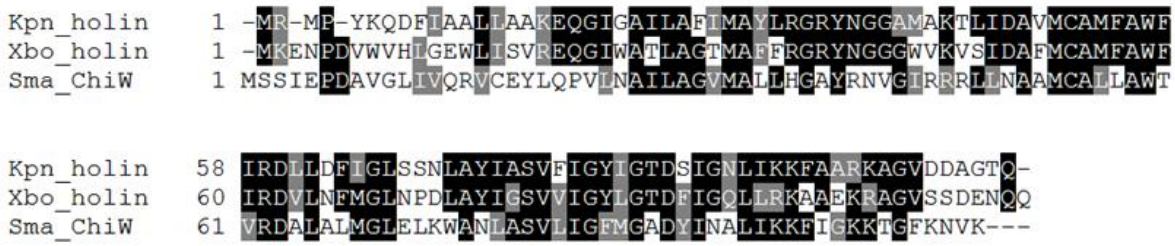


Figure 3.14 The *S. marcescens* ChiW holin exhibits similarity to prophage-encoded holins from Gram negative enteric bacteria. An alignment between *S. marcescens* holin (Sma_ChiW) and a prophage-encoded holins produced by *Klebsiella pneumoniae* 342 (Kpn_holin) (Fouts *et al.*, 2008), and *Xenorhabdus bovienii* SS-2004 (Xbo_holin) (Chaston *et al.*, 2011).

3.4.4 Analysis of structure and function of ChiX

In addition to the holin ChiW, the predicted L-alanyl-D-glutamate endopeptidase, ChiX, was shown to be an essential component in the chitinolytic system of *S. marcescens* (FIGURE 3.15).

ChiX exhibits similarity to a putative endopeptidase produced by the Gram negative pathogen *Yersinia pestis*, with 45% identity and 64% similarity. If restricted to proteins that appear in published work, the top hits for ChiX homologues include a putative phagelysin produced by *Y. enterocolitica* strain 8081 (Thomson *et al.*, 2006), which shows good similarity (61% identity, 74% similarity, and 94% sequence coverage), as well as a phage-related P7-like protein produced by *Y. pseudotuberculosis* strain 32953 (Rosso *et al.*, 2008), which has 61% identity and 75% similarity (91% coverage). Unsurprisingly, ChiX also has features similar to an endolysin produced by *Yersinia* phage PY100 (Schwudke *et al.*, 2008), which has 53% identity and 74% similarity (94% coverage) (FIGURE 3.15).

In the *Yersinia* phage, the endolysin serves as part of the lytic cycle (Schwudke *et al.*, 2008); whereas the endopeptidases produced by the *Yersinia spp.* have not been studied in isolation, but as one candidate in the genomic investigation of systemic infections caused by *Y. enterocolitica* and *Y. pseudotuberculosis* in immunocompromised individuals (Thomson *et al.*, 2006; Rosso *et al.*, 2008).

The predicted structure of ChiX, generated using the Phyre^2 on-line tool (Kelley and Sternberg, 2009), shows greatest similarity to Ply500, produced by bacteriophage A500 of the Gram positive bacterium *Listeria monocytogenes* (Korndorfer *et al.*, 2008). Ply500 is a peptidoglycan degrading endopeptidase, with predicted structural similarities to peptidases such as the D-alanyl-D-alanine dipeptidase VanX, which is involved in vancomycin resistance in *Enterococcus faecium* (Bussiere *et al.*, 1998). The ChiX protein also has predicted structural similarity with the peptidoglycan-degrading murein endopeptidase MepA from *Escherichia coli* (Marcyjaniak *et al*., 2004), and the L,D-endopeptidase CwlK produced by *Bacillus subtilis* (Fukushima *et al.*, 2007), which are members of the LAS family (Korndorfer *et al.*, 2008).

The LAS family are identified by a Zn^{2+} ion containing active site, and a highly conserved arrangement of coordinating residues, and side-chains, surrounding the active site (Korndorfer *et al.*, 2008). The L,D-endopeptidase, CwlK, produced by *B. subtilis* was the first bacterial, chromosomally encoded, peptidoglycan degrading member of the LAS family to be characterised (Fukushima *et al.*, 2007). The function of CwlK (and the predicted function of ChiX) is to cleave the base of the peptidoglycan chain containing L-alanine-D-glutamic acid (FIGURE 3.16), although the purpose this serves in *B. subtilis* remains to be tested. CwlK encodes an additional, putative, VanY D,D-carboxypeptidase motif, but is

unable to hydrolyse the D-alanine-D-alanine link (Fukushima *et al.*, 2007). CwlK is localised in the *B. subtilis* membrane and is hypothesised to be a lipoprotein, unlike the phage encoded Ply500, which is holin-dependent for export to the periplasm (Loessner *et al.*, 1995; Fukushima *et al.*, 2007). As a lipoprotein, CwlK is Sec-dependent for export, whereas *S. marcescens* ChiX does not encode any recognisable signal sequence. In this sense it is reasonable to hypothesise that the mechanism of CwlK export might be more akin to that of SAR-endolysins (such as R^{21}), since these are Sec-dependent, membrane-tethered peptidases.

The *E. coli* MepA is thought to have a role in both the removal and integration of murein in the sacculus and is the only LAS protein from the Enterobacteriaceae to have had an X-ray crystal structure solved (Goodell and Schwarz, 1983; Marcyjaniak *et al.*, 2004). Like other LAS enzymes, substitution of the Zn^{2+} coordinating residues results in a loss of its function as a D,D-carboxypeptidase (Marcyjaniak *et al.*, 2004). The fold of the MepA active site has most similarity to the N-terminal domain of sonic hedgehog (Shh-N) from *M. musculus* (Marcyjaniak *et al.*, 2004). The role of Shh-N is perhaps the most novel of all the known LAS family members: in the vertebrate embryos, sonic hedgehog plays a vital role in the induction of ventral cell types, the N-terminal (LAS) domain is responsible for signalling activities, whereas the C-terminal domain exhibits autoprocessing activities (Hall *et al.*, 1995). The Phyre^2 analysis also revealed structural similarities between ChiX and the N-terminal domain of sonic hedgehog from *Mus musculus* (ShhN), PDB entry 1VHH (Hall *et al.*, 1995).

Another LAS family member, VanX, produced by the Gram positive, antibiotic recalcitrant *Enterococcus faecium* (Gin and Zhanel, 1996) plays a crucial role in mediating resistance to vancomycin (Bussiere *et al.*, 1998). VanX is a cytoplasmic D-alanyl-D-alanine dipeptidase that clears intracellular D-alanyl-D-alanine, which increases the endogenous pool of D-alanine that is subsequently converted to D-alanine-D-lactate by VanA and is left to accumulate in the cell, since VanX exhibits strict specificity and does not cleave D-ala-D-lac (Walsh, 1993; Bussiere *et al.*, 1998). The increased production of D-ala-D-lac, as opposed to D-ala-D-ala, is crucial in mediating clinical vancomycin resistance: since vancomycin interacts with the terminal D-ala-D-ala residue of the peptidoglycan chain, by substituting this for a D-ala-D-lac moiety abolishes hydrogen bonding, and hence the affinity, between vancomycin and peptidoglycan, resulting in 1000-fold enhanced resistance to vancomycin (Nieto and Perkins, 1971; Liu *et al.*, 1994; Bussiere *et al.*, 1998). Such examples highlight the functional diversity of LAS family enzymes, and indicates that although structurally ChiX is very likely to possess a LAS-type Zn^{2+} coordinated active site, this does not give immediate insight into its role in the *S. marcescens* chitinolytic system, whether it facilitates assembly of a trans-envelope secretion system or simply facilitates the passage of chitinase remains to be tested.

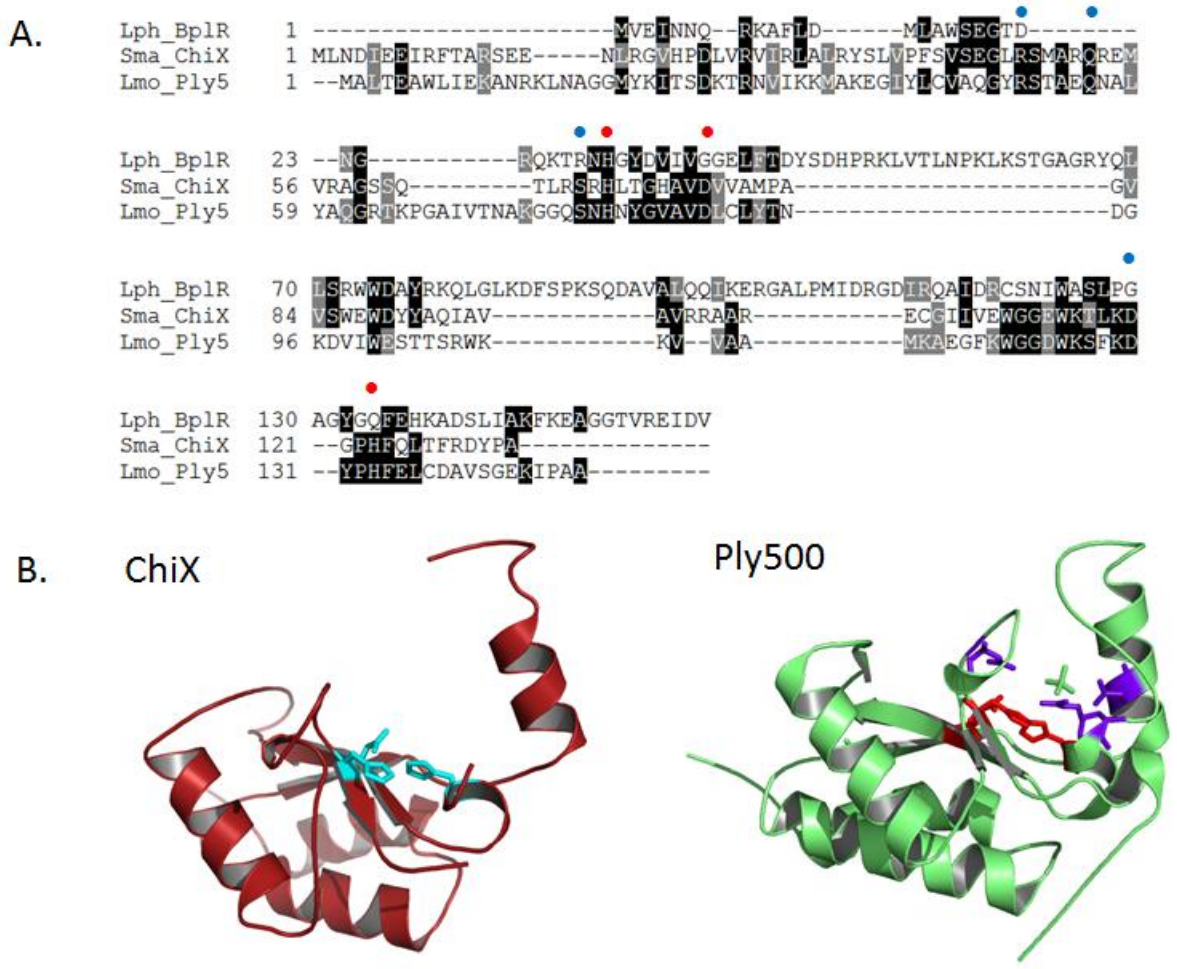


Figure 3.15 The endopeptidase ChiX has predicted structural similarity to the cell wall degrading endolysin from bacteriophage A500 of *Listeria monocytogenes*, Ply500. The amino acid sequence of the L-alanyl-D-glutamate endopeptidase ChiX (*SMA2873*) contains 133 amino acid residues and has a predicted molecular mass of 15,183 Da. (A) An alignment with Ply500 from *L. monocytogenes* bacteriophage 500 sequence, 'Lmo_Ply5' (PDB entry 2vo9). For comparison, the bacteriophage lambda R lytic transglycosylase (BplR) is also included, although this protein has only limited identity with ChiX. Zinc binding residues confirmed in the Ply500 crystal structure (His80, Asp87 and His133) are shown by the red circles. Other Ply500 active site residues (Arg50, Gln55, Ser78 and Asp130) are shown by the blue circles. Sequence alignments were performed with Clustal W2 (Larkin *et al.*, 2007), and displayed using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). (B) Phyre2 (Kelley and Sternberg, 2009) structural model of ChiX. Predicted zinc-binding side-chains are coloured cyan. The structural model of Ply500 is included for comparison, zinc-binding side-chains are coloured red and active site residues are coloured purple.

There is no published work investigating the links between a muralytic endopeptidase and chitin metabolism, there is recent work investigating the role of a muramidase, TtsA, in another enteric, *Salmonella enterica* Typhi, which has been shown to play an essential role in typhoid toxin secretion (Hodak and Galan, 2013). These authors identified a conserved N-terminal 'EGGY' motif, and

mutation of the key glutamic acid TtsA^{E14A} was found to disrupt its role in mediating secretion of typhoid toxin (Hodak and Galan, 2013). Since *Salmonella* Typhi is an exclusively human pathogen, and given that typhoid fever is a devastating systemic disease, this work provides important evidence that muramidase-mediated protein secretion is medically relevant (Hodak and Galan, 2013).

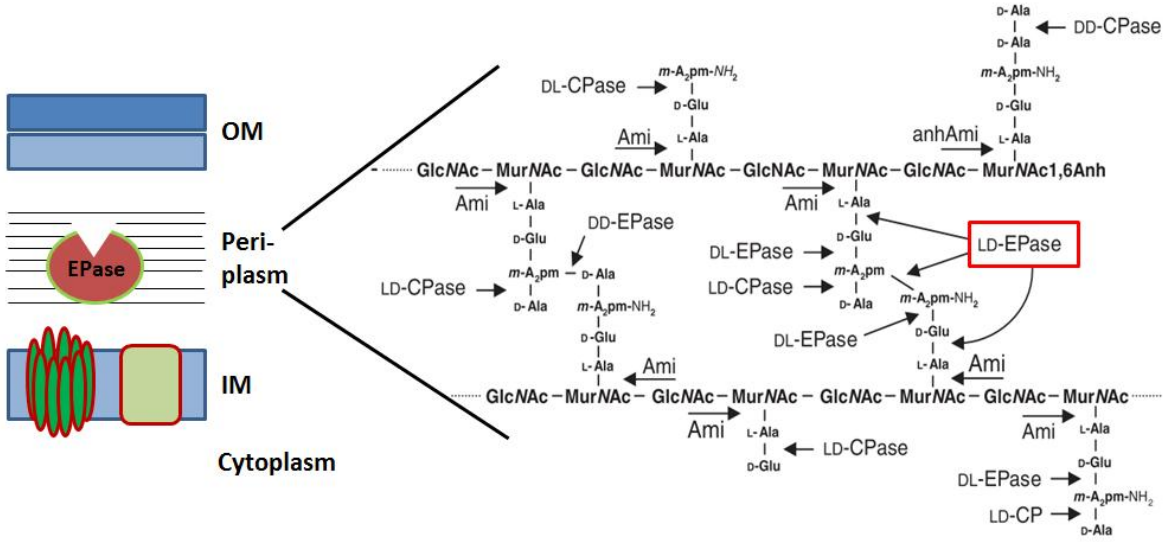


Figure 3.16 ChiX is a predicted L-alanyl-D-glutamate endopeptidase (LD-EPase). The peptidoglycan structure shown is from *E. coli* or *B. subtilis*, depicting the hydrolysis of amide and peptide bonds in peptidoglycan by endopeptidases (EPases) and carboxypeptidases (CPases) respectively. Endopeptidases (DD-EPase, LD-EPase, DL-EPase) such as Ply500 and CwlK cleave amide bonds in the peptides, whereas carboxypeptidases (DD-CPase, LD-CPase, DL-CPase) are known to cleave peptide bonds that remove C-terminal D-or L- amino acids (Vollmer *et al.*, 2008). Adapted from (Vollmer *et al.*, 2008).

Endopeptidases are known to be involved in a diverse array of cellular functions: from cell division, to peptidoglycan turnover during cell growth, to some specialised hydrolases that form pores for the assembly of large complexes that span the entire cell envelope (Vollmer, 2008). In *E. coli*, the stretched pores in the peptidoglycan can permit the passage of proteins 50-100 kDa, but not the assembly of trans-envelope structures such as Type 2, Type 3, Type 4 secretion systems, type IV pili or flagella (Vollmer *et al.*, 2008). Specialised peptidoglycan hydrolysing enzymes are often involved in the assembly of large protein structures, for example *E. coli* FlgJ has been shown to generate a localised space in the peptidoglycan layer for assembly of the flagella basal body (Nambu *et al.*, 1999). Other examples of such specialised lytic transglycosylases include VirB1 of the plant pathogen *Agrobacterium tumefaciens* that aids the assembly of the Type 4 secretion machinery (Zahrl *et al.*, 2005), and also *E. coli* PilT, which facilitates the assembly of type IV pili (Koraimann, 2003).

3.4.5 The roles of the spanins, ChiY and ChiZ

As part of the lambda phage lytic cycle the recently identified spanins, Rz and Rz1, are thought to comprise an integral inner membrane protein and an outer membrane lipoprotein, repectively. The lambda spanins have been shown to interact and contribute to the disruption of the outer membrane during host cell lysis (Berry *et al.*, 2012). Although the *S. marcescens* ChiY/ChiZ spanin-like components do not appear to form essential components of the chitinolytic secretion system (FIGURE 3.9), we cannot rule out the possibility they have subtle roles in protein externalisation.

3.4.6 A putative two-step mechanism for ChiWX-mediated secretion

Secretion systems are broadly characterised according to whether they move the substrate to the extracellular milieu *via* a one-step or a two-step mechanism. The fractionation work presented here provides sound evidence that ChiWX mediated secretion translocates chitinolytic enzymes across the inner and outer membranes separately, as part of a two-step process (FIGURE 3.10). Western analysis revealed that both ChiA and ChiC are present in the periplasm in *chiW* and *chiX* mutants, which indicates that ChiWX are both crucial for the movement of the chitinolytic set across the outer membrane (FIGURE 3.10). In support of this, the proteomic analysis confirmed that the Sec signal peptide of the Sec-targeted ChiA and Cbp21 was subject to N-terminal processing – clearly these substrates do not require ChiWX to cross the inner membrane. A further reason supporting a two-step secretion process is that ChiA, ChiB and Cbp21 contain disulfides, and the formation of disulphide bonds is catalysed in the periplasm by the Dsb system (Kadokura *et al.*, 2003). Disulfide bond formation has already been shown to be essential for proper export and folding of the *S. marcescens* nuclease NucA (Ball *et al.*, 1992).

Previous work investigating the role of ChiC in *P. aeruginosa* hypothesised that this enzyme also undergoes an unusual form of N-terminal processing (Folders *et al.*, 2001). Although ChiC in *P. aeruginosa* and *S. marcescens* are very similar, our proteomic analysis did not detect any processing of ChiC since the secreted form was shown to be intact, as was the secreted form of ChiB (FIGURE 3.7). The mode of ChiC export to the periplasm is yet to be clarified, but it is clearly not mediated by ChiW or ChiX. It could be the case that ChiC is an unusual Sec-dependent enzyme. Recent work has revealed that the SodA dismutase produced by *Rhizobium leguminosarum* is a Sec-dependent protein but is devoid of any apparent signal peptide (Krehenbrink *et al.*, 2011). Surprisingly, the ChiB protein was not detectable in the periplasm of either *chiW* or *chiX* strains: it is likely that ChiB is rapidly degraded when mislocalised to the periplasm (FIGURE 3.10). A possible way to establish whether ChiC is exported *via* Sec would be to attempt to inhibit SecA with sodium azide and assess whether export of freshly synthesised ChiC is affected.

The hypothesis that ChiW and ChiX act in conjunction to facilitate secretion of the *S. marcescens* chitinolytic proteins across the outer membrane is both compelling and perplexing. ChiW is predicted to reside in the inner membrane according to TMHMM prediction, although this remains to be proven experimentally. It seems there are three possible mechanisms that might account for the data presented here:

1. ChiW is localised in the IM; ChiX is exported *via* ChiW or Sec to the periplasm; the chitinolytic enzymes are targeted to the periplasm *via* Sec and specifically moved across the outer membrane *via* other proteins yet to be discovered (FIGURE 3.17).

This scheme (1) suggests that ChiW is embedded in the inner membrane (as TMHMM predicts), facilitates the passage of ChiX to the periplasm, and operates in partnership with other outer membrane-associated proteins to accomplish chitinase secretion. This scheme suggests a *selective* translocation - suggesting some sort of substrate recognition event - of the chitinolytic machinery across the outer membrane by a *bona fide* secretion system FIGURE 3.17.

2. ChiW is localised in the IM; ChiX is exported *via* ChiW or Sec *and* induces general periplasmic leakage thus releasing the chitinolytic substrates.

This scheme (2) also suggests that ChiW facilitates the export of ChiX to the periplasm, but that this activity either forms a localised pore in the outer membrane, or ruptures it in some way leading to non-specific release of substrate from the periplasm. This is not a secretion system but a release mechanism.

3. ChiW has an unexpected structure that allows it to interact either directly, or indirectly, with the outer membrane; ChiX is exported via Sec, ChiW facilitates movement of substrates across the OM.

This scheme (3) is the most counter intuitive and suggests that ChiW is at least partly localised in, and facilitates secretion of substrate across, the outer membrane. A secretion mechanism involving a holin-like protein forming a pore in the outer membrane would be unprecedented and nothing like holin-mediated transport across the outer membrane has been described in bacteria. There are, however, other holin-like proteins that are known to interact with the equivalent of the outer membrane in other biological systems. For example, in some eukaryotic systems it has been shown that Bax (Bcl-2 family proteins) is a functional holin (when expressed in *E. coli*) and that the protein triggers mitochondria-mediated apoptosis (Pang *et al.*, 2011; Westphal *et al.*, 2011). Prior to apoptosis, the Bax protein, which is normally present in the cytoplasm in globular form, undergoes a conformational change that exposes the transmembrane domains that targets it to form an oligomeric pore in the mitochondrial outer membrane (Westphal *et al.*, 2011). This event, known as a mitochondrial outer membrane permeabilisation (MOMP) event, causes the release of proteins from the mitochondrial intermembrane space, the resulting caspase cascade induces apoptosis (Chipuk and Green, 2008). Bax has also been shown to act as a holin in a heterologous bacterial system (Pang *et al.*, 2011). While this certainly highlights a general functional similarity between Bax and the λ-S105 holin, the data presented in this chitinase secretion study might suggest an entirely novel

mechanism: it can be hypothesised that the ChiW holin-like protein facilitates a bacterial outer membrane secretion mechanism akin to Bax-induced MOMP.

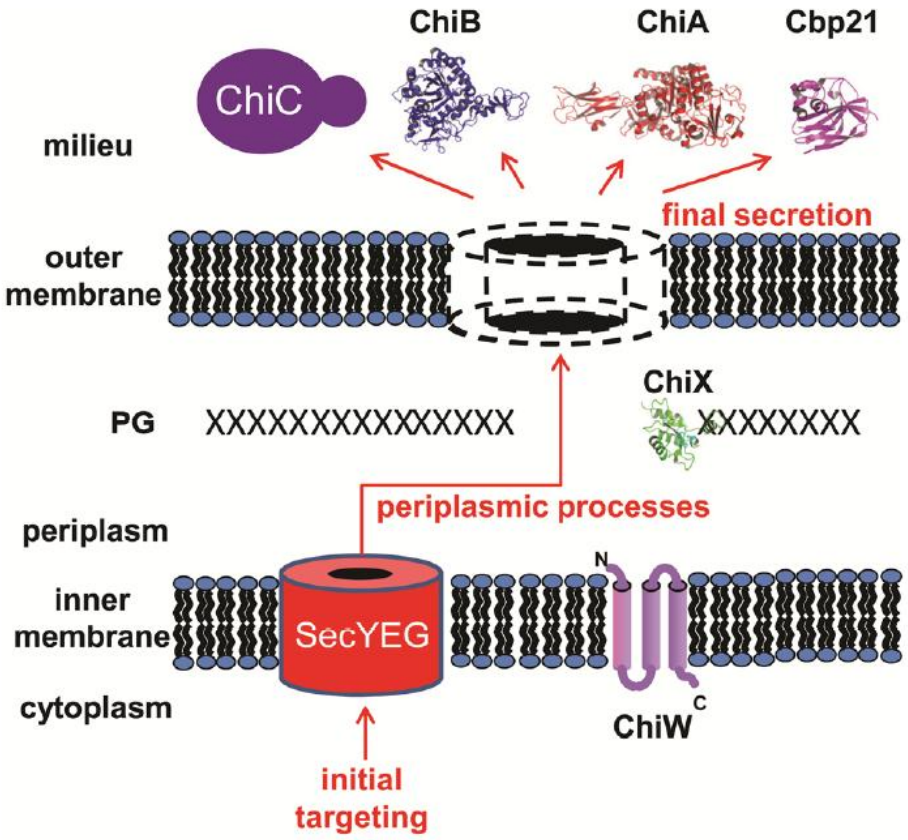


Figure 3.17 A two-step model for chitinase secretion: ChiW and ChiX facilitate movement of substrate across the outer membrane. The ChiA and Cbp21 proteins are initially targeted to the periplasm by the Sec machinery. ChiC is also initially targeted to the periplasm, although the route taken is yet to be proven. Once in the periplasm the secretion of the chitinolytic enzymes across the outer membrane is controlled by the ChiW and ChiX proteins, possibly *via* controlled permeabilisation of the outer membrane that allows specific secretion of the enzymes. There may be other, as yet unidentified, components of this secretion system involved.

3.4.7 Further characterisation of the ChiWX-dependent secretion system

For this work to progress, there are at least two very important questions that need to be answered: where in the cell envelope is ChiW and ChiX localised; and what (if anything) do they associate with? The first question can be answered by integrating N or C terminal HA-tags on to both ChiW and ChiX and then detecting their localisation by Western blot against the HA-epitope. This will provide a quick and strong indication. In the long run it would be best to generate ChiW and ChiX antisera, which would provide the same evidence and may also enable the identification of unknown binding partners that may also be involved in this mechanism. Producing ChiW antisera might prove problematic because the expression of a *bona fide* holin in a heterologous host might induce lysis. Perhaps one way to overcome this might be to overexpress a truncated version of ChiW. On going research in Dundee is focussed on identifying whether ChiX is exported to the periplasm *via* ChiW, in which case this would also provide evidence that ChiW is localised in the inner membrane.
It is also necessary to identify any potential binding partners that might associate with ChiW or ChiX to form part of a larger secretion apparatus. One approach would be to try a co-immunoprecipitation (Co-IP) experiment, whereby antibody is immobilised on agarose beads and then incubated with *S. marcescens* detergent-dispersed membrane fractions before being separated by centrifugation, washed, and analysed by SDS-PAGE. Depending on the scale of the experiment, it may be possible to submit prominent protein bands for tryptic peptide mass fingerprinting. Possible interactions could then be verified using bacterial two-hybrid experiments, before moving on to work out whether these newly identified components are essential for chitinase secretion.

It may also be valuable to visualise any ChiW-dependent pore-formation using microscopy techniques. The strains encoding an integrated ChiW or ChiX HA-tag could be exploited for cryogenic electron microscopy (cryo-EM) that would establish whether ChiW forms a single pore in the membrane, as is the case for λ-holin (Dewey *et al.*, 2010), and would also determine the average pore size. In addition to this, it would be valuable to assess ChiW pore formation in real time: designing ChiW-fluorescent protein (GFP, mCherry, mKate) chimeric constructs would make it possible to track holin expression/ accumulation throughout the *S. marcescens* growth phase and to determine the precise moment of pore-formation. Of course it will be important to assess whether the chimeric fusion disrupts ChiW function by assessing the extracellular presence of chitinase with Western blotting. If this were done in tandem with chitinase-fluorescent chimeric fusions it would be possible model pore formation in conjunction with chitinase release.

3.4.8 Non-classical protein secretion and the question of holin-mediated secretion

There is currently much interest in the question of non-classical secretion: that is secreted substrates that do not encode any clear targeting signals, and also whether any cases of non-classical secretion can be accounted for by a holin-dependent system (Desvaux, 2012). Work done on chitinase ChiC in *Pseudomonas aeruginosa* was first to address the question of non-classically secreted chitinase, and identified that it lacked a typical N-terminal signal sequence, and also that the first 11 residues of the N-terminus is cleaved off in the secreted protein (Folders *et al.*, 2001). These authors showed that expression of *Pseudomonas* ChiC is regulated by quorum sensing and that it is secreted gradually over a four day time course and was not attributable to cell lysis (Folders *et al.*, 2001).

The question of non-classically secreted proteins is still pressing even in 2011. It has been shown that heterologous expression of the carboxyl-esterase Est55 is secreted in late stationary phase in *Bacillus subtilis* despite the absence of a classic cleavable N-terminal signal sequence (Yang *et al.*, 2011). This work also identified a hydrophobic α-helical domain that contributes to the secretion of enolase, and they controlled against the question of lysis using cell density, comparing relative levels of EF-Tu and SecA, and also by including autolysin deficient mutants (Yang *et al.*, 2011). From this they concluded that large amounts of cytoplasmic substrates are secreted by an unknown non-classical mechanism, and is not attributable to lysis or membrane vesicles or the previously identified ESAT-6 system (Yang *et al.*, 2011). However, like the 2001 *Pseudomonas* paper (Folders *et al.*, 2001), they did not identify the secretion system responsible.

Recently there has been a lot of attention gathering around the idea that much non-classical secretion is holin-mediated (Tjalsma *et al.*, 2004). There are lots of details in favour of this: phage related genes are ubiquitous, and according to some studies there is a gap between exoproteins that are found within a 'secretion profile' in experimental work, and secreted proteins that are recognized by genome prediction software – so holins are currently in the lime-light to address this discrepancy (Tjalsma *et al.*, 2004). Up to now there has been some evidence that holins have a role in protein secretion, but this turned out to be self-contradictory. It was hypothesised that the holin TcdE was responsible for the secretion of toxins TcdAB in *Clostridium difficile* (Govind and Dupuy, 2012), but subsequent deletion of the *tcdE* gene was shown to have no significant effect on the secretion of TcdAB or the overall secretion profile (Olling *et al.*, 2012). Besides this, the *S. marcescens* NucE protein (*SMA0177*) was suggested to be a holin required for the secretion of the Sec-dependent NucA nuclease (Berkmen *et al.*, 1997). However, similar to the *C. difficile* story above, deletion of the *nucE* gene was found to have no effect on nuclease secretion (Strych *et al.*, 1999). Solid evidence of

holin-dependent protein secretion is yet to be published, but the holin hypothesis is still compelling, especially in light of the *Bacillus* Est55 work where cytoplasmic substrates are shown to accumulate in the exponential/ early stationary phase, and are then secreted in the late stationary phase (Yang *et al.*, 2011). In both *Vibrio harveyi* and *Pseudomonas aeruginosa* this type of late-stage accumulation and secretion of exoproteins is known to be brought about by the accumulation of auto-inducers, as part of a complex social-behaviour (Folders *et al.*, 2001; Defoirdt and Sorgeloos, 2012).

3.4.9 The role of ChiR

In this work we wanted to establish whether ChiR (encoded by *chiR SMA2876*) LysR type transcriptional regulator (LTTR) regulates expression of the *chiWXYZ* operon. In order to do this we used a basic RT-PCR to assess relative mRNA transcript levels in different Tn5-bearing strains: we used candidates TnchiB5 containing a Tn5 in *chiR*, TnchiC1 containing a Tn5 129 base pairs upstream of *chiR*, and TnchiC4 that contains a Tn5 in *chiW*. We hypothesised that ChiR would regulate the *chiWXYZ* genes involved in movement of chitinase to the extracellular supernatant, as opposed to the expression of chitinase encoding gene itself; for this reason we assessed the relative levels of *chiWX* transcript against the presence of *chiC* transcript. The result clearly shows that *chiWX* transcripts are dramatically reduced in strain TnchiB5 (FIGURE 3.12), which bears a Tn5 well within the *chiR* ORF. The level of *chiWX* transcript appears less markedly reduced in strain TnchiC1, about the same reduction as *chiC* transcript for this strain (FIGURE 3.12). This could suggest that ChiR affects both *chiWX* and *chiC* transcript levels, although the reason that *chiWX* transcript levels are less dramatically reduced for strain TnchiC1 might be attributable to the Tn5 not being embedded well within the *chiR* ORF (as it is with candidate TnchiB5). Instead it is located upstream of *chiR* in the *chiR-cbp21* intergenic region. Previous work has shown that ChiR binds to the *chiR-cbp21* intergenic region and not to the promoter regions of the chitinase encoding genes (Suzuki *et al.*, 2001). This suggests that in strain TnchiC1, ChiR binding to its own promoter and hence its function as an autoregulator is disrupted (all LTTRs are autoregulators). Since the *chiR* gene itself is not disrupted, the overall presence of ChiR in the cell is not completely lost, which gives an indication as to why the levels of *chiWX* transcripts are not so dramatically affected in TnchiC1 compared to TnchiB5 where *chiR* is completely disrupted.

Clearly this preliminary work is not sufficient to assess whether ChiR regulates *chiWXYZ* as opposed to chitinase encoding genes. To take this work further it is necessary to generate a clean Δ*chiR* strain, and to identify genes that are affected by a *chiR* deletion (microarrays). This will not only identify *chiWXYZ* vs. *chiC* regulation by ChiR, but should provide a global view that potentially reveals unidentified genes involved in the chitinolytic phenotype as well. Indeed this, in conjunction with a Δ*hfq* microarray, might give some important clues about the *S. marcescens* chitinolytic phenotype that results derived from the transposon screen may not have identified.

Instead of a basic RT-PCR, a more thorough, quantitative approach to this experiment would be a quantitative real time, qPCR. For this a Δ*chiR* strain would be necessary in order to quantify the presence of target genes in real time using fluorescent probes.

3.4.10 A specific set of ten proteins are secreted by a ChiW-dependent mechanism

In order to establish exactly what proteins are secreted in a ChiW-dependent manner, we used a whole systems approach to identify differences in the secretion profile of *S. marcescens* Db10 against a strain where the *chiW* gene had been deleted. Of the 497 proteins identified in the *S. marcescens* Db10 secretion profile, 351 proteins showed a high level of reproducibility, ten of which were shown to be diminished in a *chiW* strain (FIGURE 3.4 and FIGURE 3.5). The label free mass spec revealed the extracellular presence of the entire chitinolytic machinery is significantly affected by a *chiW* deletion. Cbp21 exhibited a 3.4 times reduction; ChiA was reduced 7 times, ChiC 14.7 times, while the presence of ChiB appeared to be most dramatically affected and was diminished 80.7 times (TABLE 3.1). This result was also confirmed by in-gel analysis of the same secretion profiles, whereby the samples were separated by SDS-PAGE and any prominent bands that appeared to be absent from the Δ*chiW* profile were identified from the *S. marcescens* Db10 profile using tryptic peptide mass fingerprinting – bands corresponding to the entire chitinolytic set, ChiA, ChiB, ChiC and Cbp21 were again shown to be lost in the Δ*chiW* profile (FIGURE 3.6). An interesting discrepancy between the two approaches is that the label-free MS revealed that ChiB was by far the most dramatically reduced in the Δ*chiW* profile (TABLE 3.1), whereas although it is clearly absent from the profile as it appears on PAGE analysis (FIGURE 3.6), it is ChiB that appears least affected.

Besides the chitinolytic machinery another six candidates were identified as potential substrates of ChiW-mediated secretion. These included a pirin metal-binding protein (encoded by *SMA3897*), anthranilate synthase TrpE (*SMA1933*), malate dehydrogenase MdhA (*SMA4522*), NADP-dependent malic enzyme MaeB (*SMA2870*), a putative haem oxygenase (*SMA2390*), and prolyl tRNA synthetase ProS (*SMA3147*). It is not immediately apparent if these proteins are credible candidates for secreted proteins. Indeed all but one was of low abundance and was just above the arbitrary 3 × difference cutoff that was imposed by us. It is notable that the gene encoding the malic enzyme MaeB (*SMA2870*) is located immediately downstream of *chiZ* (*SMA2871*), which may give an initial indication that its expression levels may have changed in the mutant strain investigated here.

In order to take this work further we would first need to determine whether these proteins are true secreted substrates of this pathway. Ideally, we would generate antisera specific to each protein for detection by Western immunoblotting, but this might not prove cost effective. For this reason we would start by integrating HA epitope tags to the native chromosomal loci and assessing subcellular localisation of the tagged proteins. Of course this approach will only be successful if protein secretion is not blocked by the addition of N- or C-terminal tags. Alternatively, the proteins could be radio-labelled and subjected to a pulse-chase experiment perhaps based on one first developed in *E. coli*

(Studier and Moffatt, 1986). These experiments will give a good indication of whether the individual proteins identified in this work are transported across the outer membrane by the ChiW secretion system. Once it is established that these proteins are true ChiW-dependent secreted substrates they can be individually characterised and understood in the wider context of both the *S. marcescens* chitinolytic machinery and its secreted proteome.

Malic enzymes, such as **MaeB** (82 kDa) in *S. marcescens*, are broadly separated into two groups depending on their associated cofactor: those that are NAD^+ or $NADP^+$ dependent. $NADP^+$ dependent malic enzymes, such as MaeB, have been isolated from both prokaryotes and eukaryotes (Takeo, 1969). $NADP^+$-dependent malic enzymes are involved in the formation of acetyl-coA (and therefore play an important role in the TCA cycle), and have also been shown to decarboxylate malate to produce CO_2 and pyruvate (Takeo, 1969). At present there is no work investigating the role of malic enzymes in *Serratia*: however in the symbiotic N_2-fixing *Sinorhizobium meliloti*, malic enzymes have been shown to be involved in the synthesis of acetyl-coA, as predicted (Driscoll and Finan, 1997). Interestingly, mammalian $NADP^+$-dependent malic enzyme has been shown to associate with pyruvate dehydrogenase complex and to be localised in the mitochondrial inner membrane (Teller *et al.*, 1992). Since mitochondrial malic enzyme is known to associate with the inner membrane, it would be interesting to establish whether *S. marcescens* MaeB is localised in the inner membrane: the production of HA-tagged MaeB will make it possible to determine this by Western blotting after separation of the membrane fractions by sucrose gradient. In addition to malic enzyme MaeB, we also identified a **malate dehydrogenase**, **MdhA**, whose extracellular presence was affected by *chiW* deletion. MdhA is a ubiquitous cellular enzyme that oxidises malate to pyruvate and CO_2. This is a 32 kDa protein with 93% sequence identity to *E. coli* malate dehydrogenase, for which there is a crystal structure that reveals a dimeric conformation, and shows significant homology to the mitochondrial enzyme (Hall *et al.*, 1992). MdhA does not have a signal peptide, even though there appears to be a short one at the N-terminus according to some bioinformatics analyses (Petersen *et al.*, 2011). If both of these proteins are the only malic enzyme and malate dehydrogenase produced by *Serratia*, it would seem very unlikely that they would be actively secreted from the cell.

The presence of 26 kDa **pirin** (*SMA3897*) was shown to be affected by the *chiW* deletion, indeed its presence was decreased 11.2 times in the *chiW* secretion profile, greater than ChiA (7 times reduction) (TABLE 3.1). The pirin superfamily of proteins are conserved between bacteria, fungi, plants and mammals. In bacteria they usually serve as transcriptional cofactors but, interestingly, in eukaryotes they have been shown to be involved in programmed cell death (Orzaez *et al.*, 2001). This is especially interesting if we consider that Bax has recently been identified as a holin-like protein involved in mitochondrial outer membrane permeabilisation (MOMP) (Pang *et al.*, 2011). Not many bacterial pirins have been properly characterised, however there has been work investigating its

function in *S. marcescens* CH-1 (Soo *et al.*, 2007). These authors identified a pyruvate dehydrogenase subunit E1 (PDH-E1) as being negatively regulated by *S. marcescens* pirin. Mutation of the pirin-encoding gene was shown to increase the PDH associated activities, such as ATP concentration, which increased 140-250% depending on the pirin gene mutation and shifted the NADH/NAD^+ ratio, which is a consequence of increased tricarboxylic acid (TCA) cycle activity (Soo *et al.*, 2007). These authors concluded that *S. marcescens* pirin has an important role in regulating the synthesis of acetyl-coA from pyruvate, which is mediated by PDH-E1, and hence pirin regulates a crucial pathway junction between the TCA-cycle and fermentative metabolism (Soo *et al.*, 2007). Thus there may be a connection between pirin, malic enzyme and malate dehydrogenase expression in *Serratia*. The crystal structure of the *E. coli* pirin YhhW has been resolved and the authors identified the antioxidant quercetin as a substrate of both human and *E. coli* pirin (Adams and Jia, 2005), before hypothesising that this may form a link to the role of pirins as transcriptional cofactors because the presence of quercetin has an inhibitory effect on DNA gyrase activity (Plaper *et al.*, 2003). The Phyre[2] predicted structure (Kelley and Sternberg, 2009) of *S. marcescens* pirin (*SMA3897*) suggests this enzyme is most similar to the RmlC-like cupins, a large superfamily of proteins with a conserved β-barrel fold and, in the majority of cases, an Fe-bound active site (FIGURE 3.18) (Dunwell *et al.*, 2004).

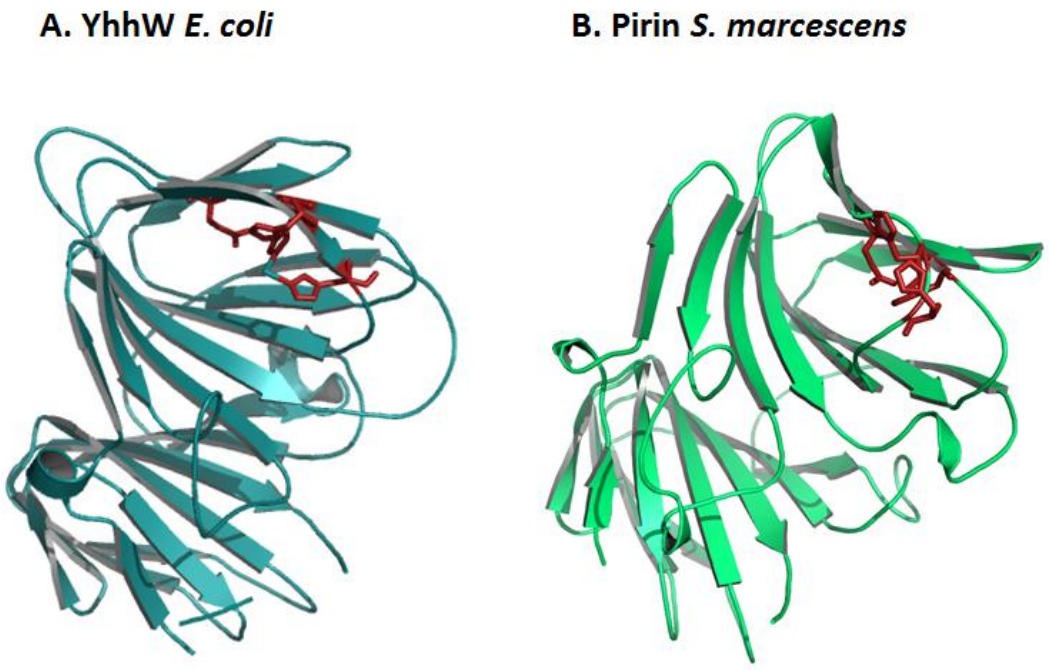


Figure 3.18 Structural prediction of *S. marcescens* pirin suggests it belongs to RmlC cupin superfamily. (A) The *S. marcescens* pirin is predicted to have the most structural similarity to *E. coli* YhhW, the archetypal RmlC protein (Adams and Jia, 2005). RmlC proteins have a characteristic bicupin fold and N-terminal metal binding site, in YhhW this is coordinated by residues His57, His59, His101 (that binds a cadmium ion) and Glu103 (together with the His residues forms the Fe binding site), these sites are represented as red sticks. (B) Predicted structure of *S. marcescens* pirin: RmlC proteins are similar between bacteria, plants and mammals – the same metal binding residues of YhhW are conserved in the *S. marcescens* pirin and are also shown as red sticks.

This work also identified the *S. marcescens* **anthranilate synthase** (AS) enzyme, **TrpE**, as being diminished in the secretome of the *chiW* deletion strain. The effect of *chiW* deletion on the presence of TrpE was shown to be greater (4.8 times) than the overall effect on the presence of chitin binding protein Cbp21 (3.4 times) (TABLE 3.1). *S. marcescens* AS is formed from a complex of two polypeptide chains TrpE2:TrpG2 - TrpG belongs to the glutamine amidotransferase family and produces chorismate from hydrolysis of glutamine, whereas the TrpE subunit produces anthranilate after binding chorismate (Zalkin, 1993; Tesmer *et al.*, 1996). The structure of *S. marcescens* anthranilate synthase has been resolved in a state with bound anthranilate to the TrpE subunit and another in the inhibitor- (tryptophan) bound state (Spraggon *et al.*, 2001). Tryptophan acts as a competitive inhibitor of chorismate and prevents the synthesis of anthranilate when bound to TrpE: from the structure these authors concluded that the inhibitor binds at a distinct site from that of the substrate and that, once bound, inactivates both TrpE subunits (Spraggon *et al.*, 2001). It is not immediately apparent why TrpE should be secreted from the cell: however, in addition to anthranilate it also produces pyruvate, as does the malic enzyme MaeB and the malate dehydrogenase MdhA. In addition to this MdhA and the pirin both have roles in the TCA cycle (MdhA in the synthesis of acetyl-coA and pirin in repressing pyruvate dehydrogenase).

The putative 25 kDa **haem oxygenase** (encoded by *SMA2390*) is predicted to be involved in haem degradation, and might possibly be a virulence factor since Fe metabolism and sequestering of Fe is a crucial factor in many important pathogens. In terms of amino acid prediction, this protein is predicted to belong to the TenA superfamily, which is a group of enzymes involved in transcriptional activation. The Phyre^2 predicted structure (Kelley and Sternberg, 2009) of *S. marcescens* haem oxygenase suggests it has structural similarities to TenA from *Helicobacter pylori* and *Bacillus subtilis*, and also to THI20 from Saccharomyces cerevisiae, which contains a TenA-like domain. The structure of TenA in Bacillus subtilis has been resolved (Toms et al., 2005) and reveals that TenA is a thiaminase that degrades thiamine, for which three active-site residues, Asp44, Cys135, and Glu205, where shown to be important for substrate binding and catalysis (Figure 3.19). Obviously the status of this enzyme as a *bona fide* secreted protein will need to be determined before properly characterising its function in *S. marcescens*.

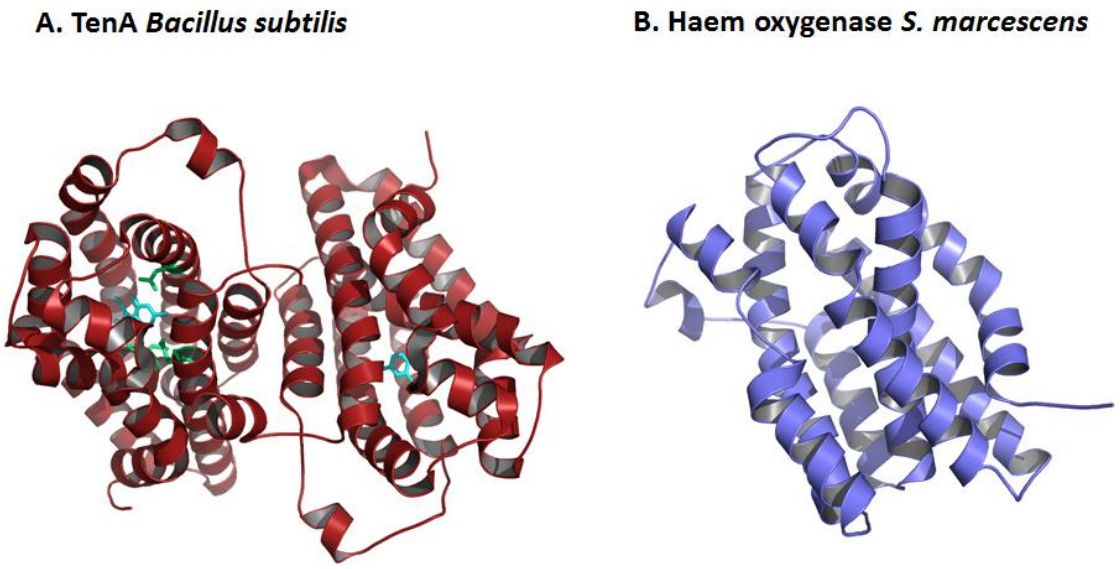


Figure 3.19 Structural prediction of *S. marcescens* haem oygenase suggests it is similar to *Bacillus subtilis* TenA. (A) The structure of *Bacillus subtilis* TenA (Jenkins *et al.*, 2008), active site residues involved in substrate binding and catalysis are shown in green and cyan as stick models . (B) The Phyre[2] predicted structure of *S. marcescens* haem oxygenase (Kelley and Sternberg, 2009).

The presence of a **proly-tRNA synthetase**, **ProS**, was also diminished in the *chiW* secretion profile: of the ten substrates identified as being significantly reduced, ProS was least affected by the *chiW* deletion (3 x reduction in relative abundance). There is no published work examining the role of the Prolyl tRNA synthetase ProS in *Serratia marcescens*; it is predicted to have structural similarity to prolyl-tRNA synthetase from *Enterococcus faecalis* (Crepin *et al*., 2006). It clearly is a putative ligase involved in translation and why it should be a substrate of ChiW-mediated secretion is not clear.

The label free mass spectrometry also identified nine substrates whose extracellular presence appears to be increased in a *chiW* deletion strain (TABLE 3.2). These were shown to be Type-1 fimbrins FimA (*SMA3915*), FimI (*SMA1250*), and SafA (*SMA1052*); 50S ribosomal proteins RplU (*SMA4526*) and RplI (*SMA4500*); a predicted fimbrial-like adhesion proteins, PmfE (*SMA3920*), and another encoded by *SMA0789*; a flagellar assembly protein FlgD (*SMA2217*) and the Type 6 secreted protein Hcp (*SMA2263*) (TABLE 3.2).

The most prominent feature of the set of proteins with increased abundance in Δ*chiW* is that most of these are fimbrial adhesins (FimA, FimI, SafA, PmfE, protein encoded by *SMA0789*). Of the remaining substrates FlgD and Hcp are associated with extracellular machineries (flagellar and Type 6 secretion system respectively), whereas RplU, RplI have clear intracellular functions and a strong reason for their extracellular presence is not immediately apparent.

Fimbrial proteins and fimbrial-like adhesion proteins have an important role in pathogenesis, particularly in facilitating adhesion/ binding, and have been linked to *S. marcescens* role in urinary

tract infections (Leranoz *et al.*, 1997), and also in wounds and ocular infections (especially in relation to contact lenses) (Parment *et al.*, 1992). In *E. coli*, fimbriation (for which FimA is a key structural component) is regulated in a bistable manner, i.e. is expressed in a subpopulation of cells within an isogenic population, and the phenotypic switching is known to be regulated by a temperature-dependent switching of the *fimS* locus encoding the promoter region of *fimA* and *fimH* (Kuwahara *et al.*, 2010). Similarly, the human pathogen *Vibrio cholera* is also known to express the *tcpA* gene, encoding the repeating subunit of toxin-coregulated pilus, in a bistable manner (Nielsen *et al.*, 2010).

3.4.11 Conclusion

This work provides a fascinating example of an apparently non-classical system dedicated to the movement of *S. marcescens* chitinolytic machinery across the cell envelope. Whether or not the ChiWX system can be regarded as a true secretion system will require further testing.

4 Evidence for bimodal and co-ordinated expression of *chiA* and *chiX*

4.1 Introduction

Since ChiW exhibits some similarities to the canonical λS-105 holin, and ChiX is a predicted endopeptidase, it is reasonable to question whether the *S. marcescens* ChiWX system will operate as a classic lambda lysis cassette. Therefore, a core problem this project needs to address is the question of whether *S. marcescens* actively secretes chitinase, or whether it releases chitinase *via* an altruistic lysis event. The work done in Chapter 3 initially addressed this problem: using immunochemistry techniques, the presence of intracellular control proteins such as cytoplasmic glutamine synthase (GS) and periplasmic maltose binding protein (MBP), were shown to be not detectable in the extracellular supernatant. In addition to this, the more sensitive and wider-ranging label-free proteomics employed in this work only detected a change in the presence of a very specific subset of proteins, the most abundant of which being the chitinolytic machinery. On the basis of the evidence gathered so far, it could be argued that if these substrates were released by a general lysis event we would expect to see many more non-specific cytoplasmic and periplasmic proteins in the extracellular milieu of a wild-type strain – whereas, in practice, only a particular small subset are affected by inactivation of the ChiWX system, which is more indicative of the substrate selectivity we might expect from a true secretion system.

As yet, however, we have not definitively ruled out the question of a lysis event. It could be argued that a subpopulation of cells may, in fact, grossly overproduce the chitinolytic machinery, while repressing all other biological pathways, and then lyse.

4.2 Aims

The aim of this Chapter was to determine whether the ChiWX system mediates a cell lysis event. We initially hypothesised that if every cell in the population was expressing *chiWX* simultaneously, then cell lysis was unlikely to be the mode of chitinase secretion. To test this hypothesis the approach chosen was that of live cell imaging with the specific objectives of collecting static images of bacterial cultures and real-time fluorescence microscopic data on growing populations. To facilitate this study, fluorescent reporter strains were constructed in collaboration with Prof Tracy Palmer and Dr Grant Buchanan (University of Dundee), and fluorescence microscopy experiments were performed in collaboration with Dr Nicola R. Stanley-Wall and Dr Victoria L. Marlow (University of Dundee).

4.3 Results

4.3.1 Expression of *chiX* is bimodal.

In order to visualise the expression of the *chiWXYZ* operon in live cells, fluorescent reporter strains were constructed using the pKNG101 suicide vector (Kaniga *et al.*, 1991) to integrate gene replacements onto the *S. marcescens* chromosome. The fluorescent protein mKate (Pletnev *et al.*, 2008) was chosen for its reported brightness and photostability**.** In this work, a gene replacement allele was prepared, Δ*chiX*::mKate. In this case, the *chiX* gene was deleted in-frame and a cassette encoding mKate, including an optimised RBS, was integrated as a replacement. As a result we have a transcriptional fusion strain, ChiXmKate (Δ*chiX*::mKate), which is also a *chiX* mutant and so defective in chitinase externalisation.

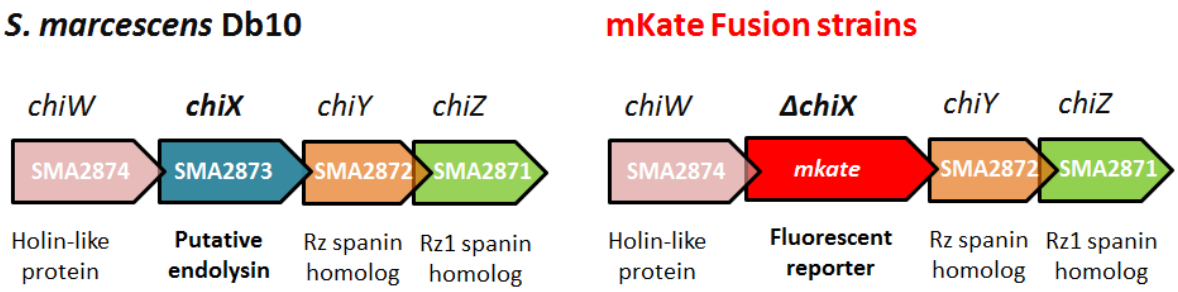


Figure 4.1 A transcriptional fusion strain, ChiXmKate, in which mKate replaces *chiX*. For strain ChiXmKate, the 732 base pair *mkate* gene was integrated at base position 3032609-3032610 on the *S. marcescens* chromosome in order to serve as a reporter of *chiX* transcription.

The ChiXmKate (Δ*chiX*::mKate) strain was designed to serve as a reporter of *chiX* expression. For this experiment we used fluorescence microscopy to observe ChiXmKate (Δ*chiX*::mKate) cells after 16 hour growth at 30°C in rich media. An initial analysis of the data shows a clear bistable expression of *chiX* in *S. marcescens* (FIGURE 4.2).

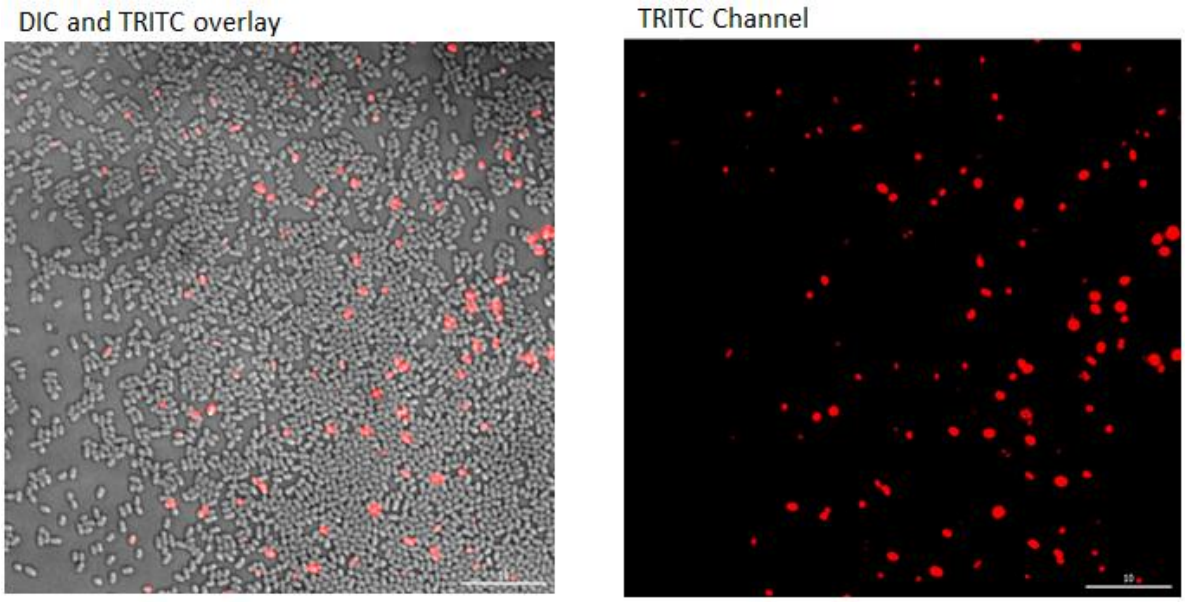


Figure 4.2 Expression of the *chiX* gene exhibits a bimodal distribution. Representative still frames (merged DIC [light microscopy] and/ or TRITC [red fluorescence] channel) of the ChiXmKate (Δ*chiX*::mKate) strain. Cells were grown for 16 hr in rich media and images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. Scale bars show 10 μm.

In order to quantify the bimodal expression data, the TRITC (red channel) fluorescence intensities corresponding to 1,500 individual cells was measured, for two separate experiments, and compared to the parent strain *S. marcescens* Db10. The ChiXmKate (Δ*chiX*::mKate) strain shows a population of bright fluorescent cells that are well above the background intensities obtained for *S. marcescens* Db10 (FIGURE 4.3). From the two separate experiments there were a total of 4% and 8%, respectively, of the population that were defined as brightly fluorescent ('ON'). The background fluorescence was calculated as the mean TRITC value of the *S. marcescens* Db10 population plus three times the standard deviation. For the first experiment the background was calculated to 132 AU and the bright ON cells were defined as those above 140 AU, which corresponded to 59 of 1,500 cells measured (4%) (FIGURE 4.3 A and B). For the second repeat experiment the background intensity was calculated to be 138 AU and the bright ON cells were defined as those above 150 AU, which corresponded to 120 of the 1500 cell population, 8% (FIGURE 4.3 C and D). The bright ON cells were determined as those that were above the background level and also well above the general population cells to ensure that cells included were emitting a clear unambiguous fluorescence.

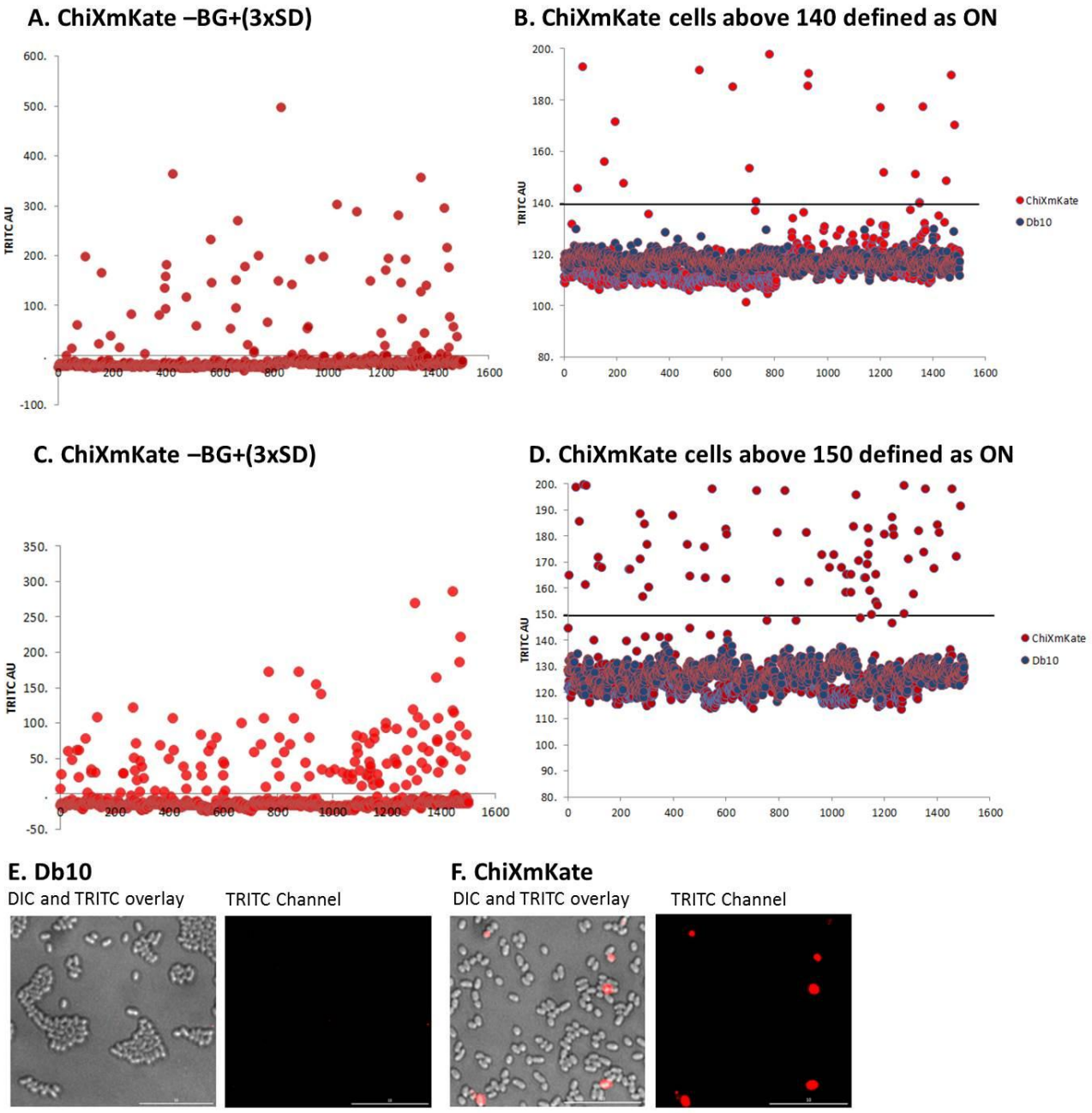


Figure 4.3 The *chiX* gene is expressed in 4 - 8% of the *S. marcescens* population. (A) First experiment. A subpopulation of cells emitting a fluorescence intensity above the background (BG) level calculated as the mean value of *S. marcescens* Db10 grown under the same conditions, plus three times the standard deviation. The BG value is set at 0 on the x axis. (B) The ON cells were defined as those well above the BG value and the general population of both ChiXmKate and the *S. marcescens* Db10 parental strain. Here, the bright cells were defined as those above 140 TRITC AU, which corresponds to 4% of the population. (C) Second Experiment. A subset of cells exhibiting greater fluorescence intensity than the general population, which corresponded (D) to 8% of the population defined as ON. (E and F) DIC and/or TRITC overlay shows mKate fluorescence was only observed for strain ChiXmKate. Scale bars show 10 µm.

4.3.2 Expression of *chiA* exhibits a bimodal distribution.

Having established that *chiX* is expressed only in a subpopulation of the cells, attention next turned to the chitinases themselves. A strain was constructed, Db10*::chiA-gfp*, that was positive for chitinase secretion (the native *chiWXYZ* operon was intact), encoded full length ChiA, but also encoded GFP 23 base pairs downstream of the *chiA* termination codon (FIGURE 4.4). The Db10*::chiA-gfp* strain was considered to be a transcriptional fusion between *chiA* and *gfp*.

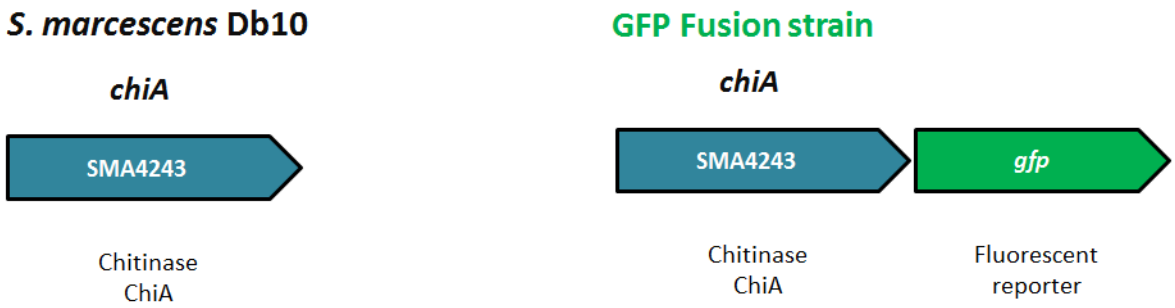


Figure 4.4 Strain Db10*::chiA-gfp* is a transcriptional fusion between *chiA* and *gfp*. For strain Db10*::chiA-gfp*, the 717 base pair *gfp* gene was integrated at base position 4540152-4540153 on the *S. marcescens* chromosome, 23 base pairs from the end of the *chiA* termination codon. In the GFP fusion strain, *chiA* is left intact and *gfp* gene is integrated 23 base pairs downstream of the *chiA* termination codon.

Next, we used fluorescence microscopy to observe the Db10*::chiA-gfp* strain cells after 16 hour growth at 30°C in rich media. It is clear from the images shown in FIGURE 4.5 that expression of *chiA* is limited to a particular subpopulation and exhibits a bimodal distribution.

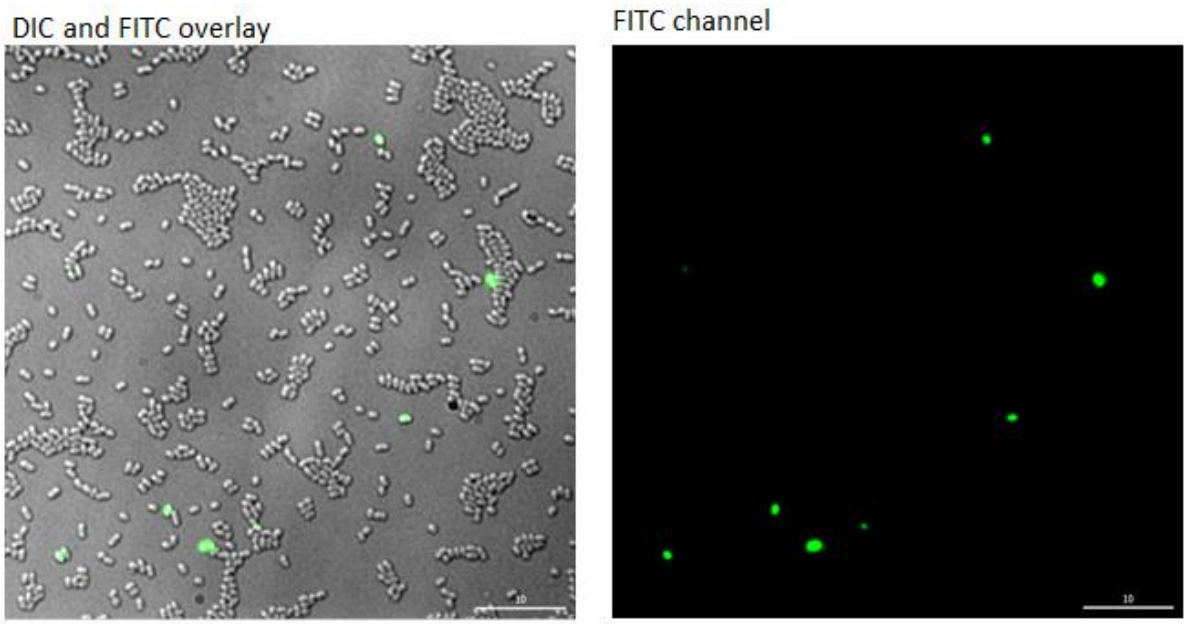


Figure 4.5 The expression of the *chiA* gene exhibits a bimodal distribution. Representative still frames (merged DIC and/ or FITC [green fluorescence] channel) of the Db10*::chiA-gfp* strain. Cells were grown for 16 hr in rich media and the images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. Scale bars show 10 µm.

It was important to quantify the bimodal expression data for the Db10*::chiA-gfp* strain, which was subjected to the same analysis as the mKate-encoding strain by measuring the fluorescence intensities corresponding to 1,500 individual cells, for two separate experiments, in comparison to the parental strain *S. marcescens* Db10. The Db10*::chiA-gfp* strain shows a population of bright fluorescent cells that are above the background intensities obtained for *S. marcescens* Db10 (FIGURE 4.6). From the two separate experiments there were a total of 3% and 1% of the population that were defined as 'ON'. The background fluorescence was calculated as the mean FITC (green channel) value of the *S. marcescens* Db10 population plus three times the standard deviation. For the first experiment the background was calculated to 119 AU and the ON cells were defined as those above 140 AU, which corresponded to 38 of 1,500 cells measured (3%) (FIGURE 4.6 A and B). For the second repeat experiment the background intensity was calculated to be 118 AU and the ON cells were defined as those above 120 AU, which corresponded to 15 of the 1500 cell population, 1% (FIGURE 4.6 C and D).

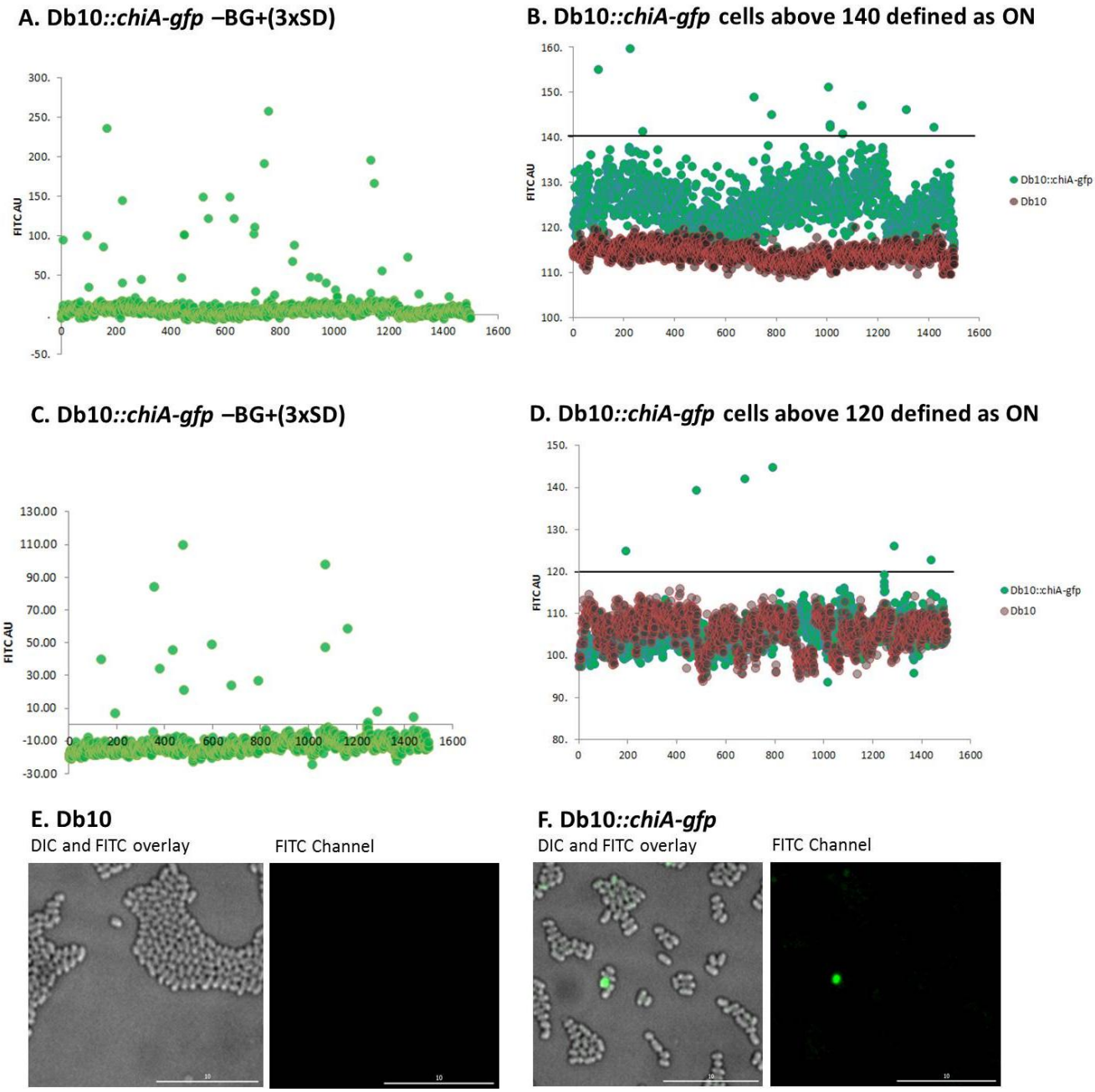


Figure 4.6 The *chiA* gene is expressed in 1 – 3% of the *S. marcescens* population. (A) First experiment. Db10*::chiA-gfp* shows a subpopulation of cells emitting a fluorescence intensity above the background (BG) level calculated as the mean value of *S. marcescens* Db10 parental strain grown under the same conditions, plus three times the standard deviation. The BG value is set at 0 on the x axis. (B) The ON cells were defined as those well above the BG value and the general population of both Db10*::chiA-gfp* and *S. marcescens* Db10 cells. Here the bright cells were defined as those above 140 TRITC AU, which corresponds to 3% of the population. (C) Second experiment. A subset of cells exhibiting greater fluorescence intensity than the general population. (D) The cells defined as ON corresponded to 1% of the population. (E and F) DIC and/or TRITC overlay shows GFP fluorescence was only observed for strain Db10*::chiA-gfp*. Scale bars show 10 µm.

4.3.3 Evidence for co-ordinated expression of *chiA* and *chiX*.

Next, a dual fusion strain was constructed that would report both *chiA* (GFP) and *chiX* (mKate) expression simultaneously. The role of the ChiXmKate*::chiA-gfp* double fusion strain was to establish whether *chiA* and *chiX* are expressed within the same subpopulation of cells.

Fluorescence microscopy was employed to observe ChiXmKate*::chiA-gfp* double fusion strain after 16 hour growth at 30^{o}C in rich media. The bimodal expression of both *chiX* and *chiA* was again evident in this experiment (FIGURE 4.7). Interestingly, the microscopy also revealed that *chiX* and *chiA* expression was co-ordinated (FIGURE 4.7). Visual inspection of the overall population revealed that most cells showed some co-expression where one fluorescence intensity appeared greater. There were relatively few cells where only one fluorescence channel (GFP or mKate) could be detected.

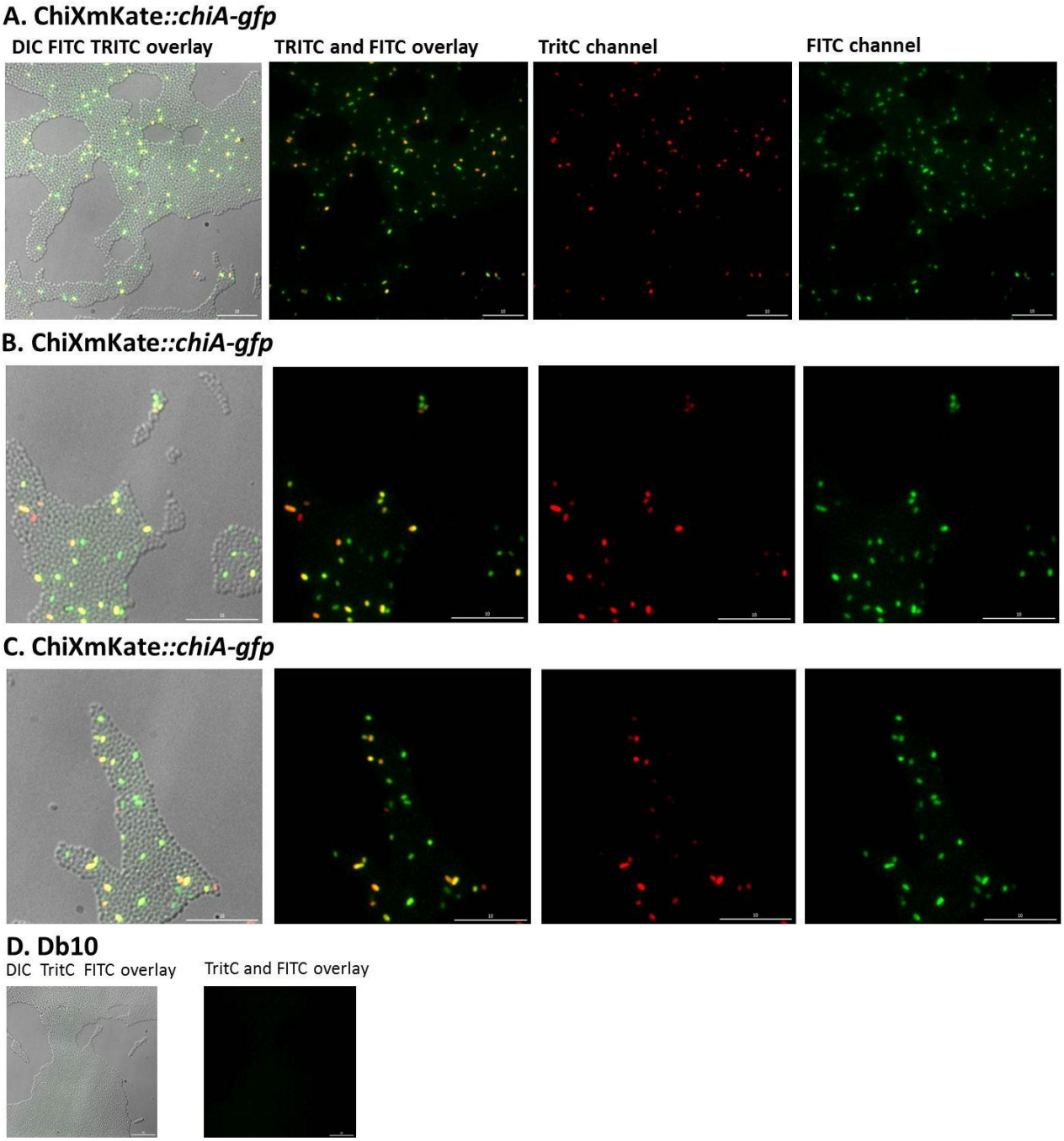


Figure 4.7 Co-ordinated expression of *chiA* and *chiX*. Representative images (DIC and/or TRITC and/or FITC channel) from three separate images (A-C) of dual fusion strain ChiXmKate*::chiA-gfp* shows co-ordinated expression of *chiA* and *chiX*. Cells where expression of *chiA* and *chiX* are both strong appear as yellow in the FITC and TRITC overlay. (D) Representative image of *S. marcescens* Db10 with no detectable fluorescence. The ChiXmKate*::chiA-gfp* cells were grown for 16 hours in rich media and images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. Scale bars show 10 µm.

It was important to quantify the respective levels of FITC (green) and TRITC (red) fluorescence intensities, as this would provide a more sensitive assessment (in addition to visual presentation) of whether *chiA* and *chiX* expression exhibited a similar distribution. For this, the fluorescence intensities of 2,000 ChiXmKate*::chiA-gfp* dual fusion strain and *S. marcescens* Db10 cells were compared and shown to exhibit a remarkably similar profile: *chiA* expression was defined as ON in 8% of the dual fusion population, whilst *chiX* expression was ON within 7% of the total population. FIGURE 4.8 shows the 7% subpopulation (147 cells of 2,000 in total) within the yellow ON box where *chiA* and *chiX* expression are co-expressed (bright ON for both intensities). Interestingly, the fluorescence emitted by the overall population of the ChiXmKate*::chiA-gfp* dual fusion strain, detected by both FITC (8E-84) and TRITC (2.6E-260) channels, was significantly different from the *S. marcescens* Db10 parental strain, suggesting a low level of basal fluorescence throughout the whole population. These cells were excluded from the ON definition (FIGURE 4.9 A and B).

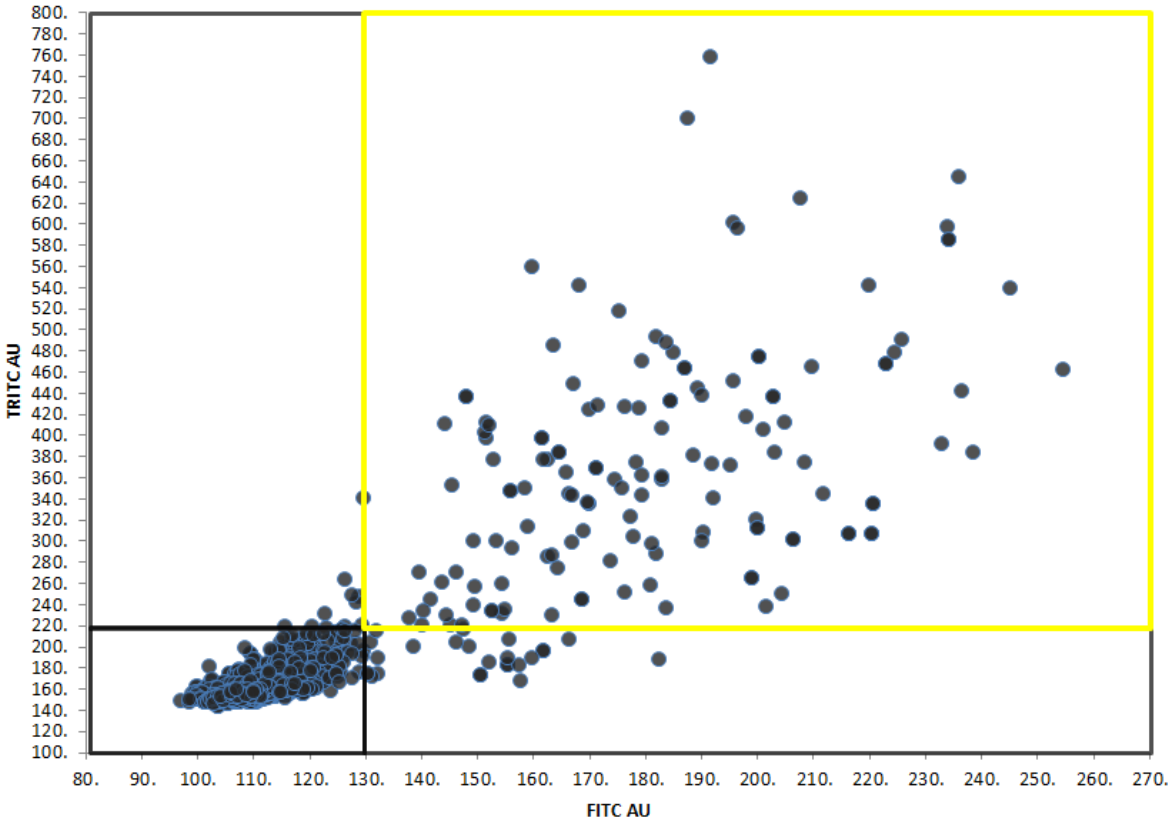


Figure 4.8 Quantification of the fluorescence intensities establishes *chiA* and *chiX* expression are co-expressed. The fluorescence intensities of a total of 2,000 cells were measured, of which 165 cells were defined as ON corresponding to *chiA* expression (8%) and 147 cells were defined as ON corresponding to *chiX* expression (7%). The 7% of the population where the expression of both *chiA* and *chiX* was significant are located within the yellow box and represent those cells for which expression of both genes can be regarded as co-ordinated.

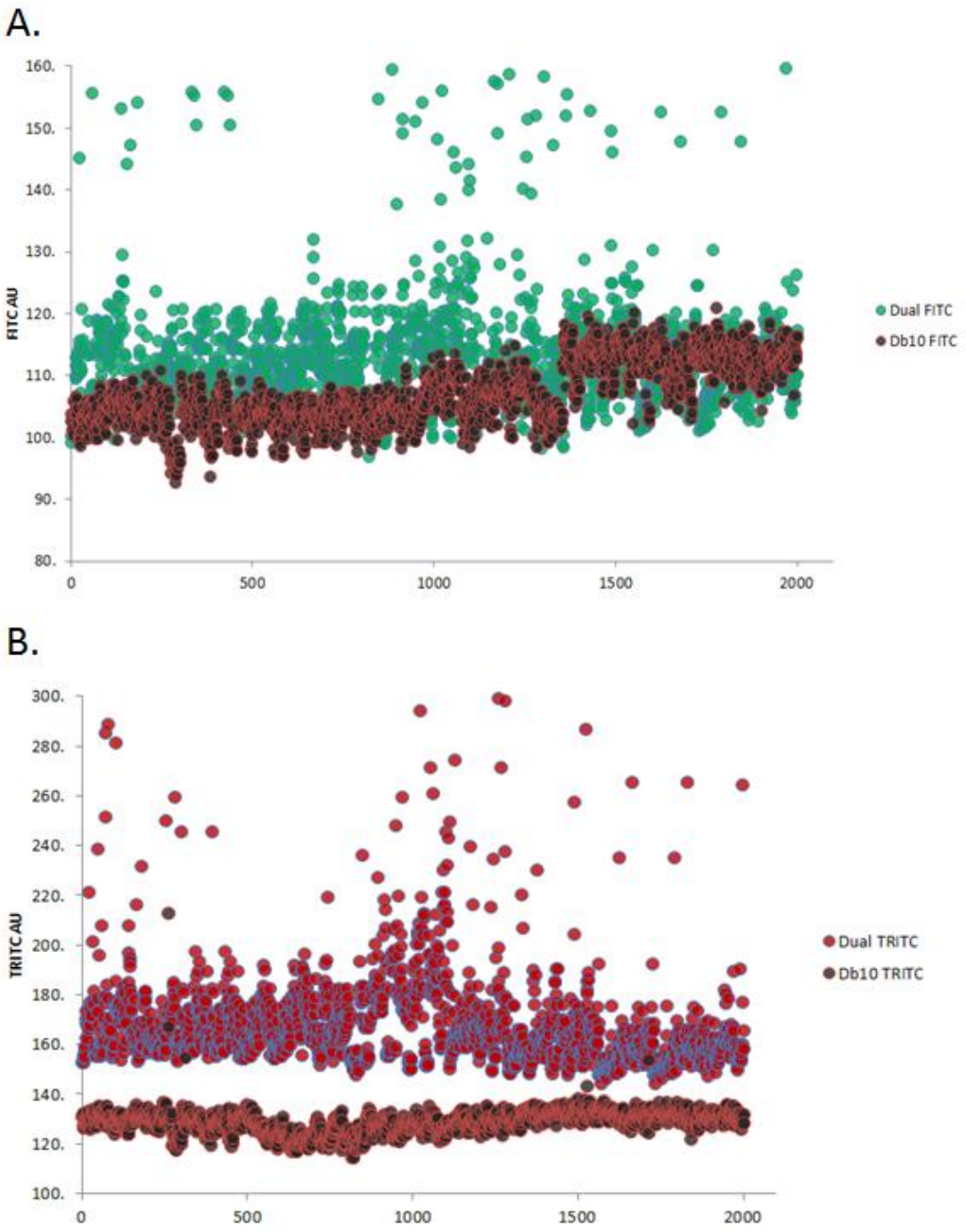


Figure 4.9 Dual fusion strain exhibits basal level of fluorescence. ChiXmKate*::chiA-gfp* cells exhibited a significantly greater level of fluorescence detected for both FITC (A) (p-value 8E-84) and TRITC (B) (p-value 2.6E-260), suggesting a basal level of fluorescence, for both intensities, throughout the whole population. The ON cells were defined as above this basal level intensity: for FITC, cells with a value greater than 130 AU were defined as ON, and for TRITC cells above 220 AU were also defined as ON.

4.3.4 Expression of *chiA* does not predispose cells to autolysis.

Having established that expression of *chiX* and *chiA* is tightly co-ordinated, attention can now turn to the important issue of whether release of chitinases is attributable to a cell lysis event. Our ChiXmKate*::chiA-gfp* dual fusion strain shows unequivocally that *chiX* is expressed at exactly the same time as *chiA*. We can therefore study the strain Db10*::chiA-gfp*, which expresses intact and active *chiX* essential for the extracellular release of chitinase, safe in the knowledge that whenever GFP production is 'ON' the cells will be in the process of releasing chitinases.

The fate of the Db10*::chiA-gfp* 'ON' population was followed using time-lapse microscopy. The Db10*::chiA-gfp* cells were grown for 18 hours in minimal media glucose and were then monitored at 15 minute intervals under the DIC (light microscopy) and FITC (green fluorescence) channels. Images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. The time lapse experiments (FIGURE 4.10 and FIGURE 4.11) showed unequivocally that fluorescent 'ON' Db10*::chiA-gfp* cells were capable of dividing and differentiating (growing) into a non-fluorescent cell population, and, less frequently, into a fluorescent subpopulation that divides again to become non-fluorescent daughter cells. The representative example of fluorescent cell division shown in (FIGURE 4.10 and FIGURE 4.11) exhibited intact cell morphology over a three hour period. This provides strong evidence that the bimodal distribution of chitinase expression is not concomitant with an altruistic lysis event. Indeed, in the first time lapse experiment, of the 39 total fluorescent cells observed throughout the twelve distinct observation points, 23 of these went on to subdivide (59%). Similarly, when the same experiment was repeated, 17 of the 28 fluorescent cells observed across twelve different observation points displayed a clear cell division event (61%). In both cases, the remaining ~40% of 'ON' cells retained an intact morphology and, while none of them lysed, most appeared quiescent (non-dividing) and were eventually subsumed within a growing colony of cells.

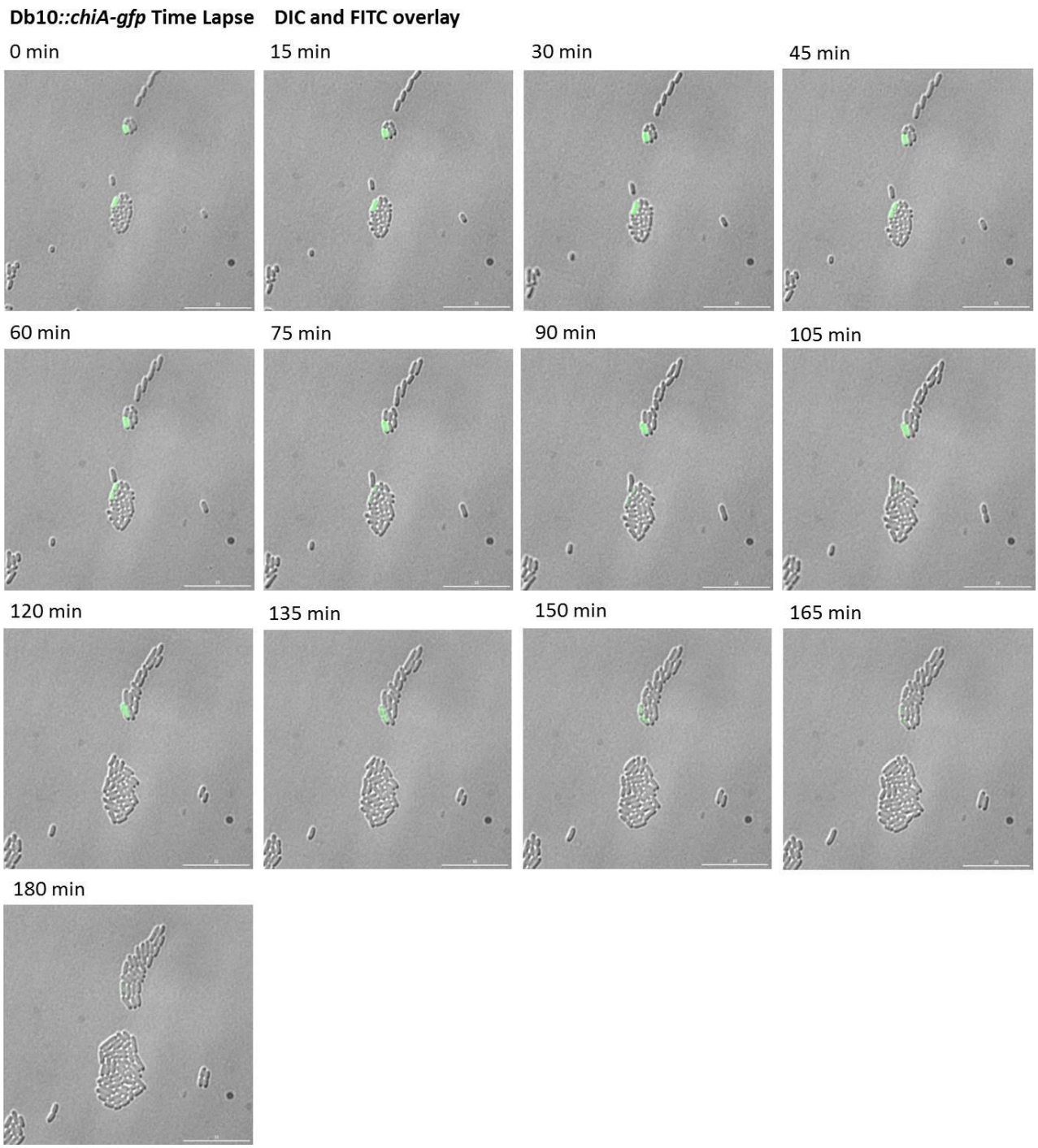


Figure 4.10 Expression of *chiA* gene is not concurrent with a lysis event. Representative images (DIC and FITC channels), taken at 15 minute intervals, from a three hour time lapse experiment using real time microscopy. Strain Db10*::chiA-gfp* is clearly able to express the *chiA* gene and subsequently undergo cell division. This behaviour is incompatible with the hypothesis that a cell lysis event is responsible for the extracellular release of chitinase, since evidently the subpopulation of cells that are expressing *chiA* at a high level retain their membrane integrity. From two separate time lapse experiments, each consisting of twelve different observation points, there were a total of 67 fluorescent cells, of which 40 cells divided (60%). Furthermore, none of the remaining 40% exhibited a clear lysis event and all appeared to retain an intact cell morphology. The Db10*::chiA-gfp* cells were grown for 18 hours in minimal media glucose and images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. Scale bars show 10 µm.

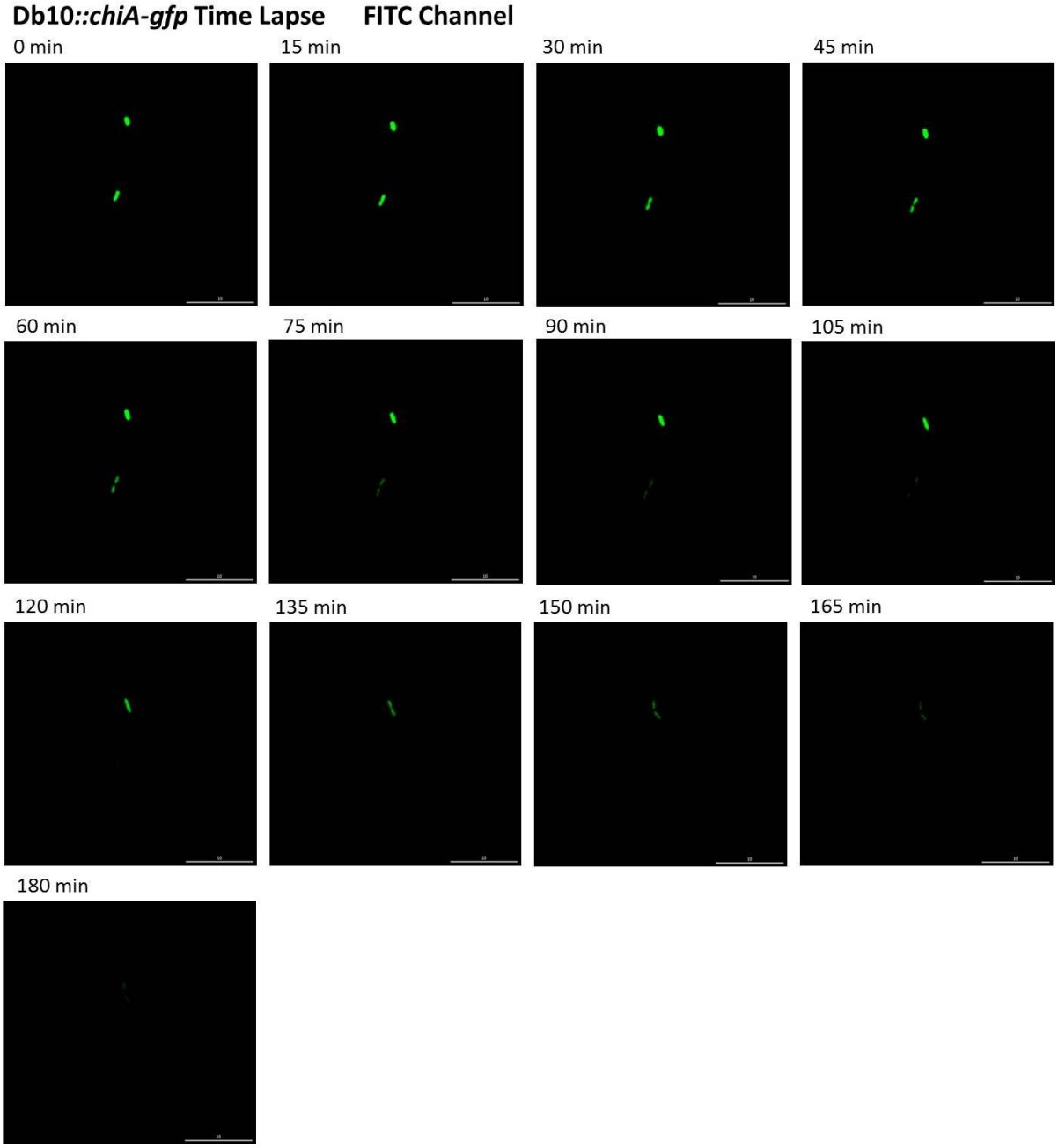


Figure 4.11 Expression of *chiA* gene is not concurrent with a lysis event. The same representative images as above showing just the FITC channel. Images were taken at 15 minute intervals, from a three hour time lapse experiment using real time microscopy. Strain Db10*::chiA-gfp* is clearly able to express the *chiA* gene and subsequently undergo cell division. This behaviour is incompatible with the hypothesis that a cell lysis event is responsible for the extracellular release of chitinase, since evidently the subpopulation of cells that are expressing *chiA* at a high level retain their membrane integrity. From two separate time lapse experiments, each consisting of twelve different observation points, there were a total of 67 fluorescent cells, of which 40 cells divided (60%). Furthermore, none of the remaining 40% exhibited a clear lysis event and all appeared to retain an intact cell morphology. The Db10*::chiA-gfp* cells were grown for 18 hours in minimal media glucose and images were taken using an Olympus x100 1.4 NA lens and CoolSNAPHQ camera. Scale bars show 10 µm.

4.4 Discussion

There is now a great appreciation of bimodal gene expression in a wide variety of biological systems, particularly in relation to virulence. It is generally accepted that the phenotypic variation provided by bimodal gene expression is favourable for survival in environments that may be prone to rapid fluctuations, such as establishing infection within a host, or in a soil environment. The work of Arkin *et al.,* (1998) was the first to study λ phage infection of *E. coli* resulting in two distinct phenotypes, lytic and lysogenic, present in an isogenic population and subject to fluctuating ratios regulated by environmental signals. More recent work in *Bacillus subtilis* has investigated bistable gene expression involved in genetic competence, sporulation (Grossman, 1995) and biofilm formation (Chai *et al.*, 2008). For example, only ~15% of the *B. subtilis* population become competent (able to take up exogenous DNA) and this has been shown to be regulated by the relative concentration of the regulator ComK, which is itself under the control of the master regulator Spo0A-P that both activates and represses *comK* transcription depending on its cellular abundance, thus creating a 'window' of ComK mediated competence (Mirouze *et al.*, 2012). There are also good examples of bimodal gene expression from enteric bacteria, such as the phase variation exhibited by pathogenic *E. coli* (Haagmans and van der Woude, 2000) and *Salmonella* (Hughes *et al.*, 1988).

Such examples serve to illustrate that phenotypic bimodality is a common phenomenon, a way for cells to pre-adapt to a possible new environment. In this work, we have shown that only a subpopulation of cells express two key genes responsible for the *S. marcescens* chitinolytic phenotype. Below is a discussion of the role of bimodal expression of the chitinolytic components, in the context of what is currently known about this behaviour.

4.4.1 Release of ChiA into the extracellular environment is not attributable to lysis

This work has shown that the release of ChiA to the extracellular environment is not attributable to ChiX-mediated lysis (FIGURE 4.10). Strain Db10*::chiA-gfp* was shown to be capable of bistable expression of *chiA* in cells that retained an intact morphology and were perfectly capable of division (FIGURE 4.10 and FIGURE 4.11). Since ChiX has already been shown to be essential for the extracellular presence of chitinase, and since it was shown in this work that expression of *chiX* is co-ordinated with expression of *chiA* (FIGURE 4.7), we can conclude that ChiX is not involved in mediating an altruistic lysis event. Instead, it is reasonable to hypothesise that ChiX facilitates the movement of chitinase to the extracellular supernatant in a way that maintains the overall integrity of the membrane. Further experiments will need to focus on testing how, in a cell producing ChiWX, the integrity of the cell envelope is kept intact, and whether ChiWX-mediated release of substrate across the outer membrane is a selective process.

Continuing research on this topic will involve integrating a *torA-gfp* fusion (encoding the signal peptide of the *E. coli* Tat substrate trimethylamine *N*-oxide reductase TorA fused to GFP) downstream of the *chiA* locus in order to test whether Tat-exported (and presumably periplasmic) GFP can then be detected in the culture supernatant. This will then enable us to discern whether the ChiX-mediated release of substrate across the outer membrane is selective. This could show whether the chitinolytic machinery is recognised by, an as yet unidentified, receptor component located in the outer membrane, or alternatively whether ChiX facilitates a non-selective leakage of the entire periplasmic contents into the culture supernatant. If the former hypothesis is correct, and the integrity of the outer membrane is kept intact, then we are justified in calling ChiWX-dependent release of chitinases a true secretion system.

Another way to test the issue of membrane integrity is to consider that if a subpopulation of cells resort to shedding their periplasmic contents, then the subset (~7%) of cells identified as expressing *chiA*/*chiX* at high levels should have a partially disrupted outer membrane that might be detectable by Gram staining. For example, the subpopulation of cells might be rendered more sensitive to retain the primary crystal violet stain as a result of any disruption in their outer membranes. The sensitivity of the outer membrane could also be tested by treating the *mkate*/ *gfp* fusion strains with bile salts or antimicrobial peptides, such as polymixin B or Bis-lentivirus lytic protein 1 (Bis-LLP1), and assessing whether this affects the bimodal distribution due to the enhanced sensitivity of the cell envelope.

Both of these experiments should establish whether ChiWX is selective and whether the integrity of the outer membrane is kept intact, which are both key features of a true secretion system.

4.4.2 Expression of *chiA* and *chiX* exhibit a co-ordinated bimodal distribution

This work has shown that expression of *chiA* and *chiX* are expressed in ~7% of the *S. marcescens* population. This sort of bimodal expression of genes is thought to act as an insurance policy against environmental fluctuations that might change too rapidly for a transcriptional response to a signal – instead, some cells in a population 'pre-adapt' to a certain change. Some researchers use the interesting term 'hysteresis' to denote this sort of behaviour based on historical exposure to a given environmental stress. Recent work with Gram negative *Pseudomonas aeruginosa* investigating the role of the LysR type transcriptional regulator (LTTR) BexR has shown that it is a bistable regulator of its target genes (including *aprA*, which encodes a secreted virulence factor alkaline protease) and, like many LTTRs, exhibits positive autoregulation (Turner *et al.*, 2009). Besides BexR-mediated bistability, there are a number of other phenotypes in *P. aeruginosa* that exhibit a bimodal distribution - these include antibiotic resistance and biofilm formation (Deziel *et al.*, 2001; Drenkard and Ausubel, 2002), which indicates the relevance of bimodality from a medical perspective.

Based on this understanding, the reason why expression of the chitinolytic machinery exhibits a bimodal distribution in *S. marcescens* becomes apparent. Chitin is quite insoluble, so with a subpopulation of cells already expressing chitinase then those cells are pre-adapted to a potential loss of an accessible carbon source and can begin to degrade complex carbohydrates, such as chitin, for the benefit of the overall population. Since bimodal expression of the chitinolytic machinery is now abundantly clear, the next question is how is this process regulated? The first candidate we would postulate as responsible for this behaviour would be the LysR regulator ChiR, and the best way to test whether ChiR is involved would be to construct a Δ*chiR* deletion in the ChiXmKate*::chiA-gfp* dual fusion strain to see whether this abrogates bimodal expression of *chiA* and/or *chiX*. If so, we would expect to see a shift from a bright ON subpopulation to a more general background basal level of fluorescence. In this regard it would also be interesting to generate a separate Δ*hfq* deletion in the dual fusion background: if the hypothesis is true that Hfq regulates chitinase transcription, whereas ChiR regulates genes involved in transport, then we would hypothesise that a Δ*hfq* strain, by contrast, would retain bimodal expression of *chiX* but would lose expression of *chiA* altogether. This work would complement a microarray transcriptional analysis of the ChiR and Hfq target genes (discussed in Chapter 2), taking Hfq regulation into account alongside ChiR is necessary since, although *chiA* and *chiX* expression are unequivocally co-ordinated, Hfq was only found to bind the mRNA of *chiA* (Sarah Murdoch & Dr Sarah Coulthurst, unpublished observation).

Pseudomonas aeruginosa BexR has only 24% sequence identity, 44% similarity (and this from only 55% query coverage) to *S. marcescens* ChiR but interestingly, like ChiR, it exhibits structural similarity to other important LTTR enzymes, such as CrgA of *Neisseria meningitidis* and to CbnR of *Ralstonia*

eutropha (Kelley and Sternberg, 2009). Applying the previous work of Turner *et al.*, (2009) on *Pseudomonas* BexR to *S. marcescens* ChiR would provide a possible direction for further characterising the putative ChiR-mediated bimodality. To take this idea further, these proposed experiments would use the dual fusion strain (ChiXmKate*::chiA-gfp*) as a reporter of *chiA* and *chiX* expression, with one strain devoid of *chiR* and another left intact. In both these strains we could integrate the promoter of *chiR* followed by a *yfp-lacZ* reporter (P_{chiR}-*yfp-lacZ*) in order to monitor *chiR* autoregulation, and at another locus we could integrate *chiR* under the control of an IPTG inducible promoter (P_{IPTG}-*chiR*) (FIGURE 4.12). To begin with, this will determine whether ChiR exhibits positive or negative autoregulation: if YFP fluorescence or *lacZ* activity increases with elevated levels of IPTG-induced *chiR* expression, then ChiR is a positive autoregulator; conversely if reporter levels decrease with elevated levels of *chiR* expression then ChiR can be said to exhibit negative autoregulation. These strains will also enable us to monitor the expression levels of the chitinolytic machinery when *chiR* expression levels are varied. If ChiR is a positive autoregulator, and enhanced levels of *chiR* expression creates a positive feedback situation (which is the case with BexR mediated regulation in *P. aeruginosa*), we would expect to see a non-linear increase in the levels of *chiX* and *chiA* expression (mKate and GFP fluorescence), the fluorescent population will increase as the expression of *chiR* is elevated (this is the hypothetical situation illustrated in FIGURE 4.12). Whereas if *chiR* acts as a repressor of its target genes we would expect to see the levels of *chiA* and *chiX* expression diminish as *chiR* expression increases.

Another interesting feature to test using these hypothetical strains would be whether the chitinolytic ON cells exhibit hysteresis. In a hysteretic system cells grown under identical conditions should exhibit different responses when regrown under the same conditions. In this situation, we could test whether blue ON cells grown on X-Gal media exhibit a greater abundance of fluorescent ON cells when inoculated and grown in fresh liquid media, compared to OFF colonies from the same plate.

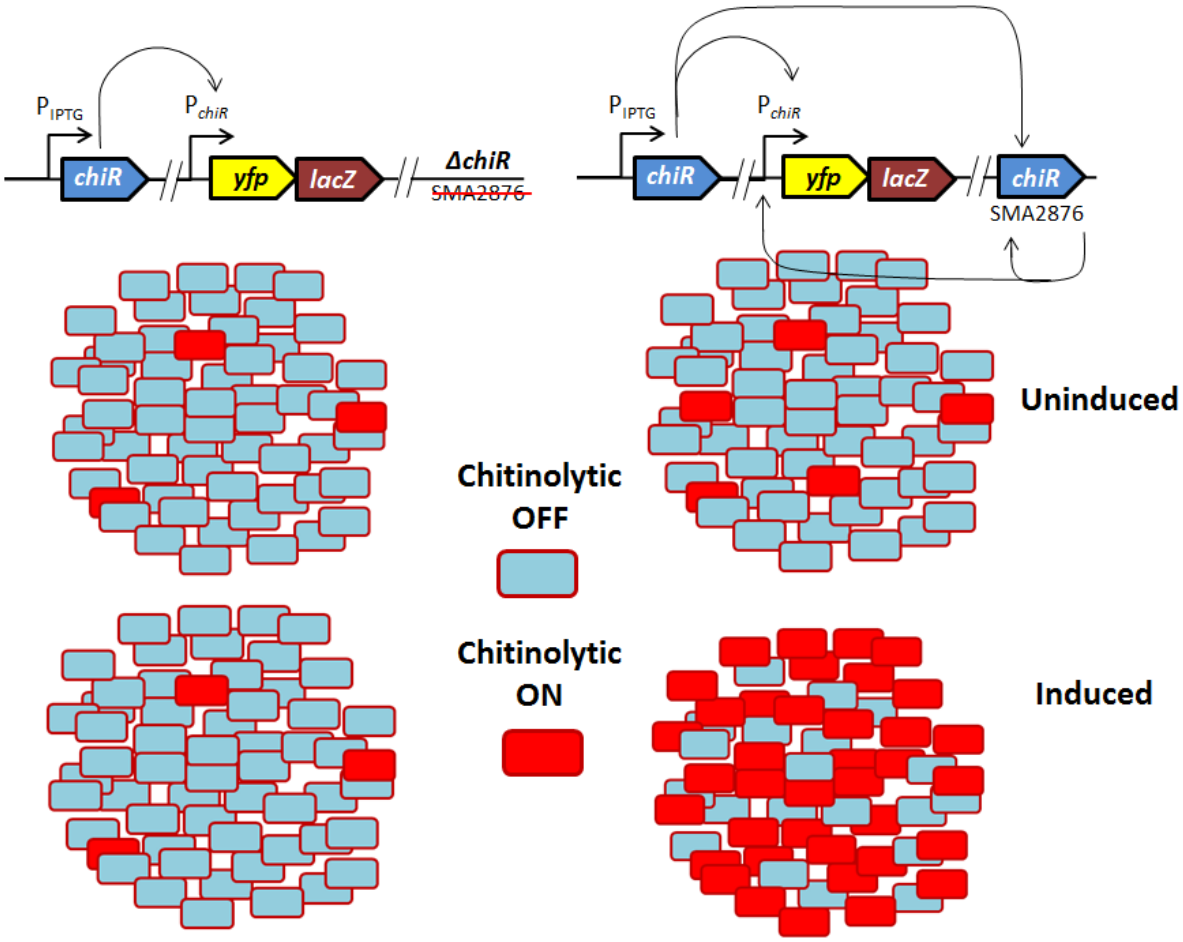


Figure 4.12 Hypothetical experiment to test whether ChiR mediates positive regulation of target genes and exhibits positive autoregulation. This experiment would use the dual fusion strain (ChiXmKate*::chiA-gfp*) as a reporter of chitinolytic gene expression, ON or OFF. One dual fusion strain would be devoid of native *chiR* (SMA2876) and the other would encode full length *chiR*. Into both dual fusion strains, the *chiR* gene under the control of an IPTG inducible promoter would be integrated onto the chromosome, and at another location a reporter of *chiR* expression would be integrated, a *yfp-lacZ* (encoding yellow fluorescent protein YFP and β-galactosidase LacZ) construct under the control of the *chiR* promoter. If, in this hypothetical situation, ChiR were shown to act as a positive regulator and to initiate a positive feedback loop as the presence of ChiR increases (with increasing IPTG concentration), we would expect to see the overall proportion of chitinolytic ON cells (shown in red) to increase. Whereas, alternatively, the Δ*chiR* (SMA2876) strain unable to initiate such a positive feedback loop would keep a relatively constant biomodal distribution, irrespective of IPTG concentration. This experiment would determine the nature of ChiR mediated regulation of the bimodal chitinolytic phenotype. Figure adapted from (Turner *et al.*, 2009).

4.4.3 Conclusions

The chitinolytic phenotype in *S. marcescens* displays a clear bimodal distribution and the extracellular secretion of chitinase is not attributable to ChiX-mediated lysis. There are good reasons for thinking that the ChiR LTTR is responsible for the bimodal chitinolytic phenotype, since disruption of *chiR* has been shown previously to result in a loss of chitinolytic phenotype and a similar LTTR is responsible for the bimodal phenotype in *P. aeruginosa* (Turner *et al.*, 2009).

Whether or not ChiWX constitutes a true secretion system requires further testing to determine whether ChiWX facilitates the selective release of chitinolytic substrates, as opposed to non-specific periplasmic shedding.

5 Future Perspectives

5.1 Do opportunistic human pathogens use chitinases to establish infection?

5.1.1 Chitinases and virulence

There has recently been a growing appreciation of the role of chitinases in pathogenesis, particularly in cases where these are used to bind 'chitinous' receptors such as mucin or carcinoembryonic antigen-related cell-adhesion molecules (CEACAM) (Tran *et al.*, 2011). Opportunist human pathogens such as *Pseudomonas aeruginosa* have been shown to up-regulate expression of genes encoding the chitinolytic ChiC and Cbp proteins (Manos *et al.*, 2009) in response to samples mimicking sputum from immunocompromised cystic fibrosis (CF) patients. Since *P. aeruginosa*, unlike *S. marcescens*, is incapable of utilising chitin as a carbon source (Folders *et al.*, 2001), it is reasonable to hypothesise that chitinolytic proteins have a putative role in virulence for this bacterium. The production of chitinolytic enzymes has been shown to be a crucial feature of the physiology of the Gram positive bacterium *Listeria monocytogenes*, especially in its ability to colonise the liver and spleen, and to actively suppress the host innate immune response in a murine infection model. Since mammalian hosts do not produce chitin *per se*, it was proposed that *L. monocytogenes* produces chitinolytic proteins to exploit glycoproteins and carbohydrate moieties as target sites for establishing infection (Chatterjee *et al.*, 2006; Chaudhuri *et al.*, 2010). In addition to the expression of chitinolytic enzymes by opportunistic pathogens to exploit chitin-like receptors in the mammalian host, the picture is further complicated if we consider that the host, in turn, also expresses chitinases/chitinase-like enzymes in response to the presence of bacterial chitinases. For example, the gene encoding chitotriosidase CHIT1 has been shown to be up-regulated in response to bacterial infection in neonates (Labadaridis *et al.*, 2005). This suggests a complex picture of host-microbe interactions mediated by chitinases/chitinase-like enzymes interacting with other chitin-like carbohydrate molecules.

Initially, as part of this study, we wanted to test whether *S. marcescens* chitinases act as virulence factors during infection of the insect host, such as wax moth larva *Galleria mellonella*. For this we performed a basic killing assay where a population of ten larvae were injected with different *Serratia marcescens* strains (FIGURE 5.1) grown overnight in rich media and were subsequently injected with 10 µl of 10^{-6} and 10^{-8} PBS dilutions of the respective strains. It was clear from this preliminary experiment that deletion of *S. marcescens* chitinase encoding genes has no effect on insect killing in this particular assay – indeed the strain completely devoid of chitinase encoding genes (Nochi) killed

more (10/10) wax moth larvae than Db10 (3/10). For this reason we did not take this line of enquiry further.

This preliminary experiment suggests that chitinases are not necessary for virulence in this particular chitin-containing insect host. It should be considered, however, that since this particular assay involved injecting bacteria beneath the chitin cuticle and into the haemocoel. A more appropriate virulence assay may have involved feeding wild-type and mutant *S. marcescens* to *Caenorhabditis elegans*, where the pathogens would have to escape the chitinous grinder in order to establish infection. Neverthelss, a Δ*hfq* deletion strain appears to be unable to kill the moth larvae in the injection assay, although it should be noted that Hfq has a pleiotropic role in the regulation of a wide spectrum of virulence factors.

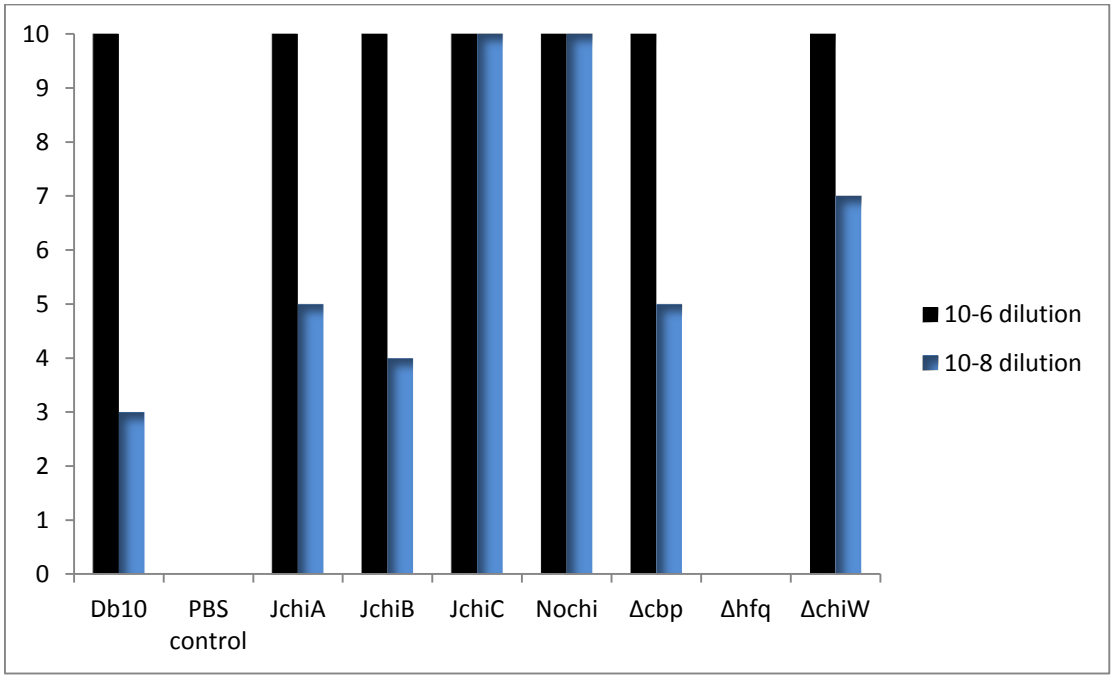


Figure 5.1 *S. marcescens* chitinases do not appear to play a role in insect virulence. A preliminary experiment testing the ability of different *S. marcescens* strains to kill wax moth larvae *G. mellonella*. Strains were grown overnight in rich media and subsequently diluted 10^{-6} or 10^{-8} in PBS: 10 µl of each culture was injected into 10 separate larvae for each strain respectively, including a PBS control, and these were incubated in the dark at 25 ^{o}C overnight. The bars represent exact numbers of dead larvae.

In *V. cholerae*, expression of the chitinolytic machinery is thought to be induced by the presence of chitin or GlcNAc (Meibom *et al.*, 2004), whereas in *V. harveyi* it has been shown to be regulated by quorum sensing as a social behaviour (Defoirdt *et al.*, 2010). Previous work studying *V. cholerae* has shown that the chitin binding protein GbpA, as well as biofilm formation, is negatively regulated by quorum sensing (Jude *et al.*, 2009). This suggests that GbpA might not be a structural component of

the biofilm, but could be used to initiate attachment to the host cell surface (Jude *et al.*, 2009), which is congruent with the work done in *V. harveyi* (Defoirdt *et al.*, 2010).

Legionnaires disease is a potentially devastating form of pneumonia caused by Gram negative *Legionella pneumophila*, and immunocompromised people are particularly susceptible to infection by this normally aquatic bacterium. In cases of infection, the bacterium is usually ingested or inhaled leading to its colonising the respiratory tract and subsequent invasion of alveolar macrophage (Fields *et al.*, 2002; Tran *et al.*, 2011). *L. pneumohila* externalises a chitinase ChiA, and a chitin binding protein CBP *via* a Type 2 secretion system. Recent work studying *L. pneumophila* ChiA has established that ChiA makes a significant contribution to *L. pneumophila* infection in the mouse lung, and that ChiA also elevates the survival of *L. pneumophila* in mammalian host cells (DebRoy *et al.*, 2006). These authors also hypothesised that ChiA is involved in degrading N-acetylated 'chitinous' proteins in the lung, and that the bacterial persistence within host cells is attributable to the host response being less capable of eradicating *L. pneumophila* cells producing ChiA (DebRoy *et al.*, 2006). This work provides further evidence of bacterial chitinases being utilised as virulence factors to target mammalian host cells.

The gene encoding chitin binding protein CbpD in *P. aeruginosa* has been shown to be regulated according to the environmental conditions, and also shows variation in expression levels between strains, i.e. clinical isolates *versus* conditioned laboratory strains (Salunkhe *et al.*, 2005). A microarray analysis of the *P. aeruginosa* transcription profiles from different strains revealed that *cbpD* expression was upregulated in a strain (LES) associated with the CF lung in comparison to a standard laboratory strain (PAO1) (Salunkhe *et al.*, 2005). In addition to this, a separate analysis of the transcription profile from different *P. aeruginosa* strains revealed that *cbpD* gene expression was elevated in a clinical CF-isolate of *P. aeruginosa* in comparison to a strain isolated from a burn wound, suggesting that CbpD might have a particular role in adherence and colonisation of the CF lung (Sriramulu *et al.*, 2005). Indeed, the work conducted by Manos *et al.,* (2009) also showed that *cbpD* and *chiC* gene expression levels are elevated in clinical CF-strain of *P. aeruginosa* in comparison to a non-CF associated strain, and it also showed that *cbpD* and *chiC* expression was greater in free planktonic cells as opposed to biofilm conditions, which indicates that these chitinolytic proteins have a possible role in establishing early acute infection and initial adherence to lung epithelial cells, rather than later stages of biofilm formation. *P. aeruginosa* has been shown to have elevated *cbpD* gene expression when grown in media containing mucin that mimicked CF-sputum (Fung *et al.*, 2010), which complements the work done in *V. cholerae* (Bhowmick *et al.*, 2008) showing that the production of GbpA was highly sensitive to the presence of mucin in the growth media.

There is also evidence that the chitinolytic phenotype of the Gram positive *Listeria monocytogenes*, causative agent of the severe form of pneumonia Listeriosis, are defective for growth in the mouse liver and spleen (Chaudhuri *et al.*, 2010), and some studies have hypothesised that *L. monocytogenes* could utlilise ChiA to exploit host immune defences within host macrophages (Chatterjee *et al.*, 2006), suggesting chitinases might have an intracellular role in infection in addition to facilitating initial attachment and adherence to target cells. Some very recent *in vivo* work has shown that *L. monocytogenes* ChiA is a key virulence factor in a mouse infection model (Chaudhuri *et al.*, 2013). One of the most interesting features of this study, in contrast to the number of times bacterial chitinases have been shown to facilitate early attachment to target sites, is that *L. monocytogenes* ChiA was shown to facilitate intracellular growth after 72-96 hours post infection (Chaudhuri *et al.*, 2013). Furthermore, *L. monocytogenes* ChiA was shown to actively change the innate immune response of the murine host, since the secretion of ChiA was shown to dramatically decrease the host expression of inducible nitric oxide synthase, which rendered the host environment more permissive to the presence of this bacterium (Chaudhuri *et al.*, 2013).

5.1.2 Chitinases and Inflammatory Bowel Disease

Many of the organisms studied for their chitinolytic abilities are human commensals and/ or (mostly opportunistic) human pathogens, such as *E. coli*, *V. cholerae*, *P. aeruginosa*, *L. monocytogenes* and *Legionella pneumophila*, and it is worth questioning why such organisms exhibit a chitinolytic phenotype, given their mammalian host is incapable of producing a chitin substrate. There is a growing body of work that is beginning to address this question, and one of the most interesting examples is provided by chitinolytic pathogens that colonise the gut. For example, the causative agent of cholera (a severe disease associated with watery diarrhoea and vomiting), *Vibrio cholerae*, encodes four chitinase genes and GbpA (a chitin binding protein). The latter was shown to facilitate binding of *V. cholerae* to intestinal epithelial cells (Kirn *et al.*, 2005) and, furthermore, to bind the 'chitinous' N-glycosylated protein mucin *in vivo* in a murine infection model (Bhowmick *et al.*, 2008). The study also demonstrates that the production of GbpA was enhanced in the presence of mucin in a concentration dependent manner and, in turn, mucin was produced at higher levels in response to the presence of *V. cholerae*, indicating a mutual positive feedback (Bhowmick *et al.*, 2008). This study has demonstrated that the chitinolytic proteins produced by *V. cholerae* can be utilised to promote

adhesion to host cells and appear to have an important role in colonisation of the gut – hence they can be regarded as key players in mediating pathogenesis in the mammalian host.

There are estimated to be 15,000-36,000 different species of bacteria living in the human gut (Frank *et al.*, 2007), and these have a beneficial role in maintaining the health of the small and large intestines, many of which associate into a thick biofilm consisting of bacteria, polysaccharides, DNA and proteins (Macfarlane *et al.*, 2005). In inflammatory bowel diseases such as Crohn's disease or ulcerative colitis, the homeostasis of the gut flora and host-microbial interactions is disrupted as is the intestinal immune balance, resulting in severe bowel disorder (Mizoguchi *et al.*, 2003). A particular variant of *E. coli*, Adherent Invasive *E. coli* (AIEC), which is present in 6% of healthy people, has been identified as having a particularly increased presence (30-50%) in the inflamed mucosa of individuals suffering from inflammatory bowel disorder (Martinez-Medina *et al.*, 2009). Since not every individual bearing AIEC necessarily has Crohn's disease, this bacterium is regarded as an opportunistic human pathogen (Martinez-Medina *et al.*, 2009). AIEC, like other *E. coli* strains, is unable to utilise chitin as a carbon source, but AIEC has also been shown to encode a family 18 chitinase ChiA (Low *et al.*, 2013). A very recent study has shown that deletion of the ChiA encoding gene in AIEC strain LF82 greatly reduced adhesion to intestinal epithelial cells, which it mediates by binding to the chitinase 3-like-1 (CHI3L1) molecule produced by mucosal tissues (Low *et al.*, 2013). The N-glycosylated N68 residue of murine CHI3L1 was shown to be a crucial target site of AIEC LF82 ChiA, and provided a fine example of a chitinolytic enzyme targeting a non-chitin substrate in a virulence model (Low *et al.*, 2013). These authors concluded that AIEC ChiA has an important role in establishing infection in a mouse infection model (Low *et al.*, 2013).

In addition to this, there has been work (Kawada *et al.*, 2008) investigating whether the *S. marcescens* chitinolytic machinery can promote adhesion to mammalian host target sites. When the *S. marcescens cbp21* gene was overexpressed in *E. coli* the adherence to CHI3L1 was greatly enhanced *in vitro* (Kawada *et al.*, 2008). From this *in vitro* study these authors hypothesise that bacterial chitin binding protein may be a key factor in facilitating invasion of colonic epithelial cells in inflammatory bowel conditions (Kawada *et al.*, 2008).

5.1.3 Possible future work in *Serratia marcescens*

Since *S. marcescens* Cbp21, expressed in *E. coli*, was shown to greatly enhance binding to colonic epithelial cells (Kawada *et al.*, 2008) , there is some evidence that the *S. marcescens* chitinolytic machinery is capable of binding to more than just a chitin substrate, and may even facilitate a role in virulence for this opportunist human pathogen. Inspired by all of the work outlined above, here are some interesting questions that could be addressed in *Serratia marcescens*:

- Is the chitinolytic phenotype of *Serratia marcescens* regulated in response to the presence of chitin, or other complex carbohydrates, or in a population dynamic fashion?

- Can *S. marcescens* utilise its chitinolytic repertoire to establish infection in mammalian cells? Or to promote binding/adherence to the surface or epithelial cells, and if so, what is the bacterial chitinase being used to target - cell surface associated GlcNAc, or a chitinase-like protein?

5.2 Harnessing chitin degrading enzymes for biotechnological applications

Chitin is the second most abundant polysaccharide on earth after cellulose. There is an estimated 10^{11} tons of chitin in the biosphere produced annually by fungi, insects, molluscs and crustaceans (Tharanathan and Kittur, 2003). In 2008 the annual commercial production of crustaceans was estimated to be 10.8 million tons (FAO, 2010) a significant proportion of which consists of chitin that is usually dumped as waste by the sea food industry. For example, shrimp makes up 45% of processed seafood and 50-70% weight of the raw material is (mostly) chitin waste (Gortari and Hours, 2013). In this respect, microbial chitinases have a crucial role in recycling environmental chitin, but they have also gathered interest for their possible exploitation in degrading commercial chitin waste. Glucose, ammonia and acetate from chitin are potentially valuable commodities.

5.2.1 Heterologous expression of *chiA* with *cbp21* confers a chitinolytic phenotype to *E. coli* K-12

In a preliminary experiment, we decided to investigate whether heterologous expression of *S. marcescens chiA* in *E. coli* would be sufficient to confer chitinolytic activity on this host. To do this the *S. marcescens chiA* gene was cloned into the pUNI-PROM vector (Jack *et al.*, 2004) for constitutive expression from the *E. coli tat* promoter. The *E. coli* K-12 wild-type strain MG1655 was transformed with pUNI-*chiA* and plated onto LB supplemented with 2% (w/v) colloidal chitin. In this case, no chitinolytic activity was observed (FIGURE 5.2). Next, the *S. marcescens cbp21* gene was cloned downstream of *chiA* on the pUNI-*chiA* vector to give pUNI-*chiA*-*cbp*. Surprisingly, this plasmid was able to confer chitinolytic activity to *E. coli* MG1655 (FIGURE 5.2). The obvious zone of clearing induced by pUNI-*chiA*-*cbp* was not as a result of CBP21 alone, since modification of this vector to encode a version of ChiA lacking its Sec signal peptide (pUNI-*chiA*NOSIG-*cbp*), exhibited a loss of chitinolytic activity (FIGURE 5.2). From this preliminary *in vivo* data it is possible that an intact *S. marsescens* ChiA protein together with an intact *S. marsescens* CBP21 protein are sufficient to confer significant chitinolytic activity to *E. coli*.

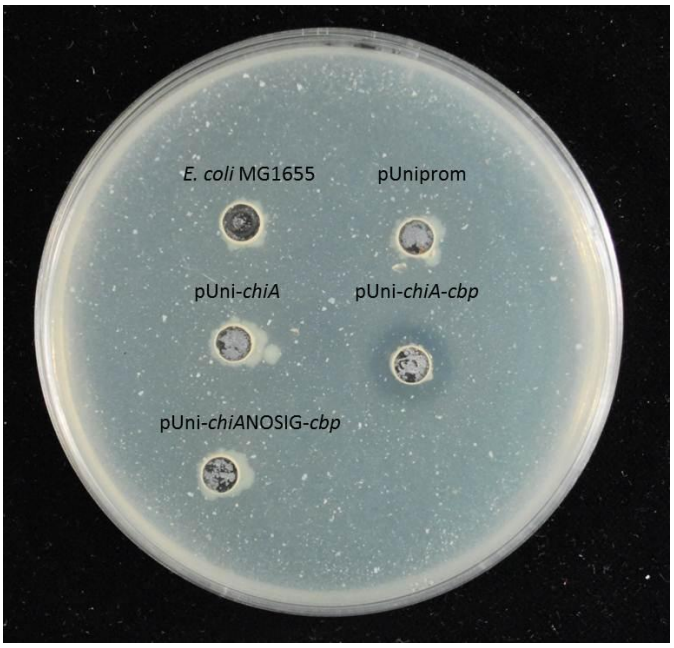


Figure 5.2 The *S. marcescens chiA and cbp21* genes confer a chitin-degrading phenotype on *E. coli* . MG1655, MG1655 + pUni-Prom empty vector, MG1655 + pUni-*chiA*, and MG1655 + pUni-*chiA-cbp21* plated on LB supplemented with 2% colloidal chitin and grown at 37°C for 16 hours.

This was an unexpected and interesting result. It is believed that the Type 2 secretion system in *E. coli* K-12 strain MG1655 is cryptic, begging the question as to how these *S. marcescens* proteins are being secreted. Moreover, since the expression of full length *chiA* in conjunction with *cbp21* is essential to confer a chitin-degrading phenotype in a heterologous host, it is surprising that the targeted removal of *cbp21* did not appear to affect the chitinolytic phenotype of *S. marcescens in vivo* (FIGURE 2.7). This final preliminary experiment brings this project full-circle. Are ChiA and Cbp21 secreted by *E. coli* K-12, or are we observing cell lysis or non-specific shedding of periplasmic contents? Transposon mutagenesis would be an appropriate place to start.

6 Materials & Methods

6.1 Media and Additives

Medium	Components	Quantity (L^{-1})
LB-agar	NaCl	10 g
	Tryptone	10 g
	Yeast Extract	5 g
	Agar	15 g
Luria Bertani medium (LB)	NaCl	10 g
	Tryptone	10 g
	Yeast Extract	5 g
Solid chitin rich media	NaCl	10 g
	Tryptone	10 g
	Yeast Extract	5 g
	Agar	15 g
	Colloidal chitin	20 g
Liquid chitin rich media	NaCl	10 g
	Tryptone	10 g
	Yeast Extract	5 g
	Colloidal chitin	20 g
Chitinase Secretion Assay Substrate Buffer Not sterilised	0.2% (w/v) chitin azure (Sigma)	0.666 g
	Succinate	11.8 g
	Adjusted to pH 6 with NaOH	
Minimal Media (MM)	50x phosphate buffer*	20 ml
	10% (w/v) $(NH_4)_2SO_4$*	10 ml
	1M $MgSO_4$*	0.41ml
	Agar	16 g

Medium	Components	Quantity (L^{-1})
MM Agarose	50x phosphate buffer*	20 ml
	10% (w/v) $(NH_4)_2SO_4$*	10 ml
	1M $MgSO_4$*	0.41ml
	20% glucose*	10 ml
	Ultra-Pure Agarose*	15 g
MM Glucose	50x phosphate buffer*	20 ml
	10% (w/v) $(NH_4)_2SO_4$*	10 ml
	1M $MgSO_4$*	0.41ml
	20% glucose*	10 ml
	Agar (for solid media only)	16 g
MM High sucrose	50x phosphate buffer*	20 ml
	10% (w/v) $(NH_4)_2SO_4$*	10 ml
	1M $MgSO_4$*	0.41ml
	50% sucrose*	200 ml
	Agar	16 g

Table 6.1 Growth media. Constituents were sterilised by autoclaving unless indicated otherwise. Components indicated by an asterisk in were sterilised individually and then added to the media afterwards.

Media Supplement	Stock concentration	Sterilised	Quantity (L^{-1})	Final concentration
DMSO	20%	filter	20 ml	0.4%
glucose	20%	filter	10 g	0.2%
glycerol	50%	autoclave	10 ml	0.5%
IPTG	1M	filter	1 ml	1 mM
SDS	20%	N/A	100 ml	2%
TMAO	20%	filter	20 ml	0.4%

Table 6.2 Media supplements.

Antibiotic	Stock concentration	Prepared in	Volume used ($100ml^{-1}$)	Final concentration
Ampicillin	125 mg ml^{-1}	Water	80 µl	100 µg ml^{-1}
Kanamycin	50 mg ml^{-1}	Water	100 µl	50 µg ml^{-1}
Streptomycin	100 mg ml^{-1}	Water	100 µl	100 µg ml^{-1}
Tetracycline	5 mg ml^{-1}	70% ethanol	200 µl	10 µg ml^{-1}

Table 6.3 Antibiotics. All filter sterilised prior to use

6.1.1 Preparation of Colloidal Chitin

Colloidal chitin was prepared by slowly adding 40g shrimp shell chitin to 1 L 37% HCl, and this was left to stir gently at 4^{o}C for 5 days, after which it was added to 2 L of distilled water at 4^{o}C and left to settle for 2 day. The pH was then adjusted to 7.5 with repeated wash steps using Tris and 50% KOH, and the colloidal chitin was finally harvested by centrifugation and autoclaved.

6.2 Buffers and Solutions

6.2.1 General Buffers and Solutions

Buffer/Solution	Component
APS	10% ammonium persulphate
Carbonate transfer buffer (CTB)	10 mM $NaCHO_3$ pH 9.9 3 mM Na_2CO_3 20% (v/v) methanol
Coomassie stain	0.1% (w/v) coomassie brilliant blue (R25)
DNA loading buffer	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol blue 40% (w/v) sucrose

Buffer/Solution	Component
EDTA	1.5 mM EDTA pH 7.5
Laemmli sample buffer (2x)	62.5 mM Tris (HCl) pH 6.8 2% (w/v) SDS 5% (v/v) β-mercaptoethanol 25% (v/v) glycerol 0.01% (w/v) bromophenol blue
Lysozyme	5 mg ml^{-1} lysozyme (from chicken egg white) Dissolved in water
50 x Phosphate buffer pH 7.0	350 g L^{-1} K_2HPO_4 100 g L^{-1} KH_2PO_4
1x Phosphate Buffered Saline (PBS)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBS-Tween (TBS-T)	1x PBS 0.1% (v/v) Tween 20
SDS-running buffer (5x)	25 mM Tris (HCl) pH 8.3

Buffer/Solution	Component
	192 mM glycine
	0.5% (w/v) SDS
Sucrose Buffer	58.8 mM Tris-acetate pH 7.8
	735 mM sucrose
TAE buffer	40 mM Tris (HCl) pH 8.0
	1.142% (v/v) acetic acid
	1 mM EDTA
Towbin Buffer (1 L)	3.03 g Tris
	14.4 g Glycine
	850 ml MeOH
	150 ml H_2O
Tris buffered saline (TBS)	50 mM Tris (HCl) pH 7.6
	150 mM NaCl
Tris-Sucrose buffer	50 mM Tris (HCl) pH 7.5
	40% (w/v) sucrose

Table 6.4 Table Buffers and solutions employed for general procedures.

6.2.2 Chromatography Buffers

Nickel affinity Buffers	Components
Nickel buffer A	50 mM Tris (HCl) pH 7.5 100 mM NaCl 12 mM Imadazole
Nickel buffer B	Buffer A plus 500 mM Imidazole
Urea Buffer	Buffer A plus 5 M Urea

Table 6.5 Nickel sepharose affinity chromatography buffers.

Size Exclusion Buffers	Components
SEC Buffer	50 mM Tris (HCl) pH 7.5 150 mM NaCl

Table 6.6 Size exclusion chromatography buffers.

6.3 Culture Conditions

6.3.1 Bacterial strains and growth conditions

The growth media and supplements used in this study are detailed in Section 6.1 and 6.2. Aerobic growth of strains in liquid culture was achieved by agitation at 200 rpm or higher and by maintaining a minimum 1:4 ratio of liquid to air in the culture vessel. *S. marcescens* was routinely grown aerobically in LB medium at 30°C. For the microscopy experiments and for the label-free mass spectrometry experiments *S. marcescens* was grown in minimal medium glucose pH 7.8. When required, media were supplemented with agar to 1.5% (w/v) and antibiotics to the final concentrations: ampicillin, 100 µg/ml; kanamycin, 50-100 µg/ml; streptomycin, 100 µg/ml; and tetracycline, 10 µg/ml.

For long term storage, cells were frozen in LB following addition of 25% (v/v) glycerol in Cryotub™ vials (Nunc) tubes, at -80˚C.

6.4 Genetic Manipulations

6.4.1 Plasmid DNA preparation

Purification of plasmid DNA was performed using a plasmid miniprep kit supplied by Qiagen. The basis of this procedure is the alkaline lysis method of plasmid extraction as described by Birnboim and Doly (1979). Cells were prepared from an overnight culture in 5 ml LB medium in addition to any required antibiotics (TABLE 6.3). Cells were harvested by centrifugation at 6238 x g for 10 minutes in an AccuSpin benchtop centrifuge (Fisher) with subsequent resuspension and alkaline lysis of the cells. The lysate, following neutralisation and subsequent centrifugation was washed over a silica membrane, which selectively absorbs plasmid DNA in the presence of high salt buffer. Bound plasmid DNA was eluted by a further wash step in the supplied elution buffer.

6.4.2 Polymerase Chain Reaction (PCR)

In this report, the DNA polymerases Gotaq (Promega) and Expand Long Template (Roche) or Herculase (Stratagene) were the standard polymerases for general work and high-fidelity amplifications, respectively. Gotaq polymerase was employed for PCR in which high fidelity was unimportant. The Expand Long Template and the Herculase enzymes possess an exonuclease activity that serves to proof-read the extending strand and increases the fidelity of the reaction. For long PCR products (>2 kb) requiring high fidelity, the Expand High Fidelity PCR system (Roche) was used. A standard PCR program used a 95˚C/30s denaturation step, a 55˚C/30s annealing step, a 72˚C elongation temperature and approximately 30 cycles. For each kilobase to be amplified an extension time of 1 min was required. For each reaction (typically 50 µl for standard PCR) a mix comprising 1 µM of each primer, 0.2 mM dNTP (supplied by Roche), 1 µl of DNA template, 1 µl of polymerase and a final volume of 1x reaction buffer, made up to the final volume with H_2O. In most cases the template for PCR was either chromosomal DNA or plasmid DNA purified using the Qiagen Blood & Tissue Kit or miniprep method respectively. When using chromosomal DNA as template or when using primers with a high degree of secondary structure it was useful to include 5% (v/v) DMSO to the reaction mixture to serve as a denaturant. The resultant PCR product, if intended for subsequent use such as cloning, was purified using either QIAquick PCR Purification or QIAquick Gel Extraction (Qiagen) using the provided kits. Gel extraction was most often used at this stage, particularly for the purification of PCR products from a plasmid template as this method enables the direct visualisation of a product band of the appropriate size following agarose gel electrophoresis, but also allows the precise excision and purification of product to the exclusion of any plasmid template.

6.4.3 Sequencing PCR

DNA sequencing was employed to ensure that the products of PCR contained no undesired mutations. The DNA sequencing service was provided by the University of Dundee in-house sequencing facility. Sequencing reactions were performed with 200-300 ng DNA template, 1 µl of 3.2 µM of the desired sequencing primer, and made up to 15 µl with H_2O. The template DNA is usually prepared by plasmid miniprep using a Qiagen kit, or gel extracted DNA following a standard PCR from a chromosomal template. The returned DNA sequence was analysed using the National Centre for Biotechnology Information (NCBI) basic local sequence alignment tool (BLAST; (Altschul *et al.*, 1990)).

6.4.4 Digestion by Restriction Endonucleases for Cloning

Digestion of DNA using restriction endonucleases was carried out in the manufacturer supplied buffer. In cases where two enzymes were used, an appropriate buffer was selected to permit efficient activity of both enzymes. If this was not possible then the two digestions were carried out separately, after the first digestion the DNA was 'cleaned' using a Strataclean resin (Agilent technologies). A typical restriction digestion used 1 µl of each enzyme (10 U/ µl), buffer diluted to 1x concentration, a variable amount of DNA template, and water up to 30 µl final volume. Digestions were carried out at 37˚C for 30 min- 3h. Following restriction enzyme digests, cut vectors were treated with alkaline phosphatase (Roche). This enzyme removes phosphate groups from the 5' end of DNA molecules, preventing re-ligation of the cut vector without the addition of insert: 3 µl of alkaline phosphatase was added to the restriction digest and incubated at 37˚C for 30 min before addition of another 3 µl of alkaline phosphatase for a further 30 min. Digested PCR products for cloning were purified by QIAquick PCR Purification (Qiagen) according to kit instructions to remove restriction endonucleases. Digested vectors for cloning were purified using the QIAquick gel-extraction kit (Qiagen): in this case, the digestion product was run on an agarose gel and the cut band of the correct size was excised from the gel and purified according to kit instructions.

6.4.5 DNA Ligation

Vector and insert were mixed in a 2 µl: 6µl ratio and incubated in 1x ligation buffer and 2 µl of T4 DNA ligase (Roche) in a final volume of 20 µl and incubated at 16˚C overnight. Following ligation, the entire 20 µl ligation mixture was transformed into the appropriate cloning strain (TABLE OF STAINS).

6.4.6 Agarose Gel Electrophoresis

DNA samples were analysed by agarose gel electrophoresis using 1% (w/v) agarose gels prepared in 1x TAE and containing 1:500,000 dilution of GelRed Nucleic Acid Stain (Biotium). DNA was mixed in a 10:1 ratio with 10x DNA loading dye and loaded into wells of the gel, which was then run in 1x TAE buffer at approximately 100 V. DNA size markers (Roche) were run alongside the samples and all were visualised after separation under UV light.

6.4.7 Transformation of Competent Cells with Plasmid DNA

Chemical transformation: 5 ml of liquid LB plus any required antibiotics was inoculated with 1:100 dilution of an overnight culture of the desired strain and incubated at 37˚C with agitation until the mid-log phase of growth (~90 min). The cell cultures were then centrifuged at 6238 x g for 10 min in an AccuSpin benchtop centrifuge (Fisher) and the resultant cell pellets resuspended in 500 µl transformation buffer (TSB). The resuspended cells were then kept at 4˚C for at least 30 minutes before storing at -80˚C or were used immediately in transformation. For each transformation, 200 µl of competent cells were used, and incubated with 1 µl of the desired plasmid DNA at 4˚C for 10 min. The cells were then heat shocked by incubation at 42˚C for 90 s before returning to 4˚C for a further 5 min. To each transformation, 1 ml of liquid LB was added followed by incubation at 37˚C for 1 h to allow for phenotypic expression of the plasmid encoded antibiotic resistance genes. Cells were then centrifuged at 16100x g for 1 min in an Eppendorf 5415 D centrifuge and the supernatant removed. Resuspended cells were then spread onto a plate of LB-agar containing any required antibiotics and incubated at 37˚C overnight.

Electroporation: 25 ml of LB broth medium supplemented with the appropriate antibiotics was inoculated 1:100 from an overnight culture and incubated at 30˚C with shaking at 200 rpm to an optical density at 600 nm (OD_{600}) of 0.4 – 0.6. Upon reaching the required OD, cell cultures were kept at 4˚C for 30-40 min before centrifugation at 3000 x g for 10 min in an AccuSpin benchtop centrifuge (Fisher) at 4˚C and the resulting pellet re-suspended gently in 10 ml ice-cold H_2O. This centrifugation and subsequent re-suspension was repeated twice, first re-suspending again in H_2O and then in 10% glycerol, both kept at 4˚C before use. For the preparation of competent cells of *Serratia marcescens*, wash steps in water were substituted by washes in ice-cold 10% glycerol. Cells were then pelleted once more before re-suspension in 250 µl of ice-cold 10% glycerol. 50 µl of electrocompetent cells were then added to 1 µl of plasmid DNA and incubated on ice for 10 min. These cells were then transferred using chilled pipette tips to a Molecular BioProducts (San Diego, CA) 2 mm electroporation cuvette (kept until this stage at -20°C). An electrical pulse with the following settings; 2.5 kV voltage, 25 µF capacitance, 200 Ω resistance, 2 mm cuvette length; was applied to the cuvette using a BioRad (Hercules, CA) GenePulser Xcell electroporator. 1 ml of LB medium at room temperature was added to the electroporation cuvette and the cells mixed by gentle pipetting. Cells were then transferred to an eppendorf tube and incubated at 37˚C for 1 h. All of the cells were pelleted and resuspended in 150 µl of LB, and then spread onto LB-agar supplemented with the required antibiotics and incubated at 30˚C overnight.

6.4.8 Chromosomal deletions using the pNKG101 system

Deletion strains of *S. marcescens* were constructed by allelic exchange using the suicide vector pKNG101 as described previously (Kaniga *et al.*, 1991). Briefly, this involved constructing the desired deletion allele in pBluescript before moving the allele onto pKNG101. The pKNG101 constructs were introduced into *S. marcescens* by conjugation followed by selection on MM streptomycin and then MM sucrose, which, following a round of PCR-based screening, allowed isolation of mutants in which the native gene had been replaced with the deletion allele.

Strains, JJH04w (Δ*chiW*), JJH05x (Δ*chiX*), JJH06y (Δ*chiY*) and JJH07z (Δ*chiZ*) were prepared as in-frame deletions that preserved all initiation and termination codons, putative ribosome binding sites and coding sequences of flanking genes. Strain JJH08p is devoid of the entire coding sequence of *chiW* and also lacks the initiation codon of *chiX*, and therefore exhibits a Δ*chiW*, Δ*chiX* genotype (Supp Table S2). The JJH01 (Δ*chiA*), JJH02 (Δ*chiB*), JJH03 (Δ*chiC*), JchiA (Δ*chiB*, Δ*chiC*), JchiB (Δ*chiA*, Δ*chiC*) and JchiC (Δ*chiA*, Δ*chiB*) strains are devoid of the coding sequence of *chiA*, *chiB* or *chiC* respectively, these were each made as in-frame deletions where the entire coding sequence was removed with the exception of the initiation and termination codons .

6.4.9 Transposon Mutagenic Screen

Transposon mutagenesis of the JchiA (*chiA*$^{+}$, Δ*chiB,* Δ*chiC*), JchiB (*chiB*$^{+}$, Δ*chiA,* Δ*chiC*) and JchiC (*chiC*$^{+}$, Δ*chiA,* Δ*chiB*) strains were performed separately using *E. coli* donor strain SM10 λpir [pUTmini-Tn5$^{Sm/Sp}$] as outlined by (de Lorenzo *et al.*, 1990). Briefly, this procedure involved patch mating *E. coli* donor strain with *S. marcescens* recipient strain and also HH26 (pNJ5000) helper strain, normalised to OD_{600} 1.0, in a 1:1:3 ratio respectively, from which 40 µl was spotted onto an LB agar plate and incubated at 30 ^{o}C for 16 hours. The patch mated colony was then suspended in 1 ml LB and diluted 10^{-2} in MM streptomycin (100 µg/ ml), from which 100 µl was spread on MM streptomycin plates (in order to select for Tn5 insertion) and incubated at 30 ^{o}C for 48 hours. Individual JchiA, JchiB and JchiC colonies that contained Tn5 were patched onto LB agar plates supplemented with 2% (w/v) colloidal chitin. Strains that exhibited a loss of chitin-degrading phenotype (no chitinolytic zone of clearing) were then patched on to fresh colloidal chitin media to check the chitinolytic phenotype (in comparison to the parental strain) had been disrupted.

6.4.10 Single Primer Specific PCR for Mapping Tn5 Insertions

Transposon insertion sites were mapped using single primer specific PCR (Shyamala and Ames, 1989) where gDNA was digested with XhoI and PstI and then ligated into pBluescript followed by a round of PCR with vector- and Tn5-specific primers. In this case, after restriction enzyme digestion, it was the gDNA that was phosphatase treated, not the vector. PCR products not present in the *S. marcescens* Db10 control gDNA were sequenced with Tn5 primers and the positions of the Tn5 insertions within the *S. marcescens* genome were located by BLAST (Altschul *et al.*, 1997) analysis of the *S. marcescens* Db11 genome, of which Db10 is the direct parent, using the publicly-available server at http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html. For further genetic and mass spectrometric analyses, the complete *S. marcescens* Db11 genome sequence and preliminary gene prediction were also obtained from the Sanger Institute at the location above.

6.5 Protein Methods

6.5.1 Overproduction of His_6-tagged protein from an IPTG-inducible promoter

SMA0468 (ChiC), SMA2875 (ChiB) and SMA4243 (ChiA) were overproduced in *E. coli* as C-terminally hexa-Histidine-tagged proteins from plasmids based on pQE60 (Qiagen). The plasmid encoding *S. marcescens* ChiA, ChiB or ChiC was transformed into *E. coli* M15 containing the pREP4 plasmid encoding the *lac* repressor. From the resultant colonies on an LB-agar plate incubated at 37˚C overnight, single colonies were used to inoculate 5 ml liquid LB including the appropriate antibiotics, which was then grown at 37˚C overnight with agitation at 200 rpm. 500 ml of liquid LB plus the appropriate antibiotics was then inoculated with a 1:100 dilution of the overnight culture and incubated at 37˚C with shaking at 200 rpm until the OD_{600} > 0.4 (~2 h). Expression of the vector-borne gene of interest was induced by the addition of 1.5 mM isopropyl β-D thiogalactopyranoside (IPTG; Sigma), activating expression from the *lac* promoter. After a further 16 h growth the cells were harvested by centrifugation at 4539.5 x g for 20 min in a Beckman JS-4.2 rotor.

6.5.2 Small Scale Solubility Tests

We also performed small scale solubility tests, whereby 5 ml cultures (as outlined in Section 6.5.1) expressing ChiA, ChiB, ChiC were overproduced for 3 hours, then resuspended in 5 ml 50 mM Tris-Hcl and lysozyme, and sonicated for 1 minute at 16 % amplitude, with pulses for 4 seconds on and 4 seconds off. The lysed cultures were then centrifuged at 3000 x g for 10 min in an AccuSpin benchtop centrifuge. The supernatant contained the soluble fraction, from which a sample was mixed 1:1 with laemmli buffer. The insoluble lysed pellet was resuspended in the same volume of 50 mM Tris-Hcl, and a sample was also mixed 1:1 with laemmli buffer. The different samples were analysed using SDS-PAGE.

6.5.3 Purification of His_6-tagged ChiA, ChiB and ChiC to generate polyclonal antisera

The pellets obtained according to Section 6.5.1 were resuspended in 10 ml 50 mM Tris (HCl) pH 7.5 per g of cell pellet. To the cell suspension, 50 µl Protease Inhibitor Cocktail 3 (Calbiochem) per g cell pellet, lysozyme and DNAase was added. Cells were broken by two passages through a French pressure cell at ~8000 psi. The French pressure effluent was then centrifuged at 17210.7x g in a Sorvall SS-34 rotor for 12 min to remove unbroken cells and other large cellular debris from the crude protein in the supernatant. For the purification of soluble ChiC, the presence of the C-terminal His_6-tag on the protein of interest allowed for the specific purification of the overproduced protein directly from a crude protein extract by nickel-affinity chromatography, whereas for insoluble ChiA and ChiB, the lysed debris (with inclusion bodies) was resuspended in 50 ml Buffer A containing 5 M Urea and gently stirred for 3 days before being applied to the nickel-affinity chromatography column. In all cases a Ni(II) column (5 ml HisTrap^{TM} HP column from Amersham Biosciences) was used in conjunction with an ÄKTA FPLC system (GE Healthcare). The column was first equilibrated with 2-3 column volumes of nickel buffer A (TABLE 6.5). The crude cell extract was then applied to the column at a flow rate of 1 ml min^{-1} and the unbound flow-through collected if desired to ascertain successful binding of the protein to the column by SDS-PAGE. Non-specifically bound proteins were eluted by further washing through the column with nickel buffer A until the elution profile displayed no further protein eluting from the column. Bound protein was then eluted in a 0-100% gradient of nickel buffer B over 30 min and collected in 2 ml fraction tubes. Fractions containing the protein of interest were concentrated using a 10 kDa cut-off spin concentrator (Vivascience) and centrifugation at 3000 x g in an AccuSpin benchtop centrifuge (Fisher) before overnight dialysis in 1.91 ml cm^{-1} volume, 8 kDa molecular weight cut-off dialysis tubing (supplied by BioDesign) into 50 mM Tris (HCl) pH 7.5, 150 mM NaCl to remove unwanted imidazole from the protein sample.

Following immobilised metal affinity chromatography the purified ChiA protein was used to raise rabbit polyclonal antisera (Eurogentec, Belgium), whereas ChiB and ChiC were put through an additional size exclusion chromatography step. Protein identification was by tryptic peptide mass fingerprinting following separation by SDS-PAGE (FingerPrints Proteomics Service, University of Dundee).

6.5.4 Size Exclusion Chromatography (SEC)

Separation by size exclusion was performed using a Superdex^{TM} column (Amersham Biosciences) in conjunction with an ÄKTA FPLC system (GE Healthcare). The column was first equilibriated with 2-3 column volumes of SEC buffer (TABLE 6.6). 500 µl of purified protein was applied to the column at a flow rate of 0.5 ml min^{-1} and eluted in 50 mM Tris (HCl) pH 7.5 / 150 mM NaCl. Fractions were collected in 5 ml tubes. The desired fractions were pooled and concentrated using a 10 kDa cut-off spin concentrator (Vivascience) and centrifugation at 3000 x g in an AccuSpin benchtop centrifuge (Fisher) and dialysed overnight against 50 mM Tris (HCl) pH 7.5 using 1.91 ml cm^{-1} volume, 8 kDa molecular weight cut-off dialysis tubing (BioDesign).

Following immobilised metal affinity chromatography and size exclusion chromatography, the purified ChiB and ChiC proteins were used to raise rabbit polyclonal antisera (Eurogentec, Belgium). Protein identification was by tryptic peptide mass fingerprinting following separation by SDS-PAGE (FingerPrints Proteomics Service, University of Dundee).

6.5.5 SDS-PAGE

SDS-PAGE was performed according to the principle described by Laemmli ((Laemmli, 1970)) using the BioRad mini protean III system. Protein samples were diluted in a 1:1 ratio with Laemmli sample buffer and boiled for 2 min. Samples were then loaded onto an SDS-PAGE gel (percentage acrylamide varies) in a BioRad gel electrophoresis tank filled with 1x SDS running buffer. The gel was run at 100 V until the dye front had crossed from the stacking gel and into the resolving gel and then increased to < 200 V. Molecular weight protein standards (BioRad) were run alongside the protein samples. Proteins were visualised by treatment with coomassie stain (InstantBlue, Expedeon) with shaking. After incubation for at least 2 hr the gel was removed from the coomassie stain. Gels were incubated in water with shaking until the background staining of the gel had sufficiently diminished.

Resolving Gel (15%)	Volume (ml)
1 M Tris (HCl) pH 8.8	2
H_2O	2.6
30% Acrylamide	3.2
10% SDS	80 µl
10% APS	80 µl
Temed	8 µl

Stacking Gel	Volume (ml)
0.5 M Tris (HCl) pH 6.8	1.25
H_2O	2.6
30% Acrylamide	1
10% SDS	50 µl
10% APS	50 µl
Temed	5 µl

Table 6.7 SDS-PAGE gels. Constituents of the resolving and stacking gels for the preparation of 12% acrylamide gels for SDS-PAGE.

6.5.6 Western Immunoblot

For Western immunoblotting protein samples were separated by SDS-PAGE on 12% (w/v) polyacrylamide gels. Samples were electroblotted at 250 mA for 90 minutes in Towbin buffer onto polyvinylidene difluoride (PVDF) (Millipore). ChiA, ChiB, ChiC, MBP (New England BioLabs), RNAP (NeoClone) or GS (Javelle *et al*., 2004) were detected by hybridization of the primary antibody (ChiA, ChiB, ChiC, MBP at 1:20,000 dilution, RNAP at 1:40,000 and GS at 1:10,000) followed by the secondary antibody, horseradish peroxidase (HRP)-conjugated goat antirabbit or antimouse antibody (BioRad; 1:10,000), and then exposed using an enhanced chemiluminescent detection kit (Millipore).

6.5.7 Protein Concentration Determination

The protein concentration of purified samples of ChiA, ChiB and ChiC was determined by measuring the absorbance at 280 nm (A_{280}) using a Nanodrop spectrophotometer (labtech) after deducting an appropriate blank (50 mM Tris-Hcl, 150 mM NaCl) reading. An accurate concentration using this method required the manual input of the predicted molecular weight and the molar extinction coefficient of the protein. These were obtained using the ExPASy ProtParam server (http://www.expasy.org/tools/protparam.html).

6.5.8 Label Free Mass Spectrometry

S. marcescens Db10 and JJH08p (Δ*chiW*, Δ*chiX*) secreted protein samples were prepared for label-free mass spectrometry by first growing cells in 50 ml minimal media at 30°C aerobically for 16 hours. Cultures were centrifuged at 5000 x *g*, 4 °C, 30 min, the supernatant decanted and the cell pellet discarded. This procedure was repeated eight times. Next, 2.6 ml of a 6.1 N trichloroacetic acid solution (approx. 100% w/v) was added to 40 ml of culture supernatant and proteins were precipitated overnight at 4 °C. The protein precipitate was collected at 5000 x g, 4 °C, 30 min and the supernatant discarded. The protein precipitates were then washed four times in 1 ml 80% acetone (–20°C) before being dried at room temperature under laminar flow hood for 45 min.

The following section was carried out by Dr Matthias Trost and Manman Guo, PPU, University of Dundee: Samples were resuspended in 500 µl 20 mM Tris-HCl pH 8.0, 4 % (w/v) SDS, 1 mM TCEP 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 FASP protocol (Manza *et al.*, 2005; Wisniewski *et al.*, 2009) and digested using trypsin (sequencing grade, Promega). About 0.5 µg of each digest was injected in an interleaved manner onto a 2 cm x 100 µm trap column and separated on a 15 cm x 75 µm Pepmap C18 reversed-phase column (Thermo Fisher Scientific) on a Dionex Ultimate 3000 RSLC. Peptides were eluted by a linear 2-hour gradient of 95% A/5% B to 35% B (A: H_2O, 0.1% Formic acid (FA); B: 80% ACN, 0.08% FA) at 300 nl/min into a LTQ Orbitrap Velos Pro (Thermo-Fisher Scientific). Data was acquired using a data-dependent "top 20" method, dynamically choosing the most abundant precursor ions from the survey scan (350-1650 Th, 60,000 resolution, AGC target value 10^6). Precursors above the threshold of 500 counts were isolated within a 2 Th window and fragmented by CID in the LTQ Velos using normalised 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 polydimethylcyclosiloxane (PCM) (Fritsch *et al.*, 2013).

Label-free quantitation was performed using MaxQuant v1.3.0.5 (Cox and Mann, 2008). Mass spectrometric runs of 4 biological replicates of Db10 and JJH08p 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 2 missed cleavage, fixed modifications were carbamidomethyl (C), variable modifications were set to Acetyl (Protein N-term) and Oxidation (M). MS/MS tolerance was set to 0.5 Da, precursor tolerance was set

to 6 ppm. Peptide and Protein FDR was set to 0.01, minimal peptide length was 7, and one unique peptide was required. Re-quantify and retention time alignment (2 min) were enabled.

6.6 Cell Fractionation

6.6.1 Separation of whole cell and extracellular supernatant fractions

For the separation of supernatant and whole cell fractions, cultures were grown for 16 hours in LB medium at 30 ^{o}C, the cells were harvested by centrifugation at 16,000 x g. The supernatant was carefully removed (supernatant fraction), and the cell pellet was resuspended in LB (whole cell fraction). Samples were mixed 1:1 with laemmli buffer and boiled for 3 min.

For small scale protein secretion assays strains were grown in 100 ml volumes with shaking 220rpm at 30 ^{o}C for a total of 120 hours. The OD_{600} for each sample was normalized before whole cell and supernatant fractions were collected by centrifugation at 16,000 x g. Culture supernatants were subjected to a further ultrafiltration step through a 0.2 μm device (Millipore). Samples were taken at 16 hr, and 24 hr, 48 hr, 72 hr intervals thereafter. Samples were boiled for 10 minutes at 1:1 ratio with Laemmli disaggregation buffer (Sigma).

6.6.2 Separation of extracellular/ cytoplasmic/ periplasmic/ total membrane fractions

For preparation of cytoplasmic, total membrane, periplasmic and secreted (culture supernatant) fractions, we performed a modified version of the protocol outlined by (Osborn and Munson, 1974). 500 ml cultures grown for 16 hours in LB medium at 30 ^{o}C, the cells were harvested by centrifugation at 8000 x g, supernatant was removed and then put through an ultrafiltration step using a 0.2 μm device (Millipore). Next, 1 g cell pellet was gently suspended in 6.8 ml sucrose buffer (58.8 mM Tris-acetate pH 7.8, 735 mM sucrose) to which 1.2 ml 5 mg/ml lysozyme was added and gently stirred for 2 minutes, 200 μl was removed (whole cell fraction), then 16 ml of cold 1.5 mM EDTA pH 7.5 was added and gently stirred for 8 minutes at 4 ^{o}C. The sample was centrifuged at 8000 x g and 5 ml supernatant (periplasmic fraction) was put through 0.2 μm filter. The sphaeroplast pellet was homogenised in 24 ml of lysis buffer (16.6 mM Tris-acetate pH 7.8, 1 mM EDTA, small crystal of DNAse, protease inhibitors) and 200 μl sample was removed (sphaeroplast fraction). The cells were then lysed and centrifuged to remove cell debris and 1 ml of the remaining crude cell extract was submitted to ultracentrifugation at 80,000 rpm for 30 mins, after which 200 μl supernatant was removed (cytoplasmic fraction). The total membrane pellet was homogenised in 1 ml buffer (50mM

Tris-HCl, 500mM NaCl) and the ultracentrifugation step was repeated. The 'washed' membrane pellet was resuspended in 800 µl of the same buffer and 200 µl was removed (membrane fraction).

6.7 Chitinolytic Activity Assays

6.7.1 Secretion Assay

Protocol adapted from (Coulthurst *et al.*, 2006). Cultures of each strain were grown in LB for 16 h. Culture samples were centrifuged at 16,000 g, and the supernatant was carefully removed at kept on ice. A 200 µl sample of culture supernatant was mixed with 400 µl of secretion assay substrate buffer (TABLE 6.1), which was incubated at 37 ^{o}C for 72 h. The samples were then centrifuged at 16,000 g for 5 min and the A_{560} of the supernatant measured. Chitinolytic activity was measured as ΔA_{560} h^{-1} ml^{-1} per OD_{600} unit with respect to a blank incubated with just LB (not culture supernatant).

6.7.2 Solid Chitin-Rich Media Plate Assay

Cultures were grown in LB for 16 hr at 30 ^{o}C. The OD_{600} of each culture was normalised, and 15 µl of each culture was spotted onto solid chitin rich media (TABLE 6.1) and incubated at 30 ^{o}C for 48 hr. Chitinolytic activity was observed as a zone of clearing around the colony.

6.7.3 Wax Moth Larvae Killing Assay

Protocol adapted from (Murdoch *et al.*, 2011). To determine whether the chitinolytic activity of *S. marcescens* is important for virulence in an insect model, overnight cultures of *S. marcescens* Db10 strains were diluted 10^{-6} and 10^{-8} in 1x PBS. Wax moth larvae (*Galleria mellonella*) were warmed from 4 ^{o}C to room temperature, and were injected with 10 µl of each dilution using a Hamilton syringe via the hind left proleg (10 larvae per dilution). Ten larvae were injected with just PBS. After the injection, the larvae were incubated at 25 oC for 24 hr, afterwhich they were scored as dead or alive. Dead larvae were very easy to score as they would turn black and harden.

6.7.4 Heterologous expression of *S. marcescens* ChiA and Cbp21 in *E. coli*

Serratia marcescens chiA was amplified using a primer that introduced *Bam*H1 and *Xba*1 restriction sites at 5' and 3' ends respectively, and *cbp21* was amplified using primers that introduced *Xba*1 and *Hind*III sites at the 5' and 3' ends respectively. The PCR product was purified by gel extraction (Qiagen). First, the *cbp21* gene product was cut with the restriction enzymes listed above, and cloned in to the expression vector pUniprom cut with the same enzymes, then chiA was cloned into the same vector using the appropriate restriction enzymes. To clone the gene encoding ChiA without the N-terminal Sec signal peptide, the region encoding the mature ChiA was amplified and cloned into the same vector above from which full length *chiA* had been removed. The absence of the N-terminal signal region was confirmed by sequencing. The constructs were transformed into *E. coli* MG1655, and grown overnight at 30 ^{o}C in LB ampicillin, and then 15 µl was spotted onto MM colloidal chitin and grown at 30 ^{o}C for 48 hr. Chitinolytic activity was observed as a zone of clearing around the colony.

6.8 Microscopy

Fluorescent reporter strains were constructed using the pKNG101 suicide vector (Kaniga *et al.*, 1991) to integrate gene replacements onto the *S. marcescens* chromosome. The fluorescent protein mKate (Pletnev *et al.*, 2008) was chosen for its reported brightness and photostability**.** In this work, a gene replacement allele was prepared, Δ*chiX::*mKate. In this case, the *chiX* gene was deleted in-frame and a cassette encoding mKate, including an optimised RBS, was integrated as a replacement. As a result we made a transcriptional fusion strain, ChiXmKate (Δ*chiX*::mKate), which is also a *chiX* mutant and so defective in chitinase externalisation. Another strain was constructed, Db10*::chiA-gfp*, that was positive for chitinase secretion (the native *chiWXYZ* operon was intact), and encoded full length ChiA, but also encoded GFP 23 base pairs downstream of the *chiA* termination codon. The Db10*::chiA-gfp* strain was considered to be a transcriptional fusion between *chiA* and *gfp*.

6.8.1 Visualisation of cells with static microscopy

Cells were grown in LB aerobically overnight at 30 ^{o}C with shaking 220 rpm. The following day the overnight culture was subcultured 25 µl in 5 ml fresh LB and grown for 16 hr at 30 ^{o}C with shaking 220 rpm. Cells were then pelleted at 8000 g, resuspended in 5 ml PBS, then spun again at 8000 g and resuspended in 10 ml PBS. Microscope slides 75 x 25 x 1.2 mm (VWR) were then prepared to visualise the strains: these were prepared by filling a 125 µl adhesive gene frame (ABgene) with molten MM agarose, which was then covered with a glass slide in order to flatten the MM agarose surface. Once the MM agarose layer had set, the cover slide was removed, and the MM agarose layer was also carefully removed with a scalpel leaving behind 2 thin strips. Then 1.2 µl of the strains were spotted onto the MM agarose strip. These were allowed to dry for ~1 minute and were then covered with a 22 x 40 mm glass cover glass plate (VWR).

The following section was carried out by Dr Victoria L. Marlow and Dr Nicola R. Stanley-Wall, University of Dundee: Images were acquired using a DeltaVision Core widefield microscope (Applied Precision) mounted on an Olympus IX71 inverted stand with an Olympus 100X 1.4 NA lens and Cascade2 512 EMCCD camera (Photometrics). Datasets (512 × 512 pixels with 13 Z sections spaced by 0.2 µm were acquired with Differential interference contrast (DIC) and fluorescence optics. DIC images were acquired with an LED Transmitted light source (Applied precision) at 32 % intensity and exposure times between 25 and 50 ms.

6.8.2 Visualisation of cells with time lapse microscopy

Cells were grown in LB aerobically at 30 °C with shaking 220 rpm for 8 hr, and then subcultured (25 µl into 5 ml) into MM glucose and grown aerobically at 30 °C with shaking 220 rpm for 18 hr. Cells were diluted to OD_{600} 0.01 in MM glucose. Microscope slides 75 x 25 x 1.2 mm (VWR) were then prepared to visualise the strains: these were prepared by filling a 125 µl adhesive gene frame (ABgene) with molten MM agarose, which was then covered with a glass slide in order to flatten the MM agarose surface. Once the MM agarose layer had set, the cover slide was removed, and the MM agarose layer was also carefully removed with a scalpel leaving behind 2 thin strips. Then 1.2 µl of the strains were spotted onto the MM agarose strip. Then 1.2 µl of the strains were spotted onto the MM agarose layer. These were allowed to dry for ~1 minute and were then covered with a 22 x 40 mm glass cover glass plate (VWR). The microscope slides were incubated at 30 °C in a temperature controlled environmental chamber (Weather Station; Applied Precision USA). Prior to the start of acquisition the cells were allowed to equilibrate on the agarose pads for 3 hours.

The following section was carried out by Dr Victoria L. Marlow and Dr Nicola R. Stanley-Wall, University of Dundee: Time-lapse imaging of microcolony development and *chiA-gfp* expression was performed using a DeltaVision Core widefield microscope (Applied Precision) mounted on an Olympus IX71 inverted stand with an Olympus 60X 1.4 NA lens and CoolSNAPHQ, camera (Photometrics) with Differential interference contrast (DIC) and fluorescence optics. For each time-lapse experiment twelve independent fields, each containing one or two cells, were manually identified and their XYZ-positions 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 up to 12 hours. GFP was imaged using a 100 W Mercury lamp and a FITC filter set (EX 490/20; EM 528/38) with an exposure time of 50 ms.

6.8.3 Image Processing

Post-acquisition data sets were rendered and analysed using OMERO software (http://openmicroscopy.org). The threshold used to define whether expression from the the transcriptional reporters Δ*chiX::mKate* and *chiA-gfp* was defined as the fluorescence intensity value greater than 3 standard deviations above the mean background fluorescence of the parental strain *S. marcescens* Db10.

7 Appendix

7.1 Strains

Strain	Relevant genotype	Source/ construction
S. marcescens		
Db10	Non pigmented Wt strain. Insect pathogen isolated from moribund *Drosophila*.	First isolated by (Flyg *et al.*, 1980) Genome sequence available at Sanger Institute, Jonathan Ewbank.
JJH01	As Db10 *ΔchiA*	This study
JJH02	As Db10 *ΔchiB*	This study
JJH03	As Db10 *ΔchiC*	This study
JchiA	As Db10 *ΔchiB ΔchiC*	This study
JchiB	As Db10 *ΔchiA ΔchiC*	This study
JchiC	As Db10 *ΔchiA ΔchiB*	This study
Nochi	As Db10 *ΔchiA ΔchiB ΔchiC*	This study
JJH04w	As Db10 Δ*chiW* (SMA2874)	This study
JJH05x	As Db10 Δ*chiX* (SMA2873)	This study
JJH06y	As Db10 Δ*chiY* (SMA2872)	This study
JJH07z	As Db10 Δ*chiZ* (SMA2871)	This study
JJH08p	As Db10 Δ*chiW, chiX*	This study
JJH09	As Db10 Δ*cbp21*	This study
JJH10	As JchiB Δ*cbp21*	This study
ChiXmKate	As JJH05x Db10 ΔchiX containing gene encoding fluorescent construct mKate (Pletnev *et al.*, 2008)	This study
Db10*::chiA-gfp*	As Db10 encoding gene encoding Green Fluorescent Protein (GFP) 23 base pairs downstream of *chiA* (in-tact full length chitinase encoding gene).	This study

ChiXmKate*::chiA-gfp*	Dual fusion strain. As ChiXmKate Δ*chiX* encoding fluorecent mKate, and as Db10*::chiA-gfp* encoding *chiA-gfp* transcriptional fusion.	This study
TnchiA1	As JchiA, containing unmapped Tn5.	This study
TnchiA2	As JchiA, containing unmapped Tn5.	This study
TnchiA3	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA4	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA5	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA6	As JchiA, containing Tn5 in SMA4482 *hfq*.	This study
TnchiA7	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA8	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA9	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA10	As JchiA, containing unmapped Tn5.	This study
TnchiA11	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA12	As JchiA, containing unmapped Tn5.	This study
TnchiA13	As JchiA, containing Tn5 in SMA4243 *chiA.*	This study
TnchiA14	As JchiA, containing Tn5 in SMA4579 *speF*	This study
TnchiB1	As JchiB, containing unmapped Tn5.	This study
TnchiB2	As JchiB, containing unmapped Tn5.	This study
TnchiB3	As JchiB, containing Tn5 in SMA2875 *chiB*	This study
TnchiB4	As JchiB, containing unmapped Tn5.	This study
TnchiB5	As JchiB, containing Tn5 in SMA2876 *chiR*.	This study
TnchiC1	As JchiC, containing Tn5 in SMA2876 *chiR*.	This study
TnchiC2	As JchiC, containing unmapped Tn5.	This study
TnchiC3	As JchiC, containing unmapped Tn5.	This study
TnchiC4	As JchiC, containing Tn5 in SMA2874 *chiW*	This study
TnchiC5	As JchiC, containing unmapped Tn5.	This study
TnchiC6	As JchiC, containing Tn5 in SMA2874 *chiW*	This study
TnchiC7	As JchiC, containing unmapped Tn5.	This study

TnchiC8	As JchiC, containing unmapped Tn5.	This study
TnchiC9	As JchiC, containing unmapped Tn5.	This study
E. coli		
Dh5α	Host strain for plasmids. ϕ80d*lac*ZΔM15 *recA1 endA1 gyrA96 thi*-1 *hsdR*17(rK -mK+) *supE44 relA1 deoR* Δ(*lacZYA-argF*) U169	Promega
MG1655	Wild type strain F- lambda- *ilvG- rfb*-50 *rph*-1	(Blattner *et al.*, 1997)
M15 pRep4	Host strain for protein expression. The plasmid pREP4 constitutively expresses the Lac repressor protein encoded by *lacI* and thus provides tight control of the *lac* promoter-mediated transcription.	Qiagen
CC118 λpir	Host for pKNG101 suicide vector. *Δ(are-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB* *argE recA1 lysogenized with λpir phage*	(Herrero *et al.*, 1990)
HH26 pNJ5000	Helper strain used in marker exchange	(Grinter, 1983)
SM10 λpir pUTmini-Tn5Sm/Sp	Donor strain used in transposon mutagenesis *thi-1, thr, leu, tonA, lacY, supE, recA*::RP4-2-Tc::Mu, 1*pir,* KmR	(de Lorenzo *et al.*, 1990)

Table 7.1 Bacterial Strains used in this study.

7.2 Vectors

Plasmid	Relevant genotype	Source/ construction
pBluescript 2	Cloning vector Amp^R	Stratagene
pUniprom	Cloning vector Amp^R	(Jack *et al.*, 2004)
pUniprom-*chiA*	pUniprom containing *chiA* from *Serratia marcescens*	This study
pUniprom-*chiAcbp21*	pUniprom containing *chiA* & *cbp21* from *Serratia marcescens*	This study
pUniprom-*chiA*NOSIG*cbp21*	*pUniprom containing chiA without N-terminal signal peptideand cbp21 from S. marcescens.*	This study
pQE60	Overproduction vector	Qiagen
pQE70	Overproduction vector	Qiagen
pQE70-*chiA*	Overproduction vector containing recombinant *chiA* fro*m S. marcescens* with C-terminal hexa-histidine tag.	This study
pQE60-*chiB*	Overproduction vector containing recombinant *chiB* fro*m S. marcescens* with C-terminal hexa-histidine tag.	This study
pQE60-*chiC*	Overproduction vector containing recombinant *chiC* fro*m S. marcescens* with C-terminal hexa-histidine tag.	This study
pKNG101	Suicide vector used to generate mutant *S. marcescens* Db10 strains lacking chitinase encoding gene/s. Sm^R, *sacB* locus mediates sucrose sensitivity.	(Kaniga *et al.*, 1991)
pKNG101-*ΔchiA*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA4243 *chiA* for generating Δ*chiA* mutant.	This study
pKNG101-*ΔchiB*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2875 *chiB* for generating Δ*chiB* mutant.	This study
pKNG101-*ΔchiC*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA0468 *chiC* for generating Δ*chiC* mutant.	This study

pKNG101-*Δcbp21*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2877 *cbp21* for generating Δ*cbp21* mutant.	This study
pKNG101-*ΔchiW*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2874 *chiW* for generating Δ*chiW* mutant.	This study
pKNG101-*ΔchiX*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2873 *chiX* for generating Δ*chiX* mutant.	This study
pKNG101-*ΔchiY*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2872 *chiY* for generating Δ*chiY* mutant.	This study
pKNG101-*ΔchiZ*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2871 *chiZ* for generating Δ*chiZ* mutant.	This study
pKNG101-*Δphl*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2874 *chiW* including overlapping region of *chiX*. For generating Δ*chiW, chiX* mutant.	This study
pKNG101-*ΔchiX::mKate*	Suicide vector containing 500bp upstream and downstream of *S. marcescens* Db10 SMA2873 *chiX* for generating Δ*chiX* mutant, containing gene encoding fluorescent mKate for integration at the *chiX* locus.	This study
pKNG101-*ΔchiA-gfp*	Suicide vector containing 500bp flanking region of *S. marcescens* Db10 SMA4243 *chiA* for integrating gene encoding green fluorescent protein 23 base pairs downstream of the *chiA* locus.	This study

Table 7.2 Plasmid vectors used in this study.

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