Characterization of a Bifunctional Cell Wall Hydrolase in the Mobile Genetic Element ICE*Bs1*

By

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Submitted to the Microbiology Graduate Program in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the Massachusetts Institute of Technology

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Abstract

Integrative and conjugative elements (ICEs) are mobile genetic elements that are normally found stably integrated into bacterial chromosomes, but under certain situations can excise and transfer to a recipient cell through conjugation. ICEs contain a set of genes that encode the molecular machinery needed for conjugative transfer. Most, if not all ICEs encode an enzyme with peptidoglycan hydrolase function that is involved in transfer. While these hydrolases are widespread, they are some of the least-studied components of conjugative transfer systems, and very little is known about their function. The integrative and conjugative element ICE*Bs1* encodes a two-domain cell wall hydrolase, CwlT, that has both muramidase and peptidase activities. I examined the role of CwlT in ICEBs1 transfer.

CwlT is required for transfer of ICEBs1. I found that deletion of *cwlT* completely abrogates ICE*Bs1* conjugation. To my knowledge, all other characterized hydrolases from conjugative systems are at least partially dispensable.

The muramidase domain of CwlT is absolutely required for transfer, while the peptidase domain is partially dispensable. I determined the effect of both of CwlT's hydrolytic activities on ICE*Bs1* transfer, using point mutations of the catalytic domains.

In order to function in conjugation, CwlT must be exported from the cytoplasm and must be able to dissociate from the cell membrane. I investigated the effect of cellular localization on CwlT activity in conjugation by using a variety of signal sequence mutants to alter CwlT's subcellular localization. Contrary to previous predictions that CwlT is a lipoprotein, I found that a deletion of its putative lipid anchor site has no effect on its role in conjugation.

CwlT acts on the cell wall of the donor and not on that of the recipient. It is unclear whether hydrolases in conjugative transfer systems work on the donor cell, the recipient cell, or both. ICE*Bs1* was able to transfer with high efficiency into species with cell wall that is indigestible to CwlT, indicating that the protein does not function on the recipient. In conjugative systems, the enzymatic specificity of the hydrolase may play an important role in determining what species mobile elements can transfer out of.

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This work would not have been possible without the support of every single one of my labmates.

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Introduction: Peptidoglycan Hydrolases in Horizontal Gene Transfer

Whereas all organisms inherit genes "vertically" from their parents, many prokaryotes are also able to acquire new genes "horizontally" from their immediate environment. This process is known as "horizontal gene transfer" (HGT), and researchers are increasingly discovering the broad impact that HGT has on bacterial evolution.

The amount of horizontally acquired DNA varies widely within bacterial species. Some organisms such as *Mycoplasma genitalium* appear to have none. Others, like vancomycin-resistant *Enterococcus faecalis* V583 and *Synechocystis* PCC6803 have acquired nearly 25% and 17% of their genomes respectively from horizontal sources (Ochman et al., 2000; Paulsen et al., 2003). In many cases, regions of horizontally acquired DNA encode traits that confer a survival advantage, such as antibiotic resistance, increased pathogenicity, ability to colonize hosts, or new metabolic capabilities (Wozniak and Waldor, 2010). Interestingly, the realization that many prokaryotic organisms contain a considerable amount of DNA from other sources could complicate certain traditional concepts such as organism and species (Goldenfeld and Woese, 2007).

There are three main mechanisms of HGT (Figure 1): transformation, transduction, and conjugation. During transformation, bacteria develop a physiological state of competence, enabling them to uptake DNA directly from the extracellular environment. In transduction, phage particles that have accidentally packaged genomic DNA from a bacterial host deliver that DNA into a new cell during infection. In conjugation, a sequence of DNA is transferred by cell-cell contact between a donor cell and a recipient cell through a process often referred to as "mating." Two types of elements commonly transfer by conjugation: conjugative plasmids, and integrative and conjugative elements (ICEs). ICEs are mobile genetic elements that are typically found

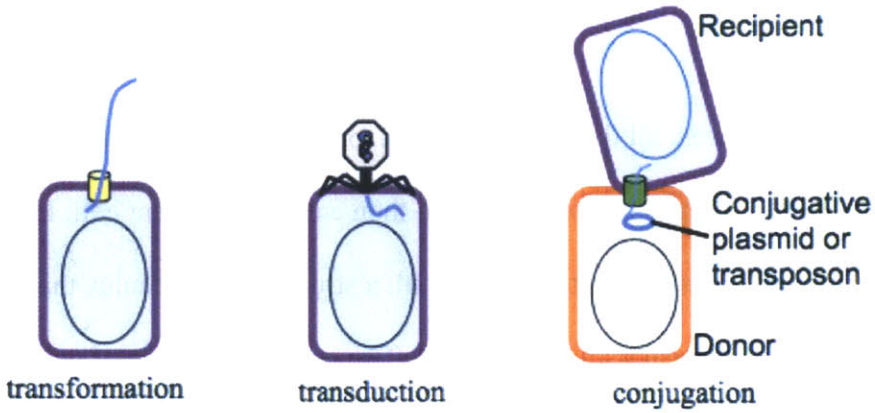

Figure 1. Modes of Horizontal Gene Transfer

In transformation, bacteria uptake DNA directly from the extracellular environment. In transduction, phage particles that have accidentally packaged genomic DNA from a bacterial host deliver that DNA into a new cell during infection. In conjugation, a sequence of DNA is transferred by cell-cell contact between a donor cell and a recipient cell. (Figure from Grossman Lab)

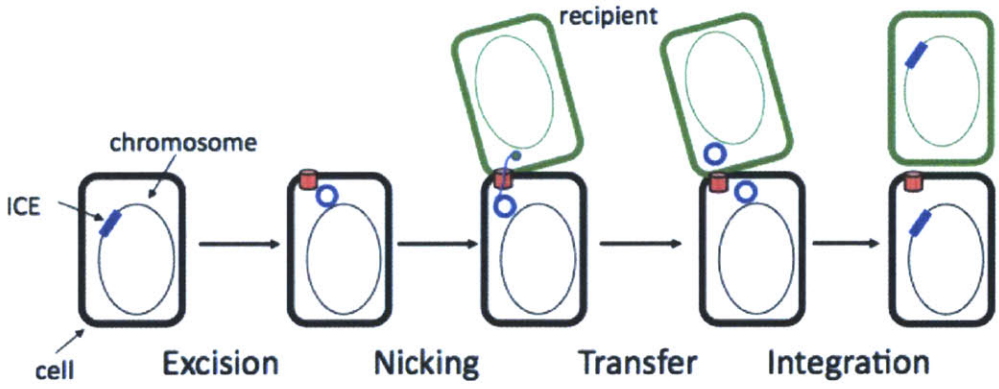

Figure 2. Lifecycle of Integrative and Conjugative Elements (ICEs)

ICEs are mobile elements typically found integrated into the chromosome of a host cell. Under certain circumstances, they can excise to form a circular plasmid intermediate, one strand of which is nicked and then transferred to a recipient cell through a multiprotein mating pore complex. After transfer, the element can recircularize and integrate into the chromosome of the recipient. (Figure from Grossman Lab)

integrated into a host cell's chromosome, but that can excise and transfer under certain circumstances (Figure 2).

Despite their differences, all three of these HGT processes comprise two fundamental DNA transfer events: the DNA leaves a donor cell and then enters a recipient cell. Each time transfer occurs, the DNA must cross the bacterial cell wall, a strong, rigid sacculus that maintains the cell's shape and resists internal osmotic pressure. The cell wall is made of peptidoglycan, a polymer of long carbohydrate chains crosslinked by short peptides (Figure 3).

Different horizontal transfer mechanisms mediate DNA passage across the cell wall in a variety of ways, but all rely to some degree on hydrolase enzymes that digest peptidoglycan. For example, in conjugation and competence, large multiprotein complexes are assembled across the cell wall, and they mediate the secretion (Bhatty et al., 2013) and uptake (Chen et al., 2005) of DNA, respectively. By contrast, phage particles carrying transducing DNA are often released from host cells by cell wall lysis that rapidly kills the host (Oliveira et al., 2013). In some cases, HGT mechanisms are thought to make use of native hydrolases in the cell, while in others, gene cassettes involved with horizontal transfer encode specialized peptidoglycan hydrolases.

In the work described here, I investigate the function of a two-domain cell wall hydrolase, CwlT, that is encoded by the integrative and conjugative element ICE*Bs1*. To provide background, this introduction discusses how cell wall hydrolases help DNA to cross the cell wall in the different mechanisms of HGT.

The introduction begins with a description of peptidoglycan structure, its various chemical modifications, and the native host cell hydrolases that digest and remodel it during normal cell growth. Then, I discuss each of the three main mechanisms of horizontal gene transfer and explain the role hydrolases are thought to play in each. As ICE*Bs1* is a conjugative element, I

emphasize aspects of conjugation, particularly its mechanisms, and associated molecular machinery and hydrolases. Finally, to give immediate context for my own work, I provide an overview of ICE*Bs1* and its cell wall hydrolase CwlT.

Peptidoglycan

Structure. Most bacteria are surrounded by a cell wall made of peptidoglycan. The cell wall forms a rigid capsule around cells, giving them shape, and resisting the high internal turgor pressure to prevent osmotic lysis (Vollmer et al., 2008a). In Gram-negative organisms, the cell wall peptidoglycan is sandwiched between the cytoplasmic membrane and the outer membrane. Gram-positive organisms do not have an outer membrane: a comparatively thicker cell wall is in direct contact with the extracellular environment.

Peptidoglycan is a covalent matrix, consisting of long glycan (carbohydrate) chains crosslinked to each other by short peptides (Figure 3). The glycan chains are made of alternating glucose derivatives, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), joined by β(1-4) glycosidic bonds. Crosslinks are formed between the strands from short peptide chains attached to the sugars, either by direct or interpeptide bridges, which can be between 1 to 7 amino acids long.

The peptidoglycan layer varies in thickness dramatically depending on organism. *E. coli* has a peptidoglycan layer approximately 6 nm thick, which is relatively standard for the Gram-negative organisms (Vollmer et al., 2008a). By contrast, the peptidoglycan of *B. subtilis* is approximately 40-50 nm thick (Hayhurst et al., 2008; Matias and Beveridge, 2005).

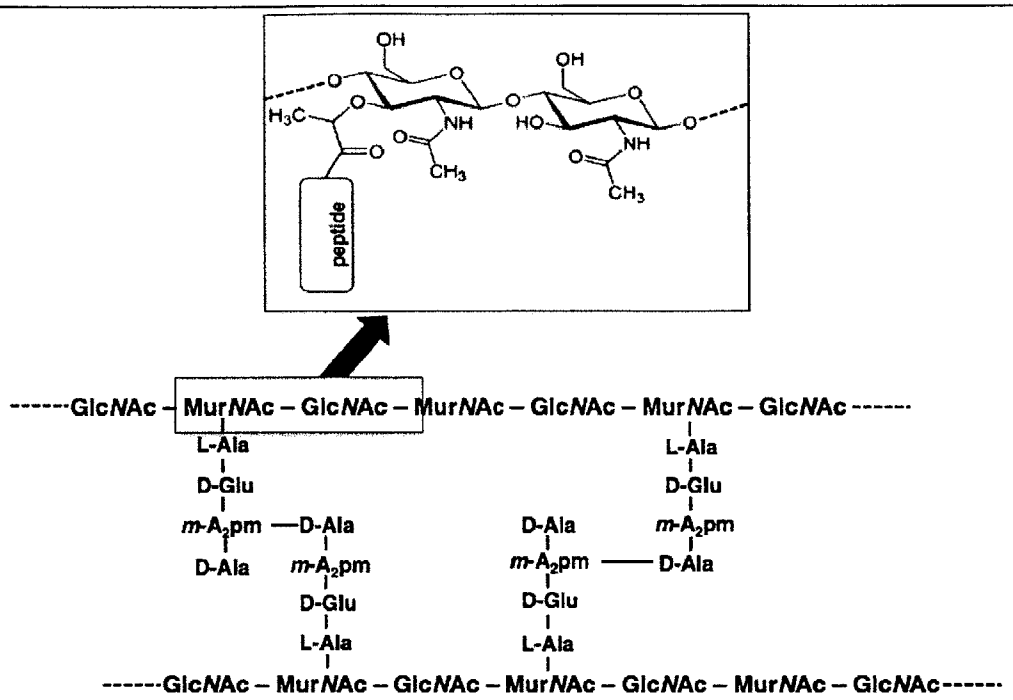

Figure 3. Structure of Peptidoglycan from *E. coli* and *B. subtilis*

The peptidoglycan matrix consists of alternating monomers of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac). The inset shows the structure of Mur*N*Ac, Glc*N*Ac, and the β(1-4) bond between them. The amino acid composition and crosslinking structure of the peptide stems are representative of peptidoglycan seen in both *E. coli* and *B. subtilis*. (Figure was adapted from Vollmer et al., 2008b, using public domain images available from Wikimedia Commons.)

Peptidoglycan as a Barrier to Transport. The peptidoglycan layer serves as a barrier to transport across the cell envelope, containing small holes 2-4 nm in diameter (Dijkstra and Keck, 1996) that allow the passage of low molecular-weight compounds but exclude those larger. Globular proteins larger than 25 to 50 kDa cannot pass through the peptidoglycan (Demchick and Koch, 1996; Dijkstra and Keck, 1996). Dedicated secretion systems are normally required to allow larger substrates through the cell wall, and localized peptidoglycan digestion is often required for their assembly; these will be discussed in more detail below.

Variations in Peptidoglycan Structure and Composition. Peptidoglycan structure can vary somewhat between species, and these variations can have important functional ramifications (reviewed in Schleifer and Kandler, 1972; Vollmer, 2008; Vollmer et al., 2008a). The most common variations are in the amino acid compositions and crosslinking patterns of the peptide side-chains. Figure 4 shows some representative examples of peptidoglycans from different species. The third residue in the peptide side-chains is the most variable, though the first also shows some diversity.

Peptide Stems and Crosslinking. There are two main types of crosslinkage between the side-chains: in the most common (3-4 linkage), the crosslinking extends from the residue at position 3 of one chain to the alanine at position 4 of the other. The second form (2-4 linkage) is found only in *Corynebacteria*, and it involves a crosslink between the second and fourth residues of connected side-chains. Crosslinks can be either direct, or they can involve a cross-bridge containing from one to seven residues. *Bacillus subtilis* contains a direct 3-4 crosslink, though direct crosslinking tends to be more common in Gram-negative species, and crossbridges are found more predominantly in Gram-positive ones.

The amount of crosslinking also varies significantly between species, from approximately 20% in *E. coli* to over 90% in *S. aureus* (Vollmer et al., 2008a). Generally, peptidoglycan from Gram-positive species tends to be more highly crosslinked. The amount of crosslinking can also change within a specific organism depending on growth phase, with the peptidoglycan tending to become more heavily crosslinked during late-exponential and stationary phases (Fordham and Gilvarg, 1974; Pisabarro et al., 1985).

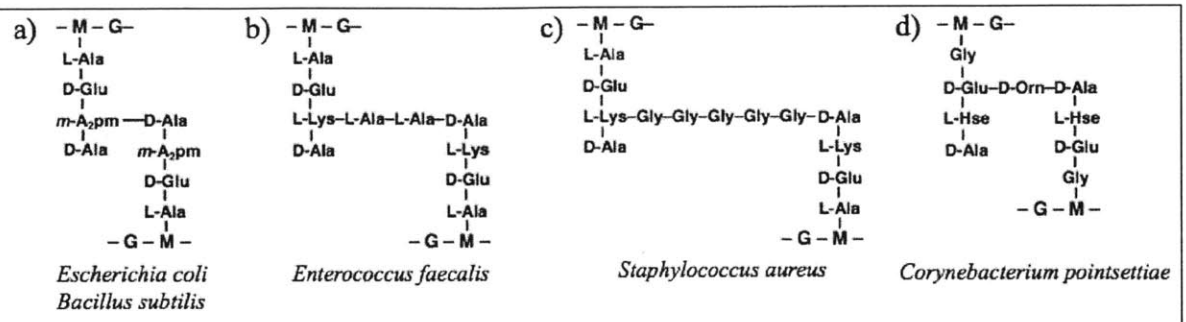

Figure 4. Variation in Peptide Composition and Crosslinking

The structure of peptide composition and crosslinking varies between species. Peptide stems are shown from a) *E. coli* and *B. subtilis*, b) *E. faecalis*, c) *S. aureus*, and d) *C. pointsettiae*. The amino acid in the third position is most variable, and crosslinks between stems can be direct, or involve bridges ranging in length from one to seven residues. D-Orn and L-Hse represent D-ornithine and L-homoserine, respectively. (Figure is adapted from Vollmer et al., 2008a, and Schleifer and Kandler, 1972).

Modifications to Glycan Strands. The monomers of the glycan strands are also sites of two common modifications involving acetyl groups: *N*-deacetylation and *O*-acetylation (Figure 5) (reviewed in Vollmer, 2008). Both of these modifications confer resistance to peptidoglycan digestion by lysozyme, and *O*-acetylation has been shown to also confer resistance to nearly all muramidases, members of the broader class of carbohydrate-digesting hydrolases to which lysozyme belongs. Since lysozyme is an important factor in the innate immune system of humans and other animals, these modifications that reduce sensitivity to its action are often linked to pathogenicity (Boneca et al., 2007; Vollmer and Tomasz, 2002).

In *N*-deacetylation, the acetyl group is removed from the amine at C-3 of the carbohydrate monomers, and this modification can be made to both *N*-acetylglucosamine and *N*-acetylmuramic acid. Peptidoglycan is highly *N*-deacetylated in species such as *B. anthracis*, *B.*

cereus, L. monocytogenes, and *S. pneumoniae*. In *O*-acetylation, the hydroxyl group normally attached to C-6 of *N*-acetylmuramic acid is replaced with an acetyl group. Species with highly *O*-acetylated peptidoglycan include *B. anthracis*, *B. cereus*, *S. aureus*, *S. pneumoniae,* and *N. gonorrhoeae*. Both *N*-deacetylation and *O*-acetylation occur after the peptidoglycan has been synthesized, and these modifications are mediated by specific enzymes.

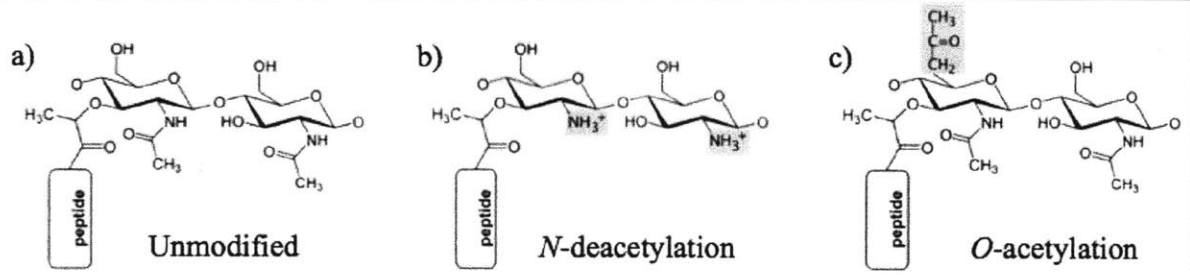

Figure 5. Modifications to the Glycan Strands

Carbohydrate monomers can be *N*-deacetylated and *O*-acetylated, two modifications that confer resistance to lysozyme and other muramidases. a) Unmodified Mur*N*Ac-Gln*N*Ac disaccharide. b) *N*-deacetylation of Mur and Gln, with deacetylated amine groups indicated by shading. c) *O*-acetylation at Mur*N*Ac, with acetyl group indicated with shading. (Figure created using public domain images available from Wikimedia Commons.)

Cell Wall Hydrolases

There are a wide variety of hydrolase enzymes that can cleave the covalent bonds in peptidoglycan. Autolysins are hydrolases encoded by bacterial genomes whose main role is to digest portions of the cell wall to allow its remodeling during the bacterial lifecycle. These will be discussed in some detail below. Hydrolases encoded by horizontal transfer systems and phage genomes will be discussed later on.

Specificities of Cell Wall Hydrolases. There are two main categories of hydrolases: glycosidases, which act on the carbohydrate chains of peptidoglycan, and peptidases, which act on the peptides. There are hydrolases that can cleave every type of bond found in peptidoglycan, and they are characterized accordingly (Figure 6). Only a subset of these hydrolase types is relevant for discussion in the context of this work.

Of the glycosidases, lytic transglycosylases (LT) and lysozymes both cleave the β(1-4) glycosidic bond between Mur*N*Ac and Glc*N*Ac. However, their products are different. Lytic transglycosylases create a 1,6-anhydro ring at Mur*N*Ac by an intramolecular transglycosylation reaction, while lysozymes create free hydroxyl groups on both Mur*N*Ac and Gln*N*Ac (Figure 7). Some of the energy of the broken glycosidic bond is retained in this ring, and it is thought that cleavages by lytic transglycosylases can be reversed relatively easily because of this (Moak and Molineux, 2000). Within the peptidase class, endopeptidases cleave peptide bonds between amino acids, either in the peptide side-chains or in the crosslinking bridges. Amidases cleave the amide bonds that link peptide side-chains to Mur*N*Ac residues.

Function of Bacterial Autolysins. Bacterial species generally have a large number of peptidoglycan hydrolases (reviewed in Smith et al., 2000; Vollmer et al., 2008b). The genomes of *E. coli* and *B. subtilis* each encode approximately 35 identified hydrolases (Sudiarta et al., 2010; Uehara and Bernhardt, 2011). Hydrolases often have overlapping functions, making it difficult to assign specific functions to certain hydrolases, as individual knockouts often do not show a phenotype.

Autolytic activity is important for many cell processes. For example, some of the covalent bonds of peptidoglycan must be broken so that new subunits can be added and the cell wall expanded. At the end of cell division, autolysins digest cell wall at the septum to allow

separation of daughter cells. During spore formation, the asymmetric septum must be digested to

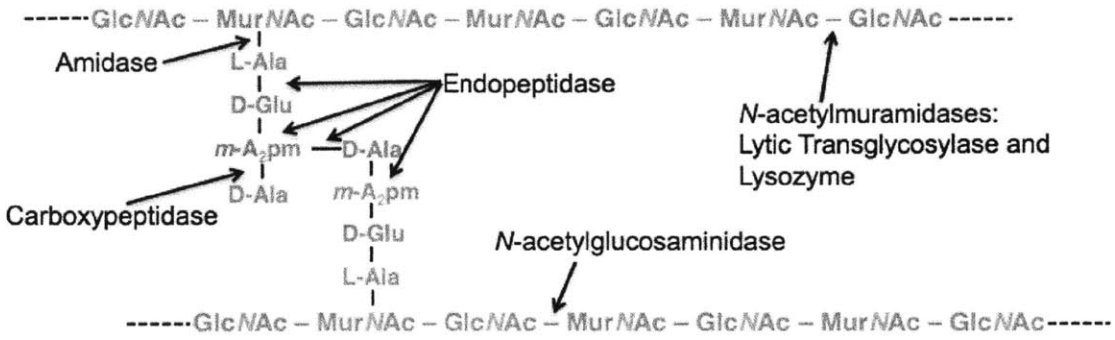

Figure 6. Hydrolase Cleavage of Bonds in Peptidoglycan
Hydrolases cleave specific bonds within peptidoglycan, and they are classified accordingly. The peptidoglycan structure shown is from *E. coli* or *B. subtilis*. Amidases cleave the amide bond between Mur*N*Ac and the peptide chain, endopeptidases cleave peptide bonds between residues within the peptide chain, and carboxypeptidases cleave peptide bonds to remove terminal amino acids. *N*-acetylglucosaminidases and *N*-acetylmuramidases both cleave glycosidic bonds within the glycan chains. Lytic transglycosylases and lysozymes both cleave the same bond, though they create different products (see Figure 5). (This diagram is adapted from Vollmer et al., 2008b).

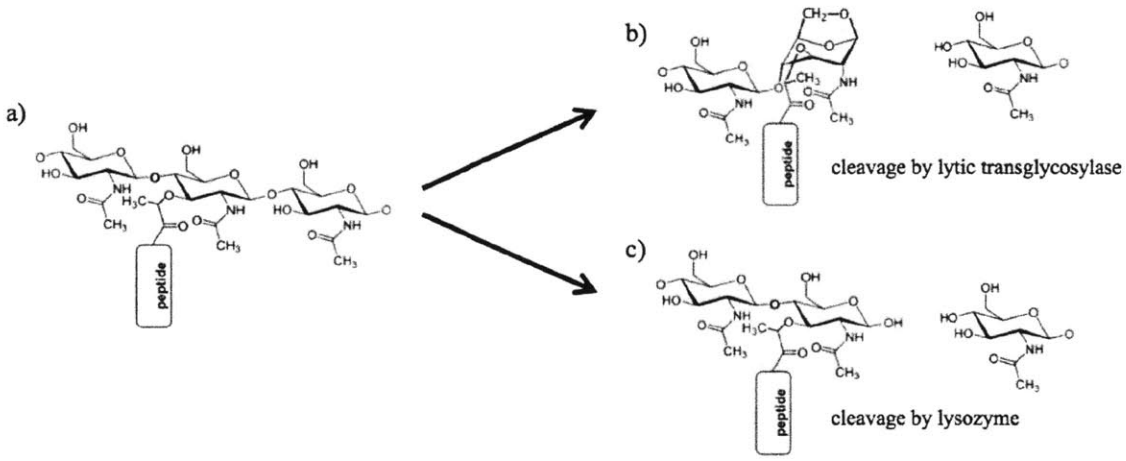

Figure 7. Comparison of Lysozyme and Lytic Transglycosylase Cleavage Products.
Lysozymes and lytic transglycosylases are both *N*-acetylmuramidases and cleaving the same β(1-4) bond, though their products are different. a) Representative Glc*N*Ac-Mur*N*Ac-Gly*N*Ac fragment. b) Lytic transglycosylases create a 1,6 anhydro ring at Mur*N*Ac by an intermolecular transglycosylation reaction. c) Lysozymes create a free hydroxyl group on C-1 of Mur*N*Ac and C-4 of Gln*N*Ac. (This diagram is adapted from Vollmer et al., 2008b, using public domain images available from Wikimedia Commons.)

allow prespore engulfment. Later, autolysin-mediated lysis of the mother cell frees the spore. Digestion of spore peptidoglycan allows germination.

Peptidoglycan hydrolases are also thought to mediate localized digestion of cell wall to allow the insertion and assembly of large protein complexes that cannot fit through the naturally occurring channels in the matrix (Dijkstra and Keck, 1996; Vollmer et al., 2008b). The clusters of genes that encode proteins for structure and assembly of these complexes also typically contain a peptidoglycan hydrolase. For example, in *E. coli* there are hydrolases are associated with Type IV pilus formation and flagellum assembly (Koraimann, 2003; Nambu et al., 1999). Hydrolases are also involved in assembly of systems that secrete conjugative DNA and other substrates, and these are discussed below.

Regulation of Cell Wall Hydrolases. Autolytic activity can kill cells if it proceeds uncontrolled. Regulation of autolysins is not well understood, but it appears to occur at a number of different levels. The first is genetic. Some hydrolases are controlled by temporally specific regulatory factors that only allow expression at points in the lifecycle when specific autolytic activity is needed. In *B. subtilis* for example, most activity of the two major autolysins, LytC and LytD occurs as cells enter stationary phase (Foster, 1992; Margot and Karamata, 1992).

Spatial localization also plays an important role. For example, *S. aureus* Atl (Yamada et al., 1996) and *E. coli* AmiC (Bernhardt and de Boer, 2003; Heidrich et al., 2001) and EnvC (Bernhardt and de Boer, 2004) are involved in cell division, and they all show localization to the septum. Cell wall binding domains on some autolysins are thought to anchor them to certain regions of peptidoglycan and prevent diffusion and indiscriminate lysis (Catalao et al., 2013). In *B. subtilis*, the autolysin LytE associates with MreB, an actin-like protein that directs its localization to certain areas of the cell wall (Carballido-Lopez et al., 2006).

Proteolytic cleavage seems to play an important role in a number of different autolysin regulatory events. In *S. aureus*, the autolysin Alt is produced in a pro-form. Its signal peptide is cleaved, and then it is further processed to yield separate glucosaminidase and amidase domains (Komatsuzawa et al., 1997). In *B. subtilis*, cell surface and extracellular proteases maintain steady-state levels of LytF and LytE by degrading unnecessary protein at the cell poles (Yamamoto et al., 2003). In *E. coli*, a soluble transglycosylase (Slt35) is released by the proteolytic cleavage of a membrane-bound lipoprotein precursor, MltB (Ehlert et al., 1995).

The energized bacterial membrane also plays a role in autolysin regulation, and this may inhibit autolysis close to the cell membrane and allow a gradient of hydrolase activity that increases with distance from it (Vollmer et al., 2008b). Disruption of membrane polarization can cause rapid autolysis of bacterial cells (Jolliffe et al., 1981).

Phage-encoded hydrolases that cause whole-cell lysis at the end of viral infection are regulated by a variety of unique mechanisms that are discussed below.

Transformation and Competence

In natural transformation (Figure 8), cells take up DNA from the extracellular environment. In order to do so, organisms must be in a physiological state of competence. Some species are always competent, while most develop competence in response to environmental conditions such as nutrient limitation or cell density. Cells may take up DNA in order to acquire genetic diversity, to repair damaged sequences in their own genome, or to obtain nutrients.

Approximately 1% of described bacterial species are known to be naturally competent (Thomas and Nielsen, 2005). Competence has been studied most extensively in *Bacillus subtilis,*

Streptococcus pneumoniae, Neisseria gonorrhoeae, and *Haemophilus influenza* (Chen et al., 2005).

Uptake Machinery. With the exception of *Helicobacter pylori*, which uses a conjugation-like apparatus for DNA uptake, both Gram-negative and Gram-positive organisms show a good deal of similarity in their mechanisms of and machinery for DNA uptake. *B. subtilis* serves as a representative organism for Gram-positive competence, and *N. gonnorhea*, for Gram-negative. (Mechanisms of transformation are reviewed in Chen et al., 2005.; Chen and Dubnau, 2004)

In Gram-negative organisms, DNA is thought to transit the outer membrane through a channel composed of secretin proteins (PilQ, in *N. gonnorhea)*, which assemble into doughnut-like multimers that can serve as aqueous channels (Collins et al., 2004). Most species take up DNA of different sequences with relatively similar affinity, though some, such as *H. influenzae* and members of the genus *Neisseriacae*, preferentially take up DNA with specific uptake sequences that show similarity to elements in their own genomes (Smith et al., 1995). In Gram-positive organisms, DNA binds to a receptor protein on the outside of the cytoplasmic membrane (ComEA in *B. subtilis*) (Provvedi and Dubnau, 1999).

Crossing the Wall and Membranes. A competence pseudopilus is a prominent structure in both Gram-negative and Gram-positive competence (Chen et al., 2006). This is a structure that shows similarity to type 4 pili, which are long, thin, hair-like appendages that play a role in cell adhesion and twitching motility. The pseudopilus spans the cell envelope: in Gram-positive organisms, it extends through the cell wall, and in Gram-negative organisms it extends through the cell wall and outer membrane. The proposed function of the competence pseudopilus is to bind DNA and drive its translocation across the outer membrane and cell wall through alternating cycles of assembly and disassembly (Cehovin et al., 2013). The DNA is delivered to

binding proteins (ComE in *N. gonnorhea* [Hamilton and Dillard, 2006], ComEA in *B. subtilis* [Provvedi and Dubnau, 1999]), and then it is translocated through a channel in the cytoplasmic membrane, composed of polytopic membrane proteins (ComA in *N. gonnorhea* [Facius et al., 1996], ComEC in *B. subtilis* [Inamine and Dubnau, 1995]). Gram-positive organisms also appear to have a membrane-associated ATP-binding protein (ComFA in *B. subtilis* [Londono-Vallejo and Dubnau, 1994]) that may be involved in DNA transport across the membrane.

Transforming DNA enters the cytoplasm as a single strand, and it is thought that a nuclease activity may be coupled to the transport process (Chen et al., 2005; Chen and Dubnau, 2004). Once in the cytoplasm of the cell, the transforming DNA is protected from degradation by various proteins. It can integrate into the bacterial chromosome in a RecA-dependent manner, or if a plasmid, it can recircularize and be maintained extrachromosomally.

Hydrolases in Competence and Transformation. A variety of somewhat scattered evidence suggests that hydrolases participate in the assembly of competence machinery. Specialized lytic transglycosylases have been shown to participate in the formation of type IV pili, which are closely related to conjugative psuedopili. These hydrolases include CofT in enterotoxigenic *E. coli* (Taniguchi et al., 2001), and the PilT proteins from plasmids R64 (Sakai and Komano, 2002) and pO113 (Srimanote et al., 2002).

Ordinary cell wall turnover mediated by the autolysins may also play a role in competence development. Readily transformable strains of *B. subtilis* contain a higher level of autolysin activity than poorly transformable ones (Young et al., 1963; Young et al., 1964), a relationship also seen in *Streptococcus* (Ranhand, 1973). Similarly, the development of competence in *B. subtilis* is co-regulated with the activity of autolysins (Guillen et al., 1989).

a) Gram-negative (*N. gonnorhea)* b) Gram-positive *(B. subtilis)*

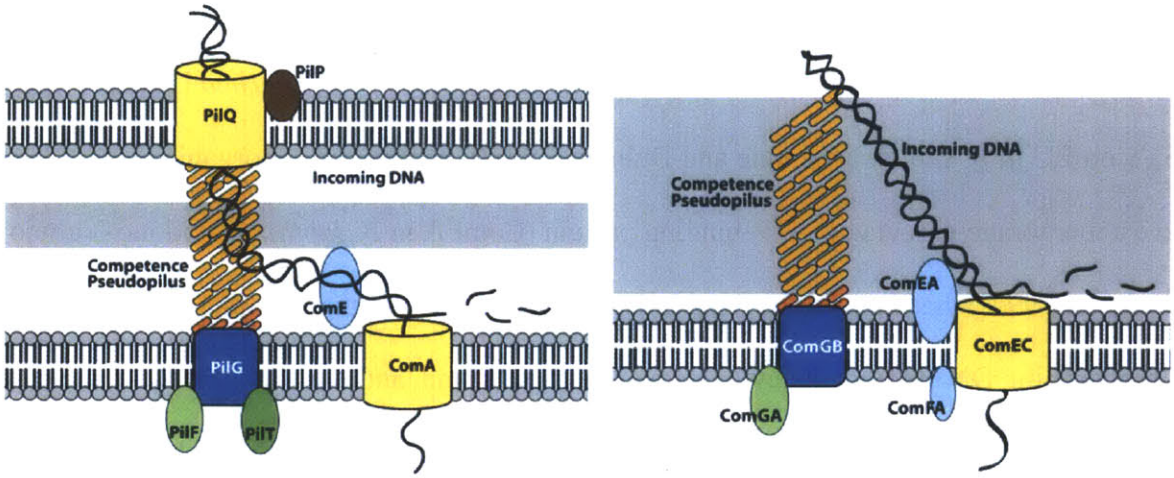

Figure 8. Transformation Machinery in Gram-negative and Gram-positive Organisms.

Not all components are represented.
a) In Gram-negative organisms (represented by *N. gonnorhea*, DNA passes through an outer membrane channel formed by the secretin PilQ, assisted by the pilot protein PilP. ComE, located in the periplasm, is involved in uptake of the DNA, and delivers it to the ComA channel in the cytoplasmic membrane. The competence psuedopilus is composed of major pilin PilE (orange) and minor pilin ComP (red).
b) In Gram-negative organisms (represented by *B. subtilis*), membrane-bound receptor ComEA delivers DNA to the ComEC channel at the cytoplasmic membrane. The competence pseudopilus is composed of major pilin ComCG (orange), and minor pseudopilins ComGD, ComGE, and ComGG (red). (Figures adapted from Chen and Dubnau, 2004)

Transduction

In transduction, foreign DNA is delivered into cells by bacteriophages (reviewed in Lang et al., 2012). This occurs because phages sometimes accidentally package sequences from the genomes of their cellular hosts, as opposed to, or in addition to, their own phage nucleic acid. This host DNA is then introduced into a new cell during infection by a transducing phage.

Transduction can be classified as either generalized or specialized, depending on the identity of the packaged host DNA. Phages such as P1 of *E. coli* perform generalized transduction,

packaging essentially random fragments of host genome. On the other hand, lysogenic phages such as Lambda may perform specialized transduction, packaging host DNA attached to the phage DNA that is adjacent to the phage attachment site on the host chromosome. The amount of DNA that can be transferred via transduction is limited by the size of the phage head, but for some phages, it is nearly 100 kb (Ochman et al., 2000).

Like the DNA delivered into cells by transformation, transduced DNA usually integrates into the genome via site-specific recombination. Occasionally, plasmids can also be transferred by transduction (Mahan et al., 1993), where they recircularize and replicate in the new host.

Transduction is unique from conjugation and transformation, because it is the only form of HGT that does not require the donor and recipient to be in close proximity, or that the foreign DNA come from the immediate environment. Thus, in both terrestrial and marine ecosystems, transducing phages are often seen as unique reservoirs of widely diverse exogenous genes (Anderson et al., 2011; Jiang and Paul, 1998; Zeph et al., 1988).

Phage Hydrolase Systems

Phage-encoded hydrolases digest cell wall peptidoglycan at different stages in the phage lifecycle, and they fall into two classes: virion-associated hydrolases that locally degrade the peptidoglycan to allow DNA insertion, and endolysins, which cause large-scale lysis of host cells to release phage particles at the end of the infection cycle. Aspects of both of these phage hydrolase systems may be similar to mechanisms in hydrolases from secretion systems and conjugative elements.

Entry into the Cell: Virion-Associated Hydrolases. Virion-associated peptidoglycan hydrolases (VAPH) are widespread in phages that infect both Gram-positive and Gram-negative bacteria (Moak and Molineux, 2004). They are usually found associated with the phage tail, and they possess many types of hydrolytic domains, including lysozymes, endopeptidases, and transglycosylases (Rodriguez-Rubio et al., 2012). Some VAPD, particularly those that infect *Staphylococcus* and *Mycobacteria*, have been shown to have multiple lytic domains, usually a muramidase and a peptidase (Oliveira et al., 2013).

Function in Infection. It is thought that virion-associated hydrolases create a small, localized opening in the cell wall peptidoglycan that allows the insertion of DNA. However, few specifics are known about their functional roles. In Gram-negative organisms, they tend not to be essential for infection, though the hydrolase (gp16) in T7 was shown to be required when host cells were grown at 20°C (Moak and Molineux, 2000). DNA internalization is less efficient and delayed in their absence, and they appear to be most important in situations such as stationary phase growth when the peptidoglycan is more heavily cross-linked (Moak and Molineux, 2000; Piuri and Hatfull, 2006; Rydman and Bamford, 2002).

A number of hydrolases have been identified in Gram-positive phages, though phenotypes of their deletions have not been examined. In the *B. subtilis* phage Φ29, the hydrolase gp13 is an essential morphogenic factor that is required for tail assembly (Xiang et al., 2008), but its functions as a hydrolase have not been explored. It is possible that phages infecting Gram-positive bacteria are more dependent on hydrolases to bypass the thick cell walls of their hosts, and that the functions of these enzymes would be essential.

Other Putative Functions of Virion-Associated Hydrolases. Virion-associated hydrolases may have other roles besides digestion of the host cell wall. The hydrolase-containing straight

tail fiber of phage T5 has been shown to also have membrane fusogenic properties and may be involved in creating a pore between the two membranes for DNA translocation (Boulanger et al., 2008). In T7, gp16 digestion allows three injected proteins to pass the cell wall and create a channel for DNA in the inner membrane. This hydrolytic enzyme may be actively involved in translocating DNA, and it is thought that while its N-terminus is anchored to peptidoglycan, its C-terminal may ratchet along DNA using a "hand-over-hand" motion, similar to how kinesin moves along actin filaments. Its C-terminus may have molecular motor ability (Boulanger et al., 2008).

Exit from the Cell: Phage Endolysins.

At the end of the phage infection cycle, progeny virions are usually released by host cell lysis, which is most often mediated by phage-encoded hydrolases known as endolysins. A variety of hydrolytic activities are found in phage endolysins: lysozymes, lytic transglycosylases, endopeptidases, and amidases (Catalao et al., 2013). The lethal activity of these enzymes is tightly regulated. Mechanisms of regulation vary somewhat, though a general strategy centers on sequestration of the hydrolase, either in the cytoplasm or in the membrane, followed by its release and rapid cell lysis.

Canonical Endolysin-Holin Systems. The most common regulation paradigm is exemplified by the lysis system in phage Lambda, and it is universal in almost all dsDNA phages, albeit with some exceptions (Catalao et al., 2013; Loessner, 2005; Wang et al., 2000). In this mechanism, phage hydrolases are synthesized and accumulate in the cytoplasm, as they lack signal sequences that would allow their secretion. Small hydrophobic accessory proteins known as holins create

pores in the cytoplasmic membrane, causing depermeabilization at a specific time; this event allows the endolysins to escape from the cytoplasm, reach the cell wall, and cause host lysis.

Signal-Arrest-Release Endolysins. In contrast to those mentioned above, members of a small class of phage endolysins are exported out of the cytoplasm, but remain anchored in the cytoplasmic membrane by a non-cleavable N-terminal transmembrane sequence known as a Signal-Arrest-Release (SAR) domain (Xu et al., 2004). This anchor is only metastable, and holin-mediated membrane permeabilization can release the endolysin. The holins associated with SAR endolysins tend to create much smaller channels than those in canonical holin-endolysin systems because endolysins do not need to pass through them, and they are referred to as pinholins to reflect this fact (Pang et al., 2009). Release of the SAR domain from the membrane often causes a refolding and conformational rearrangement of the enzyme that allows residues of the catalytic site to move into an active formation (Catalao et al., 2013). Thus far, only a few SAR endolysins have been identified, and the best-characterized examples are Lyz of P1 (Xu et al., 2004), R protein of coliphage 21 (Sun et al., 2009), and Lyz103 of the Erwinia amylovora phage ERA103 (Kuty et al., 2010). It is estimated that approximately 25% of phages contain a SAR endolysin (Park et al., 2007).

Holins are not completely required for SAR endolysin release. Since the N-terminal anchor is only metastable, SAR endolysins can slowly escape from the membrane and cause lysis on their own. The observation that SAR endolysins do not require any accessory factors has led to the proposal that they may represent the most evolutionarily primitive form of endolysins (Park et al., 2007).

Endolysins with Signal Sequences. Finally, a very small class of endolysins exemplified by the Lys44 protein from *Oenococcus Oeni* phage fOg44 have signal sequences that direct their

unhindered export from the cell (Catalao et al., 2013; Nascimento et al., 2008; Sao-Jose et al., 2000). Contrary to those of SAR endolysins, the N-terminal signal sequence of Lys44 plays an inhibitory role and its cleavage is required for activity. Additionally, depolarization of the cytoplasmic membrane appears to activate enzymatic function. In this unique case, holins do not mediate endolysin access to the cell wall, but rather are involved in creating activating conditions for it.

Conjugation

In conjugation, a sequence of DNA is transferred by cell-cell contact between a donor cell and a recipient cell through a process often referred to as "mating." Two types of genetic elements can transfer autonomously by conjugation: conjugative plasmids and integrative and conjugative elements (ICEs). Conjugative plasmids are maintained extrachromosomally, whereas ICEs are integrated into a host cell's chromosome and propagated passively along with the host genome. Under some conditions, an ICE can excise, circularize, and transfer into a recipient cell where it then reintegrates.

Both ICEs and conjugative plasmids encode the conjugation machinery needed for their own transfer. The conjugation genes are often organized into closely related modules clustered by biological function, and these clusters show evidence of frequent exchange between other mobile elements, phages, and host genomes (Burrus et al., 2002; Osborn and Boltner, 2002; Toussaint and Merlin, 2002). Usually, the transfer functions of conjugative plasmids and ICEs are repressed, but these elements can be stimulated to transfer by exposure to a wide range of inducers, including DNA damage, pheromones, antibiotics to which the elements confer

resistance, and other metabolites (Bellanger et al., 2009; Grohmann et al., 2003; Thomas and Nielsen, 2005).

Type IV Secretion Systems. Similar mechanisms appear to be involved in the transfer of both conjugative plasmids and ICEs (Alvarez-Martinez and Christie, 2009; Lee et al., 2010; Toussaint and Merlin, 2002; Zechner et al., 2012). Translocation of the transferred DNA (T-DNA) is mediated by protein machinery that comprises a type IV secretion system (T4SS). The T4SS is an elaborate, multiprotein apparatus that allows substrates to pass out of the cell through the cell membrane and the cell wall. T4SS can transport a wide range of substrates: some are involved in the conjugation of plasmids and ICEs, while others secrete virulence factors, effector proteins and other nucleoprotein complexes into either host cells or culture supernatant (Abajy et al., 2007; Cascales and Christie, 2003; Christie et al., 2005).

Most of our knowledge about type IV secretion mechanisms comes from systems in Gram-negative organisms. Some of the best-studied systems are plasmids from *Escherichia coli*, including F (Wong et al., 2012), RP4 (Rabel et al., 2003), and R388 (Vecino et al., 2011). Also, the *Legionella pneumophila* Dot/Icm (Nagai and Kubori, 2011) and *Agrobacterium tumefaciens* Ti plasmid transfer systems have been well studied. In particular, the current paradigm model for Type IV secretion is based largely on studies of the conjugation apparatus involved in the transfer of the Ti plasmid from *A. tumefaciens*, and transfer genes in other systems are often named after similar ones from *A. tumefaciens*. This model will be presented below. Much less is known about transfer in Gram-positive organisms, though there are a number of similarities to the T4SS in Gram-negative organisms.

Model for Agrobacterium Ti Plasmid Transfer. *A. tumefaciens* is an agricultural pathogen that transfers a tumorigenic conjugative element known as the Ti plasmid into plant cells

(reviewed in Pitzschke and Hirt, 2010). The plasmid integrates into the plant genome, and it encodes enzymes that promote both tumor growth and the production of opines, amino-acid-sugar conjugates that provide nutrients to the bacterium. The Ti plasmid has an extraordinarily broad host range: *in vitro*, it is able to transfer into almost any eukaryotic species, from fungi to human cells (Lacroix et al., 2006). The conjugation system can also mobilize other plasmids such as RSF1010 that do not encode their own conjugation functions (Stahl et al., 1998).

The products from at least 12 genes on the Ti plasmid (*virB1–virB11*, and *virD4*) comprise the T4SS that mediates DNA and protein transfer into plants and other organisms (Figure 9), and this is often referred to as the VirB/D4 system (reviewed in Alvarez-Martinez and Christie, 2009; Bhatty et al., 2013; Fronzes et al., 2009; Zechner et al., 2012). The VirB proteins are involved in creating the secretion channel that extends across—and allows substrates to pass through—the inner membrane, cell wall, and outer membrane of the donor cell.

Substrate Processing and Recruitment. A number of proteins are involved with the processing of the transferred DNA (T-DNA) and its delivery to the secretion channel. The relaxase VirD2 nicks one strand of the Ti plasmid at a specific repeated sequence, becoming covalently attached to its 5' end and forming the core of a complex known as the relaxosome. VirD2 is thought to remain associated with the T-DNA during its passage through the secretion channel and into the plant host. The protein has a nuclear localization signal and is involved with targeting the T-DNA to the plant nucleus (Pitzschke and Hirt, 2010). The coupling protein VirD4 interacts with the relaxosome, and transfers the T-DNA to the membrane-bound ATPases VirB4 and VirB11.

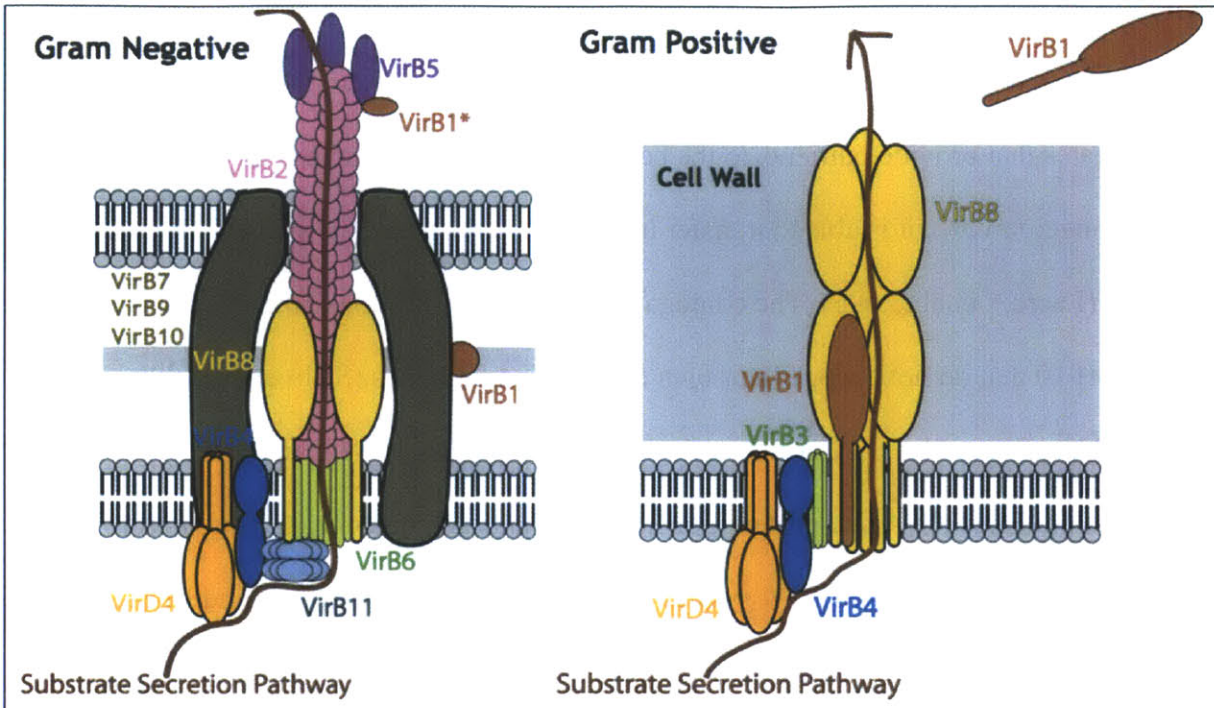

Figure 9. Comparison of Models for Gram-negative and Gram-positive Type IV Secretion A subset of the Gram-negative secretion apparatus components are conserved in Gram-positive systems. Gram-positive secretion systems lack the core complex formed by VirB7, VirB8, and Vir10, and it has been proposed that VirB8 forms a channel across the peptidoglycan. A VirB11 homolog is absent in Gram-positive systems, though ATPases VirD4 and VirB4 are conserved. While some Gram-positive systems encode surface adhesins, none are known to make conjugative pili. As the cell wall is considerably thicker in Gram-positive organisms, the hydrolase VirB1 may play a more significant role in complex assembly. (Figures adapted from Bhatty et al., 2013).

Passage Through the Cell Envelope. From VirB4 and VirB11, the substrate likely passes through a complex of membrane-bound proteins (primarily VirB6 and VirB8, as well as VirB3 and VirB10) that forms a channel in the inner membrane. VirB7, VirB9, and VirB10 create a core complex that crosses the peptidoglycan and spans the entire cell envelope. Proteins in this complex may also make a cap that protrudes from the cell surface and creates a channel through the outer membrane. A peptidoglycan hydrolase, VirB1, is thought to locally digest the

cell wall peptidoglycan to allow the assembly of this multiprotein complex; VirB1 will be discussed in greater detail below in the section on hydrolases in conjugative elements.

VirB4 and Vir11 as well as the coupling protein VirD4 all exhibit ATPase activity, and although the mechanisms are not entirely clear, it is thought that they are involved in powering substrate translocation through the secretion channel.

Interactions with Recipients: The Conjugative Pilus. A conjugative pilus extends from the cell surface and is composed primarily of subunits of the pilin protein VirB2. The protein VirB5 is found at the end of the pilus, and it has been proposed that VirB5 mediates contacts between donor and recipient cells. Although the lumen of the pilus appears wide enough to accommodate single-stranded DNA and unfolded proteins, it is unclear whether it acts as a conduit for substrates (Alvarez-Martinez and Christie, 2009). The hydrolase VirB1 is also essential for the biogenesis of the pilus, though it is unclear what role it plays in this process (Zupan et al., 2007).

Mechanisms of Gram-Positive Conjugative Transfer

Much less is known about mechanisms of conjugation in Gram-positive species. These systems need only translocate substrate across one membrane, but they must also mediate transfer across a cell wall that is much thicker than those in Gram-negative organisms. Some of the best-studied, representative Gram-positive elements are the conjugative plasmids pCW3, pIP501, pCF10, and the ICEs Tn*916* and ICE*Bs1* (Alvarez-Martinez and Christie, 2009), though none of these has yet emerged as a clear prototypical system. A core set of genes in Gram-positive systems show similarity to those in better-characterized Gram-negative systems, and comparison can provide a general picture of how DNA translocation might occur (Figure 9).

Substrate Processing and Recruitment. Gram-positive conjugation systems encode both a relaxase and a VirD4-like coupling protein similar to those in Gram-negative systems, and it is likely that substrate processing and recruitment occur in much the same ways. Studies in both pCW3 and pIP501 have shown an interaction between the relaxase and coupling protein (Abajy et al., 2007; Chen et al., 2008).

Passage Through the Cell Envelope. Of the components involved in secretion channel formation, VirB1, VirB3, VirB4, VirB6, and VirB8 appear to be at least partially conserved in Gram-positive systems (reviewed in Bhatty et al., 2013). The membrane pore structure is likely quite comparable to that in Gram-negative organisms, formed by proteins similar to VirB3, VirB6, and VirB8, (Porter et al., 2012). Other small membrane proteins may also associate with this core complex.

The structure of the envelope-spanning secretion channel must be very different from that in Gram-negative species, because none of the components that make the main core complex, VirB7, VirB9, or VirB10, are conserved in Gram-positive species. Multimerized VirB8 may create a structure that spans the cell wall (Bhatty et al., 2013; Porter et al., 2012), forming a channel to the outside of the cell through which the secretion substrates travel. Alternatively, some of the small membrane proteins found in these systems may polymerize into long fibers to create a channel across the wall (Alvarez-Martinez and Christie, 2009).

Substrate secretion is likely energized by the ATPase activities of the coupling protein and VirB4-like protein (Berkmen et al., 2010), as the VirB11 ATPase is not conserved in Gram-positive species (Bhatty et al., 2013).

Interactions with Recipients: Surface Adhesins. No Gram-positive elements are known to produce conjugative pili. The plasmid pCF10 encodes an adhesin that is anchored to the cell and promotes donor-recipient aggregation (Waters and Dunny, 2001). Surface adhesins may also be encoded by pIP501 and pCW3, but there are no signs of genes with such functions in either ICE*Bs1* or Tn916 (Alvarez-Martinez and Christie, 2009).

Role of the Hydrolase. In Gram-positive systems, the VirB1-like hydrolase has an N-terminal transmembrane domain, and may form part of the translocation complex structure. These hydrolases have been shown to interact with the coupling protein and VirB6-like subunits in the conjugal plasmids pIP501 and pCW3, (Abajy et al., 2007; Steen et al., 2009), and they may play a more active role in substrate recruitment than in Gram-negative systems. The role of hydrolases will be discussed in more detail below.

Hydrolases in conjugation systems

Specialized cell wall hydrolases are found in most, if not all, Type IV Secretion Systems, from both Gram-positive and Gram-negative backgrounds (Zahrl et al., 2005). The hydrolases are some of the least-studied components of T4SS, and their function is not well characterized. However, it is generally assumed that at least one of their roles is to create localized openings in the cell wall peptidoglycan to allow the assembly of the secretion apparatus. The peptidoglycan mesh normally contains gaps averaging approximately 2–4 nm in diameter (Demchick and Koch, 1996; Dijkstra and Keck, 1996), and it would need to be significantly remodeled to accommodate the passage of a large protein complex. Structural studies on the T4SS in *A. tumefaciens* have estimated that the core, cell-envelope spanning complex has a diameter of approximately 18.5 nm (Fronzes et al., 2009).

Hydrolase Structure. These hydrolases have some common features. VirB1 from *Agrobacterium* is the best-characterized hydrolase from a Gram-negative transfer system. At its N-terminus is a single catalytic domain with a lysozyme-like structure fold that also shows similarity to bacteriophage lytic transglycosylases (Mushegian et al., 1996). This motif is also found in P19 of plasmid R1, TraL of IncN plasmid pKM101, and TrbN of IncP plasmid RP4.

In Gram-positive conjugative systems, representative hydrolases include Orf14 of Tn916, TcpG of pCW3, CwlT of ICE*Bs1*, and Orf7 of pIP501. All of these have two catalytic domains: an N-terminal glycosidase and a C-terminal peptidase. The hydrolase PrgK from pCF10 appears to represent a slightly different class of Gram-positive hydrolase: it also has glycosidase and muramidase functions, but has an additional N-terminal peptidoglycan-binding LytM domain (Bhatty et al., 2013). Approximately 700 residues in length, PrgK is nearly as twice as large as the other Gram-positive hydrolases.

Biochemical Activities. For the most part, the biochemical activities of these hydrolases have not been thoroughly characterized. VirB1, P19, and TcpG have all been shown to degrade peptidoglycan *in vitro* (Bantwal et al., 2012; Bayer et al., 2001; Zahrl et al., 2005), though the enzymatic mechanisms and cleavage sites of these and most other T4SS hydrolases have been inferred only from homology. An exception is CwlT, which was shown to have *N*-acetylmuramidase (lysozyme-like) activity and DL-endopeptidase activity (Fukushima et al., 2008). Interestingly, experimental observations of lysozyme-like activity contradicted previous homology comparisons, which had predicted that the N-terminal region had lytic transglycosylase activity. In light of these findings, it is possible that many of the other T4SS hydrolases predicted to contain lytic transglycosylase domains may actually act as lysozymes.

Throughout this paper, carbohydrate-digesting domains of hydrolases will be referred to as muramidases, to acknowledge the uncertainty in the identity of these domains.

Phenotypes of Hydrolase Disruptions. Hydrolase function is not completely required for conjugation in any of the systems studied thus far. For Gram-negative organisms, disruption of the hydrolase seems to reduce transfer by 10–100 fold. Transfer of R1 is decreased approximately 10-fold with the deletion of its hydrolase P19 (Bayer et al., 2001), disruption of TraL from pKM101 decreased transfer by about 10–100 fold (Winans and Walker, 1985), and a VirB1 deletion reduces Ti plasmid transfer by 10-100 fold, depending on the assay used (Mushegian et al., 1996; Berger and Christie, 1994; Bohne et al., 1998).

In the Gram-positive conjugative plasmid pCW3, deletion of the hydrolase TcpG caused transfer to decrease by 1000-fold (Bantwal et al., 2012), a larger decrease than is seen in Gram-negative systems. A catalytic mutant of the peptidase domain was also constructed, which showed approximately a 10-fold decrease in transfer. This heightened requirement for hydrolytic activity may be due in part to the much thicker, more heavily crosslinked cell wall in Gram-positive organisms (Abajy et al., 2007). It may also have to do with interactions that the hydrolase makes with essential components of the secretion system; these will be discussed below.

Hydrolase Complementation. It is generally assumed that element-encoded hydrolases are not completely required for transfer because other hydrolases can substitute for their activity (Abajy et al., 2007; Bantwal et al., 2012; Baron et al., 1997; Hoppner et al., 2004). The cells' native autolysins or hydrolases from other secretion systems may be able to locally enlarge the peptidoglycan in the absence of a dedicated hydrolase.

In general, the hydrolases from many Gram-negative elements show a low degree of specificity in cross-complementation assays and are able to substitute for each other. Deletion of the hydrolase P19 from plasmid R1 can be complemented by TpgF from the T3SS of *Shigella connei*, and by TrbN from the conjugative plasmid RP4 (Zahrl et al., 2005). The conjugation deficiencies of a VirB1 deletion from *Agrobacterium* can be complemented nearly to wild-type levels by the VirB1 protein from *Brucella suis*, and partially by TraL from pKM101 (Hoppner et al., 2004). Thus far, there have been no cross-complementation studies for hydrolases in Gram-positive conjugative elements. This low level of specificity may indicate that, at least in Gram-negative organisms, the hydrolase may be a relatively late evolutionary addition to secretion systems (Zahrl et al., 2005).

Interactions with Other Secretion Channel Components. More information about the roles and other functions of these hydrolases could be inferred from studies of the other subunits that they interact with. The interactions that the peptidoglycan hydrolases make suggest that they may fulfill other roles, in addition to, or in concert, with their roles in peptidoglycan degradation. VirB1 has been shown to associate with core secretion channel proteins both in *Brucella suis* (VirB8, VirB9, and VirB11) (Hoppner et al., 2005) and in *Agrobacterium* (VirB4, VirB8, VirB9, VirB10, and VirB11) (Ward et al., 2002). It has been suggested that these interactions may restrict hydrolytic activity to the site of T4SS assembly (Hoppner et al., 2005) and/or that VirB1 may actively recruit elements of the secretion channel (Ward et al., 2002). Additionally, VirB1's peptidoglycan degradation likely also causes indirect stabilization of the channel components; as more space opens up, members of the secretion pore can promote stabilizing interactions between the different subunits and promote complex formation (Hoppner et al., 2005; Ward et

al., 2002). However, it is unlikely that these interactions are extremely specific, since hydrolases from different systems are able to functionally substitute for one another.

In Gram-positive systems, interaction studies suggest that the hydrolase may play a more active role in substrate recruitment. In the Gram-positive conjugative plasmids pCW3 and pIP501, the hydrolases (TcpG in pCW3; Orf7 into pIP501) have been shown to interact with the coupling protein (TcpA in pCW3; Orf10 into pIP501) (Abajy et al., 2007; Steen et al., 2009). This interaction may aid in transporting the coupling protein to the secretion channel components. In pIP501, the hydrolase (Orf7) has also been shown to interact with the VirB4 homolog Orf5, and with another protein, Orf14, whose function is unclear (Abajy et al., 2007). As has been suggested for Gram-negative systems, the hydrolase may play a role in recruitment of these proteins, or help to stabilize interactions between them by locally opening the cell wall.

Additional Roles of Peptidoglycan Hydrolases. At least one characterized hydrolase has other roles beyond peptidoglycan degradation. In *A. tumefaciens*, VirB1 plays an important role in formation of the conjugative pilus. The protein is processed into a C-terminal fragment, VirB1* (Baron et al., 1997) that is essential for formation of the T-pilus, which is composed of VirB2 (major subunit) and VirB5 (minor subunit) (Zupan et al., 2007). It has been proposed that the C-terminal region may have chaperone-like function, binding VirB2 and VirB5 to prevent their interaction until they are utilized at the site of T-pilus assembly (Zupan et al., 2007). The functions in T-pilus assembly are completely distinct from hydrolytic activity: the two regions are spatially separated, and they can be complemented separately (Llosa et al., 2000).

Certain parts of the C-terminal region are homologous to systems from other mobile elements, such as TraL of pKM101, TrbN of RP4, and VirB1 of *Brucella suis*, and they may be processed the same way and have similar function (Baron et al., 1997). Consistent with this

observation, VirB1 from *Brucella suis* is able to restore fully complement the pilus-deficiency in an *A. tumefaciens* strain deleted for VirB1, and TraL is able to do so partially (Hoppner et al., 2004).

Future Directions. Studies of the deletion phenotypes, cross-complementation, and interactions of these hydrolases have provided information about some of their roles in conjugation. However, a number of questions remain. While it is assumed that the hydrolases act on the cell wall of the donor, it is unclear whether they also act on the cell wall of the recipient. Particularly in Gram-positive organisms, the thick cell wall of the recipient could provide a substantial barrier, and localized digestion could aid the passage of substrates. Since cell wall hydrolases are needed for digestion of the donor cell and possibly for the recipient cell, whether the hydrolase could digest the cell wall of a particular species could have important effects on host range. Additionally, it is unclear how the potentially lethal activity of these cell wall hydrolases is regulated.

ICEBs1 and CwlT

ICE*Bs1* (Figure 10) is an integrative and conjugative element found in many isolates of *Bacillus subtilis* (Auchtung et al., 2005; Earl et al., 2008). ICE*Bs1* is approximately 21 kb in size, and it comprises 24 putative genes, largely clustered according to function, falling broadly into the categories of integration and excision, replication, conjugation, and regulation. Besides *Bacillus subtilis*, ICE*Bs1* can successfully transfer into *Bacillus anthracis*, *Bacillus licheniformis*, and *Listeria monocytogenes*, though host range has not been examined exhaustively (Auchtung et al., 2005).

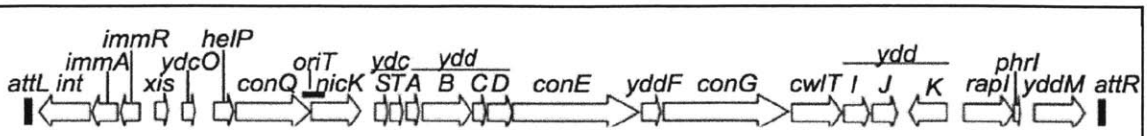

Figure 10: Genetic Map of ICE*Bs1*

Linear genetic map of ICEBs1 integrated in the chromosome. Open arrows indicate open reading frames and the direction of transcription. Gene names are indicated above or below the arrows. The small rectangles at the ends of the element represent the 60 bp direct repeats that contain the site-specific recombination sites in the left and right attachment sites, *attL* and *attR*. (Figure adapted from Menard, 2013)

Mechanism of Conjugation. A number of aspects of the ICE*Bs1* lifecycle have been investigated. The element normally incorporates into the chromosome at the 3' end of a leucine-tRNA gene, and it remains stably integrated as long as its major operon is repressed (Auchtung et al., 2007). Expression of the ICE*Bs1* genes causes excision and transfer of the element, and this can be triggered by one of two scenarios: DNA damage, or crowding by potential recipients that do not have ICE*Bs1* (Auchtung et al., 2005). To study aspects of mating, ICE*Bs1* excision and transfer can be induced very efficiently by overexpressing one of its key regulatory proteins from an exogenous locus.

After induction, ICE*Bs1* excises from the chromosome to form a double-stranded circular intermediate (Auchtung et al., 2005). Its transfer then follows steps common to both conjugative plasmids and other ICEs (Burrus et al., 2002; Lee et al., 2010; Lee and Grossman, 2007). The ICE*Bs1*-encoded relaxase NicK nicks one strand of the circular intermediate at the origin of transfer site, becoming covalently attached (Lee and Grossman, 2007). This covalent complex of the relaxase and single-stranded ICE*Bs1* DNA is then thought to associate with the coupling protein, ConQ (Iyer et al., 2004; Lee et al., 2012; Parsons et al., 2007) and transfer into the recipient (Alvarez-Martinez and Christie, 2009; Lee et al., 2012; Schroder and Lanka, 2005),

where it then likely circularizes, undergoes second-strand synthesis, and integrates into the chromosome (Berkmen, 2013). The proteins ConE, YddD, and YddB (similar to VirB4, VirB3, and VirB8 from Gram-negative T4SS) may form a membrane translocon, and the VirB6-like ATPase ConG could provide energy for the translocation process.

CwlT. The product of the ICE*Bs1* gene *yddH* was recently shown to have peptidoglycan hydrolytic activity. Accordingly, *yddH* was renamed *cwlT* (cell wall lytic) (Fukushima et al.,

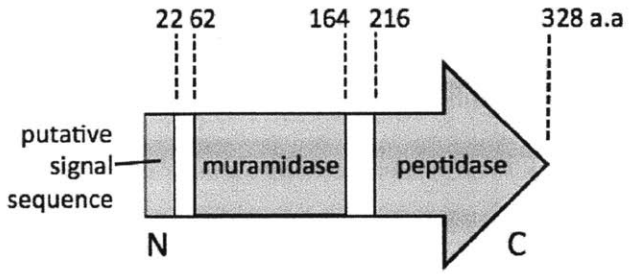

Figure 11: Map of Features in *cwlT*

Numbers represent positions of amino acid residues with respect to the initial Met codon. CwlT has a putative signal sequence (from a.a. 1 to 22), a muramidase domain (a.a. 62 to 164), and a peptidase domain (a.a. 216 to 328). (Figure adapted from Fukushima et al., 2008)

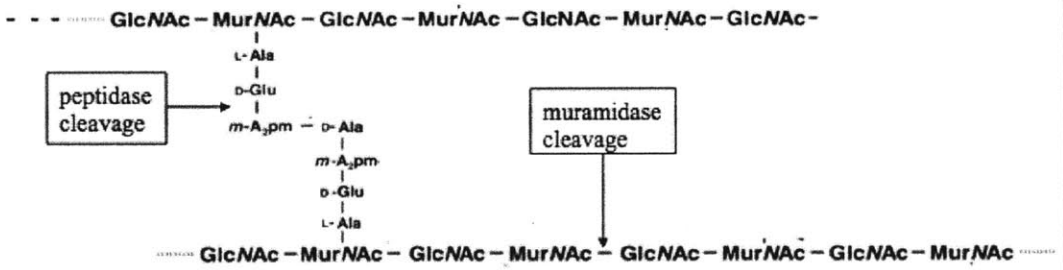

Figure 12: Biochemical Activities of CwlT

The N-terminal muramidase of CwlT cleaves the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine. The C-terminal cysteine DL-endopeptidase cleaves the bond between D-γ-glutamate and meso-diaminopimelic acid. (Figure adapted from Vollmer et al., 2008b)

2008). CwlT has two separate domains for peptidoglycan hydrolysis (Figure 11). Each of these has been characterized biochemically (Figure 12): an N-terminal muramidase cleaves the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine, and a C-terminal cysteine DL-endopeptidase cleaves the bond between D-γ-glutamate and meso-diaminopimelic acid (Fukushima et al., 2008). In this work, I describe the characterization of CwlT's role in ICE*Bs1* conjugation.

Thesis Outline

Peptidoglycan hydrolases are involved in a variety of mechanisms of horizontal gene transfer. Hydrolases are encoded by most, if not all, conjugative elements, though very little is known about their function. The experiments outlined in this thesis provide insight into the functions of a peptidoglycan hydrolase in an integrative and conjugative element.

Chapter 2 describes the examination of CwlT and its enzymatic activities on ICE*Bs1* conjugation. We investigate aspects of the localization of the protein, and whether it acts on the donor cell or recipient cell. This chapter is being prepared for publication.

Appendix A discusses the examination of *cwlT*'s downstream genes, *yddI* and *yddJ*, in conjugation, and a possible genetic interaction between *cwlT* and *yddIJ*.

Appendix B discusses preliminary experiments regarding the effect of expression of SigV, an extracellular sigma factor, on the transfer of ICE*Bs1*.

Chapter 3 discusses the work presented in Chapter 2 and Appendix A. It comments on various aspects of hydrolase function in conjugative transfer, including 1) essentiality of hydrolases, 2) diversity and function of catalytic domains, 3) localization and regulation of hydrolases, and 4) interaction between host range and enzymatic specificity.

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Chapter 2

A bifunctional cell wall hydrolase is needed in donor cells for transfer of an integrative and conjugative element

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Abstract

The mobile genetic element ICE*Bs1* is an integrative and conjugative element found in *Bacillus subtilis*. One of the ICE*Bs1* genes, *cwlT*, encodes a cell wall hydrolase with two catalytic domains, a muramidase and a peptidase. We found that *cwlT* is required for ICE*Bs1* conjugation. We examined the role of each of the two catalytic domains in conjugation and found that the muramidase is essential, whereas the peptidase is partially dispensable. We investigated the effect of the cellular localization of CwlT on conjugation. Our results indicate that in order to function correctly, CwlT must be exported from the cytoplasm and must be able to dissociate from the cell membrane. Contrary to previous predictions that CwlT is a lipoprotein, we found that alteration of its putative lipid anchor site had no effect on its role in conjugation. Finally, our results indicate that CwlT acts on the cell wall of the donor and not on the cell wall of the recipient.

Introduction

Integrative and conjugative elements (ICEs) are mobile genetic elements that are found stably integrated into a bacterial chromosome. Under certain conditions, an ICE can excise from the chromosome, circularize, and transfer to a recipient cell (Burrus and Waldor, 2004). ICEs are found in a wide variety of bacterial species, both Gram-positive and Gram-negative, and they often bestow physiologically and clinically relevant traits, including nitrogen fixation, biofilm formation, virulence, and antibiotic resistance (reviewed in Beaber et al., 2004; Wozniak and Waldor, 2010).

ICE*Bs1* is a mobile genetic element found in many isolates of *Bacillus subtilis* (Auchtung et al., 2005; Earl et al., 2008). It is approximately 21 kb in length and has 24 genes (Fig. 1a). ICE*Bs1* is found integrated in *trnS-leu2*, the gene for a leucine-tRNA, and it remains stably integrated as long as its major operon is repressed (Auchtung et al., 2007). Derepression of ICE*Bs1* gene expression and subsequent excision occur in response to DNA damage, or when the cell-cell signaling regulator RapI is produced and becomes active, usually when cells are crowding by potential recipients that do have ICE*Bs1* (Auchtung et al., 2005).

The ICE*Bs1* gene *cwlT* (cell wall lytic) encodes a bifunctional cell wall hydrolase capable of degrading peptidoglycan (Fukushima et al., 2008). Peptidoglycan is the major component of the bacterial cell wall, and it is composed of long carbohydrate chains of alternating amino sugars (*N*-acetylglucosamine and *N*-acetylmuramic acid) crosslinked by short peptide chains (Vollmer et al., 2008a). In *B. subtilis*, the cell wall is approximately 40-50 nm thick (Hayhurst et al., 2008; Smith et al., 2000), and the genome encodes a complement of hydrolases that digest the various covalent bonds in the cell wall peptidoglycan to facilitate processes such as growth, separation of

cells after division, and mother cell lysis during sporulation (Matias and Beveridge, 2005; Smith et al., 2000).

Unlike most *B. subtilis* host-encoded hydrolases, CwlT has two separate domains for peptidoglycan hydrolysis. Each domain has been characterized biochemically. The N-terminal domain is an *N*-acetylmuramidase (muramidase) that cleaves the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine. The C-terminal endopeptidase (peptidase) domain cleaves the bond between D-γ-glutamate and *meso*-diaminopimelic acid (Fukushima et al., 2008).

Peptidoglycan hydrolases are widespread in mobile genetic elements and are often found associated with Type IV secretion systems (T4SS) involved in conjugation, protein translocation, and DNA uptake (Abajy et al., 2007; Alvarez-Martinez and Christie, 2009; Koraimann, 2003; Scheurwater and Burrows, 2011; Zahrl et al., 2005). The best characterized of these is the VirB/D4 system from *Agrobacterium tumefaciens*, which encodes a large multiprotein channel that spans the cell envelope and mediates the secretion of conjugative DNA and associated proteins. Peptidoglycan hydrolases are prevalent in conjugation systems, and it is generally assumed that the hydrolases cause localized degradation of the cell wall to allow the assembly of the large secretion apparatus. However, relatively little is known about their function in conjugation. They are not essential for conjugation in Gram-negative systems and are largely uncharacterized in Gram-positive organisms.

We found that *cwlT*, the ICE*Bs1* gene encoding a bifunctional peptidoglycan hydrolase, is required for conjugation of ICE*Bs1*. Using mutations affecting each of the two domains, we found that the muramidase function is essential, and that the peptidase function is important, but partially dispensable, for ICE*Bs1* conjugation. We found that the signal sequence was involved

in secretion of CwlT and was critical for its function in conjugation. Contrary to previous predictions that CwlT is a lipoprotein (Fukushima et al., 2008; Tjalsma et al., 2000) we found that alteration of its putative lipid anchor site had no affect on its role in conjugation. We also analyzed whether CwlT functions on the donor cell, the recipient cell, or both. Our results indicate that CwlT activity is essential in the donor and that it most likely does not act on the recipient.

Materials and Methods

Media and growth conditions. Cells were grown at 37°C with agitation in LB medium (Harwood and Cutting, 1990; Sambrook and Russell, 2001) as indicated. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (5 μg/ml), kanamycin (5 μg/ml for *B. subtilis*, 25 μg/ml for *E. coli*), spectinomycin (100 μg/ml), and streptomycin (100 μg/ml). Erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) were used together to select for macrolide-lincosamide-streptogramin B (MLS) resistance. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM.

Strains and alleles. *Bacillus subtilis* strains used in this study are listed in Table 1. Standard techniques were used for cloning and strain construction (Harwood and Cutting, 1990; Sambrook and Russell, 2001). Some alleles related to ICE*Bs1* were previously described (Auchtung et al., 2005; Berkmen et al., 2010; Lee and Grossman, 2007) and are a summarized below. The spontaneous streptomycin-resistant allele (*str84*, most likely in *rpsL*) was used as a counter-selective marker in mating experiments. ICE*Bs1*^{0} indicates that the strain is cured of ICE*Bs1*. *rapI* was overexpressed from Pxyl-*rapI* inserted into *amyE*, *amyE*::{(Pxyl-*rapI*) *spc*}, to induce

ICE*Bs1* gene expression and excision. A functional ICE*Bs1* was placed at the *thrC* locus in *thrC229*::{[ICE*Bs1*Δ(*rapI-phrI*)342::*kan*] *mls*}.

Deletion of cwlT. ΔcwlT19 is an unmarked deletion that removes *cwlT* entirely and fuses the stop codon of *conG* to the intergenic region upstream of *yddI*. A 2.1 kb DNA fragment containing the *ΔcwlT19* allele was obtained by the splice-overlap-extension PCR method (Horton et al., 1989) and cloned into the EcoRI and BamHI sites of the chloramphenicol-resistant vector pEX44 (Comella and Grossman, 2005). The resulting plasmid, pTD6, was used to remove the *cwlT* gene in the chromosome of MMB970 by first integrating by single crossover and then screening for loss of the plasmid by virtue of loss of *lacZ*, and then testing by PCR for introduction of the indicated allele, essentially as described (Lee and Grossman, 2007).

Modification of muramidase and peptidase domains. Mutations in the muramidase and peptidase domains were created using a strategy similar to that for *ΔcwlT*. *cwlT-E87Q* contains an unmarked missense mutation at position 87 of *cwlT*, converting a glutamate codon to a glutamine codon. *cwlT-C237A* contains an unmarked missense mutation at position 237, converting a cysteine codon to an alanine codon. *cwlT-E87Q-C237A* contains both of these mutations. *cwlTΔ207-329* is an unmarked deletion of the entire peptidase domain, consisting of a fusion of the first 206 codons of *cwlT* to its stop codon. 1.2 kb fragments containing one or both of these mutations were constructed and cloned into pCAL1422 by isothermal assembly (Gibson et al., 2009) to yield pTD8 (*cwlT-E87Q*), pTD9 (*cwlT-C237A*), pTD10 (*cwlT-E87Q-C237A*), and pTD310 (*cwlTΔ207-329*). These plasmids were used to introduce their respective alleles into the chromosome of *ΔcwlT19* strain TD19 as described for pEX44 above. Modifications were confirmed by sequencing.

Modifications to cwlT signal sequence. cwlTΔ1-29 contains an unmarked deletion of the first 29 codons of *cwlT* and introduces a start codon at the beginning of the truncated gene. *cwlT-spoVD1-32* contains a markerless replacement of the first 29 codons of *cwlT* with the first 32 codons of *B. subtilis spoVD*, identified as a transmembrane anchor. *cwlT-C23A* contains an unmarked missense mutation that removes the putative lipoprotein anchoring site by converting the cysteine codon at position 23 to an alanine codon. These mutations were introduced into MMB970 with pCAL1422-derived plasmids pTD95 (*cwlTΔ1-29*), pTD99 (*cwlTspoVD1-32*), and pTD116 (*cwlT-C23A*) as described above.

Construction of ICEBs1-cwlT at thrC. Successful complementation of *cwlT* disruptions from an exogenous locus requires the upstream genes of ICE*Bs1* as well as *cwlT*. A complementation construct (*thrC11*) was created by starting with CAL229, which contains the entire ICE*Bs1* integrated into an attachment site (*attB*) placed at *thrC* and marked with macrolide-lincosamide-streptogramin (mls) resistance (Lee and Grossman, 2007). Genes downstream from *cwlT* were deleted by recombination with a DNA fragment containing tetracycline resistance (Lee et al., 2007), yielding strain TD11. Transformation with chromosomal DNA from TD11 was used to introduce the complementation construct to other strains.

Construction of CwlT overexpression plasmids. Plasmids for the overexpression of CwlT as well as mutant and wildtype peptidase domain fragments were constructed similarly to those previously described (Fukushima et al., 2008). The first 29 codons encoding the N-terminal signal sequence in *cwlT* were deleted for productive expression in *E. coli*. A fragment of *cwlT* containing codons for the expression of amino acids 30–329 was amplified by PCR and cloned into pET21b (Novagen) digested with NdeI and HindIII, placing a 6-Histidine sequence at the C-terminus of the protein. This yielded pTD3, which was used for overexpression of CwlT-

MurPep-6His. For expression of the peptidase domain, a fragment encoding amino acids 207 to 329 of *cwlT* was amplified by PCR either from AG174 (wildtype *cwlT*) or TD48 (*cwlT-C237A*), and cloned into pET28a (Novagen) digested with NdeI and HindIII, placing a 6-Histidine sequence at the N-terminus of the protein. This yielded plasmids pTD106 (6His-CwlT-Pep) and pTD107 (6His-CwlT-PepC237A).

Mating assays. Matings were performed essentially as previously described (Auchtung et al., 2005). Briefly, donor and recipient cells were grown in LB, and xylose (1%) was added to donor cells in mid-exponential growth (OD_{600} ~ 0.2) to induce expression of Pxyl-*rapI*. After one hour of RapI expression, equal numbers of donor and recipient cells were mixed and filtered onto sterile nitrocellulose filters. The filters were placed on plates comprised of Spizizen minimal salts (Harwood and Cutting, 1990) and 1.5% agar for 3 h. Transconjugants were identified and mating frequencies were calculated per donor cell. The reported transfer frequencies are the mean (± the standard error of mean) of at least two independent biological replicates.

Purification of CwlT Proteins. *E. coli* BL21 cells containing an arabinose-inducible copy of the T7 RNA polymerase (BL21-AI, Invitrogen) and plasmid pTD3, pTD106, or pTD107 were grown in LB containing 100 μg/mL ampicillin (pTD3) or 25 μg/mL kanamycin (pTD106 and pTD107), shaking at 37° C. At OD_{600} ~0.7 to 0.9, L-arabinose (final concentration of 0.2%) and IPTG (final concentration of 1 mM) were added to induce expression of the T7 polymerase and derepress expression of CwlT. Cells were collected after 2 hours of induction, pelleted by centrifugation, decanted and stored at -80°C.

The cell pellet was thawed on ice, re-suspended in 0.2 volumes lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by addition of CelLytic B (Sigma) and by sonication (microtip, 50% power) on ice 4 × 20 s. The lysate was incubated with DNase I (10 μg/mL) for 30 minutes, and the supernatant was separated by centrifugation at 14,000×g at 4°C for 20 minutes. CwlT-MurPep-6His, 6His-CwlT-Pep, and 6His-CwlT-PepC237A were purified by Ni-NTA column chromatography (Qiagen) according to the manufacturer's protocol for batch purification under native conditions.

Elution fractions were analyzed by SDS-PAGE. Those containing more than ~95% CwlT were pooled and exchanged into storage buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, pH 7.4) using PD-10 desalting columns (GE Healthcare). Protein concentration was determined by Bradford assay (Bio-Rad), glycerol was added to 25%, and protein was stored at -80°C.

Preparation of cell walls. Cell walls from *B. subtilis* and *B. anthracis* were prepared essentially as described previously (Fein and Rogers, 1976; Kuroda and Sekiguchi, 1990). Briefly, cells were harvested from cultures (2 L) in mid-exponential growth phase, resuspended in cold PBS (40 mL), and disrupted by sonication (microtip, 50% power) 15 × 30 s. After low-speed centrifugation (1500×g, 10 min) to remove unbroken cells, the crude cell wall was pelleted at 27,000×g for 5 min at 4°C, suspended in 20 mL of a 4% (w/v) sodium dodecyl sulfate solution (SDS) and boiled for 20 min. Pellets were washed three times with warm deionized water (to prevent precipitation of SDS), two times with 1 M NaCl, and again four times with deionized water. After each of the last four washes, the sample was first spun at low speed (1500×g, 5 min) to separate whole cells and other contaminating material from the cell wall fraction, which was then pelleted by spinning at 27,000×g for 5 min.

Determination of Hydrolytic Activities of the CwlT proteins for *B. subtilis* and *B. anthracis* Cell Walls. Hydrolytic activities were determined essentially as described (Fukushima et al., 2008). Reactions were performed in 50 mM MOPS-NaOH buffer, pH 6.5 at 32°C, with 1 mg/mL *B. subtilis* or *B. anthracis* cell wall preparations. Proteins were added to a final concentration of 10 μg/mL (CwlT-MurPep-6His) or 5 μg/mL (CwlT-Pep and 6His-CwlT-PepC237A), and the reaction mixture was agitated constantly to maintain the cell walls in suspension. Turbidity of the reaction was monitored at 540 nm using a spectrophotometer (Genesis 10, Thermo Corporation).

SDS-PAGE and Zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography were performed as previously described (Leclerc and Asselin, 1989; Sambrook and Russell, 2001). For zymography, ~1 μg of various purified CwlT proteins were electrophoresed through a 12% PAGE gel containing *B. subtilis* or *B. anthracis* cell wall preparations. Following electrophoresis, gels were soaked in deionized water for 30 minutes and then transferred into renaturation buffer (25 mM Tris-HCl, 1% Triton X-100, pH 7.2) at 30°C overnight with gentle agitation. After incubation, the gels were rinsed with deionized water, stained with 0.1% methylene blue in 0.01% KOH for 3 h, and destained with deionized water. Hydrolytic activity appeared as zones of clearing in the blue background of the stained cell walls.

Western Blot Analysis. Samples were collected from cultures after 3 hours of induction of ICE*Bs1* expression, pelleted and stored at -80°C. Pellets were thawed and resuspended in buffer (10 mM Tris, 10 mM EDTA, pH 7) containing 0.1 mg/ml lysozyme and the protease inhibitor 4-

(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) at 1 mM. The volume of buffer used to resuspend each sample of cells was adjusted to the optical density at 600 nm (OD_{600}) in order to normalize the concentration of proteins in each sample. Resuspended cells were incubated at 37°C for 30 min, SDS sample buffer was added, and samples were heated at 100°C for 10 min followed by centrifugation to remove insoluble material.

Proteins were separated by SDS-PAGE on 12% gels and transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) using a Trans-blot semidry electroblot transfer apparatus (Bio-Rad). Membranes were blocked in Odyssey Block (Li-Cor Biosciences) for 1 h, and then incubated in a 1:5,000 dilution of anti-CwlT rabbit polyclonal antisera (made commercially by Covance using CwlT-MurPep-His6 protein purified from *E. coli*) in Odyssey Block with 0.2% Tween for 1 h, and washed several times in 0.1% PBST. Membranes were then incubated with 1:5,000 goat anti-rabbit IRDye 800 CW conjugate (Li-Cor) in Odyssey Block, 0.2% Tween, and 0.01% SDS for 1 hour, and washed several times in PBST. Signals were detected using the Odyssey Infrared Imaging System (Li-Cor) according to manufacturer protocols.

Results

CwlT is required for horizontal transfer of ICE*Bs1*

We constructed a deletion of *cwlT* (Δ*cwlT19*) in ICE*Bs1* (see Materials and Methods, Table 1) and tested for the ability of ICE*Bs1*Δ*cwlT* to function in conjugation. The conjugation efficiency of wild type (*cwlT*+) ICE*Bs1* was ~5% transconjugants per donor (Table 2), similar to frequencies described previously. In contrast, there was no detectable transfer ($\leq 5\times10^{-7}$) of ICE*Bs1*Δ*cwlT* (Table 2, line 2). This phenotype was due predominantly to loss of *cwlT*, as the

mutant phenotype was largely complemented by expressing *cwlT* and all upstream ICE*Bs1* genes from an exogenous locus (Figure 1c; Table 2, line 3). We were unable to complement ICE*Bs1* Δ*cwlT* by expressing *cwlT* alone at an exogenous locus (data not shown). We suspect that proper expression of *cwlT* requires translational coupling to expression of the upstream genes. Similar issues with complementation of other ICE*Bs1* mutants have been described (Berkmen et al., 2010). Our results indicate that the ICE*Bs1*-encoded cell wall hydrolase CwlT is indispensable for conjugation.

Our results with *cwlT* contrast those for cell wall hydrolases from all other characterized mobile genetic elements. To our knowledge, hydrolases from all other characterized mobile elements are at least partially dispensable. That is, loss of the element-encoded hydrolase reduces, but does not eliminate, conjugative transfer. For example, in Gram negative bacteria, loss of the hydrolases VirB1 from the *A. tumefaciens* Ti plasmid (Berger and Christie, 1994), R19 of the P1 plasmid (Bayer et al., 1995), and TraL of pKM101 (Winans and Walker, 1985) result in an approximately 10- to 100-fold reduction in conjugative transfer. In the only other element from a Gram-positive organism, loss of the hydrolase TcpG from pCW3 causes an approximately 1000-fold decrease in conjugation, but does not eliminate it (Bantwal et al., 2012).

We suspect that the apparently greater contribution to conjugation by the element-encoded hydrolases in Gram-positive bacteria is partly due to the thicker cell wall. In addition, many hydrolases have a high degree of cross-functionality (Smith et al., 2000; Vollmer et al., 2008b; Zahrl et al., 2005). That is, there can be redundancy and the loss of one hydrolase is masked by the presence of others. For the conjugative elements, we suspect that the partial requirement for hydrolases could be due to the activities of host hydrolases or those from other resident mobile

elements (Bantwal et al., 2012; Baron et al., 1997; Hoppner et al., 2004; Zupan et al., 2007). For CwlT of ICE*Bs1*, it seems that the host hydrolases are not capable of providing sufficient function to allow any conjugative transfer.

Different effects of muramidase and peptidase mutants of CwlT

CwlT contains two peptidoglycan hydrolytic domains, a muramidase and a peptidase. To determine their respective contributions to ICE*Bs1* transfer, we made mutations in each of the two domains of CwlT and assayed for effects on the conjugation efficiency of ICE*Bs1*.

Muramidase activity is abolished by a previously characterized *cwlT-E87Q* mutation that alters the catalytic site of the muramidase domain (Fukushima et al., 2008). We introduced this mutation into *cwlT* in ICE*Bs1*. There was no detectable transfer of the ICE*Bs1 cwlT-E87Q* mutant (Table 2, line 4), indicating that muramidase activity is required for transfer of ICE*Bs1*. Levels of CwlT-E87Q protein accumulation appear comparable to those of wildtype, as measured by Western blot (data not shown). The defect in conjugation was due to the *cwlT-E87Q* mutation and not to an unexpected effect on downstream genes because the mutant phenotype was fully complemented by exogenous expression of wild type *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 5).

To investigate the role of the peptidase domain, we constructed a point mutation in *cwlT* that changes its putative catalytic cysteine (Anantharaman and Aravind, 2003), *cwlT-C237A*. We used two assays to verify that the mutant protein was defective in enzymatic function: a quantitative kinetic assay to measure the rate at which CwlT degraded purified peptidoglycan, and a zymography assay to detect hydrolase activity in purified proteins or cell lysates (Leclerc and Asselin, 1989). We purified both wild type and mutant peptidase fragments of CwlT. There

was no detectable hydrolytic activity in the C237A mutant peptidase fragment by either kinetic assay or by zymography (data not shown).

We introduced the *cwlT-C237A* mutation into ICE*Bs1* and tested for effects on conjugation. This mutant had a conjugation efficiency of ~5.3×10^{-5}, approximately 1,000-fold less than that of wild type (Table 2, line 6). Levels of CwlT-C237A protein accumulation appear comparable to those of wildtype, as measured by Western blot (data not shown). This defect was due to the *cwlT* mutation and not an unexpected effect on downstream genes because the mutant phenotype was fully complemented by exogenous expression of wild type *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 7).

The conjugation efficiency of the *cwlT-C237A* peptidase mutant (5.3×10^{-5}) was notably higher than that of the muramidase mutant ($< 4.6 \times 10^{-7}$). We were concerned that the *cwlT-C237A* mutation might not fully eliminate the peptidase activity in vivo, and that the detectable conjugation could be a result of residual peptidase activity. To test this, we constructed an allele that deletes the peptidase domain, *cwlTΔ207-329*, leaving the signal sequence and the muramidase domain. The muramidase and peptidase domains have been shown to maintain robust function when separated and purified as fragments (Fukushima et al., 2008). The deletion of the peptidase domain was introduced into *cwlT* in ICE*Bs1*. The conjugation efficiency of ICE*Bs1 cwlTΔ207-329* was ~3.0×10^{-5}, approximately 1,000-fold below that of wild type ICE*Bs1* (Table 2, line 8), and similar to that of the *cwlT-C237A* mutant (Table 2, line 6). Again, the conjugation defect was fully complemented by expression of wild type *cwlT* and the upstream ICE*Bs1* genes (Table 2, line 9). Together, our results indicate that both muramidase and peptidase functions are required for efficient transfer of ICE*Bs1*, and that the muramidase function is absolutely required for transfer, while the peptidase function is partially dispensable.

These results indicate that the cysteine at amino acid 237 is required for peptidase activity. Based on comparisons to other peptidases, C237 is likely in the active site, and histidine at amino acid 290 and the asparagine at amino acid 302 are also likely required for peptidase activity (Anantharaman and Aravind, 2003). Together, our results indicate that both muramidase and peptidase functions are required for efficient transfer of ICE*Bs1*, and that the muramidase function is absolutely required for transfer, whereas the peptidase function is partially required.

CwlT is similar to other hydrolases from well-characterized transfer systems in Gram-positive organisms (Tn916, pIP501, pCW3, pCF10), all of which have or are predicted to have two catalytic domains, a muramidase and a peptidase. Many other putative two-domain hydrolases are found in uncharacterized mobile elements from Gram-positive hosts.

The peptidase domain appears to be a unique addition to hydrolases from Gram-positive systems. Hydrolases in all Gram-negative systems appear to have only a single muramidase domain. Some phage enzymes share a similar domain structure, and it has been suggested that the peptidase domains are important in assisting digestion of highly-crosslinked Gram-positive cell wall (Navarre et al., 1999; Payne and Hatfull, 2012). In ICE*Bs1*, the muramidase function of CwlT is essential, which is consistent with the observation that such activity is conserved in conjugative systems in both Gram-negative and Gram-positive organisms. Alternatively or additionally, Gram-positive organisms often have fewer native autolysins with muramidase activity than do Gram-negatives (Abajy et al., 2007; Vollmer, 2008), so loss of peptidase function may be complemented more easily than loss of the muramidase.

Functional Requirements for CwlT localization

Subcellular localization plays an essential role in the regulation of many hydrolases. CwlT contains an N-terminal signal sequence (residues 1-29) that may determine its localization, though predictions of this region's function are discrepant. Different methods have predicted it to be either a lipoprotein signal sequence (Fukushima et al., 2008; Tjalsma et al., 2000), or a stable transmembrane domain (Juncker et al., 2003; Kall et al., 2004).

Expression of CwlT with an intact N-terminus in *E. coli* caused rapid cell lysis (data not shown). Deletion of the N-terminal 29 amino acids prevents lysis in E. coli, and this modification was required in order to express the protein in sufficient quantities for purification. This would indicate that the N-terminal region is involved in secretion of CwlT.

To examine the function of the putative signal sequence of CwlT in *B. subtilis*, we modified it in several ways. First, we deleted codons 1-29 of *cwlT* (*cwlTΔ1-29*), removing the putative signal sequence. There was no detectable transfer of ICE*Bs1 cwlTΔ1-29*, indicating that this region of CwlT is important for function (Table 3, line 2).

We also found that CwlT is most likely not a lipoprotein. The *cwlT* gene product contains an FVLC motif at amino acids 19-23, which was identified as a putative lipobox, a conserved sequence in lipoproteins (Tjalsma et al., 2000). The cysteine in this motif is where the lipid is covalently attached, and it is required for this attachment. We changed the cysteine at amino acid 23 to alanine (*cwlT-C23A*) and found that there was no detectable change in conjugation efficiency (Table 3, line 4). This result indicates that either CwlT is not a lipoprotein, or that if it is, a lipid attachment at cysteine 23 is not required for CwlT function.

We also replaced the residues 1-29 with the stable transmembrane region (residues 1-32) of SpoVD. SpoVD is a *B. subtilis* homolog of FtsI (Marchler-Bauer et al., 2013), the

transmembrane domain of which has been used previously to target heterologous proteins to the membrane (Xu et al., 2004), and this fragment should cause CwlT to be anchored in the cell membrane. This mutation, *cwlT-spoVD1-32*, completely abolished transfer of ICE*Bs1* (Table 3, line 3). Accumulation levels of all mutant proteins were examined by Western blot analysis and appear comparable to those of wildtype (data not shown). Together, these results indicate that in order to properly function in conjugation, CwlT is exported from the cell and must be able to dissociate from the membrane following secretion. Our results also indicate that CwlT is probably not a membrane-associated lipoprotein.

Overexpression of wild type *cwlT* did not induce lysis in *B. subtilis* as it did in *E. coli*. Even when CwlT was overexpressed for extended periods of time, no cell lysis was observed (data not shown). The *B. subtilis* background may provide additional regulation for CwlT. It is possible that in *B. subtilis*, the signal sequence of CwlT regulates hydrolase activity through a transient membrane association, as is observed in some phage hydrolases (Catalao et al., 2013; Xu et al., 2004), or that other factors may associate with CwlT to modulate or activate its activity in vivo. Alternatively, CwlT could be regulated by other means common in hydrolases, such proteolytic cleavage, or by cytoplasmic membrane polarization.

CwlT functions on the donor but not recipient cells

To assess whether CwlT functions on the donor, on the recipient, or both, we attempted to transfer ICE*Bs1* into recipients that have cell wall chemically different from that of *B. subtilis* (Vollmer et al., 2008a) and that cannot be digested by CwlT. Peptidoglycan from the cell wall of *B. anthracis* is different from *B. subtilis* peptidoglycan in two major ways: its glycan chains are

O-acetylated and *N*-deacetylated. Both of these modifications confer lysozyme resistance to *B. anthracis*.

We found that CwlT was not able to digest cell wall from *B. anthracis*. We purified *B. anthracis* cell wall (Methods) and tested for degradation by CwlT in solution (Figure 2) and in a polyacrylamide gel using zymography (data not shown). In both cases, there was no detectable degradation of the peptidoglycan from *B. anthracis*. To be sure that the preparation of peptidoglycan from *B. anthracis* did not contain an inhibitor of CwlT activity, we mixed the peptidoglycan from *B. anthracis* with that from *B. subtilis*. In this mixed peptidoglycan, CwlT was able to degrade about half of the material present (Fig. 2), indicating that CwlT activity is not inhibited by a factor associated with the peptidoglycan from *B. anthracis*.

Although its cell wall cannot be digested by CwlT, *B. anthracis* was a very effective recipient of ICE*Bs1*. ICE*Bs1* was able to transfer from *B. subtilis* into *B. anthracis* with an efficiency nearly identical to that of transfer from *B. subtilis* to *B. subtilis* (Table 4). Like transfer of ICE*Bs1* from *B. subtilis* to *B. subtilis*, transfer to *B. anthracis* was also dependent on *cwlT* (Table 4). Because the peptidoglycan from *B. anthracis* is different from that of *B. subtilis* and is not digested by CwlT, these results indicate that CwlT is needed to act on the cell wall of the donor, and not that of the recipient. Further, preliminary experiments have shown that whereas ICE*Bs1* is able to transfer from *B. subtilis* into *B. anthracis*, it cannot transfer in the other direction, out of *B. anthracis* and into *B. subtilis*. The element excises from the chromosome at a high frequency, but no conjugation is observed. This result is consistent with the model of CwlT being required in the donor cell: since CwlT cannot digest the cell wall of *B. anthracis*, that species cannot serve as an effective donor.

Neither of CwlT's enzymatic functions showed action on the peptidoglycan of *B. anthracis*. Since *B. anthracis* cell wall is resistant to lysozyme (Balomenou et al., 2013; Davis and Weiser, 2011; Laaberki et al., 2011), it is reasonable to assume that it would be similarly resistant to the muramidase action of CwlT.

It is less apparent why CwlT's peptidase domain cannot digest *B. anthracis* cell wall, as the peptide stems of *B. subtilis* and *B. anthracis* have the same amino acid sequence. However, in *B. subtilis*, the carboxyl group of *meso*-diaminopimellic acid (m-DAP) is amidated (Atrih et al., 1999), a modification that is not found in *B. anthracis* (Vollmer et al., 2008a). Since CwlT's peptidase domain cleaves a bond involving *m*-DAP, lack of this amidation may prevent binding or catalytic activity. CwlT inhibition may also be caused by modifications to the wall such as anionic polymers (Sudiarta et al., 2010).

We have shown that CwlT does not function on the recipient, though this was an intriguing possibility. It is largely unclear how DNA traverses the cell wall and cell membrane of the recipient (Hayes et al., 2010). Many bacteriophages use virion-associated hydrolases to penetrate the host cell wall and allow nucleic acid insertion during initial infection (Moak and Molineux, 2004; Paul et al., 2011; Poranen et al., 2002). It was conceivable that the hydrolase of a conjugative element, particularly one that transfers into Gram-positive species, could provide a similar degradation function on the recipient.

Without this function, the native autolysins in the recipient may play a role in conjugative DNA acquisition. Alternatively, the DNA could pass through the cell wall by simple diffusion. However, it is likely that the relaxase remains covalently attached to the DNA throughout the transfer process, and the size of these proteins is close to the upper limit that can pass through the

unmodified peptidoglycan mesh (Demchick and Koch, 1996; Yao et al., 1999). If the complex can freely diffuse through the cell wall, it is likely not a fast process.

Model for CwlT Activity

Our observations suggest that CwlT plays an essential role in digestion of the peptidoglycan in the donor cell. It is secreted and released from the membrane. It is likely that the protein is causing local enlargement of the peptidoglycan meshwork to allow assembly of the secretion apparatus. It is unknown what other ICE*Bs1*-encoded proteins CwlT associates with, though in the Gram-positive conjugative plasmid pIP501, the hydrolase was shown to associate with the coupling protein, as well as a putative ATPase and a putative membrane-associated protein, indicating that it may be playing a role in recruitment of these proteins, and in their incorporation into a transfer apparatus (Abajy et al., 2007). CwlT may serve a similar role, and it would be interesting to examine whether localization of homologs of these proteins is altered in a CwlT mutant.

Since CwlT activity is completely essential in the donor, the catalytic specificity of the enzyme plays an important role in determining transfer range of ICE*Bs1*. It would appear that while the element can transfer into species with cell walls that CwlT cannot digest, it would be unable to transfer out. To our knowledge, the role of hydrolase activity in determining the ability of a conjugative element to transfer out of a host has not been explored.

Table 1. Strains used

Strain	Relevant Genotype[a] (reference)
JH642	*trpC2 pheA1*
CAL85	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84* (Lee et al., 2007)
MMB970	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*}
TD19	Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19* *amyE*::{(Pxyl-*rapI*) *spc*}
TD37	Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19* *thrC11*::{*mls* ICE*Bs1* 1-311 Δ(*yddI-attR*)*11*::*tet*} *amyE*::{(Pxyl-*rapI*) *spc*}
TD46	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q amyE*::{(Pxyl-*rapI*) *spc*}
TD48	Δ(*rapI-phrI*)*342*::*kan cwlT-C237A amyE*::{(Pxyl-*rapI*) *spc*}
TD50	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A amyE*::{(Pxyl-*rapI*) *spc*}
TD52	Δ(*rapI-phrI*)*342*::*kan cwlT-C237A amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* 1-311 Δ(*yddI-attR*)*11*::*tet*}
TD57	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* 1-311 Δ(*yddI-attR*)*11*::*tet*}
TD62	Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* 1-311 Δ(*yddI-attR*)*11*::*tet*}
TD108	Δ(*rapI-phrI*)*342*::*kan cwlT-spoVD1-32 amyE*::{(Pxyl-*rapI*) *spc*}
TD123	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ*1-29 amyE*::{(Pxyl-*rapI*) *spc*}
TD221	Δ(*rapI-phrI*)*342*::*kan cwlT-C27A amyE*::{(Pxyl-*rapI*) *spc*}
TD319	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ*207-327 amyE*::{(Pxyl-*rapI*) *spc*}
TD321	Δ(*rapI-phrI*)*342*::*kan cwlT*Δ*207-327 amyE*::{(Pxyl-*rapI*) *spc*} *thrC11*::{*mls* ICE*Bs1* 1-311 Δ(*yddI-attR*)*11*::*tet*}
JMA921	*Bacillus anthracis* pXO1^{-} *ind str* derivative of UM44*r* (Hoffmaster and Koehler, 1997)

[a] All *B. subtilis* strains are derived from JH642 (Perego et al., 1988) and contain *trpC2* and *pheA1*.

Table 2. Effects of *cwlT* mutations on conjugative transfer of ICE*Bs1*.

Donor (strain number)	Mating efficiency [a]
WT *cwlT* (MMB970)	$5.9 \times 10^{-2} \pm 1.2 \times 10^{-2}$
ΔcwlT19 (TD19)	$<5 \times 10^{-7}$
ΔcwlT19 thrC37::ICEBs1ΔyddI-yddM (TD37)	$6.6 \times 10^{-2} \pm 6.4 \times 10^{-2}$
cwlT-E87Q (TD46)	$<5 \times 10^{-7}$
cwlT-E87Q thrC37::ICEBs1Δ(yddI-attR) (TD62)	$6.0 \times 10^{-2} \pm 1.1 \times 10^{-2}$
cwlT-C237A (TD48)	$5.3 \times 10^{-5} \pm 3.0 \times 10^{-5}$
cwlT-C237A thrC37::ICEBs1Δ(yddI-attR) (TD52)	$4.4 \times 10^{-2} \pm 6.0 \times 10^{-3}$
cwlTΔ(207-329) (TD319)	$3.0 \times 10^{-5} \pm 7.6 \times 10^{-6}$
cwlTΔ(207-329) thrC37::ICEBs1Δ(yddI-attR) (TD321)	$1.8 \times 10^{-2} \pm 2.1 \times 10^{-2}$

Efficiencies of transfer of ICE*Bs1* (kanamycin-resistant) from the indicated donor strains into the recipient CAL85 (streptomycin-resistant) were calculated from the number of kanamycin-resistant, streptomycin-resistant transconjugants per initial donor.

Donors WT (MMB970), Δ*cwlT* (TD19), *ΔcwlT, thrC::ICEBs1-cwlT* (TD37), *cwlT-E87Q* (TD46), *cwlT-E87Q thrC37::ICEBs1Δ(yddI-attR)* (TD62), *cwlT-C237A* (TD48), *cwlT-C237A thrC37::ICEBs1Δ(yddI-attR)* (TD52), *cwlTΔ207-329* (TD319), and *cwlTΔ207-329 thrC37::ICEBs1Δ(yddI-attR)* (TD321) were grown in LB at 37°C and expression of RapI (Pxyl-*rapI*) was induced by addition of xylose for 1 h. Mating mixtures were incubated at 37°C for 3 h. The reported transfer frequencies are the mean (±SEM) of at least two independent biological replicates. The asterisk indicates that the mating efficiency was lower than the limit of detection for this assay, $4.6 \times 10^{-7} \pm 3.0 \times 10^{-7}$

Table 3. Effects of CwlT signal peptide modification on transfer of ICE*Bs1*.

Donor (strain number)	Mating Efficiency
cwlT wild type (MMB970)	$5.6 \times 10^{-2} \pm 3.5 \times 10^{-2}$
*cwlT*Δ(*1-29*) (TD123)	$<6 \times 10^{-7}$
cwlT-spoVD1-32 (TD108)	$<6 \times 10^{-7}$
cwlT-C23A (TD221)	$6.1 \times 10^{-2} \pm 2.8 \times 10^{-2}$

Efficiencies of transfer of ICE*Bs1* from the indicated donor strains into the recipient CAL85 were calculated from the number of transconjugatns per initial donor. Mating was performed as described in Table 2. The reported transfer frequencies are the mean (±SEM) of at least two independent biological replicates.

Table 4. *Bacillus anthracis* receives ICE*Bs1* as effectively as does *Bacillus subtilis*

Donor	Recipient	Mating Efficiency
Wild type (MMB970)	*B. subtilis*	$5.5 \times 10^{-2} \pm 1.2 \times 10^{-2}$
	B. anthracis	$3.2 \times 10^{-2} \pm 5.9 \times 10^{-3}$
cwlT-E87Q (TD46)	*B. subtilis*	$<6 \times 10^{-7}$
	B. anthracis	$<6 \times 10^{-7}$
cwlT-C237A (TD48)	*B. subtilis*	$2.9 \times 10^{-5} \pm 9.2 \times 10^{-4}$
	B. anthracis	$4.3 \times 10^{-5} \pm 1.0 \times 10^{-5}$

Efficiencies of transfer of ICE*Bs1* from the indicated donor strains into either recipient CAL85 (*B. subtilis*) or JMA921 (*B. anthracis*) were calculated from the number of transconjugants per initial donor. Donor strains are WT (MMB970), *cwlT-E87Q* (TD46), and *cwlT-E87Q* (TD48). Mating was performed as described in Table 2.

The reported transfer frequencies are the mean (±SEM) of at least three independent biological replicates. The asterisk indicates that the mating efficiency was lower than the limit of detection for this assay, $5.2 \times 10^{-7} \pm 1.2 \times 10^{-7}$

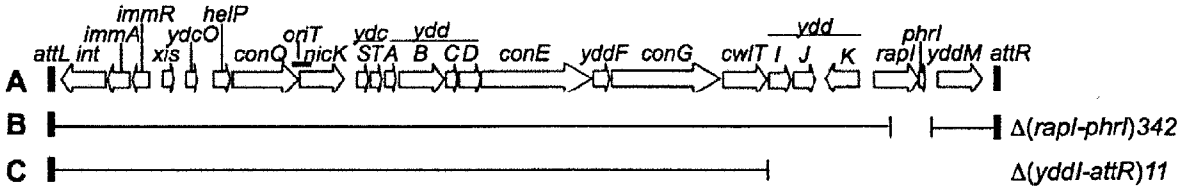

Figure 1. Map of ICE*Bs1* and its derivatives

A. Linear genetic map of ICEBs1 integrated in the chromosome. Open arrows indicate open reading frames and the direction of transcription. Gene names are indicated above or below the arrows. The small rectangles at the ends of the element represent the 60 bp direct repeats that contain the site-specific recombination sites in the left and right attachment sites, *attL* and *attR*.

B and C. Various deletions of ICE*Bs1* used in this study. Thin horizontal lines below the map of ICEBs1 represent regions that are present, and open spaces represent regions that are missing. (Figure adapted from Menard, 2013).

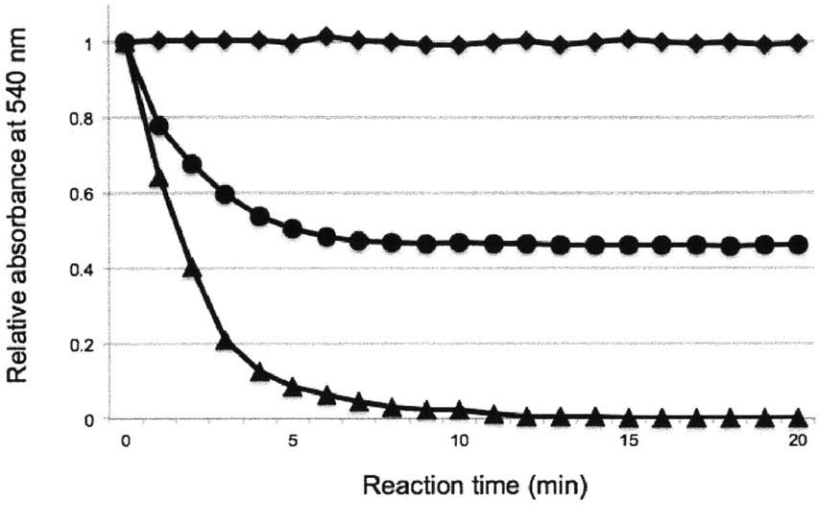

Figure 2. CwlT degrades cell wall peptidoglycan of *Bacillus subtilis* but not of *Bacillus anthracis*

Cell wall lytic activity of CwlT on peptidoglycan from *Bacillus subtilis* (triangles), *Bacillus anthracis* (diamonds), or a 1:1 mix of both types (circles). 1.5 nmol of enzyme was added to approximately 5.0 mg of purified *B. subtilis* cell wall, and the turbidity of the reaction was monitored at 540 nm at 32°C and pH 6.5.

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Appendix A

ICE*Bs1* genes *yddI* and *yddJ* function in conjugation and may show genetic interaction with *cwlT*

Background: I examined the conjugation effects of a deletion of *yddI* and *yddJ*, the two genes immediately downstream of *cwlT*. The proteins encoded by the genes *yddI* and *yddJ* show some similarity to characterized holin proteins, both in their predicted topological structure and their genomic orientation with respect to *cwlT* (Ramanculov and Young, 2001; Wang et al., 2000). Holin proteins can activate hydrolase activity by causing depermeabilization of the cell membrane, allowing hydrolases to access the cell wall (See Chapter 1).

Results: I constructed a deletion of *yddIJ* (*ΔyddIJ-265::cat*) and found that transfer of ICE*Bs1 ΔyddIJ::cat* was reduced approximately 100-fold (Figure 1). This reduction in mating efficiency could be rescued by expressing all of ICEBs1 up to *yddJ* at an exogenous locus (data not shown). To determine whether there is a genetic interaction between *cwlT* and *yddIJ*, I constructed a strain with both *ΔyddIJ* and a previously characterized point mutation in the peptidase domain of CwlT (*cwlT-C237A*) (Described in Thesis Chapter 2). Transfer of ICE*Bs1 cwlT-C237A ΔyddIJ-265::cat* was reduced approximately 1,000-fold, which is the same transfer efficiency of a *cwlT-C237A* mutation on its own. The transfer deficiencies of *cwlT-C237A* and *ΔyddIJ* are not additive. Rather, the *cwlT-C237A* mutation appears to be epistatic to *ΔyddIJ*. This suggests that there is a genetic interaction between *yddIJ* and *cwlT*, and that they may function in the same pathway.

The mating defect phenotype of a *ΔyddIJ* mutant could not be consistently reproduced. In certain replicates, mating deficiencies of *ΔyddIJ* would be nearly identical to those of TD265 in Figure 1, approximately 100-fold below wildtype. In other trials, *ΔyddIJ* would reduce transfer by an almost undetectable amount, close to 2–4 fold.

Discussion: These results indicate that *yddI* and *yddJ* may play a role in conjugation, and that they may have a genetic interaction with *cwlT*. However, before drawing conclusions from

this data, these results must be consistently reproduced. All replicate experiments were carried out as similarly as possible. However, variations in the media, growth phase, or other factors may have had influence. It would be interesting to repeat these experiments and attempt to identify experimental factors that may contribute to a Δ*yddIJ*-mediated transfer deficiency.

Methods: Media and growth conditions are as described in Chapter 2 of this thesis.

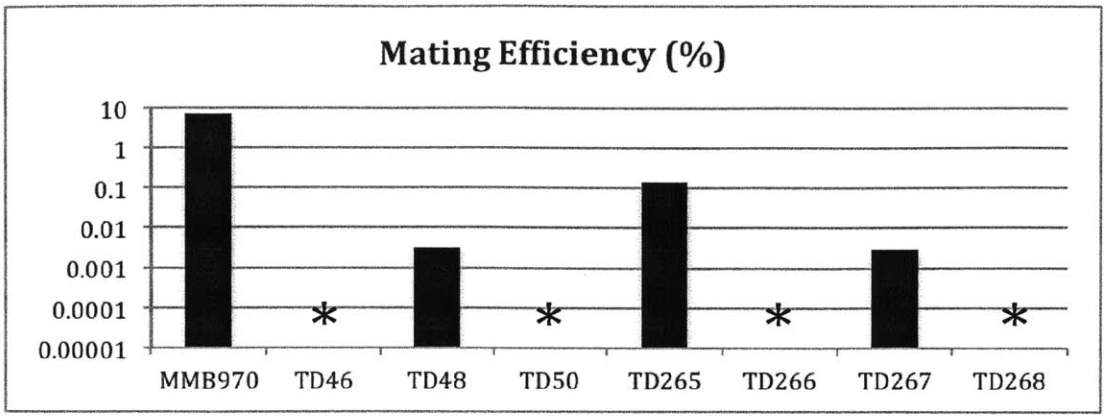
ΔyddIJ-265::cat was created with long-template PCR. Briefly, 1,000 kb upstream of *yddI* and downstream of *yddJ* were amplified in an initial reaction with primers that added homology to the chloramphenicol-resistance gene in pGEM-cat. The products of the first reaction were then used as primers to amplify the cat^R gene from pGEM-cat in a two-step long template PCR reaction. Amplification products were examined on an agarose gel, column purified, and used to transform MMB970, TD46, TD48, and TD50. Chloramphenicol-resistant mutants were obtained and backcrossed into the parent strains to create TD265, TD266, TD267, and TD268.

Mating assays were performed as described in Chapter 2 of this thesis.

Table 1. *B. subtilis* strains used.

Strain	Relevant Genotype
MMB970	Δ(*rapI-phrI*)342::*kan amyE*::{(Pxyl-*rapI*) *spc*}
CAL85	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84*
TD46	Δ(*rapI-phrI*)342::*kan cwlT-E87Q* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}
TD48	Δ(*rapI-phrI*)342::*kan cwlT-C237A* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}
TD50	Δ(*rapI-phrI*)342::*kan cwlT-E87Q-C237A* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}
TD265	Δ(*rapI-phrI*)342::*kan ΔyddIJ-265::cat amyE*::{(Pxyl-*rapI*) *spc*}
TD266	Δ(*rapI-phrI*)342::*kan ΔyddIJ-265::cat cwlT-E87Q* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}
TD267	Δ(*rapI-phrI*)342::*kan ΔyddIJ-265::cat cwlT-C237A* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}
TD268	Δ(*rapI-phrI*)342::*kan ΔyddIJ-265::cat cwlT-E87Q-C237A* (unmarked) *amyE*::{(Pxyl-*rapI*) *spc*}

All strains are derivatives of JH642 and contain *pheA1* and *trpC2* mutations (not shown).



cwlT	wt	M-	P-	M- P-	wt	M-	P-	M-P-
yddIJ	wt	wt	wt	wt	ΔIJ	ΔIJ	ΔIJ	ΔIJ

All recipients are CAL85, a str^R ICE*Bs1*^{0} recipient strain.

Figure 1: Conjugation effects of a *yddIJ* deletion

* Signifies that the number of transconjugants were lower than the limit of detection, approximately 8×10^{-6}.

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Appendix B

Expression of the extracellular sigma factor SigV inhibits transfer of ICE*Bs1*

Background: Data suggests that CwlT functions on the cell wall of the donor, and not the cell wall of the recipient (See Thesis Chapter 2). To further test this model, I attempted to modify the wall of the donor, to make it indigestible to CwlT, and assay the effect on ICE*Bs1* transfer. Recently, it was shown that the extracellular sigma factor SigV causes lysozyme resistance in *B. subtilis* by promoting expression of two peptidoglycan-modifying enzymes: *dltA*, a peptidoglycan *N*-deacetylase, and *oatA*, a peptidoglycan *O*-acetylase (Guariglia-Oropeza and Helmann, 2011; Ho et al., 2011).

While overexpression of SigV in the donor cells causes a decrease in ICE*Bs1* mating efficiency, this is not due to its effect on *dltA* or *oatA*, as deletions of these genes did not restore wildtype mating levels.

Results: I constructed donor and recipient strains that overexpressed SigV from the P*spank*(hy) promoter (P*spank*[hy]-*sigV*), and then assayed mating from donor to recipient cells. Expression of SigV in the donor cells lowered transfer by approximately 500-fold, whereas expression of SigV in the recipient cells showed no significant effect on mating (Figure 1).

To assess whether the effect of SigV was due to peptidoglycan modifications mediated by its target genes *dltA* and *oatA*, I created deletions of *dltA* (*ΔdltA-142::cat*) and oatA (*ΔoatA-146::tet)*. No rescue in mating efficiency was observed during transfer from SigV-expressing donors deleted for *dltA* (Figure 1), or *dltA oatA* (data not shown). Both SigV-expressing donor strains *dltA* and *dltA oatA* showed the same reduced transfer rates as wildtype donors expressing SigV.

Discussion: SigV overexpression in the donor significantly reduces ICE*Bs1* transfer. This reduction is not seen when SigV is expressed in the recipient. Although SigV is known to cause peptidoglycan modifications that confer cell wall resistance to lysozyme, the transfer reduction

observed in SigV overexpression is likely not due to effects on peptidoglycan, as deletion of the peptidoglycan-modifying target genes of SigV had no apparent effect on the mating deficiency. SigV has a number of other targets besides *dltA* and *oatA*, many of which are uncharacterized or incompletely characterized, and activation of one or more than one of these may affect ICE*Bs1* mating processes, either directly or indirectly.

Methods. Media and growth conditions are as described in Chapter 2 of this thesis.

The SigV-overexpressing construct P*spank*[hy]-*sigV* was created by PCR amplification of the *sigV* gene, followed by cloning into the plasmid pCAL838 (mls^R) downstream of the P*spank*(hy) promoter to yield pTD117. pTD117 was used to transform MMB970 and CAL85, integrating into the chromosome by double crossover. MLS-resistant colonies were obtained and backcrossed to yield TD130 and TD134, respectively.

ΔdltA-142::cat and *ΔoatA-146::tet* were created with long-template PCR. Briefly, 1,000 kb upstream and downstream of target genes were amplified in an initial reaction with primers that added homology to either the chloramphenicol-resistance gene in pGEM-cat (*ΔdltA-142::cat*) or the tetracycline-resistance gene in pDG1513 (*ΔoatA-146::tet*). The products of the first reaction were then used as primers to amplify the cat^R gene from pGEM-cat or the tet^R gene from pDG1513 in a two-step long template PCR reaction. Amplification products were examined on an agarose gel, column purified, and used to transform TD130 and TD134 to yield strains with various combinations of deletion alleles (see strain table).

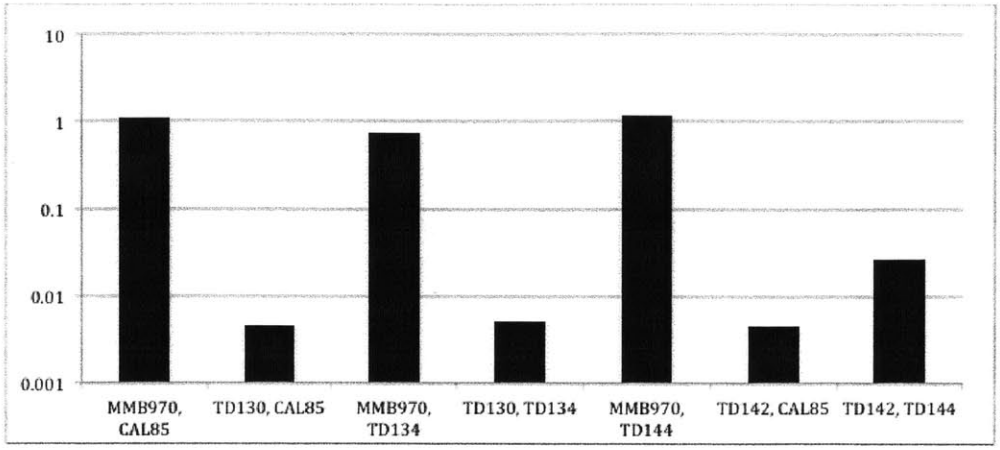
Mating was performed essentially as described in Chapter 2 of this thesis, although donors and recipients were passaged in IPTG to allow sufficient expression of P*spank*(hy)-*sigV*. Cells were first grown in LB media to OD_{600} ~ 0.2, and then diluted back 1:10 into LB medium with 1 mM IPTG. This passaging and dilution was repeated either two or three times. At the end of the

last dilution cycle, RapI expression in donors was induced with 1% xylose at OD_{600} ~ 0.2, and cells were then grown and mated according to standard protocol.

Table 1. *B. subtilis* strains used.

Strain	Relevant Genotype
MMB970	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*}
CAL85	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84*
TD130	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*} thrC::{(P*spank*{hy}-*sigV*) *mls*}
TD134	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84 thrC*::{(P*spank*{hy}-*sigV*) *mls*}
TD142	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*} thrC::{(P*spank*{hy}-*sigV*) *mls*} *dltA-142*::*cat*
TD144	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84 thrC*::{(P*spank*{hy}-*sigV*) *mls*} *dltA-142*::*cat*
TD146	Δ(*rapI-phrI*)*342*::*kan amyE*::{(Pxyl-*rapI*) *spc*} thrC::{(P*spank*{hy}-*sigV*) *mls*} *dltA-142*::*cat oatA-146*::*tet*
TD150	ICE*Bs1*^{0} (cured of ICE*Bs1*) *str84 thrC*::{(P*spank*{hy}-*sigV*) *mls*} *dltA-142*::*cat oatA-146*::*tet*

All strains are derivatives of JH642 and contain *pheA1* and *trpC2* mutations (not indicated).



Donor							
wildtype	X		X		X		
Pspank(hy)-sigV		X		X			
Pspank(hy)-sigV ΔdltA						X	X
Recipient							
wildtype	X	X				X	
Pspank(hy)-sigV			X	X			
Pspank(hy)-sigV ΔdltA					X		X

Figure 1. Expression of SigV in donor reduces ICE*Bs1* transfer efficiency

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Chapter 3

Discussion

Essentiality of CwlT in ICE*Bs1* Transfer

My results show that CwlT is essential for conjugative transfer of ICE*Bs1*. To our knowledge, hydrolases in all other characterized mobile elements are at least partially dispensable. The essentiality of CwlT may be due in part to the thick and heavily crosslinked cell wall of *B. subtilis* (Abajy et al., 2007; Alvarez-Martinez and Christie, 2009), and it may also be caused by a lack of other hydrolases in the cell that can complement CwlT activity (Smith et al., 2000; Vollmer et al., 2008b; Zahrl et al., 2005).

The only other hydrolase characterized in a Gram-positive transfer system is TcpG from the *C. difficile* conjugative plasmid pCW3, and its disruption led to a 1000-fold transfer deficiency (Bantwal et al., 2012), more than that seen in Gram-negative systems, yet still not the virtual elimination of transfer seen with a *cwlT* deletion in ICE*Bs1*. In *C. difficile*, it has been suggested that a hydrolase found on the plasmid pCP13 may provide low-level rescue of the TcpG deletion (Bantwal et al., 2012).

There may also be structural differences between the cell walls of *C. difficile* and *B. subtilis* that allow mating channel components to more easily assemble in the cell wall of *C. difficile* without additional hydrolytic function. Native autolytic activity may be higher in *C. difficile*, or the components of pCW3 may more easily assemble than those of ICE*Bs1*. Additionally, though TcpG and CwlT both have two hydrolytic domains, the proteins show virtually no similarity in structure, and it has been proposed that TcpG and CwlT represent members of different hydrolase sub-classes (Bhatty et al., 2013). In this regard, CwlT may serve additional functions that are not performed by TcpG.

In both the Gram-positive conjugative plasmids pIP501 and pCW3, the peptidoglycan hydrolases Orf7 and TcpG were shown to associate with the coupling proteins (Abajy et al.,

2007; Steen et al., 2009), which interact with the relaxosome and are thought to play an important role in recruiting the complex to the conjugation machinery. In ICE*Bs1*, CwlT may be an essential participant or intermediary in this recruitment.

More likely, CwlT may play a less direct role, in which its hydrolytic activity is required for components of the secretion apparatus to localize, and CwlT interacts with them as they move into position. In pIP501, the hydrolase Orf7 was also shown to interact with two other components of the conjugation machinery besides the coupling protein: the VirB4-like protein Orf5, and Orf14, whose function is unknown (Abajy et al., 2007). Future work could examine which ICE*Bs1* proteins are interactors of CwlT, and whether these interactions have functional consequences for localization or channel assembly *in vivo*.

Role of the Two Hydrolytic Domains

We have shown that the muramidase domain of CwlT is absolutely required for ICE*Bs1* transfer, but that the peptidase domain is partially dispensable and its disruption lowers transfer by approximately 1000-fold.

For the peptidase domain disruption, the complete domain deletion and the catalytic point mutant both caused the same reduction in transfer efficiency. This indicates that if the peptidase domain has any other roles in conjugation, they require the peptidoglycan degradation activity, and they are secondary to that function.

Two-domain hydrolases with high similarity to CwlT are found in other characterized conjugative elements from Gram-positive hosts, and they appear abundant in many other uncharacterized, putative mobile elements.

In Gram-negative organisms, hydrolases from different transfer systems are often able to substitute for one another (Hoppner et al., 2004; Zahrl et al., 2005), an observation that may imply hydrolases were late, relatively non-specific evolutionary additions to conjugative transfer (Zahrl et al., 2005). It would be interesting to examine whether two-domain hydrolases from other Gram-positive conjugative systems or even phages could complement a *cwlT* deletion, and whether these two catalytic functions must be provided on the same protein.

It would also be interesting to investigate whether the two domains of CwlT have other functions besides their peptidoglycan degradation role, or if the domains are separated by proteolytic cleavage. From Gram-negative systems, there is precedent for multifunctional hydrolases. In the Agrobacterium system, the hydrolase VirB1 is processed to form a C-terminal secreted product, VirB1* (Baron et al., 1997). It has been shown that VirB1* is required for T-pilus formation, and it has been proposed that it may serve a chaperone-like function, preventing the interactions of the pilin subunits until they are at the site of assembly (Zupan et al., 2007).

Localization and Regulation of CwlT

We have showed that CwlT's N-terminal signal peptide is required for its function in conjugation, that it is not a stable transmembrane domain, and that it is likely not a lipoprotein signal sequence. These results would suggest that CwlT must dissociate from the membrane in order to function in conjugation.

An intriguing question is how CwlT's potentially lethal lytic function is controlled. Though mechanisms of hydrolase regulation are generally not well characterized, sub-cellular localization often plays a role, particularly for phage endolysins, to which CwlT and other

mobile element hydrolases show some similarity. However, if CwlT is regulated by a phage-like mechanism, it does not fit neatly into any established regulatory paradigm.

CwlT's signal sequence appears to direct secretion. This sequence is functional for secretion in *E. coli*, as expression of full-length CwlT causes fast and complete lysis of cells. However, in *B. subtilis*, either the secretion or activity of CwlT is significantly attenuated, as overexpression for long periods of time causes no observable cell death. A small category of phage endolysins encodes N-terminal tags that direct their extracellular secretion (Oliveira et al., 2013). For at least one of these, Lys44 of the *Oenococcus oeni* phage fOg44 (Nascimento et al., 2008; Sao-Jose et al., 2000), the signal sequence acts as an inhibitory element and must be cleaved to activate enzymatic function; perhaps CwlT is regulated in a similar fashion, and this processing event occurs readily in *E. coli* but not in *B. subtilis*.

Alternatively, CwlT could be regulated by transient sequestration in the membrane, somewhat similar to a signal-arrest-release (SAR) endolyin (Kuty et al., 2010; Sun et al., 2009; Xu et al., 2004), and it may require factors that release it or mediate its activation. However, if CwlT does have such accessory factors, they are not readily apparent. Two small proteins in ICE*Bs1*, encoded by the genes *yddI* and *yddJ*, show some similarity to characterized holin proteins, both in their predicted topological structure and their genomic orientation with respect to *cwlT* (Ramanculov and Young, 2001; Wang et al., 2000). However, co-expression of holins and endolyins should cause rapid cell lysis (Park et al., 2006; White et al., 2010; Young, 2002), and none was observed when *cwlT* was over-expressed with either *yddI*, *yddJ*, or both.

Perhaps the lack of a holin or release factor causes modulation of CwlT activity. When expressed without holins, SAR-containing proteins can slowly and eventually escape from the membrane (Sun et al., 2009). If CwlT is endowed with a SAR-like domain that initially causes

its sequestration in the membrane but allows gradual release, it could be the lack of an activating factor that prevents it from lysing the cell. If this were the case, co-expression of CwlT with a characterized holin protein would likely cause lysis.

Once associated with the cell wall, it is likely that CwlT action is regulated by many of the mechanisms that regulate native autolysin activity of *B. subtilis*. In particular, the energized state of the membrane is important in controlling native autolysin activity (Smith et al., 2000; Vollmer et al., 2008b), as energy poisons such as sodium azide that cause membrane depolarization can cause rapid autolysis in *B. subtilis* (Blackman et al., 1998; Jolliffe et al., 1981).

Proteolytic cleavage seems to play an important role in a number of different autolysin regulatory events (See Thesis Introduction). CwlT may be processed into separate muramidase and peptidase forms, causing activity modulation. Alternatively or additionally, CwlT (or its processing products) may be targeted for proteolytic degradation, preventing build-up and cell lysis.

Future work could investigate CwlT's localization, determine its secretion path, and examine whether it associates with the membrane at any point, and if so, what the dynamics of this association are. In terms of the regulation of CwlT activity, it would be interesting to further examine why CwlT causes lysis in *E. coli* but not in *B. subtilis*. Examining whether CwlT could substitute for a phage endolysin and under what circumstances would give additional information about modes of regulation that may be present in *B. subtilis*.

CwlT Acts on the Donor and Not the Recipient

We have shown that CwlT catalytic function is absolutely required for ICE*Bs1* transfer, and that ICE*Bs1* can transfer with high efficiency into recipients that have cell walls that CwlT

cannot digest. Taken together, these observations suggest that CwlT functions on the cell wall of the donor, but not on the cell wall of the recipient.

The glycan strands of *B. anthracis* cell wall may be resistant to CwlT because of *N*-deacetylation and *O*-acetylation that also confer lysozyme resistance (Vollmer, 2008). The peptide stems may be resistant because they lack the amidation of *meso*-diaminopimellic acid that is seen in *B. subtilis*.

Peptidase binding may also be inhibited by the glycan strand modifications in *B. anthracis*. Unlike many cell wall hydrolases, CwlT does not have a cell wall binding domain (CBD). In the absence of a CBD, a positive charge can be necessary for hydrolases to bind strongly to peptidoglycan (Low et al., 2011). Isoelectric point calculations predict that the muramidase domain would have a charge of approximately +3 at pH 8, while the peptidase domain would have a charge of -2. It is possible that the peptidoglycan binding of the muramidase domain is essential for proper binding of the peptidase domain. If *N*-deacetylation and *O*-acetyaltion decrease binding of the CwlT muramidase domain as they do lysozyme (Vollmer, 2008), peptidase binding could also be affected.

Conjugative Hydrolases and Element Host Range

Enzymatic specificity of CwlT would appear to play an essential role in determining host range and transfer capabilities for ICE*Bs1*. Since transfer requires CwlT enzymatic activity, ICE*Bs1* is not able to transfer out of an organism with a cell wall that CwlT cannot digest.

Very little is known about the relationship between endolysin activity and host range of a horizontal element. However, studies have shown that the enzymatic domains of phage endolysins are often highly adapted to the specific peptidoglycan structure of their hosts. For

instance, in *Staphylococcus*, *Streptococcus* and *Lactococcus* species, the carbohydrate-bound peptide stems are not directly crosslinked as in most other species, but are joined by unique interpeptide bridges (Schleifer and Kandler, 1972; Vollmer et al., 2008a). Endolysins from phages that infect these species tend to have a peptidase domain that cleaves their specific interpeptide linkages (Oliveira et al., 2013). Further, lysozyme domains are found more predominantly in phages from Gram-negative species, presumably because many Gram-positive species have peptidoglycan that is resistant to lysozyme because of *N*-deacetylation or *O*-acetylation (Oliveira et al., 2013).

For conjugative elements, even less is known about the relationship between hydrolases and host range. In a pattern similar to the distribution of phage endolysins, single-domain muramidases appear most widespread in elements from Gram-negatives species, and hydrolases from Gram-positive species appear to be endowed with additional peptidase function.

Some of the most extensive information of conjugative element host range comes from studies of the ICE Tn*916*. This element is able to transfer into an extremely wide range of over 50 recipients, both Gram-negative and Gram-positive, including *E. coli* and species of *Bacillus*, *Lactobacillus*, *Streptococcus*, *Mycoplasma*, *Listeria*, and *Enterococcus* (Clewell et al., 1995; Rice, 1998). These organisms have widely differing cell walls, in terms of both modifications to their carbohydrate chains and diversity in peptide cross-linking. Though Orf7, the hydrolase encoded by Tn916, has two domains, it would be unlikely that it could digest the wide range of peptidoglycan variation seen across these recipients, which lends additional credence to the model of peptidoglycan digestion being necessary only in the donor.

Additionally, not all species that can receive Tn916 can act as donors. While the element can transfer from *Enterococcus faecalis* into *Lactococcus lactis*, it cannot transfer out, even when its

excision is induced at high levels (Marra et al., 1999; Rice, 1998). Since *L. lactis* cell wall has unique peptide crosslinks that would be unlikely substrates for an enzyme specific for *E. faecalis* peptiodoglycan, it is possible that Tn916 cannot transfer out of *L. lactis* because Orf7 cannot efficiently digest its peptidoglycan.

While extensive studies have determined which species Tn916 can transfer into, the donor is usually *E. faecalis*, and considerably less is known about which species can serve as donors themselves. Still, in many cases the rate of transfer of Tn916 is extremely low, often close to 10^{-6} and 10^{-9} transconjugants per donor (Bertram et al., 1991; Clewell et al., 1995; Franke and Clewell, 1981), which is much lower than the 10^{-1} to 10^{-2} efficiency seen for ICE*Bs1*, and is in fact very close to the limit of detection in our assays. In such a system with such low transfer, it may be difficult to tell whether conjugation defects are due to a hydrolase essentiality or not. ICE*Bs1*, with its high transfer rate, would be an ideal system in which to examine the affect of hydrolase on the ability to serve as a donor.

In general, it would be interesting to more thoroughly examine which species conjugative elements can transfer out of and whether the hydrolase specificity may affect this. This type of investigation on host range would have interesting implications for the study of phage infection, horizontal transfer, and coevolution between phages, horizontal elements, and their bacterial hosts.

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