Interactions between an integrative and conjugative element and its bacterial host

by

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Abstract

Conjugative elements are mobile genetic elements that can transfer from a donor bacterium to a recipient via an element-encoded type IV secretion system. Integrative and conjugative elements (ICEs) are an abundant class of conjugative element. ICEs are typically integrated into the bacterial host chromosome, but under certain conditions, or stochastically, they can excise from the chromosome and transfer to a recipient. ICEs likely interact with their bacterial host at every stage of their life cycle, but few of these interactions have been characterized. In this work I sought to 1) identify bacterial host factors necessary for efficient transfer of the integrative and conjugative element ICE*Bs1* to a recipient, and 2) determine whether the ICE*Bs1*-encoded cell wall-modifying enzyme CwlT acts on the cell wall of the donor bacterium, the recipient bacterium, or both.

I used CRISPR interference to induce a knockdown of individual essential *Bacillus subtilis* genes, and then screened for gene knockdowns that caused an acute defect in transfer of ICE*Bs1*. I found that wall teichoic acids were necessary in both ICE*Bs1* donors and recipients for efficient conjugative transfer. I found that depletion of wall teichoic acids caused cells involved in ICE*Bs1* conjugation to sustain lethal envelope damage caused by active conjugation machinery.

Conjugative elements must bypass the cell wall of both the donor and recipient cells in a mating pair. Conjugative elements encode cell wall hydrolases that are required for efficient transfer, which are presumed to partly degrade the cell wall of the donor bacterium during conjugation. In order to investigate the role of the ICE*Bs1*-encoded cell wall hydrolase CwlT in conjugation, I generated cell wall-less (L-form) strains of *B. subtilis* which could donate or receive ICE*Bs1*. In the absence of either the donor or recipient cell wall, CwlT was dispensable for efficient transfer. This finding indicates that CwlT acts on both the donor and recipient cell wall in a mating pair.

Thesis Supervisor: Alan D. Grossman
Title: Praecis Professor of Biology; Department Head

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Chapter 1

Introduction

Overview

Horizontal gene transfer is a fundamental force in bacterial evolution, allowing for the rapid spread of genes involved in processes as diverse as antibiotic resistance, pathogenesis, and metabolism. Horizontal gene transfer is often facilitated by conjugative elements, segments of DNA which encode the means to transfer a copy of themselves from their host to a recipient via direct cell-to-cell contact. Conjugative elements have evolved to interact with their bacterial hosts, but the nature and extent of these interactions remain poorly understood. In this work I further our understanding of how the integrative and conjugative element ICE*Bs1* interacts with its bacterial host *Bacillus subtilis*.

Significance of Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the acquisition of genetic material by an organism from an external source other than the parent (Soucy *et al.*, 2015). HGT is a fundamental force in bacterial evolution: it allows for the rapid exchange of genetic material between even distantly-related species, and it is an important source of genetic diversity (Ochman *et al.*, 2000; Gogarten *et al.*, 2002). Significant portions of some bacterial genomes appear to have been acquired via relatively recent HGT events, including ~18% of the *Escherichia coli* genome (Narra and Ochman, 2006). Many of the genes that commonly spread via HGT are of significant interest, including genes related to antibiotic resistance, pathogenesis, and metabolism. HGT is often mediated by mobile genetic elements (MGEs), segments of DNA that encode the means to transfer themselves between cells or to new sites within a genome (Frost *et al.*, 2005).

HGT drives the spread of antibiotic resistance genes and has contributed to the emergence of drug-resistant human pathogens. Methicillin-resistant *Staphylococcus aureus* is defined by the

presence of a horizontally-acquired MGE (SCC*mec*) which confers resistance to β-lactam antibiotics (Chambers and DeLeo, 2009). A large proportion of the *Clostridium difficile* genome contains horizontally acquired DNA, and different isolates contain elements conferring resistance to tetracycline, chloramphenicol, erythromycin, and aminoglycosides (Sebaihia *et al.*, 2006; He *et al.*, 2010). The problem of emergent antibiotic resistance is especially prevalent among *Enterobacteriaceae* species such as *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*. Horizontally-acquired plasmids conferring resistance to β-lactams, quinolones, aminoglycosides, and carbapenems have all been identified among these species (Huddleston, 2014).

HGT is also a major mechanism by which pathogenicity and virulence genes spread. A significant example of this is the prevalence of *S. aureus* pathogenicity islands. These pathogenicity islands can encode medically significant virulence factors, and they spread horizontally by co-opting the capsids of bacteriophages (Novick *et al.*, 2010). Many of the virulence factors associated with pathogenic *E. coli* are also encoded by MGEs, including the Shiga toxin genes from enterohemorrhagic *E. coli* (Ogura *et al.*, 2015).

HGT allows bacteria to rapidly acquire new metabolic capabilities and thereby colonize new ecological niches. Rhizobia are bacteria which can form symbiotic relationships with legumes by invading root cells and facilitating the formation of nitrogen-fixing nodules. The genes required for symbiosis are often encoded by large chromosomal or extrachromosomal MGEs, and HGT of these genes has facilitated their spread within and between genera (Remigi *et al.*, 2016).

Mechanisms of Horizontal Gene Transfer

The three major mechanisms of HGT among bacteria are transformation, transduction, and conjugation (Figure 1) (Thomas and Nielsen, 2005).

Transformation is the uptake of free DNA from the environment, and bacteria that express the DNA uptake machinery are referred to as being naturally competent. Some bacteria produce this machinery constitutively, but most regulate its expression and only enter a competent state in response to stimuli like nutrient limitation or DNA-damage. DNA binding during transformation is typically mediated by a type IV pilus or a similar structure, and a single strand of the DNA can subsequently be imported via a dedicated membrane channel (Johnsborg *et al.*, 2007; Dubnau and Blokesch, 2019).

Transduction is the introduction of foreign DNA into a bacterium via a bacteriophage vector. Generalized transduction occurs when a phage capsid is mistakenly loaded with bacterial DNA instead of a viral genome. This mispackaged bacterial DNA can subsequently be injected into a new cell and incorporated into the new bacterial genome. Specialized transduction occurs when a prophage excises improperly from the bacterial genome and mistakenly incorporates both viral and prophage-proximal host genes into the phage particle (Frost *et al.*, 2005; Chiang *et al.*, 2019).

Conjugation, which is the subject of the work described in this report, is the contact-dependent transfer of DNA between bacteria through a dedicated mating channel. Conjugation is often mediated by two classes of MGEs: conjugative plasmids or integrative and conjugative elements (ICEs) (Smillie *et al.*, 2010; Guglielmini *et al.*, 2011). These elements encode the means to transfer out of a donor bacterium and into a recipient bacterium, most commonly via a type IV secretion system (T4SS) that temporarily bridges the cytoplasm of the two bacteria

(Chandran Darbari and Waksman, 2015). In addition to facilitating their own transfer, conjugative elements can also sometimes facilitate transfer of other "mobilizable elements". Mobilizable elements lack their own functional transfer machinery and are not self-transmissible, but can instead exploit the conjugation machinery of other elements to spread horizontally (Ramsay and Firth, 2017).

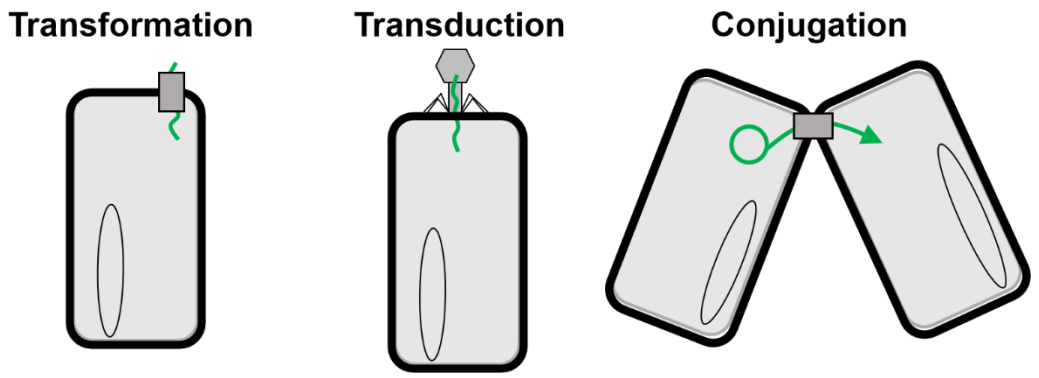

Figure 1. Mechanisms of horizontal gene transfer. The three major mechanisms of horizontal gene transfer among bacteria are transformation, transduction, and conjugation. Transformation is the uptake of DNA from the environment, transduction is the transfer of DNA to a bacterium by a bacteriophage vector, and conjugation is the contact-dependent transfer of DNA between cells via a dedicated mating channel (Thomas and Nielsen, 2005).

Conjugative Type IV Secretion Systems

Conjugation is typically mediated by T4SSs, large macromolecular protein complexes that span the bacterial cell envelope and allow for the unidirectional passage of single-stranded DNA (ssDNA) and proteins (Chandran Darbari and Waksman, 2015). Three categories of T4SS have been described: conjugative T4SSs that facilitate the delivery of ssDNA and proteins to a recipient cell, effector translocators that deliver proteins to eukaryotic cells, and release/uptake systems that allow for substrate transfer without requiring contact with a target cell (Alvarez-

Martinez and Christie, 2009; Bhatty *et al.*, 2013). The best characterized T4SS is the archetypal VirB/VirD4 system from the Ti plasmid of *Agrobacterium tumefaciens* (Christie *et al.*, 2014). This T4SS comprises 12 plasmid-encoded proteins (VirD4 and VirB1-11), and components of other T4SSs are often named in reference to their VirB/D4 homolog or functional analog (Figure 2, left) (Alvarez-Martinez and Christie, 2009; Bhatty *et al.*, 2013).

The VirB/D4 system is the prototypical example of a gram-negative conjugative T4SS (Figure 2, left). Eleven of the twelve VirB/D4 T4SS subunits are necessary for substrate transfer: VirD4 and VirB2-B11 (Grohmann *et al.*, 2018). VirD4 is the coupling protein, an ATPase that mediates the interaction between the conjugation machinery and the substrate to be transferred. VirB4 and VirB11 are also ATPases, which together with coupling protein are presumed to provide the energy for channel assembly and substrate translocation (Christie *et al.*, 2014). VirB3, VirB6, and VirB8 constitute the inner membrane platform of the secretion channel, whereas VirB7, VirB9, and VirB10 constitute the outer membrane complex (Christie *et al.*, 2014; Grohmann *et al.*, 2018). VirB2 and VirB5 are components of the pilus, an extracellular structure that protrudes from the donor to make contact with the recipient and which retracts to facilitate cell-cell contact (Christie *et al.*, 2014; Chandran Darbari and Waksman, 2015). Lastly, VirB1 is a cell wall hydrolase that is presumed to modify the layer of peptidoglycan in the periplasm to create an opening for the channel (Alvarez-Martinez and Christie, 2009).

Conjugative T4SSs from gram-positive bacteria have fewer essential components than their gram-negative counterparts (Bhatty *et al.*, 2013; Grohmann *et al.*, 2018). Each conserved component has a Vir analog: VirB1, B3, B4, B6, B8, and the T4CP (Figure 2, right). Gram-positive T4SSs lack analogs of the VirB components associated with the outer membrane core complex in gram-negatives, and they also lack the VirB11 ATPase (Grohmann *et al.*, 2018).

Furthermore, gram-positive T4SSs do not utilize conjugative pili for target cell attachment; instead, some gram-positive conjugation systems produce surface adhesins to help form a stable contact between cells (Bhatty *et al.*, 2013). The cell wall hydrolases (VirB1 analogs) from gram-positive systems also have properties that distinguish them from gram-negative hydrolases (Goessweiner-Mohr *et al.*, 2013), and these differences are discussed at length later in this chapter.

The mechanism of conjugation is broadly similar in gram-positive and gram-negative bacteria and can be divided into a series of essential steps. First, a relaxase protein binds to the transferable DNA at a site-specific origin of transfer (*oriT*). The relaxase nicks and covalently bonds to a single strand of DNA, forming a nucleoprotein complex called the relaxosome. Second, the relaxosome is recruited to the conjugation machinery by the coupling protein. Finally, the relaxase and covalently bound ssDNA are transferred through the mating channel out of the host cell (Bhatty *et al.*, 2013; Cabezón *et al.*, 2015).

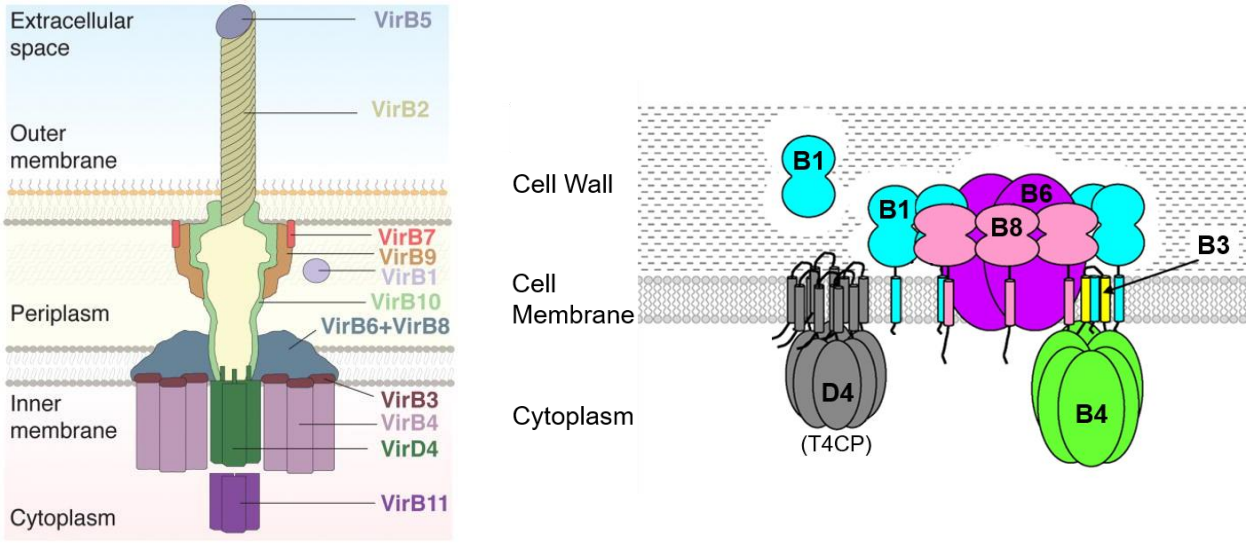

Figure 2. Conjugative type IV secretion systems from gram-negative and gram-positive bacteria.

Left: Model of the VirB/D4 T4SS from the gram-negative bacterium *A. tumefaciens*. The system comprises the Ti plasmid proteins VirD4 and VirB1-11, described in the text. Figure adapted from (Grohmann *et al.*, 2018).

Right: Model of a T4SS from a gram-positive bacterium. Proteins are labeled according to their VirB/D4 homolog or functional analog. The cell wall hydrolase B1 is depicted in both membrane-anchored and secreted forms. Figure adapted from (Auchtung *et al.*, 2016).

Integrative and Conjugative Elements

There are two classes of conjugative elements: conjugative plasmids and ICEs. Whereas plasmids are extrachromosomal segments of DNA that are replicated and inherited separately from the host chromosome, ICEs are ordinarily integrated into the chromosome of their bacterial host (Wozniak and Waldor, 2010; Smillie *et al.*, 2010). This allows ICEs to be passively replicated and inherited along with the rest of the host genome. Although conjugative plasmids are historically the most-studied class of conjugative element, ICEs are likely more abundant in nature (Guglielmini *et al.*, 2011). Like many MGEs, ICEs frequently encode cargo genes which benefit their host bacterium. ICEs encoding antibiotic resistance genes, heavy metal resistance

genes, pathogenicity factors, and metabolic pathways have all been described (Johnson and Grossman, 2015).

The ICE Life Cycle

ICEs begin their life cycle integrated into the host chromosome in an inactive state. While in this state, most ICE genes related to DNA processing and conjugation remain transcriptionally inactive. ICEs can be activated either stochastically or in response to varied stimuli or cellular conditions (Johnson and Grossman, 2015; Delavat *et al.*, 2017). Once activated, an ICE-encoded recombinase (Int) catalyzes excision of the element from the host chromosome, forming a circular intermediate which functions as a conjugative plasmid for the purposes of transfer (Figure 3) (Johnson and Grossman, 2015). Next, an ICE-encoded relaxase binds and nicks *oriT* to form the relaxasome. The relaxasome is recruited to the mating channel by the coupling protein and then transferred out of the donor and into the recipient via the mating channel (Figure 3) (Wozniak and Waldor, 2010). Transfer likely occurs simultaneously with rolling circle replication in the donor, using the new 3'-OH group created by nicking *oriT* as the primer, and using the non-transferred DNA strand as a template (Johnson and Grossman, 2015).

Once transfer into the recipient is complete, the relaxase catalyzes the ligation of the 5' and 3' ends of the transferred DNA, forming a closed ssDNA circle in the new bacterium. The ssDNA circle is then converted to double-stranded DNA via second-strand synthesis, and Int catalyzes the integration of the element into the new recipient chromosome (Figure 3) (Johnson and Grossman, 2015). The reformed double-stranded DNA element in the original donor cell can also reintegrate into the donor chromosome, preventing the element from being lost (Wozniak and Waldor, 2010). ICE recombinases vary in their site-specificity: some have a strict preference

for integration into a specific chromosomal locus (often a tRNA gene), whereas others have no site-specificity and allow for insertion at many different loci (Johnson and Grossman, 2015).

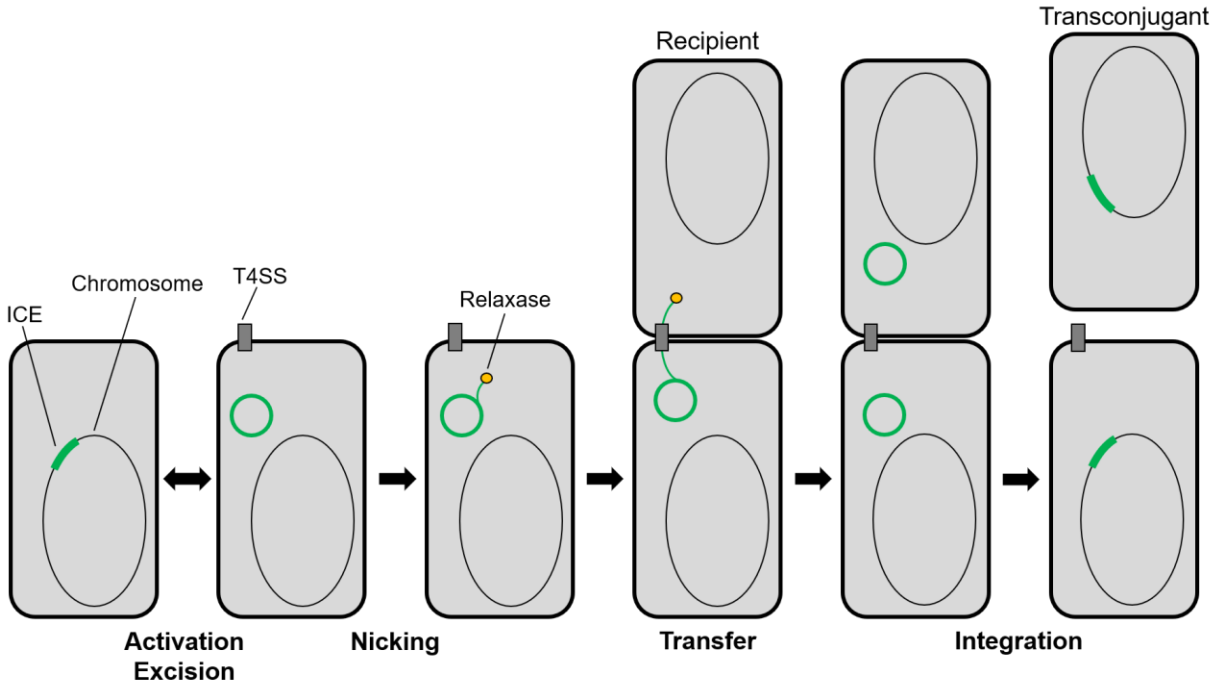

Figure 3. ICE life cycle. See text for description.

Introduction to ICE*Bs1*

ICE*Bs1* is a relatively small (~20.5 kb) ICE found in many isolates of the gram-positive bacterium *B. subtilis* (Figure 4) (Auchtung *et al.*, 2016). Identified by bioinformatic analysis in 2002, ICE*Bs1* has since become an excellent model system for the study of ICEs as a whole due

to the genetic tractability of its host organism *B. subtilis* and the ability to induce excision of the element in >90% of cells in a population (Burrus *et al.*, 2002; Auchtung *et al.*, 2005).

When inactive, ICE*Bs1* is non-disruptively integrated into the 3' end of the leucine tRNA gene *trnS-leu2* (Burrus *et al.*, 2002). While in this inactive state, expression of the operon encoding the conjugation machinery (driven by Pxis) is repressed by the ICE-encoded phage-like repressor ImmR (Auchtung *et al.*, 2007). ICE*Bs1* becomes active when the antirepressor ImmA is stimulated to degrade ImmR (Bose *et al.*, 2008). This degradation can be stimulated by one of two pathways: DNA damage to the host or the element-encoded cell-density-sensing protein RapI (Auchtung *et al.*, 2005; Bose *et al.*, 2008). In the former pathway, DNA damage to the host generates ssDNA. This activates RecA, which in turn stimulates ImmA to degrade ImmR as part of the SOS response, likely by increasing the specific activity of ImmA (Bose and Grossman, 2011). In the latter pathway, RapI stimulates the protease activity of ImmA (Bose and Grossman, 2011). Expression of *rapI* is usually repressed during log-phase growth by the transition state regulator AbrB, but expression is derepressed as the cell enters stationary phase growth (Auchtung *et al.*, 2005). RapI activity is also inhibited by PhrI, a signaling peptide that is produced and secreted by cells that contain ICE*Bs1*. This allows the presence of other ICE*Bs1*+ cells to inhibit activation of the element (Auchtung *et al.*, 2005).

Once ICE*Bs1* is activated and the Pxis operon is derepressed, the element is excised from the chromosome by the recombinase Int and the recombination directionality factor Xis (Lee *et al.*, 2007). The circularized element is nicked by the relaxase NicK at the origin of transfer (Lee and Grossman, 2007) and then transferred to a new cell via the element's T4SS, comprising the coupling protein ConQ and the components of the mating channel: ConB, ConC, ConD, ConE, and ConG (Berkmen *et al.*, 2010; Leonetti *et al.*, 2015). The cell wall hydrolase CwlT is also

necessary for transfer (DeWitt and Grossman, 2014), and this enzyme is discussed in greater detail later in this chapter.

ICE*Bs1* has recently been found to confer several advantages to its host cell. First, ICE*Bs1* encodes an exclusion mechanism (YddJ) that prevents redundant transfer of ICE*Bs1* into cells that already have the element, preventing toxic excess conjugation (Avello *et al.*, 2019). Second, the ICE*Bs1* gene *spbK* constitutes an abortive infection system that protects populations of ICE*Bs1*+ cells from predation by the bacteriophage SPβ (Johnson *et al.*, 2020). Finally, the ICE gene *devI* delays sporulation in the context of a biofilm, conferring a selective advantage to the host (Jones *et al.*, 2020).

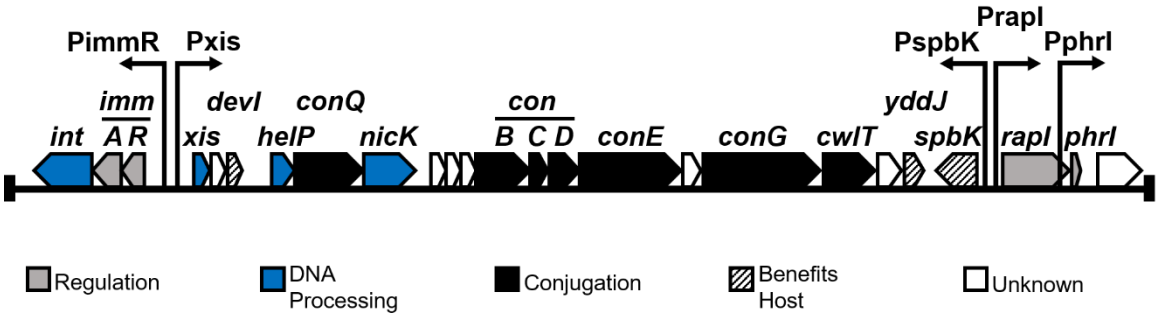

Figure 4. Genetic map of ICE*Bs1*. Genes are indicated as pentagons, with the horizontal point indicating the direction of transcription. Select promoters are indicated with vertical arrows. Text above the diagram indicates names of genes and promoters. Black boxes at either end of the map represent the flanking 60 bp direct repeats.

Bacterial Host Factors Involved in the ICE Life Cycle

Most ICE research has focused on the functions of genes encoded by the elements themselves. However, the ICE life cycle occurs entirely within bacteria, and ICEs have therefore evolved to interact with their bacterial hosts. Host factors are likely to be involved in all aspects

of the ICE life cycle, but few specific interactions between host factors and ICEs have been characterized to date (Johnson and Grossman, 2015).

Regulation of ICEs by Host Factors

Many host factors that interact with ICEs do so by regulating ICE activation. For example, multiple ICEs become activated following DNA damage to the host bacterium during the SOS response, including ICE*Bs1*, SXT of *Vibrio cholerae*, and ICE*St3* of *Streptococcus thermophilus* (Beaber *et al.*, 2004; Auchtung *et al.*, 2005; Bellanger *et al.*, 2007). DNA damage to the host results in the formation of ssDNA, which then activates RecA. Activation of RecA stimulates the degradation of certain transcriptional repressors, including the repressor ImmR of ICE*Bs1* and the repressor SetR of SXT (Beaber *et al.*, 2004; Bose and Grossman, 2011).

In other instances, host transcriptional regulators directly influence ICE activation. In the case of ICE*Bs1*, the transition state regulator AbrB represses expression of the ICE-activating protein RapI during growth. The amount of AbrB in the cell decreases as the cell exits log-phase growth, causing RapI expression to increase and making ICE*Bs1* activation more likely (Auchtung *et al.*, 2005). In ICE*clc* of *Pseudomonas knackmussii*, transcription of the element's core transfer genes depends on the stationary phase sigma factor RpoS (Miyazaki *et al.*, 2012). The nucleoid-associated protein Rok from *B. subtilis* binds and represses transcription of AT-rich regions of the genome and has been shown to inhibit activation of ICE*Bs1* (Smits and Grossman, 2010).

Host Factors Involved in ICE DNA Replication and Processing

The DNA translocase PcrA has a specific role in the life cycle of ICE*Bs1*. After ICE*Bs1* is nicked at the *oriT* by the relaxase, PcrA functions as a helicase that unwinds the element to facilitate rolling circle replication and conjugation (Lee *et al.*, 2010). PcrA is normally a poor-

functioning helicase, but the ICE*Bs1*-encoded helicase processivity factor HelP stimulates the helicase activity of PcrA and allows it to efficiently unwind ICE DNA (Thomas *et al.*, 2013). ICE*Bs1* also utilizes components of the host replication machinery to facilitate replication while in its circular form, including the DNA polymerase PolC and the beta clamp DnaN (Lee *et al.*, 2010).

ICE integration and excision reactions resemble those of lysogenic bacteriophages, and many of the ICE recombinases that catalyze these reactions are related to the site-specific recombinase from bacteriophage lambda (Wozniak and Waldor, 2010). The *E. coli* protein integration host factor (IHF) is a necessary co-factor for integration and excision of the phage lambda genome (Casjens and Hendrix, 2015), and some ICEs might likewise require host-encoded cofactors for excision and integration.

Some conjugative elements, including the F-plasmid of *E. coli*, require host factors for the relaxase to bind *oriT* (De La Cruz *et al.*, 2010). The F-plasmid *oriT* has two IHF binding sites, and IHF could facilitate relaxase binding by bending the DNA into a specific conformation (Tsai *et al.*, 1990; De La Cruz *et al.*, 2010). Activated ICEs function essentially as conjugative plasmids, and some ICEs might likewise require host cofactors to facilitate nicking. The ICE SXT of *V. cholerae* requires IHF for transfer, although its specific function in SXT transfer is not understood (McLeod *et al.*, 2006). IHF is not required for excision or integration of SXT (McLeod *et al.*, 2006), but the host protein could be required for relaxase binding to *oriT*.

Host Factors Involved in Transfer

A conjugative element must cross a bacterial cell envelope twice during transfer: once to exit the donor cell and again to enter the recipient cell. Multiple studies have shown that ICE transfer can be impacted by the composition of the host cell envelope. In the case of ICE*Bs1*, a reduction

of the positively charged lipid lysyl-phosphatidylglycerol in the membrane of either an ICE donor or recipient results in a reduction in transfer efficiency, and overproduction of the lipid in either a donor or recipient enhances transfer efficiency (Johnson and Grossman, 2016). The severity of the lipid-depletion transfer defect varies depending on the salts present in the mating environment, suggesting that lysyl-phosphatidylglycerol might be important for maintaining the proper ionic conditions needed for efficient conjugation (Johnson and Grossman, 2016). In the case of ICE*St3* of *S. thermophilus*, mutations predicted to disrupt a diverse range of cell envelope components (lipoproteins, teichoic acids, and exopolysaccharides) were investigated for their impact on ICE transfer. All mutations reduced transfer efficiency when present in an ICE*St3* donor but increased transfer efficiency when present in an ICE*St3* recipient (Dahmane *et al.*, 2018). The authors posited that the integrity of the donor cell envelope is important for the ICE*St3* conjugation machinery to function properly, but that the presence of these components in the recipient cell envelope could be a barrier to establishing a successful mating contact (Dahmane *et al.*, 2018). PAPI-1, an ICE from the gram-negative *Pseudomonas aeruginosa* (Carter *et al.*, 2010), uses common polysaccharide antigen (CPA)-capped lipopolysaccharide (LPS) in the recipient outer membrane as a receptor for the conjugation machinery (Hong *et al.*, 2017). PAPI-1+ *P. aeruginosa* produce less CPA-capped LPS and consequently are less likely to acquire redundant copies of the element (Hong *et al.*, 2017).

Host Factors Investigated in This Work

In Chapter 2 of this work, I used a genetic screen to identify bacterial host factors involved in the life cycle of ICE*Bs1*. This revealed that wall teichoic acids, a major component of the gram-positive bacterial cell wall, are important for conjugative transfer of ICE*Bs1*. In order to place this finding in context, an overview of the bacterial cell wall follows, with an emphasis placed on

the structure, synthesis, and function of wall teichoic acids. In Chapter 3, I describe the use of wall-less strains of *B. subtilis* to investigate the role of element-encoded cell wall hydrolases in bypassing the host cell wall. Cell wall hydrolases are discussed in greater detail below, as well as methods of generating stable wall-less strains of bacteria.

The Bacterial Cell Wall

Most bacteria exist in hypo-osmotic conditions and require a rigid cell envelope in order to prevent internal osmotic forces from rupturing the cell membrane. To this end, most bacteria have a peptidoglycan cell wall that surrounds the cytoplasmic membrane (Turner *et al.*, 2014). This rigid encasement, also known as the sacculus, is a single large peptidoglycan polymer. In gram-negative bacteria the cell wall is found between the inner and outer membranes, is physically connected to the outer membrane via a lipoprotein called Lpp, and is only several nanometers thick (Figure 5A, right). In gram-positive bacteria, which lack an outer membrane, the cell wall is much thicker, typically between 30 and 100 nm (Silhavy *et al.*, 2010) (Figure 5A, left).

The essential structure of peptidoglycan is largely conserved between gram-positive and gram-negative bacteria. In both, peptidoglycan is made of linear glycan strands which are crosslinked by peptide bridges (Silhavy *et al.*, 2010) (Figure 5B). The glycan strands are made of alternating *N*-acetylglucosamine (GlcNac) and *N*-acetylmuramic acid (MurNAc) subunits joined via β-1,4-glycosidic bonds (Vollmer, 2008). Peptide stems are attached to MurNAc, and these stems can be connected with each other to form the crosslinks. There is considerable species-level variation in the peptide stems and crosslinks, including variation in the identity and positions of the amino acids, the nature of the crosslinks between peptide chains, and the degree

of crosslinking (Vollmer *et al.*, 2008a). The amino acids that comprise the stems are the same in both *B. subtilis* and *E. coli*, as are the inter-stem crosslinks (Figure 5B) (Silhavy *et al.*, 2010). In both species, peptide stems are crosslinked via a bond between *meso*-diaminopimelic acid in the third position of the stem and D-alanine in the fourth position (Vollmer *et al.*, 2008a).

Teichoic acids are another major component of the gram-positive cell wall. Teichoic acids are long anionic polymers which can make up as much as 60% of the mass of the wall (Swoboda *et al.*, 2010). There are two primary classes of teichoic acids: wall teichoic acids (WTAs) and lipoteichoic acids (LTAs). LTAs are attached to the cell membrane and extend through the peptidoglycan, whereas WTAs are attached to the peptidoglycan itself and extend away from the cell wall (Figure 4B, left) (Brown *et al.*, 2013). Mutants lacking WTAs or LTAs have significant cell division and cell shape defects, and mutants lacking both WTAs and LTAs cannot be constructed (Schirner *et al.*, 2009). In this work, I found that WTAs are important for efficient transfer of an ICE, and so WTAs are discussed in greater detail below. For an overview of LTA structure and function, the reader is referred to the following review: (Percy and Gründling, 2014).

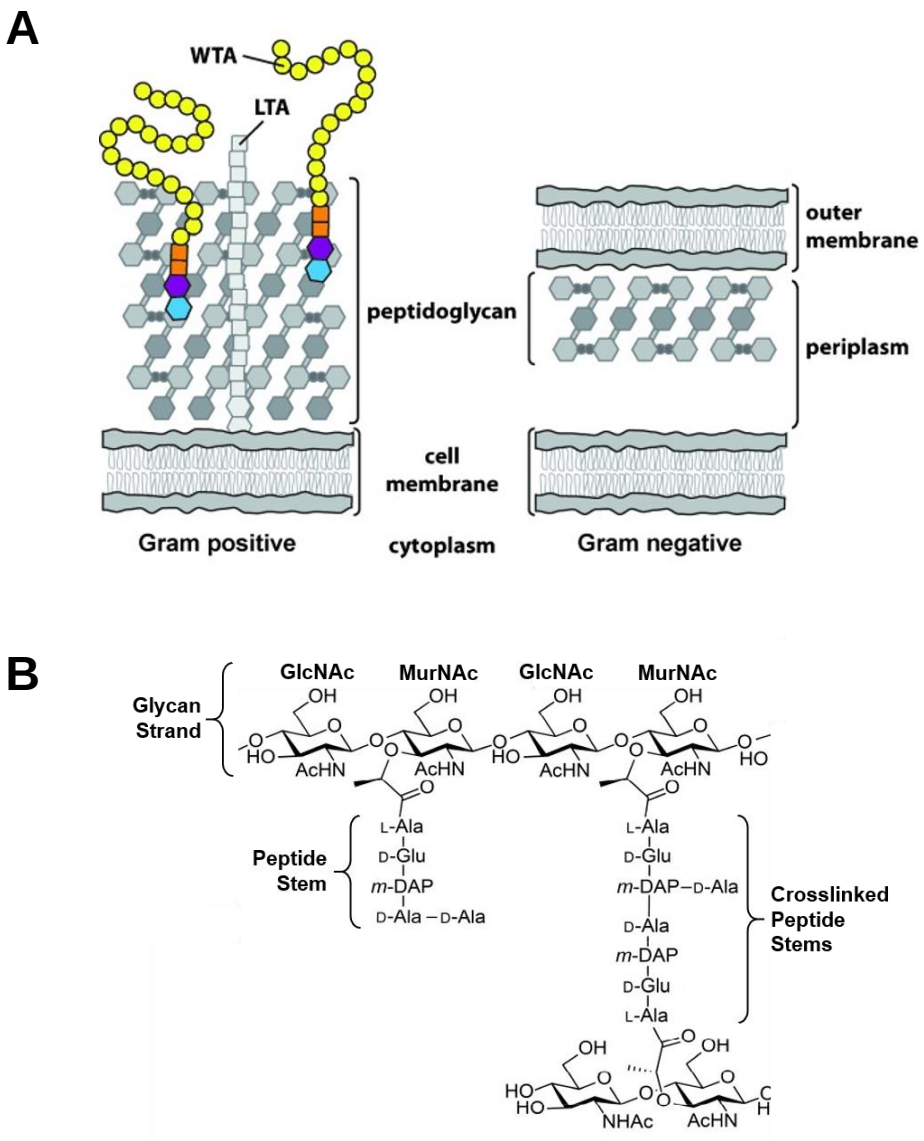

Figure 5. Structure and composition of the bacterial cell wall.

A) Schematic of the gram-positive cell wall (left) and the gram-negative cell wall (right). In gram-positive bacteria, the cell wall is made of a thick layer of peptidoglycan that encompasses the cell membrane and contains anionic polymers called teichoic acids. Wall teichoic acids (WTAs) are attached to the peptidoglycan, and lipoteichoic acids (LTAs) are anchored to the membrane. Figure adapted from (Swoboda *et al.*, 2010).

B) The basic chemical structure of peptidoglycan. Peptidoglycan is made of glycan strands, comprising alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues. The glycan strands are connected by crosslinked peptide stems attached to MurNAc. The nature of the stems and crosslinks varies between species, but this structure represents both *E. coli* and *B. subtilis.* Figure adapted from (Dik *et al.*, 2018).

Structure of Wall Teichoic Acids

Wall teichoic acids exhibit significant structural diversity across species, but they generally comprise anionic polyol-phosphodiester repeats that are covalently bound to the glycan strands of peptidoglycan. WTAs have two main components: the linkage unit and the anionic polymer chain (Figure 6A) (Neuhaus and Baddiley, 2003).

The linkage unit of WTAs is well-conserved across species and consists of a disaccharide comprising *N*-acetylmannosamine (ManNAc) and GlcNAc connected via a β-1,4-glycosidic bond. The GlcNAc in the linkage unit is covalently bound to a MurNAc residue in peptidoglycan via a phosphodiester linkage, and the ManNAc residue in the linkage unit connects to the rest of the polymer via one or two repeats of glycerol-phosphate (GroP). In *B. subtilis*, it is estimated that every ninth MurNAc subunit in peptidoglycan is modified with a WTA (Brown *et al.*, 2013).

Whereas the linkage unit of the WTA is relatively conserved, there is significant variation between and among species with respect to the repeats that make up the polymer chain. Different strains of *B. subtilis* utilize different polyol-phosphate subunits as the monomer for the WTA chain: for example, *B. subtilis* 168 uses GroP repeats whereas *B. subtilis* W23 uses ribitol-phosphate (RboP) repeats (Neuhaus and Baddiley, 2003). The negatively charged phosphate groups in these repeats are responsible for the polymer's negative charge. The polymer main chain contains approximately 40-60 repeats of these subunits, and extends out of the cell wall mesh and into the surrounding environment (Brown *et al.*, 2013).

The polymer chain of the WTA may be further modified ("tailored") by the addition of certain moieties to the monomers, most notably in *B. subtilis* by the addition of D-alanine or glucose. The degree of D-alanylation can vary under different cellular conditions, whereas the degree of glycosylation does not appear to vary significantly (Neuhaus and Baddiley, 2003;

Brown *et al.*, 2013). The D-alanyl modifications are cationic and are thought to affect the function of WTAs by lessening the extent of the polymer's overall negative charge (Neuhaus and Baddiley, 2003).

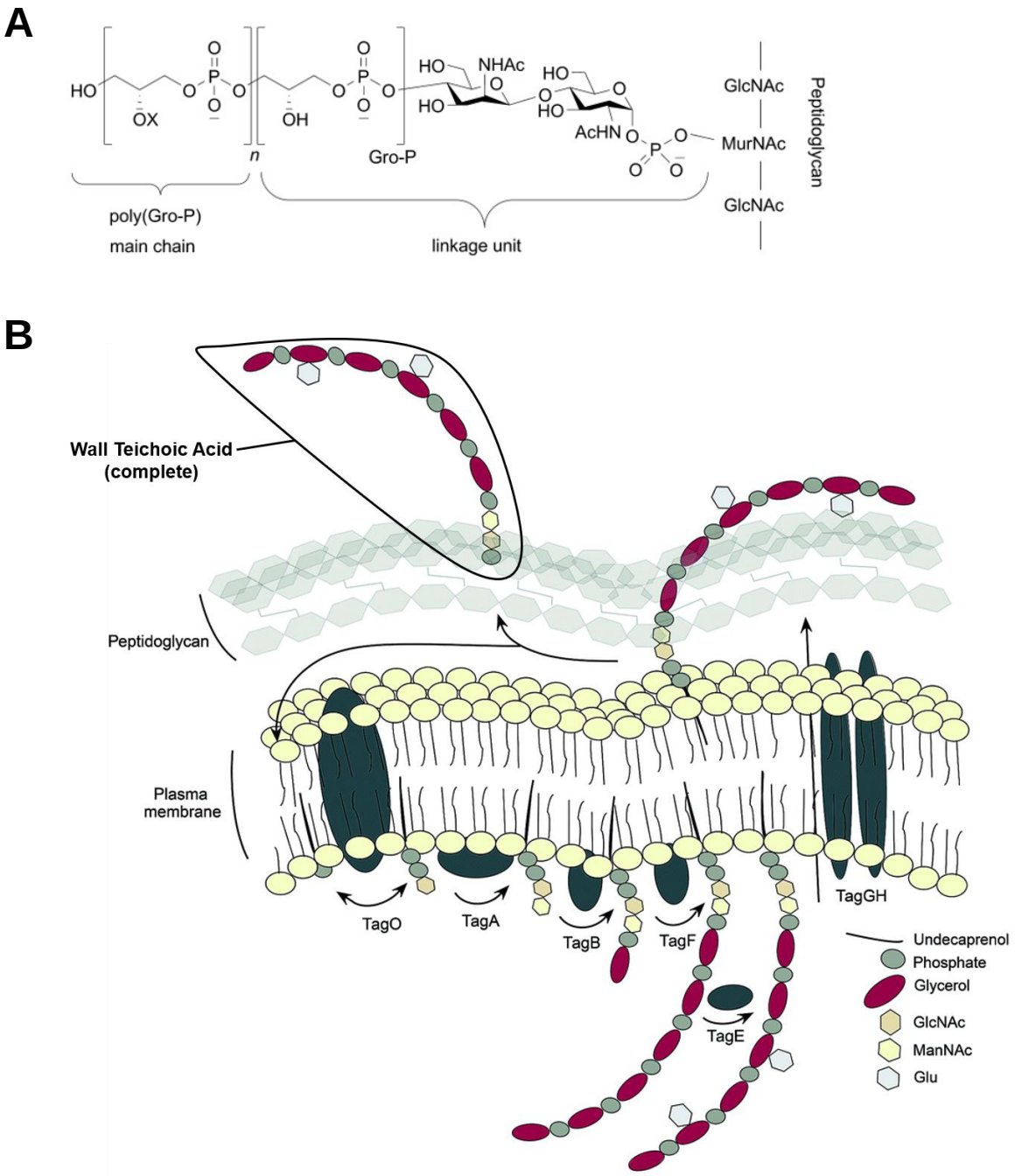

Figure 6. Structure and synthesis of wall teichoic acids.

A) Chemical structure of a glycerol-phosphate (GroP)-based wall teichoic acid from *B. subtilis* 168. X represents potential modification of the main chain with D-alanine or α-glucose, or an unmodified repeat (hydrogen). n = 40-60. Figure adapted from (Swoboda *et al.*, 2010).

B) Schematic of the wall teichoic acid biosynthesis pathway from *B. subtilis* 168, as described in the text. Figure adapted from (Gale *et al.*, 2014).

Synthesis of Wall Teichoic Acids

In *B. subtilis*, GroP-WTA biosynthesis is mediated by enzymes encoded by the *tag* operons (*tagO*, *tagAB*, *tagDEF*), whereas RboP-WTA biosynthesis is mediated by *tar* genes (Swoboda *et al.*, 2010). As *B. subtilis* 168 was the primary strain used in this body of work, a brief overview of GroP-WTA biosynthesis is described here. Reviews of RboP-based WTA biosynthesis can be found elsewhere (Swoboda *et al.*, 2010; Brown *et al.*, 2013).

WTA biosynthesis occurs in the cytoplasm at the cell membrane (Figure 6B). TagO catalyzes the first step of WTA biosynthesis, a reversible reaction wherein GlcNAc phosphate is transferred from UDP-GlcNAc to a membrane-anchored undecaprenol phosphate carrier lipid (Soldo *et al.*, 2002; D'Elia *et al.*, 2009). TagA catalyzes the second step of WTA biosynthesis, the addition of ManNAc to the lipid-GlcNAc intermediate, thereby forming a lipid-anchored disaccharide (D'Elia *et al.*, 2009). This is an irreversible reaction and is therefore the first committed step of WTA biosynthesis (D'Elia *et al.*, 2009). TagB completes the synthesis of the linkage unit by catalyzing the addition of a single GroP repeat to ManNAc (Bhavsar *et al.*, 2005). Finally, TagF repeatedly catalyzes the addition of GroP subunits to the polymer, thereby generating the negatively charged anionic chain (Schertzer and Brown, 2003). The source of the GroP subunits is CDP-glycerol, an intermediate that is produced by TagD (Pooley *et al.*, 1991). Glucose moieties are added to the lipid-bound WTA by TagE prior to export (Allison *et al.*, 2011).

The lipid-bound glycosylated WTA is transferred out of the cell via the two-component ABC transporter TagGH (Lazarevic and Karamata, 1995) and transferred from the lipid to peptidoglycan by TagTUV (Gale *et al.*, 2017). D-alanylation of the WTA occurs outside of the cell, and is mediated by the proteins encoded by the *dltABCD* operon (Perego *et al.*, 1995).

The first two genes in the *B. subtilis* WTA biosynthetic pathway (*tagO* and *tagA*) are nonessential, although the deletion mutants are slow-growing and exhibit severe cell separation and morphology defects (D'Elia *et al.*, 2006; D'Elia *et al.*, 2009). Some genes involved in the latter steps of the pathway (*tagB, tagD, tagF, tagG, tagH*) are conditionally essential, likely because their deletion results in either the accumulation of toxic intermediates or the sequestration of vital cellular resources (D'Elia *et al.*, 2006). Deletions of these downstream genes are viable if *tagO* or *tagA* is also deleted (D'Elia *et al.*, 2006; D'Elia *et al.*, 2009).

Enzymes in the WTA biosynthesis pathway have been explored as potential antibiotic targets (Brown *et al.*, 2013; Wang *et al.*, 2013; Sewell and Brown, 2014). Several WTA biosynthetic enzymes are essential, and blocking WTA production can sensitize bacteria to β-lactams even if WTA-depletion itself is not bactericidal (Wecke *et al.*, 1997; Wang *et al.*, 2013). The natural product tunicamycin blocks WTA biosynthesis in gram-positive bacteria by specifically inhibiting the activity of TagO (and the RboP-WTA analog TarO) at lower concentrations, and it also blocks peptidoglycan biosynthesis at higher concentrations (>10 μg/mL) (Pooley and Karamata, 2000; Campbell *et al.*, 2011). Although tunicamycin has been used to investigate the function of WTAs in gram-positive bacteria (Brown *et al.*, 2013; Mirouze *et al.*, 2018), the drug cannot be used as an antibiotic treatment in animals because it also inhibits the activity of an essential animal enzyme (Price and Tsvetanova, 2007). Several other inhibitors of WTA biosynthesis have also been recently described, including the TarO inhibitor ticlopidine (Farha *et al.*, 2013) and the WTA-export inhibitor targocil (Tiwari *et al.*, 2018).

Functions of Wall Teichoic Acids

WTAs are a critical component of the bacterial cell wall and have diverse functions, although their specific roles are not well understood.

WTAs regulate autolysins (cell wall-degrading enzymes) in multiple species of bacteria. In both *S. aureus* and *B. subtilis*, certain autolysins involved in cell division exhibit improper localization patterns in the absence of WTAs, and it is thought that the presence of WTAs excludes those autolysins from portions of the cell wall (Yamamoto *et al.*, 2008; Schlag *et al.*, 2010; Frankel and Schneewind, 2012). Proper localization of *B. subtilis* LytE, an autolysin required for cell elongation, is also disrupted in WTA-depleted cells (Kasahara *et al.*, 2016). The *Streptococcus pneumoniae* autolysin LytA is normally responsible for cell lysis in stationary phase. Teichoic acids have been demonstrated to be necessary for the proper localization of LytA, and depleting *S. pneumoniae* of teichoic acids sensitizes the bacteria to LytA activity (Bonnet *et al.*, 2018). WTA-lacking bacteria are also more prone to autolysis and more sensitive to the activity of peptidoglycan-lytic enzymes (Wecke *et al.*, 1997; Bera *et al.*, 2007; Schlag *et al.*, 2010). It has been speculated that negatively charged WTA polymers might either directly inhibit such enzymes by binding and sequestering them (Peschel *et al.*, 2000) or indirectly inhibit them by maintaining an ionic environment that lessens their activity (Biswas *et al.*, 2012).

WTAs are also likely involved in peptidoglycan biosynthesis. Mutants of *B. subtilis* exhibit severe cell-shape defects and have walls of irregular thickness (D'Elia *et al.*, 2006), suggesting that WTAs are important for organizing the peptidoglycan biosynthesis machinery (Brown *et al.*, 2013). Co-localization studies demonstrate that many WTA biosynthesis enzymes interact directly with peptidoglycan biosynthesis enzymes in *B. subtilis*, and it has been proposed that WTA attachment occurs at sites of nascent peptidoglycan synthesis (Formstone *et al.*, 2008).

Likewise, the enhanced β-lactam sensitivity of WTA-less bacteria suggests that the two processes are interconnected (Campbell *et al.*, 2011; Farha *et al.*, 2013).

WTAs have several other proposed functions. For example, the negative charge of WTAs is thought to be important for maintaining a proper ionic environment in the cell wall, which has consequences for proton binding, metal scavenging, and overall wall integrity (Neuhaus and Baddiley, 2003; Biswas *et al.*, 2012; Thomas and Rice, 2015). WTA-depleted cells are also more sensitive to varied environmental stressors, including high temperatures and osmotic pressure fluctuations (Vergara-Irigaray *et al.*, 2008; Wang *et al.*, 2013). WTAs are important for adhesion to both living and nonliving surfaces (Gross *et al.*, 2001; Walter *et al.*, 2007), and WTAs are an important virulence factor in some pathogenic bacteria (Weidenmaier and Peschel, 2008). WTAs also function as host surface receptors for many bacteriophages, facilitating phage attachment and adsorption. WTAs have also recently been demonstrated to have a role in *B. subtilis* competence: the polymers are likely modified with some moiety to facilitate extracellular DNA binding (Mirouze *et al.*, 2018).

Cell Wall Hydrolases

Although the cell wall is critically important for preventing lysis and maintaining cell shape, the sacculus is a dynamic structure that must undergo constant remodeling. Controlled degradation of peptidoglycan is essential for numerous cellular processes, including daughter cell separation, cell growth, wall turnover, and sporulation (Do *et al.*, 2020). Localized peptidoglycan remodeling is also required for the formation of certain large membrane complexes, including secretion systems (Vollmer *et al.*, 2008b). Accordingly, bacteria encode cell wall hydrolases which function to break bonds within peptidoglycan. Cell wall hydrolases are extremely

biochemically varied but may be broadly sorted into two categories: glycosidases, which make cuts within the glycan strands, and peptidases, which cut the peptide stems (Vermassen *et al.*, 2019; Do *et al.*, 2020).

Cell Wall Hydrolases of Conjugative Elements

The cell wall presents a significant barrier to the assembly of large membrane complexes, including T4SSs (Dijkstra and Keck, 1996). Normal openings in the sacculus are predicted to be about 4.2 nm on average in *E. coli* and *B. subtilis* (Demchick and Koch, 1996), whereas structural studies of a conjugative T4SS from the *E. coli* plasmid R388 indicate that the conjugation machinery is approximately 18.5 nm in width at the point that it crosses the cell wall (Low *et al.*, 2014), meaning that the sacculus must be remodeled to create a sufficiently wide opening for the conjugation machinery. To this end, conjugative elements of walled bacteria encode cell wall hydrolases, which are presumed to locally modify the donor peptidoglycan to enable formation of the mating channel (Bayer *et al.*, 2001; Bantwal *et al.*, 2012; DeWitt and Grossman, 2014). How the conjugation machinery broaches the cell wall of a recipient bacterium in a mating pair is not understood, although it has been speculated that element-encoded hydrolases might also remodel the recipient cell wall (Abajy *et al.*, 2007; Baidya *et al.*, 2020).

Several cell wall hydrolases of conjugative elements from gram-negative bacteria have been characterized, including VirB1 from the *A. tumefaciens* Ti plasmid, P19 from the resistance plasmid R1, and TraL from the plasmid pKM101 (Winans and Walker, 1985; Bayer *et al.*, 2001; Zupan *et al.*, 2007). All of these hydrolases encode a single glycosidase domain, and deleting each of these hydrolases results in a 10 to 100-fold transfer defect (Winans and Walker, 1985; Bohne *et al.*, 1998; Bayer *et al.*, 2001).

Several cell wall hydrolases from gram-positive conjugative elements have also been characterized, including CwlT from ICE*Bs1*, TcpG from the *Clostridium perfringens* plasmid pCW3, TraG from plasmid pIP501 of *E. faecalis*, and PrgK from plasmid pCF10 (Bantwal *et al.*, 2012; Arends *et al.*, 2013; DeWitt and Grossman, 2014). These enzymes differ from their gram-negative counterparts in several ways. First, they all have at least two peptidoglycan-degradation domains, including a glycosidase domain and a peptidase domain (Goessweiner-Mohr *et al.*, 2013). Second, the gram-positive hydrolases are more critical for transfer: deletions of CwlT (from ICE*Bs1*) and TraG (from pIP501) both eliminate transfer of their respective elements, and a deletion of TcpG (from pCW3) results in a 1000-fold transfer defect (Bantwal *et al.*, 2012; Arends *et al.*, 2013; DeWitt and Grossman, 2014). Lastly, gram-positive cell wall hydrolases have an N-terminal transmembrane domain, which might mediate interactions with other components of the conjugation machinery (Goessweiner-Mohr *et al.*, 2013).

Localization and Interactions with other T4SS Components

Element-encoded cell wall hydrolases from gram-negative bacteria localize to the periplasm. Interaction studies of VirB1 and its homolog from *Brucella suis* have shown that both interact with periplasmic core components of the T4SS (Ward *et al.*, 2002; Höppner *et al.*, 2004). These interactions might function to restrict the activity of the cell wall hydrolase to the site of the mating machinery and prevent widespread wall damage (Ward *et al.*, 2002).

Two gram-positive hydrolases, TcpG from pCW3 and TraG from pIP501, also interact with core components of the conjugation machinery as well as with the coupling proteins from their respective T4SSs (Abajy *et al.*, 2007; Steen *et al.*, 2009; Kohler *et al.*, 2017). The N-terminal transmembrane helix might be important for mediating these interactions (Goessweiner-Mohr *et al.*, 2013; Kohler *et al.*, 2017). Based on these interactions, some have speculated that gram-

positive cell wall hydrolases could have an additional role in recruiting other T4SS components to the site of the mating pore (Ward *et al.*, 2002; Abajy *et al.*, 2007; Kohler *et al.*, 2017).

In the case of some element-encoded hydrolases from gram-positive bacteria, the N-terminal transmembrane helices have inconsistently been predicted to be either signal peptides or stable transmembrane domains (Arends *et al.*, 2013; Goessweiner-Mohr *et al.*, 2013; Xu *et al.*, 2014; DeWitt and Grossman, 2014). One of these hydrolases (CwlT of ICE*Bs1*, discussed more below) is both cell-associated and present in cell-culture supernatant (DeWitt and Grossman, 2014), raising the possibility that some fraction of protein might be membrane-anchored and that some might be released from the membrane (Auchtung *et al.*, 2016).

Substitutions for Element-Encoded Cell Wall Hydrolases

Several studies have shown that element-encoded cell wall hydrolases can be functionally substituted for each other. For example, deleting the gram-negative hydrolase P19 from plasmid R1 results in a 10-fold transfer defect, but this defect can be fully complemented with the cell wall hydrolase TrbN from plasmid RP4, or even complemented with the type III secretion system cell wall hydrolase IpgF from *Shigella sonnei* (Zahrl *et al.*, 2005). Likewise, a deletion of VirB1 from the *A. tumefaciens* Ti plasmid can be fully complemented with several other hydrolases from gram-negative systems (Höppner *et al.*, 2004). The gram-positive hydrolase PrgK from pCW3 can also be partially complemented with several other element-encoded gram-positive hydrolases in conjugation assays (Laverde Gomez *et al.*, 2014).

The interchangeability of some element-encoded hydrolases might be what makes some them dispensable for transfer (Zahrl *et al.*, 2005; Laverde Gomez *et al.*, 2014). Bacteria typically encode a large number of cell wall hydrolases: both *E. coli* and *B. subtilis* encode dozens of these enzymes, many of which exhibit some degree of functional redundancy (Smith *et al.*, 2000;

Vollmer *et al.*, 2008b). In the absence of a conjugative element's ordinary cell wall hydrolase, other hydrolases in the cell might be able to serve as sufficiently functional substitutes (Zahrl *et al.*, 2005).

CwlT of ICE*Bs1*

ICE*Bs1* encodes the cell wall hydrolase CwlT, an enzyme which is indispensable for conjugative transfer (DeWitt and Grossman, 2014). CwlT contains a predicted N-terminal transmembrane helix and two catalytic domains: an *N*-acetylmuramidase domain and a DL-endopeptidase domain (Fukushima *et al.*, 2008). Eliminating the catalytic activity of the muramidase domain makes transfer undetectable. Eliminating the catalytic activity of the endopeptidase domain causes a ~1000-fold transfer defect, but does not abolish transfer entirely. A deletion of the N-terminal transmembrane helix also abolishes transfer (DeWitt and Grossman, 2014).

The N-terminal portion of CwlT has been predicted to be either a cleavable signal peptide or a stable transmembrane domain (Xu *et al.*, 2014). In other conjugative elements from gram-positive bacteria, the cell wall hydrolase transmembrane domain might mediate interactions with other T4SS components, and this could likewise be the case for CwlT (Bhatty *et al.*, 2013; Kohler *et al.*, 2017). Notably, CwlT has been detected both in cell-culture supernatant and associated with cells, indicating that at least some fraction of the protein is released from the membrane (DeWitt and Grossman, 2014). CwlT was also previously predicted to be a lipoprotein, although the proposed lipid-attachment site is not necessary for ICE*Bs1* transfer (DeWitt and Grossman, 2014). Like all cell wall hydrolases from conjugative elements, CwlT is presumed to modify the donor cell wall to facilitate conjugation, and it was previously unknown if it also a has a role in broaching the recipient cell wall.

L-Form Bacteria

The cell wall is usually essential for gram-positive and gram-negative bacteria to survive because it prevents the internal forces of the high-osmolarity cytoplasm from causing the cell to rupture. However, in osmoprotective environments (i.e. high osmolarity media) the cell wall can be rendered nonessential for viability and growth. This is exemplified by L-form bacteria, variants of normally-walled bacteria which have become cell wall-deficient but which can nevertheless survive and proliferate (Mercier *et al.*, 2014; Errington, 2017). The extent to which L-forms exist outside of laboratory environments is unclear (Onwuamaegbu *et al.*, 2005), but it has been speculated that transitioning into an L-form state could be an adaptive response to certain stressors (Markova *et al.*, 2010; Errington *et al.*, 2016), especially given that L-forms are intrinsically resistant to cell wall-targeting antibiotics (Wolf *et al.*, 2012; Kawai *et al.*, 2018). Most notably, the high osmolarity of urine might allow L-form bacteria to survive, which might allow L-forms to function as persisters in the context of recurrent urinary tract infections (Errington *et al.*, 2016; Mickiewicz *et al.*, 2019). So long as they are in their wall-deficient state, L-forms do not require the standard cell division machinery to divide (Leaver *et al.*, 2009). Instead, excess membrane production in L-forms leads to spontaneous blebbing or tubulation of the membrane, and these membrane protrusions can collapse into new, discrete L-form cells (Leaver *et al.*, 2009; Mercier *et al.*, 2013).

Recent work has demonstrated that a diverse array of bacteria can be transitioned into an L-form state by blocking biosynthesis of peptidoglycan precursors, and the genetic requirements for this transition have been best-characterized in *B. subtilis* (Mercier *et al.*, 2014). Two classes of mutations are necessary for *B. subtilis* to stably make this transition (Mercier *et al.*, 2013). The first class serves to block peptidoglycan precursor biosynthesis and increase membrane

synthesis, and this is generally accomplished with alleles that repress transcription of the *murE* operon (Leaver *et al.*, 2009; Mercier *et al.*, 2014). However, eliminating the cell wall in an aerobic environment leads to a metabolic imbalance and the generation of toxic reactive oxygen species, and so a second class of mutation is usually required to ameliorate the resulting oxidative stress (Kawai *et al.*, 2015; Kawai *et al.*, 2019). In *B. subtilis* this is most commonly accomplished via a loss-of-function mutation in the isoprenoid biosynthesis gene *ispA* (Kawai *et al.*, 2015). This mutation likely promotes *B. subtilis* L-form survival by reducing production of menaquinone, a component of the electron transport chain, thereby reducing flux through the pathway that generates reactive oxygen species (Kawai *et al.*, 2015; Kawai *et al.*, 2019). *B. subtilis* mutations that increase the efficiency of L-form generation have also been described, which primarily act by increasing the probability of successful escape from the sacculus (Domínguez-Cuevas *et al.*, 2012).

Summary of Thesis

In this report I have furthered our understanding of how ICE*Bs1* interacts with its bacterial host *B. subtilis.* In Chapter 2, I describe a screen for essential bacterial host factors that are important for transfer of ICE*Bs1* to a recipient. I show that wall teichoic acids, a major component of the gram-positive cell wall, are needed in both donors and recipients for efficient transfer of ICE*Bs1*. When bacteria depleted of wall teichoic acids are involved in ICE*Bs1* conjugation, they die from damage to the cell envelope. In Chapter 3, I use wall-deficient L-form strains of bacteria to demonstrate that the ICE*Bs1*-encoded cell wall hydrolase CwlT likely acts on both the donor cell wall and the recipient cell wall during transfer. In Appendix A, I describe

a screen to identify chromosomal mutations that reduce a strain's ability to donate ICE*Bs1*, and I describe a preliminary finding from that screen.

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Chapter 2

A CRISPR interference screen reveals a role for cell wall teichoic acids in conjugation in *Bacillus subtilis*

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Abstract

Conjugative elements are mobile genetic elements that transfer directly from a donor to a recipient bacterium via an element-encoded type IV secretion system. Conjugative elements are widespread and include plasmids and integrative and conjugative elements (ICEs). Both types of elements have evolved to interact with their bacterial hosts, although the nature and extent of these interactions are poorly understood. In this work, we sought to identify host-encoded genes with a role in the life cycle of the integrative and conjugative element ICE*Bs1* of *Bacillus subtilis*. We constructed a library of ICE*Bs1* donor strains wherein each strain contained an inducible CRISPR interference-based knockdown of a single essential *B. subtilis* gene, and identified essential gene knockdowns that caused an acute defect in transfer of ICE*Bs1* to recipient cells. This screen revealed that reducing expression of genes needed for cell wall teichoic acid synthesis caused a dramatic decrease in conjugation efficiency. Using targeted CRISPR interference, controlled decreased expression of genes needed for synthesis of wall teichoic acids, and an antibiotic that inhibits synthesis of wall teichoic acids, we found that wall teichoic acids were necessary in both ICE*Bs1* donors and recipients for efficient conjugative transfer of the element. Further, we found that depletion of wall teichoic acids caused cells involved in ICE*Bs1* conjugation to die from damage to the cell envelope. Our results indicate that wall teichoic acids help protect against envelope stress caused by active conjugation machinery.

Introduction

Horizontal gene transfer is fundamental to bacterial evolution, allowing for the rapid spread of genes involved in processes as diverse as antibiotic resistance, resource utilization, and pathogenesis (Frost *et al.*, 2005; Soucy *et al.*, 2015). Horizontal gene transfer is often facilitated by conjugative elements, which encode machinery that can transfer a copy of the element from a host to a recipient via direct cell-to-cell contact. Although most studies of conjugative elements have focused on conjugative plasmids, integrative and conjugative elements (ICEs) appear to be the most common type of conjugative element and have been identified in every bacterial clade (Guglielmini *et al.*, 2011).

ICEs normally reside integrated in a host chromosome where they are passively replicated and inherited. While in this inactive state, most ICE genes are not expressed. ICEs can be activated either stochastically or in response to certain conditions (i.e., DNA damage to the host, resource limitation), at which point a site-specific recombinase excises the ICE from the host chromosome to form a circular plasmid (Bañuelos-Vazquez *et al.*, 2017). For conjugative DNA transfer, the ICE plasmid DNA is nicked and a single strand is transferred out of the donor and into a recipient cell through the element-encoded type IV secretion system. Once transferred, the ssDNA becomes double stranded and can integrate into the chromosome of the new host, forming a stable transconjugant (Wozniak and Waldor, 2010; Johnson and Grossman, 2015; Delavat *et al.*, 2017).

ICE*Bs1* is relatively small (~20.5 kb) (Auchtung *et al.*, 2016) and found in most isolates of the gram-positive bacterium *Bacillus subtilis*. Virtually all of the ICE*Bs1* genes required for conjugative transfer have homologs or analogs in other conjugative elements (Bhatty *et al.*, 2013; Leonetti *et al.*, 2015). ICE*Bs1* is normally integrated into *trnS-leu2* (a tRNA gene). When

integrated, only a few ICE*Bs1* genes are expressed. Gene expression and subsequent excision are induced during the RecA-dependent SOS response, or in the presence of other *B. subtilis* cells lacking a copy of the element (Auchtung *et al.*, 2005; Bose *et al.*, 2008). ICE*Bs1* can be experimentally activated in >90% of cells in a population by overproducing the element-encoded regulatory protein RapI, enabling high frequencies of transfer that make it quite useful for studying conjugation (Auchtung *et al.*, 2005; Lee *et al.*, 2007). The life cycles of all mobile genetic elements depend on various host functions. In this study, we sought to identify essential host genes that are necessary for the life cycle of ICE*Bs1*. We were most interested in identifying host functions that specifically affect conjugation, rather than host functions (e.g., transcription, translation) that affect the production of ICE*Bs1* gene products or the replication of ICE DNA (Lee *et al.*, 2010).

We used CRISPR interference (CRISPRi) to block transcription of essential *B. subtilis* genes and identified host factors in an ICE*Bs1* donor that are important for transfer. Several genes were identified in this screen and we chose to focus on those involved in the synthesis of cell wall teichoic acids.

Wall teichoic acids (WTAs) are polyol-phosphate repeats that are attached to the peptidoglycan cell wall of gram-positive bacteria [reviewed in: (Swoboda *et al.*, 2010; Brown *et al.*, 2013)]. In *B. subtilis* 168, WTAs comprise 45-60 repeats of glycerol 3-phosphate (Pollack and Neuhaus, 1994). These repeats can be modified by WTA-tailoring enzymes, most notably by D-alanylation and glycosylation. In *B. subtilis* 168, WTAs are synthesized by enzymes encoded by the *tag* genes (*tagO*, *tagAB*, *tagDEF*, and *tagGH*). TagO catalyzes the first step of WTA biosynthesis (D'Elia *et al.*, 2006), and TagA catalyzes the second step and is the first committed step (D'Elia *et al.*, 2009). WTAs are not strictly required for cell growth. WTA-depleted cells are

viable but slow-growing, and have significant cell shape and cell separation defects (D'Elia *et al.*, 2006). Some WTA biosynthesis genes (*tagBDFGH*) are conditionally essential, likely because their deletion results in either the accumulation of toxic intermediates or the sequestration of vital cellular resources (D'Elia *et al.*, 2006; D'Elia *et al.*, 2009).

WTAs have multiple functions, including regulating peptidoglycan synthesis and turnover, mediating cell-cell and cell-surface adhesion, and regulating autolysin activity (Brown *et al.*, 2013). We found that wall teichoic acids are necessary in both ICE*Bs1* donor cells and recipient cells for efficient transfer of the element. The activity of the ICE*Bs1* conjugation machinery was toxic to cells that were depleted of wall teichoic acids, and these cells die from damage to the cell envelope caused by the conjugation machinery.

Results

A CRISPRi screen for essential host genes involved in conjugation

CRISPRi knockdown of essential genes. We sought to identify host genes in an ICE*Bs1* donor that are important for transfer of the element. We used a *B. subtilis* CRISPRi system that has been described (Peters *et al.*, 2016) to reduce expression of essential genes in *B. subtilis*, and screened for those that caused a defect in conjugation. Briefly, the system comprises a catalytically dead Cas9 nuclease from *Streptococcus pyogenes* (dCas9) under the regulation of the xylose-inducible promoter Pxyl and a constitutively expressed sgRNA containing a 20 nt region corresponding to a target gene of interest. When dCas9 is produced via the addition of xylose, it complexes with the sgRNA and stably binds to the host gene specified by the targeting region of the sgRNA. This interaction sterically blocks transcript elongation, thereby lowering

expression of the targeted gene or operon. The library of sgRNA alleles used in this study (a gift of Peters et al.) collectively targeted a set of 289 proposed essential *B. subtilis* genes (Peters *et al.*, 2016), 257 of which were subsequently verified to be essential in a systematic gene knockout analysis (Koo *et al.*, 2017).

CRISPRi library in ICE*Bs1*. We created a library of donor strains in which ICE*Bs1* could be activated by overproducing the ICE regulatory protein RapI. All donor strains contained a xylose-inducible Pxyl-*dcas9* allele integrated into the host chromosome. Each donor strain also had one constitutively expressed sgRNA allele (Pveg-*sgRNA*) integrated into ICE*Bs1* at a site in the element that is nonessential for transfer. In this way, we constructed a pooled library of donor strains with each individual donor strain representing a knockdown of one essential *B. subtilis* gene (Figure 1A). Importantly, the target of the knockdown is specified by the sgRNA allele contained within the element itself. As a consequence, every transconjugant generated by this library contains a genetic record of the donor strain that produced it. We can determine the relative mating efficiency of a given donor strain by pooling the population of transconjugants, collectively sequencing their sgRNA alleles, and then determining which sgRNAs differ in abundance compared to the pre-mating donor population. If a knockdown compromised ICE*Bs1* mating efficiency, then the sgRNA corresponding to that gene would be underrepresented in the resulting pool of transconjugants. Conversely, if a knockdown improved ICE*Bs1* mating efficiency, then the sgRNA allele corresponding to the that gene would be overrepresented in the pool of transconjugants.

The screen. We chose to induce a partial essential gene knockdown in the ICE*Bs1* donor population. Strong Pxyl-*dcas9* induction would likely have resulted in a significant fitness defect, which would have had the undesirable effect of eliminating knockdowns of interest from

the donor population prior to mating. To this end, we pooled and grew the library of ICE*Bs1* CRISPRi donors in rich medium containing a low concentration of xylose (0.01%) to induce a partial knockdown for approximately six doublings. We induced ICE*Bs1* in this library and mixed the donors 1:1 with an ICE*Bs1*-cured (ICE^0) recipient strain on a carbonless mating surface for two hours. We then resuspended the cells and used antibiotic resistance markers in ICE*Bs1* (*kan*) and the recipient chromosome (*str*) to select for transconjugants. We used next-generation sequencing to determine both the relative abundance of sgRNA alleles in a sample of donors harvested immediately prior to mating as well as the relative abundance of sgRNA alleles in the resulting pool of transconjugants. The overall mating efficiency of this pooled library (0.17%) was similar to that of a control strain with a knockdown targeted to a nonessential gene (MMH233, 0.36%), indicating that the partial knockdown treatment did not have a substantial global effect on conjugation.

Decreased expression of most of the essential genes had little or no effect on mating efficiency. We compared the relative abundance of each sgRNA in the transconjugant population to the pre-mating donor population and found that >80% of knockdowns resulted in less than a 4-fold change in the abundance of the sgRNA gene in the transconjugant pool relative to the starting population (Figure 1B, points above diagonal dotted line). We did not detect an increase greater than four-fold in any of the sgRNA genes in transconjugants, indicating that none of the essential genes seemed to be substantially inhibiting function of ICE*Bs1*.

We focused on knockdown strains that were well represented in the donor pool (Figure 1B, points to the right of the horizontal line, >0.01%), and that had the largest decrease in conjugation. Of this subset of knockdowns, most were involved in processes that directly affect the production of the conjugation machinery, including genes involved in translation, protein

secretion, and protein folding. We did not study these. In contrast, knockdown of *tagA* resulted in the most severe transfer defect out of all genes tested in the screen. Knockdowns of two other WTA biosynthesis genes (*tagD* and *tagF*) also resulted in >4-fold defects in conjugation (~10- and ~4.5-fold respectively; Table 2). We decided to further investigate the role of *tagA* and WTA biosynthesis in conjugation.

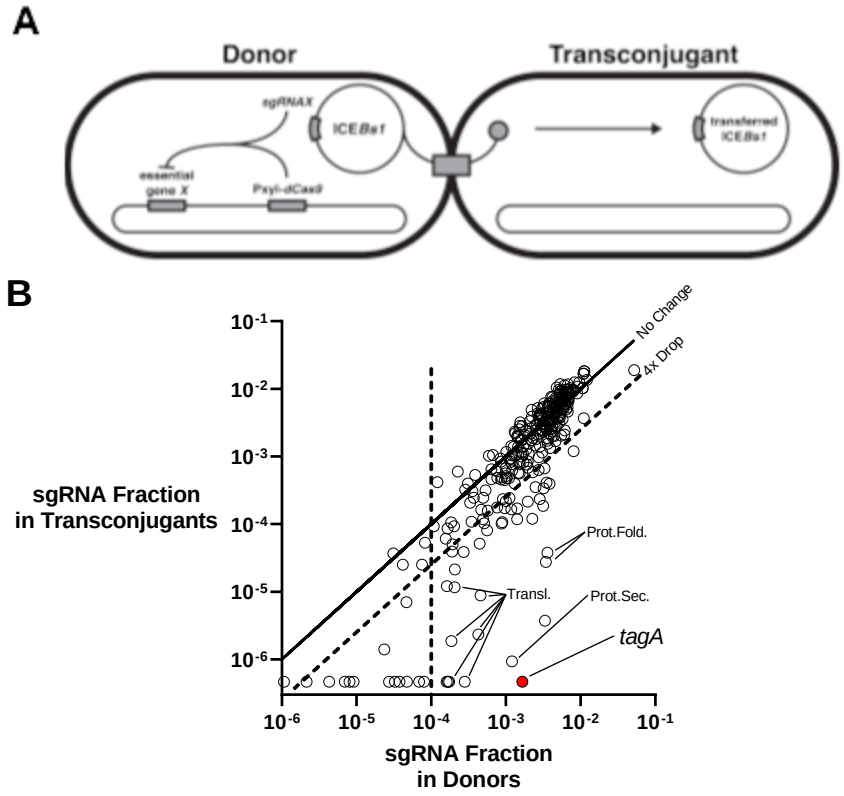

Figure 1. CRISPRi screen identifies essential host gene knockdowns that affect transfer of ICE*Bs1*.

A. Experimental design of CRISPRi screen. Each ICE*Bs1* donor contains a xylose-inducible Pxyl-*dcas9* allele integrated into a non-ICE locus of the host chromosome and a constitutively expressed sgRNA allele integrated into ICE*Bs1*. Each sgRNA allele has a unique 20 bp targeting region which specifies a knockdown of a single proposed essential gene. A pooled library of inducible ICE*Bs1* donor strains (*lacA*::{P*xyl-dcas9* (*ermR*)} Δ*amyE117*::{P*spank-rapI* (*spc*)} Δ*rapIphrI*::(*amyE kan*)::{P*veg-sgRNA*^{X} (*cat*)}), collectively representing partial knockdowns of all *B. subtilis* essential genes, was induced to mate with an ICE*Bs1*^{0} recipient strain (CAL89). If a knockdown results in a transfer defect, the corresponding sgRNA would be de-enriched in the transconjugant population relative to the pre-mating donor population.

B. Results of CRISPRi screen. The pool of sgRNA alleles in the pre-mating donor population and post-mating transconjugant population were sequenced and compared. Each point corresponds to one sgRNA allele included in the screen. The x- and y-axes correspond to the fractions of the donor and transconjugant pools, respectively, that an sgRNA allele represents. The solid diagonal line (x = y) represents no change in abundance between the two populations. Points falling below the diagonal dashed line correspond to alleles that were >4-fold depleted in the transconjugant pool. Points to the left of the vertical dashed line represent sgRNAs that were largely depleted from the donor pool prior to mating (< 0.01%). Select sgRNAs are annotated by name or functional grouping. More detailed results are reported in Table 2.

WTA biosynthesis in an ICE*Bs1* donor is necessary for efficient transfer

To validate the apparent effect of *tagA* uncovered in the CRISPRi screen, we directly tested for effects of *tagA* on ICE*Bs1* conjugation in several ways. First, we measured the conjugation efficiency {stable transconjugants / donor; each measured as colony forming units (CFUs)} from a homogenous population of donor cells in which *tagA* expression was inhibited by CRISPRi. This is in contrast to the screen which used a population of strains representing the entire CRISPRi library. As anticipated, decreasing *tagA* expression in an ICE*Bs1* donor resulted in an acute drop in conjugation (Figure 2). The severity of the defect increased as *tagA* expression decreased, with the strongest knockdown resulting in no detectable ICE*Bs1* transfer ($<5 \times 10^{-4}$).

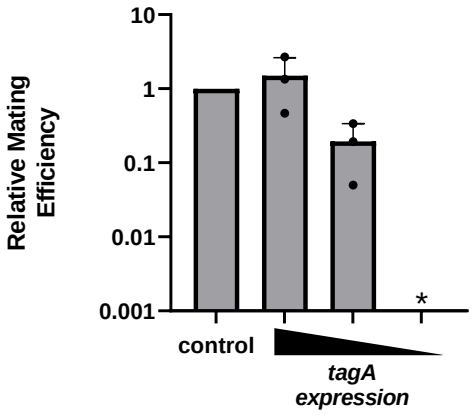

Figure 2. CRISPRi knockdown of WTA biosynthesis gene *tagA* in an ICE*Bs1* donor negatively affects transfer.

An individual ICE*Bs1* donor strain with an inducible CRISPRi knockdown of *tagA* (MMH527) was induced to mate with an ICE*Bs1*^{0} recipient strain (CAL89). Knockdowns were induced via incubation with either 0, 0.01%, or 0.1% xylose (Methods). Relative mating efficiencies are reported as number of transconjugants per pre-mating donor normalized to a same-day control. Bars represent the average of three independent biological replicates, with dots representing data each replicate. Error bars indicate standard deviation. * indicates mating efficiency was below limit of detection (< .0005 for the 0.1% xylose condition). Average mating efficiency of the control was 8.80×10^{-4}.

We inhibited WTA synthesis in ICE*Bs1* donors in two additional ways. We placed several of the endogenous WTA biosynthesis operons (*tagO*, *tagAB*, *tagDEF*) under the control of the LacI-repressible-isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter Pspank. We reduced WTA biosynthesis in an ICE*Bs1* donor by titrating down expression of a *tag* operon for approximately six generations, and then analyzed the effect on conjugation. Reducing expression of any of the three operons (*tagO*, *tagAB*, *tagDEF*) resulted in an acute drop in conjugation (Figure 3A, 3D-E). In all three cases, mating efficiency dropped more than 1,000-fold at the lowest level of expression tested.

We also inhibited WTA synthesis using a low concentration (1 μg/ml) of the antibiotic tunicamycin. At this concentration, tunicamycin specifically inhibits WTA biosynthesis in gram-positive bacteria by blocking the activity of TagO (Pooley and Karamata, 2000; Campbell *et al.*, 2011). At higher concentrations (>10 μg/ml) it inhibits peptidoglycan biosynthesis and cell growth (Price and Tsvetanova, 2007; Campbell *et al.*, 2011). Inhibiting WTA biosynthesis with tunicamycin (1 μg/ml) in an ICE*Bs1* donor decreased ICE*Bs1* transfer ~100-fold (Figure 3F). Together, our results demonstrate that WTA biosynthesis in an ICE*Bs1* donor is required for efficient transfer of the element.

WTA biosynthesis in an ICE*Bs1* recipient is also necessary for efficient transfer

We found that WTA biosynthesis in an ICE*Bs1* recipient was also necessary for efficient acquisition of ICE*Bs1*. We inhibited WTA biosynthesis in recipient cells (cured of ICE*Bs1*) by decreasing expression of *tagAB* (from a Pspank-*tagAB* fusion) by growing cells in low concentrations or the absence of IPTG for six generations. With the lowest level of expression (no IPTG), the conjugation efficiency into the WTA-depleted recipients was decreased ~200-fold relative to that of cells grown in 100 μM IPTG or wild type cells (Figure 3B). Similarly, tunicamycin treatment of recipients lowered mating efficiency by ~100-fold (Figure 3F). We also found that mating WTA-depleted donors with WTA-depleted recipients further compounded the mating defect (Figure 3C).

Based on these results, we conclude that WTAs in both donors and recipients are important for efficient conjugation of ICE*Bs1*. This could indicate a general role for WTAs in host biology, for example in enabling cell-cell contact that is needed for conjugation. Alternatively, WTAs could be important for an aspect of conjugation per se, and perhaps the high efficiency of conjugation mediated by the type IV secretion system encoded by ICE*Bs1*.

WTA biosynthesis is not necessary for transfer of the broad host range ICE Tn*916*

Tn*916* is a small (~18 kb) ICE that confers tetracycline resistance to its host. Its activity is increased several fold in the presence of tetracycline (Showsh and Andrews, 1992). Whereas the natural host of ICE*Bs1* appears to be limited to *B. subtilis*, Tn*916* is found in a broader range of gram-positive bacteria (Roberts and Mullany, 2009) and works quite well in *B. subtilis* (Christie *et al.*, 1987; Johnson and Grossman, 2014).

We found that unlike ICE*Bs1*, Tn*916* transfer was not significantly affected when WTA biosynthesis was inhibited. We inhibited WTA biosynthesis in donors or recipients with tunicamycin (1 µg/ml). The conjugation efficiency following treatment of recipients was similar to that of untreated recipients, and that of donors appeared to increase (Figure 3G). This indicates that the decrease in transfer of ICE*Bs1* in response to WTA depletion is not due to a general effect on host biology or an inability of cells to contact each other. Rather, the defect is specific and appears to highlight a difference between conjugation mediated by ICE*Bs1* compared to Tn*916*.

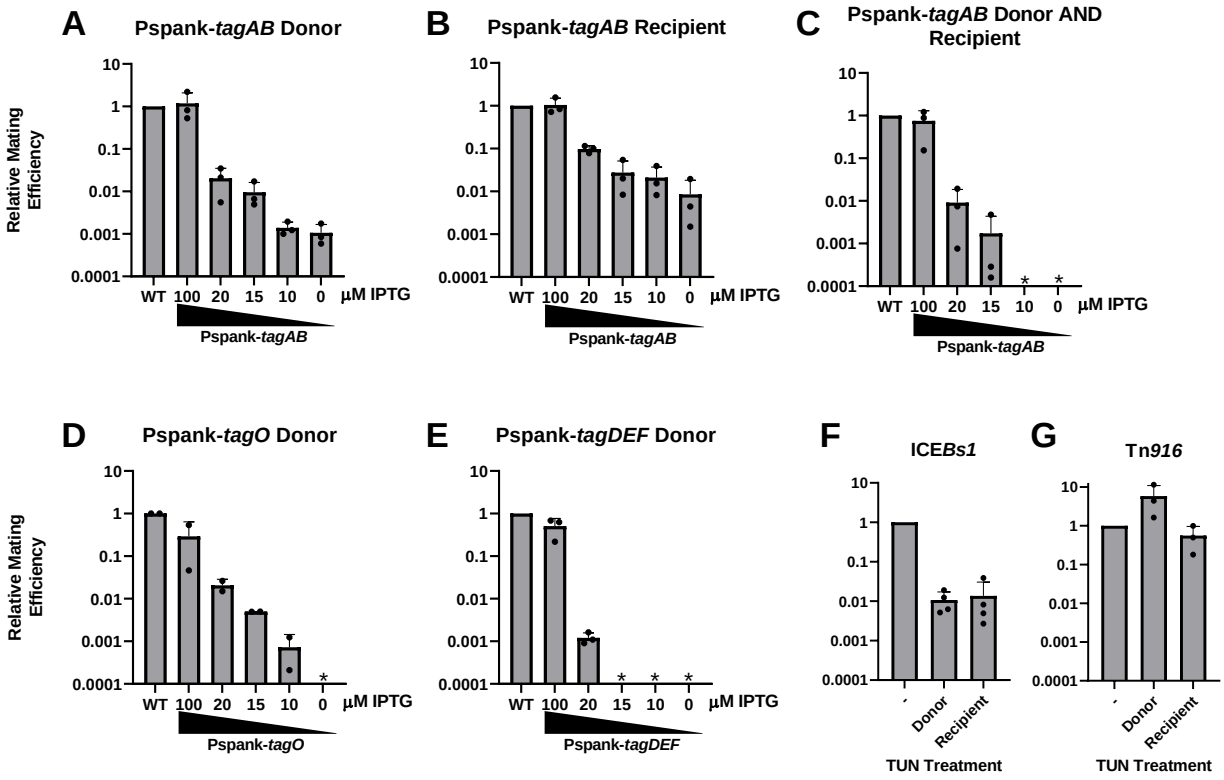

Figure 3. WTA biosynthesis is necessary in both ICE*Bs1* donors and recipients for efficient transfer of the element.

WTA biosynthesis was inhibited by various methods in ICE donors and ICE^0 recipients, and the resulting impact on mating efficiency was examined. Relative mating efficiencies are reported as described in Figure 2. Bars represent averages of two or three independent biological replicates, with points corresponding to individual replicates. Error bars indicate standard deviation.

A. An ICE*Bs1* donor in which the WTA biosynthesis operon *tagAB* had been placed under control of the IPTG-inducible promoter Pspank (MMH578) was mated with an ICE*Bs1*^{0} recipient strain (MMH676). Expression of *tagAB* was controlled by growing the donor strain at the indicated IPTG concentration. A wild type ICE*Bs1* donor (CAL874) was used as a control. Average mating efficiency of the wild type control was 7.78 x 10^{-4}.

B. An ICE*Bs1* donor (CAL874) was mated with an ICE*Bs1*^{0} recipient in which the WTA biosynthesis operon *tagAB* had been placed under the control of Pspank (MMH584). Expression of *tagAB* was controlled by growing the recipient strain at the indicated IPTG concentrations. A wild type recipient (MMH676) was used as a control. Average mating efficiency with the wild type control was 6.07 x 10^{-4}.

C. The Pspank-*tagAB* donor strain from A was mated with the *P*spank-*tagAB* recipient strain from B. Both strains were grown at the indicated IPTG concentration. A wild type donor (CAL874) and recipient (MMH676) were used as controls. * indicates mating efficiency was

below the limit of detection (< 0.0007 for 10 μM; < 0.0008 for 0 μM). Average mating efficiency of the wild type control was 1.29 x 10^{-3}.

D. An ICE*Bs1* donor in which the WTA biosynthesis operon *tagO* had been placed under the control of the IPTG-inducible promoter Pspank (MMH577) was mated with an ICE*Bs1*^{0} recipient strain (CAL89), as in A. Average mating efficiency of the wild type control (CAL874) was 6.94 x 10^{-4}.

E. An ICE*Bs1* donor in which the WTA biosynthesis operon *tagDEF* had been placed under the control of the IPTG-inducible promoter Pspank (MMH608) was mated with an ICE*Bs1*^{0} recipient, as in A and D. Average mating efficiency of the wild type control (CAL874) was 6.58 x 10^{-4}.

F. WTA biosynthesis was inhibited in an ICE*Bs1* donor (MMH550) or an ICE*Bs1*^{0} recipient (MMH676) by adding the TagO-inhibiting drug tunicamcyin at 1 μg/ml. Average mating efficiency of the untreated control was 6.92 x 10^{-3}.

G. The effect of inhibiting WTA biosynthesis on a Tn*916* donor (CMJ253) or recipient (MMH676) was tested by adding tunicamycin as in F. Average mating efficiency of the untreated control was 4.49 x 10^{-6}.

An osmo-protective mating surface rescues the WTA-depletion ICE*Bs1* transfer defect

Cell wall hydrolases encoded by conjugative elements are essential for conjugation in Gram positive bacteria (Bantwal *et al.*, 2012; Arends *et al.*, 2013; Laverde Gomez *et al.*, 2014; DeWitt and Grossman, 2014). Additionally, WTAs are important regulators of the activity of host-encoded cell wall hydrolases (autolysins) and are necessary for the proper localization of autolysins in a range of species (Yamamoto *et al.*, 2008; Schlag *et al.*, 2010; Frankel and Schneewind, 2012; Bonnet *et al.*, 2018). Bacteria depleted of WTAs are more prone to autolysis and are more sensitive to treatment with lysozyme and autolysins (Bera *et al.*, 2007; Atilano *et al.*, 2010; Tiwari *et al.*, 2018).

Based on the role of WTAs in modulating the activity of cell wall hydrolases, we hypothesized that WTA-depleted cells might have cell walls that are more sensitive to the formation of mating pairs. If true, then the decrease in conjugation efficiency should be

suppressed (conjugation restored) under osmo-protective conditions that would enable cells to survive severe defects in their walls.

We found that the conjugation defect of WTA-depleted ICE*Bs1* donors was completely suppressed when matings were done on an osmo-protective surface (Figure 4A). Matings were done on a standard mating surface (Spizizen's salts, described in Methods) or an osmo-protective mating surface that contained 20 mM MgCl2 and 0.5 M sucrose, buffered with 20 mM maleic acid pH 7 (MSM), an osmo-protective supplement that has been used to maintain protoplasts (lacking cell walls) and prevent bacterial cell death from osmotic stress (Wyrick and Rogers, 1973; Leaver *et al.*, 2009). A deletion of a sucrose metabolism gene was incorporated into all strains used in these osmo-protection mating assays to prevent degradation of the sucrose osmo-protectant (Wolf *et al.*, 2012). At the conclusion of the mating, cells were resuspended and diluted in MSM and then plated and grown on non-protective LB plates with the appropriate antibiotics to select for transconjugants.

As described above, treatment of donors with tunicamycin (1 µg/ml) to deplete WTAs caused a mating defect under standard mating conditions (Figure 4A). In contrast, this defect was fully suppressed under osmo-protective conditions and mating efficiencies were indistinguishable from those of cells without tunicamycin treatment (Figure 4A).

Similarly, we found that the conjugation defect associated with WTA-depleted recipients was largely suppressed when matings were done on an osmo-protective mating surface (Figure 4A). There was still a 5-fold drop in conjugation efficiency with WTA-depleted recipients on the osmo-protective mating surface relative to untreated cells. It seems likely that this drop was due to death of transconjugants that did not sufficiently recover from cell wall damage before the shift to non-protective conditions (LB agar).

Suppression of the conjugation defect on the osmo-protective surface (MSM) was due to osmo-protection by sucrose and not an effect of $MgCl_2$ or the malate buffer. When sucrose was omitted from the mating surface, there was no rescue of the conjugation defect caused by depletion of WTAs (Figure 4E). These results support the model that WTA-depleted cells die from osmotic stress. We conclude that WTA-depleted donors are incapable of transferring ICE*Bs1*, perhaps because they die before they can successfully participate in conjugation. This could be due to overall death of all or the vast majority of ICE*Bs1*-containing cells, or selective death of a subpopulation, perhaps those that form mating pairs with recipients.

WTA-depleted cells in ICE*Bs1* mating pairs are more likely to die

We found that there was not a large decrease in the number of viable WTA-depleted donors during mating on a non-protective surface. As above, ICE*Bs1* was activated and cells were simultaneously treated with tunicamycin. The tunicamycin treatment caused only a mild (3.7-fold) decrease in CFU concentration relative to untreated cells (1.1×10^8 CFU/ml for untreated cells versus 3.0×10^7 CFU/mL for tunicamycin-treated cells; likely due in part to a cell separation defect). We combined these donors with an ICE^0 recipient on a non-protective mating surface. We measured the percentage of viable donors recovered at the conclusion of the mating assay relative to the number of viable donors initially present in the mating mixture. The percentage of viable donors recovered after mating was similar between cells with and without tunicamycin treatment (Figure 4B), although there appeared to be a small decrease in recovery of the tunicamycin-treated cells. Based on these results, we conclude that there is not a large decrease in viability of the population of donors.

This population-level observation at the end of a mating protocol does not reflect what occurs at the single-cell level. Although the vast majority of ICE*Bs1*-containing cells are potential

donors, only a small number (~1-5%) successfully participate in conjugation under normal conditions. Based on the results above, we hypothesized that WTA-depleted donors that are part of a mating pair likely undergo cell death. This would represent death of a small fraction of the population that would not be readily observed by bulk population-based viability assays.

We used propidium iodide (PI) staining and fluorescence microscopy to monitor death (loss of cell envelope integrity) of single cells. We induced a population of ICE*Bs1* donors to mate, concentrated them at high density on an agar pad containing propidium iodide, and monitored the cells for two hours to track the number of envelope-damaged (PI-stained) cells. Because mating is ordinarily a rare event, we used a monoculture of ICE*Bs1* donors lacking the ICE gene *yddJ*, which encodes a protein that would normally block the ICE+ cell from serving as a recipient in a mating pair (Avello *et al.*, 2019). This allowed all cells to potentially serve as donors and recipients, substantially increasing the frequency of conjugation. When ICE*Bs1* was induced in WTA-depleted cells under these conditions, we observed a ~8-fold increase in the incidence of PI-stained bacteria, indicating that WTA-depleted cells are more likely to die under conditions that support ICE*Bs1* transfer (Figure 4C-D). These experiments indicate that depletion of WTAs in donors and recipients caused an increase in cell death when ICE*Bs1* is active in both donors and recipients.

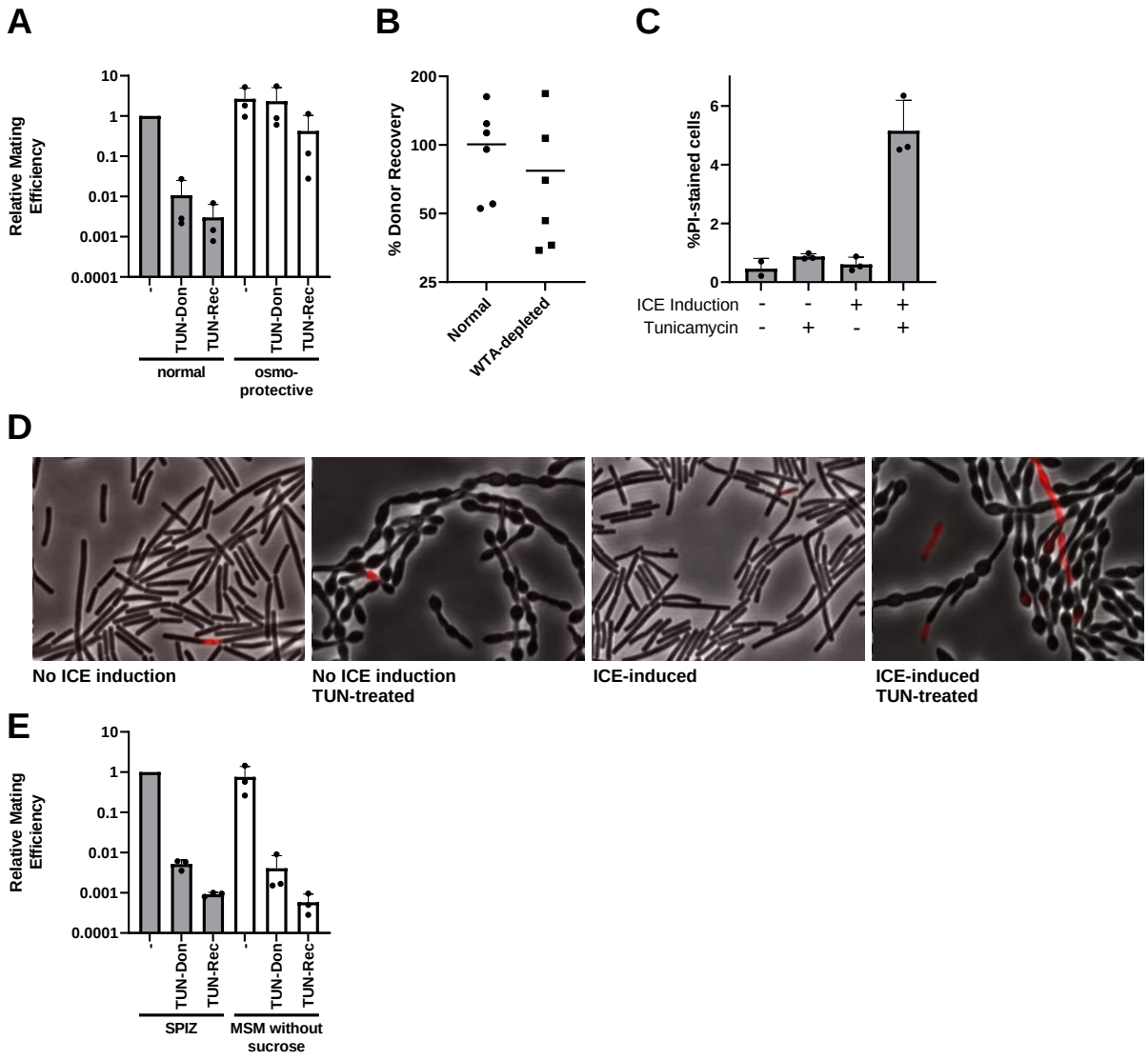

Figure 4. WTA-depleted ICE*Bs1* donors and recipients do not efficiently mate because they experience lethal cell envelope damage.

A. The use of an osmo-protective mating surface eliminates the WTA-depletion ICE*Bs1* transfer defect. WTA biosynthesis was inhibited by treating ICE*Bs1* donors (MMH862) or recipients (MMH797) with 1 μg/ml tunicamycin as in Figure 3F, and strains were mated on either a standard mating surface (1x Spizizen's salts agar) or on an osmo-protective mating surface (1x MSM agar). Bars represent the averages of 3 independent biological replicates, with points representing individual replicates. Error bars indicate standard deviation. Average mating efficiency of the wild type under non-protective conditions was 4.29×10^{-3}.

B. WTA-depleted ICE*Bs1* donors do not exhibit an observable drop in viability at the population level during mating. An ICE*Bs1* donor strain (MMH862) was or was not treated with tunicamycin and induced to mate with an ICE^0 recipient as in A, and the number of viable post-

mating donors was compared to the number of pre-mating donors. Each dot represents data from a single independent experiment (n = 6). The central bars represent the average.

C. WTA-depleted cells engaging in ICE*Bs1* mating are more likely to sustain lethal membrane damage. ICE*Bs1* Δ*yddJ* cells with (MMH794) or without (MMH788) an IPTG-inducible ICE induction allele were cultured with IPTG and +/- tunicamycin, concentrated on an agar pad containing propidium iodide, and tracked for two hours via fluorescence microscopy. The percentage of PI-stained cells was recorded. Bars represent the averages of two or three independent biological replicates, with points corresponding to individual replicates. Error bars indicate standard deviation.

D. Examples of micrographs from experiments summarized in C. PI-staining indicated as red.

E. Use of an MSM mating surface does not rescue the WTA-depletion ICE*Bs1* mating defect when the osmo-protectant sucrose is omitted. WTA biosynthesis was inhibited by treating ICE*Bs1* donors (MMH862) or recipients (MMH797) with 1 μg/ml tunicamycin as in A. Strains were mated on either a standard mating surface (1x Spizizen's salts agar) or on a 1x MSM agar surface lacking sucrose. Data reported are averages of 3 independent biological replicates. Error bars indicate standard deviation. Average mating efficiency for the untreated control (on SPIZ) was 3.34×10^{-3}.

The activity of the ICE*Bs1* conjugation machinery is sufficient to damage WTA-depleted donors and recipients

Our results indicate that WTA-depleted donors and recipients are defective in conjugation due to envelope damage. In the case of matings using WTA-depleted recipients this could be because 1) WTA-depleted recipients never acquire ICE*Bs1*, likely because forming a mating pair is lethal to WTA-depleted cells, or because 2) WTA-depleted recipients acquire ICE*Bs1* and become transconjugants but subsequently die, perhaps due to the expression of ICE*Bs1* genes or the transconjugant becoming a new donor.

We found that transfer of ICE*Bs1* into WTA-depleted recipients was not required for the decrease in conjugation efficiency. We measured mobilization of the plasmid pC194 by the ICE*Bs1* conjugation machinery into WTA-depleted recipients. In these experiments, we used a

mutant ICE*Bs1* that is unable to excise from the chromosome (Δ*attR*) and that lacks a functional origin of transfer (Δ*oriT*). When activated, this ICE mutant still expresses the conjugation machinery and is able to mobilize several plasmids that do not encode their own conjugation system, including pC194 (Lee *et al.*, 2012). We activated ICE*Bs1* gene expression and measured mobilization of pC194 into recipient cells. There was a ~100-fold decrease in mobilization efficiency of pC194 into WTA-depleted (tunicamycin-treated) recipients compared to untreated recipients (Figure 5). Based on these results, we conclude that the decrease in conjugation into WTA-depleted recipients is not due to transfer of ICE*Bs1* into recipients and subsequent death of the new transconjugant; rather, the defect in conjugation is likely due to recipient death caused by the formation of mating pairs or the act of transferring any DNA.

We performed similar experiments with WTA-depleted donor cells in which ICE*Bs1* could not transfer, but the ICE-encoded conjugation machinery could mobilize pC194. Again, there was an ~100-fold decrease in mobilization efficiency as measured by acquisition of pC194 (Figure 5). These results indicate that WTA-depleted donors are defective in transfer. Together with the results above, we infer that this defect is due to donor cell death in mating pairs.

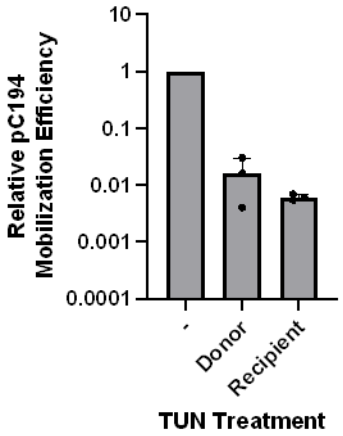

Figure 5. Mobilization of the non-conjugative plasmid pC194 by a locked-in ICE*Bs1* is negatively affected by WTA depletion. A strain containing both the mobilizable plasmid pC194 and a locked-in version of ICE*Bs1* (MMH868) was mated with an ICE*Bs1*^{0} recipient strain (MMH676). WTA biosynthesis was inhibited in either the donor or recipient strain via treatment with 1 μg/ml tunicamycin as in Figure 3F. Relative mobilization efficiency was calculated as the number of transconjugants (Cm^R $Strep^R$ CFUs) per initial donor relative to an untreated control. Bars represent averages of three independent biological replicates, with points corresponding to individual replicates. Error bars indicate standard deviation. Average mobilization efficiency of the untreated control was 1.39×10^{-3}.

Discussion

We used a CRISPRi screen to identify essential *B. subtilis* genes which cause an acute transfer defect when knocked down in an ICE*Bs1* donor. We found that WTAs are necessary in both ICE*Bs1* donors and recipients for efficient transfer of the conjugative element. The use of an osmo-protective mating surface obviates the need for WTAs, and WTA-depleted cells are more likely to die under non-protective conditions that support conjugation. Taken together, these results indicate that WTA-depleted cells fail to mate because they instead die from damage to the cell envelope. Plasmid mobilization experiments suggest that ICE*Bs1* genes do not need to be expressed in recipient cells to cause the mating defect, which implies that the activity of the conjugation machinery itself is sufficient to damage a recipient.

Possible mechanisms of conjugation-dependent toxicity in WTA-depleted cells

WTAs are important regulators of cell wall hydrolases across bacterial species, and WTA-depleted gram-positive bacteria have previously been demonstrated to be more sensitive to autolysin and lysozyme treatment (Brown *et al.*, 2013). WTAs are also important for the proper localization of autolysins. Some *B. subtilis* cell wall hydrolases appear to be excluded from binding peptidoglycan decorated by WTAs, and the enzymes are mislocalized in the absence of WTAs (Yamamoto *et al.*, 2008; Kasahara *et al.*, 2016). It is possible that WTAs similarly regulate the activity of CwlT, and that misregulation of CwlT, perhaps in combination with misregulation of other cell wall hydrolases in WTA-depleted cells, causes the lethal envelope damage during conjugation.

WTAs also have a role in cell wall biosynthesis, and WTA-depleted *B. subtilis* cells exhibit irregularities in cell wall thickness and severe cell shape defects (D'Elia *et al.*, 2006). It is possible that WTA-depletion in *B. subtilis* results in an unusually fragile cell wall, and that

WTA-depleted *B. subtilis* is consequently much more sensitive to the normal cell wall modification process that occurs during conjugation. It is challenging to test the role of CwlT on conjugation efficiencies of WTA-depleted cells because *cwlT* is required for conjugation.

If CwlT is the cause of the conjugation-dependent toxicity observed in this study, an interesting implication of these findings is that the cell wall hydrolase likely acts on both the donor and recipient cell wall during conjugation. It has been speculated that element-encoded cell-wall hydrolases are delivered from donor cells to recipient cells during conjugation in order to facilitate penetration of the recipient cell wall, but this has not been demonstrated.

Although CwlT appears to be the most likely cause of the conjugation-dependent toxicity among WTA-depleted cells, there are alternative explanations which cannot be ruled out. For example, WTA-depletion in *B. subtilis* sensitizes the cells to PBP-targeting antibiotic methicillin (Farha *et al.*, 2013), and it is possible that the ICE*Bs1* conjugation machinery comparably interferes with cell wall biosynthesis in a way that is incompatible with WTA depletion. Alternatively, ICE*Bs1* conjugation could involve inactivating or modifying teichoic acids in a way that is lethal to WTA-depleted bacteria.

ICEs respond differently to WTA depletion

We found that Tn*916* transfer was not negatively impacted by WTA-depletion, in contrast to our results with ICE*Bs1*. An important difference between the two elements that might contribute to this observation is that transfer of Tn*916* is drastically less efficient than ICE*Bs1*. It is possible that less conjugation machinery is made when Tn*916* becomes transcriptionally active, and therefore the formation of mating pairs is not as stressful with the Tn*916*-encoded conjugation machinery is it is with that from ICE*Bs1*. It is also possible that the relevant components of the Tn*916* conjugation machinery are not regulated by *B. subtilis* WTAs, perhaps reflective of the

broad host range of Tn*916*. It remains to be determined what it is about the ICE*Bs1* and Tn*916*-encoded conjugation machineries that make them respond so differently to *B. subtilis* WTAs.

Recent studies of ICE*St3* of *Streptococcus thermophilus* found that deleting a *tagO*-like gene, which might result in a decrease in WTAs, had complex effects on transfer efficiency. Deletion in donors caused a decrease in conjugation efficiency, but deletion in recipients caused an increase in conjugation efficiency (Dahmane *et al.*, 2018). It is not clear if these effects would be alleviated by osmo-protective conditions or if they are related to the effects described here for ICE*Bs1*.

Conjugation and Cell Envelope Stress

Connections between conjugation and envelope stress have long been known. One classic example is in *E. coli* where excessive transfer of F plasmids into F- recipients can lead to death of the recipient, a phenomenon called lethal zygosis (Skurray and Reeves, 1973). Subsequent studies found that radiolabeled peptidoglycan components are released into the medium when lethal zygosis occurs, indicating that the mechanism of death is due to damage of the cell envelope (Ou, 1980). Furthermore, activation of the F-plasmid sensitizes cells to certain envelope-disrupting antimicrobials (i.e. bile salts) (Bidlack and Silverman, 2004). The F plasmid has also been demonstrated to encode the means to upregulate the cell envelope stress response pathway in the host bacterium, which likely anticipates cell envelope stress from producing or utilizing the conjugation machinery (Grace *et al.*, 2015).

Our results indicate that WTAs in gram positive bacteria have an important role in protecting against envelope stress caused by conjugation machinery. In addition, our results indicate that the conjugation machinery is likely more active in mating pairs than in individual cells.

Materials and Methods

Media and Growth Conditions

B. subtilis strains were grown at 37ºC with shaking in LB medium. Experimental cultures were started from 3 ml LB exponential phase cultures inoculated from a single colony.

Where needed, *B. subtilis* strains were grown in LB at the following antibiotic concentrations for selection or maintenance of marked alleles: kanamycin (5 μg/ml), streptomycin (100 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (5 μg/ml), tetracycline (10 μg/ml) and a combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) to select for macrolide-lincosamide-streptogramin (MLS) resistance and erythromycin resistance.

Tunicamycin was used at 1 μg/ml to inhibit WTA synthesis.

The osmo-protective supplement MSM (0.5 M sucrose, 20 mM MgCl2, buffered with 20 mM maleic acid pH 7) (Wyrick and Rogers, 1973; Leaver *et al.*, 2009) was used where indicated. It was added from a 2x stock.

Strains and Alleles

Escherichia coli strain AG1111 (MC1061 F' *lacI*^{q} *lacZ*M15 Tn*10*) was used for routine cloning and plasmid construction.

The *B. subtilis* strains used in this study are listed in Table 1. Strains were constructed using natural transformation (Harwood and Cutting, 1990). All *B. subtilis* strains are derivates of JH642 and contain tryptophan and phenylalanine auxotrophies (*trpC2 pheA1*) (Smith *et al.*, 2014). Many of the alleles used in this study have been described in prior work, and are briefly summarized below.

Donor strains used in standard ICE*Bs1* mating assays typically contained the allele Δ(*rapI-phrI*)*342*::*kan* (Auchtung *et al.*, 2005). ICE*Bs1* was activated in donor strains by inducing

expression of *rapI* from one of three promoter fusions: the LacI-repressible-IPTG-inducible promoters Pspank-*rapI* or Pspank(hy)-*rapI* (Auchtung *et al.*, 2005), or the xylose-inducible promoter Pxyl-*rapI* (Berkmen *et al.*, 2010). For the first two, *rapI* expression was induced by adding Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 mM. Pxyl-*rapI* expression was induced via the addition of xylose to a final concentration of 1% w/v. ICE*Bs1*-containing strains used in live-cell microscopy also contained a deletion of *yddJ* (Avello *et al.*, 2019).

Recipient strains were derived from the ICE*Bs1*-cured (ICE*Bs1*^{0}) strain JMA222 (Auchtung *et al.*, 2005) and were streptomycin resistant (*str84*) to facilitate counterselection during mating experiments. Recipient strains also contained *spc*-marked null alleles of competence genes *comK* or *comC* to prevent natural transformation.

ICE*Bs1* donor and recipient strains that were used in osmo-protective mating assays also contained a deletion-insertion of *sacB* (Δ*sacB*::*erm*) (Koo *et al.*, 2017) to prevent degradation of sucrose. A strain containing this allele was obtained from the Bacillus Genetic Stock Center (www.bgsc.org).

ycgO::{Pspank-*rapI* (*spc*)}. We constructed a *spc*-marked Pspank-*rapI* allele integrated into the nonessential *B. subtilis* gene *ycgO*. We used a previously described Pspank-*rapI* (*spc*) allele as a template for PCR amplification (Auchtung *et al.*, 2005). The amplified allele was joined with *ycgO* flanking sequences by isothermal assembly (Gibson *et al.*, 2009), and the construct was introduced to wild type *B. subtilis* via natural transformation selecting for spectinomycin resistance.

comC::*spc*. We constructed a *comC* deletion-insertion allele, extending from 324 bp upstream and 26 bp downstream of the *comC* open reading frame, with the *aad9* (*spc*) gene from

pMagellan6. The allele was constructed by joining the appropriate *comC* flanking sequences with the amplified *aad9* gene by isothermal assembly, and was moved into wild type *B. subtilis* via natural transformation selecting for spectinomycin resistance.

Pspank-*tag* Alleles. We constructed a set of three fusions that placed each of three endogenous WTA biosynthesis operons (*tagO*, *tagAB*, *tagDEF*) under the control of the IPTG-inducible promoter Pspank. In each case, Pspank was introduced upstream of the first gene in the operon by single cross-over integration. We used a plasmid (pJCL86; lab collection) that contains Pspank and *lacI* inserted into the backbone of pAG58 (Jaacks *et al.*, 1989). A short region of the 5'UTR encompassing the predicted ribosome binding site and a few hundred bp of the 5' region of the ORFs of *tagO*, *tagA*, and *tagD* were each amplified from wild type *B. subtilis* genomic DNA. The region amplified from each gene corresponded to the following: 19 bp upstream to 357 bp downstream of the *tagO* translation start site, 24 bp upstream to 277 bp downstream of the *tagA* translation start site, and 25 bp upstream to 157 bp downstream of the *tagD* translation start site. Each segment was inserted between the SphI and HindIII sites of pJCL86 via isothermal assembly, yielding plasmids pMMH558 (*tagO*), pMMH559 (*tagA*), and pMMH605 (*tagD*). The plasmids were transformed into wild type *B. subtilis* selecting for chloramphenicol resistance in the presence of 1 mM IPTG. Proper integration of each plasmid was confirmed by diagnostic PCR and DNA (Sanger) sequencing. The resulting *B. subtilis* strains grew normally in the presence of 1 mM IPTG, and exhibited severe growth defects in the absence of IPTG. Analysis of these strains by light microscopy following a transition out of IPTG-containing growth medium confirmed that all three strains exhibited the distinctive cell shape and division defects characteristic of *B. subtilis* cells depleted of WTAs (D'Elia *et al.*, 2006).

Construction of strains for mobilization of pC194. We constructed a *B. subtilis* strain containing the plasmid pC194 and a mutant of ICE*Bs1* that is unable to excise (Δ*attR*, 'locked-in') and without a functional origin of transfer (Δ*oriT*). This strain was made by moving the *ycgO*::{Pspank-*rapI* (*spc*)} allele into the ICE*Bs1*^{0} strain JMA222, creating strain MMH863. A version of ICE*Bs1* containing three mutations, Δ*rapIphI342*::*kan*, Δ*oriT*, and Δ*attR*::*MLS*, was moved into MMH863 via natural transformation, and pC194 was subsequently introduced via natural transformation. The unmarked *oriT* deletion in this element has been described (Jones *et al.*, 2020). The Δ*attR*::*MLS* allele was constructed via isothermal assembly using the *MLS* gene from plasmid pCAL215 (Lee *et al.*, 2007) as a template, and has the same deletion boundaries as a previously reported Δ*attR*::*tet* allele (Lee and Grossman, 2007).

Construction of CRISPRi ICE*Bs1* Donor Library. The Pxyl-*dcas9* and Pveg-*sgRNA* alleles used to generate the CRISPRi knockdown library of ICE*Bs1* donor strains were previously described and a generous gift from Peters *et al*., (Peters *et al.*, 2016). The initial library contains a set of 299 plasmids, each containing a Pveg-*sgRNA* allele with a unique 20 bp targeting region, a *cat* marker conferring chloramphenicol resistance, and the appropriate flanking homology needed to integrate the sgRNA allele into the *B. subtilis* chromosome at *amyE* via double crossover. We utilized a pooled collection of these plasmids to generate our library of Pveg-*sgRNA* alleles integrated into ICE*Bs1*.

Our strategy was to insert *amyE* into ICE*Bs1*, delete *amyE* from the chromosome, and then recombine the Pveg-*sgRNA* library into *amyE* in ICE*Bs1*. We constructed a deletion-insertion of *amyE* (Δ*amyE117*::{Pspank-*rapI* (*spc*)}) that replaces the entire chromosomal gene and flanking noncoding regions with Pspank-*rapI*. This allele was constructed using a previously-described Pspank-*rapI* (*spc*) allele (Auchtung *et al.*, 2005) as a template for PCR amplification, and by

joining the amplified product to *amyE* flanking homology via isothermal assembly. The construct was transformed into *B. subtilis* selecting for resistance to spectinomycin. The allele was confirmed by sequencing PCR-amplified DNA and verified functionally to activate ICE*Bs1* following addition of IPTG.

We used isothermal assembly to construct a *kan*-marked copy of *amyE* with flanking sequences needed to integrate the allele into *rapI-phrI* of ICE*Bs1*. This allele (Δ(*rapI-phrI*)::{*amyE kan*}) was designed such that the deletion boundaries and orientation of the *kan* cassette would be identical to Δ(*rapI-phrI*)*342*::*kan*.

The pooled library of Pveg-*sgRNA* plasmids was linearized with KpnI-HF (NEB), and was incorporated into the ICE::*amyE* locus by transformation into strain MMH211 and selecting for resistance to chloramphenicol. We recovered ≥ 1.5 x 10^5 transformants in total. Transformants were resuspended and pooled in MOPS-buffered $S7_{50}$ defined minimal medium (Jaacks *et al.*, 1989) lacking a carbon source (1x $S7_{50}$ + metals) and frozen in aliquots for future use.

We constructed one Pveg-*sgRNA* allele for use as a control, with a 20 bp targeting region corresponding to the nonessential *B. subtilis* gene *cgeD*. To construct this allele we used inverse PCR as previously described (Larson *et al.*, 2013) using pJMP2 as a template for amplification (Peters *et al.*, 2016), creating plasmid pMMH221. The plasmid was linearized with KpnI-HF and transformed into the appropriate *B. subtilis* strains via selection with chloramphenicol. A copy of ICE*Bs1* with the Pveg-*sgRNA*^{cgeD} allele incorporated into the ICE::*amyE* integration site was confirmed to transfer normally.

CRISPRi Library Mating

A lawn of the CRISPRi ICE*Bs1* donor library was started from freezer stocks on the day before the experiment and grown overnight at room temperature on an LB plate. The following

day, the lawn was used to start a culture at OD600 = .02 in LB supplemented with .01% xylose to stimulate transcription of Pxyl-*dcas9* and with kanamycin to ensure maintenance of the *kan*-marked ICE*Bs1*. When cultures reached an OD600 of 0.2, ICE*Bs1* was activated by addition of IPTG (1 mM) for 1 hr to induce expression of Pspank-*rapI*.

Mating of the library into the streptomycin resistant ICE-cured recipient strain CAL89 was conducted in accordance with the standard mating protocol (see below). An aliquot of the donor culture was harvested for analysis immediately prior to combining donors and recipients. At the conclusion of the mating the cells on the filter were resuspended and plated on LB agar containing kanamycin and streptomycin to select for transconjugants and incubated overnight at 37^{o}C. Transconjugants were then resuspended in 1x $S7_{50}$ + metals and pooled, and an aliquot of the resuspension was harvested for sequence analysis.

Amplification and Sequencing of Pooled sgRNA Alleles

We used Qiagen DNeasy Blood and Tissue kits to extract DNA from the pre- and post-mating samples, following the manufacturer instructions for the use of the kit with gram-positive bacteria. We used KAPA HiFi MasterMix to amplify the *sgRNA* alleles from the DNA samples, adhering to the manufacturer protocol. We used 300 ng of sample DNA as a template and ran the reactions for 20 PCR cycles. The primers used to amplify the sgRNA alleles were oMH238 (5'-AATGATACGGCGACCACCGAGATCTACACGGGCGGGAATGGGCTCGTGTTGTACAA TAAATGT-3') and oMH239 (5'-CAAGCAGAAGACGGCATACGAGATXXXXXXGCCAGCCGATCCTCTTCTGAGATGAG TTTTTGTTCG-3'). The 5' ends of these oligos encode the Illumina adaptor sequences, and the X's correspond to multiplexing barcodes. The resulting amplicons were purified with SureSelect AMPure beads according to manufacturer instructions. The sgRNA amplicons were sequenced

with an Illumina MiSeq, using oMH240 to sequence the variable region of the sgRNA allele (5'-GGGCGGGAATGGGCTCGTGTTGTACAATAAATGT-3') and oMH241 to sequence the multiplexing barcode (5'-CGAACAAAAACTCATCTCAGAAGAGGATCGGCTGGC-3').

Mating Assays

Mating assays were conducted as previously described with minor modifications (Auchtung *et al.*, 2005; DeWitt and Grossman, 2014; Johnson and Grossman, 2014). Experimental cultures of donor and recipient strains were started in LB at an OD600 of .02 and grown with shaking at 37 ^{o}C. Kanamycin was added to cultures of strains containing a *kan*-marked ICE*Bs1* to ensure maintenance of the element. ICE*Bs1* activation was induced at an OD600 of 0.2 in donor cultures by adding either 1mM IPTG or 1% xylose. 1 hour after induction, 2.5 OD600 equivalents of donors were combined with an equal amount of recipients. The mixture of donors and recipients was collected on an nitrocellulose filter via vacuum filtration, and washed with 5 ml 1x Spizizen's salts (2 g/l $(NH_4)SO_4$, 14 g/l K_2HPO_4, 6 g/l KH_2PO_4, 1 g/l Na_3 citrate-$2H_2O$, and 0.2 g/l $MgSO_4$-$7H_2O$) (Harwood and Cutting, 1990). The filter was placed on a 1x Spizizen's salts 1.5% agar plate (without a carbon source) and incubated at 37^{o}C for 2 h. The cells were resuspended in 5 ml 1x Spizizen's. Transconjugants were quantified by serially diluting the resuspension in 1x Spizizen's salts, spreading the cells on an LB 1.5% agar plate containing kanamycin and streptomycin (for ICE*Bs1* matings), and incubating the plates overnight at 37^{o}C. Donors were quantified immediately prior to combining donor and recipient cells: an aliquot of the donor culture was serially diluted and plated on an LB 1.5% agar plate containing kanamycin and grown overnight at 37^{o}C. Mating efficiency was calculated as the number of transconjugants (kanamycin- and streptomycin-resistant post-mating CFUs/ml) per

initial donor (pre-mating donor CFUs/ml). Data reported are normalized to the mating efficiency of a wild type control strain assayed on the same day.

For mating assays done with individual strains with inducible CRISPRi-mediated knockdowns, Pxyl-*dcas9* expression was stimulated at the time of culture inoculation by supplementing the experimental cultures with 0.01%, 0.1%, or no xylose.

For mating assays with strains in which the endogenous *tag* operons had been placed under the control of the IPTG-inducible promoter Pspank, the LB starter cultures were grown in the presence of 100 μM IPTG. The starter cultures were pelleted by centrifugation and washed twice with plain LB (no IPTG). Cells were resuspended in LB containing the indicated concentration of IPTG (ranging from 0 to 100 μM), and were used to inoculate LB cultures containing IPTG at the same concentration. LB agar plates were supplemented with 1 mM IPTG to obtain CFUs for strains with an IPTG-inducible promoter fused to an essential gene.

For mating assays with tunicamycin-treated strains, tunicamycin was added concurrently with ICE*Bs1* induction to a final concentration of 1 μg/ml.

For mating assays with Tn*916*, ICE activation was stimulated with 2.5 μg/mL tetracycline, and tetracycline was used in the selection for donors and transconjugants (instead of kanamycin).

For mating assays done on an osmo-protective mating surface, the 1x Spizizen's salts 1.5% agar plate was substituted for a 1x MSM 1.5% agar plate, and the cells were resuspended and diluted post-mating in 1x MSM instead of 1x Spizizen's salts. Pre- and post-mating CFUs were grown out by plating on non-osmo-protective LB 1.5% agar plates (without osmo-protection) as described above. Sucrose was omitted from the 1x MSM solid and liquid media for the relevant experimental controls.

pC194 Mobilization Assays

pC194 mobilization assays were carried out in essentially the same manner as ICE*Bs1* mating assays. Donor cultures were grown with chloramphenicol instead of kanamycin to maintain pC194 (containing *cat*). Donors and transconjugants were measured by plating on appropriate selective media.

Live Cell Microscopy

Live-cell microscopy was done largely as previously described (Babic *et al.*, 2011) with minor modifications. Cultures of ICE*Bs1*+ Δ*yddJ* strains were grown in LB as described above. Strains either did or did not contain *amyE*::Pspank(hy)-*rapI*. All strains were treated with 1 mM IPTG for 1 hr at an OD600 of 0.2. For WTA-depletion, tunicamycin (1 μg/ml) was added concurrently with IPTG. After 1 h and at about OD600 = 0.9, cells were pelleted in a tabletop centrifuge at 14000 rpm, washed once in 1x S750 + metals, and resuspended in 50 μl 1x S750 + metals. 1 μl of the resuspension was applied to an agar pad. The agar pad comprised 1.5% Noble Agar (Difco) dissolved in carbonless 1.5% 1x S750 + metals medium and contained 30 μM propidium iodide.

The agar pad was placed on a glass coverslip (VWR) such that the cells would be in contact with the coverslip, and the coverslip was attached to a microscope slide via a frame-seal slide chamber (Bio-Rad). The cells were observed via a Nikon Ti-E inverted microscope and using a CoolSnap HQ camera (Photometrics). Propidium iodide fluorescence was generated with a Nikon Intensilight mercury illuminator through an excitation and emission filter (Chroma; filter set 49008) The cells were monitored for PI-staining at 37 ^{o}C for 2 h at 15 minute timepoints. At least 1000 cells were monitored for each biological replicate.

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Tables

Table 1. *B. subtilis* strains

Strain (reference)	Genotype
CAL874 (Lee *et al.*, 2010)	ICE*Bs1* Δ(*rapI-phrI*)*342::kan amyE::*(Pxyl-*rapI spc*)
CAL89 (Auchtung *et al.*, 2005)	ICE*Bs1*^{0} *str-84 comK*::*spc*
CMJ253 (Johnson and Grossman, 2014)	ICE*Bs1*^{0} Tn*916*+
MMH211	ICE*Bs1* Δ(*rapI-phrI*)::(*kan amyE*) Δ*amyE117*::(Pspank-*rapI spc*) *lacA*::(Pxyl-*dCas9 spc*)
MMH233	ICE*Bs1* Δ(*rapI-phrI*)::{*kan amyE*::(Pveg-*sgRNA*^{cgeD} *cat*)} Δ*amyE117*::(Pspank-*rapI spc*) *lacA*::(Pxyl-*dCas9 spc*)
MMH525	ICE*Bs1* Δ(*rapI-phrI*)*342::kan ycgO*::(Pspank-*rapI spc*) *amyE*::(Pveg-*sgRNA*^{cgeD} *cat*) *lacA*::(Pxyl-*dCas9 spc*)
MMH527	ICE*Bs1* Δ(*rapI-phrI*)*342::kan ycgO*::(Pspank-*rapI spc*) *amyE*::(Pveg-*sgRNA*^{tagA} *cat*) *lacA*::(Pxyl-*dCas9 spc*)
MMH550	ICE*Bs1* Δ(*rapI-phrI*)*342::kan ycgO*::(Pspank-*rapI spc*)
MMH577	ICE*Bs1* Δ(*rapI-phrI*)*342::kan amyE::*(Pxyl-*rapI spc*) *tagO*::(Pspank-*tagO cat*)
MMH578	ICE*Bs1* Δ(*rapI-phrI*)*342::kan amyE::*(Pxyl-*rapI spc*) *tagA*::(Pspank-*tagA cat*)
MMH584	ICE*Bs1*^{0} *str-84 tagA*::(Pspank-*tagA cat*)
MMH608	ICE*Bs1* Δ(*rapI-phrI*)*342::kan amyE::*(Pxyl-*rapI spc*) *tagD*::(Pspank-*tagD cat*)
MMH676	ICE*Bs1*^{0} *str-84 comC*::*spc*
MMH788	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342::kan*
MMH794	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342::kan amyE*::(Pspank(hy)-*rapI spc*)
MMH797	ICE*Bs1*^{0} *str-84 comC*::*spc sacB*::*mls*
MMH868	ICE*Bs1* Δ*oriT285* Δ(*rapI-phrI*)*342::kan* Δ*attR*::*mls ycgO*::(Pspank-*rapI spc*) pC194(*cat*)

Table 2. Results of CRISPRi screen. The CRISPRi screen for essential host gene knockdowns that cause an acute ICE*Bs1* defect was carried out as described in the text and in Figure 1. Relative ability of a knockdown strain to donate ICE*Bs1* was monitored by using next generation sequencing to determine the relative abundance of an sgRNA in the transconjugant population versus the abundance of the sgRNA in the population of pre-mating donors. Gene knockdowns that caused an impactful ICE*Bs1* transfer defect (>4-fold change in sgRNA abundance) and that were not depleted from the donor population prior to mating (>0.01% of pre-mating donor population) are reported here. The proportion of the starting population that each sgRNA constitutes is also listed, as well as the *Subti*wiki functional annotation for each gene (http://subtiwiki.uni-goettingen.de/v3/) (Zhu and Stülke, 2018).

Gene Name	Fold-Change After Mating (TC abundance / donor abundance)	Proportion of Pre-Mating Donor Population	Gene Function
tagA	eliminated	1.65×10^{-3}	biosynthesis of teichoic acid
rplL	eliminated	1.68×10^{-4}	translation
fusA	eliminated	1.74×10^{-4}	translation
rplF	eliminated	1.61×10^{-4}	translation
secE	7.73×10^{-4}	1.20×10^{-3}	protein secretion
mntA	1.12×10^{-3}	3.32×10^{-3}	manganese uptake
rpsC	1.66×10^{-3}	2.80×10^{-4}	translation
cysS	5.47×10^{-3}	4.26×10^{-4}	translation
groEL	8.04×10^{-3}	3.42×10^{-3}	protein folding and re-folding
infC	.0100	1.86×10^{-4}	translation
groES	.0105	3.60×10^{-3}	protein folding and re-folding
rplX	.0194	4.56×10^{-4}	translation
rny	.0531	2.25×10^{-3}	RNA processing and degradation
rplN	.0566	2.06×10^{-4}	translation
ydiO	.0588	3.14×10^{-3}	BsuM DNA modification
tufA	.0748	1.62×10^{-4}	translation
ydiP	.0822	2.85×10^{-3}	BsuM DNA modification
rplJ	.0836	1.41×10^{-3}	translation
tagD	.103	3.82×10^{-3}	biosynthesis of teichoic acid
gpsA	.104	2.07×10^{-4}	biosynthesis of phospholipids

nusA	.106	3.19×10^{-3}	transcription
secA	.110	1.96×10^{-3}	protein secretion
tsf	.115	4.46×10^{-4}	translation
glmS	.117	3.50×10^{-3}	cell wall synthesis
murAA	.117	8.72×10^{-4}	peptidoglycan precursor biosynthesis
rpsNA	.120	8.90×10^{-4}	translation
nrdI	.120	1.41×10^{-3}	synthesis of deoxyribonucleotide triphosphates
mreC	.141	1.18×10^{-3}	cell shape determination
fabF	.142	2.48×10^{-3}	fatty acid biosynthesis
murB	.143	2.71×10^{-4}	peptidoglycan precursor biosynthesis
nrdE	.144	5.61×10^{-4}	synthesis of deoxyribonucleotide triphosphates
trnI-Asn	.148	8.08×10^{-3}	translation
asnB	.178	9.67×10^{-4}	control of peptidoglycan hydrolysis
rplS	.201	5.13×10^{-4}	translation
ffh	.203	3.27×10^{-3}	presecretory protein translation
adk	.206	1.90×10^{-4}	ADP formation
rpsH	.218	1.00×10^{-3}	ribosomal protein
yneF	.218	1.11×10^{-3}	membrane protein of unknown function
tagF	.230	2.74×10^{-3}	biosynthesis of teichoic acid
alaS	.232	7.59×10^{-4}	translation
parE	.239	4.93×10^{-4}	chromosome segregation and compaction

Chapter 3

Conjugation with bacteria lacking a cell wall (L-forms) indicates that a conjugative cell wall hydrolase normally acts on the walls of both donor and recipient bacteria

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Abstract

Conjugative elements of gram-positive bacteria encode cell wall hydrolases that are required for efficient transfer. These hydrolases are presumed to be necessary to remodel or partly degrade the cell wall of the donor to enable assembly and function of the conjugation machinery. We present evidence that indicates that the cell wall hydrolase from the integrative and conjugative element ICE*Bs1* from *Bacillus subtilis* acts on the cell wall of both the donor and recipients during mating. We created *B. subtilis* cells lacking a cell wall (L-forms) and used these as donors or recipients in conjugation experiments. There was efficient transfer of ICE*Bs1* from L-form donors to walled recipients, and from walled donors to L-form recipients. The ICE*Bs1*-encoded cell wall hydrolase CwlT is required for transfer between two walled cells. We found that *cwlT* was dispensable for transfer between an L-form donor and a walled recipient, and that *cwlT* was also dispensable for transfer between a walled donor and an L-form recipient. These findings indicate that CwlT is likely involved in remodeling both the donor cell wall and the recipient cell wall for efficient mating, and that in the absence of a wall in one cell type, the hydrolases of the host likely substitute for the absence of CwlT.

Introduction

Conjugation is the direct, contact-dependent transfer of DNA from a donor cell to a recipient. Each of the two major types of conjugative elements, conjugative plasmids and integrative and conjugative elements (ICEs), encode a type 4 secretion system (T4SS) that transports DNA across the cell envelope from a donor to recipient cell (Bhatty *et al.*, 2013). Conjugative elements are important drivers of horizontal gene transfer in bacteria, and many plasmids and ICEs confer antibiotic resistances to their hosts (Burrus and Waldor, 2004; Norman *et al.*, 2009).

The bacterial cell wall is a barrier that must be crossed for the successful transfer of conjugative elements. Most bacteria have a peptidoglycan cell wall that encompasses the cytoplasmic membrane. In both gram-positive and gram-negative bacteria, peptidoglycan contains glycan strands made of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid (Vollmer, 2008). The individual glycan strands are connected to each other via crosslinked peptide stems, forming a single large macromolecule (Vollmer *et al.*, 2008). A central function of the cell wall is to bear the internal osmotic forces of the cell; if the wall is damaged while the bacterium is in a typical hypo-osmotic environment, the bacterium will rupture and die (Silhavy *et al.*, 2010). The cell wall is continually remodeled by cell wall hydrolases, enzymes that cleave and can degrade peptidoglycan (Do *et al.*, 2020).

Conjugative elements of gram-positive bacteria navigate the thick (30-100 nm) mesh of peptidoglycan to exit the donor cell and to enter the recipient cell (Silhavy *et al.*, 2010). Conjugative elements of walled bacteria encode their own cell wall hydrolases (Bhatty *et al.*, 2013), and element-encoded hydrolases are presumed to partly degrade the cell wall of the donor cell in order to facilitate the passage of the conjugation machinery (Bayer *et al.*, 2001; Bantwal *et al.*, 2012; DeWitt and Grossman, 2014). However, the mechanism by which the conjugation

machinery broaches the recipient cell wall is not understood. It has been speculated that donor-derived cell wall hydrolases might be delivered to the recipient during conjugation (Baidya *et al.*, 2020).

Cell wall hydrolases encoded by conjugative elements in gram-positive bacteria are required for transfer. Loss of the cell wall hydrolase generally results in little or no conjugation (Bantwal *et al.*, 2012; Arends *et al.*, 2013; Laverde Gomez *et al.*, 2014; DeWitt and Grossman, 2014). These hydrolases typically have two catalytic activities, a glycosidase and a peptidase. (Goessweiner-Mohr *et al.*, 2013).

ICE*Bs1* is a relatively small (~20.5 kb) ICE from the gram-positive bacterium *Bacillus subtilis* (Burrus *et al.*, 2002; Auchtung *et al.*, 2005). Prior to being activated, ICE*Bs1* is normally integrated in a tRNA gene (*trnS-leu2*). In response to DNA damage or a high concentration of cells that lack the element, ICE*Bs1* gene expression is derepressed and the element excises from the chromosome to form a circular intermediate (Auchtung *et al.*, 2005). During conjugation, a single strand is nicked by the element-encoded relaxase (Lee and Grossman, 2007) and transferred to a recipient cell via the element-encoded T4SS. The element then integrates into the new host chromosome, thereby forming a stable transconjugant (Lee *et al.*, 2007). ICE*Bs1* activation can be induced in the majority of cells in a population by overproduction of the element-encoded activator RapI (Auchtung *et al.*, 2005; Lee *et al.*, 2007).

ICE*Bs1* encodes the cell wall hydrolase CwlT, a bifunctional enzyme that contains an N-terminal transmembrane helix and two peptidoglycan-degrading domains: an acetylmuramidase (muramidase) domain and a DL-endopeptidase domain (peptidase) (Fukushima *et al.*, 2008; Xu *et al.*, 2014; DeWitt and Grossman, 2014). CwlT is essential for conjugative transfer of ICE*Bs1*. Eliminating either the entire protein, the N-terminal transmembrane helix, or the muramidase

catalytic activity makes ICE*Bs1* transfer undetectable. Eliminating the peptidase catalytic activity results in a ~1000-fold transfer defect (DeWitt and Grossman, 2014).

Although an intact cell wall is normally essential for the viability of walled bacteria, under certain osmo-protective conditions the wall becomes dispensable for survival and growth. This is exemplified by L-forms, strains of ordinarily-walled bacteria that have been genetically or chemically manipulated into abandoning their cell wall. L-forms can be stably propagated under osmo-protective conditions (Errington, 2017). A diverse variety of bacteria can be transitioned into stable L-form variants by blocking the production of certain peptidoglycan precursors under osmo-protective conditions (Mercier *et al.*, 2014), and a protocol for readily generating L-form *B. subtilis* has been described (Leaver *et al.*, 2009; Domínguez-Cuevas *et al.*, 2012).

In this work, we tested the ability of L-form *B. subtilis* to function as either conjugative donors or recipients of ICE*Bs1*. We found that ICE*Bs1* conjugation can occur relatively efficiently in the absence of either the donor cell wall or the recipient cell wall. Additionally, we found that eliminating the donor cell wall or the recipient cell wall bypassed the need for CwlT, indicating that the element-encoded cell wall hydrolase normally has a role in degrading the walls of both cells to allow conjugation and that in the absence of one of the two walls in a mating pair, the activities of cell wall hydrolases encoded by *B. subtilis* become sufficient for conjugation.

Results

Generation of L-form ICE*Bs1* donors and recipients

We made strains of *B. subtilis* that could be converted from normal walled bacteria to wall-less L-forms, and that could also serve as either ICE*Bs1* donors or recipients. In donors, ICE*Bs1* could be efficiently induced in the majority of cells in a population by overproduction of the activator RapI from the LacI-repressible-IPTG-inducible fusion Pspank(hy)-*rapI* (Auchtung *et al.*, 2005). Recipients were cured of ICE*Bs1* (ICE^0) and were resistant to an antibiotic that could be used to prevent growth of donors after mating.

To be able to convert donors and recipients to L-forms, we utilized derivatives and alleles of strain BS115 in which the *murE* operon is under the control of the xylose-inducible promoter Pxyl, and that contain a secondary copy of the xylose repressor to prevent the cells from spontaneously losing their xylose dependency (Leaver *et al.*, 2009; Domínguez-Cuevas *et al.*, 2012). These strains can be used to generate L-forms by transitioning the cells from xylose-containing rich medium to osmo-protective medium lacking xylose (NB-MSM medium; Methods). Strains that could make L-forms also contained a point mutation in *ispA* (*ispA-D92E*) that alleviates stress caused by production of reactive oxygen species produced by blocking cell wall synthesis (Kawai *et al.*, 2015). Strains also contained a null mutation in *sacB*, thereby interfering with the metabolism of sucrose and enabling the formation of defined L-form colonies on solid media (Wolf *et al.*, 2012). L-forms were maintained in xylose-free rich medium containing penicillin in order to cull any remaining rods and prevent the re-emergence of walled cells (Leaver *et al.*, 2009).

L-form *B. subtilis* can successfully donate ICE*Bs1*

We found that L-form strains of *B. subtilis* could transfer ICE*Bs1* to walled recipients, indicating that the donor cell wall is not required for ICE transfer. We generated L-form donors and activated ICE*Bs1* by overexpression of *rapI*. We concentrated and combined the L-forms with walled ICE*Bs1*-cured (ICE^0) recipients on a filter under osmo-protective conditions. We identified putative transconjugants in the mating mixture by selecting for kanamycin-resistant (ICE) and spectinomycin-resistant (recipient chromosome) colony-forming units (CFUs) under non-protective conditions that do not sustain L-forms (LB agar). We also plated a concentrated aliquot of donor cell culture on non-protective LB agar to ensure there were not walled donor cells in the mating mixture (< 2 per assay).

We reliably detected between 50 and 2000 total putative transconjugants when using an L-form donor of wild type ICE*Bs1*. We did not observe transconjugants when using an L-form donor containing a deletion of the ICE*Bs1* gene *conQ*, a gene encoding the T4SS coupling protein that is essential for conjugative transfer. Recipients used in mating assays were also non-transformable (Δ*comC*::*spc*), indicating that the cells did not acquire ICE*Bs1* by spontaneous transformation. We conclude that the observed colonies were true transconjugants, and that ICE*Bs1* transfer does not require a donor cell wall.

Quantifying mating efficiency of an L-form donor. We estimated the mating efficiency of an L-form donor of ICE*Bs1* to be approximately 6%, which is comparable to that of a walled ICE*Bs1* donor (8.5%) (Lee *et al.*, 2007). Mating efficiency is typically reported as the percent number of transconjugants per donor in the mating mixture, and so to estimate L-form mating efficiency we attempted to quantify the number of viable L-form donors (L-form kanamycin-resistant CFUs) added to the mating mixture. These estimates were prone to noise, but we

typically counted between 500 and 10,000 L-form donor CFUs. It is possible that this is an underestimation of the true number of viable L-form donors in the mating mixture, in which case the reported mating efficiency of 6% per donor would be an overestimate.

L-form *B. subtilis* can be recipients of ICE*Bs1*

We also found that L-form strains of *B. subtilis* could acquire ICE*Bs1* via conjugation, indicating that a recipient cell wall is not required for transfer. We generated ICE^0 L-form recipients, concentrated the cells, combined them with walled ICE*Bs1* donors, and transferred the mixture to a filter on an osmo-protective surface. We selected for L-form transconjugants by plating the mating mixture on osmo-protective selective medium containing kanamycin (ICE) and penicillin (recipients). We also plated a concentrated aliquot of recipient cell culture on non-protective LB agar to ensure there were not walled recipients in the mating mixture (< 2 per assay).

We reliably recovered between 500 and 10,000 L-form recipients. An average of approximately 5% of recovered recipients had acquired ICE*Bs1* (were kanamycin-resistant), which is comparable to mating efficiencies between walled cells. We did not detect transconjugants when using a Δ*conQ* donor, indicating that the cells acquired ICE*Bs1* by conjugation. We conclude that L-forms can serve both as donors and recipients, and that the ICE*Bs1* conjugation machinery can assemble and function in bacteria without a cell wall.

Unsuccessful attempts at mating L-form donors with L-form recipients

We made repeated attempts to mate L-form donors of ICE*Bs1* with L-form recipients, but were not able to reliably detect transconjugants. We could occasionally identify putative L-form recipients that had acquired ICE*Bs1* from an L-form donor, but such cells arose equally commonly in the absence of the conjugation machinery (Δ*conQ*), and the cells that arose often

had inconsistent combinations of donor and recipient chromosomal markers. We suspect that when two L-forms are pressed together tightly enough to permit conjugation, they are likely to simply fuse (protoplast fusion).

CwlT is not necessary for ICE*Bs1* transfer in the absence of the donor cell wall

The element-encoded cell wall hydrolase CwlT is required for transfer of ICE*Bs1* (DeWitt and Grossman, 2014). CwlT is thought to act on the donor cell wall in order to facilitate assembly of the mating machinery and/or the formation of a mating pore. A deletion of *cwlT*, or the use of a mutant encoding a catalytically dead enzyme, ordinarily makes ICE*Bs1* transfer undetectable (< 0.00005%) in conjugation assays with walled cells (DeWitt and Grossman, 2014). Given the presumed role of CwlT in degrading the donor cell wall, we asked whether the requirement for CwlT in conjugation would be different when the donor lacked a cell wall.

We found that CwlT was no longer required for transfer in the absence of a donor cell wall. An L-form donor lacking CwlT (Δ*cwlT*) could readily generate transconjugants, but with a 4-fold decrease in mating efficiency relative to an L-form donor of a wild type (*cwlT*$^{+}$) ICE*Bs1* (Figure 1). Because eliminating the donor cell wall makes CwlT largely unnecessary for transfer, we infer that the enzyme normally acts on the donor wall to allow conjugation. This result also indicates that CwlT is not required for ICE*Bs1* to cross the recipient cell wall, although the decrease in efficiency indicates that CwlT might contribute to crossing the recipient wall under these experimental conditions.

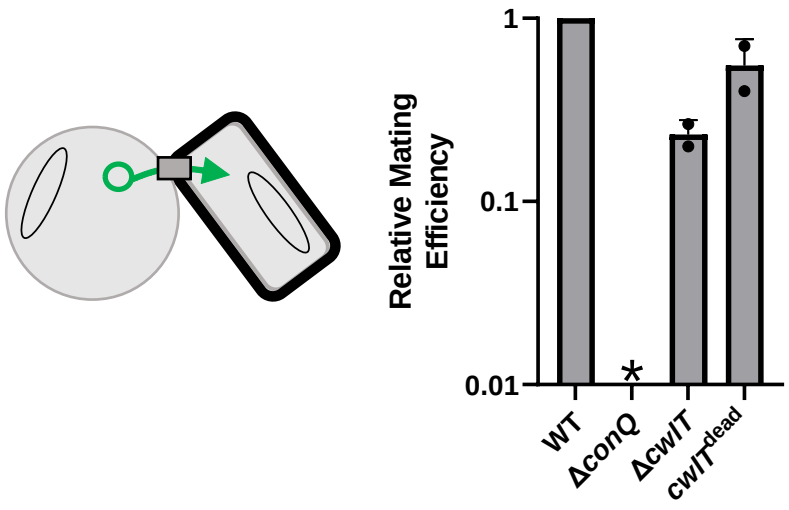

Figure 1. CwlT is not required for ICE*Bs1* transfer from an L-form donor to a walled recipient.

Left: Scheme of mating assay using L-form ICE*Bs1* donors.

Right: L-form ICE*Bs1* donors were mated with a walled recipient (MMH676) as described (Methods). L-form donors from left to right are MMH683 (WT), MMH686 (Δ*conQ*), MMH685 (Δ*cwlT*), and MMH694 (*cwlT*^{dead}). Relative mating efficiency is the number of transconjugant CFUs generated relative to input OD600 equivalents, normalized to a donor of wild type ICE*Bs1* tested on the same day. Bars represent averages of two independent biological replicates, with points representing individual replicates. Error bars represent standard deviation. * indicates mating efficiency below limit of detection (<0.013).

CwlT is not necessary for ICE*Bs1* transfer in the absence of the recipient cell wall

Cell wall hydrolases of conjugative elements might also act on the recipient cell wall. Thus, we asked if eliminating the recipient wall would change the requirement for CwlT during transfer. We found that an L-form recipient could acquire ICE*Bs1* from a walled Δ*cwlT* donor with no significant drop in transfer efficiency relative to a wild type (*cwlT*$^{+}$) donor (Figure 2). The finding that eliminating the recipient cell wall makes CwlT unnecessary for transfer indicates that CwlT normally has a role in modifying the recipient wall to enable conjugation.

This result also indicates that CwlT is not required to cross the donor cell wall under these experimental conditions.

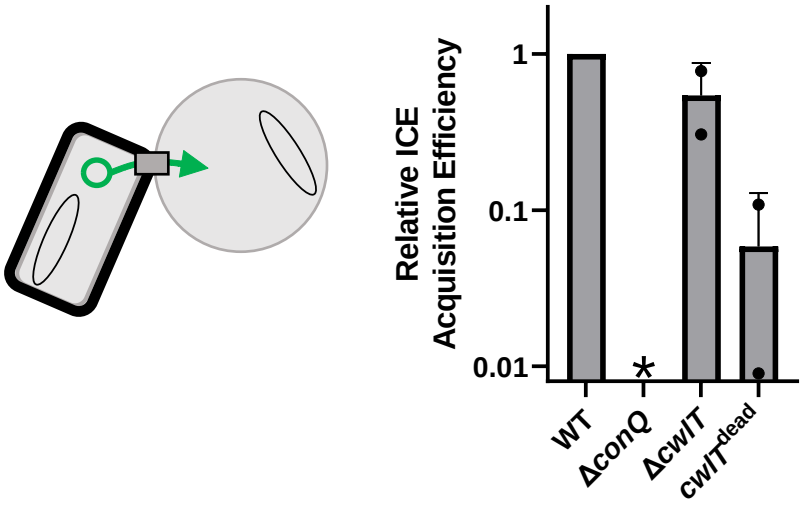

Figure 2. CwlT is not required for ICE*Bs1* transfer from a walled donor to an L-form recipient, but an ICE encoding a catalytically dead CwlT does not mate efficiently.

Left: Scheme of mating assay using L-form ICE*Bs1* recipients.

Right: Walled donor strains of ICE*Bs1* were mated with an L-form ICE*Bs1*^{0} recipient (MMH688) as described (Methods). Donors from left to right are MMH702 (WT), MMH701 (Δ*conQ*), MMH700 (Δ*cwlT*), and MMH705 (*cwlT*^{dead}). Relative ICE acquisition efficiency is the number of ICE+ recipient CFUs generated relative to total recovered recipients, normalized to acquisition efficiency of the L-form recipient mated with a wild type ICE*Bs1* donor tested on the same day. Bars indicate average of two independent biological replicates, with points representing individual replicates. * indicates acquisition efficiency below limit of detection (<0.007).

Navigating the remaining wall without CwlT

Our results indicate that eliminating either the donor or recipient cell wall in a mating pair makes CwlT dispensable for transfer, but this raises the question of how an element lacking a cell wall hydrolase can navigate the remaining wall. Notably, CwlT is one of over 35 cell wall

hydrolases encoded by *B. subtilis*, including 4 paralogs of CwlT (Sudiarta *et al.*, 2010). Bacterial cell wall hydrolases are required for many cellular functions, including growth, cell division, sporulation, and the formation of other cell wall-spanning membrane structures. These hydrolases ensure that the sacculus is constantly subjected to controlled degradation and remodeling (Do *et al.*, 2020). We speculate that in the absence of CwlT other cell wall hydrolases could substitute for its activity, or naturally-occurring gaps in the sacculus might suffice as openings for conjugation. We suspect that these alternative hydrolases or openings are ordinarily poor substitutes for CwlT activity, but suffice for efficient transfer in the absence of one of the two walls. Microscopic observations of mixed cultures of L-forms and rods lend credence to this idea. L-forms are very malleable, and are capable of tightly cohering to rod-shaped cells (Figure 3). Such tight cohesion could make it more likely that the L-form membrane will contact the walled cell at an uncommon segment of unprotected membrane.

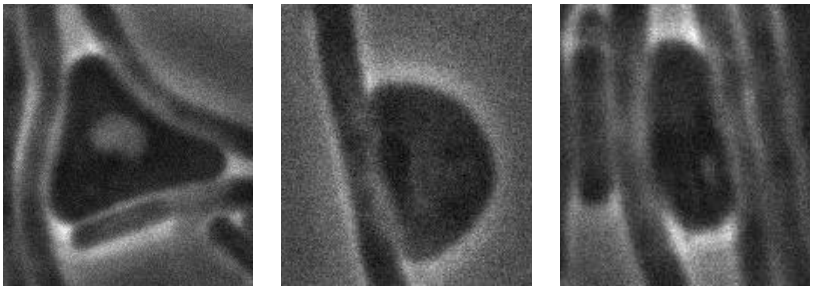

Figure 3. Malleable L-forms can form close associations with walled bacteria. Cultures of L-forms (MMH688) and exponentially-growing walled bacteria (AG174) were mixed 1:1 by volume and observed on an agar pad by phase contrast microscopy.

A catalytically dead CwlT does not allow efficient passage through the donor cell wall

Cell wall hydrolases from other conjugative elements specifically interact with components of the mating channel, and these interactions might be needed for proper formation of the mating pore (Abajy *et al.*, 2007; Steen *et al.*, 2009; Kohler *et al.*, 2017). Our results indicate that in the absence of CwlT, other cell wall hydrolases encoded by *B. subtilis* might suffice for navigating one cell wall. We postulated that these putative substitutes might also need to interact with the conjugation machinery, and wondered whether the presence of a mutant CwlT that is altered in each of the two catalytic sites (CwlT-E87Q-C237A; CwlT^{dead}) and is incapable of degrading peptidoglycan (DeWitt and Grossman, 2014) might inhibit the ability of the putative substitute hydrolases to function.

We found that ICE*Bs1* encoding CwlT^{dead} transferred relatively efficiently out of an L-form donor into a walled recipient, comparably to a Δ*cwlT* ICE*Bs1* donor (Figure 1). This indicates that if there are other hydrolases acting on the recipient, the dead enzyme does not block these. However, we found that in matings between a walled donor and L-form recipient, ICE*Bs1* encoding CwlT^{dead} had an ~50-fold decrease in transfer efficiency (Figure 2). This result is consistent with the notion that the ICE*Bs1* conjugation machinery interacts with a cell wall hydrolase for efficient passage through the donor cell wall and that in the absence of CwlT a different hydrolase may substitute. In this model, the presence of the inactive CwlT effectively prevents other hydrolases from serving as a substitute, probably by blocking their interaction with the conjugation machinery, leading to the observed difference between the Δ*cwlT* and *cwlT*^{dead} mutants.

Absence of a cell wall does not bypass the need for other components of the conjugation system

There are components of the ICE*Bs1*-encoded conjugation system other than CwlT and ConQ that are normally required for conjugation, including: ConB, ConC, ConD, ConE, and ConG. (Berkmen *et al.*, 2010; Leonetti *et al.*, 2015). Unlike CwlT, we found that all of these components were still required for transfer in the absence of either a donor or recipient cell wall. We did not detect ICE*Bs1* transfer in any mating experiments with L-forms when any one of these components was deleted (≤ .003 when using L-form donors, ≤ .001 when using L-form recipients).

Discussion

In this work, we used cell wall-deficient L-form strains of *B. subtilis* to investigate the role of the ICE*Bs1* cell wall hydrolase CwlT in conjugation. We found that a cell wall was not required in an ICE*Bs1* donor or recipient for efficient transfer of the element, and that eliminating either the donor or recipient wall makes CwlT unnecessary for transfer. Because eliminating the donor or recipient wall obviated the need for the enzyme, we infer that CwlT likely has a role in degrading the wall of both cells in a mating pair. We speculate that the activity of other host-encoded cell wall hydrolases likely suffice as substitute for CwlT in the absence of one of the two cell walls, and that these substitutes likely interact with the conjugation machinery to facilitate passage through the donor cell wall.

Substitutions for CwlT activity

The idea that alternative cell wall hydrolases could serve as substitutes during conjugation has precedent. Multiple studies have shown that deletions of conjugative cell wall hydrolases can be complemented with hydrolases from other elements, both in gram-positive and gram-negative systems (Höppner *et al.*, 2004; Zahrl *et al.*, 2005; Laverde Gomez *et al.*, 2014). Furthermore, cell wall hydrolases from gram-negative conjugative systems are partially dispensable for transfer; one proposed explanation for this is that other cell wall hydrolases substitute for the element-encoded enzymes in their absence (Höppner *et al.*, 2004; Zahrl *et al.*, 2005).

Role of CwlT in an ICE*Bs1* donor

Cell wall hydrolases from other conjugative elements have been shown to interact with components of the conjugation channel. The cell wall hydrolase VirB1 from the gram-negative *A. tumefaciens* Ti plasmid makes contacts with several components of the T4SS, including VirB4 (an ATPase), VirB8 (an inner membrane protein), and VirB10 (a component of the outer membrane core complex) (Ward *et al.*, 2002). The cell wall hydrolase TraG from the gram-positive-associated plasmid pIP501 likewise interacts with VirB4 and VirB8 analogs (Abajy *et al.*, 2007; Kohler *et al.*, 2017). In the gram-positive conjugative plasmids pIP501 and pCW3, the hydrolases interact with the coupling protein (Abajy *et al.*, 2007; Steen *et al.*, 2009). Based on these interactions, some have speculated that cell wall hydrolases in gram-positive systems might serve as essential scaffolding components that promote the assembly of the conjugation channel (Abajy *et al.*, 2007; Kohler *et al.*, 2017). However, we observed efficient conjugation in the absence of *cwlT*, provided either the donor or recipient lacked a cell wall. That CwlT was not needed under these conditions indicates that it is not required for assembly of the conjugation machinery, nor is it a critical structural component of the conjugation machinery encoded by

ICE*Bs1*. We suspect that interactions between cell wall hydrolases and the conjugation machinery primarily serve to localize the hydrolase and direct and limit peptidoglycan-degradation activity.

Conjugation among wall-less bacteria

The extent to which L-forms might exist outside of the laboratory is unclear (Onwuamaegbu *et al.*, 2005), but they have been proposed to be an adaptive mechanism that allows bacteria to survive otherwise-lethal cell-wall stress, including high concentrations of antibiotics that inhibit cell wall biosynthesis (Errington *et al.*, 2016). Accordingly, L-forms might be functional persistors in the context of certain infections. In particular, the high osmolarity of urine might allow L-forms to arise in the context of urinary tract infections (Mickiewicz *et al.*, 2019). To the extent that L-forms do exist in nature, our results indicate that they are readily capable of donating or acquiring genetic material via conjugation.

Materials and methods

Media and Growth Conditions

Walled *B. subtilis* cells were grown at 37°C either in LB liquid medium with aeration, or on LB agar (1.5%) plates. Experimental cultures of walled *B. subtilis* were started from a 3 ml LB exponential phase culture inoculated from a single colony. For xylose-addicted walled strains, media were supplemented with xylose (0.5% w/v). For D-alanine auxotrophs, media were supplemented with D-alanine (100 μg/ml).

L-form *B. subtilis* cells were grown largely as described (Leaver *et al.*, 2009; Domínguez-Cuevas *et al.*, 2012). Liquid cultures were grown at 30°C without shaking in the rich medium

NB-MSM, which contains Nutrient Broth (NB; Oxoid) and the osmo-protective supplement MSM. MSM was added from a 2x stock (2x = 1 M sucrose, 40 mM $MgCl_2$, buffered with 20 mM maleic acid pH 7) to a final concentration of 1x. Solid-surface cultures of L-form *B. subtilis* were grown on NA-MSM medium {NB supplemented with 1x MSM and 1.5% bacto-agar (Difco)}. All L-form cultures were supplemented with 0.5 mg/ml Penicillin G (PenG; GoldBio) to prevent the re-emergence of walled cells.

B. subtilis strains were grown with antibiotics at the following concentrations for the selection or maintenance of marked alleles: kanamycin (5 μg/ml), streptomycin (100 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (5 μg/ml), tetracycline (12.5 μg/ml), and a combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) to select for macrolide-lincosamide-streptogramin (MLS) resistance.

Strains and Alleles

Escherichia coli strain AG1111 (MC1061 F' *lacI*^{q} *lacZ*M15 Tn*10*) was used for routine cloning and plasmid construction.

The *B. subtilis* strains used in this study are listed in Table 1. Strains were constructed using natural transformation (Harwood and Cutting, 1990). Strains used for generating L-forms are derivatives of BS115, obtained from the Bacillus Genetic Stock Center (BGSC; www.bgsc.org) (Leaver *et al.*, 2009). BS115 contains a xylose-inducible *murE* operon (*spoVD*::(*cat* Pxyl-*murE*)) and encodes a second copy of the xylose repressor (*amyE*::(*tet xylR*)). Strains for generating L-forms also contained the mutation *ispA-D92E,* which stabilizes L-forms by mitigating stress caused by reactive oxygen species that derives from eliminating the cell wall (Kawai *et al.*, 2015). This mutation was reconstructed *de novo* for this work. All other strains are derivatives of AG174 (Smith *et al.*, 2014). All strains that were grown in the 1x MSM supplement contained a

disruption of the sucrose metabolism gene *sacB* to prevent degradation of the supplement and enable the formation of defined L-form colony forming units (CFUs) (Wolf *et al.*, 2012). One of these alleles {*sacB*::*erm*, (Koo *et al.*, 2017)} was obtained from the Bacillus Genetic Stock Center (www.bgsc.org).

ICE*Bs1* donor strains contained *sacB*::(Pspank(hy)-*rapI mls*) and Δ(*rapI-phrI*)*342*::*kan* (Auchtung *et al.*, 2005). ICE*Bs1* was experimentally activated by inducing expression of *rapI* via the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 mM. Walled donors were also D-alanine auxotrophs (Δ*alr-ndoAI*::*cat*) to enable cleaner selection for recipients and transconjugants (Brophy *et al.*, 2018). The deletions and point mutations of *cwlT* have been described (DeWitt and Grossman, 2014). The deletions of individual ICE*Bs1* conjugation genes (*conQBCDEG*) have also been described (Berkmen *et al.*, 2010; Leonetti *et al.*, 2015).

Recipients were ICE*Bs1*^{0} and contained Δ*comC*::*spc* to prevent transformation and enable selection with spectinomycin. Recipients in the AG174 background were derived from the ICE*Bs1*^{0} JMA222 (Auchtung *et al.*, 2005). ICE*Bs1*^{0} strains in the BS115 background were generated using a strategy similar to what was previously described (Auchtung *et al.*, 2005), by transforming an ICE*Bs1*+ strain with a deletion-insertion of the gene encoding the ICE*Bs1* repressor *immR* (Δ*immR*::*mls*), passaging the cells, and patching for loss of MLS resistance. Some recipients also contained *str-84*, conferring streptomycin resistance (Lee and Grossman, 2007).

Δ*sacB*::(Pspank(hy)-*rapI mls*). We constructed a deletion-insertion allele in which codons 16-466 of *sacB* are replaced with {Pspank(hy)-*rapI* (*mls*)}. This was constructed by first replacing *spc* from pJMA31, which contains Pspank(hy)-*rapI spc* (Auchtung *et al.*, 2005), with

mls from pCAL215 (Auchtung *et al.*, 2007), creating plasmid pMMH675. The resulting {Pspank(hy)-*rapI mls*} was amplified by PCR, joined to sequences upstream and downstream of *sacB* by isothermal assembly (Gibson *et al.*, 2009), and introduced to *B. subtilis* via natural transformation selecting for MLS resistance.

ispA-D92E. The *ispA-D92E* mutant contains a missense mutation at nucleotide 276 that converts the aspartic acid to a glutamic acid residue. This mutation is needed to stabilize L-form strains, and alleviates reactive oxygen species stress which results from eliminating the cell wall (Kawai *et al.*, 2015). To construct this mutant, we used the same strategy for constructing markerless point mutants that has been previously described (DeWitt and Grossman, 2014; Jones *et al.*, 2020). Briefly, a 1 kb fragment containing the *ispA-D92E* allele was generated by isothermal assembly and inserted into the plasmid pMMH597 (a derivative of pCAL1422, containing *spc* and a constitutively expressed *lacZ*), creating plasmid pMMH598. The resulting construct was transformed into *B. subtilis* and integrated into the chromosome at *ispA* by single-crossover selecting for spectinomycin resistance. The resulting strain was repeatedly passaged and isolates were screened for loss of *lacZ* and *spc*, indicating loss of the construct from the chromosome. Individual isolates were screened for the point mutation by sequencing a PCR-amplified DNA fragment.

Δ*alr-ndoAI*::*cat*. A deletion allele was constructed that replaces the open reading frames of *alr*, *ndoAI*, and *ndoA* with *cat*. This mutation causes a requirement for D-alanine for growth in both minimal and rich media. Including this allele in ICE donor strains enables easy selection for recipients and transconjugants during mating assays (Brophy *et al.*, 2018). *ndoA* and *ndoAI* (encoding a toxin-antitoxin system) were included in the deletion out of concern that disrupting expression of the operon could result in toxicity. The allele was constructed by first using PCR

amplification and isothermal assembly to create the plasmid pJAB403 (containing *cat* joined on either end to approximately 1 kb of *alr-ndoAI-ndoA* upstream and downstream flanking homology). The plasmid was linearized and introduced to *B. subtilis* via natural transformation selecting for chloramphenicol resistance in the presence of supplemental D-alanine.

Generation of L-form *B. subtilis*

L-form *B. subtilis* were generated as previously described with minor modifications (Leaver *et al.*, 2009; Domínguez-Cuevas *et al.*, 2012). Briefly, a single colony of a walled L-form-generation strain (a BS115-derivative) was used to inoculate 3 ml of LB supplemented with chloramphenicol and 0.5% xylose. This culture was grown to exponential phase (OD600 = 0.6) at 37°C with aeration. Part (1 ml) of this culture was pelleted, washed twice with NB-MSM, and resuspended in 1 ml NB-MSM and 30 µl of the resuspension was used to inoculate 10 ml NB-MSM and incubated for 18 h at 30°C without shaking. Analysis of the culture by light microscopy revealed a mixture of L-forms and rods. 30 µl of this culture was used to inoculate either NA-MSM + PenG, or 10 ml of NB-MSM + PenG. Pure L-form cultures would arise after several days of growth at 30°C. These were then used to inoculate experimental cultures. For strains with ICE*Bs1* containing *kan*, cultures were supplemented with kanamycin at every step of L-form generation and maintenance.

We were unable to efficiently recover L-forms that had been frozen and stored in glycerol at -80°C, so a new L-form culture was generated for each independent biological replicate done during the course of this work.

ICE*Bs1* mating assays between L-form and walled bacteria

The mating assay protocol used in this work was based on previously published protocols (Auchtung *et al.*, 2005), but was modified to accommodate osmotically-sensitive L-forms. Two

days prior to mating, 150 ml cultures of L-form *B. subtilis* were started at OD600 = 0.002. Cultures of walled bacteria were started at OD600 = 0.02 on the day of the experiment. For L-form donors, ICE*Bs1* was activated by adding IPTG (1 mM) 24 h prior to harvesting for mating. For walled donors, ICE*Bs1* was activated by adding IPTG (1 mM) at OD600 = 0.2 for 1 hr prior to harvesting.

Once the culture of walled cells reached OD600 = 0.9, L-form cells and walled cells were harvested for mating. Cultures of L-form and walled *B. subtilis* were pelleted via centrifugation in a swinging-bucket centrifuge (5000 rpm for 5 min), washed twice with plain NB-MSM, and resuspended in a small volume of NB-MSM. Aliquots of concentrated L-form cultures were plated on plain LB agar and incubated overnight to ensure no walled cells had been present in the mating mixture.

Donor cells and recipient cells (2 OD600 equivalents of each) were combined to a total volume of 700 µl, gently mixed, and pipetted onto to an analytical nitrocellulose filter (Nalgene, pore size 0.22 µm). Vacuum filtration was found to decrease L-form recovery, so the liquid was instead allowed to slowly diffuse through the filter and dry for 1 hr at 30°C, at which point the filter was transferred to a 1x MSM 1.5% agar plate and allowed to mate for 3 hr at 30°C.

For mating assays between L-form donors and walled recipients, the mating mixture was resuspended in 5 ml Spizizens salts (2 g/l $(NH_4)SO_4$, 14 g/l K_2HPO_4, 6 g/l KH_2PO_4, 1 g/l Na_3 citrate-$2H_2O$, and 0.2 g/l $MgSO_4$-$7H_2O$) (Harwood and Cutting, 1990), concentrated by centrifugation (5000 rpm for 5 min), and plated on LB agar containing kanamycin and spectinomycin to select for transconjugants. Select transconjugants were validated by patching for additional recipient-specific markers and sensitivities (*amyE*+, streptomycin resistant; tetracycline and MLS sensitive). Relative mating efficiency was reported as the total number of

transconjugants per input OD600 donor equivalents, normalized to the mating efficiency of a wild type ICE*Bs1* L-form donor assayed on the same day. To estimate absolute mating efficiency (normalized to L-form donors), an aliquot of pre-mating, concentrated L-form donors was serially diluted in 1x MSM and plated on NA-MSM + Kan + PenG to quantify L-form donor CFUs.

For mating assays between walled donors and L-form recipients, the mating mixture was gently resuspended by repeatedly running 5 ml 1x MSM over the mating filter in a 50 ml conical tube, concentrated via centrifugation (5000 rpm for 5 min), serially diluted in 1x MSM, and plated on NA-MSM + PenG and NA-MSM + PenG + Kan to select for recipients and transconjugants, respectively. Relative ICE acquisition efficiency was reported as the percentage of kanamycin-resistant recipients, normalized to a wild-type ICE*Bs1* mating done on the same day.

Live Cell Microscopy

Live cell microscopy was done largely as previously described (Babic *et al.*, 2011) with modifications to the growth medium to accommodate L-forms. 1.5 μl of cultures of walled cells, L-forms, or both, were applied to an agar pad. The agar pad comprised 1.5% Noble agar (Difco) dissolved in NB and supplemented with 1x MSM. The agar pad was placed on a glass coverslip (VWR) such that the cells would be in contact with the coverslip, and the coverslip was attached to a microscope slide via a frame-seal slide chamber (Bio-Rad). The cells were observed via a Nikon Ti-E inverted microscope and with a CoolSnap HQ camera (Photometrics).

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Tables

Table 1. *B. subtilis* strains.

Strain (reference)	Genotype
AG174 (Smith *et al.*, 2014)	*trpC2 pheA1*
BS115 (Leaver *et al.*, 2009)	*trpC2 spoVD*::(*cat* Pxyl-*murE*) *amyE*::(*tet xylR*)
MMH676	AG174 ICE*Bs1*^{0} *str-84* Δ*comC*::*spc*
MMH683	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan* Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH685	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19* Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH686	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan* Δ*conQ848* Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH688	BS115 *ispA-D92E* ICE*Bs1*^{0} *sacB*::*mls* Δ*comC*::*spc*
MMH694	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A* Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH700	AG174 Δ(*rapI-phrI*)*342*::*kan* Δ*cwlT19* Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH701	AG174 Δ(*rapI-phrI*)*342*::*kan* Δ*conQ848* Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH702	AG174 Δ(*rapI-phrI*)*342*::*kan* Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH705	AG174 Δ(*rapI-phrI*)*342*::*kan cwlT-E87Q-C237A* Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH712	AG174 Δ(*rapI-phrI*)*342*::*kan conE*Δ(*88-808*) Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH713	AG174 Δ(*rapI-phrI*)*342*::*kan conC*Δ(*5-81*) Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH715	AG174 Δ(*rapI-phrI*)*342*::*kan conD*Δ(*5-131*) Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH716	AG174 Δ(*rapI-phrI*)*342*::*kan conB*Δ(*9-350*) Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH717	AG174 Δ(*rapI-phrI*)*342*::*kan conG*Δ(*5-805*) Δ*sacB*::(Pspank(hy)-*rapI mls*) Δ*alr-ndoAI*::*cat*
MMH718	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan conE*Δ(*88-808*) Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH719	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan conD*Δ(*5-131*) Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH720	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan conB*Δ(*9-350*) Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH721	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan conG*Δ(*5-805*) Δ*sacB*::(Pspank(hy)-*rapI mls*)
MMH726	BS115 *ispA-D92E* Δ(*rapI-phrI*)*342*::*kan conC*Δ(*5-81*) Δ*sacB*::(Pspank(hy)-*rapI mls*)

Appendix A

A transposon insertion screen identifies bacterial host genes with a role in the ICE*Bs1* life cycle

I designed a screen to identify *Bacillus subtilis* transposon insertion mutants with a reduced ability to donate ICE*Bs1*. I utilized a library of selectable transposon insertion mutants that had been constructed in an ICE*Bs1*-cured (ICE*Bs1*^{0}) background (Johnson and Grossman, 2014). I moved this library into an ICE*Bs1* donor strain containing a selectable copy of ICE (CMJ338). The ICE antibiotic marker (Δ(*rapI-phrI*)*342::kan*) disrupted the native *rapI* regulatory gene, and ICE*Bs1* activation was induced by ectopic overexpression of *rapI* from a xylose-inducible promoter. The result was a library of approximately 50,000 inducible ICE*Bs1* donors with non-ICE*Bs1* transposon insertions. Because most transposon insertion mutations are loss-of-function alleles, I primarily expected this screen to identify nonessential genes with non-redundant functions. However, the antibiotic resistance cassette in the transposon lacks a transcriptional terminator and can drive expression of downstream genes (Johnson *et al.*, 2020).

I screened individual mutant donors from this library for a reduced ability to transfer ICE*Bs1* to an ICE*Bs1*^{0} recipient. I grew cultures of individual mutant donors in minimal medium (1x $S7_{50}$ + 1% arabinose) (Jaacks *et al.*, 1989) to stationary phase and then mixed each donor culture 1:1 with a culture of an ICE*Bs1*^{0} recipient strain (CAL89) grown under identical conditions. After combining donor and recipient, I induced ICE*Bs1* via the addition of 1% xylose and allowed mating to proceed for 3 hours. I then plated the mating mixture on medium selective for transconjugants. I returned to the donor cultures to isolate any mutants which produced visibly fewer transconjugants than a wild type control donor, and manually confirmed that the mutants had a mating defect under more controlled experimental conditions.

I expected to isolate uninformative mutant ICE donors which either had a growth defect under the experimental conditions, or which did not produce RapI because of an insensitivity to xylose. I eliminated mutants with a growth defect by manually confirming growth under the

experimental conditions. I eliminated xylose-insensitive mutants by moving the marked transposon insertion into an ICE*Bs1* donor strain which could be induced to mate by expressing *rapI* from an IPTG-inducible promoter, and validating the mating defect phenotype. For the remaining mutants, I used inverse PCR to identify the location of the transposon insertion.

I screened 8000 mutant donors, and ultimately identified two transposon insertion mutants of interest with mild (<10-fold) mating defects. Both insertions were in the *ytr* operon (Figure 1A), which encodes a putative ATP-binding cassette (ABC) transporter (YtrBCDEF; Figure 1B), a short peptide of unknown function (YtrG), and a transcriptional repressor (YtrA) (Yoshida *et al.*, 2000). YtrA represses transcription of the *ytr* operon, and the repression is alleviated in the presence of Lipid II-binding antibiotics (Salzberg *et al.*, 2011). The function of the ABC transporter is not understood.

One transposon insertion identified in this screen disrupted the YtrA repressor binding site in the *ytr* operon promoter, and the insertion was oriented such that the antibiotic resistance cassette could drive expression of the operon (Figure 1A). The second insertion was in *ytrD*, and was also oriented co-directionally with the operon in a way that could drive expression of *ytrE* and *ytrF* (Figure 1A). The mutations in ICE*Bs1* donors caused very mild mating defects when assayed in exponential phase (Figure 1C), but appeared to cause a more significant mating defect when assayed in stationary phase (Figure 1D).

In the time since this screen was conducted, a recent non-peer-reviewed report found that overexpression of the ABC transporter YtrBCDEF causes defects in both competence and biofilm formation, and also results in a thickening of the cell wall (Benda *et al.*, 2020). The authors found that overexpression of *ytrF* (encoding the putative substrate-binding protein) and *ytrCD* (encoding the transmembrane proteins) was sufficient to cause the competence defect

(Benda *et al.*, 2020). One of the transposon insertions identified in this work is positioned to drive expression of *ytrF*, suggesting that the mechanisms by which competence and conjugation are inhibited might be similar.

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Figures and Tables

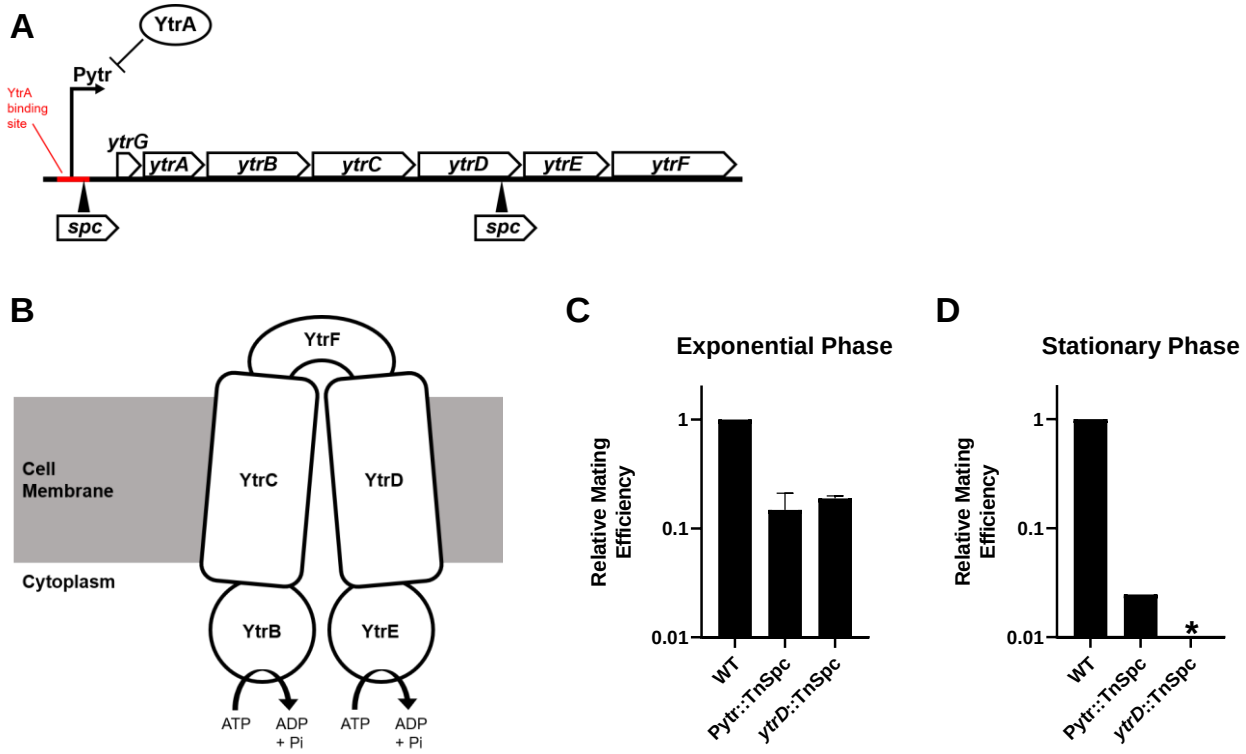

Figure 1. Transposon insertion mutations in the *ytr* operon cause an ICE*Bs1* transfer defect.

A) Genetic map of the *ytr* operon. Open pentagons represent genes, with the horizontal point indicating direction of transcription. Horizontal arrow represents Pytr promoter. Black triangles indicate sites of *spc*-marked transposon insertions in isolated mutants. Open circle represents the transcriptional repressor YtrA, which represses transcription of Pytr. Red horizontal line represents YtrA binding site.

B) Scheme of the ABC transporter encoded by *ytrBCDEF*. YtrB and YtrE are ATPases, YtrC and YtrD are transmembrane proteins, and YtrF is a putative substrate-binding protein.

C) ICE*Bs1* donors containing a transposon insertion in Pytr (MMH97) or *ytrD* (MMH101) were mated with an ICE*Bs1*^{0} recipient CAL89. Matings were done in minimal medium (1x $S7_{50}$

+ 1% arabinose) in exponential phase (OD600 = 0.9). Relative mating efficiency is reported as the number of transconjugants (kanR strepR) generated per donor, normalized to a wild type strain (CMJ338) assayed on the same day. Data reported are averages of 2-4 independent biological replicates, and error bars represent standard deviation. Mating efficiency of wild type was .045 transconjugants per donor.

D) ICE*Bs1* donors containing a *ytr* operon transposon insertion were assayed for ICE*Bs1* mating efficiency as in C, except cells were grown to stationary phase (OD600 = 2.0). * indicates mating was below limit of detection (< .008). Mating efficiency of WT was .0000057 transconjugants per donor. Data presented are preliminary results and represent one biological replicate.

Table 1. *B. subtilis* strains

Strain (reference)	Genotype
CAL89 (Auchtung *et al.*, 2005)	ICE*Bs1*^{0} *str-84 comK*::*spc*
CMJ338	*trpC2 pheA1* Δ(*rapI-phrI*)*342::kan amyE*::(Pxyl-*rapI* MLS)
MMH97	*trpC2 pheA1* Δ(*rapI-phrI*)*342::kan amyE*::(Pxyl-*rapI* MLS) Pytr::*spc*
MMH101	*trpC2 pheA1* Δ(*rapI-phrI*)*342::kan amyE*::(Pxyl-*rapI* MLS) *ytrD*::*spc*

Chapter 4

Discussion

Conjugative elements are ubiquitous in bacteria. They often facilitate transfer of genes involved in diverse processes, including antibiotic resistance, pathogenesis, and resource utilization (Johnson and Grossman, 2015; Cabezón *et al.*, 2015). Integrative and conjugative elements (ICEs) are likely the most abundant class of conjugative element in nature, and have been identified in every bacterial clade (Guglielmini *et al.*, 2011). Conjugative elements have evolved to interact with their bacterial hosts, but the nature and extent of these interactions remain poorly characterized.

Through this work, I have sought to advance our understanding of how conjugative elements interact with their bacterial hosts. In Chapter 2, I used a CRISPR interference screen to identify essential bacterial host factors necessary for efficient transfer of ICE*Bs1* to a recipient. In Chapter 3, I used wall-less L-form bacteria to clarify the target of the ICE*Bs1*-encoded cell wall hydrolase during transfer. The conclusions and implications of each project are discussed below.

Wall teichoic acids are important for efficient transfer of ICE*Bs1*

In Chapter 2, I used a CRISPR interference screen to block expression of individual essential genes and operons in *Bacillus subtilis* and identify knockdowns that cause an acute defect in ICE*Bs1* transfer. I found that wall teichoic acids (WTAs) are necessary in both ICE*Bs1* donors and recipients for efficient conjugative transfer of the element. In contrast, transfer of another small ICE (Tn*916*) is not negatively impacted by WTA depletion, indicating that the defect is specific to ICE*Bs1*.

Conducting mating assays with WTA-depleted cells on an osmo-protective surface rescues the ICE*Bs1* transfer defect. Furthermore, single-cell observations revealed that WTA-depleted cells engaging in conjugation on a non-protective surface are more likely to die. Together these

findings indicate that WTA-depleted cells do not successfully complete ICE*Bs1* transfer because they sustain lethal envelope damage. Plasmid mobilization experiments indicated that ICE*Bs1* does not need to be present or expressed in WTA-depleted recipient cells to cause the mating defect, implying that the activity of the conjugation machinery itself is sufficient to damage a recipient.

CwlT activity as a possible explanation of the WTA-depletion transfer defect

My findings from Chapter 2 suggest that one or more components of the conjugation machinery cause lethal envelope damage to WTA-depleted cells, but the results do not specifically identify said component(s). One possible explanation is that the element's cell wall hydrolase CwlT lethally damages WTA-depleted donors and recipients. The finding that CwlT acts on both the donor and recipient cell wall (Chapter 3) lends credence to this model, as both donors and recipients require WTAs to prevent conjugation-dependent envelope damage.

WTAs regulate the localization and activity of some autolysins in *B. subtilis* (Yamamoto *et al.*, 2008; Kasahara *et al.*, 2016), and they might also regulate CwlT localization in order to constrain its activity. WTAs also help maintain the proper ionic environment within the cell wall, and disrupting this environment could cause autolysins like CwlT to become hyperactive (Biswas *et al.*, 2012). WTAs could alternatively have an indirect effect on sensitivity to CwlT: WTA-depleted cells exhibit severe morphological defects and have irregularities in cell wall thickness (D'Elia *et al.*, 2006), which could make the sacculus more sensitive to remodeling during conjugation. None of these explanations are mutually exclusive.

Several experiments could be done to test these hypotheses. For example, localization studies of CwlT could reveal if the enzyme becomes mislocalized in response to WTA depletion.

Glycosylated WTAs can also be stained with a fluorescent lectin (fluorescein-labeled Concanavalin A) (Mirouze *et al.*, 2018), which could be used to determine if WTAs do or do not co-localize with either CwlT or the conjugation machinery generally. It would also be interesting to determine if WTA-depletion makes *B. subtilis* more sensitive to treatment with purified CwlT.

Although CwlT is normally essential for conjugation, it is unnecessary for transfer in the absence of either a donor or recipient cell wall (Chapter 3). This presents an opportunity to test the hypothesis that CwlT is the cause of the WTA-depletion transfer defect: if CwlT is responsible for damaging WTA-depleted cells, then WTA-depletion of a walled recipient should not affect acquisition of a Δ*cwlT* ICE from an L-form donor.

Although CwlT is the most intuitive source of the damage to WTA-depleted cells during conjugation, other possible explanations have not been ruled out. For example, WTA depletion sensitizes cells to treatment with penicillin binding protein-targeting beta-lactam antibiotics (Farha *et al.*, 2015), and ICE transfer could comparably interfere with wall synthesis in a way that is lethal to WTA-depleted cells. WTA elimination is also incompatible with lipoteichoic acid elimination (Schirner *et al.*, 2009), and ICE*Bs1* could potentially inactivate or modify lipoteichoic acids in way that harms WTA-depleted cells.

WTAs and horizontal gene transfer

WTAs have roles in horizontal gene transfer aside from the impact on ICE*Bs1* conjugation described here. For example, WTAs are critical for adsorption of many *B. subtilis*-targeting bacteriophages, some of which use glycosylated WTAs as their sole host receptor (Young, 1967; Yasbin *et al.*, 1976). A recent study has also implicated WTAs in transformation: *B. subtilis* WTAs must be modified by a putative sugar transferase to enable exogenous DNA to bind to the

surface of naturally competent cells (Mirouze *et al.*, 2018). With the findings described in Chapter 2, WTAs have now been implicated in every major mechanism of horizontal gene transfer in *B. subtilis*.

WTA biosynthesis enzymes have recently been explored as a potential antibiotic targets, and several candidate drugs that target these enzymes have been described in recent years (Farha *et al.*, 2015; Tiwari *et al.*, 2018). These drugs might also help reduce the amount of horizontal gene transfer that occurs within the targeted bacterial population.

A conjugative element-encoded cell wall hydrolase acts on both the donor and recipient cell wall

In Chapter 3, I found that wall-less (L-form) *B. subtilis* are capable of donating and receiving ICE*Bs1*, demonstrating that neither the donor nor recipient cell wall is required for conjugative transfer. I found that eliminating the cell wall of an ICE*Bs1* donor makes CwlT dispensable for transfer, indicating that CwlT normally acts on the donor cell wall. I also found that eliminating the recipient cell wall makes CwlT dispensable for transfer, indicating that CwlT normally acts on the recipient cell wall. These findings are the first experimental evidence demonstrating that a conjugative element-encoded cell wall hydrolase acts on both the donor and recipient cell wall in a mating pair. CwlT is structurally and functionally similar to other hydrolases from conjugative elements of gram-positive bacteria, including TcpG from pCW3, TraG from pIP501, and Orf14 from Tn*916* (Bantwal *et al.*, 2012; Arends *et al.*, 2013; Xu *et al.*, 2014). Based on their similarity, I speculate that many (if not all) hydrolases of gram-positive bacterial conjugation systems likewise act on the recipient cell wall in a mating pair.

Substitutes for CwlT activity

The findings from Chapter 3 suggest that CwlT is 1) not needed to navigate the donor wall in the absence of a recipient wall, and 2) not needed to navigate the recipient wall in the absence of a donor wall. How does ICE*Bs1* manage to cross the one remaining cell wall in the absence of an element-encoded cell wall hydrolase? The simplest explanation is that other cell wall hydrolases, or other naturally-occurring openings in the peptidoglycan, are capable of substituting for CwlT activity once one of the walls is no longer an obstacle. *B. subtilis* encodes dozens of cell wall hydrolases, and the sacculus is constantly subjected to controlled degradation by these enzymes (Smith *et al.*, 2000; Do *et al.*, 2020). Whereas the probability of two cells spontaneously making contact at a site with mutually unprotected membranes might be exceedingly rare under normal circumstances, such contacts could be more common when one of the two cells lacks a wall entirely.

Future experiments will focus on identifying hydrolases that can substitute for CwlT activity during ICE*Bs1* transfer to or from L-forms. *B. subtilis* encodes more than 35 cell wall hydrolases (Smith *et al.*, 2000). Many of these hydrolases can be stably deleted, and it would be interesting to determine if using walled cells with substantially reduced hydrolase activity would restore the requirement for CwlT in ICE*Bs1* transfer to or from an L-form.

Another finding from Chapter 3 is that an ICE encoding a catalytically dead CwlT can transfer efficiently from an L-form donor to a walled recipient, but cannot transfer efficiently from a walled donor to an L-form recipient. One interpretation of this result is that the ICE*Bs1* conjugation machinery needs to interact with a hydrolase to exit the donor, and that the dead enzyme blocks the interaction between the conjugation machinery and the substitute. If this model is correct, the substituting hydrolase might need to closely resemble CwlT in order to

successfully interact with the conjugation machinery. *B. subtilis* encodes four paralogs of *cwlT* (*yomI, yqbO, xkdO,* and *cwlQ*) (Sudiarta *et al.*, 2010). Three of these genes (*yomI, yqbO, xkdO*) are associated with functional or defective mobile genetic elements (Sudiarta *et al.*, 2010). The function of the fourth (*cwlQ*) is not understood, although its expression is highly correlated with flagellar protein expression (Nicolas *et al.*, 2012). It would be interesting to delete these genes in an ICE*Bs1* donor and determine if this restores the requirement for *cwlT* during transfer from a walled cell to an L-form.

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