Specificity and Benefits of an Exclusion Mechanism for a Mobile Genetic Element in *Bacillus subtilis*

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ABSTRACT

The horizontal transfer of mobile genetic elements, including Integrative and Conjugative Elements (ICEs), plays an essential role in bacterial evolution by helping to promote the spread of genes involved in antibiotic and heavy metal resistance, metabolism, symbiosis, and pathogenicity. Like conjugative plasmids, ICEs spread to new hosts by conjugative transfer through Type 4 Secretion Systems (T4SSs) encoded in the ICE DNA, however unlike plasmids, ICEs are usually found integrated into the host chromosome except for immediately prior to, during and after conjugative transfer. Almost all plasmids and some ICEs have an exclusion mechanism, which prevents acquisition of a second copy of the element via conjugative transfer. ICE*Bs1* has an exclusion mechanism in which the ICE*Bs1* exclusion protein YddJ targets the conjugation machinery protein ConG, the VirB6 homolog in the ICE*Bs1* T4SS, to prevent transfer from a would-be donor cell. My work described in this thesis involves a mutagenesis and enrichment screen which isolated exclusion-resistant, transfer-competent mutations in ConG, and swap experiments with ICE*Bs1* and ICE*Bat1* ConG and YddJ homologs demonstrating that YddJ targets its cognate ConG for exclusion, and that YddJ and ConG together determine the specificity of exclusion. I identified regions of ConG and YddJ that are essential for exclusion specificity, and found that YddJ-mediated exclusion protects donor cells from serving as recipients during or immediately after they serve as donors. These findings further our understanding of regulation of horizontal gene transfer, particularly in Gram-positive bacteria. They provide further evidence of a conserved target in exclusion, the VirB6 homologs, and indicate that different mobile genetic elements can employ exclusion systems for different reasons.

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Chapter 1

Introduction

Overview

Horizontal gene transfer is a fundamental driving force in bacterial evolution and in the spread of various phenotypes, including antibiotic resistance, alternative metabolic capabilities, and pathogenicity islands. One major form of horizontal gene transfer is the spread of mobile genetic elements, including conjugative plasmids and integrative and conjugative elements (ICEs) by conjugation, through conjugation machinery encoded by the element. This allows a plasmid or ICE to spread through a population of cells, by moving from its host (donor) into a new recipient cell (called a transconjugant after it has received the element). The process of transfer and establishment in a new host cell is regulated in different ICEs and plasmids by various mechanisms. One such mechanism, called entry exclusion, is a way for an ICE or plasmid to prevent its host cell from receiving a second identical copy of the ICE or plasmid from a would-be donor cell. This process involves a protein in the element that specifically recognizes its cognate conjugation machinery in a would-be donor cell and physically prevents DNA transfer through said machinery. In this work I further our understanding of the exclusion mechanism of ICE*Bs1*, an ICE found in the Gram-positive bacterium *Bacillus subtilis*, by identifying the target of the exclusion protein in the conjugation machinery and delineating regions in the target and exclusion protein that confer specificity of exclusion. I also demonstrate that ICE*Bs1* exclusion protects ICE*Bs1* donors from serving as recipients for transfer from new transconjugants. The specificity and viability benefits of ICE*Bs1* exclusion allow for the protection of both ICE*Bs1* and its host during critical periods of high transfer, while providing minimal disruption to the spread of genetic diversity through the conjugative transfer of other related elements.

Introduction to Horizontal Gene Transfer

Horizontal gene transfer (HGT), also called lateral gene transfer (LGT), is the movement of DNA "laterally" into a new host from the environment or a non-parent organism (Wozniak and Waldor, 2010). HGT can occur between two prokaryotes, between two eukaryotes, and between a prokaryote and a eukaryote (in either direction). Two major examples of prokaryotic to eukaryotic HGT are the emergence of mitochondria and plastids from endosymbiotic events involving α-proteobacteria and cyanobacteria, respectively (Keeling and Palmer, 2008). The opportunistic transfer of DNA from the soil-dwelling *Agrobacterium tumefaciens* to its plant host, by T4SS conjugation machinery encoded by the pTI plasmid in *A. tumefaciens*, causes crown gall disease in the plant host and is one of the most well-studied examples of conjugative DNA transfer (Lacroix and Citovsky, 2013). There are also reports of DNA integration from bacterial endosymbionts, such as *Wolbachia pipientis*, into the genomes of their hosts; in the case of *W. pipientis* hosts include insects and certain nematodes (Hotopp, 2011). Examples of eukaryotic to prokaryotic HGT include bacterial acquisition of DNA from the human genome. In 11% of *Neisseria gonorrhoeae* strains, a DNA fragment with 98–100% identity to the human L1 element was identified, which was presumably acquired via non-homologous end-joining (Anderson and Seifert, 2011). Eukaryotic horizontal gene transfer is thought to have introduced the genes *btubA* and *btubB* into *Prosthecobacter dejongeii;* these genes are co-transcribed along with a kinesin light chain gene homolog (Jenkins et al., 2002). BtubA and BtubB have higher sequence homology to eukaryotic tubulin than to the bacterial tubulin homolog FtsZ, and although they differ from eukaryotic tubulin in folding and strength of dimerization, they are structurally similar to eukaryotic tubulin and they form tubulin-like

protofilaments (Schlieper et al., 2005). Examples of eukaryotic to eukaryotic HGT are historically less well documented than prokaryotic HGT, due in part to nuclear gene duplication events that confound the search for HGT events, but there are still plenty of reported examples (Keeling and Palmer, 2008). These include examples of acquisition of new traits in fungi, such as the acquisition of *toxA*, which encodes a host-specific toxin, by *Stagonospora nodorum* from *Pyrenophora tritici-repentis* (Friesen et al., 2006). Phylogenetic analysis indicates that fHANT-AC, a cluster of three high affinity nitrate assimilation genes that are often coordinately regulated, was transferred horizontally from a lineage leading to Oomycetes to both Ascomycota and Basidiomycota (Slot and Hibbett, 2007).

The earliest and by far the best characterized types of HGT occur between bacterial species. Although amounts vary, DNA from HGT comprises over 20% of some bacterial genomes (Ochman et al., 2000). There are three traditionally recognized major types of horizontal gene transfer in bacteria: transformation, transduction, and conjugation. Transformation is the uptake of free DNA from the environment; this process requires that the cells be competent, and received DNA must be integrated into the chromosome, or re-circularized if the received DNA is a plasmid (von Wintersdorff et al., 2016). Some bacterial species such as *Neisseria* spp. are always competent, while others can have competence induced by changes in nutritional status, the presence of peptides or autoinducers, or various stressors (Johnston et al., 2014). Transduction occurs when a bacteriophage packages some of its host's chromosomal DNA into the phage capsid, and then inserts this chromosomal DNA into its new host upon entry (Frost et al., 2005). Conjugation is the transfer of DNA from a donor to a recipient cell, requiring cell-cell contact through a multi-protein complex known as the conjugation machinery, assembled at

the donor cell surface (**Fig. 1**) (Frost et al., 2005). A fourth type of bacterial horizontal gene transfer is the fusion of two bacterial cells, i.e. protoplast fusion, which is less frequent than transformation, transduction and conjugation, but can be induced in the laboratory with the addition of Polyethylene glycol (PEG) (Lévi-Meyrueis et al., 1980).

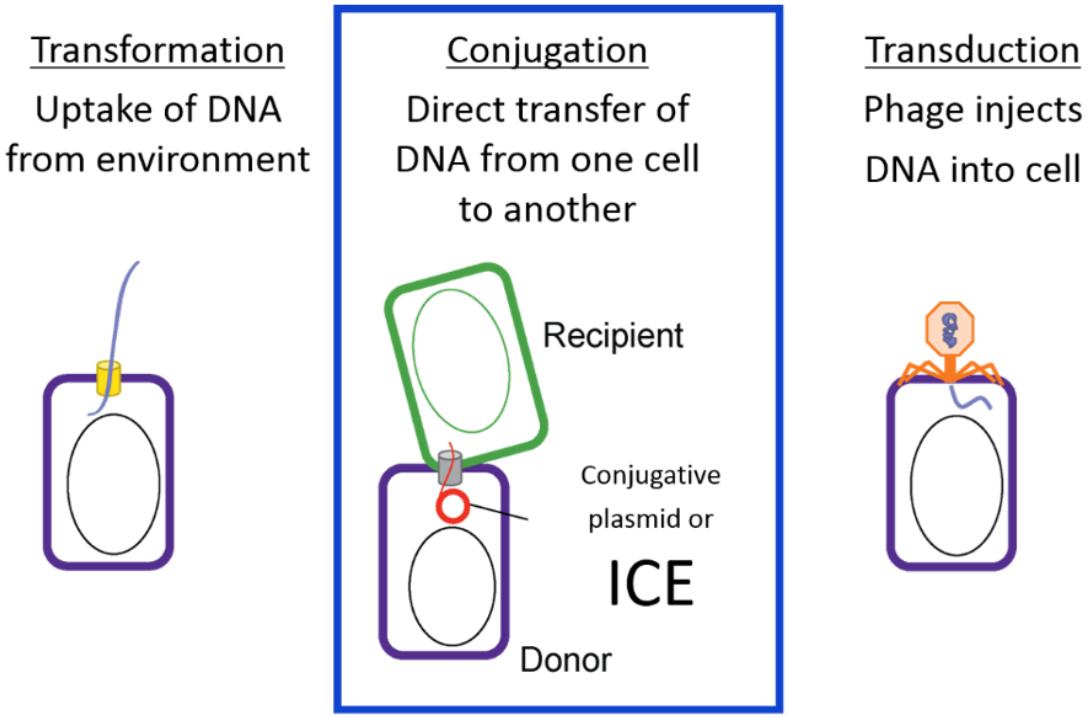

Fig. 1: The three major types of HGT in bacteria. Transformation, Conjugation and Transduction are the three major types of horizontal gene transfer that occur in bacteria. See text for more details.

Each of these three types of HGT helps drive bacterial evolution by spreading antibiotic resistance genes and other phenotypes beneficial to bacterial hosts. The spread of antibiotic resistance genes can occur through transduction and transformation, although conjugation is most often considered responsible; conjugation is a more efficient mechanism of transfer and provides better protection from the environment for the transferred DNA (von Wintersdorff et

al., 2016). Antibiotic resistance is a wide-spread problem, and of particular clinical concern is antibiotic resistance among members of the so-called ESKAPE group, consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.; as well as *Escherichia coli* (Partridge et al., 2018). *Staphylococci* can transfer resistance to gentamicin, methicillin, penicillin, and other β-lactam antibiotics through conjugation; these resistances are often found in MRSA strains (Partridge et al., 2018). *P. aeruginosa* strains are also able to transfer carbapenem resistance, in the form of a carbapenemase cassette, through conjugation (Partridge et al., 2018).

Aside from genes conferring antibiotic resistance, HGT is also responsible for the spread of genes conferring other useful phenotypes, including genes for utilizing alternative carbon sources. For example, the capability of utilizing chlorocatechols as a carbon source can be transferred from *Pseudomonas* sp. strain B13 to other strains via conjugation (Ravatn et al., 1998). *Salmonella senftenberg* (Hochhut et al., 1997) and *Lactococcus lactis* (Rauch and De Vos, 1994) can transfer by conjugation genes for sucrose fermentation. Biofilm communities found in acid mine drainages, which rely on several microorganisms to facilitate the breakdown of pyrite into an acidic solution rich in metals, are found to have extensive heterogeneity developed over short time scales in part by extensive HGT (Koechler et al., 2015).

Introduction to Conjugative Plasmids and ICEs

A large amount of HGT in bacteria is due to the movement of mobile genetic elements, which include conjugative plasmids and Integrative and Conjugative Elements (ICEs) – these elements are responsible for the majority of HGT by conjugation observed in bacteria. Plasmids

are typically extrachromosomal circular double-stranded DNA molecules that replicate independently of the host chromosome. Conjugative plasmids are generally large plasmids that exist in low copy number in their host cells, and contain MOB (mobility) genes for conjugative DNA processing, and MPF (mating pair formation) genes for DNA translocation from the donor into the recipient (Getino and de la Cruz, 2018). In terms of their mobility, plasmids can be considered conjugative, mobilizable, or non-mobilizable. Mobilizable plasmids are relatively smaller and have a higher copy number than conjugative plasmids, and while they contain MOB genes, they rely on some conjugative plasmids and ICEs for transfer into new hosts (Getino and de la Cruz, 2018). Plasmids are often classified based on incompatibility groups - incompatibility is defined as the inability for two plasmids occupying the same host to be stably inherited without the pressure of external selection (Novick, 1987). Evidence indicates that this inability is due to the two plasmids having at least one replication or partitioning system element in common, which usually results in one of the plasmids being unable to correct for fluctuations in its copy number (Novick, 1987).

Like their name suggests, ICEs are DNA molecules that are normally found integrated into their bacterial host's chromosome, and replicate passively along with the chromosome and are passed to both daughter cells during cell division. ICEs range in size from ~20 kb to >500 kb, and contain functional groups of genes from different sources – these functional groups of genes are typically clustered on the element (Johnson and Grossman, 2015). Many ICEs contain what are called cargo genes – genes that confer a beneficial phenotype to the host, such as antibiotic or heavy metal resistance, or the ability to utilize an alternative carbon source. Plenty of ICEs have been identified based on the ability of their host to transfer a phenotype of

interest to other cells (Johnson and Grossman, 2015). ICEs in this category include Tn*916*, which carries a tetracycline resistance gene (Franke and Clewell, 1981; Gawron-Burke and Clewell, 1982), and ICE*bph-sal*, which carries genes for biphenyl and salicylate metabolism in *Pseudomonas putida* (Nishi et al., 2000). Besides transfer of their cargo genes to new recipients, ICEs can increase genetic diversity in themselves as well as their hosts through several types of genetic recombination events (Johnson and Grossman, 2015). If an ICE's normal attachment site (att site) in the chromosome is occupied by another copy of that ICE or a different ICE, some ICEs are able to insert next to this already-present ICE, forming a tandem array. The ICE*clc* can form tandem arrays with itself, and these multiple copies of ICE*clc* allows its host to utilize chlorobenzene as a substrate (Ravatn et al., 1998). The related ICEs SXT and R391 can be found in tandem arrays in donor cells; these donors give rise to transconjugants containing hybrid SXT/R391 elements formed from *recA*-independent recombination between the ICEs (Burrus and Waldor, 2004). Finally, some ICEs, including Tn*916* (Naglich and Andrews, 1988) and ICE*Bs1* (Lee et al., 2012), can use their conjugation machinery to transfer certain plasmids, a process known as plasmid mobilization. SXT can mobilize plasmids (Hochhut et al., 2000) and genomic islands from *Vibrio* and related species (Daccord et al., 2010; Daccord et al., 2013; Daccord et al., 2012). CTnDOT-ERL ICEs can mobilize plasmids (Shoemaker et al., 1986; Valentine et al., 1988) as well as non-replicating Bacteroides units (NBUs), a type of genomic islands (Shoemaker et al., 1993; Stevens et al., 1992).

ICEs vary in their requirements for integration sites. Some ICEs integrate into specific sites on their host chromosome, such as the ICEs in the family SXT in *Vibrio cholera* and *Providencia alcalifaciens*, which integrate into the 5' end of the *prfC*, a gene that codes for peptide chain

release factor 3 (Hochhut et al., 2001; Hochhut and Waldor, 1999). Other ICEs are far more flexible about where on the chromosome they integrate, such as the ICE Tn*916*, which does not integrate in a site-specific manner in most hosts (Roberts and Mullany, 2009).

Under normal conditions when an ICE is integrated into its host chromosome, the ICE genes for DNA excision, processing and transfer are not expressed. However, under certain circumstances such as host cell stress, these ICE genes are expressed, and ICE is excised from the chromosome, forming a dsDNA intermediate (**Fig. 2**). ICE-encoded and host proteins recognize the oriT, nick the dsDNA, and form a ssDNA-protein complex for transfer, which is pumped from the donor into the recipient through mating pair complex formed by other ICE proteins (Johnson and Grossman, 2015). In the recipient, the ICE re-circularizes, is replicated to generate dsDNA, then is integrated into the host chromosome using an ICE-encoded recombinase (integrase) protein (Johnson and Grossman, 2015). ICEs that maintain a copy of themselves in the original donor must undergo replication to convert the ICE ssDNA still in the donor to dsDNA, so it can be reintegrated back into the chromosome (Johnson and Grossman, 2015). Many ICEs transfer linear ssDNA as described here, but ICEs from Actinomycetes transfer dsDNA by an FtsK-like ATPase, through a different conjugation system (te Poele et al., 2008). The ICEs described here, and as well as conjugative plasmids, use what is known as a type IV secretion system for conjugative transfer.

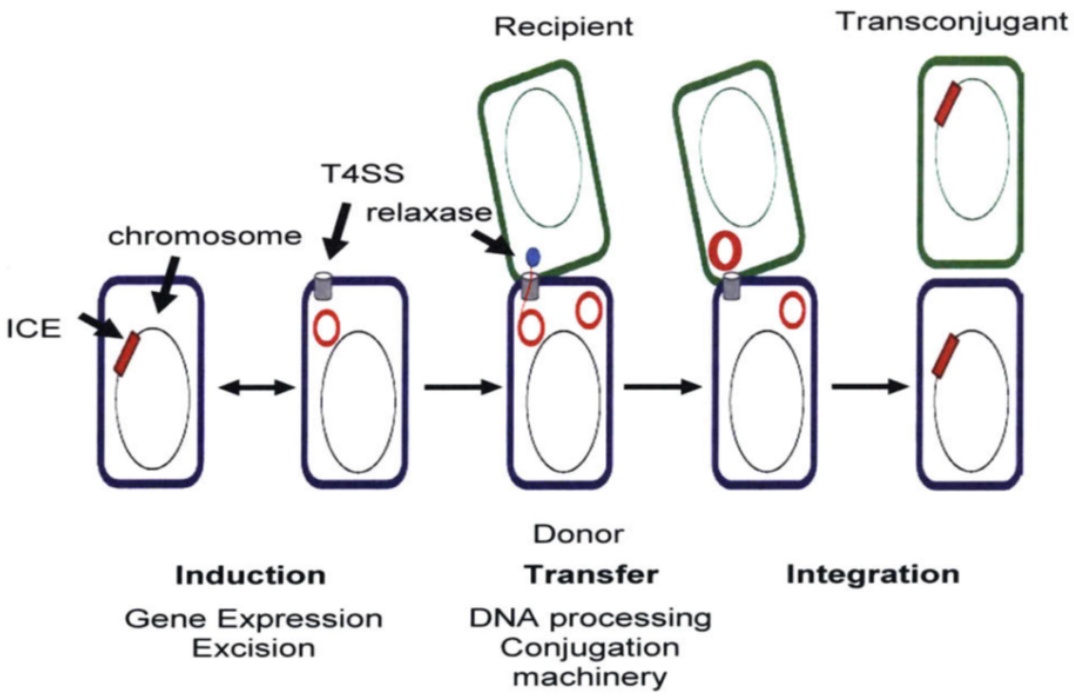

Fig. 2: ICE life cycle. Adapted from (Auchtung et al., 2016). ICEs are normally stably integrated into their host chromosome, but under certain conditions will excise from the chromosome, circularize, and transfer from the donor into a new host (recipient) through T4SS conjugation machinery encoded by the element. The ICE then integrates into the new recipient chromosome, which is now called a transconjugant. If the ICE undergoes replication in the donor, it can also reintegrate into the donor chromosome. See text for further description.

Conjugative Transfer of ICEs and Plasmids – Type IV Secretion Systems

While ICEs and plasmids can move into a new host cell in multiple ways, including transformation or transduction, conjugation is a way for ICEs and plasmids to move themselves intentionally to take advantage of a new available host. Conjugative transfer of plasmids and ICEs through an ssDNA intermediate, as described in the previous section, occurs through a Type IV Secretion System (T4SS). Besides conjugation systems in Gram-positive and Gram-negative bacteria, which transport conjugative DNA and probably one or more proteins (de la Cruz et al., 2010; Frost, 2009; Garcillán-Barcia and de la Cruz, 2008), other T4SSs include

release/uptake systems for substrate import and export (Cascales and Christie, 2003; Zechner et al., 2012), and effector translocators which transport protein effectors into the cytosols of eukaryotic cell targets for bacterial colonization (Cascales and Christie, 2003).

Despite the differences in their host cell envelope structure, T4SSs in Gram-positive and Gram-negative hosts follow the same basic four steps for conjugative transfer, and the first two steps are fairly similar in Gram-positive and Gram-negative hosts (Bhatty et al., 2013). The first step consists of a DNA relaxase and accessory factors binding and recognizing the origin of transfer (oriT) on the ICE or plasmid, forming a protein-DNA complex called the relaxosome (de la Cruz et al., 2010; Wong et al., 2012). The relaxase nicks the DNA strand for transfer (the T-strand) and stays bound with an active site tyrosine to the 5' end of the T-strand. The second step involves the relaxosome engaging with the Type IV Coupling Protein (T4CP) (Zechner et al., 2012). Characterized T4CPs have non-specific DNA binding capability, and there is evidence for ATP hydrolysis capabilities dependent on DNA binding. T4CPs are believed to form oligomers, with each monomer containing an N-terminal transmembrane region (Chen et al., 2008; Tato et al., 2007), and the C-terminal domains of the monomers together forming a central channel that DNA may pass through to cross the cytoplasmic membrane (Gomis-Rüth et al., 2001; Gomis-Rüth et al., 2004). The relaxase and accessory factors carry translocation signals, so the transfer intermediate can be translocated by the T4CP/mpf complex (César et al., 2006; Grandoso et al., 2000). In the third step, the donor engages with the recipient and forms a junction, and a signal is transmitted to start the process of substrate transfer (S. Lang et al., 2011). The fourth and final step consists of the substrate moving across the donor and recipient cell envelopes through a translocation channel made up of mating pair formation

proteins (Schröder and Lanka, 2005; Zechner et al., 2012). Relaxase leads the T-DNA in the 5'->3' direction across the mpf (César et al., 2006; Grandoso et al., 2000).

Much is known about the Gram-negative T4SSs from studies of Gram-negative conjugative plasmids including F, R388, pKM101, RP4, and especially the pTI plasmid from *Agrobacterium tumefaciens*. Because the pTI plasmid conjugation machinery, also referred to as the VirB/D system, is so well studied, homologs in other conjugative systems are often referred to by the names of their counterparts in the VirB/D system (Bhatty et al., 2013). Gram-negative conjugation machinery includes several categories of subunits – 3 ATPases, plus four additional types of subunits making up a translocon through which substrates pass through to enter the recipient cell, a core complex that houses the translocon, and additional proteins associated with the conjugative pilus that forms donor-recipient cell junctions (**Fig. 3A**). The three ATPases are the coupling protein VirD4 (described in the previous paragraph), and VirB4 and VirB11. VirB4 homologs are ATPases with predicted roles in substrate transfer and conjugation machine assembly (Bhatty et al., 2013), including assembly of the conjugative pilus (Kerr and Christie, 2010). VirB11 homologs are double-ring-shaped hexamers that are found associated with Gram-negative conjugative systems, but not with Gram-positive ones (Alvarez-Martinez and Christie, 2009). The activity of VirB11 is probably coordinated with the two other ATPases, and important early in the process of substrate transfer (Atmakuri et al., 2004). The translocon is made up of VirB3, VirB6, VirB8, and VirB2 pilin proteins, along with the ATPases (Bhatty et al., 2013). VirB3 is a small hydrophobic protein found with VirB4; VirB3 and VirB4 subunits are a single protein in some conjugation systems, suggesting that their functions are linked (Alvarez-Martinez and Christie, 2009). There are two subtypes of VirB6 homologs. The shorter VirB6

subtype, like VirB6 from the pTI plasmid, consists of homologs that are ~300 residues long, with around five transmembrane segments and a large central domain localized to the periplasmic space (Bhatty et al., 2013). The extended VirB6 subtype consists of homologs that are longer than 500 residues, with the N-terminal half having 5-8 transmembrane segments, and the C-terminal half having a large hydrophilic domain (Alvarez-Martinez and Christie, 2009). In the SXT ICE (J. Marrero and Waldor, 2007) and F-plasmid (Audette et al., 2007) VirB6 homologs, there is evidence that this C-terminal region may protrude from the donor cell surface, and possibly interact with the recipient cell. VirB8 has a short N-terminal domain, a transmembrane domain, and a large C-terminal periplasmic domain (Baron, 2006). VirB8 proteins are all globular proteins with extended beta-sheets and five alpha helices, packed as dimers or trimers in the VirB8 homologs that have been crystallized so far (Bailey et al., 2006; Porter et al., 2012; Terradot et al., 2005). Mutational analysis suggests that this oligomerization is important for VirB8 function (Paschos et al., 2006).

The core complex, which houses the Gram-negative translocon, is made up of VirB7, VirB9, and VirB10. Cryoelectron microscopy at 15 angstrom resolution of the pKM101 transfer system shows the core complex to be a 1MDa barrel that is 185 Angstroms tall and 185 Angstroms wide, composed of 14 copies of VirB7, VirB9, and VirB10 (Fronzes et al., 2009). The core complex is divided into two subsections, one closest to the cytoplasmic membrane and one closest to the outer membrane (Fronzes et al., 2009). VirB7 is a small outer membrane lipoprotein, and VirB9 is a periplasmic subunit connected with VirB10. VirB10 has a small cytoplasmic domain, a transmembrane segment, and a large periplasmic domain consisting of a coiled-coiled or praline-rich domain and a large globular C- terminal domain, part of which

spans the outer membrane, so that VirB10 reaches across the entire cell envelope (Jakubowski et al., 2009). Last but not least, there are three pilus-associated proteins – the VirB2 pilin proteins (also associated with the translocon, as described above), VirB5, and VirB1. VirB2 and VirB5 are required for substrate transfer, although their roles are not well understood (Bhatty et al., 2013). VirB1 is a cell wall hydrolase that is dispensable for conjugative transfer in Gram-negative systems (Berger and Christie, 1994), but essential for conjugative pilus biogenesis, along with VirB2 and VirB5 (Fullner et al., 1996). The conjugative pilus serves to connect the donor and recipient cells for the establishment of a cell-cell junction, but doesn't have a role in actual transfer of the conjugative DNA. It is not clear whether the conjugative pilus and translocation channel are physically linked or separate organelles, though some experimental evidence suggests that they are linked (Bhatty et al., 2013).

Thorough investigations have been made concerning Gram-positive plasmid and ICE-encoded relaxases (Alvarez-Martinez and Christie, 2009; Grohmann et al., 2003), as well as relaxase and coupling protein interactions (Abajy et al., 2007; Chen et al., 2008). Results from these investigations indicate that initial stages of substrate processing and substrate-T4CP docking reactions in Gram-positive conjugation systems are very similar to those in Gram-negative systems (Bhatty et al., 2013). Furthermore, substrate transfer across the plasma membrane is probably similar in Gram-positive and Gram-negative conjugation systems, as most Gram-positive conjugation systems have homologs to the VirD4 coupling protein, VirB1, VirB3, VirB4, VirB6, and VirB8 (Bhatty et al., 2013) (**Fig. 3B**). Interestingly, the cell wall hydrolase VirB1 homologs are required for transfer in Gram-positive systems, unlike in Gram-negative systems (Bhatty et al., 2013). Gram-positive systems do not have homologs for VirB7, VirB9, or VirB10,

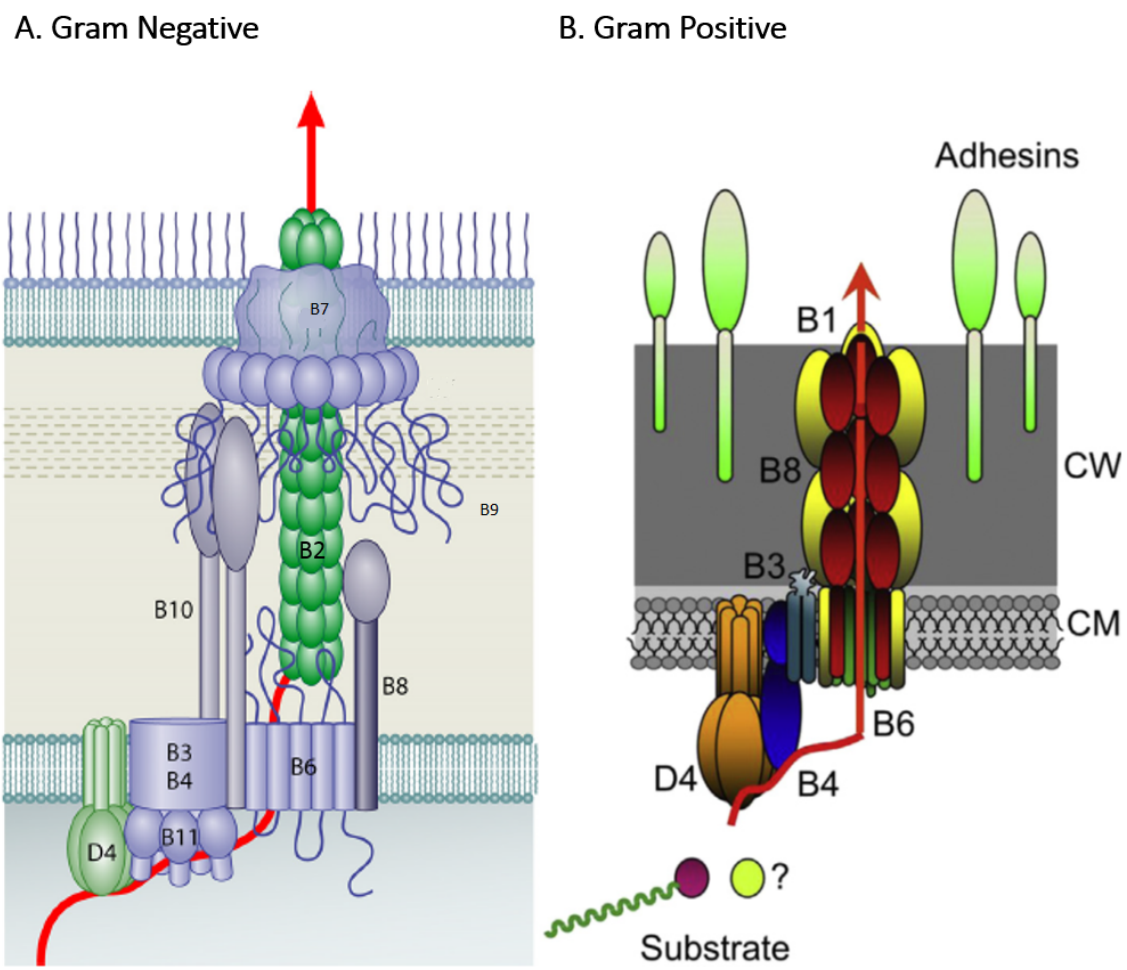

Fig. 3: Gram-negative and Gram-positive T4SS conjugation machinery. Models of the components and path of substrate transfer for Gram-negative and Gram-positive T4SS conjugation systems. **(A)** The Gram-negative conjugation machinery consists of a translocon made up of 3 ATPases (VirB4, VirB11 and the coupling protein VirD4) and 3 transmembrane proteins (VirB3, VirB6 and VirB8) and VirB2 pilin proteins, housed within a core complex consisting of VirB7, VirB9, and VirB10 subunits. (Adapted from (Alvarez-Martinez and Christie, 2009)). **(B)** The Gram-positive conjugation machinery includes homologs of the coupling protein VirD4, the ATPase VirB4, the transmembrane proteins VirB3, VirB6 and VirB8, and also is dependent on the action of cell wall hydrolase VirB1 homologs (whereas these are dispensable for Gram-negative conjugative transfer). Some Gram-positive systems also include adhesions to mediate donor-recipient cell junctions (Adapted from (Bhatty et al., 2013)).

and thus do not have a core complex, and no evidence for a conjugative pilus has been found in any Gram-positive systems (Bhatty et al., 2013). Instead, some Gram-positive systems have known or predicted adhesion proteins, such as PrgB and possibly PrgC and PgrA from the

conjugative plasmid pCF10, and TraO from the conjugative plasmid pIP501, which may mediate donor-recipient cell contacts (Alvarez-Martinez and Christie, 2009). In the Gram-positive ICEs Tn916 and ICE*Bs1*, it has been suggested that the C-terminal domains of the extended VirB6 homologs in these systems (Or15 and ConG, respectively) could play roles in mediating donor-recipient cell contacts (Bhatty et al., 2013).

Regulation of HGT in ICEs and Plasmids

Conjugative transfer of plasmids is regulated by a variety of processes, depending on the identity of the plasmid or ICE, its host cell and the host cell conditions and environment. Conjugative transfer of Rhizobial plasmids, including the pTi plasmid of *A. tumefaciens* (Lang and Faure, 2014), and the symbiotic plasmids of *Rhizobium leguminosarum* bv *viciae* 8401 (Danino et al., 2003; McAnulla et al., 2007) and *Sinorhizobium fredii* NBR234 (He et al., 2003), are controlled by a quorum sensing mechanism in which a signal molecule (usually homoserine-lactone) produced by the cells accumulates in high density conditions, then diffuses back into the cell and forms a complex with transcriptional regulators, to control expression of plasmid genes required for conjugative transfer. In some cases, sub-inhibitory concentrations of multiple antibiotics can induce conjugative plasmid transfer. One example is the transfer of plasmids PRK2013, pSU2007, and RP4 from *E. coli* DH5α to *E. coli* HB101, which is increased with low concentrations of Kanamycin and Streptomycin (Zhang et al., 2013). Another example, although the mechanism is not completely understood, is the increase in conjugative transfer in biofilms when those biofilms are exposed to antibiotics to which some of the bacteria in the biofilm are resistant (Bañuelos-Vazquez et al., 2017; Ma and Bryers, 2013).

Evidence (increase in transcription of genes involved in conjugation) suggests an increase in transfer of conjugative plasmids pMOL28 and pMOL30 (which each confer resistance to different heavy metals) in the presence of high metal concentrations - these plasmids are found in *Cupriavidus metallidurans* CH34 (Bañuelos-Vazquez et al., 2017; Monchy et al., 2007). An increase in 2,4-dichlorophenoxyacetic acid concentration increases the number of transconjugants in soil mating experiments with plasmids pEMT1 and pEMT3, both of which carry genes for degradation of this compound, so it can be used as an alternative carbon source.

Like for conjugative plasmids, ICE conjugative transfer is controlled by a variety of different mechanisms depending on the type of ICE, but a few common mechanisms of control are quorum sensing, stress-response, and the presence of certain antimicrobials, usually the element in question includes resistance genes for these antimicrobials (Bañuelos-Vazquez et al., 2017). ICE*Ml*Sym^{R7A} transfer is regulated by homoserine lactone-based quorum sensing, which controls the expression of ICE*Ml*Sym^{R7A} genes *msi171* and *msi172* and leads to increased transfer at high cellular density (Ramsay et al., 2009; Ramsay et al., 2006). The presence of tetracycline causes an increase in conjugative transfer of Tn916 (Showsh and Andrews, 1992), Tn925 (Torres et al., 1991), and CTnDOT (Cheng et al., 2001; Sutanto et al., 2004; Whittle et al., 2001; Whittle et al., 2002; Wood and Gardner, 2015); Tn916 and CTnDOT both carry tetracycline resistance genes. The transfer of the ICEs SXT-R391 (Wozniak and Waldor, 2010) and ICE*Bs1* (see Introduction to ICE*Bs1*) (Auchtung et al., 2016), are both regulated by RecA as part of an SOS DNA damage response.

Exclusion

One additional form of conjugative transfer regulation found in almost all conjugative plasmids and some ICEs is called exclusion. Exclusion is a way for a mobile genetic element to prevent its host cell from receiving a second copy of the element through conjugative transfer. There are two categories of exclusion that function at different points during the process of conjugative DNA transfer. Surface exclusion, a feature of the F-plasmid and related plasmids, is mediated by the F-plasmid protein TraT and works by preventing the formation of stable mating aggregates which are necessary for conjugative transfer, although the exact mechanism remains unknown (Achtman et al., 1977; Garcillán-Barcia and de la Cruz, 2008). Entry exclusion, or hereafter just referred to as exclusion, prevents conjugative DNA transfer by inhibiting DNA transfer through its cognate conjugation machinery from a would-be donor cell (Garcillán-Barcia and de la Cruz, 2008). In the F-plasmid and related plasmids, entry exclusion is mediated by the F-plasmid protein TraS (Achtman et al., 1977), which recognizes its target TraG in the F-plasmid conjugation machinery to prevent conjugative transfer (Anthony et al., 1999; Audette et al., 2007). Different elements vary considerably in the nature of their exclusion proteins, with limited homology among exclusion proteins from different elements (Garcillán-Barcia and de la Cruz, 2008).

However, there are some thematic similarities among different exclusion systems. In general, exclusion is mediated by a single protein encoded by the element, that is localized to the plasma membrane of the host cell, where it is in position to inhibit cognate conjugation machinery (Garcillán-Barcia and de la Cruz, 2008). For the exclusion protein TrbK of the plasmid

RP4 (Haase et al., 1996), and possibly also the exclusion proteins (both called Eex) of the plasmids pKM101 and R388 (Pohlman et al., 1994), this localization is accomplished by lipoprotein modification at a cysteine residue near the N-terminus of the exclusion protein. In other cases, such as for TraS of the F-plasmid (Achtman et al., 1979), and EexA and EexB of the R27 plasmid (Gunton et al., 2008), there is no evidence for lipoprotein modification, but the exclusion protein is predicted to contain one or more transmembrane segments (Garcillán-Barcia and de la Cruz, 2008). Exclusion proteins almost always function in the recipient – this has been clearly demonstrated for the exclusion protein Eex of the SXT/R391 family of ICEs (Marrero and Waldor, 2005) and the exclusion protein TrbK of the RP4 plasmid (Haase et al., 1996; Haase et al., 1995), among others. Furthermore, with the entry and surface exclusion genes TraS and TraT in the F-plasmid (Achtman et al., 1977; Skurray et al., 1976) and with the exclusion protein Eex of the pKM101 plasmid (Pohlman et al., 1994), exclusion has been shown to be dose-dependent, with higher levels of exclusion seen with more expression of the exclusion proteins. Exclusion proteins are often not required in the donor for conjugative transfer, as is seen with TraS in the F-plasmid (Achtman et al., 1980). Thus, despite the trend of limited sequence conservation among exclusion proteins from different families of mobile genetic elements, structural and functional trends among exclusion proteins – membrane localization, dose-dependent effect, and function in the recipient – reflect their shared role of preventing conjugative DNA transfer from a would-be donor cell.

The presence of at least one exclusion system is considered a standard characteristic of conjugative plasmids, however this is not the case for integrative and conjugative elements. In fact, various lines of evidence indicate that the presence of one or more exclusion systems is

essential for the stability of a conjugative plasmid (Garcillán-Barcia and de la Cruz, 2008). For example, exclusion-deficient F-plasmid mutants could not be isolated by chemical mutagenesis (Achtman et al., 1977; Achtman et al., 1980). In fact, pMA373, a sex pheromone plasmid, is the only conjugative plasmid reported without an exclusion gene (de Boever and Clewell, 2001). While exclusion has been documented in some ICEs, such as the Gram-negative ICE SXT/R391 family (J. Marrero and Waldor, 2005), plenty of ICEs do not show any exclusion activity. No exclusion has been documented in ICEs from low GC Gram-positive bacteria; this might be a way to increase diversity by setting the stage for intracellular transposition to generate large multidrug resistance composite transposons (Ayoubi et al., 1991; McDougal et al., 1998; Rice and Carias, 1998).

Exclusion has been shown and postulated to have a few key benefits for the elements that encode it, as well as the elements' host bacteria. One demonstrated benefit is that, in conjugative plasmids, exclusion confers protection from lethal zygosis by preventing redundant conjugative transfer events. First characterized with F-plasmids, lethal zygosis is the phenomenon in which many F- recipients die when they are mated with an excess amount of Hfr donors (Alfoldi et al., 1957). Pili-mediated contact between donor and recipient cell during conjugation causes the lethality (Clowes, 1963; Gross, 1963; Skurray and Reeves, 1973), and DNA transfer, i.e. the process of conjugation, is responsible for some but not all of the killing during lethal zygosis (Ou, 1980). Evidence indicates that increased permeability of the recipient cell membrane due to excessive conjugation is the major cause of recipient cell death in lethal zygosis (Ou, 1980; Viljanen, 1987). Another benefit of exclusion is that by preventing host occupancy by multiple copies of the same element, it could prevent harmful recombination

events that result in non-functional versions of the element (Garcillán-Barcia and de la Cruz, 2008). Finally, a mathematical model suggests that a type of conjugative plasmids with an exclusion mechanism can make headway into a population of bacteria that harbor a population of incompatible conjugative plasmids that lack an exclusion system (Nelly, 1985). Other models predict that incompatible conjugative plasmids that are unable to exclude each other must develop different survival strategies if they are to coexist within a bacterial population (N Hoeven, 1986; Nelly van der Hoeven, 1984).

There are two conjugative plasmids with exclusion systems for which the exclusion protein's target in the conjugation machinery has been identified. These are the F/R100 family of plasmids (Anthony et al., 1999; Audette et al., 2007) and the R64/R621a family of plasmids (Sakuma et al., 2013). In the F/R100 family of plasmids, the exclusion protein TraS is a small hydrophobic protein, except for a hydrophilic region in residues 10-24 (Jalajakumari et al., 1987). It is localized to the inner membrane, and shows no evidence of a signal sequence or lipoprotein modification (Achtman et al., 1979), but it does have 3 or 4 predicted transmembrane domains (depending on the computational program used) (Garcillán-Barcia and de la Cruz, 2008). The target of TraS in the conjugation machinery is TraG (Anthony et al., 1999; Audette et al., 2007). The N-terminal portion of TraG is homologous to the large periplasmic region of VirB6, while the C-terminal portion is believed to be in the periplasm, where it interacts with TraN to stabilize mating pairs (Firth and Skurray, 1992). Identification of TraG as the target was done using swap experiments with the R100-1 TraG homolog (Anthony et al., 1999), and chimeras of the two were made to narrow exclusion specificity to residues 610-673 on the TraG homolog from the F-plasmid, part of the protein which is predicted to be in the

periplasm (Audette et al., 2007). It has been predicted that TraG may be sent to the recipient cell to initiate transfer at the recipient inner membrane, where exclusion would take place when TraG comes into contact with TraS (Audette et al., 2007).

In the IncI1 plasmid R64 the gene responsible for entry exclusion, *excAB*, codes for two proteins: the protein ExcA which is essential for exclusion and localizes to both the inner membrane and the cytoplasm, and the protein ExcB which is likely an inner membrane protein (Furuya and Komano, 1994; Hartskeerl et al., 1985a; Hartskeerl et al., 1985b; Hartskeerl et al., 1986). On the other hand, the IncI*γ* plasmid R621a *excA* gene only encodes one protein, ExcA. The R64 ExcA and R621a ExcA show no shared sequence identity in the N-terminal region of the protein (60 residues for R64 ExcA, 44 residues for R621a ExcA), 39% identity in the middle 120 residues, and 95% identity in the final C-terminal 40 residues (Sakuma et al., 2013). R64 and R621a exclude each other (Takahashi et al., 2011), and for both plasmids ExcA's target in the conjugation machinery is TraY (Sakuma et al., 2013). Both the R64 TraY and R621 TraY proteins are 745 residues, with the majority of the two proteins sharing >86% sequence identity (Sakuma et al., 2013). However, the homologs have an internal variable segment with 49% shared sequence identity and a C-terminal variable segment with 41% sequence identity (Sakuma et al., 2013). The R64 ExcA protein recognizes the internal variable segment of the R64 TraY protein, and the R621a ExcA protein recognizes the C-terminal variable segment of the R621a TraY protein (Sakuma et al., 2013). Possibly unique among exclusion systems, the R27 plasmid has an exclusion system in which the exclusion proteins EexA in the cytoplasmic membrane and EexB in the outer membrane function in both the donor and the recipient (Gunton et al., 2008). It may be that recognition of the exclusion proteins in the donor by the

exclusion proteins in the recipient is sufficient to prevent conjugative transfer, and there is no other target of the exclusion proteins in the conjugation machinery itself.

There is also one family of ICEs, the SXT/R391 family, for which an exclusion protein and its target within the conjugation machinery have been identified (Marrero and Waldor, 2005). In the SXT/R391 family, the exclusion protein Eex is sufficient for exclusion in the recipient, and is localized to the inner membrane (Marrero and Waldor, 2005). Its target in the conjugation machinery is TraG, which is localized to the inner membrane of the donor cell (Marrero and Waldor, 2005). SXT ICEs and R391 ICEs do not exclude each other, despite good sequence conservation between the elements; swaps and chimeric proteins from ICEs from the two families were used to identify regions key for specificity of exclusion on TraG (residues 606-608), and on Eex (residues 121-132 of R391 EeXs and residues 121-137 on SXT EeXs) (Marrero and Waldor, 2007). Interestingly, TraG and Eex residues involved in exclusion are predicted to be in the cytoplasm (Marrero and Waldor, 2007), leading to theories including membrane inversion and protein translocation to explain how TraG and Eex could interact.

As indicated earlier, no exclusion system has been reported in a Gram-positive ICE, until now. I am studying exclusion identified in an ICE called ICE*Bs1*, found in the Gram-positive soil-dwelling bacterium *Bacillus subtilis*.

Introduction to ICE*Bs1*

ICE*Bs1* (**Fig. 4**) is an approximately 20 kb ICE containing 25 open reading frames (**Table 1**) that was identified less than 20 years ago in the Gram-positive soil-dwelling bacterium *Bacillus subtilis* (Auchtung et al., 2005; Burrus et al., 2002). ICE*Bs1* is an excellent model for studying

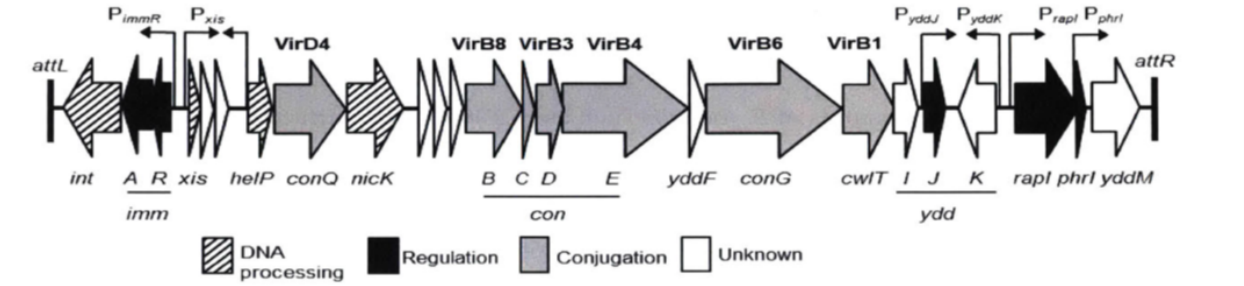

Fig. 4: ICE*Bs1* genetic map. Genes are shown as horizontal arrows indicating the direction of transcription. Genes are categorized as functioning in DNA processing (diagonal stripes), conjugation (solid gray), or regulation (black), or are shown as white if their function is unknown. The VirB/D homolog (or analog) names are listed above those genes with roles in conjugation. Vertical arrows (with the arrowhead indicating transcription direction) mark the positions of the promoters for *immR, xis, yddI, yddK, rapI, phr,* and an uncharacterized small antisense RNA. The 60 bp *att* repeats marking the ends of the element are shown as black boxes (Auchtung et al., 2016). See **Table 1** for a detailed list of ICE*Bs1* gene functions.

ICEs in Gram-positive organisms due to the convenience of genetic manipulations in *B. subtilis*, the relatively small size of ICE*Bs1*, and the high rates of conjugative transfer which can be induced easily in the laboratory (Auchtung et al., 2016). The host range of ICE*Bs1* has not been fully determined, but it includes *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus anthracis*, and *Listeria monocytogenes* (Auchtung et al., 2005). An engineered *B. subtilis* donor strain, in which inducible expression of ICE*Bs1* conjugation machinery drives transfer of a miniaturized ICE*Bs1*, is capable of transferring into undomesticated human gut, skin and soil bacterial species from the genera *Bacillus*, *Enterococcus*, *Lysinibacillus*, *Oceanobacillus*, *Paenibacillus, Rummeliibacillus*, *Sporosarcina*, *Streptococcus* and *Staphylococcus* (Brophy et al., 2018). ICE*Bs1* genes are homologous to genes from other ICEs, and its conjugation machinery is homologous to the conjugation machinery characterized in the Gram-positive conjugative plasmids pCW3 of *Clostridium perfringens*, pCF10 of *E. faecalis*, and pIP501 of *Streptococcus agalactiae* (Alvarez-Martinez and Christie, 2009; Bhatty et al., 2013). There have been no published reports of any

Table 1: ICE*Bs1* genes and functions.

ORF	Function	References
int	Site-specific recombinase (integration and excision)	(Lee et al., 2007)
immA	Anti-repressor protease (ImmR degradation)	(Bose et al., 2008; Bose and Grossman, 2011)
immR	Transcriptional repressor; Immunity repressor (Int inhibition)	(Auchtung et al., 2007)
xis	Recombination-directionality-factor (promotes Int towards excision)	(Lee et al., 2007)
ydzL	Unknown	
ydcO	Unknown	
helP	Helicase processivity factor (unwinding of DNA for replication/transfer)	(Thomas et al., 2013)
conQ	Coupling protein ATPase (VirD4-like)	(Lee et al., 2012)
nicK	Relaxase (nicks DNA at oriT for replication/transfer)	(Lee and Grossman, 2007)
ydcS	Unknown	
ydcT	Unknown	
yddA	Unknown	
conB	Membrane channel (VirB8-like)	(Leonetti et al., 2015)
conC	Membrane channel	(Leonetti et al., 2015)
conD	Membrane channel (VirB3-like)	(Berkmen et al., 2010; Leonetti et al., 2015)
conE	ATPase (VirB4-like)	(Berkmen et al., 2010; Leonetti et al., 2015)
yddF	Unknown; Mild effect on mobilization	(Leonetti et al., 2015)
conG	Membrane channel (VirB6-like); Exclusion target	(Avello et al., 2019; Leonetti et al., 2015), this thesis
cwlT	Cell wall hydrolase (VirB1-like)	(DeWitt and Grossman, 2014; Fukushima et al., 2008)
yddI	Unknown	(Leonetti et al., 2015)
yddJ	Exclusion protein (Inhibits transfer through ICE*Bs1* T4SS)	(Avello et al., 2019), this thesis
yddK	Unknown	
rapI	Cell-cell signaling (Activates ImmA)	(Auchtung et al., 2005; Bose et al., 2008)
phrI	Cell-cell signaling (Inhibits RapI)	(Auchtung et al., 2005)
yddM	Unknown	

ICE*Bs1* genes providing any advantage or benefit for the host (Auchtung et al., 2016). ICE*Bs1* can mobilize plasmids lacking dedicated mobilization functions (Lee et al., 2012), which suggests a way for ICE*Bs1* to further contribute to dissemination of genetic material (Auchtung et al., 2016).

Typically, ICE*Bs1* remains integrated into a leucine-tRNA gene (*trnS-leu2*) in the *B. subtilis* chromosome (Auchtung et al., 2005). There are two main conditions which will cause ICE*Bs1* to excise from its host chromosome and transfer to a new host cell. The first condition is DNA damage to the host chromosome; this leads to activation of the SOS response, whose key mediator RecA (Marrero and Yasbin, 1988) promotes the activity of the ICE*Bs1* anti-repressor protein ImmA, a site-specific protease which cleaves the ICE*Bs1* immunity repressor ImmR, causing its degradation and preventing it from repressing transcription from the ICE*Bs1* P*xis* promoter (Auchtung et al., 2005; Bose et al., 2008; Bose and Grossman, 2011) (**Fig. 5**). The second condition is a high concentration of ICE0 potential recipient cells. ICE*Bs1* excision is induced by high cell density but repressed if surrounding cells already contain ICE*Bs1* and thus are unsuitable ICE*Bs1* recipients. High cell density leads to a decrease in AbrB-mediated inhibition of *rapI* transcription (Phillips and Strauch, 2002), and the increasing RapI levels can then promote ImmA-mediated degradation of ImmR (Bose et al., 2008). However, high density of ICE*Bs1*-containing cells leads to an increase in extracellular concentrations of the ICE*Bs1* peptide PhrI, which is translated as a pre-pro-PhrI peptide, probably secreted by host machinery and cleaved by host-encoded proteases, and then released from ICE*Bs1* host cells (Auchtung et al., 2005; Lazazzera, 2001; Pottathil and Lazazzera, 2003). It is then imported through the oligopeptide permease Opp into would-be ICE*Bs1* donor cells, where the mature

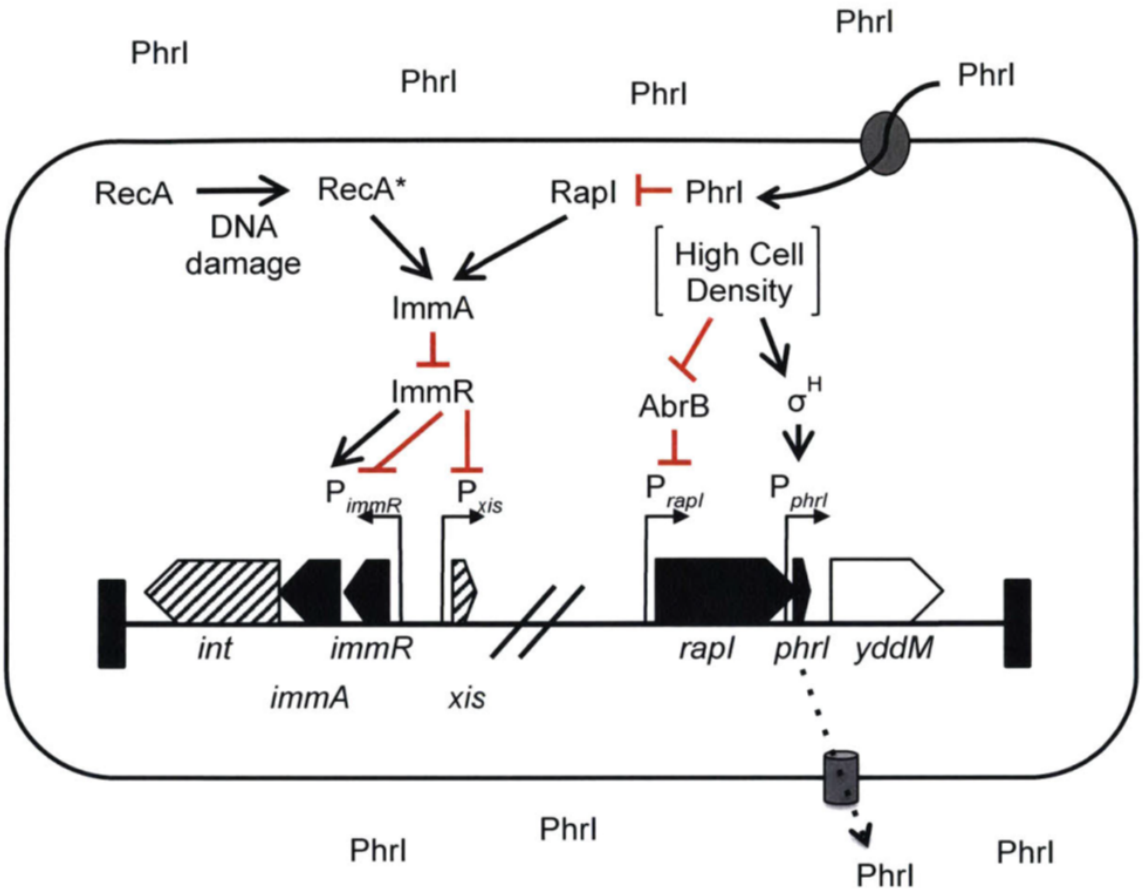

Fig. 5: ICE*Bs1* regulation. Diagram of an ICE*Bs1* donor cell, with two paths for de-repression of Pxis and subsequent ICE*Bs1* gene expression. See the text for a description of ICE*Bs1* regulation. An abbreviated diagram of ICE*Bs1* is shown with horizontal arrows indicating genes, either black for regulation, striped for DNA processing, or white for unknown function. Vertical arrows indicate promoters, with the arrowheads denoting the direction of transcription. In the genetic pathway, inhibition is denoted with red bars, while black arrows indicate activation. Figure adapted from (Auchtung et al., 2016).

PhrI inhibits RapI and thus prevents ICE*Bs1* excision and transfer in a high density of ICE*Bs1*-containing cells (Auchtung et al., 2005).

In either the condition of DNA damage or the condition of high density of ICE0 cells, transcription from the ICE*Bs1* P*xis* promoter allows for expression of Xis, and Xis along with another ICE*Bs1* protein Int are necessary and sufficient for excision of ICE*Bs1* from the

chromosome (Lee et al., 2007). ICE*Bs1* excision generates a double-stranded circular ICE*Bs1* intermediate, and the repaired chromosome (Lee et al., 2007). This intermediate is nicked at its origin of Transfer (oriT) by the ICE*Bs1*-encoded relaxase NicK in a site-specific manner, and like other homologous relaxases NicK probably attaches to the 5' end of the nicked DNA by a phosphotyrosol linkage (Lee and Grossman, 2007). The NicK-DNA complex, called the relaxosome, is delivered by the ICE*Bs1* T4CP ConQ (Lee et al., 2012) to the translocon, which likely consists of 4 ICE*Bs1* proteins: ConB, ConC, ConD, and ConG (Leonetti et al., 2015) (**Fig. 6**).

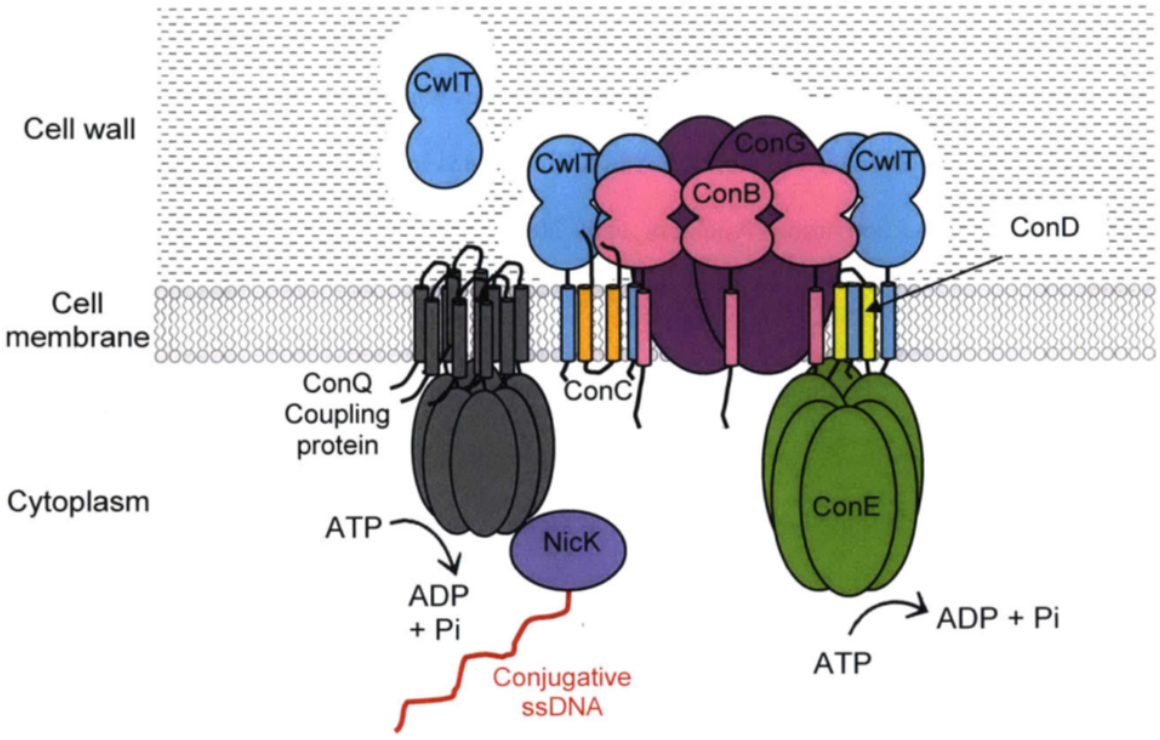

Fig. 6: Model of the ICE*Bs1* conjugation machinery. Hypothetical model of the ICE*Bs1* conjugation machinery, with protein arrangements based on information gathered for homologous conjugation systems. Adapted from (Auchtung et al., 2016). See the text for a description of conjugative transfer and conjugation machinery components of ICE*Bs1*.

ConB, a 354 residue protein with one predicted transmembrane segment (Leonetti et al., 2015), is homologous to structural analogs of VirB8 (Goessweiner-Mohr et al., 2013; Porter et al., 2012). ConC is 82 residues with two predicted transmembrane segments (Leonetti et al., 2015), and it does not have a homolog in the VirB/D system and may be specific to Gram-positive conjugation systems (Alvarez-Martinez and Christie, 2009). ConD is a 174 residue VirB3 homolog with 2 predicted transmembrane segments (Leonetti et al., 2015). ConG, an 815-residue VirB6 homolog, has 7 predicted transmembrane domains in the N-terminal half of the protein, with a large predicted extracellular C-terminal domain (Leonetti et al., 2015). Besides these transmembrane proteins, conjugative transfer of ICE*Bs1* also requires two other ICE*Bs1* proteins: VirB1 homolog CwlT, a bifunctional cell wall hydrolase with a muramidase and a peptidase domain (DeWitt and Grossman, 2014), and the VirB4 homolog ATPase ConE (Leonetti et al., 2015).

ICE*Bs1* has multiple mechanisms for preventing its host cell from acquiring a second copy of the element, and these mechanisms work at different steps of the conjugative transfer process. The first mechanism is the *rapI-phrI* signaling system. As mentioned earlier in this section, high density of ICE*Bs1*-containing cells leads to the generation of PhrI, which inhibits RapI in a would-be donor cell and thus prevents de-repression of ICE*Bs1* gene expression (Auchtung et al., 2005). This prevents ICE*Bs1*-containing cells from serving as donors when they are surrounded by other cells that already contain ICE*Bs1*. The second mechanism is an immunity system, mediated by the ICE*Bs1* protein ImmR. Early experiments demonstrated that transfer of ICE*Bs1* to ICE0 recipients is ~500-fold higher than transfer to recipients already containing ICE*Bs1* (Auchtung et al., 2007; Auchtung et al., 2005). This inhibition is partly mediated by the

expression of ImmR in the recipient (Auchtung et al., 2007), which prevents stable integration of the element by reducing the activity or production of Int, which is needed for stable integration. However, this ImmR-mediated immunity – that is, expression of ImmR in the recipient – did not fully replicate the effect of having ICE*Bs1* in the recipient, suggesting a third mechanism was preventing ICE*Bs1* host cells from acquiring a second copy of ICE*Bs1* via conjugative transfer (Auchtung et al., 2016).

This third mechanism was identified as exclusion by Monika Avello. Chapter 2 includes her work demonstrating that ICE*Bs1* has an exclusion mechanism, in which the ICE*Bs1* protein YddJ is necessary and sufficient for exclusion, and that exclusion provides a selective advantage for ICE*Bs1* host cells. Also described in Chapter 2 is my screen for exclusion-resistant mutants, identification of ConG as YddJ's target in the donor conjugation machinery, and my demonstration of ConG and YddJ specificity. Appendix A describes mating assays demonstrating that eight uncharacterized, non-essential ICE*Bs1* genes each had no effect on exclusion. Appendix B explains a mutagenesis and enrichment screen that was done to search for exclusion-resistant mutations outside of the ICE*Bs1* gene *conG;* no mutants were identified from this screen. Appendix C details experiments demonstrating that *conG* cannot complement a deletion of its homolog *orf15* in the ICE Tn*916*, and that *orf15* cannot complement a Δ*conG* ICE*Bs1*. Chapter 3 expands on the work in Chapter 2, and shows how I identified the regions of ConG and YddJ that confer specificity of exclusion, and how exclusion serves to protect ICE*Bs1* donors from excessive back-transfer from new transconjugants. Appendix D explores the effect of a few mutations made in a region of ConG showing an interesting similarity to a motif identified in the ConG homolog TcpH in the conjugative plasmid pCW3.

References

Abajy, M. Y., Kopeć, J., Schiwon, K., Burzynski, M., Döring, M., Bohn, C., & Grohmann, E. (2007). A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria. *Journal of Bacteriology*, *189*(6), 2487–2496. https://doi.org/10.1128/JB.01491-06

Achtman, M., Kennedy, N., & Skurray, R. (1977). Cell-cell interactions in conjugating Escherichia coli: role of traT protein in surface exclusion. *Proceedings of the National Academy of Sciences*, *74*(11), 5104–5108. https://doi.org/10.1073/pnas.74.11.5104

Achtman, M., Manning, P. A., Edelbluth, C., & Herrlich, P. (1979). Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor tra cistrons in Escherichia coli minicells. *Proceedings of the National Academy of Sciences*, *76*(10), 4837–4841. https://doi.org/10.1073/pnas.76.10.4837

Achtman, Mark, Manning, P. A., Kusecek, B., Schwuchow, S., & Neil, W. (1980). Genetic analysis of F sex factor cistrons needed for surface exclusion in Escherichia coli. *Journal of Molecular Biology*, *138*(4), 779–795. https://doi.org/10.1016/0022-2836(80)90065-0

Alfoldi, L., Jacob, F., & Wollman, E. L. (1957). Lethal zygosis in crossing between colicinogenic and non-colicinogenic strains of Escherichia coli. *Comptes Rendus Hebdomadaires Des Séances de l'Académie Des Sciences*, *244*(24), 2974–2977.

Alvarez-Martinez, C. E., & Christie, P. J. (2009). Biological Diversity of Prokaryotic Type IV Secretion Systems. *Microbiology and Molecular Biology Reviews*, *73*(4), 775–808. https://doi.org/10.1128/mmbr.00023-09

Anderson, M. T., & Seifert, H. S. (2011). Opportunity and means: Horizontal gene transfer from the human host to a bacterial pathogen. *MBio*, *2*(1). https://doi.org/10.1128/mBio.00005-11

Anthony, K. G., Klimke, W. A., Manchak, J., & Frost, L. S. (1999). Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100-1: Insights into the mechanism of conjugation. *Journal of Bacteriology*, *181*(17), 5149–5159.

Atmakuri, K., Cascales, E., & Christie, P. J. (2004). Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. *Molecular Microbiology*, *54*(5), 1199–1211. https://doi.org/10.1111/j.1365-2958.2004.04345.x

Auchtung, J. M., Aleksanyan, N., Bulku, A., & Berkmen, M. B. (2016). Biology of ICEBs1, an integrative and conjugative element in Bacillus subtilis. *Plasmid*, Vol. 86, pp. 14–25. https://doi.org/10.1016/j.plasmid.2016.07.001

Auchtung, J. M., Lee, C. A., Garrison, K. L., & Grossman, A. D. (2007). Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICEBs1 of Bacillus subtilis. *Molecular Microbiology*, *64*(6), 1515–1528. https://doi.org/10.1111/j.1365-2958.2007.05748.x

Auchtung, J. M., Lee, C. a, Monson, R. E., Lehman, A. P., & Grossman, A. D. (2005). Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. *Proceedings of the National Academy of Sciences*, *102*(35), 12554–12559. https://doi.org/10.1073/pnas.0505835102

Audette, G. F., Manchak, J., Beatty, P., Klimke, W. A., & Frost, L. S. (2007). Entry exclusion in F-

like plasmids requires intact TraG in the door that recognizes its cognate TraS in the recipient. *Microbiology*, *153*(2), 442–451. https://doi.org/10.1099/mic.0.2006/001917-0

Avello, M., Davis, K. P., & Grossman, A. D. Identification, characterization, and benefits of an exclusion system in an integrative and conjugative element of Bacillus subtilis. Submitted with revisions to *Molecular Microbiology* on June 7th, 2019.

Ayoubi, P., Kilic, A. O., & Vijayakumar, M. N. (1991). Tn5253, the pneumococcal Ω (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn5251 and Tn5252. *Journal of Bacteriology*, *173*(5), 1617–1622. https://doi.org/10.1128/jb.173.5.1617-1622.1991

Bailey, S., Ward, D., Middleton, R., Grossmann, J. G., & Zambryski, P. C. (2006). Agrobacterium tumefaciens VirB8 structure reveals potential protein-protein interaction sites. *Proceedings of the National Academy of Sciences*, *103*(8), 2582–2587. https://doi.org/10.1073/pnas.0511216103

Bañuelos-Vazquez, L. A., Torres Tejerizo, G., & Brom, S. (2017). Regulation of conjugative transfer of plasmids and integrative conjugative elements. *Plasmid*, Vol. 91, pp. 82–89. https://doi.org/10.1016/j.plasmid.2017.04.002

Baron, C. (2006). VirB8: a conserved type IV secretion system assembly factor and drug targetThis paper is one of a selection of papers published in this Special Issue, entitled CSBMCB — Membrane Proteins in Health and Disease. *Biochemistry and Cell Biology*, *84*(6), 890–899. https://doi.org/10.1139/o06-148

Berger, B. R., & Christie, P. J. (1994). Genetic complementation analysis of the Agrobacterium tumefaciens virB operon: virB2 through virB11 are essential virulence genes. *Journal of Bacteriology*, *176*(12), 3646–3660. https://doi.org/10.1128/jb.176.12.3646-3660.1994

Berkmen, M. B., Lee, C. A., Loveday, E. K., & Grossman, A. D. (2010). Polar positioning of a conjugation protein from the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *192*(1), 38–45. https://doi.org/10.1128/JB.00860-09

Bhatty, M., Laverde Gomez, J. A., & Christie, P. J. (2013). The expanding bacterial type IV secretion lexicon. *Research in Microbiology*, *164*(6), 620–639. https://doi.org/10.1016/j.resmic.2013.03.012

Bose, B., Auchtung, J. M., Lee, C. A., & Grossman, A. D. (2008). A conserved anti-repressor controls horizontal gene transfer by proteolysis. *Molecular Microbiology*, *70*(3), 570–582. https://doi.org/10.1111/j.1365-2958.2008.06414.x

Bose, B., & Grossman, A. D. (2011). Regulation of horizontal gene transfer in Bacillus subtilis by activation of a conserved site-specific protease. *Journal of Bacteriology*, *193*(1), 22–29. https://doi.org/10.1128/JB.01143-10

Brophy, J. A. N., Triassi, A. J., Adams, B. L., Renberg, R. L., Stratis-Cullum, D. N., Grossman, A. D., & Voigt, C. A. (2018). Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nature Microbiology*, *3*(9), 1043–1053. https://doi.org/10.1038/s41564-018-0216-5

Burrus, V., Pavlovic, G., Decaris, B., & Guédon, G. (2002). The ICESt1 element of Streptococcus thermophilus belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration. *Plasmid*, *48*(2), 77–97. https://doi.org/10.1016/S0147-619X(02)00102-6

Burrus, V., & Waldor, M. K. (2004). Formation of SXT Tandem Arrays and SXT-R391 Hybrids.

Journal of Bacteriology, *186*(9), 2636–2645. https://doi.org/10.1128/JB.186.9.2636-2645.2004

Cascales, E., & Christie, P. J. (2003). The versatile bacterial type IV secretion systems. *Nature Reviews Microbiology*, *1*(2), 137–149. https://doi.org/10.1038/nrmicro753

César, C. E., Machón, C., De La Cruz, F., & Llosa, M. (2006). A new domain of conjugative relaxase TrwC responsible for efficient oriT-specific recombination on minimal target sequences. *Molecular Microbiology*, *62*(4), 984–996. https://doi.org/10.1111/j.1365-2958.2006.05437.x

Chen, Y., Zhang, X., Manias, D., Yeo, H. J., Dunny, G. M., & Christie, P. J. (2008). Enterococcus faecalis PcfC, a spatially localized substrate receptor for type IV secretiontinn of the pCF10 transfer intermediate. *Journal of Bacteriology*, *190*(10), 3632–3645. https://doi.org/10.1128/JB.01999-07

Cheng, Q., Sutanto, Y., Shoemaker, N. B., Gardner, J. F., & Salyers, A. A. (2001). Identification of genes required for excision of CTnDOT, a Bacteroides conjugative transposon. *Molecular Microbiology*, *41*(3), 625–632. https://doi.org/10.1046/j.1365-2958.2001.02519.x

Clowes, R. C. (1963). Colicin factors and Episomes. *Genetical Research*, *4*(01), 162. https://doi.org/10.1017/s0016672300003529

Curtiss, R. (2003). Bacterial Conjugation. *Annual Review of Microbiology*, *23*(1), 69–136. https://doi.org/10.1146/annurev.mi.23.100169.000441

Daccord, A., Ceccarelli, D., & Burrus, V. (2010). Integrating conjugative elements of the SXT/R391 family trigger the excision and drive the mobilization of a new class of Vibrio genomic islands. *Molecular Microbiology*, *78*(3), 576–588. https://doi.org/10.1111/j.1365-2958.2010.07364.x

Daccord, A., Ceccarelli, D., Rodrigue, S., & Burrus, V. (2013). Comparative analysis of mobilizable Genomic Islands. *Journal of Bacteriology*, *195*(3), 606–614. https://doi.org/10.1128/JB.01985-12

Daccord, A., Mursell, M., Poulin-Laprade, D., & Burrus, V. (2012). Dynamics of the setCD-regulated integration and excision of genomic islands mobilized by integrating conjugative elements of the SXT/R391 family. *Journal of Bacteriology*, *194*(21), 5794–5802. https://doi.org/10.1128/JB.01093-12

Danino, V. E., Wilkinson, A., Edwards, A., & Downie, J. A. (2003). Recipient-induced transfer of the symbiotic plasmid pRL1JI in Rhizobium leguminosarum bv. viciae is regulated by a quorum-sensing relay. *Molecular Microbiology*, *50*(2), 511–525. https://doi.org/10.1046/j.1365-2958.2003.03699.x

De Boever, E. H., & Clewell, D. B. (2001). The Enterococcus faecalis pheromone-responsive plasmid pAM373 does not encode an entry exclusion function. *Plasmid*, *45*(1), 57–60. https://doi.org/10.1006/plas.2000.1494

De La Cruz, F., Frost, L. S., Meyer, R. J., & Zechner, E. L. (2010). Conjugative DNA metabolism in Gram-negative bacteria. *FEMS Microbiology Reviews*, Vol. 34, pp. 18–40. https://doi.org/10.1111/j.1574-6976.2009.00195.x

DeWitt, T., & Grossman, A. D. (2014). The bifunctional cell wall hydrolase cwlT is needed for conjugation of the integrative and conjugative element ICEBs1 in Bacillus subtilis and B. anthracis. *Journal of Bacteriology*, *196*(8), 1588–1596. https://doi.org/10.1128/JB.00012-14

Dunning Hotopp, J. C. (2011). Horizontal gene transfer between bacteria and animals. *Trends in Genetics*, Vol. 27, pp. 157–163. https://doi.org/10.1016/j.tig.2011.01.005

Firth, N., & Skurray, R. (1992). Characterization of the F plasmid bifunctional conjugation gene, traG. *MGG Molecular & General Genetics*, *232*(1), 145–153. https://doi.org/10.1007/BF00299147

Franke, A. E., & Clewell, D. B. (1981). Evidence for a Chromosome-Borne Resistance Transposon (Tn916) in Streptococcus faecalis That Is Capable of "Conjugal" Transfer in the Absence of a Conjugative Plasmid. *Journal of Bacteriology*, *145*(1). 494-502

Friesen, T. L., Stukenbrock, E. H., Liu, Z., Meinhardt, S., Ling, H., Faris, J. D., … Oliver, R. P. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, *38*(8), 953–956. https://doi.org/10.1038/ng1839

Fronzes, R., Schäfer, E., Wang, L., Saibil, H. R., Orlova, E. V., & Waksman, G. (2009). Structure of a type IV secretion system core complex. *Science*, *323*(5911), 266–268. https://doi.org/10.1126/science.1166101

Frost, L. S., Leplae, R., Summers, A. O., & Toussaint, A. (2005). Mobile genetic elements: The agents of open source evolution. *Nature Reviews Microbiology*, Vol. 3, pp. 722–732. https://doi.org/10.1038/nrmicro1235

Fukushima, T., Kitajima, T., Yamaguchi, H., Ouyang, Q., Furuhata, K., Yamamoto, H., … Sekiguchi, J. (2008). Identification and Characterization of Novel Cell Wall Hydrolase CwlT. *Journal of Biological Chemistry*, *283*(17), 11117–11125. https://doi.org/10.1074/jbc.m706626200

Fullner, K. J., Lara, J. C., & Nester, E. W. (1996). Pilus assembly by agrobacterium T-DNA transfer genes. *Science*, *273*(5278), 1107–1109. https://doi.org/10.1126/science.273.5278.1107

Furuya, N., & Komano, T. (1994). Surface Exclusion Gene of IncI1 Plasmid R64: Nucleotide Sequence and Analysis of Deletion Mutants. *Plasmid*, *32*, 80–84.

Garcillán-Barcia, M. P., & de la Cruz, F. (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid*, Vol. 60, pp. 1–18. https://doi.org/10.1016/j.plasmid.2008.03.002

Gawron-Burke, C., & Clewell, D. B. (1982). A transposon in Streptococcus faecalis with fertility properties. *Nature*, *300*(5889), 281–284. https://doi.org/10.1038/300281a0

Getino, M., & de la Cruz, F. (2018). Natural and Artificial Strategies To Control the Conjugative Transmission of Plasmids. *Microbiology Spectrum*, *6*(1). https://doi.org/10.1128/microbiolspec.mtbp-0015-2016

Goessweiner-Mohr, N., Grumet, L., Arends, K., Pavkov-Keller, T., Gruber, C. C., Gruber, K., … Keller, W. (2013). The 2.5A Structure of the enterococcus conjugation protein TraM resembles VirB8 type IV secretion proteins. *Journal of Biological Chemistry*, *288*(3), 2018–2028. https://doi.org/10.1074/jbc.M112.428847

Gomis-Rüth, F. X., Moncalián, G., Pérez-Luque, R., González, A., Cabezón, E., De La Cruz, F., & Coll, M. (2001). The bacterial conjugation protein TrwB resembles ring helicases and F1-atpase. *Nature*, *409*(6820), 637–641. https://doi.org/10.1038/35054586

Gomis-Ruth, F. X., Sola, M., Cruz, F., & Coll, M. (2005). Coupling Factors in Macromolecular Type-IV Secretion Machineries. *Current Pharmaceutical Design*, *10*(13), 1551–1565. https://doi.org/10.2174/1381612043384817

Grandoso, G., Avila, P., Cayón, A., Hernando, M. A., Llosa, M., & De la Cruz, F. (2000). Two active-site tyrosyl residues of protein TrwC act sequentially at the origin of transfer during

plasmid R388 conjugation. *Journal of Molecular Biology*, *295*(5), 1163–1172. https://doi.org/10.1006/jmbi.1999.3425
Grohmann, E., Muth, G., & Espinosa, M. (2003). Conjugative Plasmid Transfer in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews*, *67*(2), 277–301. https://doi.org/10.1128/mmbr.67.2.277-301.2003
Gross, J. D. (1963). Cellular damage associated with multiple mating in E. coli. *Genetical Research*, *4*(03), 463. https://doi.org/10.1017/s0016672300003852
Gunton, J. E., Ussher, J. E. R., Rooker, M. M., Wetsch, N. M., Alonso, G., & Taylor, D. E. (2008). Entry exclusion in the IncHI1 plasmid R27 is mediated by EexA and EexB. *Plasmid*, *59*(2), 86–101. https://doi.org/10.1016/j.plasmid.2007.11.004
Haase, J., Kalkum, M., & Lanka, E. (1996). TrbK, a small cytoplasmic membrane lipoprotein, functions in entry exclusion of the IncPα plasmid RP4. *Journal of Bacteriology*, *178*(23), 6720–6729. https://doi.org/10.1128/jb.178.23.6720-6729.1996
Haase, J., Lurz, R., Grahn, A. M., Bamford, D. H., & Lanka, E. (1995). Bacterial Conjugation Mediated by Plasmid RP4: RSF1010 Mobilization, Donor-Specific Phage Propagation, and Pilus Production Require the Same Tra2 Core Components of a Proposed DNA Transport Complex. *Journal of Bacteriology,* 177(16), 4779–4791.
Hartskeerl, R., Zuidweg, E., Van Geffen, M., & Hoekstra, W. (1985). The IncI Plasmids R144, R64 and ColIb Belong to One Exclusion Group. *Microbiology*, *131*(6), 1305–1311. https://doi.org/10.1099/00221287-131-6-1305
Hartskeerl, Rudy, Overduin, P., Hoekstra, W., & Tommassen, J. (1986). Nucleotide sequence of the exclusion-determining locus of IncI plasmid R144. *Gene*, *42*(1), 107–111. https://doi.org/10.1016/0378-1119(86)90156-3
Hartskeerl, Rudy, Tommassen, J., & Hoekstra, W. (1985). Relationship between the proteins encoded by the exclusion determining locus of the IncI plasmid R144 and the cellular localization of these proteins in Escherichia coli K-12. *MGG Molecular & General Genetics*, *200*(1), 138–144. https://doi.org/10.1007/BF00383327
He, X., Chang, W., Pierce, D. L., Seib, L. O., Wagner, J., & Fuqua, C. (2003). Quorum sensing in Rhizobium sp. Strain NGR234 regulates conjugal transfer (tra) gene expression and influences growth rate. *Journal of Bacteriology*, *185*(3), 809–822. https://doi.org/10.1128/JB.185.3.809-822.2003
Hochhut, B., Beaber, J. W., Woodgate, R., & Waldor, M. K. (2001). Formation of chromosomal tandem arrays of the SXT element and R391, two conjugative chromosomally integrating elements that share an attachment site. *Journal of Bacteriology*, *183*(4), 1124–1132. https://doi.org/10.1128/JB.183.4.1124-1132.2001
Hochhut, Bianca, Jahreis, K., Lengeler, J. W., & Schmid, K. (1997). CTnscr94, a conjugative transposon found in enterobacteria. *Journal of Bacteriology*, *179*(7), 2097–2102. https://doi.org/10.1128/jb.179.7.2097-2102.1997
Hochhut, Bianca, Marrero, J., & Waldor, M. K. (2000). Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in Vibrio cholerae O139. *Journal of Bacteriology*, *182*(7), 2043–2047. https://doi.org/10.1128/JB.182.7.2043-2047.2000
Hochhut, Bianca, & Waldor, M. K. (1999). Site-specific integration of the conjugal Vibrio cholerae SXT element into prfC. *Molecular Microbiology*, *32*(1), 99–110.

https://doi.org/10.1046/j.1365-2958.1999.01330.x
Hoeven, N. (1986). Coexistence of incompatible plasmids in a bacterial population living under a feast and famine regime. *Journal of Mathematical Biology*, *24*(3), 313–325. Retrieved from http://www.springerlink.com/index/Q0500L34GWWPU553.pdf
Hoeven, Nelly van der. (1984). A mathematical model for the co-existence of incompatible, conjugative plasmids in individual bacteria of a bacterial population. *Journal of Theoretical Biology*, *110*(3), 411–423. https://doi.org/10.1016/S0022-5193(84)80183-6
Jakubowski, S. J., Kerr, J. E., Garza, I., Krishnamoorthy, V., Bayliss, R., Waksman, G., & Christie, P. J. (2009). Agrobacterium VirB10 domain requirements for type IV secretion and T pilus biogenesis. *Molecular Microbiology*, *71*(3), 779–794. https://doi.org/10.1111/j.1365-2958.2008.06565.x
Jalajakumari, M. B., Guidolin, A., Buhk, H. J., Manning, P. A., Ham, L. M., Hodgson, A. L. M., … Skurray, R. A. (1987). Surface exclusion genes traS and traT of the F sex factor of Escherichia coli K-12. Determination of the nucleotide sequence and promoter and terminator activities. *Journal of Molecular Biology*, *198*(1), 1–11. https://doi.org/10.1016/0022-2836(87)90452-9
Jenkins, C., Samudrala, R., Anderson, I., Hedlund, B. P., Petroni, G., Michailova, N., … Staley, J. T. (2002). Genes for the cytoskeletal protein tubulin in the bacterial genus Prosthecobacter. *Proceedings of the National Academy of Sciences*, *99*(26), 17049–17054. https://doi.org/10.1073/pnas.012516899
Johnson, C. M., & Grossman, A. D. (2015). Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annual Review of Genetics*, *49*(1), 577–601. https://doi.org/10.1146/annurev-genet-112414-055018
Johnston, C., Martin, B., Fichant, G., Polard, P., & Claverys, J. P. (2014). Bacterial transformation: Distribution, shared mechanisms and divergent control. *Nature Reviews Microbiology*, Vol. 12, pp. 181–196. https://doi.org/10.1038/nrmicro3199
Keeling, P. J., & Palmer, J. D. (2008). Horizontal gene transfer in eukaryotic evolution. *Nature Reviews Genetics*, Vol. 9, pp. 605–618. https://doi.org/10.1038/nrg2386
Kerr, J. E., & Christie, P. J. (2010). Evidence for VirB4-mediated dislocation of membrane-integrated VirB2 pilin during biogenesis of the Agrobacterium VirB/VirD4 type IV secretion system. *Journal of Bacteriology*, *192*(19), 4923–4934. https://doi.org/10.1128/JB.00557-10
Koechler, S., Farasin, J., Cleiss-Arnold, J., & Arsène-Ploetze, F. (2015). Toxic metal resistance in biofilms: Diversity of microbial responses and their evolution. *Research in Microbiology*, *166*(10), 764–773. https://doi.org/10.1016/j.resmic.2015.03.008
Lacroix, B., & Citovsky, V. (2013). The roles of bacterial and host plant factors in Agrobacterium-mediated genetic transformation. *International Journal of Developmental Biology*, *57*(6–8), 467–481. https://doi.org/10.1387/ijdb.130199bl
Lang, J., & Faure, D. (2014). Functions and regulation of quorum-sensing in Agrobacterium tumefaciens. *Frontiers in Plant Science*, *5*. https://doi.org/10.3389/fpls.2014.00014
Lang, S., Kirchberger, P. C., Gruber, C. J., Redzej, A., Raffl, S., Zellnig, G., … Zechner, E. L. (2011). An activation domain of plasmid R1 TraI protein delineates stages of gene transfer initiation. *Molecular Microbiology*, *82*(5), 1071–1085. https://doi.org/10.1111/j.1365-2958.2011.07872.x
Lazazzera, B. A. (2001). The intracellular function of extracellular signaling peptides. *Peptides*,

22(10), 1519–1527. https://doi.org/10.1016/S0196-9781(01)00488-0
Lee, C. A., Auchtung, J. M., Monson, R. E., & Grossman, A. D. (2007). Identification and characterization of int (integrase), xis (excisionase) and chromosomal attachment sites of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Molecular Microbiology*, *66*(6), 1356–1369. https://doi.org/10.1111/j.1365-2958.2007.06000.x
Lee, C. A., & Grossman, A. D. (2007). Identification of the origin of transfer (oriT) and DNA relaxase required for conjugation of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *189*(20), 7254–7261. https://doi.org/10.1128/JB.00932-07
Lee, C. A., Thomas, J., & Grossman, A. D. (2012). The Bacillus subtilis conjugative transposon ICEBs1 mobilizes plasmids lacking dedicated mobilization functions. *Journal of Bacteriology*, *194*(12), 3165–3172. https://doi.org/10.1128/JB.00301-12
Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15
Lévi-Meyrueis, C., Fodor, K., & Schaeffer, P. (1980). Polyethyleneglycol-induced transformation of Bacillus subtilis protoplasts by bacterial chromosomal DNA. *MGG Molecular & General Genetics*, *179*(3), 589–594. https://doi.org/10.1007/BF00271749
Ma, H., & Bryers, J. D. (2013). Non-invasive determination of conjugative transfer of plasmids bearing antibiotic-resistance genes in biofilm-bound bacteria: Effects of substrate loading and antibiotic selection. *Applied Microbiology and Biotechnology*, *97*(1), 317–328. https://doi.org/10.1007/s00253-012-4179-9
Marrero, J., & Waldor, M. K. (2005). Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Developmental Cell*, *8*(6), 963–970. https://doi.org/10.1016/j.devcel.2005.05.004
Marrero, J., & Waldor, M. K. (2007). Determinants of entry exclusion within Eex and TraG are cytoplasmic. *Journal of Bacteriology*, *189*(17), 6469–6473. https://doi.org/10.1128/JB.00522-07
Marrero, R., & Yasbin, R. E. (1988). Cloning of the Bacillus subtilis recE+ gene and functional expression of recE+ in B. subtilis. *Journal of Bacteriology*, *170*(1), 335–344. https://doi.org/10.1128/jb.170.1.335-344.1988
McAnulla, C., Edwards, A., Sanchez-Contreras, M., Sawers, R. G., & Downie, J. A. (2007). Quorum-sensing-regulated transcriptional initiation of plasmid transfer and replication genes in Rhizobium leguminosarum biovar viciae. *Microbiology*, *153*(7), 2074–2082. https://doi.org/10.1099/mic.0.2007/007153-0
McDougal, L. K., Tenover, F. C., Lee, L. N., Rasheed, J. K., Patterson, J. E., Jorgensen, J. H., & Leblanc, D. J. (1998). Detection of Tn917-like sequences within a Tn916-like conjugative transposon (Tn3872) in erythromycin-resistant isolates of Streptococcus pneumoniae. *Antimicrobial Agents and Chemotherapy*, *42*(9), 2312–2318.
Monchy, S., Benotmane, M. A., Janssen, P., Vallaeys, T., Taghavi, S., Van Der Lelie, D., & Mergeay, M. (2007). Plasmids pMOL28 and pMOL30 of Cupriavidus metallidurans are specialized in the maximal viable response to heavy metals. *Journal of Bacteriology*, *189*(20), 7417–7425. https://doi.org/10.1128/JB.00375-07

Naglich, J. G., & Andrews, R. E. (1988). Tn916-dependent conjugal transfer of PC194 and PUB110 from Bacillus subtilis into Bacillus thuringiensis subsp. israelensis. *Plasmid*, *20*(2), 113–126. https://doi.org/10.1016/0147-619X(88)90014-5

Nelly, van der H. (1985). Evolution of bacterial surface exclusion against incompatible plasmids. *Journal of Theoretical Biology*, *117*(3), 431–452. https://doi.org/10.1016/S0022-5193(85)80153-3

Nishi, A., Tominaga, K., & Furukawa, K. (2000). A 90-kilobase conjugative chromosomal element coding for biphenyl and salicylate catabolism in Pseudomonas putida KF715. *Journal of Bacteriology*, *182*(7), 1949–1955. https://doi.org/10.1128/JB.182.7.1949-1955.2000

Novick, R. P. (1987). Plasmid incompatibility. *Microbiological Reviews*, *51*(4), 381–395. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3325793%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC373122

Ochman, Howard, Lawrence, Jeffrey G., & Groisman, Eduardo A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature*, *405*(6784), 299–304. Retrieved from http://adsabs.harvard.edu/abs/2000Natur.405..299O

Ou, J. T. (1980). Role of surface exclusion genes in lethal zygosis in Escherichia coli K12 mating. *MGG Molecular & General Genetics*, *178*(3), 573–581. https://doi.org/10.1007/BF00337863

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews*, *31*(4). https://doi.org/10.1128/cmr.00088-17

Paschos, A., Patey, G., Sivanesan, D., Gao, C., Bayliss, R., Waksman, G., … Baron, C. (2006). Dimerization and interactions of Brucella suis VirB8 with VirB4 and VirB10 are required for its biological activity. *Proceedings of the National Academy of Sciences*, *103*(19), 7252–7257. https://doi.org/10.1073/pnas.0600862103

Phillips, Z. E. V, & Strauch, M. A. (2002). Bacillus subtilis sporulation and stationary phase gene expression. *Cellular and Molecular Life Sciences*, Vol. 59, pp. 392–402. https://doi.org/10.1007/s00018-002-8431-9

Pohlman, R. F., Genetti, H. D., & Winans, S. C. (1994). Entry exclusion of the IncN plasmid pKM101 is mediated by a single hydrophilic protein containing a lipid attachment motif. *Plasmid*, *31*(2), 158–165. https://doi.org/10.1006/plas.1994.1017

Porter, C. J., Bantwal, R., Bannam, T. L., Rosado, C. J., Pearce, M. C., Adams, V., … Rood, J. I. (2012). The conjugation protein TcpC from Clostridium perfringens is structurally related to the type IV secretion system protein VirB8 from Gram-negative bacteria. *Molecular Microbiology*, *83*(2), 275–288. https://doi.org/10.1111/j.1365-2958.2011.07930.x

Pottathil, M., & Lazazzera, B. A. (2003). The extracellular PHR peptide-Rap phosphatase signaling circuit of bacillus subtilis. *Frontiers in Bioscience*, *8*(4), d32-45. https://doi.org/10.2741/913

Ramsay, J. P., Sullivan, J. T., Jambari, N., Ortori, C. A., Heeb, S., Williams, P., … Ronson, C. W. (2009). A LuxRI-family regulatory system controls excision and transfer of the Mesorhizobium loti strain R7A symbiosis island by activating expression of two conserved hypothetical genes. *Molecular Microbiology*, *73*(6), 1141–1155. https://doi.org/10.1111/j.1365-2958.2009.06843.x

Ramsay, J. P., Sullivan, J. T., Stuart, G. S., Lamont, I. L., & Ronson, C. W. (2006). Excision and transfer of the Mesorhizobium loti R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS. *Molecular Microbiology*, *62*(3), 723–734. https://doi.org/10.1111/j.1365-2958.2006.05396.x

Rauch, P. J. G., & De Vos, W. M. (1994). Identification and characterization of genes involved in excision of the Lactococcus lactis conjugative transposon Tn5276. *Journal of Bacteriology*, *176*(8), 2165–2171. https://doi.org/10.1128/jb.176.8.2165-2171.1994

Ravatn, R., Studer, S., Springael, D., Zehnder, A. J. B., & Van Der Meer, J. R. (1998). Chromosomal integration, tandem amplification, and deamplification in Pseudomonas putida F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from Pseudomonas sp. strain B13. *Journal of Bacteriology*, *180*(17), 4360–4369.

Rice, L.B., & Carias, L.L., (1998). Transfer of Tn5385, a composite, multiresistance chromosomal element from Enterococcus faecalis. *Journal of Bacteriology*, *180*(3), 714–721. Retrieved from http://jb.asm.org/

Roberts, A. P., & Mullany, P. (2009). A modular master on the move: the Tn916 family of mobile genetic elements. *Trends in Microbiology*, Vol. 17, pp. 251–258. https://doi.org/10.1016/j.tim.2009.03.002

Sakuma, T., Tazumi, S., Furuya, N., & Komano, T. (2013). ExcA proteins of IncI1 plasmid R64 and IncIγ plasmid R621a recognize different segments of their cognate TraY proteins in entry exclusion. *Plasmid*, *69*(2), 138–145. https://doi.org/10.1016/j.plasmid.2012.11.004

Schlieper, D., Oliva, M. A., Andreu, J. M., & Lowe, J. (2005). Structure of bacterial tubulin BtubA/B: Evidence for horizontal gene transfer. *Proceedings of the National Academy of Sciences*, *102*(26), 9170–9175. https://doi.org/10.1073/pnas.0502859102

Schröder, G., & Lanka, E. (2005). The mating pair formation system of conjugative plasmids - A versatile secretion machinery for transfer of proteins and DNA. *Plasmid*, Vol. 54, pp. 1–25. https://doi.org/10.1016/j.plasmid.2005.02.001

Shoemaker, N. B., Getty, C., Guthrie, E. P., & Salyers, A. A. (1986). Regions in Bacteroides plasmids pBFTM10 and pB8-51 that allow Escherichia coli-Bacteroides shuttle vectors to be mobilized by IncP plasmids and by a conjugative Bacteroides tetracycline resistance element. *Journal of Bacteriology*, *166*(3), 959–965. Retrieved from http://jb.asm.org/

Shoemaker, N. B., Wang, G.-R., Stevens, A. M., & Salyers, A. A. (1993). Excision, Transfer, and Integration of NBU1, a Mobilizable Site-Selective Insertion Element Downloaded from. *Journal of Bacteriology, 175*(20). Retrieved from http://jb.asm.org/

Showsh, S. A., & Andrews, R. E. (1992). Tetracycline enhances Tn916-mediated conjugal transfer. *Plasmid*, *28*(3), 213–224. https://doi.org/10.1016/0147-619X(92)90053-D

Skurray, R. A., & Reeves, P. (1973). Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12. *Journal of Bacteriology*, *113*(1), 58–70.

Skurray, R. A., Willetts, N., & Reeves, P. (1976). Effect of tra mutations on F factor-specified immunity to lethal zygosis. *MGG Molecular & General Genetics*, *146*(2), 161–165. https://doi.org/10.1007/BF00268084

Slot, J. C., & Hibbett, D. S. (2007). Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: A phylogenetic study. *PLoS ONE*, *2*(10). https://doi.org/10.1371/journal.pone.0001097

Stevens, A. M., Sanders, J. M., Shoemaker, N. B., & Salyers, A. A. (1992). Genes involved in

production of plasmidlike forms by a Bacteroides conjugal chromosomal element share amino acid homology with two-component regulatory systems. *Journal of Bacteriology*, *174*(9), 2935–2942. https://doi.org/10.1128/jb.174.9.2935-2942.1992

Sutanto, Y., DiChiara, J. M., Shoemaker, N. B., Gardner, J. F., & Salyers, A. A. (2004). Factors required in vitro for excision of the Bacteroides conjugative transposon, CTnDOT. *Plasmid*, *52*(2), 119–130. https://doi.org/10.1016/j.plasmid.2004.06.003

Takahashi, H., Shao, M., Furuya, N., & Komano, T. (2011). The genome sequence of the incompatibility group Iγ plasmid R621a: Evolution of IncI plasmids. *Plasmid*, *66*(2), 112–121. https://doi.org/10.1016/j.plasmid.2011.06.004

Tato, I., Matilla, I., Arechaga, I., Zunzunegui, S., De La Cruz, F., & Cabezon, E. (2007). The ATPase activity of the DNA transporter TrwB is modulated by protein TrwA: Implications for a common assembly mechanism of DNA translocating motors. *Journal of Biological Chemistry*, *282*(35), 25569–25576. https://doi.org/10.1074/jbc.M703464200

te Poele, E. M., Bolhuis, H., & Dijkhuizen, L. (2008). Actinomycete integrative and conjugative elements. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, Vol. 94, pp. 127–143. https://doi.org/10.1007/s10482-008-9255-x

Terradot, L., Bayliss, R., Oomen, C., Leonard, G. A., Baron, C., & Waksman, G. (2005). Structures of two core subunits of the bacterial type IV secretion system, VirB8 from Brucella suis and ComB10 from Helicobacter pylori. *Proceedings of the National Academy of Sciences*, *102*(12), 4596–4601. https://doi.org/10.1073/pnas.0408927102

Thomas, J., Lee, C. A., & Grossman, A. D. (2013). A Conserved Helicase Processivity Factor Is Needed for Conjugation and Replication of an Integrative and Conjugative Element. *PLoS Genetics*, *9*(1). https://doi.org/10.1371/journal.pgen.1003198

Torres, O. R., Korman, R. Z., Zahler, S. A., & Dunny, G. M. (1991). The conjugative transposon Tn 925: enhancement of conjugal transfer by tetracycline in Enterococcus faecalis and mobilization of chromosomal genes in Bacillus subtilis and E. faecalis. *MGG Molecular & General Genetics*, *225*(3), 395–400. https://doi.org/10.1007/BF00261679

Valentine, P. J., Shoemaker, N. B., & Salyers, A. A. (1988). Mobilization of Bacteroides plasmids by Bacteroides conjugal elements. *Journal of Bacteriology*, *170*(3), 1319–1324. Retrieved from http://jb.asm.org/

Viljanen, P. (1987). Polycations which disorganize the outer membrane inhibit conjugation in Escherichia coli. *The Journal of Antibiotics*, *40*(6), 882–886. https://doi.org/10.7164/antibiotics.40.882

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., … Wolffs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, *7*, 173. https://doi.org/10.3389/fmicb.2016.00173

Whittle, G., Hund, B. D., Shoemaker, N. B., & Salyers, A. A. (2001). Characterization of the 13-Kilobase ermF Region of the Bacteroides Conjugative Transposon CTnDOT. *Applied and Environmental Microbiology*, *67*(8), 3488–3495. https://doi.org/10.1128/AEM.67.8.3488-3495.2001

Whittle, G., Shoemaker, N. B., & Salyers, A. A. (2002). Characterization of genes involved in modulation of conjugal transfer of the Bacteroides conjugative transposon CTnDOT. *Journal of Bacteriology*, *184*(14), 3839–3847. https://doi.org/10.1128/JB.184.14.3839-

3847.2002
Wong, J. J. W., Lu, J., & Glover, J. N. M. (2012). Relaxosome function and conjugation regulation in F-like plasmids - a structural biology perspective. *Molecular Microbiology*, Vol. 85, pp. 602–617. https://doi.org/10.1111/j.1365-2958.2012.08131.x
Wood, M. M., & Gardner, J. F. (2015). The Integration and Excision of CTnDOT. *Microbiology Spectrum*, *3*(2), MDNA3-0020–2014. https://doi.org/10.1128/microbiolspec.mdna3-0020-2014
Wozniak, R. A. F., & Waldor, M. K. (2010). Integrative and conjugative elements: Mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nature Reviews Microbiology*, Vol. 8, pp. 552–563. https://doi.org/10.1038/nrmicro2382
Zechner, E. L., Lang, S., & Schildbach, J. F. (2012). Assembly and mechanisms of bacterial type IV secretion machines. *Philosophical Transactions of the Royal Society B: Biological Sciences*, Vol. 367, pp. 1073–1087. https://doi.org/10.1098/rstb.2011.0207
Zhang, P. Y., Xu, P. P., Xia, Z. J., Wang, J., Xiong, J., & Li, Y. Z. (2013). Combined treatment with the antibiotics kanamycin and streptomycin promotes the conjugation of Escherichia coli. *FEMS Microbiology Letters*, *348*(2), 149–156. https://doi.org/10.1111/1574-6968.12282

Chapter 2

Identification, characterization, and benefits of an exclusion system in an integrative and conjugative element of *Bacillus subtilis*

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CONTRIBUTIONS

[1A]MA identified exclusion in ICE*Bs1*, determined that YddJ is the exclusion protein, and demonstrated the selective advantage provided by exclusion

[1B]KPD carried out the mutagenesis screen for exclusion-resistant mutations, determined that ConG is the target of YddJ in the donor, and showed that YddJ and ConG determine exclusion specificity

ABSTRACT

Integrative and conjugative elements (ICEs) are mobile genetic elements that transfer from cell to cell by conjugation (like plasmids) and integrate into the chromosomes of bacterial hosts (like lysogenic phages or transposons). ICEs are prevalent in bacterial chromosomes and play a major role in bacterial evolution by promoting horizontal gene transfer. Exclusion prevents redundant transfer of conjugative elements into host cells that already contain a copy of the element. Exclusion has been characterized mostly for conjugative elements of Gram-negative bacteria. Here, we report the identification and characterization of an exclusion mechanism in ICE*Bs1* from the Gram-positive bacterium *Bacillus subtilis*. We found that cells containing ICE*Bs1* inhibit the activity of the ICE*Bs1*-encoded conjugation machinery in other cells. This inhibition (exclusion) was specific to the cognate conjugation machinery and the ICE*Bs1* gene *yddJ* was both necessary and sufficient to mediate exclusion by recipient cells. Through a mutagenesis and enrichment screen, we identified exclusion-resistant mutations in the ICE*Bs1* gene *conG*. Using genes from a heterologous but related ICE, we found that exclusion specificity was determined by ConG and YddJ. Finally, we found that under conditions that support conjugation, exclusion provides a selective advantage to the element and its host cells.

INTRODUCTION

Integrative and conjugative elements (ICEs), also known as conjugative transposons, are self-transmissible genetic elements whose life cycle combines characteristics of lysogenic phages or transposons and conjugative plasmids. ICEs integrate into the host chromosome and are passively replicated with the rest of the genome (like some lysogenic phages and transposons), and under specific conditions, or stochastically, excise and transfer to other cells via self-encoded conjugation machinery (like conjugative plasmids) (Johnson and Grossman, 2015). ICEs are remarkably prevalent and thought to outnumber conjugative plasmids across major bacterial clades (Guglielmini et al., 2011). ICEs contribute to bacterial evolution by enabling the acquisition of various phenotypes by horizontal gene transfer. These phenotypes are conferred to host cells by so-called 'cargo genes' that are within an ICE but that are not required for the ICE life cycle. Some of the phenotypes conferred by ICEs include antibiotic resistances, pathogenicity, symbiosis, and metabolic capabilities (reviewed in Burrus and Waldor, 2004; Johnson and Grossman, 2015). In addition, many ICEs can mobilize other genetic elements, typically plasmids, that do not encode their own transfer machinery (e.g., Naglich and Andrews, 1988; Valentine et al., 1988; Hochhut et al., 2000; Lee et al, 2012).

ICE*Bs1* (Fig. 1) is approximately 20 kb and found in the chromosome of various isolates of the Gram-positive bacterium *Bacillus subtilis* (Burrus et al., 2002; Auchtung et al., 2005; Earl et al., 2007). ICE*Bs1* integrates into a specific site in *trnS-leu2* (encoding a leucyl-tRNA) in the host chromosome. Integration does not disrupt the tRNA gene. When stably integrated, most of the ICE*Bs1* genes are repressed. Multiple mechanisms control induction (de-repression) and transfer of ICE*Bs1*. The ICE*Bs1*-encoded RapI-PhrI signaling system controls induction by sensing

cues about growth phase, the concentration of potential recipients, and whether or not the neighboring cells already contain a copy of ICE*Bs1* (Auchtung et al., 2005). RecA also controls induction of ICE*Bs1* in response to DNA damage, independently of the RapI-PhrI signaling system (Auchtung et al., 2005). Both the RapI and RecA pathways cause the ICE*Bs1* repressor ImmR to be cleaved by the protease and anti-repressor ImmA (Bose et al., 2008).

The *rapI-phrI* signaling system and ImmR both help to limit acquisition of ICE*Bs1* by cells that already contain a copy of the element. PhrI-mediated inhibition of RapI activity inhibits SOS-independent de-repression of ICE*Bs1* gene expression (Auchtung et al., 2005), thereby inhibiting the earliest step in activation of the element in potential donor cells. ImmR mediates a phage-like immunity that inhibits integration of additional copies of ICE*Bs1* that enter a host cell that already contains the element (Auchtung et al., 2007). Thus, if ICE*Bs1* is activated in some cells and actually does transfer to a recipient that already contains a copy, then integration of the newly acquired element is inhibited by ImmR-mediated immunity (Auchtung et al., 2007). Similar to repressor-mediated immunity in some lysogenic phages, ImmR-mediated immunity is bypassed by overproduction of the ICE*Bs1* integrase Int (Auchtung et al., 2007). Thus, these two mechanisms that inhibit acquisition of a second copy of ICE*Bs1* work at the first and last steps in acquisition.

The conjugation machinery encoded by ICE*Bs1* is a type IV secretion system (T4SS) that resembles other well characterized T4SSs encoded by conjugative elements found in Gram-positive bacteria, such as pCW3, pIP501, and pCF10 (Goessweiner-Mohr et al., 2014; Guglielmini, et al., 2014; Leonetti et al., 2015). The T4SS of ICE*Bs1* is classified as a member of the MPF_{FA} class of T4SSs, which are found in Firmicutes and Actinobacteria and do not contain

dedicated adhesion proteins (Guglielmini et al., 2014). T4SSs involved in conjugation transfer ssDNA that is attached to a relaxase protein from a donor cell to a recipient cell when the two cells are in direct contact (Bhatty et al., 2013; Goessweiner-Mohr et al., 2014; Grohmann et al., 2018). All T4SSs appear to share a conserved set of proteins (Bhatty et al., 2013; Guglielmini, et al., 2014). Using nomenclature from the *Agrobacterium tumefaciens* Ti plasmid, six of these proteins and the ICE*Bs1* counterparts are, respectively: VirB1/CwlT (cell wall hydrolase), VirD4/ConQ (the coupling protein), VirB4/ConE (an ATPase), and VirB3/ConD, VirB6/ConG, and VirB8/ConB (three membrane channel components) (Fig. 1).

Exclusion is a mechanism encoded by conjugative elements to prevent redundant transfer; i.e., the entry of an identical or highly similar element into a cell that already contains a copy of the element (Garcillan-Barcia and de la Cruz, 2008). Exclusion systems (also called entry exclusion) have been described for many of the major conjugative plasmid groups. They prevent transfer of DNA by the cognate conjugation machinery from a cell that has the plasmid to another cell that also has the plasmid. The general mechanism for exclusion in Gram-negative bacteria involves an exclusion protein present in the inner membrane of the "recipient" cell that already contains the element. The exclusion protein recognizes a component of the cognate conjugation machinery in a would-be donor, and through means yet unknown, prevents successful transfer of DNA. To date, the exclusion protein and its target in the conjugation machinery have been identified for three different conjugative systems, all from elements found in Gram-negative bacteria: 1) the F/R100 plasmids (Anthony et al., 1999; Audette et al., 2007); 2) the SXT/R391 ICEs (Marrero et al., 2005); and 3) the R64/R621a plasmids (Sakuma et al., 2013).

Selective advantages for elements with exclusion have been proposed and are mostly theoretical. For example, mathematical modeling of competition between incompatible conjugative plasmids with and without exclusion predicts an exclusion-competent plasmid can penetrate a cell population containing an exclusion-less plasmid, potentially expelling it from the population (van der Hoeven, 1985). Experimental evidence for the advantage of exclusion has come from studies of the F plasmid in *E. coli*, where it has been shown that exclusion prevents lethal zygosis, a phenomenon where F- recipient cells are killed in the presence of excess Hfr or F+ donors that lack the exclusion functions (Skurray et al., 1973, 1974, 1976; Ou, 1980).

In this study, we describe an exclusion mechanism in ICE*Bs1* that specifically inhibits its cognate conjugation machinery. We identified the ICE*Bs1* gene *yddJ* as necessary and sufficient for exclusion in the recipient cell. Through a mutagenesis and enrichment screen, we identified exclusion-resistant mutations in the ICE*Bs1* gene *conG*, an essential component of the conjugation machinery. Using homologs of *conG* and *yddJ* from a heterologous ICE, we found that *conG* and *yddJ* determine specificity of exclusion. We provide evidence that exclusion protects ICE*Bs1* and its host cell from cell death caused by redundant transfer, providing experimental evidence that there is selective pressure to maintain exclusion, at least for some conjugative elements.

RESULTS

Rationale

Cells containing the mobile element ICE*Bs1* have multiple element-encoded mechanisms for limiting acquisition of a second copy of ICE*Bs1*. One mechanism involves RapI-PhrI mediated cell-cell signaling (Auchtung et al., 2005). A second mechanism is element-encoded immunity, analogous to phage immunity, and is mediated by the ICE*Bs1* repressor ImmR (Auchtung et al., 2007). In the course of analyzing ImmR-mediated immunity, it seemed that there was at least one additional mechanism by which cells that contain ICE*Bs1* limit acquisition of another copy of the element (Auchtung et al., 2007). To uncover and analyze this third mechanism, we used conditions and assays that bypassed the effects of cell-cell signaling and repressor-mediated immunity. Cell-cell signaling was bypassed by inducing ICE*Bs1* by over-production of the activator RapI (Auchtung et al., 2005). ImmR-mediated immunity was bypassed by assaying transfer of a mobilizable plasmid (Lee et al., 2012) that does not need to integrate into the chromosome of the transconjugant and is not affected by ICE*Bs1*-mediated immunity. Results described below demonstrate that ICE*Bs1* has a mechanism of exclusion that functions to inhibit transfer of DNA through the ICE*Bs1*-encoded conjugation machinery.

The presence of ICE*Bs1* in recipient cells inhibits acquisition of a plasmid mobilized by the ICE*Bs1* conjugation machinery

We used a plasmid mobilization assay to monitor the efficiency of conjugation through the ICE*Bs1*-encoded type IV secretion system. The conjugation machinery encoded by ICE*Bs1* can mobilize at least three different rolling circle replicating (RCR) plasmids that do not encode their own conjugation machinery (Lee et al., 2012). We measured the ability of recipient cells with or

without ICE*Bs1* (strain CAL88 or CAL89, respectively) to obtain the RCR plasmid pC194 from donor cells via the ICE*Bs1*-encoded conjugation machinery. Donor cells containing ICE and pC194 (ICE*Bs1* Δ(*rapI*-*phrI*)*342*::*kan*, Pxyl-*rapI*, pC194, Str^S; MA116) were grown in defined minimal medium to mid-exponential phase in the presence of chloramphenicol to select for pC194. Pxyl-*rapI* expression was induced with xylose, thereby causing induction of ICE*Bs1*, and these donor cells were mixed with recipients and placed on filters to allow efficient mating (Materials and Methods). The transfer efficiency of pC194 into recipients was calculated as the number of transconjugants (determined by Cm^R Str^R CFUs) per initial number of donors at the time of cell mixing, converted to percent transconjugants per donor (Materials and Methods).

The transfer efficiency of pC194 into recipient cells that did not contain ICE*Bs1* was ~1% transconjugants (containing pC194) per donor (Fig. 2), similar to previous findings (Lee et al., 2012). In contrast, the efficiency of acquisition of pC194 by recipients that contained ICE*Bs1* was ~0.05% transconjugants per donor (Fig. 2), a reduction of ~20-fold. Transfer of the plasmid from donor to recipient by transformation with free or released plasmid was unlikely because recipient cells were defective in competence due to a *comK* null mutation. These results indicate that ICE*Bs1* likely has an exclusion mechanism that inhibits acquisition of plasmid DNA through the ICE*Bs1*-encoded conjugation machinery.

ICE*Bs1*-mediated exclusion is specific for the ICE*Bs1* conjugation machinery

We found that the inhibition of acquisition of pC194 by ICE*Bs1* in recipients was specific to the ICE*Bs1* conjugation machinery in donors. We used Tn*916*, an ICE that can also mobilize pC194 (Naglich and Andrews, 1988) and that does not encode an exclusion function (Norgren and Scott, 1991) to test specificity of ICE*Bs1*-mediated exclusion. Donor cells containing Tn*916*

and pC194 (ICE*Bs1*^{0} Tn*916*; pC194, Spc^{S}; MA1100) were grown in defined minimal medium with chloramphenicol to mid-exponential phase, induced with 2.5 µg/mL tetracycline for 2 hours (to boost activation of Tn*916*), mixed with recipients with or without ICE*Bs1*, filtered and incubated for 3 hours and then harvested, as above.

The efficiency of transfer of pC194 by the Tn*916*-encoded conjugation machinery was ~1x10^{-4} % transconjugants per donor (Fig. 2), similar to previously reported results (Naglich and Andrews, 1988). The presence of ICE*Bs1* in recipient cells did not reduce the efficiency of transfer of pC194 by the Tn*916*-encoded conjugation machinery (Fig. 2). Although transfer of pC194 by Tn*916* was low, our limit of detection was ~10^{-6} %, so we would have easily detected inhibition by ICE*Bs1*-containing recipients. Based on these results, we conclude that 1) pC194 is not the target of the inhibition by ICE*Bs1* in the recipient, and 2) ICE*Bs1* has an exclusion mechanism that inhibits acquisition of DNA that is transferred through the ICE*Bs1*, but not the Tn*916*, conjugation machinery.

In recipient cells, the ICE*Bs1* gene *yddJ* is both necessary and sufficient for exclusion

We expected the exclusion gene(s) to be transcriptionally active in recipient cells containing ICE*Bs1* that is integrated in the chromosome. In the integrated state, most ICE*Bs1* genes are repressed and only a few genes are expressed, including genes at the left and right ends of the element (Fig. 1). We tested the ability of various recipient cells containing deletions within ICE*Bs1* to exclude pC194 mobilized by ICE*Bs1* from donor cells. Because the functions of all three genes (*immR*, *immA*, *int*) at the left end of ICE*Bs1* are known, we focused on genes at the right end (Fig. 1A). Preliminary analyses of deletion-insertion mutations in *yddM*, *rapI-phrI*, and *yddI* indicated that none of these genes was required for exclusion. In contrast, deletion-

insertion mutations that removed *yddJ*, alone or in combination with other genes, caused a defect in exclusion. Based on these preliminary analyses, we tested directly the effects of *yddJ*.

We found that *yddJ* in recipient cells was necessary for exclusion. We constructed a deletion of *yddJ* and compared the ability of recipients without ICE*Bs1* (ICE*Bs1*^{0} *str-84*; CAL89), with ICE*Bs1* containing *yddJ+* (ICE*Bs1 yddJ+ str-84*; CAL88) or not containing *yddJ* (ICE*Bs1 ΔyddJ str-84*; MA665) to acquire pC194 by mobilization through the ICE*Bs1* conjugation machinery in donor cells. As expected, cells with ICE*Bs1* (*yddJ*+) had reduced acquisition of pC194 (~0.2% transconjugants per donor) (Fig. 3A). In contrast, cells with ICE*Bs1 ΔyddJ* acquired pC194 at ~3% transconjugants per donor, a frequency similar to that of cells with no ICE*Bs1* (Fig. 3A). These results indicate that *yddJ* in recipients is necessary for exclusion.

We also found that *yddJ* alone, of all the ICE*Bs1* genes, was sufficient to cause exclusion. We expressed *yddJ*, from its own promoter, in cells that were missing ICE*Bs1* (ICE*Bs1*^{0} *lacA*::{PyddJ-*yddJ*} *str-84*; MA996). This strain was used as a recipient and compared to recipients without ICE*Bs1*, and therefore no *yddJ* (ICE*Bs1*^{0} *str-84*; CAL89). The mobilization of pC194 from donor cells into recipients without *yddJ* was ~2%. In contrast, the mobilization of pC194 was reduced ~100-fold to ~0.03% into recipients expressing *yddJ* (Fig. 3A). We also expressed *yddJ* from the strong promoter Pspank(hy) in cells that were missing ICE*Bs1* (ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*} *str-84*; MA982). The mobilization of pC194 from donor cells into these recipients was reduced ~500-fold to ~0.004% (Fig. 3A). Based on these results, we conclude that *yddJ* is sufficient to cause exclusion in the absence of all other ICE*Bs1* genes, and that exogenous expression (from an exogenous locus, using its own or an exogenous strong promoter) causes more exclusion than normal levels of expression of *yddJ* from its endogenous

locus within ICE*Bs1*. In most of the experiments described below, we used strains over-expressing *yddJ* to cause exclusion.

Assays described above used mobilization of pC194 as a read-out for conjugation, largely to bypass the effects of repressor-mediated immunity that inhibits stable acquisition of ICE*Bs1* (Auchtung et al., 2007). Since recipients over-expressing *yddJ* do not have ICE*Bs1*, there is no *immR* (repressor)-mediated immunity. Therefore, we could determine if *yddJ* also inhibited acquisition of ICE*Bs1* itself.

We found that *yddJ* in recipients inhibited acquisition of ICE*Bs1* from donor cells. We monitored transfer of ICE*Bs1* from donor cells (ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*, Str^S; MMB970) into recipient cells without or with over-expression of *yddJ*, as above. The transfer efficiency of ICE*Bs1* was calculated as the number of transconjugants (Kan^R Str^R CFUs) per initial number of donors at the time of cell mixing, converted to percent transconjugants per donor. We found the transfer of ICE*Bs1* into a *yddJ* over-expressing recipient was ~0.01%, a reduction of ~1000-fold compared to ~14% into a recipient without *yddJ* (Fig. 3B). These results indicate that *yddJ* is sufficient for exclusion of ICE*Bs1*. In experiments below, we use transfer of ICE*Bs1* into recipients with or without over-expression of *yddJ* as an assay for exclusion.

***yddJ* in donor cells is not required for transfer or exclusion**

We wondered if *yddJ* in donor cells was needed for *yddJ*-mediated exclusion coming from recipients. In some exclusion systems, the gene mediating exclusion in the recipient is also needed in the donor. For example, in plasmid R27, the genes *eexA* and *eexB* are needed in both the recipient and donor cells for exclusion (Gunton et al., 2008).

We found that loss of *yddJ* in donor cells had virtually no effect on transfer efficiency or exclusion. In the experiment above, we also monitored transfer of ICE*Bs1* from donor cells that contained a deletion of *yddJ* in ICE*Bs1* (ICE*Bs1* Δ*yddJ* Δ(*rapI*-*phrI*)*342*::*kan*, Pxyl-*rapI*; MA11) into recipient cells with or without over-expression of *yddJ*. Deletion of *yddJ* in donors did not affect the ability of ICE*Bs1* to transfer into ICE*Bs1*^{0} recipient cells, indicating *yddJ* is not required for transfer. The *ΔyddJ* donor was as sensitive as the *yddJ*+ donor to exclusion by the presence of *yddJ* in recipient cells (Fig. 3B). Based on these results, we conclude that *yddJ* is needed only in recipients to mediate exclusion.

YddJ is a putative lipoprotein with a cystatin-like fold

yddJ is predicted to encode a 126 aa lipoprotein (Zhou et al., 2008). Based on what is known about *B. subtilis* lipoproteins, the N-terminal 18 aa should serve as a signal peptide that is cleaved at the cell surface. The cysteine at amino acid position 19 would serve as the site of lipid modification, resulting in a mature 108 aa form that is tethered to the cell membrane by the lipid anchor (Simonen and Palva, 1993).

Previous findings indicate that YddJ is a lipoprotein. In proteomic studies with glucose-starved *B. subtilis* cells (Otto et al., 2010), YddJ was detected in the enriched membrane fraction, but not in the biotinylation enrichment (which purifies membrane proteins containing an extracellular cysteine) or membrane-shaved (which purifies integral membrane proteins) fractions. These findings are consistent with the predicted lack of exposed, unmodified cysteines in mature YddJ available for biotinylation, and the fact that YddJ does not have any predicted transmembrane domains.

We searched for conserved motifs and structural similarity between YddJ and other proteins. Analysis using Phyre2 (Kelley et al., 2015) indicated that the residues 5-49 of the processed, mature YddJ (36% of the protein) modeled with 95.1% confidence to the crystal structure of a protein from *S. aureus* from the DUF4467 protein family. KEGG's SSDB Motif Search (Kanehisa et al., 2017) also identified DUF4467 as matching residues 24-118 of YddJ with an E-value of $4x10^{-31}$. DUF4467 is a large family of Gram-positive lipoproteins with a cystatin-like fold (Finn et al., 2017). Cystatins are a superfamily of proteins which act as inhibitors of C1 and C13 cysteine peptidase families (Brown and Dziegielewska, 1997). ICE*Bs1* contains a cell wall hydrolase, CwlT, with a peptidase domain belonging to the C1 family (Fukushima et al., 2008; Dewitt and Grossman, 2014). This similarity indicated that YddJ in the recipient cell might target the peptidase domain of CwlT in the donor cell.

CwlT is not the target of YddJ-mediated exclusion

CwlT is a bifunctional cell wall hydrolase, containing an N-terminal muramidase domain and a C-terminal peptidase domain (Fukushima et al., 2008; Dewitt and Grossman, 2014). Muramidase activity is virtually required for transfer, and peptidase activity is important, but partially dispensable; mutating the active site or deleting the peptidase domain results in a mutant ICE*Bs1* that has an approximately 1,000-fold decrease in transfer efficiency (Dewitt and Grossman, 2014).

If the peptidase domain and peptidase activity of CwlT was the target of YddJ-mediated exclusion, then CwlT mutants that lack peptidase activity or the peptidase domain should be insensitive to exclusion. We found the contrary. We monitored transfer of ICE*Bs1* from donor cells that contained wild type *cwlT* (MMB970) or a deletion of the peptidase domain of *cwlT* in

ICE*Bs1* (MA980) into recipient cells with or without over-expression of *yddJ*. ICE*Bs1* donors with the mutant *cwlT* were excluded by *yddJ* over-expressing recipients ~2,000-fold (0.02% transconjugants/donor into recipient cells without *yddJ*, compared to $2x10^{-5}$ % transconjugants/donor into recipient cells with *yddJ*), nearly the same extent (~3000-fold) as ICE*Bs1* containing wild type *cwlT* (~18% transconjugants/donor into recipient cells without *yddJ*, compared to $6x10^{-3}$ % transconjugants/donor into recipient cells with *yddJ*), revealing that the peptidase domain of CwlT is not the target of YddJ.

Isolation of exclusion-resistant mutations in ICE*Bs1*

We hypothesized that YddJ on recipient bacteria interacted with some part of the ICE*Bs1*-encoded conjugation machinery in the donor. If so, then mutations in the target gene(s) encoding the ICE*Bs1* conjugation machinery might make donors resistant to YddJ-mediated exclusion. To identify potential target(s) of *yddJ*-mediated exclusion in ICE*Bs1*, we performed a mutagenesis and enrichment screen to identify exclusion-resistant, transfer-competent mutants of ICE*Bs1*. We randomly mutagenized a pool of ICE*Bs1* donor cells with ethyl methanesulfonate to generate point mutations throughout the chromosome. We used this pool of mutagenized cells as donors, and selected for transfer of ICE*Bs1* into recipients that were over-expressing *yddJ*. We selected for and pooled transconjugants and then used the transconjugant pool as donors in successive rounds of mating with recipients that over-expressed *yddJ* **(**Fig. 4A**)**. We expected that any exclusion-resistant ICE*Bs1* mutants that were fully functional for conjugation would transfer up to 1000-fold more efficiently than wild type (exclusion-sensitive) ICE*Bs1*. After several rounds of conjugation, such an exclusion-resistant mutant should be enriched in the transconjugant population. We mutagenized 16 independent

cultures of donor cells and enriched for mutants with increased transfer efficiency from each separate culture. We purified transconjugants and sequenced ICE*Bs1* to identify exclusion-resistant mutations.

From 16 independently mutagenized cultures, we identified three different point mutations in *conG* of ICE*Bs1* that caused an exclusion-resistant phenotype without causing a defect in transfer efficiency (Fig. 4B). A glutamate-to-lysine mutation at residue 288 (*conG-E288K*) was isolated from 14 of the independent cultures. Two other mutations, an aspartate-to-tyrosine and aspartate-to-glycine mutation at residue 292 (*conG-D292Y*; *conG-D292G*) were each isolated once. We compared transfer and exclusion of wild type and *conG-E288K* ICE*Bs1* donors and confirmed that the *conG-E288K* mutation fully abolished exclusion by *yddJ* without affecting transfer efficiency (Fig. 4C). In preliminary testing, we found that ICE*Bs1* donors containing *conG-D292Y* or *conG-D292G* mutations behaved similarly to *conG-E288K*.

ConG is essential for ICE*Bs1* conjugation and is predicted to have seven transmembrane domains that form part of the mating channel (Babic et al., 2011; Leonetti et al., 2015). The exclusion-resistant point mutations were in a region predicted to form part of an extracellular loop between the third and fourth transmembrane domains (Fig. 4B). The existence of exclusion-resistant mutations in *conG* strongly indicates that ConG in the donor is the target of YddJ in the recipient.

ConG and YddJ determine exclusion specificity

We postulated that YddJ specifically recognizes and targets its cognate ConG, as was indicated by the inability of YddJ to inhibit Tn*916*-mediated transfer, despite the presence of a *conG* homolog in Tn*916*. However, Tn*916* does not have a homolog of *yddJ*, nor does it have

exclusion. We identified a homolog of *yddJ* in ICE*Bat1*, a putative ICE found in *Bacillus atrophaeus*.

Proteins from ICE*Bat1* compared to those from ICE*Bs1*. ICE*Bat1* is similar to ICE*Bs1*. It contains homologs for all ICE*Bs1* genes needed for regulation and conjugation, including *conG*. Most conjugation proteins are virtually identical (>95%); however, ConG is less so (only 87% identical), and the differences are concentrated in two regions (Fig. 5A): the loop region between the predicted third and fourth transmembrane domains and the predicted extracellular C-terminal region. Notably, the loop region between the third and fourth transmembrane domains is the location of the mutations in $ConG_{ICE\mathit{Bs1}}$ that confer resistance to YddJ-mediated exclusion.

We also found that YddJ is more divergent (only 67% identical) between these two ICEs than most of the other ICE proteins (Fig. 5B). There are four regions of sequence divergence with at least two consecutive non-identical residues: 1) residues 30-48 on ICE*Bs1* YddJ and 30-50 on ICE*Bat1* YddJ, 2) residues 57-58 on ICE*Bs1* YddJ and 59-60 on ICE*Bat1* YddJ, 3) residues 65-81 on ICE*Bs1* YddJ and 67-82 on ICE*Bat1* YddJ, and 4) 86-95 on ICE*Bs1* YddJ and 87-96 on ICE*Bat1* YddJ. These differences are consistent with the notion that ConG and YddJ determine exclusion specificity.

ConG from ICE*Bat1* functions in the context of the ICE*Bs1* conjugation machinery. *B. atrophaeus* is difficult to manipulate genetically and little is known about the function of ICE*Bat1*. Therefore, to test function and specificity, we introduced *conG* and *yddJ* from ICE*Bat1* into *B. subtilis*.

We first determined that ConG from ICE*Bat1* was able to function with the ICE*Bs1*-encoded conjugation machinery. We constructed donor strains with ICE*Bs1* containing *conG* from either ICE*Bs1* or ICE*Bat1*. In both cases, *conG* was deleted from its native locus within ICE*Bs1*, and a copy of $conG_{ICEBs1}$ (ICE*Bs1* Δ*conG* Δ(*rapI-phrI*)*342*::*kan*, *thrC*::{Pspank(hy)-$conG_{ICEBs1}$ *mls*}; KPD225) or $conG_{ICEBat1}$ (ICE*Bs1* Δ*conG* Δ(*rapI-phrI*)*342*::*kan*, *thrC*::{Pspank(hy)-$conG_{ICEBat1}$ *mls*}; KPD224) was expressed ectopically from Pspank(hy) located at *thrC*. Both strains contained Pxyl-*rapI* for xylose-inducible activation of ICE.

We found that both $conG_{ICEBs1}$ and $conG_{ICEBat1}$ complemented the transfer defect caused by loss of *conG* in ICE*Bs1*. As determined previously, $conG_{ICEBs1}$ fully restored transfer to wild type levels (Babic et al., 2011; Leonetti et al., 2015). $conG_{ICEBat1}$ largely restored conjugation, but to levels ~10-fold lower than those with $conG_{ICEBs1}$ (Fig. 5C). Based on these results, we conclude that $conG_{ICEBat1}$ is largely functional in the context of the ICE*Bs1*-encoded conjugation machinery.

YddJ inhibited transfer only when the conjugation machinery contained the cognate ConG. We monitored ICE transfer from donors expressing either $conG_{ICEBs1}$ or $conG_{ICEBat1}$ into recipient strains that expressed *yddJ* from either ICE*Bs1* (MA982) or ICE*Bat1* (KPD219). Donor cells expressing $conG_{ICEBs1}$ as part of the conjugation machinery were sensitive to exclusion by recipient cells expressing $yddJ_{ICEBs1}$ (Fig. 5C), recapitulating results presented above. However, they were not sensitive to exclusion by recipients expressing $yddJ_{ICEBat1}$ (Fig. 5C). The failure of $YddJ_{ICEBat1}$ to inhibit the conjugation machinery containing ConG from ICE*Bs1* is intriguing, but could simply reflect a non-functional $yddJ_{ICEBat1}$, due perhaps to lack of expression, misfolding,

or a defective gene. Alternatively, if YddJ$_{ICEBat1}$ is functional, then it could indicate that the ConG-YddJ pair determines specificity of exclusion.

We found that YddJ$_{ICEBat1}$ was indeed functional and was able to inhibit the conjugation machinery that contained ConG from ICE*Bat1*. Donor cells expressing *conG*$_{ICEBat1}$ as part of the conjugation machinery were able to transfer ICE*Bs1* at a frequency of $6x10^{-2}$ % into cells without *yddJ*. Transfer was reduced ~2,000-fold into recipients that expressed *yddJ*$_{ICEBat1}$, but there was no significant reduction into recipient cells that expressed *yddJ*$_{ICEBs1}$ (Fig. 5C).

To summarize these results: 1) YddJ$_{ICEBat1}$ is functional and capable of inhibiting transfer from the ICE*Bs1* conjugation machinery that contains ConG$_{ICEBat1}$, but does not inhibit transfer from the ICE*Bs1* conjugation machinery containing ConG$_{ICEBs1}$; 2) YddJ$_{ICEBs1}$ is capable of inhibiting transfer from the ICE*Bs1* conjugation machinery that contains the ConG$_{ICEBs1}$, but does not inhibit transfer from the ICE*Bs1* conjugation machinery that contains ConG$_{ICEBat1}$. Together, these results demonstrate that the specificity for exclusion resides with ConG and YddJ, and that YddJ-mediated exclusion is specific for its cognate element, even between highly related ICEs.

Exclusion is beneficial to ICE*Bs1* and its host cells by preventing loss of viability due to redundant transfer

We tested whether exclusion conferred any benefit to ICE*Bs1* and/or its host cells by measuring the viability of cells containing ICE*Bs1* with and without exclusion. Cells containing ICE*Bs1* (with or without a functional exclusion system) were grown in defined minimal medium, ICE*Bs1* was activated by over-expression of *rapI*, and ~$8x10^8$ cells were filtered and then incubated as in a mating experiment (Materials and Methods). Cells were then recovered from

the filters and the number of viable cells (CFUs) was determined and compared to that of the initial input onto the filter.

We found that cells containing ICE*Bs1* with a defective exclusion system had decreased viability relative to cells with a functional exclusion system. This decrease in viability was dependent on conditions that favor conjugation, including activation of ICE*Bs1*, the presence of a functional conjugation system, and a sufficient cell density (see below). In all cases, the number of exclusion-competent cells (ICE*Bs1 yddJ*+; MA1049) recovered from the filter was ~80-90% of the initial input. In contrast, in the absence of a functional exclusion system (ICE*Bs1* Δ*yddJ*; MA1050, and ICE*Bs1 conG-E288K*; MA1089) the number of cells recovered was <25% of the input (Fig. 6A). These results indicate that something about exclusion was likely preventing cell death under the conditions tested.

The cell death observed in the absence of a functional exclusion system could be due to cell autonomous effects of *yddJ*, or possibly excessive conjugation, or both. To test this, we used three different conditions that would limit or eliminate conjugation and monitored the effects of a functional exclusion system on cell death. We found that cell death in the absence of exclusion was dependent on conditions that favor conjugative transfer. The three conditions tested were:

1) No activation of ICE*Bs1*. In experiments in which ICE*Bs1* was not activated (no expression of *rapI*), there was no detectable effect of the presence or absence of a functional exclusion system on cell viability (Fig. 6B).

2) An ICE*Bs1* mutant that is incapable of transfer. We introduced a *conQ* null mutation (*ΔconQ*) into ICE*Bs1* with *yddJ* (MA1070) or without *yddJ* (MA1069). *conQ* encodes the coupling

protein that 'brings' the DNA substrate for transfer to the conjugation machinery and is essential for transfer (Lee et al., 2012). In the *ΔconQ* mutant, the absence of *yddJ* (no exclusion) did not cause a decrease in viability compared to *ΔconQ, yddJ+* (Fig. 6C).

3) Conditions of low cell density. Conjugative transfer between cells is dependent on cell-cell contact. At low cell densities, there will be few mating pairs formed and low transfer frequencies. We measured the viability of cells with activated ICE*Bs1* at high and low cell density. ICE*Bs1*-containing cells were grown and activated by over-expression of *rapI* as described above. After 2 hours of activation, ~$8x10^8$ or ~$3x10^6$ cells were filtered and incubated for 3 hours. Viable cells were recovered and quantified as described above.

We found that cells containing defective exclusion systems had increased viability under low cell density (conditions that prevent conjugation), compared to high cell density (conditions that promote conjugation). Cells containing exclusion-competent ICE*Bs1* (ICE*Bs1 yddJ+*; MA1049) were recovered >100% (indicative of cell growth) of initial input at low cell density, and ~90% of initial input at high cell density. In contrast, cells containing exclusion-deficient ICE*Bs1* (ICE*Bs1* Δ*yddJ*; MA1050, and ICE*Bs1 conG-E288K*; MA1089) were recovered >100% of initial input at low cell density, compared to <30% of initial input at high cell density (Fig. 6D). Together, these results indicate that exclusion confers a benefit to ICE*Bs1* and its host cell by preventing redundant transfer, thus protecting host cell viability under conditions of high transfer.

DISCUSSION

Experiments described here demonstrate that ICE*Bs1* encodes an exclusion mechanism that is beneficial to cells with the element. The ICE*Bs1* gene *yddJ* causes exclusion in recipient cells by inhibiting transfer from the ICE*Bs1* conjugation machinery in donor cells. The target of YddJ-mediated exclusion is the essential conjugation protein ConG, a conserved protein with seven predicted transmembrane domains. Exclusion protects ICE*Bs1* and its host cells against cell death caused by redundant transfer. Together with previous findings, we conclude that ICE*Bs1* has three distinct mechanisms to inhibit host cells from acquiring a second copy of the element. 1) RapI-dependent, SOS-independent activation under conditions of high population density, the earliest step in the ICE*Bs1* life cycle, is inhibited by PhrI-mediated cell signaling if a host cell is surrounded by other cells containing ICE*Bs1* (Auchtung et al., 2005). 2) Exclusion (described here), inhibits transfer of DNA through the ICE*Bs1* conjugation machinery. This inhibition appears to be independent of the DNA substrate to be transferred. 3) If ICE*Bs1* is transferred to a cell that already has a copy, then repressor-mediated immunity inhibits integration and stable acquisition of that copy of the element (Auchtung et al., 2007). We postulate that these mechanisms all contribute to the stability of ICE*Bs1* in a host genome and that introduction of a second copy will lead to recombination between and instability of the linked elements.

Selective advantage of exclusion

To date, experimental evidence demonstrating the selective advantages of exclusion has come from the characterization of lethal zygosis in the F plasmid (Skurray et al., 1973, 1974, 1976; Ou, 1980). Recipients lacking the F plasmid (F-) are killed when mixed with an excess of Hfr or F+ exclusion-null donors. F+ exclusion-null cells are also susceptible to lethal zygosis

when used as recipients in matings with excess Hfr donors. These results indicate that exclusion is important for protecting the recipient cell from excess transfer, whether it is an established host or an unoccupied cell, and that unidirectional transfer from donor to recipient is sufficient for killing.

Our findings are similar to the lethal zygosis studies above, in that under conditions that support conjugative transfer, exclusion protects the element and host cell by preventing excessive transfer and cell death. We observe killing in the absence of exclusion with donors that are induced to transfer ICE amongst themselves. These experiments do not distinguish whether it is mutual or unidirectional transfer between donor cells that results in killing, though we speculate that the former is likely. The mechanism of killing in lethal zygosis was proposed to be excessive recipient membrane damage. Experiments in which Hfr donors were mixed with F- recipients whose cell walls were labeled with radioactive DAP resulted in a release of the recipient's cell wall material into the medium (Ou, 1980). This may also be the case in the context of ICE*Bs1* and *B. subtilis,* where assembling the type IV secretion system and transferring to/from donor cells at high frequencies results in extensive degradation of the thick cell wall.

Under what natural scenarios would a conjugative element and its host cell encounter such conditions of extreme transfer that exclusion protects against? One possibility is when an element transfers rapidly through chains of recipient cells, as has been demonstrated for ICE*Bs1* and *B. subtilis* (Babic et al., 2011), or complex bacterial communities such as biofilms **(**Lécuyer et al., 2018). It is not hard to imagine that exclusion in such contexts would be the main mechanism to prevent excessive transfer and maintain host cell viability. In addition to its

protective role under conditions of high transfer, it is also likely that exclusion contributes to the efficient propagation of an element through a cell population by preventing futile transfer into a cell already occupied by a copy of the element.

Diverse proteins mediating exclusion

YddJ is a member of the DUF4467 family consisting of ~250 lipoproteins found in various Gram-positive bacteria (multiple species of *Bacillus*, Staphylococcus, Streptococcus, Listeria, etc.) (Finn et al., 2017). YddJ is one of the first DUF4467 members with an established function, that of an exclusion protein. We suspect that other YddJ-like proteins associated with conjugative elements are exclusion proteins as well. It is also possible that there are YddJ-like proteins that, like cystatins, target cysteine peptidases. This was the scenario we initially hypothesized for ICE*Bs1* exclusion; our initial hypothesis was that YddJ inhibited the peptidase domain of the cell wall hydrolase CwlT from ICE*Bs1*. Such peptidase inhibitors, if they exist, may regulate hydrolases from conjugative elements and modulate their transfer. It is also possible that such peptidase inhibitors have a role in regulating cell wall metabolism.

Genes that mediate exclusion have been identified in many conjugative elements, mostly from the major incompatibility groups of conjugative plasmids (Garcillan-Barcia and de la Cruz, 2008). Exclusion proteins are generally diverse with no consistently conserved domains. Any similarity is typically between proteins from the same plasmid groups. Exclusion proteins do have a few general features in common. They tend to be relatively small transmembrane proteins or lipoproteins. Most are not required in donor cells, either for conjugation or exclusion, with the noted exception of R27 (Gunton et al., 2008), and are sufficient for exclusion in the recipient cells.

A conserved target for exclusion proteins?

The target of the exclusion system of ICE*Bs1* is ConG, an essential component of the conjugation machinery. ConG is predicted to have seven transmembrane domains and a large extracellular C-terminal region. *orf15* of Tn*916* encodes a similar protein (Burrus et al., 2002), although some isolates of Tn*916* appear to encode a truncated form of Orf15 that is missing the large extracellular C-terminal region (Browne et al., 2015). ConG is thought to be part of the channel through which substrate is transferred (Auchtung et al., 2016) and is homologous to VirB6, a five transmembrane domain inner membrane protein predicted to form the channel of the Ti plasmid of *A. tumefaciens* (Jakubowski et al., 2004).

All type IV secretion systems have a polytopic protein homologous or analogous to VirB6 that performs a similar function (Bhatty et al., 2013), and these proteins have been identified as the donor targets for the exclusion systems of the F/R100 plasmids, SXT/R391 ICEs, and R64/R621a plasmids (isolated from Gram-negative bacteria and characterized in *E.coli*). The targets for both F/R100 and SXT/R391 exclusion are TraG proteins, VirB6 homologs (Beaber et al., 2002; Lawley et al., 2003) predicted to have 3-5 transmembrane domains (Audette et al., 2007; Marrero et al., 2007). The target for R64/R621a exclusion is TraY, proposed to be a VirB6 analog (Guglielmini et al., 2014) predicted to have seven transmembrane domains (Komano et al., 2000).

The targets of exclusion in the donor likely share the function of forming the channel of the secretion system through which the substrate travels. The fact that diverse exclusion proteins with no obvious sequence similarity converge upon targeting this function indicates that this is a conserved strategy for exclusion.

Even though all type IV secretion systems probably have a ConG/VirB6 homolog or analog, they do not all have exclusion systems. For example, Tn*916*, an ICE whose conjugation machinery is closely related to that of ICE*Bs1*, does not have an exclusion system (Norgren and Scott, 1991). This could be due to some fundamental difference in the components of the T4SS, for example, differences in sequence or in key contacts made between those components. Alternatively, we believe that most conjugation systems likely have the potential for exclusion, and the presence or absence of exclusion depends on some significant selective advantage to having an exclusion system. Variables that might contribute to selective pressures could include the efficiency of activation of an element, the efficiency of transfer, and the potential cost to the host and/or element to having multiple copies of an element in a single cell.

MATERIALS and METHODS

Media and growth conditions

Cells were grown at 37°C with shaking in $S7_{50}$ defined minimal medium (Jaacks et al., 1989) supplemented with auxotrophic requirements (40 μg/ml tryptophan, 40 μg/ml phenylalanine, 200 μg/ml threonine, as needed), or LB medium as indicated. Antibiotics were used at the following concentrations for growth on LB agar plates: chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), streptomycin (100 μg/ml), spectinomycin (100 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. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM to induce expression from the Pspank(hy) promoter.

When 1mM IPTG was added to cells without ICE*Bs1* and with no genes under the control of the Pspank(HY) promoter, no deleterious effects on growth were observed. Tetracycline was used at a final concentration of 2.5 μg/ml to stimulate Tn*916* gene expression and excision in donor cells.

Strains and alleles

The *B. subtilis* strains used are listed in Table 1. Standard techniques were used for cloning and strain construction (Harwood and Cutting, 1990). Some alleles related to ICE*Bs1* were previously described and are summarized below. Donor strains contained a derivative of ICE*Bs1* that contains a deletion of *rapI-phrI* and a kanamycin-resistance cassette inserted, Δ(*rapI-phrI*)*342*::*kan* (Auchtung et al., 2005). *rapI* was over-expressed from Pxyl-*rapI* to induce ICE*Bs1* gene expression and excision in donor cells. Alleles were integrated into *amyE* with various antibiotic resistances, and included: *amyE*::{Pxyl-*rapI spc*} (Berkmen et al., 2010), *amyE*::{Pxyl-*rapI cat*}, and *amyE*::{Pxyl-*rapI mls*} (Johnson and Grossman, 2014). ICE*Bs1*^{0} indicates that the strain is cured of ICE*Bs1*. Recipients were typically streptomycin-resistant (*str-84*) (Auchtung et al., 2005; Lee et al., 2007), and streptomycin was used as a counter-selective marker in mating experiments unless otherwise indicated. The donor strain MA980 contains the peptidase deletion of *cwlT*, *cwlT*Δ(*207-327*), described previously (Dewitt and Grossman, 2014). Donor strains containing a deletion of *conG*, Δ*conG*(*5-805*), were derived from MMB1283 (Leonetti et al., 2015). Donor strains containing a deletion of *conQ*, Δ*conQ848*, were derived from CAL848 (Lee et al., 2012).

Construction of *comK* and *comC* null mutations. Null mutations in *comK* and *comC* were used to prevent transformation. The *comK*::*spc* allele has been described (Auchtung et al.,

2005). The *comK*::*tet* allele replaced the *comK* open reading frame with the *tet* cassette from pDG1513 (Guerout-Fleury et al., 1995). The *comK*::*cat* allele was derived from CAL419 and has been described (Lee et al., 2007). The *comC*::*mls* allele replaced from 324 bp upstream to 26 bp downstream of the *comC* open reading frame with the *mls* cassette from pHP13 (Lee et al., 2012).

Construction of pC194-containing ICE*Bs1* and Tn*916* donor strains for mobilization assays. pC194-containing ICE*Bs1* strain MA116 was derived by transforming pC194 into the ICE*Bs1* donor strain MMB970 (ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*) and selecting for chloramphenicol resistance. pC194-containing Tn*916* strain MA1100 was derived by first transforming JMA222 (ICE*Bs1*^{0}) (Auchtung et al., 2005) with chromosomal DNA from BS49 (Browne et al., 2015; Haraldsen et al., 2003) and selecting for tetracycline resistance to introduce Tn*916*. The *comC*::*mls* allele was introduced by transformation. pC194 was introduced by mobilization (conjugation) from strain MA116 and selecting for chloramphenicol and tetracycline resistance.

Deletion of *yddJ*. We constructed a deletion of *yddJ* that extends from the first base pair downstream from the *yddI* open reading frame (61 base pairs upstream from the *yddJ* start codon to the second bp downstream from the *yddJ* open reading frame). This deletion was constructed by amplifying two ~0.5 kb fragments containing DNA flanking the deletion endpoints by PCR and cloning them in pCAL1422 (a plasmid that contains *E. coli lacZ*) via isothermal assembly (Gibson et al., 2009), essentially as previously described for other alleles (Thomas et al., 2013; Wright et al., 2015). The resulting plasmid, pTD113, was integrated into the chromosome via single-crossover recombination. Transformants were screened for loss of

lacZ, indicating loss of the integrated plasmid, and PCR was used to identify a clone that contained the Δ*yddJ* allele.

Construction of PyddJ-*yddJ* and Pspank(hy)-*yddJ* at *lacA*. We expressed *yddJ* from its own promoter by inserting the region spanning 600 bp upstream to the end of the *yddJ* open reading frame into *lacA* to generate PyddJ-*yddJ*, present in strain MA996. We also fused *yddJ* to the LacI-repressible IPTG-inducible promoter Pspank(hy) to test *yddJ* function. Constructs included Pspank(hy)-*yddJ* (from ICE*Bs1*), present in strain MA982, and Pspank(hy)-$yddJ_{ICEBat1}$, (*yddJ* from ICE*Bat1*) present in strain KPD219. *yddJ* from ICE*Bs1* was PCR amplified from genomic DNA from strain AG174. $yddJ_{ICEBat1}$ was amplified by PCR from genomic DNA from *B. atrophaeus* strain 11A1 (from the *Bacillus* Genetic Stock Center; www.bgsc.org). For the PyddJ-*yddJ* construct, the PCR fragment was inserted by isothermal assembly between the PacI and SacI sites of pCJ80, a cloning vector that contains Pspank(hy), *lacI*, an *mls* cassette, and flanking homology for insertion by double-crossover into the chromosome at *lacA* (Wright and Grossman, 2016). For the Pspank(hy)-*yddJ* constructs, the PCR fragments were inserted by isothermal assembly between the SphI and SacI sites of pCJ80. The alleles were integrated by double cross-over into the chromosome by transformation and selecting for MLS resistance, generating the alleles *lacA*::{Pspank(hy)-*yddJ mls*} or *lacA*::{Pspank(hy)-$yddJ_{ICEBat1}$ *mls*}.

Construction of Pspank(hy)-*conG* at *thrC*. *conG* was amplified by PCR from *B. subtilis* or *B. atrophaeus* genomic DNA and fused to Pspank(hy) essentially as described (Leonetti et al., 2015). Constructs included Pspank(hy)-$conG_{ICEBs1}$, present in strain KPD225, and Pspank(hy)-$conG_{ICEBat1}$, present in strain KPD224. These alleles were used to complement the Δ*conG*(*5-805*) deletion in ICE*Bs1*.

Construction of isogenic strains used for determining effects of exclusion on survival.

Transfer-competent wild type and exclusion-deficient mutants of ICE*Bs1* (containing the Δ(*rapI-phrI*)*342*::*kan* allele) were transferred by conjugation into MA1027 (ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *comC*::*mls*). The corresponding transfer-deficient mutants of ICE*Bs1* were transformed into BOSE986 (ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*}), then transformed with DNA from MA1012 (ICE*Bs1*^{0} *comC*::*mls*) selecting for *mls*, thereby making the cells defective in competence. MA1049 (ICE*Bs1*), MA1050 (ICE*Bs1* Δ*yddJ*), and MA1089 (ICE*Bs1 conG-E288K*) were derived by transferring ICE from MMB970, MA11, and KPD80 into MA1027, and selecting for kanamycin and MLS resistance. MA1069 (ICE*Bs1* Δ*yddJ* Δ*conQ*) and MA1070 (ICE*Bs1* Δ*conQ*) were derived by transforming chromosomal DNA from CAL848 with and without Δ*yddJ* into BOSE986, selecting for kanamycin resistance, and introducing *comC*::*mls* by transforming chromosomal DNA from MA1012.

Mating assays

Mating assays for ICE*Bs1* were performed essentially as described (Auchtung et al., 2005; Lee et al., 2007). Briefly, donor and recipient cells were grown in $S7_{50}$ defined minimal medium containing 0.1 % glutamate and 1% arabinose until they reached mid-exponential growth phase. At an OD600 of 0.2, 1% xylose was added to donors to induce expression of Pxyl-*rapI*, causing induction of ICE*Bs1*. For recipients containing Pspank(hy)-*yddJ*, 1 mM IPTG was added as indicated. After 2 hours of growth in the presence of xylose, equal numbers (~$4x10^8$ cells each) of donor and recipient cells were mixed and collected by vacuum filtration onto a nitrocellulose filter. Filters were incubated at 37°C for 3 hours on 1.5% agar plates containing 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-$2H_2O$, 0.2 g/l

$MgSO_4$-$7H_20$) (Harwood and Cutting, 1990). Cells were resuspended from the filters, diluted and plated on LB agar plates containing the appropriate antibiotics to select for transconjugants. Plates were incubated at 37°C overnight to allow for colony growth. The number of donor cells (CFU/ml) was determined at the time of cell mixing (after growth in xylose for 2 hours). Mating efficiency was calculated as the percent transconjugants CFU/ml per initial donor CFU/ml. The number of initial donors, rather than the number of viable donors post-mating, was used in these calculations for two reasons: 1) there is limited growth of cells on filters during mating; and importantly, 2) there is some loss of viability of donor cells on the filters during mating. This loss of viability leads to an overestimate of mating efficiencies per initial donor.

Mutagenesis and enrichment screens

For the initial round of the screen, 16 independent cultures of ICE*Bs1* donor cells (strain KPD38) were grown in LB medium to mid-exponential phase, and mutagenized with 1.2% ethyl methylsulfonate for 40 minutes, resulting in ~50% killing and a ~100-fold increase in the frequency of streptomycin resistant mutants, essentially as described (Grossman et al., 1992).Cells were pelleted, washed twice with LB, then resuspended to an OD600 of 0.125 and allowed to continue growing. At an OD600 of 0.5, *rapI* expression was induced by adding 1% xylose, and cells were grown for 30 minutes to an OD600 of ~1. Recipient cells (KPD36: ICE*Bs1*^{0}, Pxyl-*rapI cat*, Pspank(hy)-*yddJ*, *comK*::*tet*) were grown in LB to mid-exponential phase. At an OD600 of 0.1, *yddJ* expression in recipients was induced with 1mM IPTG, and cells were grown to an OD600 of ~1.0. Equal numbers of donor and recipient cells were mixed and collected by vacuum filtration onto a nitrocellulose filter. Filters were incubated at 37°C for 3 hours on 1.5%

agar plates containing 1x Spizizen's salts. Cells were resuspended from the filters and plated on LB agar containing tetracycline and kanamycin to select for transconjugants.

For the second round of the screen, transconjugants from the previous round (now donors) were scraped off of the selection plates (above), resuspended in LB containing kanamycin and tetracycline, and diluted to an OD600 of 0.125. These donor cells were grown to an OD600 of 0.5 and induced as described above. Recipient cells (KPD35: ICE*Bs1*^{0}, Pxyl-*rapI spc*, Pspank(hy)-*yddJ*, *str-84 comK*::*tet*) for the second round were grown and induced as described above. As before, donor and recipient cells were mixed, filtered, and incubated at 37°C for 3 hours on 1.5% agar plates containing 1x Spizizen's salts. Cells were resuspended from the filters and plated on LB agar containing spectinomycin and kanamycin to select for transconjugants.

Rounds three and four of the screen repeated the process described for round two with alternating recipient strains: In round three, KPD36 was used as the recipient strain and transconjugants were selected with chloramphenicol and kanamycin. In round four, KPD35 was used as the recipient strain and transconjugants were selected with spectinomycin and kanamycin. After four rounds of the screen, exclusion-resistant mutants were sufficiently enriched in the transconjugant population that exclusion by *yddJ* was no longer observed. At this point, transconjugants were restreaked to purity, genomic DNA was isolated from two colonies from each of the 16 independent parallel enrichments, and ICE*Bs1* was sequenced to identify the mutations that were likely causing the exclusion-resistant phenotype.

Mating survival assays

Cells were grown in $S7_{50}$ defined minimal medium containing 0.1% glutamate and 1% arabinose until they reached mid-exponential growth phase. At an OD600 of 0.2, 1% xylose was

added to induce expression of Pxyl-*rapI*, causing induction of ICE*Bs1*. After 2 hours of growth in the presence of xylose, ~$8x10^8$ cells were mixed with 5 ml of medium and filtered onto a nitrocellulose filter and incubated at 37°C for 3 hours on 1.5% agar plates containing 1x Spizizen's salts, as if for a mating experiment. Cells were resuspended from the filters, diluted and plated on LB agar to determine post-mating viable cell counts. The number of viable cells was also determined prior to filtering the cells.

For survival assays testing the effect of high and low cell density, cells were prepared as described above, with the exception that after 2 hours of induction with xylose, either ~$8x10^8$ or ~$3x10^6$ cells were mixed with 5 ml or 10 ml of medium, respectively, before sampling and filtering.

DATA AVAILABILTY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

None. We have no conflicts of interests.

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REFERENCES

Anthony, K. G., Klimke, W. A., Manchak, J., & Frost, L. S. (1999). Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100-1: Insights into the mechanism of conjugation. *J Bacteriol*, *181*(17), 5149–5159.

Auchtung, J. M., Aleksanyan, N., Bulku, A., & Berkmen, M. B. (2016). Biology of ICE*Bs1*, an integrative and conjugative element in *Bacillus subtilis*. *Plasmid*, *86*, 14–25. https://doi.org/10.1016/j.plasmid.2016.07.001

Auchtung, J. M., Lee, C. a, Garrison, K. L., & Grossman, A. D. (2007). Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICE*Bs1* of *Bacillus subtilis*. *Mol Microbiol*, *64*(6), 1515–1528. https://doi.org/10.1111/j.1365-2958.2007.05748.x

Auchtung, J. M., Lee, C. a, Monson, R. E., Lehman, A. P., & Grossman, A. D. (2005). Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response. *PNAS*, *102*(35), 12554–12559. https://doi.org/10.1073/pnas.0505835102

Audette, G. F., Manchak, J., Beatty, P., Klimke, W. a, & Frost, L. S. (2007). Entry exclusion in F-like plasmids requires intact TraG in the donor that recognizes its cognate TraS in the recipient. *Microbiology*, *153*(Pt 2), 442–451. https://doi.org/10.1099/mic.0.2006/001917-0

Babic, A., Berkmen, M. B., Lee, C. A., & Grossman, A. D. (2011). Efficient Gene Transfer in Bacterial Cell Chains. *MBio*, *2*(2), e00027-11. https://doi.org/10.1128/mBio.00027-11.Editor

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2002). Genomic and Functional Analyses of SXT , an Integrating Antibiotic Resistance Gene Transfer Element Derived from Vibrio cholerae Genomic and Functional Analyses of SXT , an Integrating Antibiotic Resistance Gene Transfer Element Derived from Vibrio choler. *J Bacteriol*, *184*(15), 4259–4269. https://doi.org/10.1128/JB.184.15.4259

Berkmen, M. B., Lee, C. A., Loveday, E., & Grossman, A. D. (2010). Polar Positioning of a Conjugation Protein from the Integrative and Conjugative Element ICE*Bs1* of *Bacillus subtilis*. *J Bacteriol*, *192*(1), 38–45. https://doi.org/10.1128/JB.00860-09

Bhatty, M., Laverde Gomez, J. A., & Christie, P. J. (2013). The Expanding Bacterial Type IV Lexicon. *Res Microbiol*, *164*(6). https://doi.org/10.1037/a0018493.Understanding

Bose, B., Auchtung, J. M., Lee, C. A., & Grossman, A. D. (2008). A conserved anti-repressor controls horizontal gene transfer by proteolysis. *Mol Microbiol*, *70*(3), 570–582. https://doi.org/10.1111/j.1365-2958.2008.06414.x.A

Brown, W. M., & Dziegielewska, K. M. (1997). Friends and relations of the cystatin superfamily--new members and their evolution. *Protein Science : A Publication of the Protein Society*, *6*(1), 5–12. https://doi.org/10.1002/pro.5560060102

Browne, H. P., Anvar, S. Y., Frank, J., Lawley, T. D., Roberts, A. P., & Smits, W. K. (2015). Complete genome sequence of BS49 and draft genome sequence of BS34A, *Bacillus subtilis* strains carrying Tn*916*. *FEMS Microbiol Lett*, *362*(3), 1–4. https://doi.org/10.1093/femsle/fnu050

Burrus, V., Pavlovic, G., Decaris, B., & Guédon, G. (2002). The ICESt1 element of Streptococcus thermophilus belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration. *Plasmid*, *48*(2), 77–97. https://doi.org/10.1016/S0147-619X(02)00102-6

Burrus, V., & Waldor, M. K. (2004). Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol*, *155*(5), 376–386. https://doi.org/10.1016/j.resmic.2004.01.012

Dewitt, T., & Grossman, A. D. (2014). The Bifunctional Cell Wall Hydrolase CwlT Is Needed for Conjugation of the Integrative and Conjugative Element ICE*Bs1* in *Bacillus subtilis* and B. anthracis. *J Bacteriol*, *196*(8), 1588–1596. https://doi.org/10.1128/JB.00012-14

Earl, A. M., Losick, R., & Kolter, R. (2007). *Bacillus subtilis* Genome Diversity. *J Bacteriol*, *189*(3), 1163–1170. https://doi.org/10.1128/JB.01343-06

Finn, R. D., Attwood, T. K., Babbitt, P. C., Bateman, A., Bork, P., Bridge, A. J., … Mitchell, A. L. (2017). InterPro in 2017-beyond protein family and domain annotations. *Nucleic Acids Res*, *45*(Database issue), D190–D199. https://doi.org/10.1093/nar/gkw1107

Fukushima, T., Kitajima, T., Yamaguchi, H., Ouyang, Q., Furuhata, K., Yamamoto, H., … Sekiguchi, J. (2008). Identification and characterization of novel cell wall hydrolase CwlT: a two-domain autolysin exhibiting n-acetylmuramidase and DL-endopeptidase activities. *J Biol Chem*, *283*(17), 11117–11125. https://doi.org/10.1074/jbc.M706626200

Garcillan-Barcia, M. P., & de la Cruz, F. (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid*, *60*(1), 1–18. https://doi.org/10.1016/j.plasmid.2008.03.002

Gibson, D. G., Young, L., Chuang, R., Venter, J. C., Iii, C. A. H., Smith, H. O., & America, N. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*, *6*(5), 12–16. https://doi.org/10.1038/NMETH.1318

Goessweiner-Mohr, N., Arends, K., Keller, W., & Grohmann, E. (2014). Conjugation in Gram-Positive Bacteria. *Plasmids: Biology and Impact in Biotechnology and Discovery*, 237–256. https://doi.org/10.1128/microbiolspec.PLAS-0004-2013

Grohmann, E., Christie, P. J., Waksman, G., & Backert, S. (2018). Type IV secretion in Gram-negative and Gram-positive bacteria. *Mol Microbiol*, *107*(4), 455–471. https://doi.org/10.1111/mmi.13896

Grossman, A. D., Lewis, T., & Devivo, R. (1992). Suppressors of a *spo0A* missense mutation and their effects on sporulation in *Bacillus subtilis*. *Biochimie*, *74*, 679–688.

Guerout-Fleury, A.-M., Shazand, K., Frandsen, N., & Stragier, P. (1995). Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene*, *167*, 335–336.

Guglielmini, J., Néron, B., Abby, S. S., Garcillán-Barcia, M. P., La Cruz, D. F., & Rocha, E. P. C. (2014). Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. *Nucleic Acids Res*, *42*(9), 5715–5727. https://doi.org/10.1093/nar/gku194

Guglielmini, J., Quintais, L., Garcillán-Barcia, M. P., de la Cruz, F., & Rocha, E. P. C. (2011). The repertoire of ice in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. *PLoS Genet*, *7*(8). https://doi.org/10.1371/journal.pgen.1002222

Gunton, J. E., Ussher, J. E. R., Rooker, M. M., Wetsch, N. M., Alonso, G., & Taylor, D. E. (2008). Entry exclusion in the IncHI1 plasmid R27 is mediated by EexA and EexB. *Plasmid*, *59*(2), 86–101. https://doi.org/10.1016/j.plasmid.2007.11.004

Haraldsen, J. D., & Sonenshein, A. L. (2003). Efficient sporulation in *Clostridium difficile* requires disruption of the s K gene. *Mol Microbiol*, *48*(3), 811–821.

Harwood, C. R., & Cutting, S. M. (1990). *Molecular Biological Methods for Bacillus*. Chichester: John Wiley & Sons.

Hochhut, B., Marrero, J., & Waldor, M. K. (2000). Mobilization of Plasmids and Chromosomal

DNA Mediated by the SXT Element , a Constin Found in Vibrio cholerae O139. *J Bacteriol*, *182*(7), 2043–2047.
Jaacks, K. J., Healy, J., Losick, R., & Grossman, A. D. (1989). Identification and Characterization of Genes Controlled by the Sporulation-Regulatory Gene *spo0H* in *Bacillus subtilis*. *J Bacteriol*, *171*(8), 4121–4129.
Jakubowski, S. J., Krishnamoorthy, V., Cascales, E., & Christie, P. J. (2004). *Agrobacterium tumefaciens* VirB6 Domains Direct the Ordered Export of a DNA Substrate Through a Type IV Secretion System. *J Mol Biol*, *341*(4), 961–977. https://doi.org/10.1037/a0017530.A
Johnson, C. M., & Grossman, A. D. (2014). Identification of host genes that affect acquisition of an integrative and conjugative element in *Bacillus subtilis*. *Mol Microbiol*, *93*(6), 1284–1301. https://doi.org/10.1111/mmi.12736
Johnson, C. M., & Grossman, A. D. (2015). Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annu Rev Genet*, *49*, 577–601. https://doi.org/10.1146/annurev-genet-112414-055018.
Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., & Morishima, K. (2017). KEGG : new perspectives on genomes , pathways , diseases and drugs. *Nucleic Acids Res*, *45*, 353–361. https://doi.org/10.1093/nar/gkw1092
Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modelling , prediction and analysis. *Nat Protoc*, *10*(6), 845–858. https://doi.org/10.1038/nprot.2015.053.The
Komano, T., Yoshida, T., Narahara, K., & Furuya, N. (2000). The transfer region of Incl1 plasmid R64: Similarities between R64 tra and Legionella icm/dot genes. *Mol Microbiol*, *35*(6), 1348–1359. https://doi.org/10.1046/j.1365-2958.2000.01769.x
Lawley, T. D., Klimke, W. A., Gubbins, M. J., & Frost, L. S. (2003). F factor conjugation is a true type IV secretion system. *FEMS Microbiol Lett*, *224*(1), 1–15. https://doi.org/10.1016/S0378-1097(03)00430-0
Lécuyer, F., Bourassa, J., Gélinas, M., Charron-Lamoureux, V., Burrus, V., Beauregard, P.B. (2018). Biofilm Formation Drives Transfer of the Conjugative Element ICE*Bs1* in *Bacillus subtilis*. *mSphere, 3*(5), e00473-18. https://doi.org/10.1128/mSphere.00473-18
Lee, C. A., & Grossman, A. D. (2007). Identification of the origin of transfer (*oriT*) and DNA relaxase required for conjugation of the integrative and conjugative element ICE*Bs1* of *Bacillus subtilis*. *J Bacteriol*, *189*(20), 7254–7261. https://doi.org/10.1128/JB.00932-07
Lee, C. A., Thomas, J., & Grossman, A. D. (2012). The *Bacillus subtilis* conjugative transposon ICE*Bs1* mobilizes plasmids lacking dedicated mobilization functions. *J Bacteriol*, *194*(12), 3165–3172. https://doi.org/10.1128/JB.00301-12
Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICE*Bs1* of *Bacillus subtilis*. *J Bacteriol*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15
Marrero, J., & Waldor, M. K. (2005). Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Dev Cell*, *8*(6), 963–970. https://doi.org/10.1016/j.devcel.2005.05.004
Marrero, J., & Waldor, M. K. (2007). Determinants of entry exclusion within Eex and TraG are cytoplasmic. *J Bacteriol*, *189*(17), 6469–6473. https://doi.org/10.1128/JB.00522-07

Naglich, J. G., & Andrews, R. E. (1988). Tn*916*-Dependent Conjugal Transfer of pC194 and pUB110 from *Bacillus subtilis* into *Bacillus thuringiensis* subsp. *israelensis*. *Plasmid*, *20*, 113–126.

Norgren, M., & Scott, J. R. (1991). The presence of conjugative transposon Tn*916* in the recipient strain does not impede transfer of a second copy of the element. *J Bacteriol*, *173*(1), 319–324. https://doi.org/10.1128/jb.173.1.319-324.1991

Otto, A., Bernhardt, J., Meyer, H., Schaffer, M., Herbst, F. A., Siebourg, J., … Becher, D. (2010). Systems-wide temporal proteomic profiling in glucose-starved *Bacillus subtilis*. *Nat Commun*, *1*(9). https://doi.org/10.1038/ncomms1137

Ou, J. T. (1980). Role of surface exclusion genes in lethal zygosis in Escherichia coli K12 mating. *Mol Gen Genet*, *178*(3), 573–581. https://doi.org/10.1007/BF00337863

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator , abrB : regulator synthesis is controlled by the spoOA sporulation gene in *Bacillus subtilis*. *Mol Microbiol*, *2*(6), 689–699.

Sakuma, T., Tazumi, S., Furuya, N., & Komano, T. (2013). ExcA proteins of IncI1 plasmid R64 and IncIγ plasmid R621a recognize different segments of their cognate TraY proteins in entry exclusion. *Plasmid*, *69*(2), 138–145. https://doi.org/10.1016/j.plasmid.2012.11.004

Simonen, M., & Palva, I. (1993). Protein Secretion in *Bacillus* Species. *Microbiol Rev*, *57*(1), 109–137.

Skurray, R. A., & Reeves, P. (1973). Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12. *J Bacteriol*, *113*(1), 58–70.

Skurray, R. A., & Reeves, P. (1974). F Factor-Mediated Immunity to Lethal Zygosis in Escherichia coli K-12 F Factor-Mediated Immunity to Lethal Zygosis in Escherichia coli K-12. *J Bacteriol*, *117*(1), 100–106.

Skurray, R. A., Willetts, N., & Reeves, P. (1976). Effect of tra mutations on F factor-specified immunity to lethal zygosis. *Mol Gen Genet*, *146*(2), 161–165. https://doi.org/10.1007/BF00268084

Thomas, J., Lee, C. A., & Grossman, A. D. (2013). A Conserved Helicase Processivity Factor Is Needed for Conjugation and Replication of an Integrative and Conjugative Element. *PLoS Genet*, *9*(1). https://doi.org/10.1371/journal.pgen.1003198

Valentine, P. J., Shoemaker, N. B., & Salyers, A. A. (1988). Mobilization of Bacteroides Plasmids by Bacteroides Conjugal Elements. *J Bacteriol*, *170*(3), 1319–1324.

van der Hoeven, N. (1985). Evolution of bacterial surface exclusion against incompatible plasmids. *J Theor Biol*, *117*(3), 431–452. https://doi.org/10.1016/S0022-5193(85)80153-3

Wright, L. D., & Grossman, A. D. (2016). Autonomous Replication of the Conjugative Transposon Tn *916*. *J Bacteriol*, *198*(24), 3355–3366. https://doi.org/10.1128/JB.00639-16.Editor

Wright, L. D., Johnson, C. M., & Grossman, A. D. (2015). Identification of a Single Strand Origin of Replication in the Integrative and Conjugative Element ICE *Bs1* of *Bacillus subtilis*. *PLoS Genet*, *11*(10), e1005556. https://doi.org/10.1371/journal.pgen.1005556

Zhou, M., Boekhorst, J., Francke, C., & Siezen, R. J. (2008). LocateP : Genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinformatics*, *9*(173), 1–17. https://doi.org/10.1186/1471-2105-9-173

The data that support the findings of this study are available from the corresponding author

upon reasonable request.

Figures

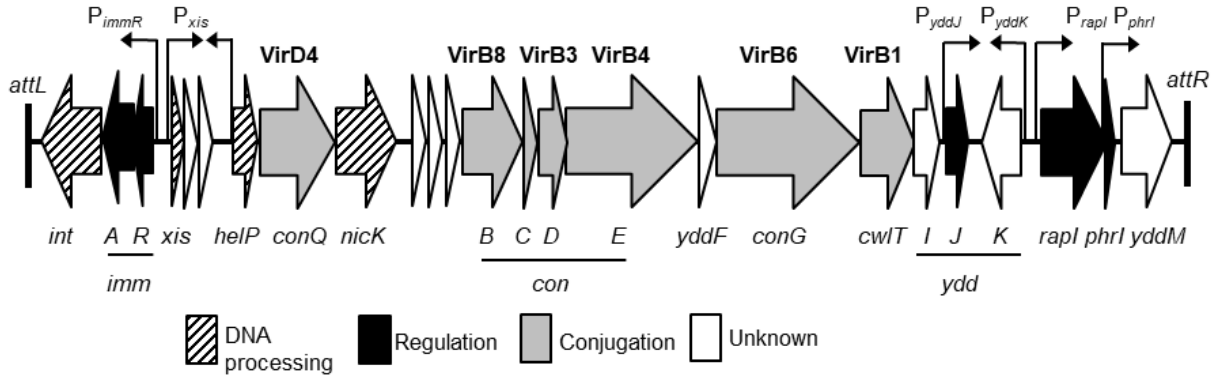


Fig.1. Genetic map of ICE*Bs1*. Organization of ICE*Bs1* open reading frames, indicated by horizontal arrows pointing in the direction of transcription, with the name of the gene indicated below. The color and patterns of each arrow indicates the gene's function as DNA processing (diagonal stripes), regulation (black), conjugation (gray), and unknown (white). Conjugation genes encoding proteins homologous/analogous to the VirB/D type IV secretion system are indicated by the corresponding protein names in bold above the arrows. The positions of the promoters for *immR*, *xis*, *yddJ*, *yddK*, *rapI, phrI,* and an uncharacterized small antisense RNA are indicated by vertical arrows with the arrow head pointing in the direction of transcription. Black boxes indicate the 60 bp repeats marking the ends of the element (Auchtung et al., 2016).

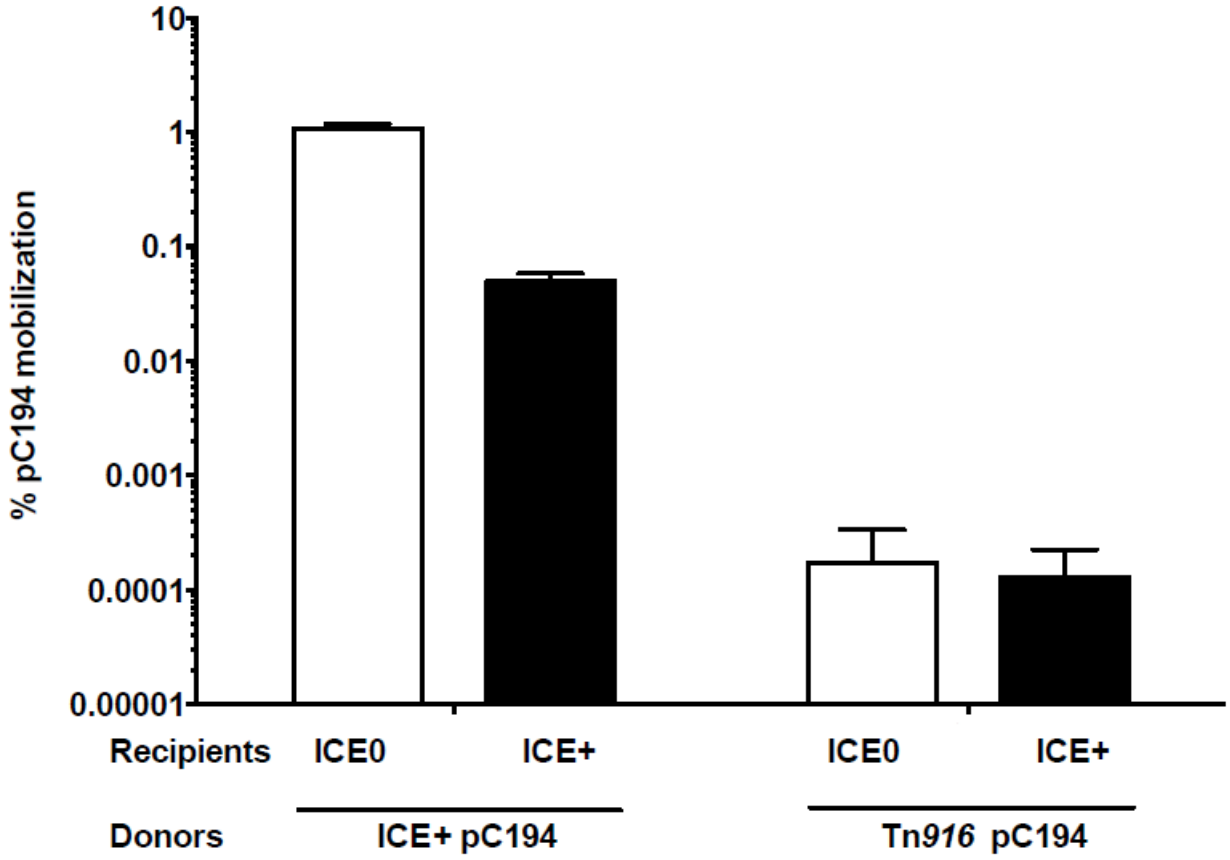


Fig. 2. ICE*Bs1* in recipient cells inhibits acquisition of pC194 mobilized by ICE*Bs1*, but not by Tn*916*. The percent mobilization of pC194 (Cm^R) by indicated donors and recipients. Left two bars: donors with ICE*Bs1* (MA116; ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*; pC194(*cat*), Str^S). Right two bars: donors with Tn*916* (MA1100; ICE*Bs1*^{0} Tn*916*; pC194(*cat*), Spc^S). **White bars:** mobilization into recipients without ICE*Bs1* (CAL89; ICE*Bs1*^{0} *str-84 comK*::*spc*). **Black bars:** mobilization into recipients with ICE*Bs1* (CAL88; ICE*Bs1 str-84 comK*::*spc*). Mobilization was calculated as the percent number of transconjugants (Cm^R Str^R cells for ICE*Bs1* donors, and Cm^R Spc^R cells for Tn*916* donors) per number of initial donors. Data presented are averages from three independent experiments, with error bars depicting standard deviations.

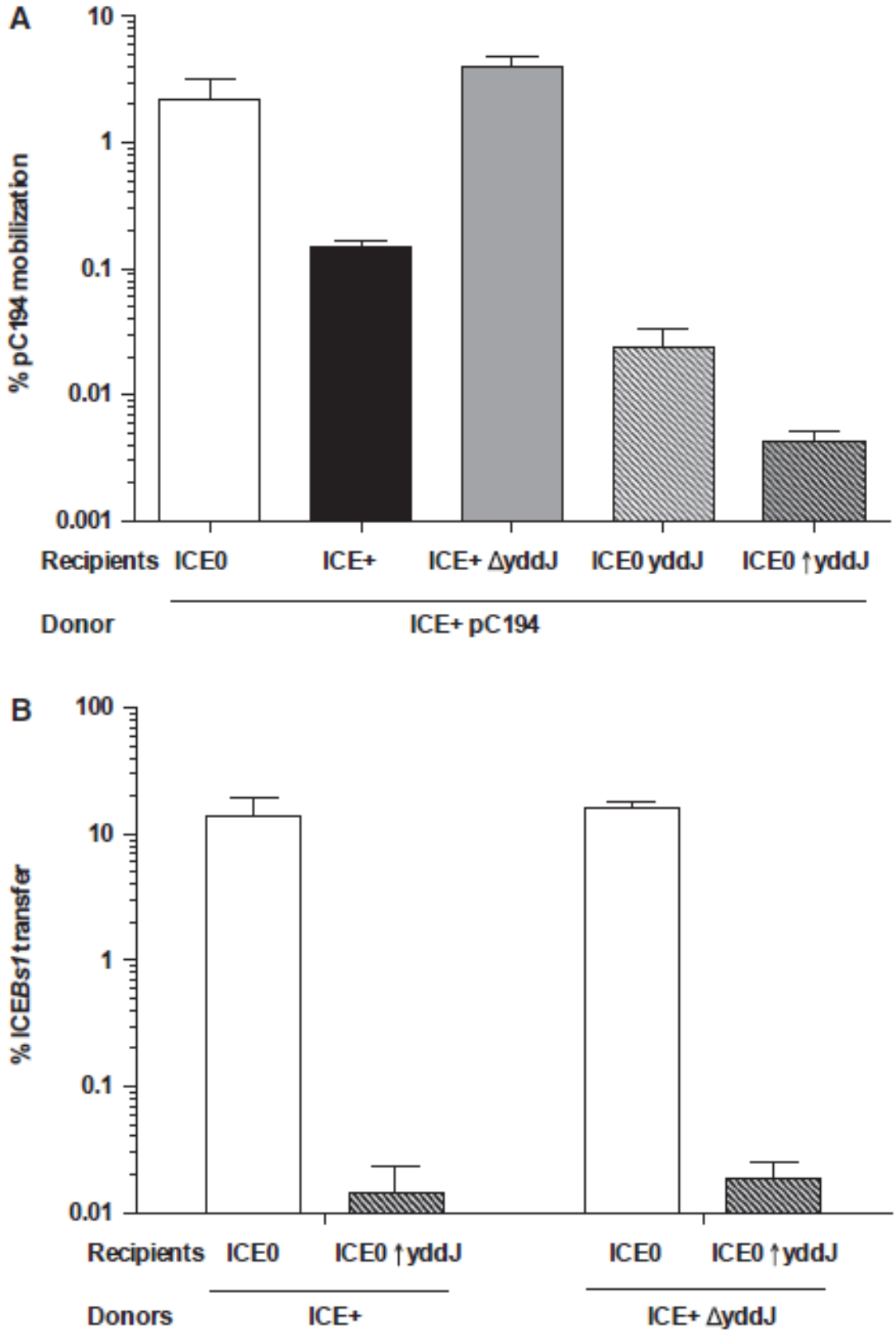

A
10
1
0.1
0.01
0.001
% pC194 mobilization
Recipients ICE0
ICE+
ICE+ ΔyddJ
ICE0 yddJ
ICE0 ↑yddJ
Donor
ICE+ pC194
B
100
10
1
0.1
0.01
% ICEBs1 transfer
Recipients ICE0
ICE0 ↑yddJ
ICE0
ICE0 ↑yddJ
Donors
ICE+
ICE+ ΔyddJ

Fig. 3. In recipient cells, ICE*Bs1* gene *yddJ* is necessary and sufficient for exclusion. A. *yddJ* is necessary and sufficient in the recipient cell for exclusion of pC194 mobilized by ICE*Bs1.* The percent mobilization of pC194 by ICE*Bs1* (MA116) donors into various recipients: without ICE*Bs1* (CAL89), white bars; with ICE*Bs1* (CAL88), black bars; with ICE*Bs1* with *yddJ* deleted (MA665; ICE*Bs1* Δ*yddJ str-84*), gray bars; without ICE*Bs1* and *yddJ* expressed from its own promoter (MA996; ICE*Bs1*^{0} *lacA*::PyddJ-*yddJ str-84*), light dashed bars; without ICE*Bs1* and *yddJ* over-expressed from the Pspank(hy) promoter (MA982; ICE*Bs1*^{0} *lacA*::Pspank(hy)-*yddJ str-84*), dark dashed bars. Mobilization was calculated as the percent number of transconjugants (Cm^R Str^R cells) per number of initial donors. Data presented are averages from three independent experiments, with error bars depicting standard deviations. **B.** *yddJ* is sufficient in the recipient cell and not required in the donor cell for exclusion of ICE*Bs1*. Left two bars: percent transfer of ICE*Bs1* with *yddJ* (MMB970; ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*). Right two bars: percent transfer of ICE*Bs1* without *yddJ* (MA11; ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*). White bars: transfer into recipients without ICE*Bs1* (CAL89). Black bars: transfer into recipients without ICE*Bs1* and overexpressing *yddJ* (MA982). Transfer was calculated as the percent number of transconjugants (Kan^R Str^R cells) per number of initial donors. Data presented are averages from three independent experiments, with error bars depicting standard deviations.

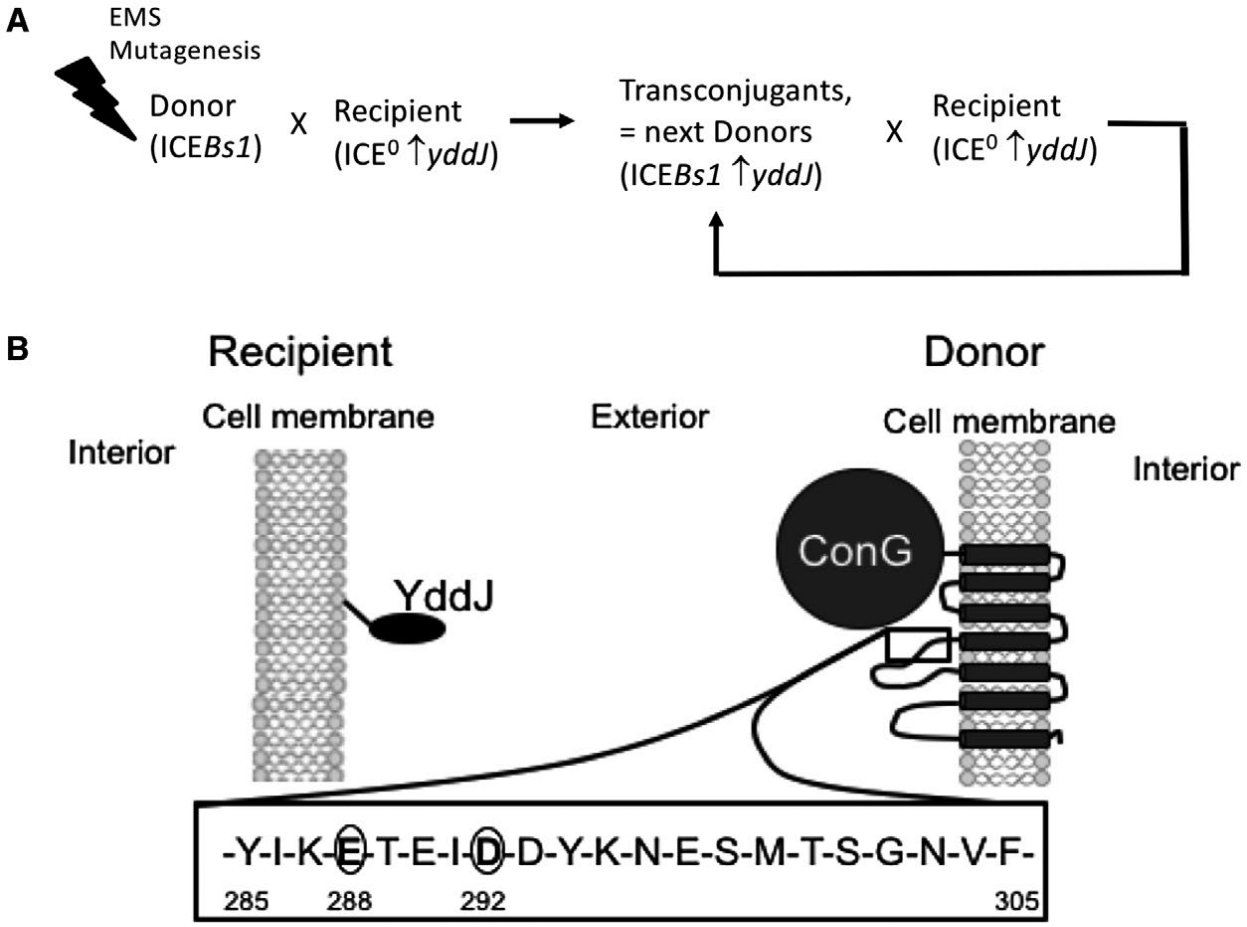
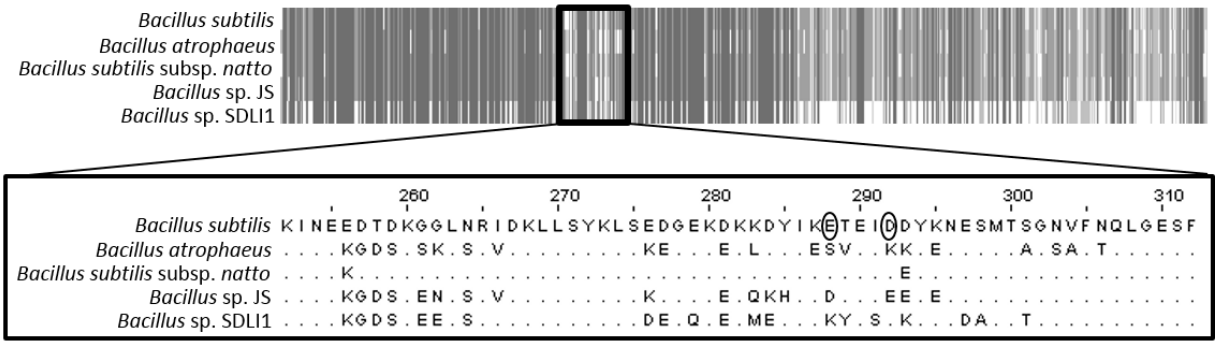
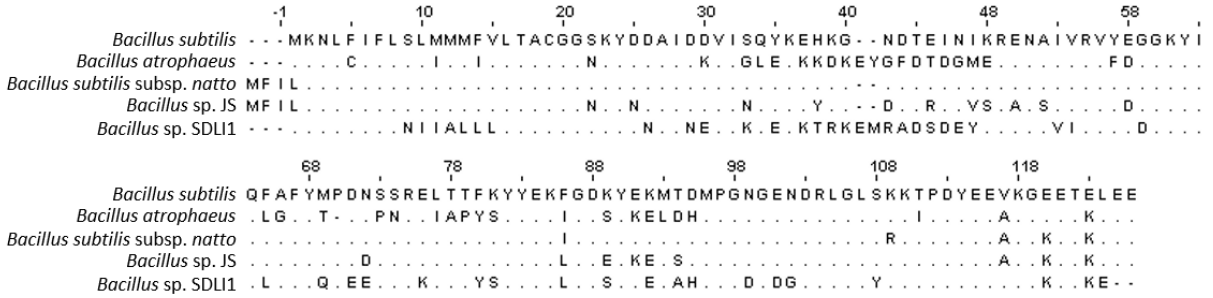

A
EMS
Mutagenesis
Donor
(ICEBs1)
X
Recipient
(ICE0 ↑yddJ)
Transconjugants,
= next Donors
(ICEBs1 ↑yddJ)
X
Recipient
(ICE0 ↑yddJ)
B
Recipient
Donor
Cell membrane
Exterior
Cell membrane
Interior
Interior
YddJ
ConG
-Y-I-K-E-T-E-I-D-D-Y-K-N-E-S-M-T-S-G-N-V-F-
285
288
292
305
C
% ICEBs1 transfer
100
10
1
0.1
0.01
0.001
Recipients
ICE0
ICE0 ↑yddJ
ICE0
ICE0 ↑yddJ
Donors
ICE+
ICE+ conG-E288K

Fig. 4. Isolation of exclusion-resistant *conG* mutations in ICE*Bs1*. A. Schematic of the mutagenesis and enrichment screen for exclusion-resistant mutations in ICE*Bs1* (described in the text and Methods). **B.** Schematic of YddJ and ConG predicted topologies. YddJ is a putative lipoprotein. Results from proteomic fractionation studies indicated that YddJ is associated with the cell membrane but that it is not a transmembrane protein (Otto et al., 2010). ConG is predicted to have seven transmembrane regions. Residues 285-305 of the extracellular loop between the third and fourth transmembrane regions are shown with the residues (288 and 292) identified in the screen for exclusion-resistance circled. **C.** ICE*Bs1 conG-E288K* donors are resistant to *yddJ*-mediated exclusion. Left two bars: percent transfer of ICE*Bs1* (MA1049; ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*). Right two bars: percent transfer of exclusion-resistant ICE*Bs1* (MA1089; ICE*Bs1 conG-E288K* Δ(*rapI-phrI*)*342*::*kan*, Pxyl-*rapI*). White bars: recipients without ICE*Bs1* (CAL89). Dashed bars: recipients without ICE*Bs1* and overexpressing *yddJ* (MA982). Transfer was calculated as the percent number of transconjugants (Kan^R Str^R cells) per number of initial donors. Data presented are averages from three independent experiments, with error bars depicting standard deviations.

A ConG Homologs



B YddJ Homologs



C

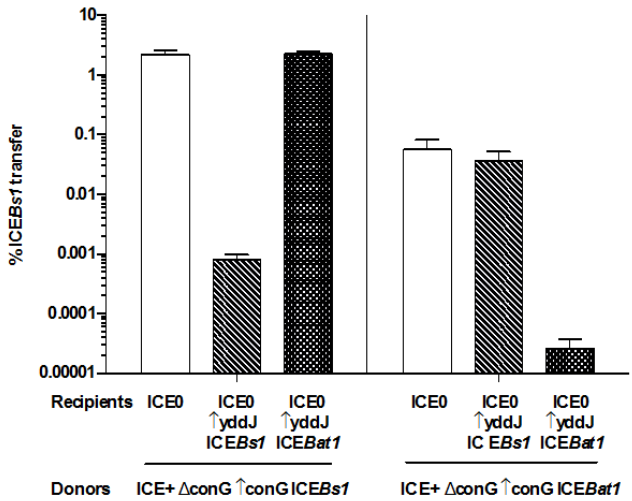


Fig. 5. ICE*Bs1* and ICE*Bat1* homology and exclusion specificity. Alignments of ConG (**A**) and YddJ (**B**) homologs from ICE*Bs1*-like elements from five *Bacillus* species, including *B. subtilis* (ICE*Bs1*) and *B. atrophaeus* (ICE*Bat1*), were generated using Jalview (www.jalview.org). **A.** Schematic alignment of ConG homologs. Gray indicates regions that are identical, while white indicates regions that are dissimilar. Alignment of the dissimilar internal region that includes the residues mutated in exclusion-resistant ConG from ICE*Bs1* (circled) is shown in detail. **B.** Alignment of YddJ homologs. **C.** The percent transfer of ICE*Bs1* strains with *conG* from either ICE*Bs1* (left panel; donor strain KPD225) or ICE*Bat1* (right panel; donor strain KPD224). White bars: recipients without ICE*Bs1* (CAL89). Dashed bars: recipients with *yddJ* from ICE*Bs1* over-expressed (MA982). Hatched bars: recipients with *yddJ* from ICE*Bat1* over-expressed (KPD219; ICE*Bs1*^{0} *lacA*::Pspank(hy)-*yddJ*$_{\text{ICE}Bat1}$ *str-84*). Transfer was calculated as the percent number of transconjugants (Kan^{R} Str^{R} cells) per number of initial donors. Data presented are averages from three independent experiments, with error bars depicting standard deviations.

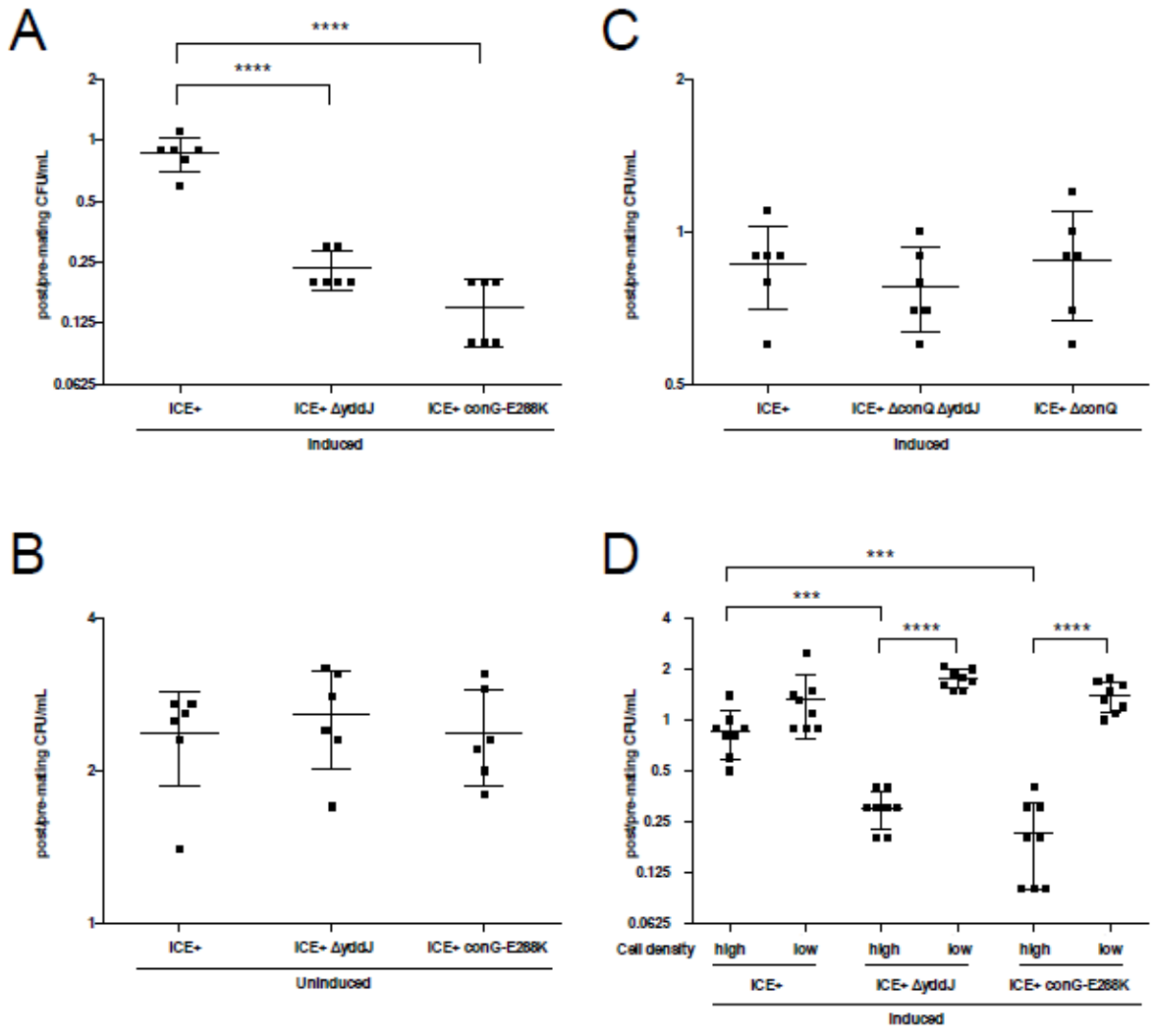


Fig. 6. Exclusion is beneficial to ICE*Bs1* and its host cells by preventing loss of viability due to redundant transfer. Data depicted in **A-C.** are from experiments where cells (monocultures) were grown in minimal medium, induced (as indicated) with 1% xylose for 2 hours, and placed at high cell density on filters for 3 hours. Cells were sampled before and after plating on filters to determine CFU/ml pre- and post-mating conditions. The y-axis shows the number of viable cells recovered post-mating per number of input cells pre-mating on a $\log_2$ scale. Each dot represents a value from an independent experiment (n=6). The middle bars represent averages and the shorter bars depict standard deviations. P-values were calculated by an ordinary one-way ANOVA with Dunnett's correction for multiple comparisons (**** indicates p-value <0.0001) using GraphPad Prism version 6. **A and B.** Cells containing exclusion-defective ICE*Bs1* exhibit a loss of viability under conditions that favor conjugation. Cell recovery is shown for cells containing wildtype (MA1049; ICE*Bs1*, Pxyl-*rapI*) or exclusion-deficient ICE*Bs1* (MA1050; ICE*Bs1* Δ*yddJ*, Pxyl-*rapI*, and MA1089; ICE*Bs1 conG-E288K*, Pxyl-*rapI*). **A.** Cell viability following activation (induction) of ICE*Bs1*. **B.** Cell viability with NO activation (uninduced) of ICE*Bs1*. **C.** Loss of viability from defective exclusion depends on a functional conjugation machinery. Cell recovery is shown for induced

cells containing wildtype (MA1049) and transfer-deficient ICE*Bs1* with exclusion (MA1070; ICE*Bs1* Δ*conQ*, Pxyl-*rapI*) and without exclusion (MA1069; ICE*Bs1* Δ*conQ* Δ*yddJ*, Pxyl-*rapI*). **D.** Loss of viability depends on high cell density. Cells containing wildtype (MA1049) and exclusion-deficient ICE*Bs1* (MA1050 and MA1089) were grown in minimal medium, induced with 1% xylose for 2 hours, and placed at high and low cell density on filters for 3 hours. CFU/ml were determined before and after incubation on the filter. The y-axis depicts the number of viable cells recovered from the filter per number of input cells pre-filtration on a $\log_2$ scale. Each dot represents a value from an independent experiment (n=8). The middle bars represent averages and the shorter bars depict standard deviations. P-values were calculated by an unpaired two-tailed t-test with Welch's correction (*** indicates p-value<0.0005; **** indicates p-value <0.0001) using GraphPad Prism version 6.

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype (reference)
BOSE986	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*}
CAL88	*str-84 comK*::*spc* (Auchtung et al., 2005)
CAL89	ICE*Bs1*^{0} *str-84 comK*::*spc* (Auchtung et al., 2005)
KPD35	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*tet*
KPD36	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI cat*} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*tet*
KPD38	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan*, *amyE*::{Pxyl-*rapI mls*} *comK*::*cat*
KPD80	ICE*Bs1 conG-E288K* Δ(*rapI-phrI*)*342*::kan *amyE*::{Pxyl-*rapI cat*} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*tet*
KPD219	ICE*Bs1*^{0} *lacA*::{Pspank(hy)- *yddJ*$_{ICE\mathit{Bat1}}$ *mls*} *str-84 comK*::*spc*
KPD224	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)- *conG*$_{ICE\mathit{Bat1}}$ *mls*}
KPD225	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)- *conG mls*}
MMB970	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} (Dewitt & Grossman, 2014)
MA11	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*}
MA116	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*}; pC194 (*cat*)
MA980	ICE*Bs1 cwlT*Δ(*207-327*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} (Dewitt & Grossman, 2014)
MA982	ICE*Bs1*^{0} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*spc*
MA996	ICE*Bs1*^{0} *lacA*::{PyddJ- *yddJ mls*} *str-84 comK*::*spc*
MA997	ICE*Bs1* Δ*yddJ str-84 comK*::*spc*
MA1027	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1049	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1050	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1069	ICE*Bs1* Δ*conQ848* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1070	ICE*Bs1* Δ*conQ848* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1089	ICE*Bs1 conG-E288K* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls*
MA1100	ICE*Bs1*^{0} Tn*916* (*tet*) *comC*::*mls*; pC194 (*cat*)

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

Appendix A

No effect of non-essential ICE*Bs1* genes on exclusion

As part of a search for exclusion-resistant mutations in ICE*Bs1* outside of ConG, I decided to test whether deletion of any of the nine ICE*Bs1* genes with no known function had any effect on exclusion. This list of ICE*Bs1* genes includes *ydzL*, *ydcO*, *ydcS*, *ydcT*, *yddA*, *yddF*, *yddI*, *yddK*, and *yddM* (Auchtung et al., 2016). Marker-less deletions of *ydzL* (ELS25), *ydcO* (ELS17), *ydcS* (ELS19), and *yddF* (MMB1273) had previously been made by Emma L. Sedivy (ELS) and Melanie M. Berkmen (MMB). A marked deletion of *yddM* (CAL420) had been made by Catherine A. Lee, a marked deletion of *yddA* (KPD208) had been made by Emma L. Sedivy, and a marked double-deletion of *ydcS* & *ydcT* (KPD209) had also been made by Emma L. Sedivy. As for *yddI* and *yddK*, previous unpublished results by Monika Avello indicated that deletion of *yddI* in the donor did not have any effect on transfer or exclusion, and *yddK* has been implicated in protecting against lytic growth of the bacteriophage SPß following induction (Grossman lab, unpublished results), so I did not personally test either of these genes for their effect on transfer or exclusion.

I performed an LB mating assay in which donor strains MMB970 (WT ICE*Bs1* donor), ELS25, ELS17, ELS19, MMB1273, CAL420, KPD208 and KPD209 were each mated with ICE0 recipients (KPD51) and with ICE0, *yddJ*-overexpressing recipients (KPD35). I followed the standard ICE*Bs1* LB mating assay protocol for our lab; briefly, 3ml LB cultures of each donor and recipient were grown to mid-log, then used to inoculate LB cultures to OD=0.01. Cultures were grown for ~1hr until they reached OD=0.1, then donor cultures were induced for 1 hour with 1% Xylose or 1mM IPTG, for overexpression of *rapI* under the Pxyl promoter or Pspank(hy) promoter, respectively. Concurrently, the ICE0 *yddJ*-overexpressing recipients (KPD35) were induced with 1mM IPTG to drive *yddJ* overexpression under the Pspank(hy) promoter. Donor and recipient cultures were mixed at OD ratio of 1:1, and poured over a filter, and the filter was incubated on

a Spizizen Salt plate for 3 hours at 37°C, to allow for mating. Cells were then resuspended off the filter in Spizizen Salts liquid media, and serial dilutions and plating on selective media was performed to quantify the numbers of donor, recipient, and transconjugant CFU. Percent transfer was calculated as CFU/ml of transconjugants divided by the CFU/ml of donors, and exclusion was calculated as the fold-difference between mating into an ICE0 recipient and mating into an ICE0 *yddJ*-overexpressing recipient. The results are presented in Fig. 1.

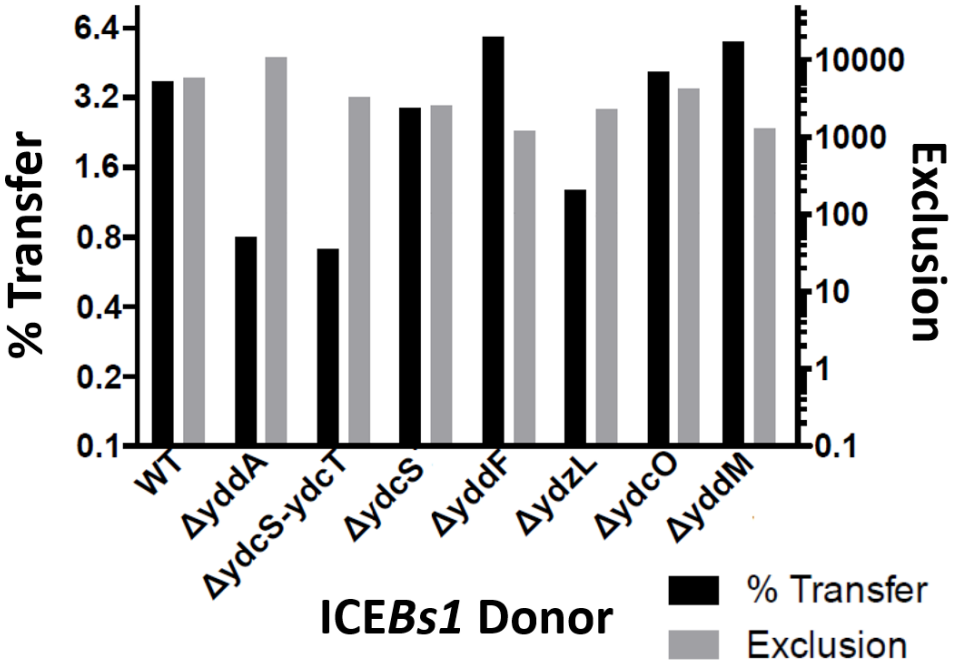


Fig. 1: 8 non-essential ICE*Bs1* genes have no considerable impact on ICE*Bs1* transfer and exclusion. Eight ICE*Bs1* donor strains, with deletions in *yddA*, *ydcS*, *ydcS* & *ydcT*, *yddF*, *ydzL*, *ydcO*, and *yddM*, as shown above, all showed % transfer within 6-fold of that of WT ICE*Bs1*. These donors also showed exclusion by ICE*Bs1* YddJ within 5-fold of that of WT ICE*Bs1*. These results are from a single mating assay and should not be assigned any statistical significance.

Only one replicate of this mating assay was performed, so no statistical significance can be assigned to the results, however even based only on this one replicate, it is clear that deletions

in *ydzL* (ELS25), *ydcO* (ELS17), *ydcS* (ELS19), *yddF* (MMB1273), *yddM* (CAL420), and *yddA* (KPD208), and the double deletion of *ydcS* & *ydcT* (KPD209), did not have any considerable effect on ICE*Bs1* transfer or exclusion by YddJ. The % transfer of all donors was within 6-fold of that of the WT ICE*Bs1* donor (MMB970), and the exclusion of all donors was within 5-fold of that of the WT ICE*Bs1* donor (Fig. 1). These results indicate that *ydzL*, *ydcO*, *ydcS*, *ydcT*, *yddF*, *yddM*, and *yddA* do not play any major role in exclusion under our standard mating assay conditions.

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype (Reference)
KPD51	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *str-84 comK*::*tet*
KPD35	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*tet*
MMB970	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} (DeWitt and Grossman, 2014)
ELS25	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} Δ*ydzL(5-85)*
ELS17	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} Δ*ydcO(5-85)*
ELS19	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} Δ*ydcS(5-83)*
MMB1273	ICE*Bs1* Δ*yddF*(*5-103*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} (Leonetti et al., 2015)
CAL420	ΔICE-318::*kan amyE*::{Pspank(hy)-*rapI spc*}
KPD208	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} Δ*yddA*::*cat*
KPD209	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *ΔydcS-ydcT::cat*

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

References:

Auchtung, J. M., Aleksanyan, N., Bulku, A., & Berkmen, M. B. (2016). Biology of ICEBs1, an integrative and conjugative element in Bacillus subtilis. *Plasmid*, Vol. 86, pp. 14–25. https://doi.org/10.1016/j.plasmid.2016.07.001

DeWitt, T., & Grossman, A. D. (2014). The bifunctional cell wall hydrolase cwlT is needed for conjugation of the integrative and conjugative element ICEBs1 in Bacillus subtilis and B. anthracis. *Journal of Bacteriology*, *196*(8), 1588–1596. https://doi.org/10.1128/JB.00012-14

Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator, abrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus subtilis. *Molecular Microbiology*, *2*(6), 689–699. https://doi.org/10.1111/j.1365-2958.1988.tb00079.x

Appendix B

Screen to identify exclusion-resistant mutations outside of ConG

After 16 repetitions of the screen for exclusion-resistant mutations all yielded mutations in *conG*, I designed a mutagenesis and enrichment screen with slightly modified original donor and two recipient strains, designed to isolate any exclusion-resistant ICE*Bs1* mutations outside of ConG. The initial donor was Δ*conG* ICE*Bs1* complemented with inducible *conG* expression under Pspank(hy) at *thrC* (I tried two versions, KPD167 and KPD68). Both the modified chloramphenicol-resistant recipient (KPD74, in place of KPD36 used in the original screen) and the spectinomycin-resistant recipient (KPD73, in place of KPD35 used in the original screen) also have inducible ConG expression under Pspank(hy) at *thrC*, so that transconjugants could effectively serve as donors of a Δ*conG* ICE*Bs1*. Both KPD73 and KPD74 also have inducible *yddJ* overexpression under *lacA* from Pspank(hy), and KPD73, KPD74, KPD167, and KPD168 were *comK* knockouts to ensure cells did not become competent during mating assays.

The screen was carried out in essentially the same way as the original screen for exclusion-resistant ICE*Bs1* mutations, except for adjustments to allow for complementation of Δ*conG* ICE*Bs1*. For the initial mating assay, initial donor cultures were grown to mid-log in LB, mutagenized with 1.2% EMS for 40 minutes, then washed and allowed to recover, except this time donor cultures were diluted back further and induced with 1mM IPTG and 1% xylose for 1.5 hours, to drive *conG* and *rapI* overexpression, respectively, but prevent the donor cultures from going much over OD=1.0 before mating. Concurrently, recipient cultures were induced with 1mM IPTG to drive YddJ overexpression. Just like for the original screen, transconjugants were scraped off the plates and grown in LB with selective media, and used as donors in a round of mating with the other strain of recipients, and in each subsequent round of mating, transconjugants from the previous mating were used as donors, with the only difference being that donors were always diluted back further before induction than in the original screen, and induced with 1mM IPTG and 1% xylose for 1.5 hours. Seven replicates of this modified screen were attempted, and with the limit of detection being < 1 in 272 initial transconjugants, <1 in 308 initial transconjugants, <1 in 254 initial transconjugants, and <1 in 335 initial transconjugants, for the

four replicates done with the KPD167 initial donor. For the three replicates attempted with the KPD68 donor, the limits of detection were <1 in 92 initial transconjugants, <1 in 100 initial transconjugants, and <1 in 137 initial transconjugants. With these seven attempts of the screen, I was unable to get successful enrichment of any exclusion-resistant mutations after four rounds of mating. This indicates that any mutation to generate an exclusion-resistant mutant outside of ConG, if such a mutation is possible, is quite rare. This aligns with later identification of ConG being sufficient for determining the specificity of exclusion (see Chapter 3).

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype
KPD167	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} *comK*::*phleo*
KPD68	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} *comK*::*cat*
KPD73	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)- *yddJ tet*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} *str-84 comK*::*phleo*
KPD74	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI cat*} *lacA*::{Pspank(hy)- *yddJ tet*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} *str-84 comK*::*phleo*

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

References

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator, abrB : regulator synthesis is controlled by the spoOA sporulation gene in Bacillus subtilis. *Mol Microbiol*, *2*(6), 689–699.

Appendix C

Non-interchangeability of ICE*Bs1* ConG and its Tn*916* homolog Orf15

After identifying exclusion-resistant mutations in $ConG_{Bs1}$, I began looking for ConG homologs that (if they could complement a Δ*conG* ICE*Bs1* donor and restore transfer) I could use to test whether ConG and YddJ together determined the specificity of exclusion. Ultimately, I chose to use the ConG homolog (and YddJ homolog) from ICE*Bat1*, but before that, I tried using Orf15, the ConG homolog from Tn*916* in *Bacillus subtilis*. Tn*916* in *Bacillus subtilis* is part of a larger family of Tn*916* elements, found in a broad host range (Roberts and Mullany, 2009). Tn*916* encodes a T4SS similar to ICE*Bs1*, containing a relaxase, coupling protein, a cell wall hydrolase, a ConE-like ATPase (Orf16), and homologs to ConB (Orf13), ConD (Orf17), as well as to ConG (Orf15) (Bhatty et al., 2013). Orf15 has 8 predicted transmembrane domains, and topology predictions suggest that the region of Orf15 corresponding to $ConG_{Bs1}$ residues 276-295 (the region of ConG that determines exclusion specificity, see Chapter 3) is in a loop connecting the third and fourth predicted transmembrane segments of Orf15, just like $ConG_{Bs1}$ residues 276-295 are in what is predicted to be a loop connecting the third and fourth transmembrane segments of $ConG_{Bs1}$.

A strain (MMB1393) previously made by Melanie M. Berkmen clearly demonstrated that expression of $ConG_{Bs1}$ from an exogenous locus (thrC) under the control of the inducible promoter Pspank(hy) was able to complement a Δ*conG* deletion in ICE*Bs1* and restore transfer. Restoration of transfer is complete in LB media, and several-fold less than WT ICE*Bs1* when matings are done in minimal S750 media. To confirm that the same complementation would be possible with Orf15 in Tn*916*, I first made a Δ*orf15* Tn*916* donor strain (KPD101), and confirmed that this deletion abolished Tn916 transfer. I then made a Δ*orf15* Tn*916* donor strain where I complemented the deletion by overexpressing Orf15 at thrC under the inducible

PspankHY promoter (KPD108). For this strain, transfer into an ICE0 recipient (CAL419) was partially restored, approximately ~30-fold lower than for WT Tn*916* in LB matings, but still well above the limit of detection for transfer. I then made a Δ*orf15* Tn*916* donor with $ConG_{Bs1}$ expressed at thrC under the inducible promoter PspankHY (KPD107), and a Δ*conG* ICE*Bs1* donor with Orf15 expressed at thrC under the inducible promoter PspankHY (KPD106). Neither of these strains were able to transfer at all when mated with an ICE0 recipient (CAL419), indicating that both ConG and Orf15 are completely unable to function in place of each other to complement deletions in their respective elements. This could be due to differences in sequence, or due to predicted differences in topology and number of transmembrane domains, that could leave corresponding parts of the two proteins positioned differently relative to the cell membrane, which could prevent Orf15 from making key interactions that ConG does with other ICE*Bs1* Conjugation machinery proteins, and vice versa. Whatever the case, after being unable to complement Δ*conG* ICE*Bs1* with Orf15 and vice versa, I tested whether $ConG_{Bat1}$ would complement a Δ*conG* ICE*Bs1* donor, and found that it could, and ultimately used these swaps to determine the specificity of exclusion (see Chapter 2).

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype
KPD101	*ICEBs1*^{0} Tn*916 (tet)* Δ*orf15*
KPD108	*ICEBs1*^{0} Tn*916 (tet)* Δ*orf15 thrC*::{Pspank(hy)-*orf15 mls*}
KPD107	*ICEBs1*^{0} Tn*916 (tet)* Δ*orf15 thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*}
KPD106	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)-*orf15 mls*}
MMB1393	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} (Leonetti et al., 2015)
CAL419	ICE*Bs1*^{0} *str-84 comK*::*cat* (Auchtung et al., 2007)

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

References

Auchtung, J. M., Lee, C. A., Garrison, K. L., & Grossman, A. D. (2007). Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICEBs1 of Bacillus subtilis. *Molecular Microbiology*, *64*(6), 1515–1528. https://doi.org/10.1111/j.1365-2958.2007.05748.x

Bhatty, M., Laverde Gomez, J. A., & Christie, P. J. (2013). The expanding bacterial type IV secretion lexicon. *Research in Microbiology*, *164*(6), 620–639. https://doi.org/10.1016/j.resmic.2013.03.012

Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator , abrB : regulator synthesis is controlled by the spoOA sporulation gene in Bacillus subtilis. *Mol Microbiol*, *2*(6), 689–699.

Roberts, A. P., & Mullany, P. (2009). A modular master on the move: the Tn916 family of mobile genetic elements. *Trends in Microbiology*, Vol. 17, pp. 251–258. https://doi.org/10.1016/j.tim.2009.03.002

Chapter 3

Specificity and selective advantage of an exclusion system in the integrative and conjugative element ICE*Bs1* of *Bacillus subtilis*

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Abstract

Integrative and conjugative elements (ICEs) are mobile genetic elements capable of transferring their own and other DNA. They contribute to the spread of antibiotic resistances and other important traits for bacterial evolution. Exclusion is a mechanism used by many conjugative plasmids and a few ICEs to prevent their host cell from acquiring a second copy of the cognate element. An element-encoded exclusion protein inhibits the activity of its cognate conjugation machinery. ICE*Bs1* of *Bacillus subtilis* has an exclusion mechanism whereby the exclusion protein YddJ in a potential recipient inhibits the activity of the ICE*Bs1*-encoded conjugation machinery in a potential donor. The target of YddJ-mediated exclusion is the conjugation protein ConG (a VirB6 homolog). Here we defined the regions of YddJ and ConG proteins that confer exclusion specificity and determined the consequences to donor and recipient cells in the absence of exclusion. Using chimeras that had parts of ConG from ICE*Bs1* and the closely related ICE*Bat1* we identified a specific region of ConG that is needed for exclusion by the cognate YddJ. Using chimeras of YddJ from ICE*Bs1* and ICE*Bat1* we identified three regions in YddJ needed for exclusion specificity. In addition, we found that YddJ-mediated exclusion protected donor cells from being killed following conjugation into recipients. Additional results demonstrated that the death of donors was dependent on the ability of transconjugants to themselves become donors. This type of killing is in marked contrast to the lethal zygosis described for the F plasmid of *Escherichia coli*.

Importance

Horizontal transfer of mobile genetic elements, including Integrative and Conjugative Elements (ICEs), is a major driving force of bacterial evolution, responsible for the spread of antibiotic and heavy metal resistances and other traits. Exclusion mechanisms allow mobile genetic elements to prevent their host from acquiring additional copies of the element. In this work, we characterize the specificity of ICE*Bs1* exclusion and its benefits for ICE*Bs1* donor cells. The specificity of exclusion allows transfer of genetic material while inhibiting transfer of identical elements, thus promoting ICE evolution and spread of new genetic information. Exclusion protects donors when they are especially vulnerable during periods of high transfer, and this could be essential in allowing ICE*Bs1* to break into new populations of host cells.

Introduction

Integrative and conjugative elements (ICEs) play a major role in bacterial evolution by contributing to the spread of genetic material, including genes for antibiotic resistances, pathogenesis, symbiosis, and metabolic functions (Bañuelos-Vazquez et al., 2017; Brown-Jaque et al., 2015). Also called conjugative transposons, ICEs are typically found integrated into the host chromosome. Under certain conditions they can excise from the chromosome and transfer to a new host cell through conjugation machinery encoded by the element (Johnson and Grossman, 2015), thus enabling their spread through a population of bacterial cells. The conjugation machinery encoded by most ICEs is known as a Type 4 Secretion System, or T4SS (Bañuelos-Vazquez et al., 2017). ICEs often encode genes, known as cargo genes, that confer useful phenotypes for their host cell; these include genes for antibiotic resistance, genes for

heavy metal resistance, and genes for utilizing alternate carbon sources (reviewed in (Burrus and Waldor, 2004)). Plenty of ICEs are also capable of utilizing their own conjugation machinery to transfer other plasmids to new host cells, allowing for dissemination of plasmids that do not encode conjugation machinery (Hochhut et al., 2000; Lee et al., 2012; Naglich and Andrews, 1988).

ICE*Bs1* is a model for ICEs in Gram-positive bacteria. Originally found in the Gram-positive *Bacillus subtilis*, ICE*Bs1* is relatively small (~20 kb), can be activated in >90% of cells in a population and, under appropriate conditions, has a high frequency of conjugative transfer (Auchtung et al., 2016). ICE*Bs1* is normally integrated into a specific site in *trnS-leu2* (encoding a leucyl-tRNA) in the *B. subtilis* chromosome; this integration does not disrupt the tRNA gene (Auchtung et al., 2005). DNA damage to its host cell, or crowding by *B. subtilis* cells that do not contain ICE*Bs1* both lead to de-repression of transcription of ICE*Bs1* genes, and subsequent excision and transfer of the element.

Some ICEs, including ICE*Bs1*, have a mechanism called exclusion that prevents their host cell from acquiring a second copy of the element. Exclusion systems are also found in conjugative plasmids; in fact, almost every conjugative plasmid encodes one or more exclusion system, and they are considered an essential part of conjugative plasmid biology (Garcillán-Barcia and de la Cruz, 2008). Studies of the F-plasmid have shown that exclusion protects host cells against lethal zygosis, a phenonemon in which host cells that serve as recipients during excessive transfer events die, likely due to cell wall damage (Ou, 1980; Skurray and Reeves, 1973, 1974; Skurray et al., 1976). Other studies with the F-plasmid indicate that exclusion prevents recombination events that result in deletions and defective plasmid copies (Hopkins et al.,

1980; Peters and Benson, 1995; Syvanen et al., 1986). In general, exclusion systems are mediated by a single protein encoded by the element, that is localized to the membrane of the host cell, where it is in position to inhibit cognate conjugation machinery (Garcillán-Barcia and de la Cruz, 2008). Identified exclusion proteins tend to be fairly small, and membrane attachment is in the form of one or more transmembrane domains, or lipid modification, or both. The target protein in the donor has been identified for exclusion systems from the F/R100 family of plasmids (Anthony et al., 1999; Audette et al., 2007), the R64/R62Ia plasmids (Sakuma et al., 2013), and the SXT/R391 ICEs (Marrero and Waldor, 2007; Marrero and Waldor, 2005). ICE*Bs1* is the only ICE from Gram-positive bacteria that is known to have an exclusion system (Avello et al., 2019).

In the ICE*Bs1* exclusion system, the ICE*Bs1*-encoded exclusion protein YddJ specifically inhibits its cognate conjugation machinery by targeting the conjugation protein ConG in would-be donor cells (Fig. 1A), thereby inhibiting transfer of DNA into a cell that already contains ICE*Bs1* (Avello et al., 2019). YddJ is a putative lipoprotein, with a cysteine at amino acid position 19 predicted to serve as the site of lipid modification for the membrane anchor (Simonen and Palva, 1993; Zhou et al., 2008). Proteomic studies detected YddJ in the enriched membrane fraction, indicating its membrane localization (Otto et al., 2010). ConG, a homolog of VirB6 in the pTI system from *A. tumefaciens*, is a membrane protein with seven predicted transmembrane segments, and essential member of the ICE*Bs1* T4SS conjugation system (Leonetti et al., 2015). Previous experiments demonstrated that exclusion protects the viability of ICE*Bs1* host cells under conditions that promote conjugation, although from these experiments it was not clear whether ICE*Bs1* donors, recipients, or both were being protected

(Avello et al., 2019). Here we identify the regions in YddJ and ConG that determine the specificity of exclusion, and demonstrate how exclusion promotes ICE*Bs1* donor viability by limiting ICE*Bs1* transfer from new transconjugants back into the original donors.

Results

Rationale

ICE*Bs1* has three mechanisms – recognition of self by cell-cell signaling, exclusion, and immunity– for inhibiting its host cell from receiving an additional copy of the same or a similar element. Self-recognition by cell-cell signaling relies on the ICE*Bs1* peptide PhrI, which is secreted by ICE*Bs1* host cells in conditions of high cell density, and taken up by nearby cells (Auchtung et al., 2005). Once inside the cell in its mature form, PhrI inhibits RapI from promoting ImmA-mediated degradation of ImmR, thus preventing de-repression of ICE*Bs1* gene expression, excision and transfer (Auchtung et al., 2005; Bose et al., 2008). In the event that an ICE*Bs1* host cell does receive an additional copy of ICE*Bs1* DNA, the ICE*Bs1* immunity mechanism relies on expression of the ICE*Bs1* immunity repressor ImmR to inhibit integration of this second copy of ICE*Bs1* DNA (Auchtung et al., 2007).

In order to study the effects of the third mechanism, ICE*Bs1* exclusion, separate from these other two mechanisms, we needed to analyze ICE*Bs1* conjugation in a setup that bypasses both cell-cell signaling and immunity. To bypass cell-cell signaling, we overexpressed *rapI* in the donors under the control of an inducible promoter at an exogenous locus (Pxyl promoter at the *amyE* locus), prior to mixing donor and recipient cells for mating. To bypass immunity, but still observe the effects of exclusion, we mated ICE*Bs1* donors with ICE0 recipient cells that

expressed *yddJ* from an exogenous locus (Pspank(hy) at the *lacA* locus). Without ImmR, these recipients could not demonstrate immunity, showing that expression of *yddJ* alone in the recipient is necessary and sufficient for ICE*Bs1* exclusion (Avello et al., 2019).

Identification of regions of ConG that are essential for exclusion specificity

ICE*Bat1* (from *B. atrophaeus*) is similar to ICE*Bs1* (Avello et al., 2019) (Fig. 1B). YddJ from ICE*Bat1* ($YddJ_{Bat1}$) is capable of inhibiting the ICE*Bs1* conjugation machinery, but only if the ConG homolog present in the machinery is from ICE*Bat1* ($ConG_{Bat1}$). Similarly, YddJ from ICE*Bs1* ($YddJ_{Bs1}$) inhibits the conjugation machinery only if it contains the cognate ConG (Avello et al., 2019). Key residues for exclusion specificity must be divergent between the two ConG homologs. Alignment of the homologs (Fig. 1B) reveals two main regions of divergence. One region, including residues 276-295 of both $ConG_{Bs1}$ and $ConG_{Bat1}$, is predicted to be a loop that connects the putative third and fourth transmembrane regions and exclusion-resistant mutations in *conG* are in this loop region (Avello et al., 2019). The other divergent region is at the C-terminal half of the ConG homologs and predicted to be a large extracellular domain.

We made chimeras between $ConG_{Bs1}$ and $ConG_{Bat1}$ to test if these regions were sufficient to confer exclusion specificity. We decided initially to focus on residues 276-295 because that is where exclusion-resistant mutations were found. Experiments described below demonstrate that residues 276-295 from ConG are sufficient to confer specificity with the cognate exclusion protein YddJ.

Previous experiments have demonstrated that overexpression of $conG_{Bs1}$ from an ectopic locus (*thrC*) can restore transfer of a Δ*conG* ICE*Bs1* (Leonetti et al., 2015). Furthermore, overexpression of $conG_{Bat1}$ from *thrC* can also restore transfer of Δ*conG* ICE*Bs1* (Avello et al.,

2019), however transfer is approximately 68-fold less than when *conG*$_{Bs1}$ is used for complementation - 0.0019+/-0.0010% (donor strain KPD224) compared to 0.13+/-0.033% (donor strain KPD225). To investigate the importance of ConG residues 276-295, we made one chimeric construct, Con*G*$_{Bs1}${*Bat1*(276-295)}, where we substituted ConG$_{Bat1}$ residues 276-295 for residues 276-295 in ConG$_{Bs1}$. When con*G*$_{Bs1}${*Bat1*(276-295)} was used to complement a ICE*Bs1* Δ*conG*(5-805) donor (strain KPD136), this donor yielded 0.048+/-0.021% transfer into an ICE0 recipient (CAL89). We made another chimeric construct, ConG$_{Bat1}${*Bs1*(276-295)}, where we substituted ConG$_{Bs1}$ residues 276-295 for residues 276-295 in ConG$_{Bat1}$ (Fig. 2). When *conG*$_{Bat1}${*Bs1*(276-295)} was used to complement a ICE*Bs1* Δ*conG*(5-805) donor (strain KPD135), this donor yielded 0.0014+/-0.0010% transfer into an ICE0 recipient (CAL89). While complementation was lower than observed with *conG*$_{Bs1}$, the transfer of both donors (KPD135 and KPD1336) containing chimeric constructs was high enough above the limit of detection that exclusion could be measured.

Exclusion is calculated as the transfer into an ICE0 recipient divided by the transfer into an ICE+ recipient or a recipient overexpressing YddJ; overexpression of YddJ is sufficient for exclusion (Avello et al., 2019). To test whether Con*G*$_{Bs1}${*Bat1*(276-295)} is excluded by YddJ$_{Bs1}$ or YddJ$_{Bat1}$, we mated the donor strain overexpressing con*G*$_{Bs1}${*Bat1*(276-295)} (KPD136) with an ICE0 recipient (CAL89), an ICE0 recipient overexpressing *yddJ*$_{Bs1}$ (MA982), and an ICE0 recipient overexpressing *yddJ*$_{Bat1}$ (KPD219). Con*G*$_{Bs1}${*Bat1*(276-295)} was excluded by YddJ$_{Bat1}$, but not by YddJ$_{Bs1}$ (Fig. 2). To test whether Con*G*$_{Bat1}${*Bs1*(276-295)} is excluded by YddJ$_{Bs1}$ or YddJ$_{Bat1}$, we mated the donor strain overexpressing *conG*$_{Bat1}${*Bs1*(276-295)} (KPD135) with an ICE0 recipient (CAL89), an ICE0 recipient overexpressing *yddJ*$_{Bs1}$ (MA982), and an ICE0 recipient

overexpressing *yddJ*$_{Bat1}$ (KPD219). ConG$_{Bat1}${*Bs1*(276-295)} was excluded by YddJ$_{Bs1}$, but not by YddJ$_{Bat1}$ (Fig. 2). In fact, YddJ$_{Bs1}$ exclusion of the donor with ConG$_{Bat1}${*Bs1*(276-295)} (KPD135) was not significantly different than YddJ$_{Bs1}$ exclusion of donor with Con*G*$_{Bs1}$ (KPD225), and YddJ$_{Bat1}$ exclusion of the donor with Con*G*$_{Bs1}${*Bat1*(276-295)} (KPD136) was not significantly different than YddJ$_{Bat1}$ exclusion of the donor with Con*G*$_{Bat1}$ (KPD224) (Fig. 2). Taken together, these results indicate that residues 276-295 of both ConG$_{Bs1}$ and ConG$_{Bat1}$ confer specificity of exclusion. They may not be the only residues that contribute to specificity of exclusion, but without these key residues from the cognate ConG, no exclusion by YddJ is observed, and with these key residues, exclusion is almost as high as observed for donors expressing the WT cognate ConG protein.

Identification of YddJ Regions Essential for Exclusion Specificity

To identify regions of YddJ$_{Bs1}$ and YddJ$_{Bat1}$ essential for exclusion specificity, we used a similar approach of generating chimeric constructs, and testing whether ICE0 recipient strains expressing these chimeric constructs could exclude an ICE*Bs1* Δ*conG*(5-805) donor expressing Con*G*$_{Bs1}$ (KPD225) or expressing ConG$_{Bat1}$ (KPD224) (Fig. 3). An alignment of YddJ$_{Bs1}$ and YddJ$_{Bat1}$ shows 4 regions that each have at least 2 consecutive non-identical residues (Fig. 3). We made and tested four chimeric YddJ constructs. For the first construct, we substituted YddJ$_{Bat1}$ residues 30-50, 67-82, and 87-96 for their corresponding YddJ$_{Bs1}$ residues (30-48, 65-81, and 86-95, respectively), to make YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)}. For the second construct, we substituted YddJ$_{Bs1}$ residues 30-48 and 65-81 for their corresponding YddJ$_{Bat1}$ residues (30-50 and 67-82, respectively) to make YddJ$_{Bat1}${*Bs1*(30-48)(65-81)}. For the third construct, we substituted YddJ$_{Bs1}$ residues 30-48, 65-81, and 86-95 for their corresponding YddJ$_{Bat1}$ residues

(30-50, 67-82, and 87-96, respectively) to make YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)}. These three chimeric YddJ constructs are shown in Fig. 3. For each construct, we tested whether it could exclude a ICE*Bs1* Δ*conG*(5-805) donor with ConG$_{Bs1}$ (KPD225) or a donor with ConG$_{Bat1}$ (KPD224).

YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} excluded a donor with Con*G*$_{Bs1}$ (exclusion = 1646+/-1132), but not a donor with ConG$_{Bat1}$. YddJ$_{Bat1}${*Bs1*(30-48)(65-81)} also excluded a donor with Con*G*$_{Bs1}$ (exclusion = 1021+/-663), but also did not exclude a donor with ConG$_{Bat1}$. YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} exclusion of a donor with ConG$_{Bs1}$ was not significantly different than YddJ$_{Bs1}$ exclusion of a donor with ConG$_{Bs1}$ (exclusion = 2831+/-975) (Fig. 3), however YddJ$_{Bat1}${*Bs1*(30-48)(65-81)} did show significantly less exclusion of a donor with ConG$_{Bs1}$. On the other hand, YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} excluded a donor with Con*G*$_{Bat1}$ (exclusion = 514+/-335) essentially as well as YddJ$_{Bat1}$ did (exclusion = 673+/-197). However, YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} did not exclude a donor with Con*G*$_{Bs1}$. We also made a fourth YddJ chimeric construct, YddJ$_{Bs1}${*Bat1*(30-50)(67-82)}, where we substituted YddJ$_{Bat1}$ residues 30-50 and 67-82 for residues 30-48 and 65-81, respectively, in YddJ$_{Bs1}$. YddJ$_{Bs1}${*Bat1*(30-50)(67-82)} showed very little exclusion of a donor with Con*G*$_{Bat1}$ (exclusion = 38+/-55), and no exclusion of a donor with Con*G*$_{Bs1}$. Thus, YddJ$_{Bat1}$ residues 30-50, 67-82, and 87-96 are necessary for exclusion specificity and/or proper folding of the chimeric YddJ construct to generate a functional protein. On the other hand, YddJ$_{Bs1}$ residues in regions 30-48, 65-81, and to some extent 86-95, are necessary for exclusion specificity and/or proper folding of the chimeric YddJ construct. It was only possible to interpret results for YddJ chimeric constructs that were able to show some exclusion against a donor with ConG$_{Bs1}$ and/or a donor

with ConG$_{Bat1}$ – constructs which showed no exclusion of either donor might have residues key for specificity, but just not be folded correctly.

Essential ConG and YddJ Regions are Sufficient for Exclusion

Finally, we tested whether YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} and YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} could exclude ICE*Bs1* Δ*conG*(5-805) donors with Con*G*$_{Bs1}${*Bat1*(276-295)} or with Con*G*$_{Bat1}${*Bst1*(276-295)}. The results are shown in Fig. 4. YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} was able to exclude the ICE*Bs1* Δ*conG*(5-805) donor with Con*G*$_{Bat1}${*Bs1*(276-295)}, to a level not significantly different than YddJ$_{Bs1}$ exclusion of a donor with ConG$_{Bs1}$. However, YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} was not able to exclude the ICE*Bs1* Δ*conG*(5-805) donor with Con*G*$_{Bs1}${*Bat1*(276-295)}. YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} was able to exclude the ICE*Bs1* Δ*conG*(5-805) donor with Con*G*$_{Bs1}${*Bat1*(276-295)} to a level not significantly different than YddJ$_{Bat1}$ exclusion of a donor with ConG$_{Bat1}$. However, YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} was not able to exclude the ICE*Bs1* Δ*conG*(5-805) donor with Con*G*$_{Bat1}${*Bs1*(276-295)}. Taken together, these results demonstrate that the key residues identified in ConG$_{Bs1}$ and ConG$_{Bat1}$, and in YddJ$_{Bs1}$ and YddJ$_{Bat1}$, are sufficient to generate the exclusion effect observed by their counterpart WT proteins.

Exclusion protects ICE*Bs1* Donors against Back-Transfer from New Transconjugants

ICE*Bs1*-mediated exclusion protects host cells from cell death due to excessive transfer (Avello et al., 2019). In conditions that support mating, there was a significant drop in viability in mutants that were defective in exclusion (Avello et al., 2019). In these experiments, it was not possible to determine if death was due to cells acting as donors, recipients, or both. The

following experiments demonstrate that decreased viability of exclusion-null mutants is due to donor cells acting as recipients during or shortly after they act as donors.

To test whether exclusion protects ICE*Bs1* donor cells against serving as recipients during or shortly after mating, we performed mating assays with WT ICE*Bs1* donors (MA1049) and with Δ*yddJ* ICE*Bs1* donors (MA1050). In both cases, mating was done with ICE0 recipients (CAL419) at a 1:100 donor:recipient ratio. We monitored percent donor survival (pre-mating CFU divided by post-mating CFU, coverted to a percentage) by selection using an antibiotic marker in the donor chromosome. In these matings, any donor cell is entirely or almost entirely surrounded by ICE0 recipients and thus unlikely to act as recipient itself, except from ICE*Bs1* back-transfer from a new transconjugant. When comparing pre-/post-mating donor viability of both WT ICE*Bs1* donors and Δ*yddJ* ICE*Bs1* donors, WT ICE*Bs1* donors showed a noticeable amount of donor death (donor survival = 1.7+/-0.46%), but Δ*yddJ* ICE*Bs1* donors showed significantly more donor death (donor survival = 0.5+/-0.29%) (Fig. 5). This indicates that exclusion by YddJ is helping to protect the donor cells when they are entirely or almost entirely surrounded by ICE0 recipients. We hypothesized that this protection was due to YddJ-mediated exclusion preventing redundant back-transfer of ICE*Bs1* from the new transconjugants into the donor cells. This redundant back-transfer, especially when the donor cells were or had just been involved in mating, could lead to increased levels of donor death due to excessive cell wall lysis.

To test whether limiting back-transfer rescued the survival decrease observed in exclusion-deficient donors, we measured the pre-mating and post-mating donor viability of a Δ*yddJ* ICE*Bs1* donor overexpressing *yddJ* (KPD210), in a 1:100 donor:recipient ratio mating with ICE0 recipients (CAL419). In this donor strain, YddJ can function in the donor, limiting the donor's

ability to act as a recipient for ICE*Bs1* back-transfer, but the ICE*Bs1* transferred to the transconjugants is Δ*yddJ*. The donor survival for this mating was 3.5+/-0.88% (Fig. 5). Ectopic overexpression of *yddJ* in the donors fully restores the survival defect observed in the exclusion-deficient Δ*yddJ* ICE*Bs1* strain compared to the WT ICE*Bs1* strain. This indicates that exclusion by YddJ functioning in the donors protects the donors from cell death due to redundant back-transfer of ICE*Bs1* from the new transconjugants.

Discussion

Protection of donors by ICE*Bs1* exclusion represents an interesting, different example than the previously characterized phenomenon lethal zygosis. Lethal zygosis was first identified as recipient death occurring when recipients lacking the F plasmid (F-) are killed when mixed with an excess of either Hfr donors or F+ exclusion-null donors; recipient death by lethal zygosis also occurs when F+ exclusion-null recipients are mixed with an excess of Hfr donors (Ou, 1980; Skurray and Reeves, 1973, 1974; Skurray et al., 1976). In lethal zygosis, recipients are killed when they serve as recipients during multiple conjugation events; this killing is probably caused by increased permeability of the cell wall due to multiple matings (Ou, 1980). In the case of the Hfr x F- matings, the recipients are almost always F-, since they do not receive the integrated F-plasmid DNA, and are therefore unable to act as donors themselves, and yet they still die do to lethal zygosis. Furthermore, none of these studies report anything about donor death, even in the cases where an excess of F+ exclusion-null strains are mixed with F- recipients, and there was nothing to stop the F+ exclusion-null cells from attempting to transfer into each other. So, while F+ exclusion-null cells attempting to transfer into each other may result in donor death

that was not mentioned, lethal zygosis does not depend on recipients also functioning as donors. This is clearly different than the protective benefit of ICE*Bs1* exclusion, which protects donors from serving as recipients during or shortly after they serve as donors.

The protective benefit of ICE*Bs1* exclusion probably serves an important role when ICE*Bs1* is breaking into a new population of host cells, a situation that is mimicked by our 1:100 donor:recipient ratio matings. Once a cell receives ICE, it is ready to quickly donate it to other cells, and this feature gives ICE*Bs1* some distinct advantages, like being able to move quickly through cell chains via conjugation (Babic et al., 2011). However, this rapid pace at which transconjugants can become donors does make transconjugant attempts at back-transfer likely, which presents a problem for both ICE*Bs1* and its host. The results reported in this paper suggest that it is taxing for ICE*Bs1* host cells to serve as donors. Not only are they vulnerable to death by serving as recipients during or right after they have served as donors (which occurs when donors are exclusion-null), but there is a considerable amount of donor death even when the donors are capable of exclusion. We know that overexpression of *yddJ* in an ICE0 recipient causes approximately two thousand-fold decrease in transfer, and yet still in the KPD210 strain, pre/post-mating donor viability was 0.035+/-0.0088. Given that serving as a recipient during or right after serving as a donor even further decreases donor viability, it is likely that being a donor causes cell wall or membrane damage, and then serving as a recipient further exacerbates this damage, since the process of being a donor and the process of being a recipient both involve penetration of the cell wall and membrane. Whatever the mechanism of killing, it seems that exclusion is essential in protecting ICE*Bs1* donor cells when they are already in the vulnerable state of serving or having just served as donors.

Our identification of key regions for exclusion specificity in ConG and YddJ also highlight important aspects of ICE*Bs1* biology, and how ICEs contribute to bacterial evolution by spreading genetic material. The fact that exclusion (or lack thereof) can be based on differences of a few residues in the exclusion protein or target protein demonstrates that exclusion by a copy of ICE*Bs1* can very selectively allow slightly different elements (such as ICE*Bat1*) to enter the host cell, while significantly reducing the number of conjugation attempts by other would-be ICE*Bs1* donors. ICEs play an important role in bacterial evolution by contributing to the spread of genetic material, and one way in which an ICE gains or loses genetic material (which it can then transfer along with itself) is through genetic rearrangement events with other ICEs and plasmids (Johnson and Grossman, 2015). It has been theorized (Garcillán-Barcia and de la Cruz, 2008) that the lack of exclusion systems in some ICEs allows for more rapid evolution of the ICE, but this could be harmful for ICE*Bs1* given its strict requirement for integration site. Having an exclusion system that allows for as much exposure to other elements as possible, while limiting the number of identical elements that enter, would allow ICE*Bs1* to have the chance to be exposed to as many other ICEs as possible and benefit from the genetic diversity, while avoiding suffering the ill effects.

As noted previously (Avello et al., 2019), there are 3 systems where the target of the exclusion protein has been identified; in the case of the F/R100 conjugative plasmids (Lawley et al., 2003) and the SXT/R391 family of Gram-negative ICEs (Beaber et al., 2002), the target proteins are VirB6 homologs like ConG, and in the case of the R64/R621a conjugative plasmids (Guglielmini et al., 2014), the target protein is a VirB6 analog. For the F plasmid, the target protein TraG is predicted to have 3-5 inner-membrane spanning domains (Firth and Skurray,

1992; Frost et al., 1994) with large periplasmic regions located approximately at residues 75–325 and at residues 445–938, the C-terminus of the protein which encompasses the region (residues 610-673) that determines the specificity of exclusion (Audette et al., 2007). For the SXT/R391 family of ICEs, the target protein is also called TraG and localized to the inner membrane of the donor cell (Marrero and Waldor, 2005) with a few transmembrane domains, and both computational programs and experimental results indicate that the C-terminal half of the protein (residues 510 to 1189) is in the cytoplasm (Marrero and Waldor, 2007). Residues 606-608, which determine the specificity of exclusion, are in this C-terminal cytoplasmic half of the protein (Marrero and Waldor, 2005). Finally, for the R64/R621a conjugative plasmids, the target protein TraY is predicted to have seven transmembrane domains (Komano et al., 2000; Roy and Isberg, 1997). In the R64 TraY protein, an internal segment (residues 430-522) determines exclusion specificity, and in the R621a TraY protein, a C-terminal segment (residues 716-745) determines exclusion specificity (Sakuma et al., 2013). Using topology predictions generated by CCTOP (Dobson et al., 2015), the internal segment is predicted to localize to the cytoplasm, and the external segment is predicted to localize outside the plasma membrane. In each of these systems, while the target protein is a VirB6 homolog like ConG, the identified regions for exclusion specificity can be found in the middle or end of the protein, localized to either the periplasm or the cytoplasm, suggesting that the exact mechanism of exclusion may differ among the systems.

Despite the theme of VirB6 homolog or analog target proteins, the likelihood that exclusion mechanisms differ among conjugative ICEs and plasmids is also supported by the variety of exclusion proteins. In the case of the F/R100 family of plasmids, the exclusion protein TraS is a

small hydrophobic protein except for a short hydrophilic region (Jalajakumari et al., 1987), predicted to be localized to the inner membrane with 4 (F-plasmid TraS) or 3 (R100-plasmid TraS) transmembrane segments (Audette et al., 2007), and shows no evidence of lipoprotein modification or a signal sequence (Beutint and Achtman, 1979). It has been theorized that TraG may be sent to the recipient cell to initiate transfer at the recipient inner membrane, where TraS would inhibit TraG to cause exclusion (Audette et al., 2007). In the case of the SXT/R391 family of ICEs, the 143 residue exclusion protein Eex is localized to the inner membrane (Marrero and Waldor, 2005), and the regions essential for exclusion specificity are residues 121-132 of the R391 EeX homolog, and residues 121-137 of the SXT EeX homolog (Marrero and Waldor, 2007). In both homologs, the regions essential for exclusion specificity are localized to the cytoplasm, and different mechanisms including membrane inversion and protein translocation have been proposed for how Eex homologs can interact with their cognate TraG proteins to mediate exclusion (Marrero and Waldor, 2007). In the plasmid R64, the gene responsible for exclusion, *excAB*, codes for two proteins: the protein ExcA which is essential for exclusion and localizes to both the inner membrane and the cytoplasm, and the protein ExcB which is likely an inner membrane protein (Furuya and Komano, 1994; Hartskeerl et al., 1985a; Rudy Hartskeerl et al., 1986; Rudy Hartskeerl et al., 1985b). On the other hand, the plasmid R621a *excA* gene only encodes one protein, ExcA. The R64 ExcA and R621a ExcA show no shared sequence identity in the N-terminal region of the protein (60 residues for R64 ExcA, 44 residues for R621a ExcA), 39% identity in the middle 120 residues, and 95% identity in the final C-terminal 40 residues (Sakuma et al., 2013). Given that the sequence dissimilarity between the homologs is in the N-terminal and middle residues, it is probable that residues key for

exclusion specificity are located in these parts of the protein. Along with differences in what part of their cognate ConG homologs or analogs determine exclusion specificity, the exclusion proteins from these systems differ in their topology and where they have or may contain residues that determine exclusion specificity. This suggests the possibility that, while the target is shared, the physical mechanisms mediating exclusion are different in each system. This could allow the exclusion proteins to take advantages of subtle differences in conjugation machinery architecture to achieve different levels of conjugative transfer inhibition.

Materials and Methods:

Cell Growth Conditions and Media

During mating assays, cells were grown in $S7_{50}$ defined medium supplemented with needs for auxotrophic growth (40 µg/ml tryptophan, 40 µg/ml phenylalanine, and 200 µg/ml threonine for strains containing alleles inserted at the *thrC* locus). Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM to induce expression from the Pspank(hy) promoter, where indicated. LB plates contained antibiotics, where indicated, at the following concentrations: kanamycin (5 µg/ml), spectinomycin (100 µg/ml), streptomycin (100 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

Table 1 contains the *B. subtilis* strains used in this study. Cloning and generation of strains was done following standard techniques (Harwood and Cutting, 1990). All recipient strains

(KPD219, CAL89, MA982, KPD128, KPD131, KPD132, KPD137, CAL419) did not contain ICE*Bs1* (were ICE0), contained null mutations in *comK* or *comC* (see below), and were streptomycin-resistant (*str-84*) (Auchtung et al., 2005; Lee and Grossman, 2007). Streptomycin was used as a counter-selective marker in mating assays (see more on mating assays below). All ICE*Bs1* donor strains contain a version of ICE*Bs1* which has a kanamycin-resistance cassette inserted in place of *rapI-phrI*: Δ(*rapI-phrI*)*342*::*kan* (Auchtung et al., 2005). *rapI* was over-expressed from Pxyl-*rapI* in donor cells, to achieve inducible ICE*Bs1* gene expression and excision. Pxyl-*rapI* alleles were integrated into *amyE* with *spc* or cat antibiotic resistance: *amyE*::{Pxyl-*rapI spc*} (Berkmen et al., 2010) or *amyE*::{Pxyl-*rapI cat*} (Berkmen et al., 2010; Menard and Grossman, 2013). Any ICE*Bs1* donor strains containing a deletion of *conG*, Δ*conG*(*5-805*), were derived from MMB1283 (Leonetti et al., 2015). KPD210, a donor strain containing a complete deletion of *yddJ*, was derived from MA11, the construction of which was described previously (Avello et al., 2019).

Construction of *comK* and *comC* null mutations. Null mutations in *comK* and *comC* were used to prevent transformation in all recipient strains, and in donor strains (MA1049, MA1050, KPD210) used in experiments where even low levels of transformation could significantly alter donor CFU counts. The *comK*::*cat* allele was derived from CAL419 and has been described (Lee and Grossman, 2007). The *comK*::*spc* allele (Auchtung et al., 2005) and the *comC::mls* allele (Avello et al., 2019) were also previously described.

Construction of Pspank(hy)-*yddJ* and Pspank(hy)-*yddJ* chimeras at *lacA.* All *yddJ* overexpression constructs consist of *yddJ* fused to the *lacI*-repressible IPTG-inducible promoter Pspank(hy), and integrated at *lacA* with an MLS resistance cassette. Pspank(hy)-$yddJ_{Bs1}$ (*yddJ*

from ICE*Bs1*) present in strain MA982, and Pspank(hy)-*yddJ*$_{Bat1}$, (*yddJ* from ICE*Bat1*) present in strain KPD219, were both made as described previously (Avello et al., 2019). To make the yddJ chimera overexpression constructs, *yddJ*$_{Bat1}$ DNA was amplified by PCR from genomic DNA from *B. atrophaeus* strain 11A1 (from the Bacillus Genetic Stock Center; www.bgsc.org) and *yddJ*$_{Bs1}$ DNA was amplified by PCR from genomic DNA from *B. subtilis* strain AG174. Fragments from the two *yddJ* genes were amplified and fused by isothermal assembly as necessary to make the following four chimeric constructs: (1) YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)}, in which YddJ$_{Bat1}$ amino acids 30-50, 67-82, and 87-96 were substituted for their corresponding YddJ$_{Bs1}$ amino acids (30-48, 65-81, and 86-95, respectively); (2) YddJ$_{Bat1}${*Bs1*(30-48)(65-81)}, in which YddJ$_{Bs1}$ residues 30-48 and 65-81 were substituted for their corresponding YddJ$_{Bat1}$ residues (30-50 and 67-82, respectively); (3) YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)}, in which YddJ$_{Bs1}$ residues 30-48, 65-81, and 86-95 were substituted for their corresponding YddJ$_{Bat1}$ residues (30-50, 67-82, and 87-96, respectively); and (4) YddJ$_{Bs1}${*Bat1*(30-50)(67-82)}, in which YddJ$_{Bat1}$ residues 30-50 and 67-82 were substituted for residues 30-48 and 65-81, respectively, in YddJ$_{Bs1}$. For all Pspank(hy)-*yddJ* constructs, the *yddJ* PCR fragments were joined together, and then joined to two fragments amplified from pCJ80 by isothermal assembly. One fragment from pCJ80 included the pCJ80 SphI cut site and the adjacent 2409 bp upstream of this cut site, which includes homology to the 5' end of *lacA*. The other fragment included the pCJ80 SacI cut site and the adjacent 2299 bp downstream of this cut site, which includes homology to the 3' end of *lacA*. These two fragments were digested with SphI and SacI, respectively, before isothermal assembly with the yddJ PCR DNA. The 5' and 3' primers end primers for amplifying yddJ DNA for each construct contained sequence homology to pCJ80 upstream of the SphI cut site and

downstream of SacI cut site, to facilitate joining by isothermal assembly. The resulting isothermal assembly product was integrated by double cross-over into the chromosome by transformation and selecting for MLS resistance, to generate the *yddJ* overexpression alleles.

Construction of Pspank(hy)-*conG* and Pspank(hy)-*conG* chimeras at *thrC.* All *conG* overexpression constructs consist of *conG* fused to the *lacI*-repressible IPTG-inducible promoter Pspank(hy), and integrated at *thrC* with an MLS resistance cassette. These alleles were used to complement the Δ*conG*(*5-805*) deletion in ICE*Bs1*. Pspank(hy)-*conG*$_{Bs1}$ (*conG* from ICE*Bs1*) present in strain KPD225, and Pspank(hy)-*conG*$_{Bat1}$, (*conG* from ICE*Bat1*) present in strain KPD224, were both made as described previously (Avello et al., 2019; Leonetti et al., 2015). To make the *conG* chimera overexpression constructs, *conG*$_{Bat1}$ DNA was amplified by PCR from genomic DNA from *B. atrophaeus* strain 11A1 (from the Bacillus Genetic Stock Center; www.bgsc.org) and *conG*$_{Bs1}$ DNA was amplified by PCR from genomic DNA from *B. subtilis* strain AG174. Fragments from the two *conG* genes were amplified and fused by isothermal assembly as necessary to make the following two chimeric constructs: Con*G*$_{Bs1}${*Bat1*(276-295)}, in which ConG$_{Bat1}$ residues 276-295 were substituted for residues 276-295 in ConG$_{Bs1}$; and ConG$_{Bat1}${*Bs1*(276-295)}, in which ConG$_{Bs1}$ residues 276-295 were substituted for residues 276-295 in ConG$_{Bat1}$. For all Pspank(hy)-*conG* constructs, the *conG* PCR fragments were joined together, and then joined to two fragments amplified from pMMB1341 (Leonetti et al., 2015) by isothermal assembly. One fragment from pMMB1341 included the HindIII cut site and the adjacent 2330 bp upstream of this cut site, which includes homology to the end of *thrC*. The other fragment included the SphI cut site and the adjacent 1867 bp downstream, which includes homology to the start of *thrC*. These two fragments were digested with HindIII and

SphI, respectively, before isothermal assembly with the *conG* PCR DNA. The 5' and 3' primers end primers for amplifying *conG* DNA for each construct contained sequence homology to pMMB1341 upstream of the HindIII cut site and downstream of SphI cut site, to facilitate joining by isothermal assembly. The resulting isothermal assembly product was integrated by double cross-over into the chromosome by transformation and selecting for MLS resistance, to generate the *conG* overexpression alleles.

Mating Assays

Mating assays were performed essentially as described previously (Auchtung et al., 2005; Lee and Grossman, 2007). Donor and recipient cultures were grown in $S7_{50}$ defined minimal medium supplemented with 0.1 % glutamate and 1% arabinose until they reached mid-exponential growth phase, then diluted back to an OD of 0.1. At this point 1% xylose was added to donor cultures to induce expression of Pxyl-*rapI*. After 2 hours of xylose induction, donor and recipient cells were mixed, poured over a nitrocellulose filter and the media was drained off by vacuum filtration. Unless otherwise indicated in the results section, equal numbers of donor and recipient cells were used (~$4x10^8$ cells of each). Filters were incubated for 3 hours at 37°C on 1.5% agar plates containing 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-$2H_2O$, 0.2 g/l $MgSO_4$-$7H_20$) (Harwood and Cutting, 1990). Cells were re-suspended from the filters, serially diluted in 1X Spizizen's salts and plated on LB agar plates containing kanamycin and streptomycin to select for transconjugants. The number of viable ICE*Bs1* donor cells (CFU/ml) was determined at the time of donor and recipient cell mixing, by serial dilution in 1X Spizizen's salts and plating on LB Media containing kanamycin. All LB plates from the mating assay were incubated at 37°C overnight to allow for colony

growth. Mating efficiency was calculated as the percent transconjugants CFU/ml per initial donor CFU/ml. Exclusion was calculated as the percent transfer into an ICE0 recipient divided by the percent transfer into an ICE0, *yddJ*-overexpressing recipient.

References:

Anthony, K. G., Klimke, W. A., Manchak, J., & Frost, L. S. (1999). Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100-1: Insights into the mechanism of conjugation. *Journal of Bacteriology*, *181*(17), 5149–5159.

Auchtung, J. M., Aleksanyan, N., Bulku, A., & Berkmen, M. B. (2016). Biology of ICEBs1, an integrative and conjugative element in Bacillus subtilis. *Plasmid*, Vol. 86, pp. 14–25. https://doi.org/10.1016/j.plasmid.2016.07.001

Auchtung, J. M., Lee, C. A., Garrison, K. L., & Grossman, A. D. (2007). Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICEBs1 of Bacillus subtilis. *Molecular Microbiology*, *64*(6), 1515–1528. https://doi.org/10.1111/j.1365-2958.2007.05748.x

Auchtung, J. M., Lee, C. A., Monson, R. E., Lehman, A. P., & Grossman, A. D. (2005). Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. *Proceedings of the National Academy of Sciences*, *102*(35), 12554–12559. https://doi.org/10.1073/pnas.0505835102

Audette, G. F., Manchak, J., Beatty, P., Klimke, W. A., & Frost, L. S. (2007). Entry exclusion in F-like plasmids requires intact TraG in the door that recognizes its cognate TraS in the recipient. *Microbiology*, *153*(2), 442–451. https://doi.org/10.1099/mic.0.2006/001917-0

Avello, M., Davis, K. P., & Grossman, A. D. (2019). Identification, characterization, and benefits of an exclusion system in an integrative and conjugative element of Bacillus subtilis. *Molecular Microbiology*, 1–17. https://doi.org/10.1111/mmi.14359

Babic, A., Berkmen, M. B., Lee, C. A., & Grossman, A. D. (2011). Efficient Gene Transfer in Bacterial Cell Chains. *MBio*, *2*(2). https://doi.org/10.1128/mbio.00027-11

Bañuelos-Vazquez, L. A., Torres Tejerizo, G., & Brom, S. (2017). Regulation of conjugative transfer of plasmids and integrative conjugative elements. *Plasmid*, *91*(January), 82–89. https://doi.org/10.1016/j.plasmid.2017.04.002

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2002). Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from Vibrio cholerae. *Journal of Bacteriology*, *184*(15), 4259–4269. https://doi.org/10.1128/JB.184.15.4259-4269.2002

Berkmen, M. B., Lee, C. A., Loveday, E. K., & Grossman, A. D. (2010). Polar positioning of a conjugation protein from the integrative and conjugative element ICEBs1 of Bacillus

subtilis. *Journal of Bacteriology*, *192*(1), 38–45. https://doi.org/10.1128/JB.00860-09
Beutint, L., & Achtman, M. (1979). Two Escherichia coli Chromosomal Cistrons, sfrA and sfrB, Which Are Needed for Expression of F Factor tra Functions. *Journal of Bacteriology*, 139(3), 730-737.
Bose, B., Auchtung, J. M., Lee, C. A., & Grossman, A. D. (2008). A conserved anti-repressor controls horizontal gene transfer by proteolysis. *Molecular Microbiology*, *70*(3), 570–582. https://doi.org/10.1111/j.1365-2958.2008.06414.x
Brown-Jaque, M., Calero-Cáceres, W., & Muniesa, M. (2015). Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid*, Vol. 79, pp. 1–7. https://doi.org/10.1016/j.plasmid.2015.01.001
Burrus, V., & Waldor, M. K. (2004). Shaping bacterial genomes with integrative and conjugative elements. *Research in Microbiology*, Vol. 155, pp. 376–386. https://doi.org/10.1016/j.resmic.2004.01.012
Dobson, L., Reményi, I., & Tusnády, G. E. (2015). CCTOP: A Consensus Constrained TOPology prediction web server. *Nucleic Acids Research*, *43*(W1), W408–W412. https://doi.org/10.1093/nar/gkv451
Firth, N., & Skurray, R. (1992). Characterization of the F plasmid bifunctional conjugation gene, traG. *MGG Molecular & General Genetics*, *232*(1), 145–153. https://doi.org/10.1007/BF00299147
Frost, L. S., Ippen-Ihler, K., & Skurray, R. A. (1994). Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiological Reviews*, *58*(2), 162–210. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7915817
Furuya, N., & Komano, T. (1994). Surface Exclusion Gene of IncI1 Plasmid R64: Nucleotide Sequence and Analysis of Deletion Mutants. *Plasmid*, *32*, 80–84.
Garcillán-Barcia, M. P., & de la Cruz, F. (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid*, Vol. 60, pp. 1–18. https://doi.org/10.1016/j.plasmid.2008.03.002
Guglielmini, J., Néron, B., Abby, S. S., Garcillán-Barcia, M. P., La Cruz, D. F., & Rocha, E. P. C. (2014). Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. *Nucleic Acids Research*, *42*(9), 5715–5727. https://doi.org/10.1093/nar/gku194
Hartskeerl, R., Zuidweg, E., Van Geffen, M., & Hoekstra, W. (1985). The IncI Plasmids R144, R64 and ColIb Belong to One Exclusion Group. *Microbiology*, *131*(6), 1305–1311. https://doi.org/10.1099/00221287-131-6-1305
Hartskeerl, Rudy, Overduin, P., Hoekstra, W., & Tommassen, J. (1986). Nucleotide sequence of the exclusion-determining locus of IncI plasmid R144. *Gene*, *42*(1), 107–111. https://doi.org/10.1016/0378-1119(86)90156-3
Hartskeerl, Rudy, Tommassen, J., & Hoekstra, W. (1985). Relationship between the proteins encoded by the exclusion determining locus of the IncI plasmid R144 and the cellular localization of these proteins in Escherichia coli K-12. *MGG Molecular & General Genetics*, *200*(1), 138–144. https://doi.org/10.1007/BF00383327
Harwood, C. R., & Cutting, S. M. (1990). *Molecular Biological Methods for Bacillus*. Chichester: John Wiley & Sons.
Hochhut, B., Marrero, J., & Waldor, M. K. (2000). Mobilization of plasmids and chromosomal

DNA mediated by the SXT element, a constin found in Vibrio cholerae O139. *Journal of Bacteriology*, *182*(7), 2043–2047. https://doi.org/10.1128/JB.182.7.2043-2047.2000

Hopkins, J. D., Clements, M. B., Liang, T. Y., Isberg, R. R., & Syvanen, M. (1980). Recombination genes on the Escherichia coli sex factor specific for transposable elements. *Proceedings of the National Academy of Sciences*, *77*(5), 2814–2818. https://doi.org/10.1073/pnas.77.5.2814

Jalajakumari, M. B., Guidolin, A., Buhk, H. J., Manning, P. A., Ham, L. M., Hodgson, A. L. M., … Skurray, R. A. (1987). Surface exclusion genes traS and traT of the F sex factor of Escherichia coli K-12. Determination of the nucleotide sequence and promoter and terminator activities. *Journal of Molecular Biology*, *198*(1), 1–11. https://doi.org/10.1016/0022-2836(87)90452-9

Johnson, C. M., & Grossman, A. D. (2015). Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annual Review of Genetics*, *49*(1), 577–601. https://doi.org/10.1146/annurev-genet-112414-055018

Komano, T., Yoshida, T., Narahara, K., & Furuya, N. (2000). The transfer region of Incl1 plasmid R64: Similarities between R64 tra and Legionella icm/dot genes. *Molecular Microbiology*, *35*(6), 1348–1359. https://doi.org/10.1046/j.1365-2958.2000.01769.x

Lawley, T. D., Klimke, W. A., Gubbins, M. J., & Frost, L. S. (2003, July 15). F factor conjugation is a true type IV secretion system. *FEMS Microbiology Letters*, Vol. 224, pp. 1–15. https://doi.org/10.1016/S0378-1097(03)00430-0

Lee, C. A., & Grossman, A. D. (2007). Identification of the origin of transfer (oriT) and DNA relaxase required for conjugation of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *189*(20), 7254–7261. https://doi.org/10.1128/JB.00932-07

Lee, C. A., Thomas, J., & Grossman, A. D. (2012). The Bacillus subtilis conjugative transposon ICEBs1 mobilizes plasmids lacking dedicated mobilization functions. *Journal of Bacteriology*, *194*(12), 3165–3172. https://doi.org/10.1128/JB.00301-12

Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15

Marrero, J., & Waldor, M. K. (2005). Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Developmental Cell*, *8*(6), 963–970. https://doi.org/10.1016/j.devcel.2005.05.004

Marrero, J., & Waldor, M. K. (2007a). Determinants of entry exclusion within Eex and TraG are cytoplasmic. *Journal of Bacteriology*, *189*(17), 6469–6473. https://doi.org/10.1128/JB.00522-07

Marrero, J., & Waldor, M. K. (2007b). The SXT/R391 family of integrative conjugative elements is composed of two exclusion groups. *Journal of Bacteriology*, *189*(8), 3302–3305. https://doi.org/10.1128/JB.01902-06

Menard, K. L., & Grossman, A. D. (2013). Selective Pressures to Maintain Attachment Site Specificity of Integrative and Conjugative Elements. *PLoS Genetics*, *9*(7). https://doi.org/10.1371/journal.pgen.1003623

Naglich, J. G., & Andrews, R. E. (1988). Tn916-dependent conjugal transfer of PC194 and

PUB110 from Bacillus subtilis into Bacillus thuringiensis subsp. israelensis. *Plasmid*, *20*(2), 113–126. https://doi.org/10.1016/0147-619X(88)90014-5

Otto, A., Bernhardt, J., Meyer, H., Schaffer, M., Herbst, F. A., Siebourg, J., … Becher, D. (2010). Systems-wide temporal proteomic profiling in glucose-starved Bacillus subtilis. *Nature Communications*, *1*(9), 1–9. https://doi.org/10.1038/ncomms1137

Ou, J. T. (1980). Role of surface exclusion genes in lethal zygosis in Escherichia coli K12 mating. *MGG Molecular & General Genetics*, *178*(3), 573–581. https://doi.org/10.1007/BF00337863

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator, abrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus subtilis. *Molecular Microbiology*, *2*(6), 689–699. https://doi.org/10.1111/j.1365-2958.1988.tb00079.x

Peters, J. E., & Benson, S. A. (1995). Redundant transfer of F' plasmids occurs between Escherichia coli cells during nonlethal selections. *Journal of Bacteriology*, *177*(3), 847–850. https://doi.org/10.1128/jb.177.3.847-850.1995

Roy, C. R., & Isberg, R. R. (1997). Topology of Legionella pneumophila DotA: An inner membrane protein required for replication in macrophages. *Infection and Immunity*, *65*(2), 571–578.

Sakuma, T., Tazumi, S., Furuya, N., & Komano, T. (2013). ExcA proteins of IncI1 plasmid R64 and IncIγ plasmid R621a recognize different segments of their cognate TraY proteins in entry exclusion. *Plasmid*, *69*(2), 138–145. https://doi.org/10.1016/j.plasmid.2012.11.004

Simonen, M., & Palva, I. (1993). Protein secretion in Bacillus species. *Microbiological Reviews*, *57*(1), 109–137. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8464403%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC372902

Skurray, R. A., & Reeves, P. (1973). Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12. *Journal of Bacteriology*, *113*(1), 58–70.

Skurray, R. A., & Reeves, P. (1974). F factor mediated immunity to lethal zygosis in Escherichia coli K 12. *Journal of Bacteriology*, *117*(1), 100–106.

Skurray, R. A., Willetts, N., & Reeves, P. (1976). Effect of tra mutations on F factor-specified immunity to lethal zygosis. *MGG Molecular & General Genetics*, *146*(2), 161–165. https://doi.org/10.1007/BF00268084

Syvanen, M., Hopkins, J. D., Griffin IV, T. J., Liang, T. Y., Ippen-Ihler, K., & Kolodner, R. (1986). Stimulation of precise excision and recombination by conjugal proficient F' plasmids. *MGG Molecular & General Genetics*, *203*(1), 1–7. https://doi.org/10.1007/BF00330376

Zhou, M., Boekhorst, J., Francke, C., & Siezen, R. J. (2008). LocateP: Genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinformatics*, *9*(173), 1–17. https://doi.org/10.1186/1471-2105-9-173

Figures

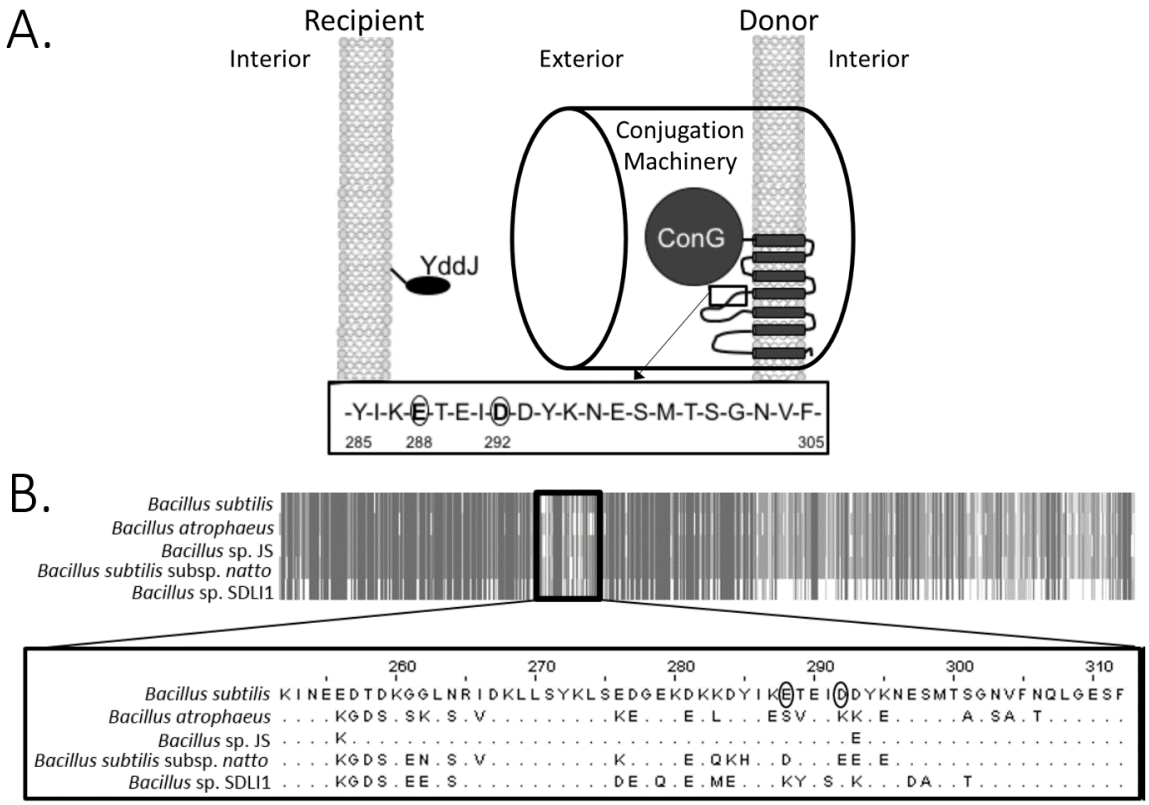

Fig. 1: ConG is the target of YddJ in ICE*Bs1* exclusion. 1A: YddJ, a putative lipoprotein, in an ICE*Bs1* host cell ("Recipient") targets its cognate ConG in the ICE*Bs1* conjugation machinery of a would-be ICE*Bs1* donor cell to mediate exclusion. Exclusion-resistant mutations (marked with circles in the inset) were found at ConG residues 288 and 292, in what is predicted to be a loop region exterior to the membrane connecting the third and fourth transmembrane domains (Avello et al., 2019). Figure adapted from (Avello et al., 2019). 1B: Alignment of ConG homologs from *Bacillus subtilis* (ICE*Bs1*), *Bacillus atrophaeus* (ICE*Bat1*), as well as *Bacillus* sp. JS, *Bacillus subtilis* subsp. *natto*, and *Bacillus* sp. SDLI1. These alignments show two main regions of sequence divergence: the C-terminal half of the protein, which is predicted to be exterior to the membrane, and a region highlighted in the black box, which includes the residues where exclusion-resistant ConG mutations were found (marked with circles) (Avello et al., 2019). Figure adapted from (Avello et al., 2019).

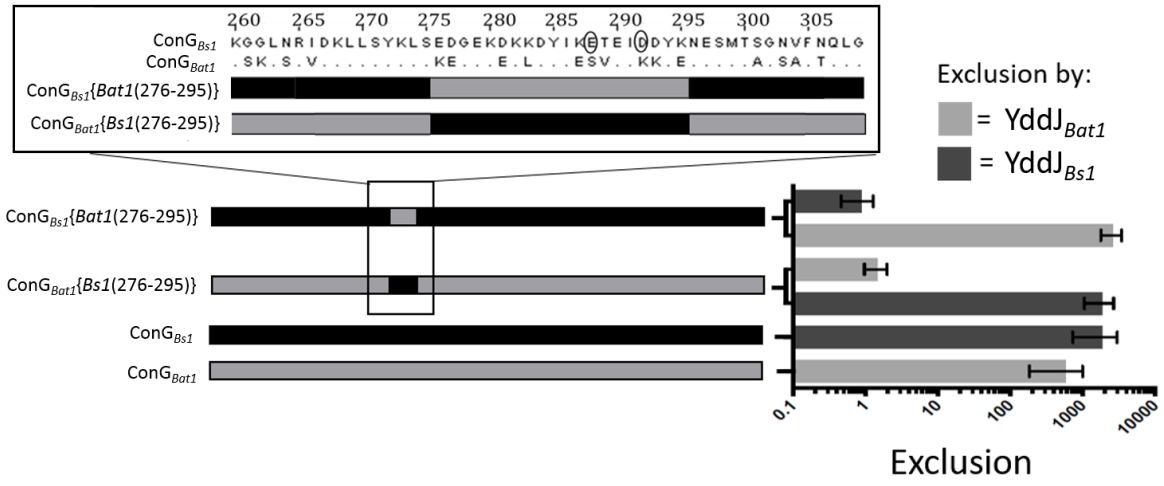

Fig. 2: Regions of ConG$_{Bs1}$ and ConG$_{Bat1}$ that confer specificity of exclusion. Exclusion of ICE*Bs1* Δ*conG(5-805)* donors expressing ConG$_{Bat1}${*Bs1*(276-295)} (KPD135), ConG$_{Bs1}${*Bat1*(276-295)} (KPD136), ConG$_{Bs1}$ (KPD225), or ConG$_{Bat1}$ (KPD224), by YddJ$_{Bat1}$ (KPD219) or YddJ$_{Bs1}$ (MA982). Three replicate mating panels were done in which both KPD135 and KPD136 were each mated with an ICE0 recipient (CAL89), an ICE0 recipient expressing YddJ$_{Bs1}$ (MA982), and an ICE0 recipient expressing YddJ$_{Bat1}$ (KPD219); KPD224 was mated with CAL89 and KPD219; and KPD225 was mated with CAL89 and MA982. Exclusion of a donor by a YddJ construct or homolog is calculated as the fold-decrease in transfer into an ICE0 recipient compared to transfer into an ICE0 recipient expressing that construct or homolog of YddJ. Transfer is calculated as the CFU/ml of transconjugants divided by the CFU/ml of pre-mating donors, converted to a percentage, for each mating assay. Bars represent averages from three independent experiments, with error bars depicting standard deviations.

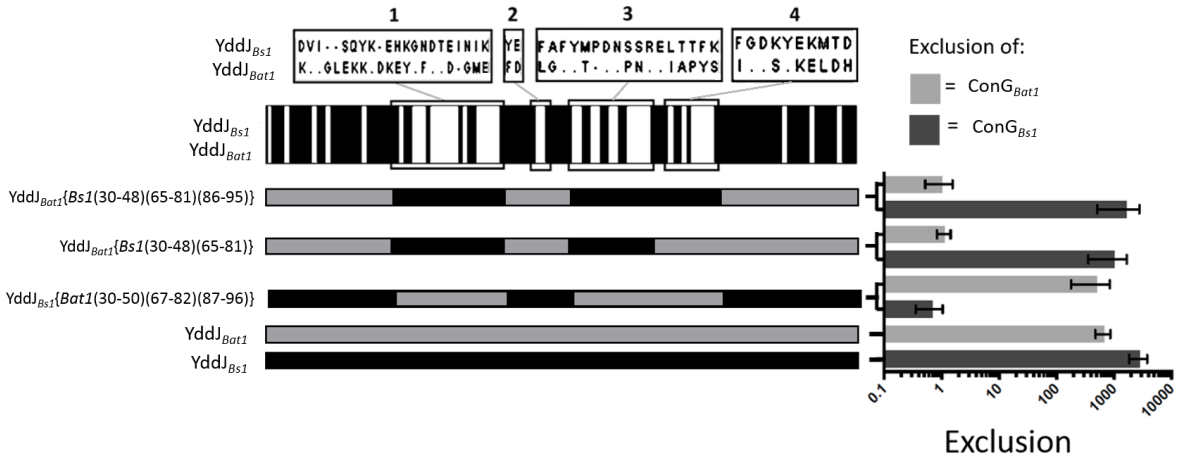

Fig. 3: Regions of YddJ$_{Bs1}$ and YddJ$_{Bat1}$ that confer specificity of exclusion. Exclusion of ICE*Bs1* Δ*conG(5-805)* donors expressing ConG$_{Bs1}$ (KPD225) or ConG$_{Bat1}$ (KPD224) by YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} (KPD128), YddJ$_{Bat1}${*Bs1*(30-48)(65-81)} (KPD131), YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} (KPD132), YddJ$_{Bat1}$ (KPD219), or YddJ$_{Bs1}$ (MA982). Three replicate mating panels were done in which ICE*Bs1* Δ*conG(5-805)* donors expressing ConG$_{Bs1}$ (KPD225) or ConG$_{Bat1}$ (KPD224) were mated with an ICE0 recipient (CAL89), and each of the ICE0 recipients expressing a homolog or chimeric construct of YddJ. Exclusion of a donor by any YddJ homolog or chimeric construct is calculated as the fold-decrease in transfer into an ICE0 recipient (CAL89) compared to transfer into an ICE0 recipient expressing that YddJ homolog or chimeric construct. Transfer is calculated as the CFU/ml of transconjugants divided by the CFU/ml of initial donors, converted to a percentage, for each mating assay. Bars represent averages from three independent experiments, with error bars depicting standard deviations.

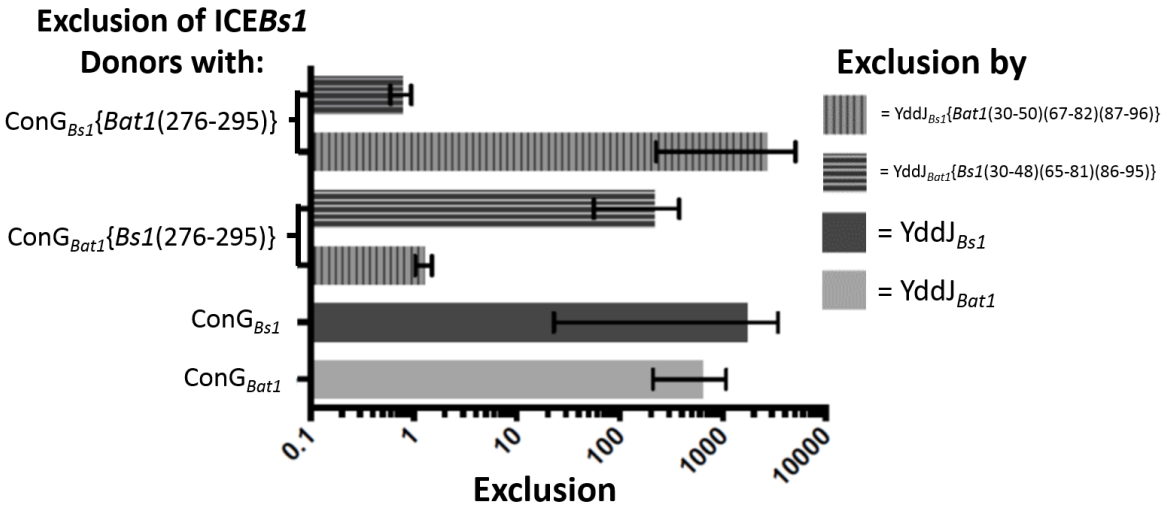

Fig. 4: Residues conferring specificity of exclusion in ICE*Bs1* and in ICE*Bat1* are sufficient for exclusion in ConG and YddJ chimera constructs. Three replicate mating panels were done in which both chimeric ConG construct donors (KPD135 and KPD136) were mated with an ICE0 recipient (CAL89), an ICE0 recipient expressing YddJ$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} (KPD128), and an ICE0 recipient expressing YddJ$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} (KPD132). As part of the same panel, both WT ConG construct donors (KPD224 and KPD225) were mated with an ICE0 recipient, (CAL89), and an ICE0 recipient expressing their cognate YddJ. Exclusion of a donor by any YddJ homolog or chimeric construct is calculated as the fold-decrease in transfer into an ICE0 recipient (CAL89) compared to transfer into an ICE0 recipient expressing that YddJ homolog or chimeric construct. Transfer is calculated as the CFU/ml of transconjugants divided by the CFU/ml of initial donors, converted to a percentage, for each mating assay. Bars represent averages from the three independent replicates, with error bars depicting standard deviations.

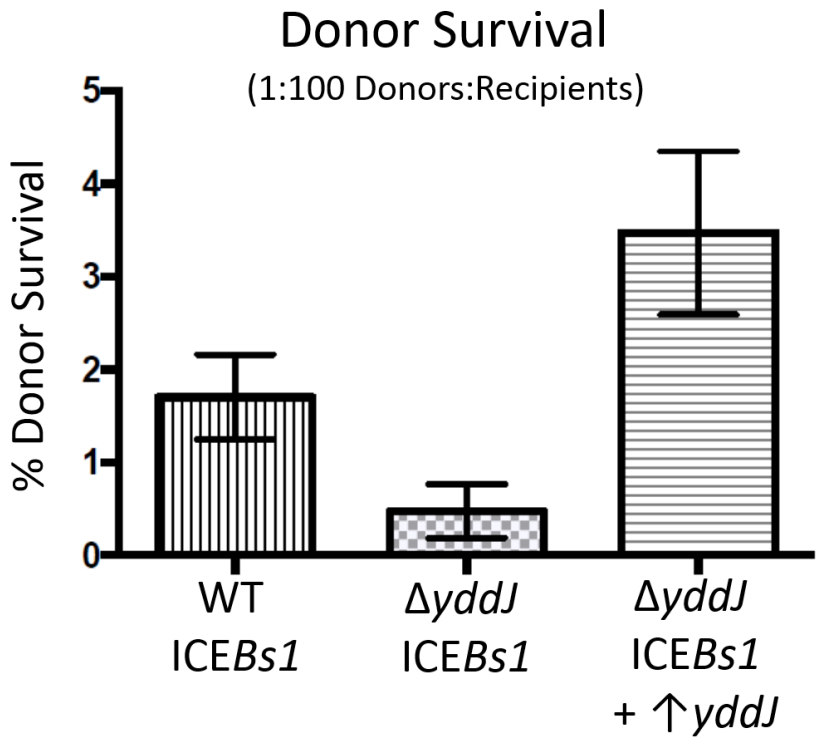

Fig. 5: YddJ-mediated exclusion protects ICE*Bs1* donors from back-transfer from new transconjugants. WT ICE*Bs1* donors (MA1049), Δ*yddJ* ICE*Bs1* donors (MA1050), and Δ*yddJ* ICE*Bs1* donors overexpressing *yddJ* from an exogenous locus (KPD210) were each mated at a 1:100 donor:recipient ratio with ICE0 recipients (CAL419). Percent donor survival was calculated by dividing post-mating donor CFU by pre-mating donor CFU and converting the result to a percentage. Percent donor survival for each donor is shown on the graph above. Bars represent averages from the three replicate mating assays for each donor, with error bars depicting standard deviations.

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype (reference)
KPD137	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}${*Bat1*(30-50)(67-82)} *mls*} *str-84 comK*::*spc*
KPD224	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bat1}$ *mls*}
KPD225	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*}
KPD135	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bat1}${*Bs1*(276-295)} *mls*}
KPD136	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}${*Bat1*(276-295)} *mls*}
KPD219	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}$ *mls*} *str-84 comK*::*spc* (Avello et al., 2019)
CAL89	ICE*Bs1*^{0} *str-84 comK*::*spc* (Auchtung et al., 2005)
MA982	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}$ *mls*} *str-84 comK*::*spc* (Avello et al., 2019)
KPD128	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} *mls*} *str-84 comK*::*spc*
KPD131	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}${*Bs1*(30-48)(65-81)} *mls*} *str-84 comK*::*spc*
KPD132	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} *mls*} *str-84 comK*::*spc*
MA1049	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls* (Avello et al., 2019)
MA1050	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*mls* (Avello et al., 2019)
CAL419	ICE*Bs1*^{0} *str-84 comK*::*cat* (Auchtung et al., 2007)
KPD210	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}$ *mls*} *comK*::*cat*

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

Appendix D

Effect of charge-flip mutations in key ConG region on exclusion and transfer

After discovering that $ConG_{Bs1}$ had some amount of sequence conservation with the DEREK motif identified in the ConG homolog TcpH in pCW3 (Teng et al., 2008), I designed some mutated $ConG_{Bs1}$ constructs to test the importance of the pattern of charged residues in this region. A conjugative plasmid found in the Gram-positive *Clostridium perfringens*, pCW3 uses a T4SS similar to ICE*Bs1*, that contains a relaxase, a coupling protein, a cell wall hydrolase, and functional homologs to ConB (TcpC), ConD (TcpE), and ConE (TcpF), as well as the ConG homolog TcpH (Bhatty et al., 2013). Similar to predictions for ConG, TcpH is found in the cell membrane and has eight putative transmembrane domains in its N-terminal half, is essential for conjugative transfer of pCW3 and is predicted to play an important structural role in the mating pair formation complex (Teng et al., 2008). In an alignment with TcpH, Orf15 from Tn*916*, and other homologs, Teng et al. found 3 conserved regions, TcpH 242VQQPW246 which was essential for conjugative transfer, 545DTK547 which was mostly dispensable and 287DEREK291 which was partially dispensable for conjugative transfer (Teng et al., 2008). Teng et al. reported that 287DEREK291 was not required for TcpH self-interaction or interaction with TcpC in a bacterial 2-hybrid assay, but otherwise did not give any predictions as to this region's functional role.

In a multiple sequence alignment with TcpH, Orf15, $ConG_{Bs1}$, and $ConG_{Bat1}$, TcpH 287DEREK291 is aligned with $ConG_{Bs1\ 281}DKKDY_{285}$ and $ConG_{Bat1\ 281}EKLDY_{285}$. In both $ConG_{Bs1}$ and $ConG_{Bat1}$, residue 279 is E and 280 is K, extending the pattern of almost-alternating positively and negatively charged residues. Residues 279-285 are within the region of residues ($ConB_{Bs1}$ and $ConG_{Bat1}$ 276-295) that I identified as conferring the specificity of exclusion in ConG, by swaps between $ConG_{Bs1}$ and $ConG_{Bat1}$ (see Chapter 3). To investigate the importance

of the pattern of positively and negatively charged residues in $ConG_{Bs1}$ 279-285, I made two mutant ConG constructs: $ConG_{Bs1}\{_{280}DKD_{282}\}$ where I changed $ConG_{Bs1}$ residue 280K to D, 281D to K, and 282K to D; and also $ConG_{Bs1}\{_{280}DKKD_{283}\}$ where I changed $ConG_{Bs1}$ residue 280K to D, 281D to K, and 283K to D. To investigate the importance of sequence identity in this region, I made the construct $ConG_{Bs1}${Orf15(289-301)}, where I replaced $ConG_{Bs1}$ residues 280-292 (KDKKDYIKETEID) with Orf15 residues 289-301 (GEDREKIVAEEIE).

I used each of these three ConG constructs to complement Δ*conG* ICE*Bs1* donors (complementation was done by expression under the inducible promoter PspankHY at the exogenous locus thrC), and tested these three donor strains (KPD145, KPD146, and KPD147) along with a Δ*conG* ICE*Bs1* donor complemented with WT $ConG_{Bs1}$ (MMB1393), for their ability to transfer into an ICE0 recipient (KPD51), and into an ICE0, $YddJ_{Bs1}$-overexpressing recipient (KPD35). This was done following a typical LB Mating assay protocol, adjusted for a longer induction time for inducing ConG overexpression under PspankHY. Briefly, 3ml LB cultures of each donor and recipient were grown to mid-log, then used to inoculate LB cultures to OD=0.001. Cultures were grown for ~1hr until they reached OD=0.01, then donor cultures were induced for 2 hours with 1mM IPTG, for overexpression of *rapI* and *conG* (or a mutant *conG* construct), each under their own Pspank(hy) promoter. Concurrently, the ICE0 *yddJ*-overexpressing recipients (KPD35) were induced with 1mM IPTG to drive *yddJ* overexpression under the Pspank(hy) promoter. Donor and recipient cultures were mixed at OD ratio of 1:1, and poured over a filter, and the filter was incubated on a Spizizen Salts plate for 3 hours at 37^{o}C, to allow for mating. Cells were then re-suspended off the filter in Spizizen Salts liquid media, and serial dilutions and plating on selective media was performed to quantify the

numbers of donor, recipient, and transconjugant CFU. Percent transfer was calculated as CFU/ml of transconjugant divided by the CFU/ml of donors, and exclusion was calculated as the fold-difference between mating into an ICE0 recipient and mating into an ICE0 *yddJ*-overexpressing recipient. The results are presented in Fig. 1.

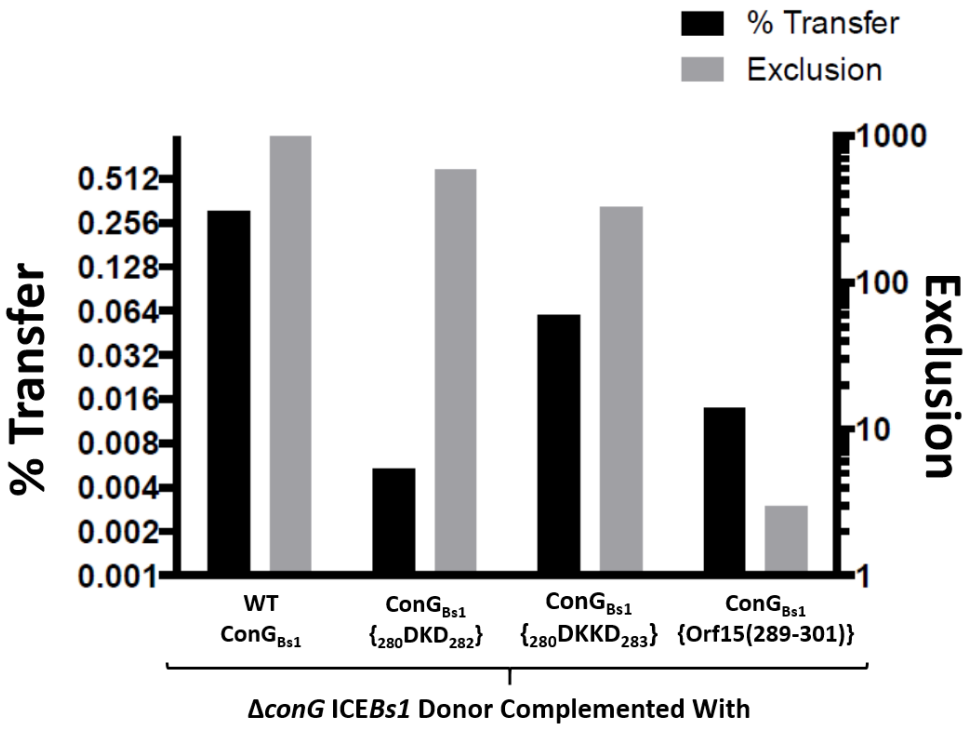

Fig. 1: Effect of mutations in key ConG region on exclusion and transfer. In this experiment, Δ*conG* ICE*Bs1* donors complemented with WT ConG$_{Bs1}$ (MMB1393), ConG$_{Bs1}$ {$_{280}$DKD$_{282}$} (KPD145), ConG$_{Bs1}$ {$_{280}$DKKD$_{283}$} (KPD146), and ConG$_{Bs1}$ {Orf15(289-301)} (KPD147) were mated with ICE0 recipients (KPD51) and ICE0, YddJ$_{Bs1}$-overexpressing recipients (KPD35) in an LB Mating Assay. % Transfer (into ICE0 recipients), and exclusion (fold-difference in transfer into ICE0 recipients vs. transfer into ICE0 YddJ$_{Bs1}$-overexpressing recipients) was calculated for each donor. These results are from a single mating assay and should not be assigned any statistical significance.

Because this mating assay panel was only done once, no statistical significance can be assigned to these results; however, these results do give us a general sense for the behavior of donors with these ConG constructs. The donor complemented with ConG$_{Bs1}${$_{280}$DKD$_{282}$}

(KPD145) showed a 58-fold decrease in transfer (into ICE0) and 14-fold decrease in exclusion, compared to the donor complemented with WT ConG (MMB1393); while the donor complemented with $ConG_{Bs1}\{_{280}DKKD_{283}\}$ (KPD146) showed only a 5-fold decrease in transfer and a 25-fold decrease in exclusion, compared to MMB1393. On the other hand, the donor complemented with $ConG_{Bs1}$\{Orf15(289-301)\} (KPD147) showed a 22-fold decrease in transfer (into ICE0) compared to MMB1393, and almost no exclusion (exclusion = 3, compared to exclusion = 8295 for MMB1393).

It is clear from these results both exclusion and transfer can be affected by even small perturbations in the order of charged residues in this region of $ConG_{Bs1}$, and that constructs with larger decreases in transfer do not necessarily have larger decreases in exclusion, compared to WT $ConG_{Bs1}$. This suggests that this region in $ConG_{Bs1}$, and also likely in $ConG_{Bat1}$, does have a functional role in conjugation beyond just exclusion, as is evident for TcpH in pCW3, however further investigation will be required to determine the nature of this role.

Table 1. *B. subtilis* strains[a]

Strain	Relevant genotype
KPD145	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)- *conG*$_{Bs1}$($_{280}$DKD$_{282}$) *mls*}
KPD146	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)- *conG*$_{Bs1}$($_{280}$DKKD$_{283}$) *mls*}
KPD147	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)- *conG*$_{Bs1}${Orf15(289-301)} *mls*}
KPD51	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *str-84 comK*::*tet*
KPD35	ICE*Bs1*^{0} *amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)- *yddJ mls*} *str-84 comK*::*tet*
MMB1393	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pspank(hy)-*rapI spc*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} (Leonetti et al., 2015)

[a]all strains derived from JH642 and contain *pheA1*, *trpC2* mutations (Perego et al., 1988)

References

Bhatty, M., Laverde Gomez, J. A., & Christie, P. J. (2013). The expanding bacterial type IV secretion lexicon. *Research in Microbiology*, *164*(6), 620–639. https://doi.org/10.1016/j.resmic.2013.03.012

Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15

Perego, M., Spiegelman, G. B., & Hoch, J. A. (1988). Structure of the gene for the transition state regulator , abrB : regulator synthesis is controlled by the spoOA sporulation gene in Bacillus subtilis. *Mol Microbiol*, *2*(6), 689–699.

Teng, W. L., Bannam, T. L., Parsons, J. A., & Rood, J. I. (2008). Functional characterization and localization of the TcpH conjugation protein from Clostridium perfringens. *Journal of Bacteriology*, *190*(14), 5075–5086. https://doi.org/10.1128/JB.00386-08

Chapter 4

Discussion

Summary

ICE*Bs1* has an exclusion mechanism, in which the exclusion protein YddJ specifically recognizes its target ConG in the conjugation machinery. YddJ is necessary and sufficient in the recipient for exclusion, and is not required in the donor for exclusion or transfer (Chapter 2). A mutagenesis and enrichment screen identified exclusion-resistant mutations in ConG, and swap experiments with ICE*Bs1* and ICE*Bat1* homologs demonstrated that YddJ targets its cognate ConG in the conjugation machinery, and YddJ and ConG together determine the specificity of exclusion (Chapter 2). Experiments with chimeric versions of ConG and YddJ, generated from ICE*Bs1* and ICE*Bat1* homologs, demonstrate that residues 276-295 of both ICE*Bs1* ConG and ICE*Bat1* ConG confer specificity of exclusion. Residues 30-50, 67-82, and 87-96 of ICE*Bat1* YddJ, and the corresponding residues 30-48, 65-81, and to a lesser extent 86-95 of ICE*Bs1* YddJ are necessary for specificity of exclusion and/or generation of a functional YddJ chimeric construct. Exclusion confers a protective benefit to ICE*Bs1* host cells, under conditions that support conjugative transfer (Chapter 2). Specifically, exclusion protects ICE*Bs1* donors from becoming recipients during or immediately after they serve as donors (Chapter 3).

Protective Advantage and Mechanism of ICE*Bs1* Exclusion

For an ICE*Bs1* host cell, what is so detrimental about serving as a recipient during or right after serving as a donor? How does ICE*Bs1* exclusion protect against whatever damage is caused by this scenario? One likely possibility is that serving as a donor inflicts sufficient membrane and/or cell wall damage on an ICE*Bs1* host cell to make it particularly vulnerable to damage that occurs when it serves as a recipient. The assembly of a transmembrane channel

to move conjugative DNA and proteins across both the donor and the recipient cell membranes likely causes stress to either or both membranes. The function of both the muramidase and peptidase domains of CwlT contribute to ICE*Bs1* transfer efficiency, indicating that cell wall hydrolysis of the donor, the recipient or both is necessary for transfer (DeWitt and Grossman, 2014). Planned future experiments include testing whether the use of osmotic protective conditions used to sustain *Bacillus subtilis* L-forms (Leaver et al., 2009) in mating assays can rescue the donor survival decrease observed in exclusion-null donors. This would indicate that exclusion is protecting ICE*Bs1* donors from excessive cell wall damage sustained when they also serve as recipients. If indeed exclusion is protecting against cell wall damage or membrane penetration in the would-be recipient, then exclusion must stop the conjugative process before either of those occur – that is, before penetration of the would-be recipient cell membrane, or before hydrolysis of the recipient cell wall by CwlT and/or any other host hydrolases.

Regarding the mechanism of ICE*Bs1* exclusion, we know that ConG residues 276-295 and ICE*Bs1* YddJ residues 30-48, 65-81, and to a lesser extent 86-95 are necessary for exclusion specificity (or in the case of YddJ, necessary for creating a functional chimeric YddJ protein) (see Chapter 3). Topological predictions (Avello et al., 2019; Leonetti et al., 2015) indicate the region necessary for exclusion specificity in ConG is in a loop exterior to the donor cell membrane. Since YddJ is a putative lipoprotein and experimental evidence indicates its association with the membrane (Avello et al., 2019; Otto et al., 2010; Zhou et al., 2008), it is possible that YddJ and ConG could directly interact, with the residues necessary for exclusion specificity forming the interaction interface. It is unknown if this is the case, and if so, whether

any other essential ICE*Bs1* or host proteins are necessary for this interaction, or how this interaction would prevent conjugative transfer.

One intriguing hypothesis, generated from a comparison of ConG and ConB to their homologs VirB6 and VirB8, respectively, is that YddJ interaction with ConG could prevent ConG from making productive interactions with the T-DNA or from "passing" the T-DNA to ConB. VirB6, the ConG homolog in the VirB/D T4SS, has 5 transmembrane segments, with a large central periplasmic loop (residues 84-165) between the second and third transmembrane segments, that is necessary for VirB6 interaction with conjugative transfer DNA (T-DNA) (Jakubowski et al., 2004). VirB6 residues 165-245, which encompass transmembrane segments 3, 4 and 5 of the protein, are necessary for T-DNA transfer from VirB6 to VirB8 (Jakubowski et al., 2004). An alignment between VirB6 and ICE*Bs1* ConG indicated that the VirB6 residue 84-165 periplasmic loop aligns with a ConG region predicted to be a loop, exterior to the cytoplasm, connecting the third and fourth transmembrane domains. This region includes residues 276-295, which are necessary for exclusion specificity in ICE*Bs1* and ICE*Bat1* ConG (see Chapter 3). Polyphobius, an online transmembrane topology prediction tool (Käll et al., 2005), was used to predict the location and orientation of transmembrane segments in ICE*Bat1* ConG and three other ConG homologs (in *Bacillus subtilis* subsp. *natto*, *Bacillus* sp. JS, and *Bacillus* sp. SDLI1) in addition to ICE*Bs1* ConG. An alignment of the predicted exterior loop regions from each of these ConG homologs and the periplasmic loop from VirB6 is shown in Fig. 1. It is possible that this exterior loop region in ConG performs a similar function to the periplasmic loop of VirB6, and if YddJ directly interacts with ConG at or near ConG residues 276-295, YddJ-mediated exclusion could prevent ConG from interacting with the T-DNA, or it could prevent

ConG from passing the T-DNA to ConB, just like VirB6 passes the T-DNA to VirB8. Experiments are currently underway to test this hypothesis.

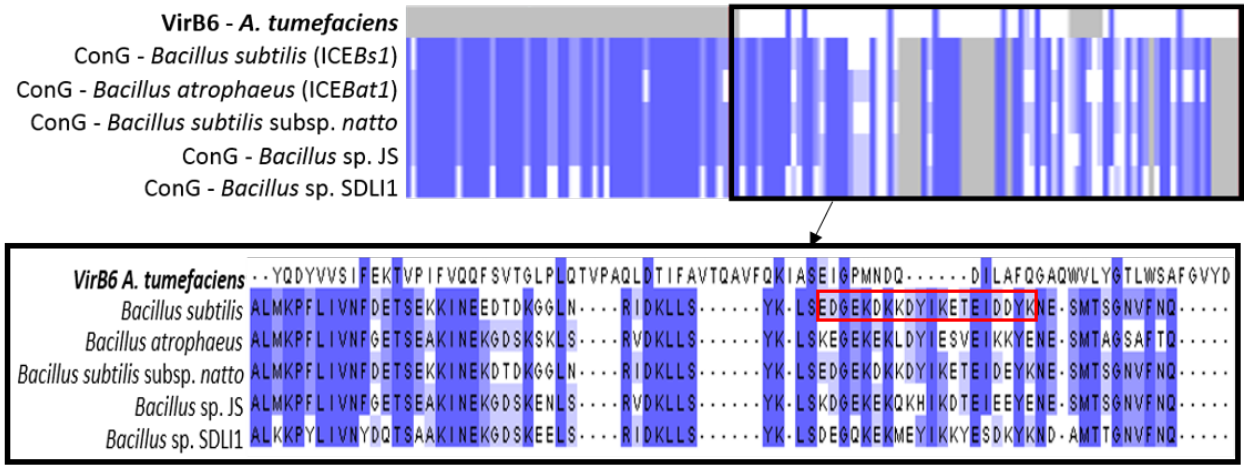

Fig. 1: Alignments of the predicted periplasmic loop region of VirB6, and corresponding loop in ConG homologs. Polyphobius (Käll et al., 2005) was used to predict transmembrane domains and topology of 5 ConG homologs. Whole-protein alignments between VirB6 and the ConG homologs indicated that the VirB6 periplasmic loop region spanning residues residue 84-165 aligns with C-terminal end of the predicted exterior loop region between the third and fourth transmembrane segments in the ConG homologs. This exterior loop contains residues 276-295 (marked with the red box), which are essential for exclusion specificity in ICE*Bs1* ConG and ICE*Bat1* ConG. The top colored panel above shows an alignment between this exterior loop in several ConG homologs, and the periplasmic loop in VirB6. Blue indicates sequence identity, white indicates non-identity, and gray indicates gaps in the alignment. Below the alignment is a zoomed-in version of the C-terminal part of this alignment, showing the protein sequences where the VirB6 loop region actually aligns.

If indeed YddJ mediates exclusion by preventing ConG interaction with the T-DNA (or by preventing ConG passing the T-DNA to ConB), then this provides not only essential information about the ICE*Bs1* exclusion mechanism, but also about the ICE*Bs1* conjugation process. It indicates that this step with the T-DNA occurs before whatever damage-causing process exclusion protects against. As mentioned earlier, this damage-causing process is likely either degradation of the recipient cell wall by CwlT or another hydrolase, or penetration of the

recipient cell membrane by the mating apparatus. This suggests that engagement of ConG with the T-DNA, or ConG passing the T-DNA to ConB, could somehow either trigger the mating apparatus to penetrate the recipient cell membrane, or cause CwlT or another recipient hydrolase to begin cutting through the recipient cell wall. In this way, further investigation into the mechanism of ICE*Bs1* exclusion will likely yield valuable insight into the nature and function of ICE*Bs1* and other Gram-positive conjugation machinery.

The Purpose of Exclusion in ICE*Bs1*

The question of why ICE*Bs1* has an exclusion system is particularly interesting given that ICE*Bs1* already has two mechanisms – ImmR-mediated immunity and PhrI degradation of RapI (see Chapter 1: Introduction) – to prevent redundant ICE*Bs1* transfer. Exclusion prevents ICE*Bs1* donor cells from serving as recipients during or immediately after serving as donors, possibly to prevent additional cell wall damage by CwlT or other hydrolases. Since immunity works after conjugation has occurred, it would not protect ICE*Bs1* hosts from cell wall damage or any other harmful effects of conjugation itself. On the other hand, uptake of PhrI and subsequent PhrI degradation of RapI does prevent conjugation from occurring. In the experiments described in Chapter 3, exclusion is protecting ICE*Bs1* donors in a situation where they start off the three hour mating period surrounded by ICE0 recipients, and none of these recipients receive *rapI* when they become transconjugants (because the copy of ICE*Bs1* transferred is Δ(*rapI-phrI*)*342*::*kan*). However, even if these recipients were receiving a WT copy of ICE*Bs1* containing *rapI* and *phrI*, it would likely not prevent new transconjugants from being able to serve as donors, as least for a short window of time. Previous work indicates that

initially there is no ImmR present in a new transconjugant, to repress transcription from P*xis*, and this allows transconjugants to rapidly be ready to act as donors, particularly in the context of cell chains (Babic et al., 2011). Eventually sufficient ImmR will accumulate in the new transconjugant that RapI and PhrI would have an effect on ICE*Bs1* regulation, however this is likely too late to prevent transfer of ICE*Bs1* back into the initial donor (Babic et al., 2011). Transfer of ICE*Bs1* from a new transconjugant back into the original donor may mimic the situation of cell chains, given that the transconjugant and original donor are already in contact. Thus, exclusion is likely serving to protect ICE*Bs1* host cells during a window of time in which the donors are particularly vulnerable and the other two mechanisms to prevent redundant transfer are unable to provide adequate protection.

What happens if cell-cell signaling, exclusion, and immunity all fail, and a second copy of ICE*Bs1* is able to integrate into the cell? Given the existence of three mechanisms each designed to prevent this, it suggests that integration of a second copy would be seriously detrimental to either ICE*Bs1* (the new copy and/or the original copy), the host cell, or both. Indeed, for a host cell to maintain two copies of ICE*Bs1*, the host cell must be kept under constant selective pressure. When ICE*Bs1* integrates into the chromosome, it integrates into a specific site in *trnS-leu2* (Auchtung et al., 2005). Known as the AttB site, this 17bp sequence containing an inverted 5bp repeat on either end is sufficient for ICE*Bs1* integration, and after ICE*Bs1* integration this 17bp sequence is present in both the left attachment site (AttL) and the right attachment site (AttR) of ICE*Bs1* (Lee et al., 2007). This means that if a second copy of ICE*Bs1* integrated into the chromosome, it would likely form a tandem array with the first copy of ICE*Bs1*. Imperfect recombination events could lead to one or both elements becoming non-

functional. Furthermore, the integration of each additional copy of ICE*Bs1* would recreate another AttB site, allowing tandem elements to accumulate, and it is unknown what effect this could have on the host chromosome, but it could very well be quite detrimental.

The Purpose of Exclusion in Other Mobile Genetic Elements

How does the purpose of ICE*Bs1* exclusion compare to the purpose of exclusion in other mobile genetic elements? In the case of conjugative plasmids, results indicate that exclusion is an essential part of their biology (Garcillán-Barcia and de la Cruz, 2008). So far, it has proved impossible to stably maintain exclusion-deficient, transfer-competent F plasmid mutants in the laboratory, and exclusion in F-plasmids is essential for resisting lethal zygosis (Ou, 1980; Skurray and Reeves, 1974). Exclusion also helps ensure plasmid stability and maintenance, by preventing recombination events that result in nonfunctional plasmid copies (Hopkins et al., 1980; Peters and Benson, 1995; Syvanen et al., 1986). On the other hand, many ICEs do not have exclusion, and it has been proposed (Garcillán-Barcia and de la Cruz, 2008) that this allows for rapid evolutionary development. So why do some ICEs have exclusion, and what determines whether or not an ICE harbors an exclusion system? ICEs reported to contain exclusion systems include the SXT/R391 family of ICEs, found in Gram-negative hosts (Marrero and Waldor, 2005, 2007). Exclusion systems are also found in two integrative and conjugative plasmid systems, SLP1 (Hagège et al., 1999) and pSAM2 (Possoz et al., 2003) in mycelium-forming Streptomycetes; like ICEs these integrative and conjugative plasmids integrate into the recipient chromosome after transfer, although like for all Actinomycetes conjugative transfer is by a different mechanism than T4SS transfer of ssDNA described in Chapter 1 (te Poele et al.,

2008). SLP1 and pSAM2 (Hagège et al., 1994; Omer and Cohen, 1984; Possoz et al., 2001), and SXT and R391 ICEs (Hochhut et al., 2001) all integrate into the chromosome in a site-specific manner, just like ICE*Bs1*, which suggests the immediate hypothesis that site-specific integration is what necessitates exclusion in these elements. However, SXT elements and R391 elements integrate at an identical attachment site in the 5' end of the *prfC* gene, and when both elements were found in a cell, they were integrated in tandem fashion in the chromosome (Hochhut et al., 2001), just like ICE*Bs1* could presumably do, if one or more additional copies of ICE*Bs1* managed to evade cell-cell signaling, exclusion and immunity. Since SXT and R391 can't exclude each other, integration into the same chromosomal site alone is not enough to necessitate an exclusion system. In the case of pSAM2 and SLP1, conjugative transfer occurs by a different mechanism (te Poele et al., 2008), so clearly something other than the effect of donating or receiving transfer through a T4SS can necessitate an exclusion system in an ICE. Given the differences between SXT/R391 ICEs, ICE*Bs1*, and pSAM2 and SLP1, it is likely that the reasons behind an exclusion system are different in the case of each element.

Ultimately, it seems that ICEs maintain an exclusion system when there is selective pressure to do so. This selective pressure could be pressure to achieve a favorable outcome, as is possibly the case for SXT and R391. Genetic analysis suggests extensive recombination among members of the SXT and R391 families of ICEs, which allows for exchange of genetic material, in particular the trading of genes in variable genetic regions, many of which confer some benefit to the ICE host cells (Wozniak et al., 2009). Alternatively, the selective pressure on an ICE to have an exclusion system could be to avoid a negative outcome. This appears to be the case for ICE*Bs1* – both the process of a donor cell serving as a recipient for a second copy of ICE*Bs1*, and

integration of this second copy, are both unfavorable events. It would be interesting to see if an ICE that did not have an exclusion system could be forced to evolve one in a laboratory setting by having selective pressure applied to it – perhaps by genetically modifying it to only be able to integrate into a single location on the chromosome, or by adding some genetic component to the ICE DNA that caused two or more copies in one cell to be toxic. This exercise could not only be informative concerning the conjugative transfer mechanism and pressures on the element, but it could also have practical applications, such as designing an exclusion protein to express in a bacterial cell line to keep out unwanted copies of a particular mobile genetic element.

References

Auchtung, J. M., Lee, C. A., Monson, R. E., Lehman, A. P., & Grossman, A. D. (2005). Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. *Proceedings of the National Academy of Sciences*, *102*(35), 12554–12559. https://doi.org/10.1073/pnas.0505835102

Avello, M., Davis, K. P., & Grossman, A. D. (2019). Identification, characterization, and benefits of an exclusion system in an integrative and conjugative element of Bacillus subtilis. *Molecular Microbiology*, 1–17. https://doi.org/10.1111/mmi.14359

Babic, A., Berkmen, M. B., Lee, C. A., & Grossman, A. D. (2011). Efficient Gene Transfer in Bacterial Cell Chains. *MBio*, *2*(2). https://doi.org/10.1128/mbio.00027-11

DeWitt, T., & Grossman, A. D. (2014). The bifunctional cell wall hydrolase cwlT is needed for conjugation of the integrative and conjugative element ICEBs1 in Bacillus subtilis and B. anthracis. *Journal of Bacteriology*, *196*(8), 1588–1596. https://doi.org/10.1128/JB.00012-14

Garcillán-Barcia, M. P., & de la Cruz, F. (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid*, Vol. 60, pp. 1–18. https://doi.org/10.1016/j.plasmid.2008.03.002

Hagège, J., Boccard, F., Smokvina, T., Pernodet, J. L., Friedmann, A., & Guérineau, M. (1994). Identification of a gene encoding the replication initiator protein of the streptomyces integrating element, pSAM2. *Plasmid*, *31*(2), 166–183. https://doi.org/10.1006/plas.1994.1018

Hagege, J. M., Brasch, M. A., & Cohen, S. N. (1999). Regulation of transfer functions by the imp locus of the Streptomyces coelicolor plasmidogenic element SLP1. *Journal of Bacteriology*,

181(19), 5976–5983.
Hochhut, B., Beaber, J. W., Woodgate, R., & Waldor, M. K. (2001). Formation of chromosomal tandem arrays of the SXT element and R391, two conjugative chromosomally integrating elements that share an attachment site. *Journal of Bacteriology*, *183*(4), 1124–1132. https://doi.org/10.1128/JB.183.4.1124-1132.2001
Hopkins, J. D., Clements, M. B., Liang, T. Y., Isberg, R. R., & Syvanen, M. (1980). Recombination genes on the Escherichia coli sex factor specific for transposable elements. *Proceedings of the National Academy of Sciences*, *77*(5), 2814–2818. https://doi.org/10.1073/pnas.77.5.2814
Jakubowski, S. J., Krishnamoorthy, V., Cascales, E., & Christie, P. J. (2004). Agrobacterium tumefaciens VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. *Journal of Molecular Biology*, *341*(4), 961–977. https://doi.org/10.1016/j.jmb.2004.06.052
Käll, L., Krogh, A., & Sonnhammer, E. L. L. (2005). An HMM posterior decoder for sequence feature prediction that includes homology information. *Bioinformatics*, *21*(SUPPL. 1). https://doi.org/10.1093/bioinformatics/bti1014
Leaver, M., Domínguez-Cuevas, P., Coxhead, J. M., Daniel, R. A., & Errington, J. (2009). Life without a wall or division machine in Bacillus subtilis. *Nature*, *457*(7231), 849–853. https://doi.org/10.1038/nature07742
Lee, C. A., Auchtung, J. M., Monson, R. E., & Grossman, A. D. (2007). Identification and characterization of int (integrase), xis (excisionase) and chromosomal attachment sites of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Molecular Microbiology*, *66*(6), 1356–1369. https://doi.org/10.1111/j.1365-2958.2007.06000.x
Leonetti, C. T., Hamada, M. A., Laurer, S. J., Broulidakis, M. P., Swerdlow, K. J., Lee, C. A., … Berkmen, M. B. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. *Journal of Bacteriology*, *197*(15), 2558–2567. https://doi.org/10.1128/JB.00142-15
Marrero, J., & Waldor, M. K. (2005). Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Developmental Cell*, *8*(6), 963–970. https://doi.org/10.1016/j.devcel.2005.05.004
Marrero, J., & Waldor, M. K. (2007). The SXT/R391 family of integrative conjugative elements is composed of two exclusion groups. *Journal of Bacteriology*, *189*(8), 3302–3305. https://doi.org/10.1128/JB.01902-06
Omer CA, & Cohen SN. (1984). Plasmid formation in Streptomyces: excision and integration of the SLP1 replicon at a specific chromosomal site. *Molecular & General Genetics*, *196*(3), 429–438.
Otto, A., Bernhardt, J., Meyer, H., Schaffer, M., Herbst, F. A., Siebourg, J., … Becher, D. (2010). Systems-wide temporal proteomic profiling in glucose-starved Bacillus subtilis. *Nature Communications*, *1*(9), 1–9. https://doi.org/10.1038/ncomms1137
Ou, J. T. (1980). Role of surface exclusion genes in lethal zygosis in Escherichia coli K12 mating. *MGG Molecular & General Genetics*, *178*(3), 573–581. https://doi.org/10.1007/BF00337863
Peters, J. E., & Benson, S. A. (1995). Redundant transfer of F' plasmids occurs between Escherichia coli cells during nonlethal selections. *Journal of Bacteriology*, *177*(3), 847–850.

https://doi.org/10.1128/jb.177.3.847-850.1995
Possoz, C., Gagnat, J., Sezonov, G., Guérineau, M., & Pernodet, J. L. (2003). Conjugal immunity of Streptomyces strains carrying the integrative element pSAM2 is due to the pif gene (pSAM2 immunity factor). *Molecular Microbiology*, *47*(5), 1385–1393. https://doi.org/10.1046/j.1365-2958.2003.03380.x
Possoz, C., Ribard, C., Gagnat, J., Pernodet, J. L., & Guérineau, M. (2001). The integrative element pSAM2 from Streptomyces: Kinetics and mode of conjugal transfer. *Molecular Microbiology*, *42*(1), 159–166. https://doi.org/10.1046/j.1365-2958.2001.02618.x
Skurray, R. A., & Reeves, P. (1974). F factor mediated immunity to lethal zygosis in Escherichia coli K 12. *Journal of Bacteriology*, *117*(1), 100–106.
Syvanen, M., Hopkins, J. D., Griffin IV, T. J., Liang, T. Y., Ippen-Ihler, K., & Kolodner, R. (1986). Stimulation of precise excision and recombination by conjugal proficient F' plasmids. *MGG Molecular & General Genetics*, *203*(1), 1–7. https://doi.org/10.1007/BF00330376
te Poele, E. M., Bolhuis, H., & Dijkhuizen, L. (2008). Actinomycete integrative and conjugative elements. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, Vol. 94, pp. 127–143. https://doi.org/10.1007/s10482-008-9255-x
Wozniak, R. A. F., Fouts, D. E., Spagnoletti, M., Colombo, M. M., Ceccarelli, D., Garriss, G., … Waldor, M. K. (2009). Comparative ICE genomics: Insights into the evolution of the SXT/R391 family of ICEs. *PLoS Genetics*, *5*(12), 1–13. https://doi.org/10.1371/journal.pgen.1000786
Zhou, M., Boekhorst, J., Francke, C., & Siezen, R. J. (2008). LocateP: Genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinformatics*, *9*(173), 1–17. https://doi.org/10.1186/1471-2105-9-173