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

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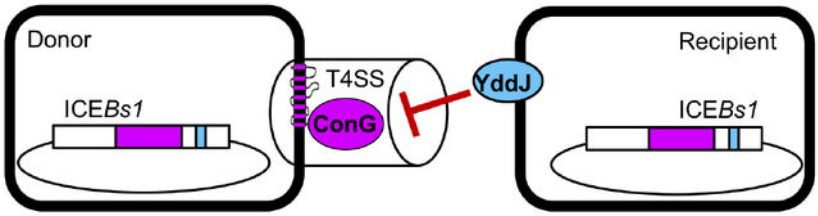
Abstract

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.

Abbreviated Summary



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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.

Type IV secretion systems encoded by plasmids and integrative and conjugative elements (ICEs) mediate transfer of DNA from donor to recipient cells, enabling bacteria to acquire new traits, including antibiotic resistances. Exclusion systems in conjugative elements inhibit the activity of the cognate conjugation machinery. We identified and characterized an exclusion system in ICE*Bs1* of *Bacillus subtilis*: the exclusion protein YddJ targets ConG (VirB6 homolog) in its cognate conjugation machinery inhibiting conjugative DNA transfer and providing 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 & 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 & Waldor, 2004; Johnson & Grossman, 2015). In addition, many ICEs can mobilize other genetic elements, typically plasmids, that do not encode their own transfer machinery (e.g., Naglich & 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 a 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 & 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 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 & Andrews, 1988) and that does not encode an exclusion function (Norgren & 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 ~1×10^{-4} % transconjugants per donor (Fig. 2), similar to previously reported results (Naglich & 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 & 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 4×10^{-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 & Dziegielewska, 1997). ICE*Bs1* contains a cell wall hydrolase, CwlT, with a peptidase domain belonging to the C1 family (Fukushima et al., 2008; Dewitt & 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 & 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 & 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 2×10^{-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 6×10^{-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 $ConG_{ICE\,Bs1}$ 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 $conG_{ICE\,Bs1}$ (ICE*Bs1* Δ*conG* Δ(*rapI-phrI*)*342*::*kan*, *thrC*::{Pspank(hy)-$conG_{ICE\,Bs1}$ *mls*}; KPD225) or $conG_{ICE\,Bat1}$ (ICE*Bs1* Δ*conG* Δ(*rapI-phrI*)*342*::*kan*, *thrC*::{Pspank(hy)-$conG_{ICE\,Bat1}$ *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 $conG_{ICE\,Bs1}$ and $conG_{ICE\,Bat1}$ complemented the transfer defect caused by loss of *conG* in ICE*Bs1*. As determined previously, $conG_{ICE\,Bs1}$ fully restored transfer to wild type levels (Babic et al., 2011; Leonetti et al., 2015). $conG_{ICE\,Bat1}$ largely restored conjugation, but to levels ~10-fold lower than those with $conG_{ICE\,Bs1}$ (Fig. 5C). Based on

these results, we conclude that *conG*$_{ICE Bat1}$ 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*$_{ICE Bs1}$ or *conG*$_{ICE Bat1}$ into recipient strains that expressed *yddJ* from either ICE*Bs1* (MA982) or ICE*Bat1* (KPD219). Donor cells expressing *conG*$_{ICE Bs1}$ as part of the conjugation machinery were sensitive to exclusion by recipient cells expressing *yddJ*$_{ICE Bs1}$ (Fig. 5C), recapitulating results presented above. However, they were not sensitive to exclusion by recipients expressing *yddJ*$_{ICE Bat1}$ (Fig. 5C). The failure of YddJ$_{ICE Bat1}$ to inhibit the conjugation machinery containing ConG from ICE*Bs1* is intriguing, but could simply reflect a non-functional *yddJ*$_{ICE Bat1}$, due perhaps to lack of expression, misfolding, or a defective gene. Alternatively, if YddJ$_{ICE Bat1}$ is functional, then it could indicate that the ConG-YddJ pair determines specificity of exclusion.

We found that YddJ$_{ICE Bat1}$ was indeed functional and was able to inhibit the conjugation machinery that contained ConG from ICE*Bat1*. Donor cells expressing *conG*$_{ICE Bat1}$ as part of the conjugation machinery were able to transfer ICE*Bs1* at a frequency of 6×10^{-2} % into cells without *yddJ*. Transfer was reduced ~2,000-fold into recipients that expressed *yddJ*$_{ICE Bat1}$, but there was no significant reduction into recipient cells that expressed *yddJ*$_{ICE Bs1}$ (Fig. 5C).

To summarize these results: 1) YddJ$_{ICE Bat1}$ is functional and capable of inhibiting transfer from the ICE*Bs1* conjugation machinery that contains ConG$_{ICE Bat1}$, but does not inhibit transfer from the ICE*Bs1* conjugation machinery containing ConG$_{ICE Bs1}$; 2) YddJ$_{ICE Bs1}$ is capable of inhibiting transfer from the ICE*Bs1* conjugation machinery that contains the ConG$_{ICE Bs1}$, but does not inhibit transfer from the ICE*Bs1* conjugation machinery that contains ConG$_{ICE Bat1}$. 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 ~8×10^{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, ~8×10^8 or ~3×10^6 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 peptidases 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 & 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 & 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 minimum 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 & 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 & 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 & 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 into 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)-$yddJ_{\text{ICE}Bat1}$, (*yddJ* from ICE*Bat1*) present in strain KPD219. *yddJ* from ICE*Bs1* was PCR amplified from genomic DNA from strain AG174. $yddJ_{\text{ICE}Bat1}$ 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 & 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-phr1*)*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 (~4×10^8 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 & 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, ~8×10^8 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 ~8×10^8 or ~3×10^6 cells were mixed with 5 ml or 10 ml of medium, respectively, before sampling and filtering.

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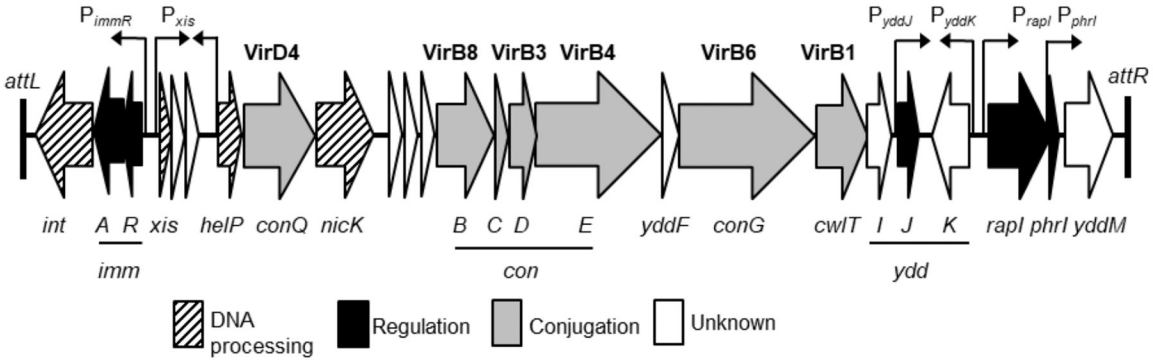

Fig.1. Genetic map of ICE*Bs1*.

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).

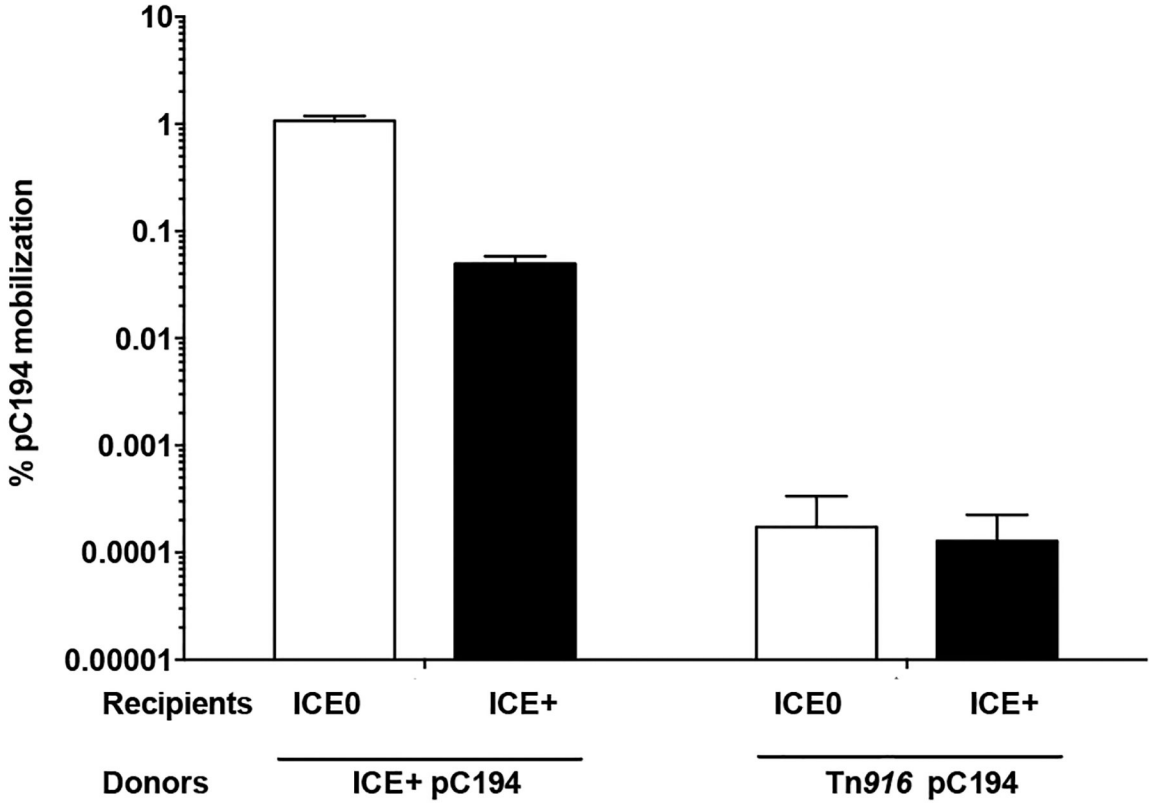

Fig. 2. ICE*Bs1* in recipient cells inhibits acquisition of pC194 mobilized by ICE*Bs1*, but not by Tn*916*.

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.

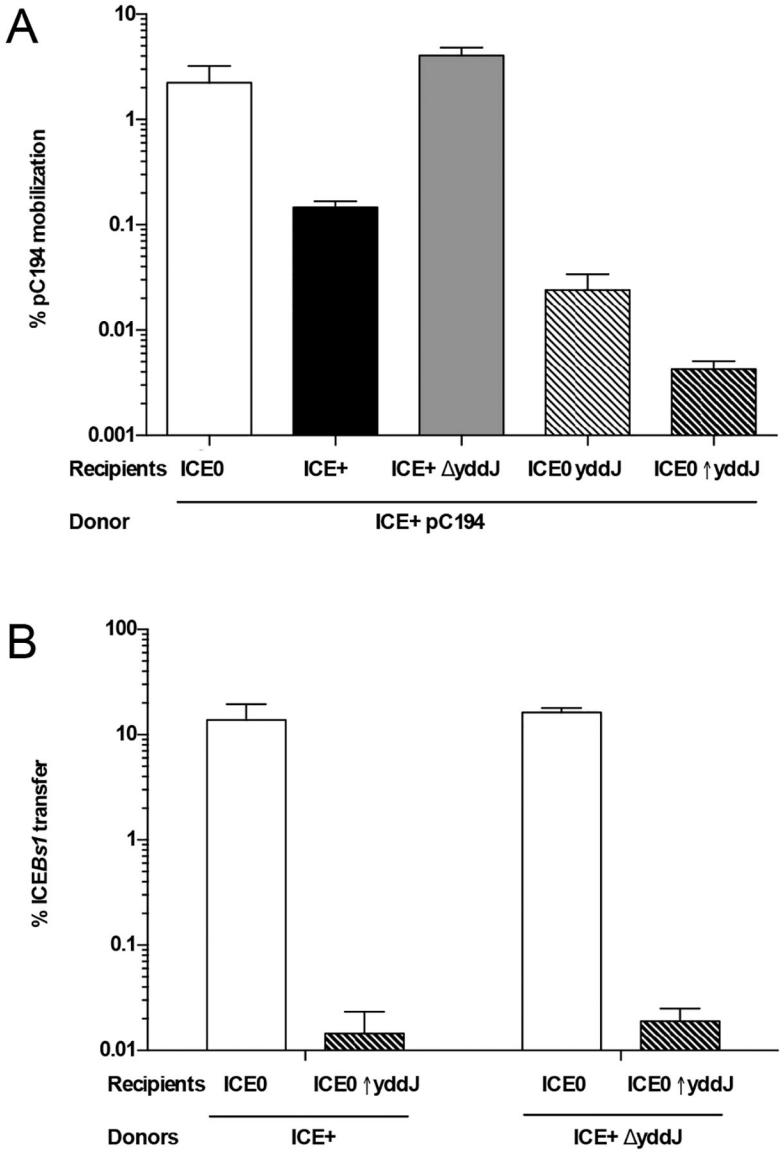

Fig. 3. In recipient cells, ICE*Bs1* gene *yddJ* is necessary and sufficient for exclusion.

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.

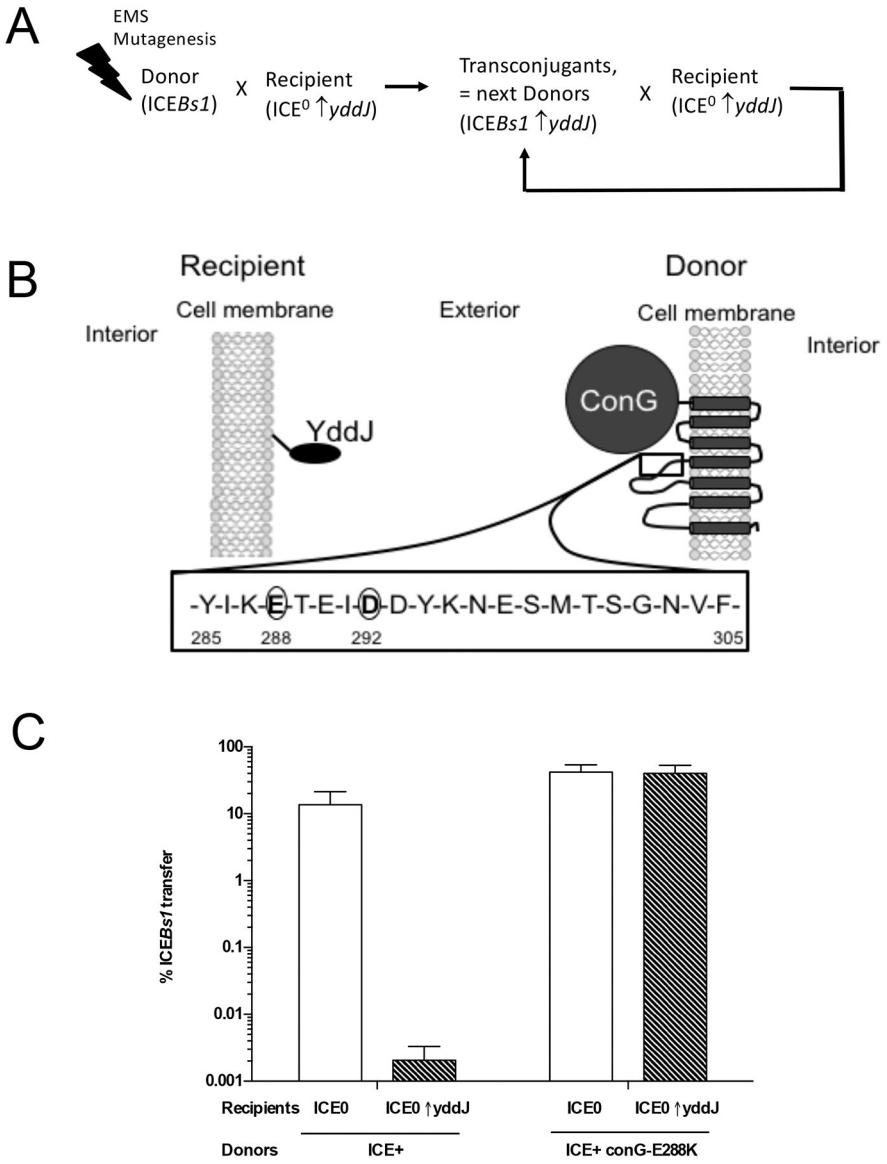

Fig. 4. Isolation of exclusion-resistant *conG* mutations in ICE*Bs1*.

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.

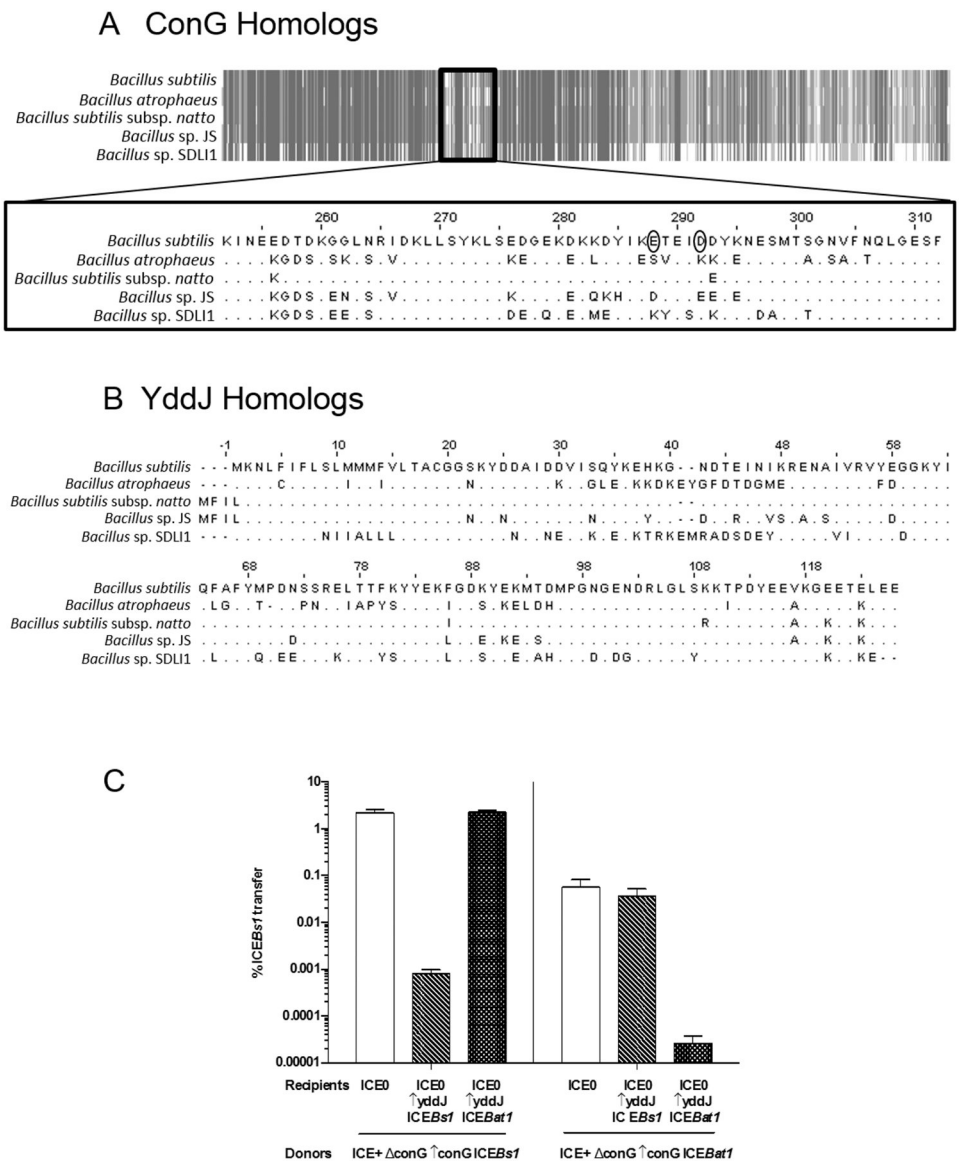

Fig. 5. ICE*Bs1* and ICE*Bat1* homology and exclusion specificity.

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.

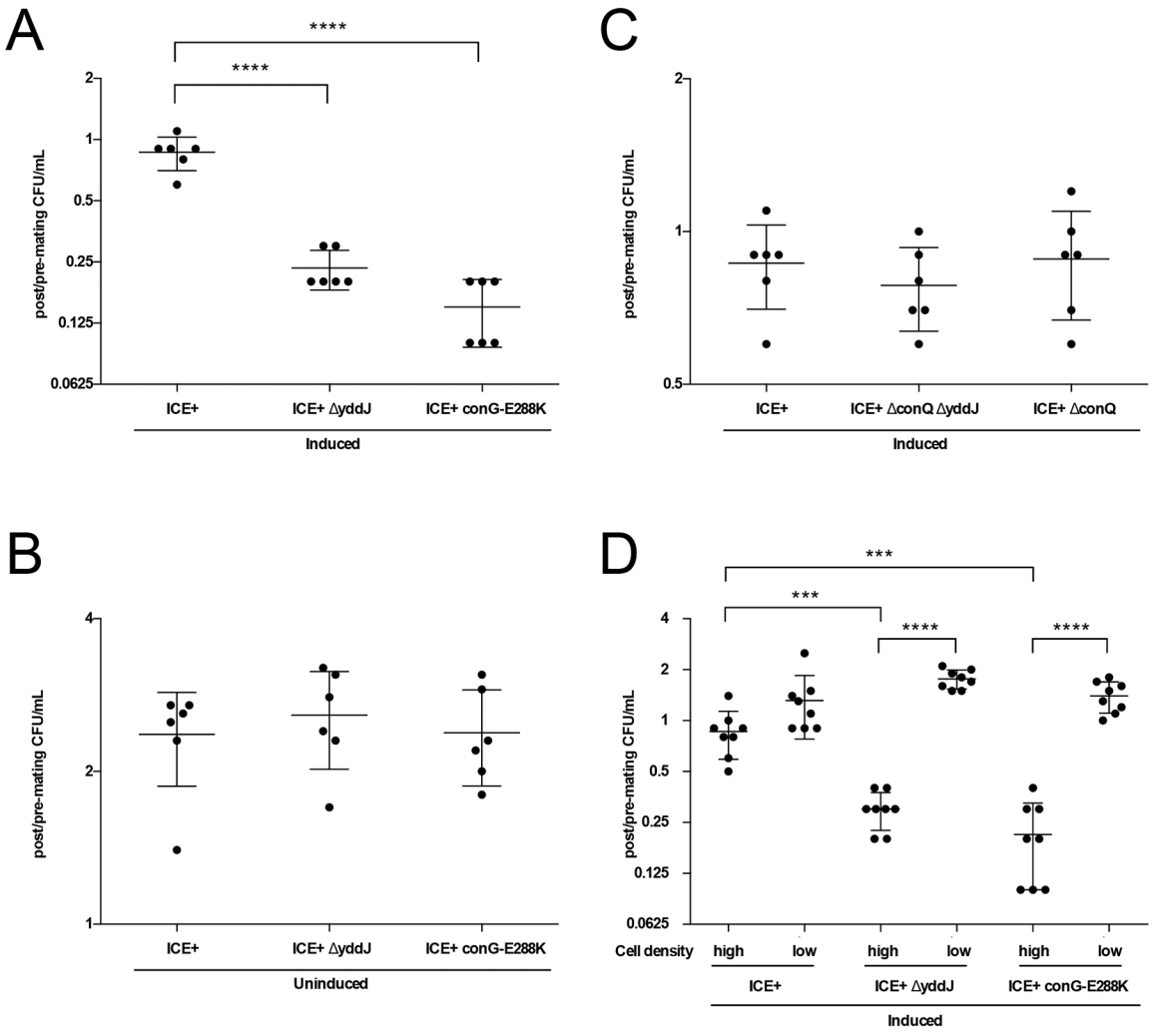

Fig. 6. Exclusion is beneficial to ICE*Bs1* and its host cells by preventing loss of viability due to redundant transfer.

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*$_{\text{ICE}Bat1}$ *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*$_{\text{ICE}Bat1}$ *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)