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

Kathleen P. Davis[!] and Alan D. Grossman[*]

Department of Biology

Massachusetts Institute of Technology

Cambridge, MA 02139

Running title: Exclusion of an integrative & conjugative element

[!] Current address: Tufts University School of Medicine; Arnold 5; 136 Harrison Ave. Boston, MA 02111

[2]Corresponding author:
Department of Biology
Building 68-530
Massachusetts Institute of Technology
Cambridge, MA 02139
phone: 617-253-1515
email: adg@mit.edu

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. 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 that confer exclusion specificity and determined the importance of exclusion to host cells. Using chimeras that had parts of ConG from ICE*Bs1* and the closely related ICE*Bat1* we identified a putative extracellular loop of ConG that conferred specificity for exclusion by the cognate YddJ. Using chimeras of YddJ from ICE*Bs1* and ICE*Bat1* we identified two regions in YddJ needed for exclusion specificity. We also found that YddJ-mediated exclusion reduced death of donor cells following conjugation into recipients. Donor death was dependent on the ability of transconjugants to themselves become donors and was reduced under osmo-protective conditions, indicating that death was likely due to alterations in the donor cell envelope caused by excessive conjugation. We postulate that elements that can have high frequencies of transfer likely evolved exclusion mechanisms to protect the host cells from excessive death.

52 **Importance**

53 Horizontal gene transfer is a driving force in bacterial evolution, responsible for the spread of
54 many traits, including antibiotic and heavy metal resistances. Conjugation, one type of horizontal
55 gene transfer, involves DNA transfer from donor to recipient cells through conjugation
56 machinery and direct cell-cell contact. Exclusion mechanisms allow conjugative elements to
57 prevent their host from acquiring additional copies of the element, and are highly specific
58 enabling hosts to acquire heterologous elements. We defined regions of the exclusion protein and
59 its target in the conjugation machinery that convey high specificity of exclusion. We found that
60 exclusion protects donors from cell death during periods of high transfer. This is likely important
61 for the element to enter new populations of cells.
62
63
64

Introduction

Integrative and conjugative elements (ICEs, also called conjugative transposons) 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 (1–3). ICEs are typically found integrated into the host chromosome. Under certain conditions they can excise and transfer to a new host through conjugation machinery encoded by the element (4,5), thus enabling their spread through a population of bacterial cells. The conjugation machinery encoded by most ICEs is a type 4 secretion system (T4SS) (1) and the genes that confer various phenotypes to the host cells are typically not required for conjugation and are called cargo genes. The conjugation machineries from many ICEs are also capable of transferring (mobilizing) other elements, notably plasmids, to new host cells, allowing for dissemination of elements that do not encode their own conjugation machinery (6–8).

ICE*Bs1* is relatively small (~20 kb) and present in a unique site (in *trnS-leu2*) in most strains of *Bacillus subtilis* (9,10). 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 potential transfer of the element. ICE*Bs1* can be activated in >90% of cells in a population by overproduction of the element-encoded activator protein RapI, making the element readily amenable to population-based studies (9,11,12). ICE*Bs1* has three known mechanisms for inhibiting its host cell from receiving an additional copy of element: 1) inhibition of ICE*Bs1* activation by cell-cell signaling from neighboring cells that already contain a copy of the element (9); 2) repressor-mediated immunity (13); and 3) exclusion (12). Exclusion is a key part of conjugative plasmid biology and most conjugative plasmids appear to have an exclusion system (14). In the F-plasmid of *E. coli*, 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 (15–18). In addition, exclusion prevents cells from having recombination events that result in deletions and defective plasmid copies (19–21).

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 (14). 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 system systems from the F/R100 family of plasmids (22,23), the R64/R62Ia plasmids (24), and the SXT/R391 ICEs (25,26) and ICE*Bs1* (12). ICE*Bs1* is the only ICE from Gram-positive bacteria that is known to have exclusion system.

In ICE*Bs1*, the element-encoded exclusion protein YddJ specifically inhibits its cognate conjugation machinery by targeting the conjugation protein ConG in would-be donor cells, thereby inhibiting transfer of DNA into a cell that already contains ICE*Bs1* (12). ConG, a homolog of VirB6 in the pTI conjugation system from *A. tumefaciens*, is a membrane protein with seven predicted transmembrane segments, and essential for function of the ICE*Bs1* conjugation system (27). Exclusion protects the viability of ICE*Bs1* host cells under conditions that promote conjugation, although it was not clear whether ICE*Bs1* donors, recipients, or both were being protected (12).

Here, we identify the regions in YddJ and ConG that determine the specificity of exclusion. We found that exclusion promotes viability of ICE*Bs1* donor cells by limiting ICE*Bs1* transfer from new transconjugants back into the original donors.

Results

Rationale and experimental approach

Exclusion specificity in ICE*Bs1* was established using *conG* and *yddJ* from ICE*Bat1* in place of their homologues in ICE*Bs1* (12). Here, to define the regions of each gene needed to confer specificity, we made chimeras between *conG* or *yddJ* from ICE*Bs1* and ICE*Bat1*. To study the effects of exclusion we used experimental conditions that bypass both cell-cell signaling and immunity. Cell-cell signaling is bypassed by overexpressing *rapI* from an inducible promoter (Pxyl-*rapI*) in ICE-containing donor cells (9). Repressor-mediated immunity in potential recipient cells is bypassed by expressing *yddJ* from an exogenous locus under control of a strong promoter {Pspank(hy)-*yddJ*} in the absence ICE*Bs1* (12).

Identification of regions of ConG that are essential for exclusion specificity

Regions and resides of ConG and YddJ that are needed for exclusion specificity must be divergent between the proteins from ICE*Bs1* and ICE*Bat1*. There are two main regions of divergence in ConG (12). One includes residues 276-295 of both $ConG_{Bs1}$ and $ConG_{Bat1}$ (Fig. 1), and is predicted to be a loop between the putative third and fourth transmembrane regions. Exclusion-resistant mutations in *conG* are in this loop region (12). The C-terminal region of ConG is also divergent between the two elements. This region is predicted to be a large extracellular domain.

We found that amino acids 276-295 in ConG were sufficient to confer specificity. We replaced amino acids 276-295 in $ConG_{Bs1}$ with the corresponding residues from $ConG_{Bat1}$, generating $ConG_{Bs1}$-Bat1(276-295), referred to as $ConG_{Bs1}$-H1 (Fig. 1A, B). This hybrid protein was functional in conjugation with the ICE*Bs1* conjugation machinery. The conjugation efficiency was ~5% (~5 transconjugants per 100 initial donors) into recipients that did not

contain YddJ (or ICE*Bs1*). When recipients produced $YddJ_{Bat1}$, the conjugation efficiency was reduced by a factor of ~10^{-3}, which we refer to as ~1,000-fold exclusion (Fig. 1B). Exclusion is the ratio of transconjugants into recipients without (no exclusion) versus with *yddJ*. This level of exclusion is similar to that observed when both ConG and YddJ were from ICE*Bat1* (Fig. 1D). In contrast, when recipients produced $YddJ_{Bs1}$, there was no detectable change in the conjugation efficiency giving exclusion of ~1 (no exclusion) (Fig. 1B). Based on these results, we conclude that amino acids 276-295 of ConG from ICE*Bat1* are sufficient to confer exclusion specificity to YddJ from ICE*Bat1*.

We also made the reciprocal replacement, replacing residues 276-295 from $ConG_{Bat1}$ with those from $ConG_{Bs1}$ (Fig. 1A, C). This hybrid $ConG_{Bat1}$-Bs1(276-295), referred to as $ConG_{Bat1}$-H2, was functional in conjugation with the ICE*Bs1* conjugation machinery, but less so than wild type or the other hybrid. The reduced transfer efficiency was expected based on previous analyses substituting $ConG_{Bat1}$ for $ConG_{Bs1}$ in the context of the ICE*Bs1* conjugation machinery (12). The conjugation efficiency was ~0.1% transconjugants per donor into recipients that did not contain YddJ. When recipients produced YddJ from ICE*Bs1*, exclusion was ~1,000-fold, similar that when both ConG and YddJ were from ICE*Bs1* (Fig. 1C). In contrast, when recipients produced $YddJ_{Bat1}$, there was no detectable exclusion (Fig. 1C). Based on these results, we conclude that amino acids 276-295 of ConG from ICE*Bs1* are sufficient to confer exclusion specificity to YddJ from ICE*Bs1*.

Together, the results above indicate that residues 276-295 of both $ConG_{Bs1}$ and $ConG_{Bat1}$ confer specificity of exclusion. These might 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. With these key residues, exclusion is virtually indistinguishable from that for donors expressing the wild type cognate ConG protein.

Identification of YddJ Regions Essential for Exclusion Specificity

To identify regions of $YddJ_{Bs1}$ and $YddJ_{Bat1}$ that confer exclusion specificity, we used a similar approach of generating hybrids and testing whether recipient strains expressing these hybrid proteins could exclude ICE*Bs1* using $ConG_{Bs1}$ or $ConG_{Bat1}$. There are two main regions of sequence dissimilarity between the YddJ homologs (Fig. 2A). We made and tested three functional hybrid proteins, focusing on these regions (Fig. 2B).

Hybrid J1, $YddJ_{Bat1}$-Bs1(30-48)(65-81)(86-95). We made constructs that replaced amino acids from regions 1, 2a and 2b (amino acids 30-50, 67-82, and 87-96) in $YddJ_{Bat1}$ with the corresponding residues (30-48, 65-81, and 86-95, note slightly different numbering) from $YddJ_{Bs1}$ to make hybrid J1, formally known as $YddJ_{Bat1}$-Bs1(30-48)(65-81)(86-95) (Fig. 2B). Hybrid J1 was able to exclude an element that had conjugation machinery with $ConG_{Bs1}$ (exclusion ~1600), but unable to exclude conjugation machinery with $ConG_{Bat1}$ (Fig. 2B). These results indicate that the specificity of $YddJ_{Bat1}$ had been switched to that of $YddJ_{Bs1}$ and that these three regions were sufficient to confer specificity.

Hybrid J2, $YddJ_{Bat1}$-Bs1(30-48)(65-81). We made a construct similar to hybrid J1, but only replaced amino acids in regions 1 and 2a (amino acids 30-50 and 67-82) in $YddJ_{Bat1}$ with the corresponding residues (30-48 and 65-81, respectively) from $YddJ_{Bs1}$ to generate hybrid J2, formally known as $YddJ_{Bat1}$-Bs1(30-48)(65-81) (Fig. 2B). Hybrid J2 excluded a donor with $ConG_{Bs1}$ (exclusion ~1000), but did not exclude a donor with $ConG_{Bat1}$ (Fig. 2B). These results indicate that the specificity of $YddJ_{Bat1}$ had been switched to that of $YddJ_{Bs1}$ and that these two

regions were sufficient to change the specificity. They also indicate that amino acids in region 2b (resides 86-95) of YddJ from ICE*Bs1* contributed little if anything to specificity in this context.

Hybrid J3, YddJ$_{Bs1}$-Bat1(30-50)(67-82)(87-96). We made a construct that replaced amino regions 1, 2a and 2b (amino acids 30-50, 67-82, and 87-96) in YddJ from ICE*Bs1* with the corresponding residues from YddJ from ICE*Bat1* to make hybrid J3, formally known as YddJ$_{Bs1}$-Bat1(30-50)(67-82)(87-96) (Fig. 2B). This hybrid is essentially the reciprocal of hybrid J1. Hybrid J3 was able to exclude an element that had conjugation machinery with ConG$_{Bat1}$, but unable to exclude conjugation machinery with ConG$_{Bs1}$ (Fig. 2B). These results indicate that the specificity of YddJ$_{Bs1}$ had been switched to that of YddJ$_{Bat1}$ and that these three regions were sufficient to confer specificity.

We made another hybrid (J4), similar to hybrid J3, but that replaced only regions 1 and 2a (not region 2b) of YddJ$_{Bs1}$ with the corresponding two regions from YddJ$_{Bat1.}$ Formally, this hybrid is known as YddJ$_{Bs1}$-Bat1(30-50)(67-82). This hybrid had very little if any exclusion of a donor with ConG$_{Bat1}$ and no detectable exclusion of a donor with Con*G*$_{Bs1}$. These results are largely uninterpretable. It is possible that key residues needed for exclusion were missing. It is also possible that the protein is not functional (perhaps mis-folded). Nonetheless, together, our results indicate that regions 1, 2a, and 2b, and in the context of YddJ$_{Bat1}$, regions 1 and 2a from YddJ$_{Bs1}$ are sufficient to confer exclusion specificity.

YddJ hybrid proteins can exclude conjugation machinery with ConG hybrid proteins

As a final demonstration of specificity, we tested the ability of hybrid J1 and J3 to exclude conjugation machinery containing the ConG hybrids H1 and H2. Hybrid J1 (YddJ$_{Bat1}$ with regions 1, 2a and 2b from YddJ$_{Bs1}$) was able to inhibit ConG$_{Bat1}$-H2 (ConG$_{Bat1}$ with amino acids 276-295 from ConG$_{Bs1}$), but not ConG$_{Bat1}$ (Fig. 3). Likewise, hybrid J3 (YddJ$_{Bs1}$ with regions 1,

2a and 2b from $YddJ_{Bat1}$) was able to inhibit $ConG_{Bat1}$-H1, but not $ConG_{Bs1}$ (Fig. 3). Together, our results demonstrate that the key residues in $ConG_{Bs1}$ and $ConG_{Bat1}$, and in $YddJ_{Bs1}$ and $YddJ_{Bat1}$, are sufficient to generate the exclusion specificity of their counterpart wild type proteins.

Death of ICE*Bs1* donors due to excessive mating

Previous work found that loss of exclusion leads to a drop in cell viability under conditions that support mating (12). However, it is not known if the drop in viability was due to killing of donors, recipients, or both. Experiments described below demonstrate that decreased viability of exclusion-defective mutants occurs when cells function concurrently as both donors and recipients.

There was considerable death of ICE*Bs1* host cells (initial donors) when these cells were surrounded by an excess of recipient cells. We mixed ICE*Bs1* donors that had exclusion (KPD154) with recipient cells at a ratio of 1 donor to ~100 recipients. After mating on filters, there was a dramatic drop in viability of the original donor cells such that only ~5% (4.6 ± 2.0%) of the original donors survived post-mating (Fig**.** 4). In the absence of exclusion (Δ*yddJ*, strain KPD155), only ~2% (1.8 ± 0.5%) of the original donors survived (Fig. 4), a significant difference based on the one-tailed t test ($p = 0.0174$).

The decrease in donor survival was due to loss of exclusion and not the absence of YddJ per se. We analyzed survival of donors that express YddJ, but that contain a missense mutation in *conG* that make them insensitive to exclusion (12). This mutant also had decreased survival (1.7 ± 0.5%), similar to that of cells without *yddJ* (Fig. 4). Together, these results indicate that 1) there is significant donor death when the donors are surrounded by a vast excess of recipients

and likely to be transferring to multiple cells; and 2) that exclusion provides ~2-3-fold protection from this death.

The protection conferred by YddJ was due to its function in the original donors. We expressed *yddJ* from an ectopic locus in donor cells that contained ICE*Bs1*Δ*yddJ* (KPD156). The original donor has exclusion, but a transconjugant will not because the transferred element (ICE*Bs1*Δ*yddJ*) is missing *yddJ*. Survival of donors ectopically expressing *yddJ* was ~4% (3.6 ± 0.9%) (Fig. 4) in mating experiments analogous to those above with 1 donor to ~100 recipients. These results indicate that in the absence of exclusion, some of the donor death is likely due to donors acting as recipients in conjugation and that the transconjugants are likely transferring DNA back to the original donors. In donors capable of excluding entry of a second copy of ICE*Bs1*, donor death is likely from mating events with many recipients.

The drop in viability of donor cells was due to the presence of the mating machinery and close proximity of recipients. Donor death was dependent on overexpression of *rapI* to induce ICE*Bs1*. Without *rapI* induction, there was no detectable drop in donor cell viability under conditions that mimic the mating described above. Furthermore, donor death was not simply due to overexpression of *rapI* and activation of ICE*Bs1*. Matings done with both wild type ICE*Bs1* donors (KPD154) and Δ*yddJ* ICE*Bs1* donors (KPD155) at a ratio of 1 donor to ~100 recipients, with overexpression of *rapI*, but at a cell concentration low enough to reduce mating (~4x10^5 rather than ~8x10^8 total cells for mating) had 63 ± 16% and 89 ± 7% survival of wild type and *yddJ* donors, respectively. Thus, the large drop in viability was dependent on activation of ICE*Bs1* and conditions that support multiple mating events. We postulate that excessive mating, and serving as both a donor and recipient (in the absence of YddJ-mediated exclusion), likely causes cell wall damage that leads to cell death.

246 **Osmo-protective conditions increase survival during excessive mating**

247 We found that osmo-protective conditions increased donor survival under conditions of
248 excessive mating, both with and without exclusion. Mating assays were done using donors with
249 (KPD154) and without (ICE*Bs1*Δ*yddJ*, strain KPD155) exclusion at a ratio of 1 donor to ~100
250 recipients (CAL419) in osmo-protective conditions. These osmo-protective conditions consisted
251 of replacing 1X Spizizen's salts used as the support for mating filters and used to recover cells
252 from the mating filters, with a solution of 20mM MgCl2 and 0.5M sucrose and buffered with
253 20mM maleic acid (see Materials and Methods). This solution (called MSM) has been used for
254 the propagation *B. subtilis* cells with no cell wall, so-called L-forms (28).

255 In matings with osmo-protective conditions, survival of wild type donors was 51.9 ± 11.0%**,**
256 and survival of donors without exclusion (ICE*Bs1*Δ*yddJ*) was 17.7 ± 6.3%, compared to 4.6 ±
257 2.0% and 1.8 ± 0.5%, respectively, in matings without osmo-protection (Fig. 4). Thus, a
258 significant amount of donor death for both wild type and exclusion-deficient donors was
259 eliminated by osmo-protection. These results indicate that donors surrounded by an excess of
260 recipients are likely dying from cell wall damage due to excessive mating, either into a single
261 recipient or into multiple recipients. There was still lower survival of donors without exclusion,
262 even under the osmo-protective conditions used here. We suspect that this is due to the shift from
263 osmo-protection to LB agar plates, and that the donors without exclusion have more envelope
264 damage and lower survival following the shift.

265

266

Discussion

Results presented here show that *B. subtilis* cells that transfer ICE*Bs1* can die from excessive transfer. This death is exacerbated by loss of exclusion, which likely enables transfer from a transconjugant back into the original donor. Death of the donors is largely relieved under osmo-protective conditions, indicating that death is due to alterations in the integrity of the cell envelope. If there is death of recipients we would not have detected it in our assays.

Death of ICE*Bs1* donors compared to lethal zygosis in *E. coli*

Death of ICE*Bs1* donors and the protection by exclusion is different from the previously characterized phenomenon of lethal zygosis in *E. coli*. In lethal zygosis, cells are killed when they serve as recipients during multiple conjugation events. This killing occurs when recipients lacking the F plasmid (F-) are mixed with an excess of either Hfr donors or F+ exclusion-deficient donors. Recipient death by lethal zygosis also occurs when F+ exclusion-deficient recipients are mixed with an excess of Hfr donors (15–18). This killing is probably caused by increased permeability of the cell wall due to multiple matings (15). During Hfr transfer, the transconjugants do not become donors because the entire conjugative element is not transferred. This is in contrast to the situation with ICE*Bs1* in which transconjugants acquire the entire element and quickly become donors (29). It is not known if *E. coli* donors also die under conditions of excess mating.

Benefits of ICE*Bs1* exclusion

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 likely mimicked by matings with 1 donor to ~100 recipients. 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 (29) and spread in a biofilm (30,31). That transconjugants quickly become donors indicates that if a mating pair is reasonably stable, then there is the likelihood that a transconjugant could transfer ICE back to the original donor. Our results indicate that it is taxing for ICE*Bs1* host cells to serve as donors. There is a considerable amount of donor death with multiple transfer opportunities (1 donor per 100 recipients at high cell concentrations), and there is more death in the absence of exclusion. Whatever the mechanism of killing, it seems that exclusion can protect ICE*Bs1* donor cells when they are already in the vulnerable state of serving or having just served as donors.

Comparison of exclusion proteins

Exclusion proteins from different families of conjugative elements have limited sequence similarity, but still have some common features. In general, exclusion proteins are relatively small and found on the surface of the host cell where it is in position to inhibit cognate conjugation machinery in a potential donor (14). Exclusion proteins are usually not required for conjugative transfer, except for that of R27 (32), often function in a dose-dependent manner, and target a VirB6 (ConG) homolog or analog in the cognate secretion system, as discussed above.

In the case of the F/R100 family of plasmids (*E. coli*, *S. flexneri*), the exclusion protein TraS a small hydrophobic protein except for a short hydrophilic region (33), predicted to be localized to the inner membrane (23). In the SXT/R391 family of ICEs (*V. cholerae*, *P. rettgeri*), the exclusion protein Eex is in the inner membrane (26), and paradoxically, the regions essential for exclusion specificity are in a cytoplasm region of the protein (34). For ICE*Bs1* (*B. subtilis*), the exclusion protein YddJ is predicted to be extracellular and attached to the cell surface via a lipid modification (12).

312 **Targets of exclusion proteins**

313 The targets of exclusion proteins from four different families of conjugative elements, all
314 from gram-negative bacteria, have been identified. In each case, the exclusion protein targets its
315 cognate VirB6 homologue or analogue. The region conferring specificity of exclusion appears to
316 be either periplasmic (23) or cytoplasmic (24,26,34). Likewise, the region of ConG of ICE*Bs1*
317 (and ICE*Bat1*) that confers exclusion specificity is predicted to be cytoplasmic. Together, these
318 analyses indicate that either this normally periplasmic or cytoplasmic region can be present on
319 the cell surface, or that the cognate exclusion protein has access to part of the periplasm or
320 cytoplasm.

321 **Specificity of exclusion and contributions to ICE biology**

322 Identification of key regions for exclusion specificity in ConG and YddJ also highlight
323 important aspects of ICE*Bs1* biology, and how ICEs contribute to bacterial evolution by
324 spreading genetic material. The fact that exclusion (or lack thereof) can be based on differences
325 of a few residues in the exclusion protein or target protein demonstrates that exclusion by a copy
326 of ICE*Bs1* can very selectively allow slightly different elements (such as ICE*Bat1*) to enter the
327 host cell, while significantly reducing the number the number conjugation attempts by other
328 would-be ICE*Bs1* donors.

329 ICEs play an important role in bacterial evolution by contributing to the spread of genetic
330 material, and one way in which an ICE gains or loses genetic material (which it can then transfer
331 along with itself) is through genetic rearrangement events with other ICEs and plasmids (5). It
332 has been theorized (14) that the lack of exclusion systems in some ICEs allows for more rapid
333 evolution of the ICE, but this could be harmful for ICE*Bs1* given its strict requirement for
334 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.

Materials and Methods

Media and growth conditions

Cells were typically grown in $S7_{50}$ defined medium (35) supplemented as needed for auxotrophic requirements (40 μg/ml tryptophan, 40 μg/ml phenylalanine, and 200 μg/ml threonine for strains containing alleles inserted at *thrC*). Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM to induce expression from the promoter Pspank(hy). 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

B. subtilis strains used in this study are listed in Table 1. Cloning and generation of strains was done following standard techniques (36). All strains (KPD219, CAL89, MA982, KPD128, KPD131, KPD132, KPD137, CAL419) used as recipients in mating experiments did not contain ICE*Bs1* (ICE^0), contained null mutations in *comK* or *comC* (described below), and were streptomycin-resistant (*str-84*) (9,37). 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* that has a kanamycin-resistance gene inserted in place of *rapI-phrI*: Δ(*rapI-phrI*)*342*::*kan* (9). *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 genes: *amyE*::{Pxyl-*rapI spc*} (38) or *amyE*::{Pxyl-*rapI cat*} (38,39). Any ICE*Bs1* donor strains containing a deletion of *conG*, Δ*conG*(*5-805*), were derived from MMB1283 (27). KPD210, a donor strain containing a complete deletion of *yddJ*, was derived from MA11 (12).

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 (KPD154, KPD155, KPD156, KPD157) used in experiments where even low levels of transformation could significantly alter donor CFU counts. *comK*::*cat* was from CAL419 (37), *comK*::*spc* (9) and *comC::mls* (12) 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 gene. 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 described previously (12).

To make the *yddJ* hybrids, $yddJ_{Bat1}$ 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 of each *yddJ* were amplified and fused by isothermal assembly as necessary to make four chimeric constructs.

1) Hybrid J1: formally known as $YddJ_{Bat1}${*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).

2) Hybrid J2: formally known as $YddJ_{Bat1}${*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) Hybrid J3: formally known as $YddJ_{Bs1}${*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).

(4) Hybrid J4: formally known as $YddJ_{Bs1}${*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 (a vector for making fusions to Pspank(hy) and integration at *lacA*) (40) by isothermal assembly. One fragment from pCJ80 included the pCJ80 SphI cut site and 2409 bp upstream of this cut site, including homology to the 5' end of *lacA*. The other fragment included the pCJ80 SacI cut site and 2299 bp downstream of this cut site, including 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 primers used to amplify *yddJ* contained sequences complementary to sequences in the primers used to amplify regions of *lacA*, thereby enabling 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*. *conG* was expressed ectopically from the LacI-repressible IPTG-inducible promoter Pspank(hy), from constructs integrated at *thrC* with an *mls* resistance gene. The Pspank(hy)-*conG* alleles were used to complement the Δ*conG*(*5-805*) deletion in ICE*Bs1*. Pspank(hy)-$conG_{Bs1}$ (*conG* from ICE*Bs1*)

404 present in strain KPD225, and Pspank(hy)-*conG*$_{Bat1}$, (*conG* from ICE*Bat1*) present in strain
405 KPD224, have been described (12,27).

406 To make the *conG* hybrids, *conG*$_{Bat1}$ DNA was amplified by PCR from genomic DNA from
407 *B. atrophaeus* strain 11A1 (from the Bacillus Genetic Stock Center; www.bgsc.org) and *conG*$_{Bs1}$
408 DNA was amplified by PCR from genomic DNA from *B. subtilis* strain AG174. Fragments of
409 each *conG* were amplified and fused by isothermal assembly as necessary to make two chimeric
410 constructs.

411 1) ConG$_{Bs1}$-H1: formally known as Con*G*$_{Bs1}${*Bat1*(276-295)}, in which ConG$_{Bat1}$ residues
412 276-295 were substituted for residues 276-295 in ConG$_{Bs1}$.

413 2) ConG$_{Bat1}$-H2: formally known as ConG$_{Bat1}${*Bs1*(276-295)}, in which ConG$_{Bs1}$ residues
414 276-295 were substituted for residues 276-295 in ConG$_{Bat1}$.

415 For all Pspank(hy)-*conG* constructs, the *conG* PCR fragments were joined together, and then
416 joined to two fragments amplified from pMMB1341 (27) by isothermal assembly. One fragment
417 from pMMB1341 included the HindIII cut site and the adjacent 2330 bp upstream of this cut site,
418 which includes sequences from the 3' end of *thrC*. The other fragment included the SphI cut site
419 and the adjacent 1867 bp downstream, which includes sequences from the 5' end of *thrC*. These
420 two fragments were digested with HindIII and SphI, respectively, before isothermal assembly
421 with the *conG* PCR DNA. The primers used to amplify *conG* contained sequences
422 complementary to sequences in the primers used to amplify regions of *thrC*, thereby enabling
423 joining by isothermal assembly. The resulting isothermal assembly product was integrated by
424 double cross-over into the chromosome by transformation and selecting for MLS resistance, to
425 generate the *conG* overexpression alleles.

426 **Mating Assays**

427 Mating assays were performed essentially as described (9,37). Donor and recipient cultures
428 were grown in $S7_{50}$ defined minimal medium supplemented with 0.1 % glutamate and 1%
429 arabinose until they reached mid-exponential growth phase, then diluted back to an optical
430 density at 600 nm (OD600) of 0.1. At this point 1% xylose was added to donor cultures to induce
431 expression of Pxyl-*rapI*. After two hours of xylose induction, donor and recipient cells were
432 mixed, and poured over a nitrocellulose filter under vacuum filtration. Unless otherwise
433 indicated, equal numbers of donor and recipient cells were used (~$4x10^8$ cells of each). Filters
434 were incubated for 3 hours at 37°C on 1.5% agar plates containing 1x Spizizen's salts (2 g/l
435 $(NH_4)SO_4$, 14 g/l K_2HPO_4, 6 g/l KH_2PO_4, 1 g/l Na_3 citrate-$2H_2O$, 0.2 g/l $MgSO_4$-$7H_20$) (36).
436 Cells were re-suspended from the filters, serially diluted in 1X Spizizen's salts and plated on LB
437 agar plates containing kanamycin and streptomycin to select for transconjugants. For matings
438 done in osmo-protective conditions, 1X Spizizen's salts (in mating plates and resuspension
439 media) was replaced with MSM (20mM MgCl2, 0.5M sucrose and buffered with 20mM maleic
440 acid) (28). The number of viable ICE*Bs1* donor cells (CFU/ml) was determined at the time of
441 donor and recipient cell mixing, by serial dilution plating on LB agar plates containing
442 kanamycin. Mating efficiency was calculated as the percent transconjugants CFU/ml per donor
443 CFU/ml (at the time of mixing donors with recipients). The fold-exclusion was calculated as the
444 percent transfer into an ICE^0 recipient divided by the percent transfer into an ICE^0 recipient that
445 was expressing *yddJ*.
446
447

Acknowledgements

We thank Janet Smith for helpful comments on the manuscript. This research was supported, in part, by the National Institute of General Medical Sciences of the National Institutes of Health under award number R01 GM050895 and R35 GM122538 to ADG. KPD was also supported, in part, by the NIGMS predoctoral training grant T32 GM007287. Any opinions, findings, and conclusions or recommendations expressed in this report are those of the authors and do not necessarily reflect the views of the National Institutes of Health.

References

1. Bañuelos-Vazquez LA, Torres Tejerizo G, Brom S. 2017. Regulation of conjugative transfer of plasmids and integrative conjugative elements. Plasmid. 91:82-89.
2. Brown-Jaque M, Calero-Cáceres W, Muniesa M. 2015. Transfer of antibiotic-resistance genes via phage-related mobile elements. Plasmid. 79:1–7.
3. Burrus V, Waldor MK. 2004. Formation of SXT Tandem Arrays and SXT-R391 Hybrids. J Bacteriol. 186:2636–2645.
4. Bhatty M, Laverde Gomez JA, Christie PJ. 2013. The expanding bacterial type IV secretion lexicon. Res Microbiol. 164:620–639.
5. Johnson CM, Grossman AD. 2015. Integrative and Conjugative Elements (ICEs): What They Do and How They Work. Annu Rev Genet. 49:577–601.
6. Hochhut B, Marrero J, Waldor MK. 2000. Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in Vibrio cholerae O139. J Bacteriol. 182:2043–2047.
7. Lee CA, Thomas J, Grossman AD. 2012. The Bacillus subtilis conjugative transposon ICEBs1 mobilizes plasmids lacking dedicated mobilization functions. J Bacteriol. 194:3165–3172.
8. Naglich JG, Andrews RE. 1988. Tn916-dependent conjugal transfer of PC194 and PUB110 from Bacillus subtilis into Bacillus thuringiensis subsp. israelensis. Plasmid. 20:113–126.
9. Auchtung JM, Lee C a, Monson RE, Lehman AP, Grossman AD. 2005. Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. Proc Natl Acad Sci. 102:12554–12559.
10. 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. 48:77–97.
11. Auchtung JM, Aleksanyan N, Bulku A, Berkmen MB. 2016. Biology of ICEBs1, an integrative and conjugative element in Bacillus subtilis. Plasmid. 86:14-25.
12. Avello M, Davis KP, Grossman AD. 2019. Identification, characterization and benefits of an exclusion system in an integrative and conjugative element of Bacillus subtilis. 112:1066–1082.
13. Auchtung JM, Lee CA, Garrison KL, Grossman AD. 2007. Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICEBs1 of Bacillus subtilis. Mol Microbiol. 64:1515–1528.
14. Garcillán-Barcia MP, de la Cruz F. 2008. Why is entry exclusion an essential feature of conjugative plasmids? Plasmid. 60:1-18.
15. Ou JT. 1980. Role of surface exclusion genes in lethal zygosis in Escherichia coli K12 mating. MGG Mol Gen Genet. 178:573–581.
16. Skurray RA, Reeves P. 1973. Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12. J Bacteriol. 113:58–70.
17. Skurray RA, Reeves P. 1974. F factor mediated immunity to lethal zygosis in Escherichia coli K 12. J Bacteriol. 117:100–106.
18. Skurray RA, Willetts N, Reeves P. 1976. Effect of tra mutations on F factor-specified immunity to lethal zygosis. MGG Mol Gen Genet. 146:161–165.
19. Hopkins JD, Clements MB, Liang TY, Isberg RR, Syvanen M. 1980. Recombination genes on the Escherichia coli sex factor specific for transposable elements. Proc Natl Acad Sci.

77:2814–2818.
20. Peters JE, Benson SA. 1995. Redundant transfer of F' plasmids occurs between Escherichia coli cells during nonlethal selections. J Bacteriol. 177:847-850.
21. Syvanen M, Hopkins JD, Griffin IV TJ, Liang TY, Ippen-Ihler K, Kolodner R. 1986. Stimulation of precise excision and recombination by conjugal proficient F′ plasmids. MGG Mol Gen Genet. 203:1–7.
22. Anthony KG, Klimke WA, Manchak J, Frost LS. 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. 181:5149–5159.
23. Audette GF, Manchak J, Beatty P, Klimke WA, Frost LS. 2007. Entry exclusion in F-like plasmids requires intact TraG in the door that recognizes its cognate TraS in the recipient. Microbiology. 153:442–451.
24. 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. 69:138–145.
25. Marrero J, Waldor MK. 2007. The SXT/R391 family of integrative conjugative elements is composed of two exclusion groups. J Bacteriol. 189:3302–3305.
26. Marrero J, Waldor MK. 2005. Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. Dev Cell. 8:963–970.
27. Leonetti CT, Hamada MA, Laurer SJ, Broulidakis MP, Swerdlow KJ, Lee CA. Grossman AD, Berkmen MB. 2015. Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of Bacillus subtilis. J Bacteriol. 197:2558–2567.
28. Leaver M, Domı´nguez-Cuevas P, Coxhead JM, Daniel RA, Errington J. 2009. Life without a wall or division machine in Bacillus subtilis. Nature. 457: 849-853.
29. Babic A, Berkmen MB, Lee CA, Grossman AD. 2011. Efficient Gene Transfer in Bacterial Cell Chains. MBio. 2:1-7.
30. Lécuyer F, Bourassa J-S, Gélinas M, Charron-Lamoureux V, Burrus V, Beauregard PB. 2018. Biofilm Formation Drives Transfer of the Conjugative Element ICEBs1 in Bacillus subtilis. mSphere. 3:1-12.
31. Jones JM, Grinberg I, Eldar A, Grossman AD. 2020. A mobile genetic element increases bacterial host fitness by manipulating development. bioRxiv https://doi.org/10.1101/2020.10.09.328104
32. Gunton JE, Ussher JER, Rooker MM, Wetsch NM, Alonso G, Taylor DE. 2008. Entry exclusion in the IncHI1 plasmid R27 is mediated by EexA and EexB. Plasmid. 59:86–101.
33. Jalajakumari MB, Guidolin A, Buhk HJ, Manning PA, Ham LM, Hodgson ALM Cheah KC, Skurray RA. 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. J Mol Biol. 198:1–11.
34. Marrero J, Waldor MK. 2007. Determinants of entry exclusion within Eex and TraG are cytoplasmic. J Bacteriol. 189:6469–6473.
35. Jaacks KJ, Healy J, Losick R, Grossmant AD. 1989. Identification and Characterization of Genes Controlled by the Sporulation-Regulatory Gene spoOH in Bacillus subtilis. J Bacteriol. 171:4121–4129.
36. Harwood CR, Cutting SM. Molecular Biological Methods for Bacillus. Chichester: John Wiley & Sons; 1990.

549 37. Lee CA, Grossman AD. 2007. Identification of the origin of transfer (oriT) and DNA
550 relaxase required for conjugation of the integrative and conjugative element ICEBs1 of
551 Bacillus subtilis. J Bacteriol. 189:7254–7261.
552 38. Berkmen MB, Lee CA, Loveday EK, Grossman AD. 2010. Polar positioning of a
553 conjugation protein from the integrative and conjugative element ICEBs1 of Bacillus
554 subtilis. J Bacteriol. 192:38–45.
555 39. Menard KL, Grossman AD. 2013. Selective Pressures to Maintain Attachment Site
556 Specificity of Integrative and Conjugative Elements. PLoS Genet. 9:1-14.
557 40. Wright LD, Grossman AD. 2016. Autonomous Replication of the Conjugative Transposon
558 Tn 916. J Bacteriol. 198:3355–3366.
559 41. Perego M, Spiegelman GB, Hoch JA. 1988. Structure of the gene for the transition state
560 regulator, abrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus
561 subtilis. Mol Microbiol. 2:689–99.
562 42. Smith JL, Goldberg JM, Grossman AD. 2014. Laboratory Strains JH642 (AG174) and
563 AG1839. 2:3–4.
564
565
566

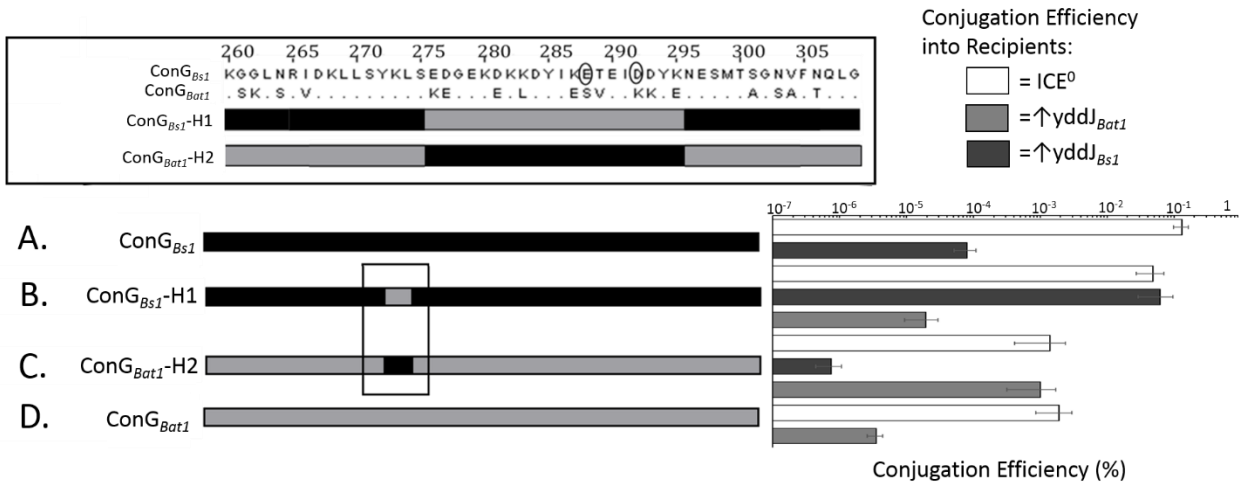
Table 1. *B. subtilis* strains used[a]

Strain	Relevant genotype (reference)
CAL419	ICE*Bs1*^{0} *str-84 comK*::*cat* (13)
CAL89	ICE*Bs1*^{0} *str-84 comK*::*spc* (9)
KPD128	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}${*Bat1*(30-50)(67-82)(87-96)} *mls*} *str-84 comK*::*spc* (aka Hybrid J3)
KPD131	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}${*Bs1*(30-48)(65-81)} *mls*} *str-84 comK*::*spc* (aka Hybrid J2)
KPD132	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}${*Bs1*(30-48)(65-81)(86-95)} *mls*} *str-84 comK*::*spc* (aka Hybrid J1)
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*} (aka ConG$_{Bat1}$-H2)
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*} (aka ConG$_{Bs1}$-H1)
KPD137	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}${*Bat1*(30-50)(67-82)} *mls*} *str-84 comK*::*spc*
KPD154	ICE*Bs1* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*tet*
KPD155	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *comC*::*tet*
KPD156	ICE*Bs1* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}$ *mls*} *comC*::*tet*
KPD157	ICE*Bs1* E288K-*conG* Δ*yddJ* Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI spc*} *lacA*::{Pspank(hy)- *yddJ*$_{Bs1}$ *mls*} *comC*::*tet*
KPD219	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bat1}$ *mls*} *str-84 comK*::*spc* (12)
KPD224	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bat1}$ *mls*} (12)
KPD225	ICE*Bs1* Δ*conG*(*5-805*) Δ(*rapI-phrI*)*342*::*kan amyE*::{Pxyl-*rapI cat*} *thrC*::{Pspank(hy)-*conG*$_{Bs1}$ *mls*} (12)
MA982	ICE*Bs1*^{0} *lacA*::{Pspank(hy)-*yddJ*$_{Bs1}$ *mls*} *str-84 comK*::*spc* (12)

[a]All strains are derived from our lab strain AG174 (JH642) and contain *pheA1*, *trpC2* mutations (not listed) (41,42).

572

573



574

575 **Figure 1. Regions of ConG$_{Bs1}$ and ConG$_{Bat1}$ that confer specificity of exclusion.**

576 **Top left.** Comparison of the indicated regions ConG from ICE*Bs1* and ICE*Bat1*. Amino
577 acids that differ between the two proteins in this region are indicated. The two circled residues
578 are sites of mutation that makes ConG insensitive to exclusion (12). The bars below the sequence
579 indicate the regions in the hybrid proteins from ICE*Bs1* (black) and ICE*Bat1* (gray).

580 **Bottom. Left.** Cartoon of ConG present in the donor strains. Regions of ConG from ICE*Bs1*
581 (black) and ICE*Bat1* (gray) are indicated. **A)** ConG$_{Bs1}$ (KPD225); **B)** ConG$_{Bs1}$-H1 (KPD136); **C)**
582 ConG$_{Bat1}$-H2 (KPD135); and **D)** ConG$_{Bat1}$ (KPD224). **Right, top and bottom.** Conjugation
583 efficiencies of the indicated donors (left) into recipients with no YddJ (open, white bars;
584 CAL89); YddJ from ICE*Bs1* (black bars; strain MA982); YddJ from ICE*Bat1* (gray bars;
585 KPD219). Conjugation efficiency is calculated as the CFU/ml of transconjugants divided by the
586 CFU/ml of donors at the start of mating, and is multiplied by 100% to convert to a percentage.
587 Data bars represent averages from three independent experiments, with error bars depicting
588 standard deviations.

589

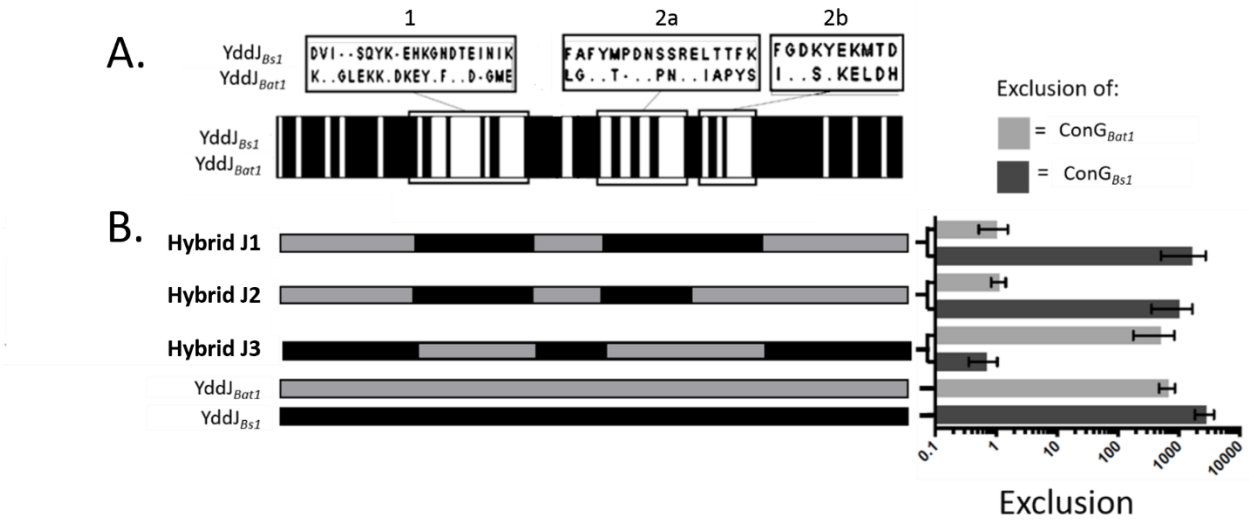
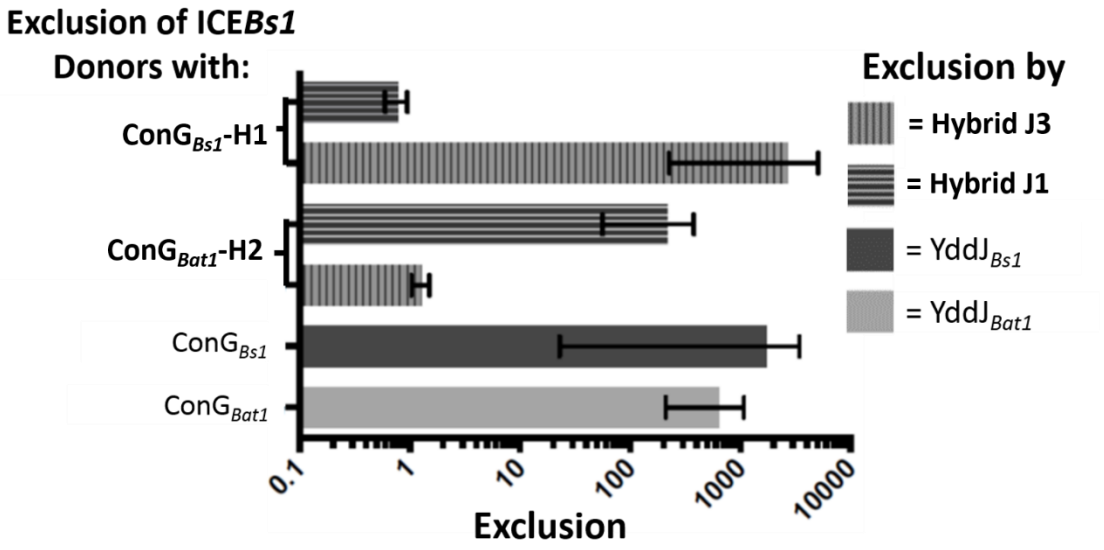


Figure 2. Regions of $YddJ_{Bs1}$ and $YddJ_{Bat1}$ that confer specificity of exclusion.

A) Protein sequence of the indicated regions of YddJ from ICE*Bs1* and ICE*Bat1*. Amino acids that differ between the two proteins in these regions are indicated. The bars below the sequence compare the proteins across their entire lengths. Black and white indicate sequence identity and divergence, respectively.

B) Exclusion (right) of the ICE*Bs1* conjugation machinery that contains $ConG_{Bs1}$ (black bars; KPD225) or $ConG_{Bat1}$ (gray bars; KPD224) by the indicated YddJ proteins (left): hybrid J1 (KPD132); hybrid J2 (KPD131); hybrid J3 (KPD128); $YddJ_{Bat1}$ (KPD219); $YddJ_{Bs1}$ (MA982). Exclusion was calculated as conjugation efficiency into a recipient without YddJ (no exclusion; CAL89) divided by that into a recipient expressing the indicated YddJ. Data bars represent averages from three independent experiments, with error bars depicting standard deviations.

606
607



608
609

610 **Figure 3. YddJ hybrid proteins can exclude conjugation machinery with ConG hybrid**
611 **proteins.**

612 Conjugation machinery in the donor contained ConG$_{Bs1}$-H1 (KPD136); ConG$_{Bat1}$-H2
613 (KPD135); ConG$_{Bs1}$ (KPD225); ConG$_{Bat1}$ (KPD224). Recipients expressed YddJ$_{Bs1}$ (black bars;
614 MA982); YddJ$_{Bat1}$ (gray bars; KPD219); hybrid J3 (vertical stripes; KPD128); hybrid J1
615 (horizontal stripes; KPD132). Exclusion was calculated as for Fig. 2. with results from matings
616 into recipients that did not contain *yddJ* (CAL89) that were done in parallel. Data bars represent
617 averages from three independent experiments, with error bars depicting standard deviations.

618
619
620
621
622

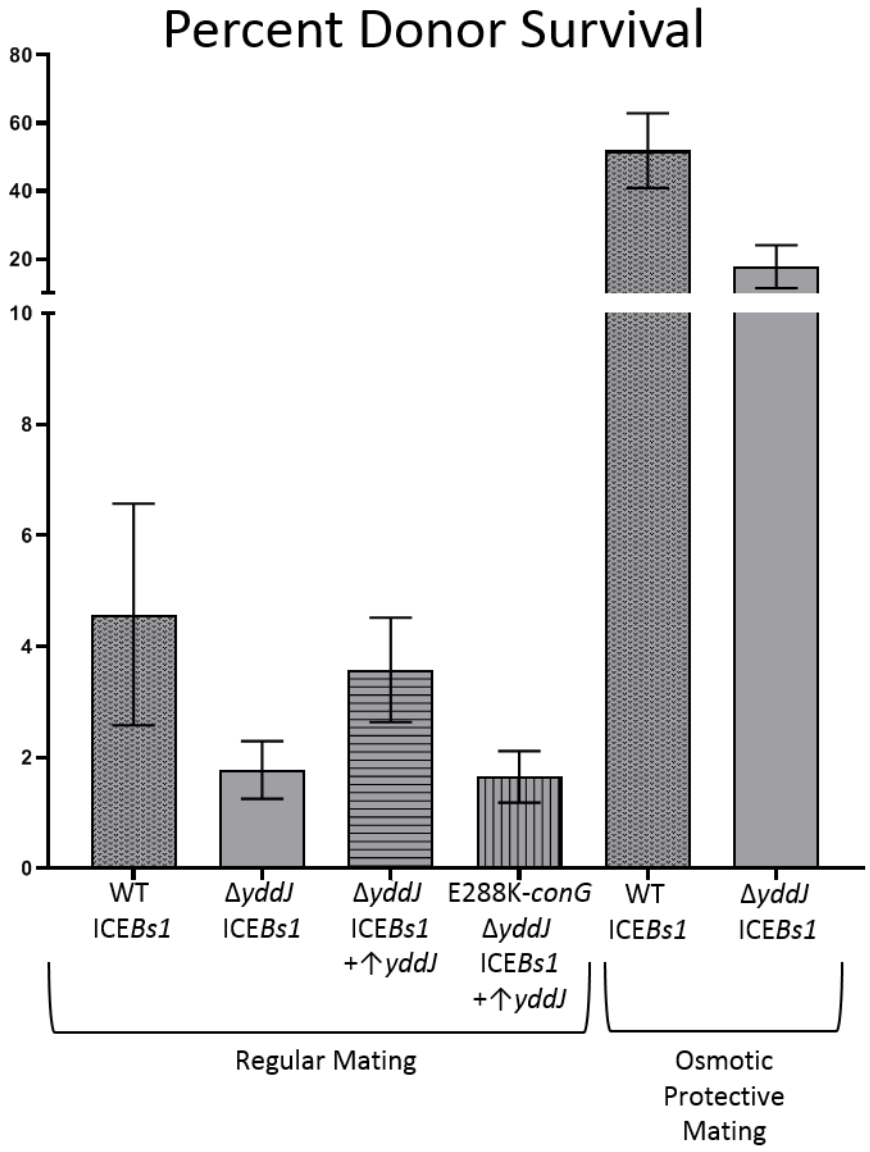
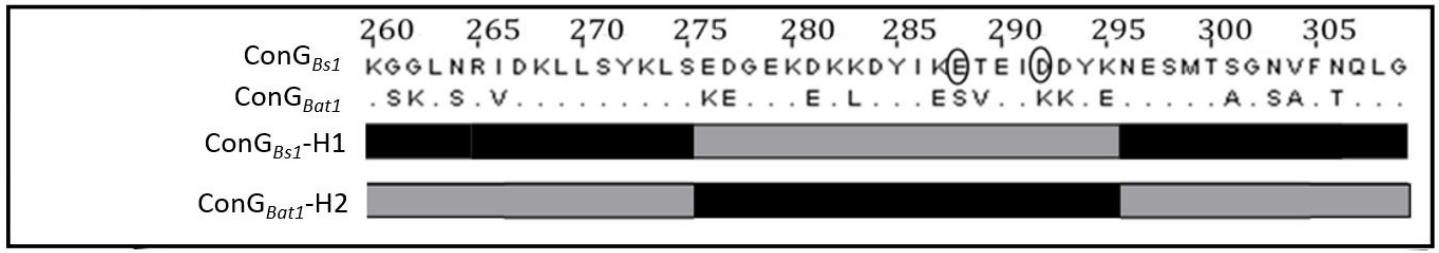


Figure 4. Death of donors by excessive mating is exacerbated by loss of exclusion and largely alleviated under osmo-protective conditions. WT ICE*Bs1* donors were mixed with recipients that lacked ICE (CAL419) at a ratio of 1 donor to ~100 recipients and put through mating conditions described in Fig. 1. In this case, mating filters were placed on agar plates as above (regular mating) or with osmo-protection. After incubation, mating mixtures were resuspended either without (regular mating) or with osmo-protection. Percent donor survival was determined by measuring CFUs/ml after mating compared to that prior to mating. Donors included: ICE*Bs1* (wt; KPD154); ICE*Bs1* Δ*yddJ* (KPD155); ICE*Bs1* Δ*yddJ* overexpressing *yddJ* from an ectopic locus (KPD156); ICE*Bs1* Δ*yddJ conG*E288K (resistant to exclusion), also overexpressing *yddJ* from an ectopic locus (KPD157). Data bars represent averages from the three replicate mating assays for each donor, with error bars depicting standard deviations. p-values from one-tailed t test were: 0.0174 for wild type compared to Δ*yddJ*; 0.0052 for Δ*yddJ* with over-expressed *yddJ* compared to *conGE288K* (exclusion-resistant) with over-expressed *yddJ*; and 8.43×10^{-4} for the pair compared under osmo-protective conditions.

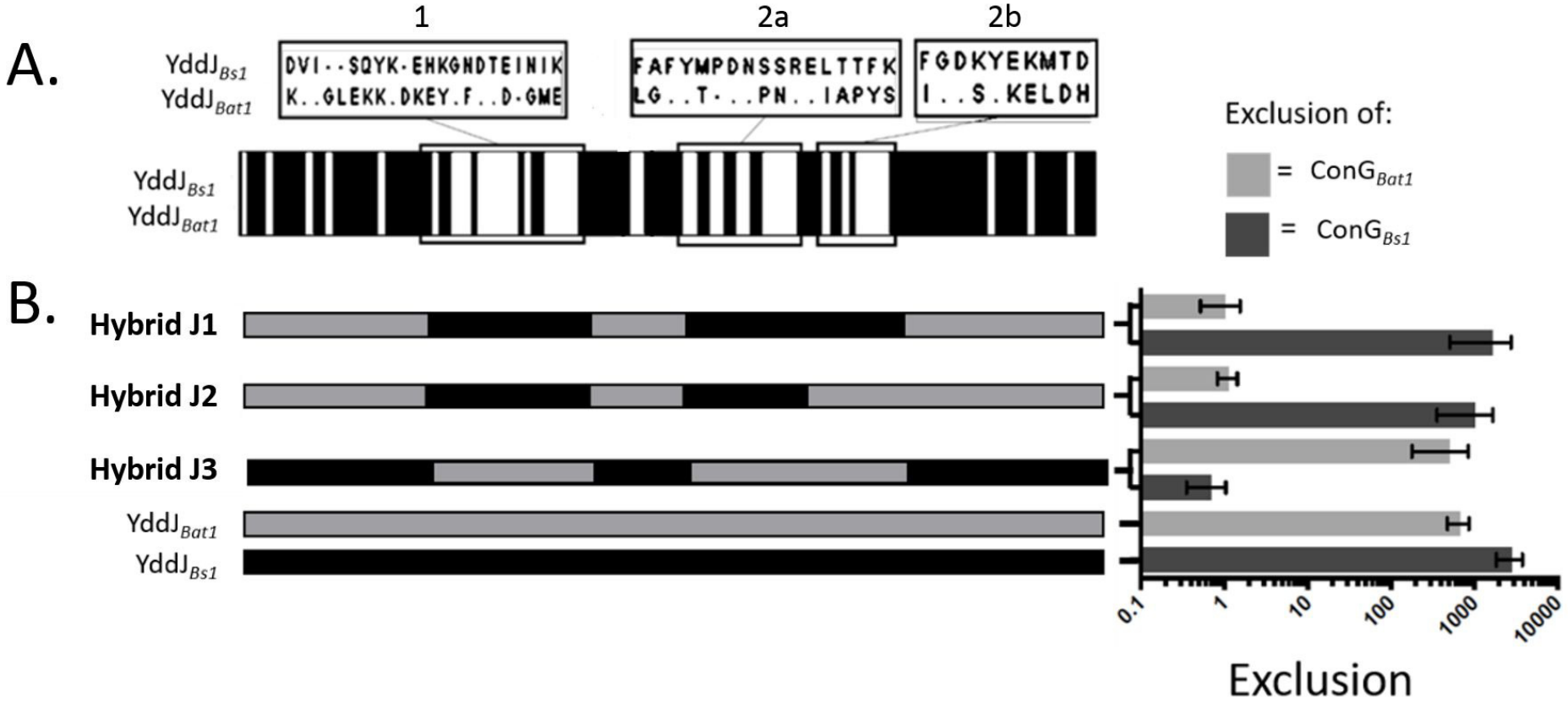


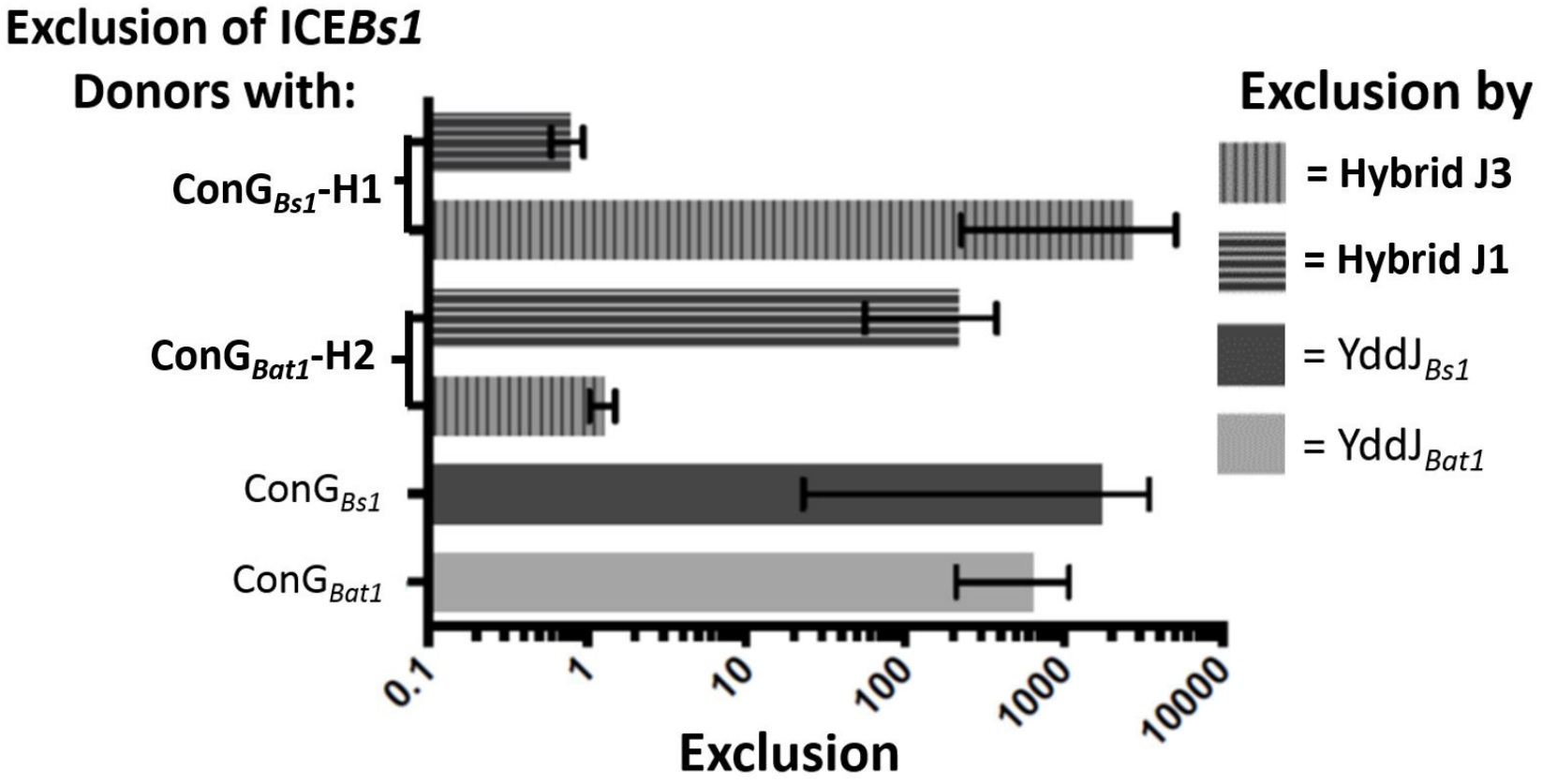
Conjugation Efficiency into Recipients:

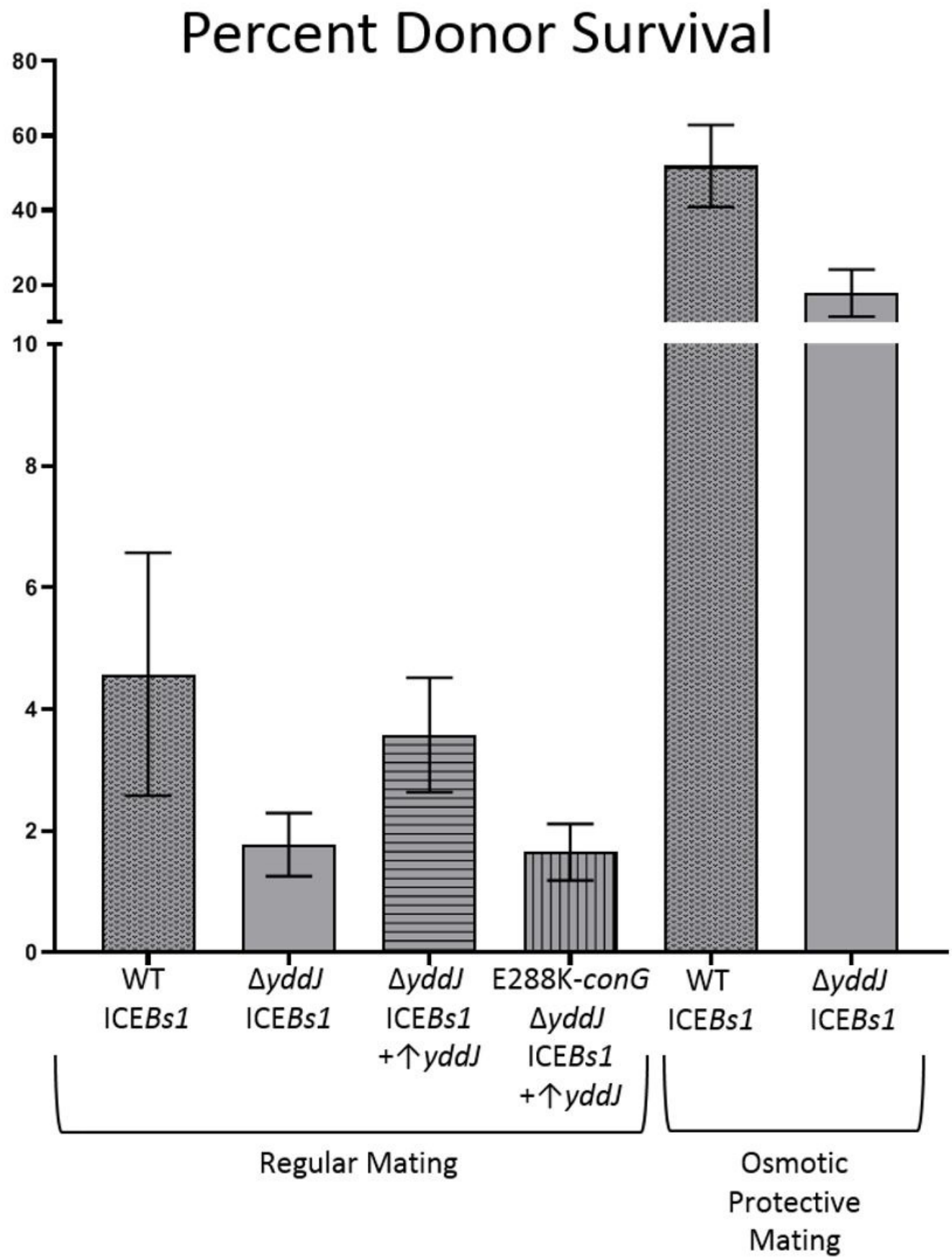
= ICE^0

=↑yddJ$_{Bat1}$

=↑yddJ$_{Bs1}$


A.
1
2a
2b
YddJBs1
YddJBat1
DVI··SQYK·EHKGHDTEINIK
K..GLEKK.DKEY.F..D·GME
FAFYMPDNSSRELTTFK
LG..T···PN..IAPYS
FGDKYEKMTD
I..S.KELDH
YddJBs1
YddJBat1
Exclusion of:
= ConGBat1
= ConGBs1
B.
Hybrid J1
Hybrid J2
Hybrid J3
YddJBat1
YddJBs1
0.1
1
10
100
1000
10000
Exclusion


Exclusion of ICEBs1
Donors with:
ConG Bs1 -H1
ConG Bat1 -H2
ConG Bs1
ConG Bat1
0.1
1
10
100
1000
10000
Exclusion
Exclusion by
= Hybrid J3
= Hybrid J1
= YddJ Bs1
= YddJ Bat1


Percent Donor Survival
80
60
40
20
10
8
6
4
2
0
WT ICEBs1
ΔyddJ ICEBs1
ΔyddJ ICEBs1 +↑yddJ
E288K-conG ΔyddJ ICEBs1 +↑yddJ
WT ICEBs1
ΔyddJ ICEBs1
Regular Mating
Osmotic Protective Mating