INTRODUCTION

Horizontal gene transfer is fundamental to bacterial evolution, allowing for the rapid spread of genes involved in processes as diverse as antibiotic resistance, resource utilization, and pathogenesis (Frost et al., 2005; Soucy et al., 2015; Wiedenbeck & Cohan, 2011). Horizontal gene transfer is often mediated by conjugative elements which encode machinery to transfer a copy of the element from a host to a recipient via direct cell-to-cell contact. Although most studies of conjugative elements have focused on plasmids, integrative and conjugative elements (ICEs) appear to be the most abundant type of conjugative element and have been identified in every bacterial clade (Guglielmini et al., 2011). ICEs normally reside integrated in a host chromosome where they are passively replicated and inherited. While in this inactive state, most ICE genes are not expressed. ICEs can be activated either stochastically or in response to certain conditions (i.e., DNA damage to the host, resource limitation), at which point a site-specific recombinase excises the ICE from the host chromosome to form a circular plasmid (Bañuelos-Vázquez et al., 2017). For conjugative
DNA transfer, the ICE plasmid DNA is nicked and a single strand is transferred out of the donor and into a recipient cell through the element-encoded type IV secretion system. Once transferred, the ssDNA becomes double stranded and can integrate into the chromosome of the new host, forming a stable transconjugant (Delavat et al., 2016). Mutants of the ICE B1 donor that are important for transfer. Several genes were identified in this screen and we chose to focus on those involved in the synthesis of cell wall teichoic acids.

Wall teichoic acids (WTAs) are polyol-phosphate repeats that are attached to the peptidoglycan cell wall of gram-positive bacteria [reviewed in: (Brown et al., 2013; Swoboda et al., 2010)]. In B. subtilis 168, WTAs comprise 45-60 repeats of glycerol 3-phosphoate (Pollack & Neuhaus, 1994). These repeats can be modified by WTA-tailoring enzymes, most notably by D-alanylation and glycosylation (Allison et al., 2011; Perego et al., 1995). In B. subtilis 168, WTAs are synthesized by enzymes encoded by the tag genes (tagO, tagAB, tagDEF, and tagGH). TagO catalyzes the first step of WTA biosynthesis (D’Elia et al., 2006). TagA catalyzes the second step and is the first committed step (D’Elia, Henderson et al., 2009). WTAs are not strictly required for cell growth. WTA-depleted B. subtilis cells are viable but slow-growing, and have significant alterations in cell shape and cell separation (D’Elia et al., 2006). Some WTA biosynthesis genes (tagBDFGH) are conditionally essential, likely because their deletion results in either the accumulation of toxic intermediates or the sequestration of vital cellular resources (D’Elia, Millar et al., 2006, 2009).

WTAs are a critical component of the bacterial cell wall and have diverse functions, although their specific roles are not well understood. WTAs regulate autolysins (cell wall-degrading enzymes) in multiple species of bacteria. In both S. aureus and B. subtilis, certain autolysins involved in cell division exhibit improper localization patterns in the absence of WTAs, and it is thought that the presence of WTAs may exclude those autolysins from portions of the cell wall (Frankel & Schneewind, 2012; Schlag et al., 2010; Yamamoto et al., 2008). Proper localization of B. subtilis LytE, an autolysin required for cell elongation, is also disrupted in WTA-depleted cells (Kasahara et al., 2016). WTA-lacking bacteria are also more prone to autolysis and more sensitive to the activity of peptidoglycan-lactam enzymes (Bera et al., 2007; Schlag et al., 2010; Wecke et al., 1997).

WTAs may also have a role in peptidoglycan biosynthesis. Mutants of B. subtilis exhibit cell-shape defects and have walls of irregular thickness (D’Elia et al., 2006), suggesting that WTAs may be important for organizing the peptidoglycan biosynthesis machinery (Brown et al., 2013). Depletion of WTAs can sensitize bacteria to β-lactams, which may suggest that the two processes are interconnected (Campbell et al., 2011; Farha et al., 2013). WTAs have been proposed to have several other functions, including in virulence (Weidenmaier & Peschel, 2008), bacteriophage attachment (Yasbin et al., 1976; Young, 1967), competence (Miroouze et al., 2018), and cell-cell and cell-surface adhesion (Gross et al., 2001; Walter et al., 2007).

In work described here, we found that wall teichoic acids are necessary in both ICE B1 donor and recipient cells for efficient transfer of the element. The activity of the ICE B1 conjugation machinery was toxic to cells that were depleted of wall teichoic acids, and these cells appeared to die from damage to the cell envelope caused by the conjugation machinery.

2 | RESULTS

2.1 | A CRISPRi screen for essential host genes involved in conjugation

2.1.1 | Rationale

We sought to identify host genes in an ICE B1 donor that are important for transfer of the element. Previously, we used Tn-seq to identify genes in recipients that are involved in acquisition of ICEB1 via conjugation (Johnson & Grossman, 2014). Several of the genes identified were also important in donors (Johnson & Grossman, 2014). In preliminary experiments, we screened individual insertion mutants from a transposon insertion library and were unable to reliably identify candidate genes in donors that affected conjugation. Therefore, we focused on a screen that would readily identify essential genes that have a role in conjugation.

2.1.2 | CRISPRi knockdown of essential genes

We used a B. subtilis CRISPRi system (Peters et al., 2016) to reduce expression of essential genes in B. subtilis, and screened for those
that caused a defect in conjugation. Briefly, the system comprises a catalytically dead Cas9 nuclease from Streptococcus pyogenes (dCas9) under the regulation of the xylose-inducible promoter P\textsubscript{xyl} and a constitutively expressed single-guide RNAs (sgRNA) containing a 20 nt region corresponding to the target gene of interest. When dCas9 is produced via the addition of xylose, it complexes with the sgRNA and stably binds to the host gene specified by the targeting region of the sgRNA. This interaction sterically blocks transcript elongation, thereby lowering expression of the targeted gene or operon. The library of sgRNAs used in this study (a gift of Peters et al.) collectively targeted a set of 289 proposed essential \textit{B. subtilis} genes (Peters et al., 2016), 257 of which were subsequently verified to be essential in a systematic gene knockout analysis (Koo et al., 2017).

### 2.1.3 CRISPRi library in ICEBs1

We created a library of donor strains in which ICEBs1 could be induced by overproducing its activator protein Rapl. All donor strains contained a xylose-inducible P\textsubscript{xyl}-dCas9 integrated into the host chromosome. Each donor strain also had one constitutively expressed sgRNA allele (Pveg-sgRNA) integrated into a site in ICEBs1 that is not needed for transfer. In this way, we constructed a pooled library of donor strains with each individual donor strain representing a knockdown of one essential \textit{B. subtilis} gene (Figure 1a).

### 2.1.4 The screen

We used the CRISPRi system in ICEBs1 to partially decrease expression of essential genes. We used partial (0.01% xylose) rather than full expression of dCas9 because we were concerned that high levels, in combination with an sgRNA targeting an essential gene, would cause a significant fitness defect of each donor strain and would have strongly biased or interfered with the screen. Expression of ICEBs1 was activated and the donor library was mixed with recipients and transconjugants were selected (Experimental Procedures). The overall mating efficiency of this pooled library (0.17%) was similar to that of a control strain with a knockdown targeted to a nonessential gene (MMH233, 0.36%), indicating that the partial knockdown treatment did not have a substantial global effect on conjugation.

We used next-generation sequencing to determine the relative abundance of sgRNA alleles in a sample of: (1) donors harvested immediately prior to mating and (2) the pool of transconjugants. Importantly, the target of a gene knockdown is specified by the sgRNA encoded within ICEBs1 itself. As a consequence, every transconjugant generated by this library contains a genetic record of the donor strain that produced it. We can determine the relative mating efficiency of a given donor strain by pooling the population of transconjugants, collectively sequencing their sgRNA alleles, and then determining which sgRNAs differ in abundance compared with the pre-mating donor population. If a knockdown compromised ICEBs1 mating efficiency, then the sgRNA corresponding to that gene would be underrepresented in the resulting pool of transconjugants. Conversely, if a knockdown improved ICEBs1 mating efficiency, then the sgRNA allele corresponding to the that gene would be overrepresented in the pool of transconjugants.

Decreased expression of most of the essential genes had little or no effect on mating efficiency (Figure 1b). We compared the relative abundance of each sgRNA in the transconjugant population to the pre-mating donor population and found that >80% of knockdowns resulted in less than a four-fold change in the abundance of the sgRNA gene in the transconjugant pool relative to the starting population (Figure 1b, points above diagonal dotted line). We did not detect an increase greater than four-fold in any of the sgRNA genes in transconjugants, indicating that none of the essential genes seemed to be substantially inhibiting the function of ICEBs1.

We chose to focus on those genes that caused less than or equal to four-fold effects to increase the chances that observed effects were robust and significant. We also focused on knockdown strains that were well represented in the donor pool (Figure 1b, points to the right of the horizontal line, >0.01%), and that had the largest decrease in conjugation. Of this subset of knockdowns, most were involved in processes that directly affect the production of the conjugation machinery, including genes involved in translation, protein secretion, and protein folding. We did not study these.

Knockdown of \textit{tagA} resulted in the most severe transfer defect out of all genes tested in the screen. \textit{tagA} is needed for biosynthesis of WTAs. Strains with knockdowns of other WTA biosynthesis genes (\textit{tagO}, \textit{tagB}, \textit{tagD}, \textit{tagF}, \textit{tagG}, and \textit{tagH}) were also present in the library, and two of these knockdowns (\textit{tagD} and \textit{tagF}) also resulted in greater than four-fold defects in conjugation (~10- and ~4.5-fold, respectively; Table S1). We decided to further investigate the role of \textit{tagA} and WTA biosynthesis in conjugation.

We note that several other genes were also identified as candidates for affecting conjugation, including genes whose products are involved in peptidoglycan synthesis, cell shape, restriction-modification, and other cellular functions (Table S1). For example, reduced expression of \textit{rny} (encoding the endoribonuclease RNase Y) and \textit{mnt} (encoding a component of an ABC manganese transporter) appeared to cause an ~20-fold and ~1000-fold decrease in conjugation, respectively, in the context of the CRISPRi screen (Table S1). Although we have not validated these findings, some of these genes may have interesting roles in the life cycle of ICEBs1.

### 2.2 WTAs are necessary in an ICEBs1 donor for efficient transfer

To validate the apparent effect of \textit{tagA} revealed in the CRISPRi screen, we directly tested for effects of \textit{tagA} on ICEBs1 conjugation in several ways. First, we measured the conjugation efficiency [stable transconjugants per donor; each measured as colony-forming units (CFUs)] from a homogenous population of donor cells in which \textit{tagA} expression was inhibited by CRISPRi. This is in contrast to the screen which used a population of strains representing the entire
CRISPRi library. As anticipated, decreasing tagA expression in an ICEBs1 donor resulted in an acute drop in conjugation (Figure 2a). The severity of the defect increased as tagA expression decreased, with the strongest knockdown resulting in no detectable ICEBs1 transfer (<1 × 10⁻³).

We inhibited WTA synthesis in ICEBs1 donors in two additional ways. In one, we made three different donor strains, each with one of three tag operons (tagO, tagAB, or tagDEF) under the control of the LacI-repressible isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter Pspank. With low expression levels (little or no IPTG), these strains exhibited cell shape defects characteristic of WTA depletion (Figure S1) (D’Elia et al., 2006; Mirouze et al., 2018). We grew these strains in different IPTG concentrations (ranging from 0 to 100 μM) for approximately six generations and then measured effects on conjugation. Reducing expression of any of the three operons (tagO, tagAB, tagDEF) resulted in an acute drop in conjugation (Figure 2b–d). In all three cases, mating efficiency dropped more than 1000-fold at the lowest level of expression tested (no IPTG). Whereas disruption of downstream WTA biosynthesis genes (tagB, tagD, tagF) can impact Lipid II production by sequestering the
cellular supply of undecaprenyl phosphate in the blocked WTA synthesis pathway, reducing tagO expression or inhibiting TagO (the first step of WTA biosynthesis) does not result in this sequestration (D’Elia, Millar, et al., 2009). Accordingly, these results demonstrate that the ICEBs1 transfer defect is not a consequence of Lipid II depletion.

We also inhibited WTA synthesis using a low concentration (1 μg/ml) of the antibiotic tunicamycin. At this concentration, tunicamycin specifically inhibits WTA biosynthesis in gram-positive bacteria by blocking the activity of TagO (Campbell et al., 2011; Pooley & Karamata, 2000). At higher concentrations (>10 μg/ml) it inhibits peptidoglycan biosynthesis and cell growth (Campbell et al., 2011;
Importantly, inhibiting WTA biosynthesis with tunicamycin (1 μg/ml) for approximately three generations (60 min) in an ICEBs1 donor decreased ICEBs1 transfer ~150-fold (Figure 3a). Based on these results, we infer that WTAs are likely necessary for efficient transfer of ICEBs1. Alternatively, it is formally possible that the biosynthetic activity of TagO and WTA biosynthesis per se is important and not accumulation of WTAs themselves.

We found that WTAs themselves are necessary for efficient ICE transfer, rather than WTA biosynthesis. We inhibited WTA biosynthesis without substantially depleting cells of WTAs by treating ICEBs1 donors with tunicamycin for 1 min prior to mating. Under these conditions, there would be no substantial decrease in the amount of WTAs, but biosynthesis should be inhibited. There was little or no defect in conjugation of ICEBs1 under these conditions.

**FIGURE 3** WTAs are necessary in both ICEBs1 donors and recipients for efficient transfer of the element. WTA biosynthesis or WTA D-alanine modification was inhibited or prevented in ICE donors and ICE0 recipients, and the resulting impacts on mating efficiencies were examined. Relative mating efficiencies are reported as described in Figure 2. Graphs show averages and standard deviations of log-transformed data from three biological replicates. * indicates average relative mating efficiency was significantly different from the wild type or untreated control (one-way ANOVA and Dunnett’s multiple comparisons test, \( p < .05 \)). (a) WTA biosynthesis was inhibited in an ICEBs1 donor (MMH550), an ICEBs10 recipient (MMH676), or both by adding the TagO-inhibiting drug tunicamycin (1 μg/ml). The drug was added either 60 min prior to mating to block synthesis and deplete WTAs, or 1 min prior to mating to block WTA synthesis without substantial WTA depletion. Average mating efficiency of the untreated control was \( 3.6 \times 10^{-3} \) with a standard deviation of \( 2.3 \times 10^{-3} \). (b) An ICEBs1 donor (CAL874) was mated with an ICEBs10 recipient in which the WTA biosynthesis operon tagAB had been placed under the control of Pspank (MMH584). Expression of tagAB was controlled by growing the recipient strain at the indicated IPTG concentrations. A wild type recipient (CAL89) was used as a control. Average mating efficiency with the wild type control was \( 6.1 \times 10^{-4} \) with a standard deviation of \( 2.3 \times 10^{-4} \). (c) The Pspank-tagAB donor strain from Figure 2b (MMH578) was mated with the Pspank-tagAB recipient strain from 3b (MMH584). Both strains were grown at the indicated IPTG concentration. ND indicates relative mating efficiency was below the limit of detection (<0.0007 and <0.0008 for the 10 μM and 0 μM treatments, respectively). Average mating efficiency of the wild type control was \( 1.3 \times 10^{-3} \) with a standard deviation of \( 8.2 \times 10^{-4} \). (d) Donors (MMH541) or recipients (MMH545) that were defective in D-alanylation of WTAs (ΔdltABCDE::cat) were used in conjugation experiments. ΔdltABCDE donors (MMH541) were mated with a wild type recipient (CAL85). ΔdltABCDE recipients (MMH545) were mated with a wild type donor (CAL874). The wild type donor (CAL874) was mated with the wild type recipient (CAL85) as a control. Average mating efficiency with the control was \( 2.4 \times 10^{-3} \) with a standard deviation of \( 1.6 \times 10^{-3} \). (e) The effect of inhibiting WTA biosynthesis on a Tn916 donor (CMJ253) or recipient (MMH676) was tested by adding tunicamycin to growing cells 60 min prior to mating. Average mating efficiency of the untreated control was \( 4.5 \times 10^{-6} \) with a standard deviation of \( 2.1 \times 10^{-6} \).
Together, our results demonstrate that WTAs are required in donor cells for efficient transfer of ICEBs1.

### 2.3 WTAs are necessary in an ICEBs1 recipient for efficient transfer

We wished to determine if WTAs are also needed in recipients for efficient acquisition of ICEBs1. We treated recipients with tunicamycin for 1 h and found that acquisition of ICEBs1 was reduced ~50-fold (Figure 3a). As with donors, blocking WTA biosynthesis in the recipient with tunicamycin treatment for only 1 min prior to mating caused little or no defect in acquisition of ICEBs1 (Figure 3a), indicating that WTAs themselves (and not simply WTA biosynthesis) are necessary in the recipient for efficient mating.

We also reduced WTAs in recipients by decreasing expression of tagAB (from a Psplank-tagAB fusion) by growing cells in low concentrations (or in the absence) of IPTG for approximately six generations. With the lowest level of expression (no IPTG), the mating efficiency into the WTA-depleted recipients was decreased ~200-fold relative to that of cells grown in 100 μM IPTG or wild type cells (Figure 3b).

Reducing WTAs in both donors and recipients caused a defect in mating that was more severe than reduction in either donor or recipient alone (Figure 3a,c). This was true following treatment of donors and recipients with tunicamycin (Figure 3a) or growing both donors and recipients containing Psplank-tagAB in low concentrations of IPTG (Figure 3c).

Based on these results, we conclude that WTAs in both donors and recipients are important for efficient conjugation of ICEBs1. WTAs could be needed for the proper function, assembly, or regulation of the conjugation machinery, for establishing or maintaining cell-cell contact to enable mating, or for cell viability during mating.

### 2.4 D-alanlylation of wall teichoic acids is not needed for efficient transfer of ICEBs1

In B. subtilis, WTA polymers may be tailored by the addition of D-alanine moieties. The degree of WTA D-alanlylation varies under different cellular conditions, and the cationic D-alanine moieties are thought to affect the function of WTAs by lessening the extent of the polymer’s negative charge (Neuhaus & Baddiley, 2003). WTA D-alanlylation occurs outside the cell and is mediated by the proteins encoded by the dltABCDE operon (Perego et al., 1995). We constructed ICEBs1 donor and recipient strains that lacked the dltABCDE operon to determine whether D-alanine-modified WTAs are needed for efficient transfer of ICEBs1.

We found that D-alanlylation of WTAs is not necessary for efficient transfer or acquisition of ICEBs1. The mating efficiency of ICEBs1 was not significantly affected by the use of either a ΔdltABCDE ICEBs1 donor or recipient (Figure 3d). Based on these results, we conclude that the presence of D-alanine moieties on either ICEBs1 donor or recipient WTAs is not necessary for efficient transfer of the element.

### 2.5 WTAs are not necessary for transfer of the broad host range ICE Tn916

We tested for effects of WTAs on conjugative transfer of Tn916, a small (~18 kb) ICE that confers tetracycline resistance to its host. Activity of Tn916 is increased several fold in the presence of tetracycline (Showsh & Andrews, 1992). Whereas the natural host of ICEBs1 appears to be limited to B. subtilis, Tn916 is found in a broader range of gram-positive bacteria (Roberts & Mullany, 2009) and, although not its natural host, works quite well in B. subtilis (Celli & Trieu-Cuot, 1998; Christie et al., 1987; Johnson & Grossman, 2014).

(Figure 3a).
We found that unlike ICEBs1, Tn916 transfer was not significantly affected when WTA biosynthesis was inhibited. We depleted WTAs in Tn916 donors or recipients with tunicamycin (1 μg/ml) for 60 min. The mating efficiency following treatment of recipients was similar to that of untreated recipients, and that of donors appeared to increase slightly (Figure 3e). This indicates that the decrease in transfer of ICEBs1 in response to WTA depletion is probably not due to a general effect on host physiology, or an inability of cells to contact each other, unless the presence of Tn916 compensates for these alterations. This differential effect of WTAs on ICEBs1 compared with Tn916 highlights a difference between conjugation mediated by these elements.

2.6 | An osmoprotective mating surface bypasses the need for WTAs in ICEBs1 conjugation

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

Based on the role of WTAs in modulating the activity of cell wall hydrolases, we hypothesized that WTA-depleted cells might have cell walls that are more sensitive to the formation of mating pairs. If true, then the decrease in conjugation efficiency should be suppressed (conjugation restored) under osmoprotective conditions that would enable cells to survive severe defects in their walls.

We found that the conjugation defect of WTA-depleted ICEBs1 donors was completely suppressed when matings were done on an osmoprotective surface (Figure 4a). Matings were done on a standard mating surface (Spizizen’s salts, described in Experimental Procedures) or an osmoprotective mating surface that contained 20 mM MgCl₂ and 0.5 M sucrose, buffered with 20 mM maleic acid pH 7 (MSM), an osmoprotective supplement that has been used to maintain protoplasts (lacking cell walls) and prevent bacterial cell death from osmotic stress (Leaver et al., 2009; Wyrick & Rogers, 1973). A deletion of a sucrose metabolism gene was incorporated into all strains used in these osmoprotection mating assays to prevent degradation of the sucrose osmoprotectant (Wolf et al., 2012). At the conclusion of the mating, cells were resuspended and diluted in MSM and then plated and grown on non-protective LB plates with the appropriate antibiotics to select for transconjugants.

As described above, treatment of donors with tunicamycin (1 μg/ml) for 60 min to deplete WTAs caused a mating defect under standard mating conditions (Figure 4a). In contrast, this defect was fully suppressed under osmoprotective conditions and mating efficiencies were indistinguishable from those of cells without tunicamycin treatment (Figure 4a).

Similarly, we found that the mating defect associated with WTA-depleted recipients was largely suppressed when matings were done on an osmoprotective mating surface (Figure 4a). There was still an approximately 5- to 10-fold drop in conjugation efficiency with WTA-depleted recipients on the osmoprotective mating surface relative to untreated cells (Figure 4a). This drop was likely due to death of transconjugants that did not sufficiently recover from cell wall damage before the shift to non-protective conditions (LB agar).

Suppression of the mating defect on the osmoprotective surface (MSM) was due to osmoprotection by sucrose and not an effect of MgCl₂ or the malate buffer. When sucrose was omitted from the mating surface, death of transconjugants that did not sufficiently recover from cell death occurred (Figure 4a). This drop was likely due to suppression of conjugation under osmoprotective conditions that would enable cells to survive severe defects in their walls.

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Suppression of the mating defect on the osmoprotective surface (MSM) was due to osmoprotection by sucrose and not an effect of MgCl₂ or the malate buffer. When sucrose was omitted from the mating surface, there was no rescue of the conjugation defect caused by depletion of WTAs (Figure 4b). These results support the model that WTA-depleted cells die from osmotic stress. Furthermore, we conclude that WTA-depleted donors have significantly decreased ability to transfer ICEBs1, perhaps because they die before they can successfully participate in conjugation. This could be due to overall death of all or the vast majority of ICEBs1-containing cells, or selective death of a subpopulation, perhaps those that form mating pairs with recipients.

2.7 | WTA-depleted cells in ICEBs1 mating pairs are more likely to die

We found that there was not a large decrease in the number of viable WTA-depleted donors during mating under standard (non-osmoprotective) conditions. As above, ICEBs1 was activated and cells were simultaneously treated with tunicamycin. This tunicamycin treatment caused a mild (3.7-fold) decrease in CFUs relative to untreated cells (1.1 × 10⁶ CFU/ml for untreated cells versus 3.0 × 10⁷ CFU/ml). We suspect that this drop in CFUs following tunicamycin treatment was due, at least in part, to defects in cell separation after division. We combined these donors with an ICE₀ recipient on a (non-protective) mating surface. The percentage of viable donors recovered after mating was similar between cells with and without tunicamycin treatment (Figure 4c), although there appeared to be a small drop in recovery of the tunicamycin-treated cells. Based on these results, we conclude that there is not a large drop in viability of the population of donors.

This population-level observation from the conclusion of a mating protocol does not reflect what occurs at the single-cell level. Although the vast majority of ICEBs1-containing cells are potential donors, only a small number (~1%) successfully participate in conjugation under conditions used here. Based on the results above, we hypothesized that WTA-depleted donors that are part of a mating pair likely undergo cell death. This would represent death of a small fraction of the population that would not be readily observed by bulk population-based viability assays.

We used propidium iodide (PI) staining and fluorescence microscopy to monitor death (loss of cell envelope integrity) of single cells. We induced a population of ICEBs1 donors to mate, concentrated them at high density on an agar pad containing propidium iodide, and monitored the cells for 2 h, and counted the number of envelope-damaged (PI-stained) cells after 2 h. Because mating is ordinarily a rare event, we used a monoculture of ICEBs1 donors lacking the ICE gene yddJ, which encodes a protein that would normally block the ICE+ cell from serving as a recipient in a mating pair (Avello et al., 2019). This allowed all cells to potentially serve as donors and recipients, thereby significantly increasing the frequency of conjugation. When ICEBs1 was induced in WTA-depleted cells under these conditions, we observed an approximately eightfold increase in the incidence of PI-stained bacteria, indicating that WTA-depleted cells are more likely to die under conditions that support ICEBs1 transfer (Figures 4d and S2).

2.8 | The activity of the ICEBs1 conjugation machinery is sufficient to kill WTA-depleted donors and recipients

Our results indicate that WTA-depleted donors and recipients are defective in ICEBs1 mating due to envelope damage. In the case of matings using WTA-depleted recipients, this could be because (1) WTA-depleted recipients never acquire ICEBs1, likely because forming a mating pair is lethal to WTA-depleted cells, or because (2) WTA-depleted recipients acquire ICEBs1 and become transconjugants but subsequently die, perhaps due to the expression of ICEBs1 genes or the transconjugant becoming a new donor.
We found that transfer of ICEBs1 into WTA-depleted recipients was not required for the decrease in conjugation efficiency. We measured mobilization of the plasmid pC194 by the ICEBs1 conjugation machinery into WTA-depleted recipients. In these experiments, we used a mutant ICEBs1 that is unable to excise from the chromosome (ΔattR) and that lacks a functional origin of transfer (ΔoriT). When activated, this ICE mutant still expresses the conjugation machinery and is able to mobilize several plasmids that do not encode their own conjugation system, including pC194 (Lee et al., 2012). We activated ICEBs1 gene expression and measured mobilization of pC194 into recipient cells. There was a ~100-fold decrease in mobilization efficiency of pC194 into WTA-depleted (tunicamycin treated) recipients compared with untreated recipients (Figure 5). Based on these results, we conclude that the decrease in conjugation efficiency into WTA-depleted (tunicamycin treated) recipients is not due to transfer of ICEBs1 into recipients and subsequent death of the new transconjugant; rather, the defect is likely due to death of transconjugants caused by the formation of mating pairs or the act of transferring any DNA.

We performed similar experiments with WTA-depleted donor cells in which ICEBs1 could not transfer, but the ICE-encoded conjugation machinery could mobilize pC194. Again, there was an ~100-fold decrease in mobilization efficiency as measured by acquisition of pC194 (Figure 5). These results indicate that WTA-depleted donors are defective in transfer. Together with the results above, we infer that this defect is due to donor cell death in mating pairs.

3 | DISCUSSION

We used a CRISPRi screen to identify essential genes in B. subtilis that are needed for efficient conjugation of ICEBs1. We found that WTA-s are necessary in both donor and recipient cells for efficient transfer of ICEBs1. Under mating conditions, likely in mating pairs, there was significant death of WTA-depleted cells. Cell death and the need for WTAs for conjugation were reduced or eliminated when matings were done in osmoprotective conditions. Together, our results indicate that WTA-depleted cells fail to mate because they die from damage to their cell envelope.

The defect in conjugation of WTA-depleted cells was dependent on the ICEBs1 conjugation machinery, but did not depend on ICEBs1 DNA being transferred. We found that WTA-depleted cells were defective in transfer of a plasmid that normally can be transferred by the ICEBs1 conjugation machinery. In contrast, there was little or no defect in transfer of Tn916. These results indicate that the primary effect of WTAs is likely not to enable cell-cell contact (unless Tn916 has some unknown function that does this). Rather, WTAs are important for proper function or control of the conjugation machinery, and that there is something fundamentally different about the activity of the ICEBs1 conjugation machinery compared with that of Tn916 (see below).

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

WTAs are important regulators of cell wall hydrolases across bacterial species, and WTA-depleted gram-positive bacteria have previously been demonstrated to be more sensitive to autolysin and lysozyme treatment (Brown et al., 2013). WTAs are also important for the proper localization of autolysins. Some B. subtilis cell wall hydrolases appear to be excluded from binding peptidoglycan decorated by WTAs, and the enzymes are mislocalized in the absence of WTAs (Kasahara et al., 2016; Yamamoto et al., 2008). It is possible that WTAs similarly regulate the activity of the bifunctional cell wall hydrolase CwlT that is encoded by ICEBs1, and that mis-regulation of CwlT, perhaps in combination with mis-regulation of other cell wall hydrolases in WTA-depleted cells, causes the lethal envelope damage during conjugation. It is challenging to test the role of CwlT on conjugation efficiencies of WTA-depleted cells because cwlT is required for conjugation (DeWitt & Grossman, 2014).

WTAs also have a role in cell wall biosynthesis, and WTA-depleted B. subtilis cells exhibit irregularities in cell wall thickness and severe cell

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**Figure 5** Mobilization of the non-conjugative plasmid pC194 by a locked-in ICEBs1 is negatively affected by WTA depletion. A strain containing both the mobilizable plasmid pC194 and a locked-in version of ICEBs1 (MMHB68) was mated with an ICEBs1<sup>Δ</sup> recipient strain (MMH676). WTAs were depleted in either the donor or recipient strain via treatment with 1 μg/ml tunicamycin for 60 min as in Figure 3a. Relative mobilization efficiency was calculated as the number of transconjugants (Cm<sup>R</sup> Strp<sup>R</sup> CFUs) per initial donor relative to an untreated control. Graphs shows averages and standard deviations of log-transformed data from three biological replicates. * indicates relative mobilization efficiency was significantly different from 1 (one-way ANOVA and Dunnets’s multiple comparisons test, p < .05). Average mobilization efficiency of the untreated control was 1.4 × 10<sup>-3</sup> with a standard deviation of 6.2 × 10<sup>-4</sup>.

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shape defects (D’Elia et al., 2006). It is possible that WTA-depletion in B. subtilis results in an unusually fragile cell wall, and that WTA-depleted B. subtilis is consequently much more sensitive to the normal cell wall modification process that occurs during conjugation.

The strains investigated in this study were all derived from B. subtilis 168, which utilizes glycerol 3-phosphate-(GroP) based WTAs. In contrast, some bacteria utilize ribitol 3-phosphate- (RboP) based WTAs in lieu of GroP-based WTAs. Prior reports indicate that ICEBs1 is capable of efficient transfer into a wide variety of recipients, including Listeria monocytogenes (Auchtung et al., 2005; Brophy et al., 2018), which is reported to utilize RboP-based WTAs (Eugster & Loessner, 2011). ICEBs1 is also capable of transfer (albeit less efficiently) into Staphylococcus aureus (Brophy et al., 2018), which also utilizes RboP-based WTAs (Brown et al., 2013). Based on these results, we speculate that the requirement for WTAs in an ICEBs1 recipient is likely not specific to GroP-WTAs versus RboP-WTAs.

3.2 Activity of the conjugation machinery

Our results have interesting implications concerning the activity of the conjugation machinery and its effects on donor and recipient cells. First, cell death of WTA-depleted donors occurs only during mating conditions, and in a small subpopulation of cells. This indicates that the conjugation machinery is only activated in the presence of recipients, likely only in mating pairs. This is consistent with analyses of other elements that indicate the conjugation machinery is activated by cell-cell contact [reviewed in: (Christie et al., 2014)].

Additionally, if the decrease in acquisition of ICEBs1 by WTA-depleted recipients is due to killing that is mediated by the ICEBs1-encoded cell wall hydrolase CwIT, then this indicates that the donor-encoded hydrolase is acting on recipient cells. There are other interpretations of this result: for example, the WTA-depleted cell’s own autolysins could be hyper-active, and active conjugation machinery from the donor could exacerbate the effects of hyper-active autolysins in recipients in mating pairs, thereby causing cell death.

Although CwIT is one possible cause of the conjugation-dependent toxicity among WTA-depleted cells, there are alternative explanations which cannot be ruled out. For example, WTA-depletion in B. subtilis sensitizes the cells to PBP-targeting antibiotic methicillin (Farha et al., 2013), and it is possible that the ICEBs1 conjugation machinery comparably interferes with cell wall biosynthesis in a way that is incompatible with WTA depletion. Alternatively, ICEBs1 conjugation could involve inactivating or modifying teichoic acids in a way that is lethal to WTA-depleted bacteria.

3.3 Different ICEs respond differently to WTA depletion

We found that Tn916 transfer was not negatively impacted by WTA-depletion, in contrast to our results with ICEBs1. ICEBs1 and Tn916 are closely related elements. The conjugation and replication machinery of ICEBs1 is homologous to that of Tn916, with common genes between the two elements having about 30% sequence identity (Churchward, 2008). Additionally, both elements have a modular organization, with genes related to conjugation, and regulation of activation organized into distinct groups (Burrus & Waldor, 2004). Like CwIT, the cell wall hydrolase encoded by Tn916 (Orf14) is predicted to be a bifunctional hydrolase containing both muramidase and peptidase domains (Xu et al., 2014), and CwIT and Orf14 are about 43% identical.

An important difference between ICEBs1 and Tn916 that might contribute to this observation is that transfer of Tn916 is less efficient than that of ICEBs1. It is possible that less conjugation machinery is made when Tn916 becomes transcriptionally active, and therefore the formation of mating pairs is not as stressful with the Tn916-encoded conjugation machinery as it is with that from ICEBs1. It is also possible that the relevant components of the Tn916 conjugation machinery are not regulated by B. subtilis WTAs, perhaps reflective of the broad host range of Tn916. It remains to be determined what it is about the ICEBs1 and Tn916-encoded conjugation machineries that make them respond so differently to B. subtilis WTAs.

Recent studies of ICESt3 of Streptococcus thermophilus found that deleting a tagO-like gene, which might result in a decrease in WTAs, resulted in complex effects on transfer efficiency. Deletion in donors caused a decrease, but deletion in recipients caused an increase in conjugation efficiency (Dahmane et al., 2018). It is not clear if these effects are alleviated by osmoprotective conditions and if they are related to the effects described here for ICEBs1.

3.4 Conjugation and cell envelope stress

Connections between conjugation and envelope stress have long been known. One classic example is in E. coli where excessive transfer of F plasmids into F- recipients can lead to death of the recipient, a phenomenon called lethal zygosis (Skurray & Reeves, 1973). Subsequent studies found that radiolabeled peptidoglycan components are released into the medium when lethal zygosis occurs, indicating that the mechanism of death is due to damage of the cell envelope (Ou, 1980). Furthermore, activation of the F-plasmid sensitizes cells to certain envelope-disrupting antimicrobials (i.e. bile salts) (Bidlack & Silverman, 2004). The F plasmid has also been demonstrated to encode the means to upregulate the cell envelope stress response pathway in the host bacterium, which likely helps protect host cells from envelope stress caused by the F-encoded conjugation machinery (Grace et al., 2015). Activation of ICEBs1 may also result in the upregulation of liaH (Auchtung et al., 2005), previously named yqH, that encodes a homolog of phage shock protein A. liaH is activated by the LiaRS cell envelope response system and is thought to have a role in maintaining the integrity of the cell envelope, although its specific function is not well understood (Radeck et al., 2017).

Our results indicate that WTAs have an important role in protecting B. subtilis against envelope stress caused by the ICEBs1
conjugation machinery. We suspect that WTAs in other gram-positive bacteria also have a role in conjugation, and that role very likely will depend on aspects of the specific conjugation machinery assembled in the cell envelope.

4 | EXPERIMENTAL PROCEDURES

4.1 | Media and growth conditions

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

Where needed, *B. subtilis* strains were grown in LB at the following antibiotic concentrations for selection or maintenance of marked alleles: kanamycin (5 μg/ml), streptomycin (100 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (5 μg/ml), and a combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) to select for macrolide-lincosamide-streptogramin (MLS) resistance and erythromycin resistance. Tunicamycin was used at 1 μg/ml to inhibit WTA synthesis.

The osmoprotective supplement MSM (0.5 M sucrose, 20 mM MgCl2, buffered with 20 mM maleic acid pH 7) (Leaver et al., 2009) was used where indicated. It was added to a 2x stock.

4.2 | Strains and alleles

*Escherichia coli* strain AG1111 (MC1061 F' lacQ lacZM15 Tn10) was used for routine cloning and plasmid construction.

The *B. subtilis* strains used in this study are listed in Table 1. Strains were constructed using natural transformation (Harwood & Cutting, 1990). All *B. subtilis* strains are derivatives of JH642 and contain tryptophan and phenylalanine auxotrophies (trpC2 pheA1) (Brehm et al., 1973; Smith et al., 2014). Many of the alleles used in this study have been described previously and are briefly summarized below.

Donor strains used in standard ICEBs1 mating assays contained the allele Δ(rapI-phi)342::kan (Auchtung et al., 2005). ICEBs1 was activated in donor strains by inducing expression of rapI from one of three promoter fusions: the LacI-repressible-IPTG-inducible promoters Pspank-rapl or Pspank(hy)-rapl (Auchtung et al., 2005), or the xylose-inducible promoter Pxyl-rapl (Berkmen et al., 2010). For the first two, rapl expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 mM. Pxyl-rapl expression was induced via the addition of xylose to a final concentration of 1% w/v. ICEBs1-containing strains used in live cell microscopy also contained a deletion of yddJ (Avello et al., 2019).

Recipient strains were derived from the ICEBs1-cured (ICEBs1Δ1) strain JMA222 (Auchtung et al., 2005) and were streptomycin resistant (str84) to facilitate counterselection during mating experiments. Recipient strains also contained spc-marked null alleles of competence genes comK or comC to prevent natural transformation.

ICEBs1 donor and recipient strains that were used in osmoprotective mating assays also contained a deletion-insertion of sacB (ΔsacB::erm) (Koo et al., 2017) to prevent degradation of sucrose (Wolf et al., 2012). A strain containing this allele was obtained from the Bacillus Genetic Stock Center (www.bgsc.org).

4.2.1 | ycgO::[Pspank-rapl (spc)]

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

4.2.2 | comC::spc

We constructed a comC deletion-insertion allele, extending from 324 bp upstream and 26 bp downstream of the comC open reading frame, with the aad9 (spc) gene from pMagellan6. The allele was constructed by joining the appropriate comC flanking sequences with the amplified aad9 gene by isothermal assembly, and was moved into wild type *B. subtilis* via natural transformation selecting for spectinomycin resistance.

4.2.3 | DltABCDE::cat

We constructed a dltABCDE deletion-insertion allele: The deletion extends from 1 bp upstream of the dltA open reading frame to 22 bp upstream of the 3’ end of the dltE open reading frame, and cat from pGEMcat (Youngman et al., 1989) is inserted. The allele was constructed by joining the appropriate dlt operon flanking sequences with the amplified cat by isothermal assembly, and was introduced into wild type *B. subtilis* via natural transformation selecting for chloramphenicol resistance.

4.2.4 | Pspank-tag alleles

We constructed a set of three fusions that placed each of three endogenous WTA biosynthesis operons (tagO, tagAB, tagDEF) under the control of the IPTG-inducible promoter Pspank. In each case, Pspank was introduced upstream of the first gene in the operon by single crossover integration. We used a plasmid (pJCL86; lab collection) that contains Pspank and lacI inserted into the backbone of pAGS8 (Jaacks et al., 1989). A short region of the 5’UTR encompassing the predicted ribosome binding site and a few hundred
generations and were subsequently analyzed by light microscopy.

grown in the reduced IPTG concentration for approximately six μg/ml.

Briefly, the Pspank::lacA followed by the tagD

translation start site. Each segment was inserted between the tagD

start site, and 25 bp upstream to 157 bp downstream of the tagO

amplified from each gene corresponded to the following: 19 bp up

amplified from wild type B. subtilis (D’Elia et al., 2006) when grown in medium containing 20 μM, 10 μM, or 0 μM IPTG (Figure S1).

TABLE 1. B. subtilis strains

| Strain | Genotype† (reference) |
|--------|------------------------|
| CAL85  | ICEB1Δ str-84 (Lee et al., 2007) |
| CAL89  | ICEB1Δ str-84 comK::spc (Auchtung et al., 2005) |
| CMU253 | ICEB1Δ Tn916+ (Johnson & Grossman, 2014) |
| CAL874 | ICEB1 ΔrapI::kan amyE::Pxyl::rapI spc (Lee et al., 2010) |
| MMH211 | ICEB1 ΔrapI::kan amyE ΔamyE117::Pspank-rapl spc lacA::(Pxyl-dcas9 spc) |
| MMH233 | ICEB1 Δ(rapI-phrI)::kan amyE::(Pveg-sgRNA::cat) ΔamyE117::(Pspank-rapl spc) lacA::(Pxyl-dcas9 spc) |
| MMH525 | ICEB1 Δ(rapI-phrI)342::kan ycgO::(Pspank-rapl spc) amyE::(Pveg-sgRNA::cat) lacA::(Pxyl-dcas9 spc) |
| MMH527 | ICEB1 Δ(rapI-phrI)342::kan ycgO::(Pspank-rapl spc) amyE::(Pveg-sgRNA::cat) lacA::(Pxyl-dcas9 spc) |
| MMH541 | ICEB1 Δ(rapI-phrI)342::kan amyE::(Pxyl-rapl spc) ΔdltABCDE::cat |
| MMH545 | ICEB1Δ str-84 ΔdltABCDE::cat |
| MMH550 | ICEB1 Δ(rapI-phrI)342::kan ycgO::(Pspank-rapl spc) |
| MMH577 | ICEB1 Δ(rapI-phrI)342::kan amyE::(Pxyl-rapl spc) tagO::(Pspank-tagO cat) |
| MMH578 | ICEB1 Δ(rapI-phrI)342::kan amyE::(Pxyl-rapl spc) tagA::(Pspank-tagA cat) |
| MMH584 | ICEB1Δ str-84 tagA::(Pspank-tagA cat) |
| MMH608 | ICEB1 Δ(rapI-phrI)342::kan amyE::(Pxyl-rapl spc) tagD::(Pspank-tagD cat) |
| MMH676 | ICEB1Δ str-84 comC::spc |
| MMH788 | ICEB1 Δ(yddJ Δ(rapI-phrI))342::kan |
| MMH794 | ICEB1 Δ(yddJ Δ(rapI-phrI))342::kan amyE::(Pspank-hy::rapI spc) |
| MMH797 | ICEB1Δ str-84 comC::spc sacB::mls |
| MMH862 | ICEB1 Δ(rapI-phrI)342::kan ycgO::(Pspank-rapl spc) sacB::mls |
| MMH868 | ICEB1 ΔoriT285 Δ(rapI-phrI)342::kan ΔattR::mls ycgO::(Pspank-rapl spc) pC194(cat) |

†All strains are derived from lab strain AG174 (JH642) (Brehm et al., 1973; Smith et al., 2014) and contain the trpC2 and pheA1 alleles (not show).

bp of the 5’ region of the ORFs of tagO, tagA, and tagD were each amplified from wild type B. subtilis genomic DNA. The region amplified from each gene corresponded to the following: 19 bp upstream to 357 bp downstream of the tagO translation start site, 24 bp upstream to 277 bp downstream of the tagA translation start site, and 25 bp upstream to 157 bp downstream of the tagD translation start site. Each segment was inserted between the SpHl and HindIII sites of pJCLB6 via isothermal assembly, yielding plasmids pMMH558 (tagO), pMMH559 (tagA), and pMMH605 (tagD). The plasmids were transformed into wild type B. subtilis selecting for chloramphenicol resistance in the presence of 1 mM IPTG. Proper integration of each plasmid was confirmed by diagnostic PCR and DNA (Sanger) sequencing. The resulting B. subtilis strains grew normally in the presence of 1 mM IPTG and exhibited severe growth defects in the absence of IPTG. Briefly, the Pspank::lacA strains were grown in medium containing 1 mM IPTG and then transitioned into growth medium containing different IPTG concentrations (100 μM, 20μM, 10 μM, or 0 μM IPTG). Strains were grown in the reduced IPTG concentration for approximately six generations and were subsequently analyzed by light microscopy. All Pspank-tag strains exhibited the distinctive cell shape and separation defects characteristic of B. subtilis cells depleted of WTAs (D’Elia et al., 2006) when grown in medium containing 20 μM, 10 μM, or 0 μM IPTG (Figure S1).

4.2.5 Construction of strains for mobilization of pC194

We constructed a B. subtilis strain containing the plasmid pC194 and a mutant of ICEB1 that is unable to excise (∆attR, “locked-in”) and without a functional origin of transfer (∆oriT). This strain was made by moving the ycgO::(Pspank-rapl [spc]) allele into the ICEB1Δ strain JMA222, creating strain MMH863. A version of ICEB1 containing three mutations, ∆(rapI-phrI)342::kan, ∆oriT, and ∆attR::mls, was moved into MMH863 via natural transformation, and pC194 was subsequently introduced via natural transformation. The unmarked oriT deletion in this element has been described (Jones et al., 2021). The ∆attR::mls allele was constructed via isothermal assembly using mls from pCAL215 (Lee et al., 2007) as a template, and has the same
deletion boundaries as a previously reported ΔattR::tet allele (Lee & Grossman, 2007).

4.2.6 | Construction of CRISPRi ICEBs1 donor library

The Pxyl-dcas9 and Pveg-sgRNA alleles used to generate the CRISPRi knockdown library of ICEBs1 donor strains were previously described and a generous gift from Peters et al. (2016). The library of sgRNAs used in this study (a gift of Peters et al.) collectively targeted a set of 289 proposed essential B. subtilis genes (Peters et al., 2016), 257 of which were subsequently verified to be essential in a systematic gene knockout analysis (Koo et al., 2017). The initial library contains a set of 299 plasmids, each containing a Pveg-sgRNA allele with a unique 20 bp targeting region, a cat marker conferring chloramphenicol resistance, and the appropriate flanking homology needed to integrate the sgRNA allele into the B. subtilis chromosome at amyE via double crossover. We utilized a pooled collection of these plasmids to generate our library of Pveg-sgRNA alleles integrated into ICEBs1.

Our strategy was to insert amyE into ICEBs1, delete amyE from the chromosome, and then recombine the Pveg-sgRNA library into amyE in ICEBs1. We constructed a deletion-insertion of amyE (ΔamyE17::(Pspank-rapl (spc))) that replaces the entire chromosomal gene and flanking noncoding regions with Pspank-rapl. This allele was constructed using a previously described Pspank-rapl (spc) allele (Auchtung et al., 2005) as a template for PCR amplification, and by joining the amplified product to amyE flanking homology via isothermal assembly. The construct was transformed into B. subtilis selecting for resistance to spectinomycin. The allele was confirmed by sequencing PCR-amplified DNA and verified functionally to activate ICEBs1 following addition of IPTG.

We used isothermal assembly to construct a kan-marked copy of amyE with flanking sequences needed to integrate the allele into rapl-phr1 of ICEBs1. This allele (Δ(rapl-phr1)::(amyE kan)) was designed such that the deletion boundaries and orientation of the kan cassette would be identical to Δ(rapl-phr1)342::kan.

The pooled library of Pveg-sgRNA plasmids was linearized with KpnI-HF (NEB), and was incorporated into the ICE::amyE locus by transformation into strain MMH211 and selecting for resistance to chloramphenicol. We recovered ≥1.5 transformation into strain MMH211 and selecting for resistance to KpnI-HF (NEB), and was incorporated into the ICE::amyE locus by transformation into strain MMH211 and selecting for resistance to chloramphenicol. We recovered ≥1.5

4.3 | CRISPRi library mating

We used the CRISPRi system in ICEBs1 to partially decrease expression of essential genes in the ICEBs1 donor library. We used partial rather than full expression of dcas9 because pilot experiments with maximal expression indicated that full expression of dcas9 (1% xylose) in combination with an sgRNA targeting an essential gene caused a significant fitness defect in many donor library strains that would strongly bias or interfere with the screen. Similarly, we opted not to use leaky expression of dcas9 (0% xylose) because pilot experiments suggested that the knockdowns would not be strong enough to generate notable variations in sgRNA allele frequency in the resulting transconjugant population.

A lawn of the CRISPRi ICEBs1 donor library was started from freezer stocks on the day before the experiment and grown overnight at room temperature on an LB plate. The following day, the lawn was used to start a culture at OD600 = 0.02 in LB supplemented with 0.01% xylose to stimulate transcription of Pxyl-dcas9 and with kanamycin to ensure maintenance of the kan-marked ICEBs1. When cultures reached an OD600 of 0.15, ICEBs1 was activated by addition of IPTG (1 mM) for 1 h to induce expression of Pspank-rapl.

Mating of the library into the streptomycin-resistant, ICE-cured recipient strain CAL89 was conducted using a standard mating procedure (see below). An aliquot of the donor culture was harvested for analysis immediately prior to mixing donors and recipients. At the conclusion of the mating, the cells on the filter were resuspended and plated on LB agar containing kanamycin and streptomycin to select for transconjugants and incubated overnight at 37°C. Transconjugants were then resuspended in 1x S750 + metals and pooled, and an aliquot of the resuspension was harvested for sequence analysis.

4.4 | Amplification and sequencing of pooled sgRNA alleles

We used Qiagen DNaseasy Blood and Tissue kits to extract DNA from the pre- and post-mating samples, following the manufacturer instructions for the use of the kit with gram-positive bacteria. We used KAPA HiFi MasterMix to amplify the genes encoding the sgRNA from the DNA samples, adhering to the manufacturer protocol. We used 300 ng of sample DNA as a template and ran the reactions for 20 PCR cycles. The primers used to amplify the sgRNA alleles were oMH238 (5′-AATGATACGGCGACCACTACGG AGATXXXXXX TCTACACGGGCGGGAATGGGCTCGTGTTGTACAATAAATGT-3′) and oMH239 (5′-CAAGCAGAAGACGGCATACGAGATXXXXXX GCCAGCGGATCTCTTCTGAGATGAGTTTTTGTTCG-3′). The 5′ ends of these oligos encode the Illumina adaptor sequences, and the X’s correspond to multiplexing barcodes. The resulting amplicons were purified with Select AMPure beads according to manufacturer instructions. DNA quality was checked with a bioanalyzer. Indexed samples were pooled and sequenced at the MIT BioMicro Center. The variable regions of the sgRNA ampiclons
were sequenced with an Illumina MiSeq, using a 20 bp read length and primer oMH240 (5’-GGGCGGGATAAGGCCTGTGTTGACATAAAATGTG-3’) to sequence the variable region of the sgRNA allele and oMH241 (5’-CGAACAAAAACTCTACTCAGAAAGGACTCGCGTCG-3’) to sequence the multiplexing barcode. FastQC (version 0.11.5) was used to assess quality of raw sequences. The 20 nt reads were matched to the respective variable regions from the sgRNA library using the R (version 3.4.4) package ShortRead, with allowance for up to 2 mismatches per read. Raw sequencing data are available at the Sequence Read Archive (accession numbers SAMN25690317 and SAMN25690318 for the donor and transconjugant read data, respectively).

4.5 | Mating assays

Mating assays were conducted as previously described (Auchtung et al., 2005; DeWitt & Grossman, 2014; Johnson & Grossman, 2014), with minor modifications. Experimental cultures of donor and recipient strains were started in LB at an OD600 of 0.02 and grown with shaking at 37°C. Kanamycin was added to cultures of strains containing a kan-marked ICEBs1 to ensure maintenance of the element. ICEBs1 activation was induced via overexpression of rapI, at an OD600 of 0.15 in donor cultures by adding either 1mM IPTG (to induce expression of Pspank-rapI or Pspank(hy)-rapI) or 1% xylose (to induce expression of Pxyll-rapI).

One hour after induction, 2.5 OD600 equivalents of donors and recipients were combined with an equal amount of recipients. The mixture of donors and recipients was collected on an nitrocellulose filter via vacuum filtration and washed with 5 ml 1× Spizizen’s salts (2 g/L (NH₄)₂SO₄, 14 g/L K₂HPO₄, 6 g/L KH₂PO₄, 1 g/L Na₃ citrate-2H₂O, and 0.2 g/L MgSO₄·7H₂O) (Harwood & Cutting, 1990). The filter was placed on a 1× Spizizen’s salts 1.5% agar plate (without a carbon source) and incubated at 37°C for 2 h. The cells were resuspended in 5 ml 1× Spizizen’s salts. Transconjugants were quantified by serially diluting the resuspension in 1× Spizizen’s salts, spreading the cells on an LB 1.5% agar plate containing kanamycin and streptomycin (for ICEBs1 matings), and incubating the plates overnight at 37°C. Donors were quantified immediately prior to combining donor and recipient cells: an aliquot of the donor culture was serially diluted and plated on an LB 1.5% agar plate containing kanamycin and grown overnight at 37°C. Mating efficiency was calculated as the number of transconjugants (kanamycin- and streptomycin-resistant post-mating CFUs/ml) per initial donor (pre-mating donor CFUs/ml). Data reported are normalized to the mean mating efficiency of a wild type control strain.

For mating assays done with individual strains with inducible CRISPRi-mediated knockdowns, Pxyll-dcas9 expression was stimulated at the time of culture inoculation by supplementing the experimental cultures with 0.01% or 0.1% xylose.

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

For mating assays with tunicamycin-treated strains, tunicamycin was added concurrently with ICEBs1 induction to a final concentration of 1 μg/ml.

For mating assays with Tn916, ICE activation was stimulated with 2.5 μg/ml tetracycline, and tetracycline was used in the selection for donors and transconjugants (instead of kanamycin).

For mating assays done on an osmoprotective mating surface, the 1× Spizizen’s salts 1.5% agar plate was substituted for a 1× MSM 1.5% agar plate, and the cells were resuspended and diluted post-mating in 1× MSM instead of 1× Spizizen’s salts. Pre- and post-mating CFUs were grown out by plating on non-osmoprotective LB 1.5% agar plates as described above. Sucrose was omitted from the 1× MSM solid and liquid media for the relevant experimental controls.

4.6 | pC194 mobilization assays

pC194 mobilization assays were carried out in essentially the same manner as ICEBs1 mating assays. Donor cultures were grown with chloramphenicol instead of kanamycin to maintain pC194 (containing cat). Donors and transconjugants were measured by plating on appropriate selective media.

4.7 | Live cell microscopy

Live cell microscopy was done largely as previously described (Babic et al., 2011) with minor modifications. Cultures of ICEBs1+ ΔyyddJ strains were grown in LB as described above. Strains either did or did not contain amyE::Pspank(hy)-rapI. All strains were treated with 1 mM IPTG for 1 h at an OD600 of 0.15. For WTA-depletion, tunicamycin (1 μg/ml) was added concurrently with IPTG. After 1 h, cells were pelleted in a tabletop centrifuge at 14,000 rpm, washed once in 1× S750 + metals, and resuspended in 50 μl 1× S750 + metals. One microliter of the resuspension was applied to an agar pad. The agar pad comprised 1.5% Noble Agar (Difco) dissolved in carbonless 1.5% 1× S750 + metals medium and contained 30 μM propidium iodide.

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

**AUTHOR CONTRIBUTIONS**

MMH and ADG conceived and designed the study. MMH and MEA acquired data. MMH, MEA, and ADG analyzed and interpreted data, and wrote and edited the manuscript.

**ETHICAL STATEMENT**

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

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

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