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The Composition of the Cell Envelope Affects Conjugation in *Bacillus subtilis*

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ABSTRACT

Conjugation in bacteria is the contact-dependent transfer of DNA from one cell to another via donor-encoded conjugation machinery. It is a major type of horizontal gene transfer between bacteria. Conjugation of the integrative and conjugative element ICE*Bs1* into *Bacillus subtilis* is affected by the composition of phospholipids in the cell membranes of the donor and recipient. We found that reduction (or elimination) of lysyl-phosphatidylglycerol caused by loss of *mprF* caused a decrease in conjugation efficiency. Conversely, alterations that caused an increase in lysyl-phosphatidylglycerol, including loss of *ugtP* or overproduction of *mprF*, caused an increase in conjugation efficiency. In addition, we found that mutations that alter production of other phospholipids, e.g., loss of *clsA* and *yfnI*, also affected conjugation, apparently without substantively altering levels of lysyl-phosphatidylglycerol, indicating that there are multiple pathways by which changes to the cell envelope affect conjugation. We found that the contribution of *mprF* to conjugation was affected by the chemical environment. Wild-type cells were generally more responsive to addition of anions that enhanced conjugation, whereas *mprF* mutant cells were more sensitive to combinations of anions that inhibited conjugation at pH 7. Our results indicate that *mprF* and lysyl-phosphatidylglycerol allow cells to maintain relatively consistent conjugation efficiencies under a variety of ionic conditions.

IMPORTANCE

Horizontal gene transfer is a driving force in microbial evolution, enabling cells that receive DNA to acquire new genes and phenotypes. Conjugation, the contact-dependent transfer of DNA from a donor to a recipient by a donor-encoded secretion machine, is a prevalent type of horizontal gene transfer. Although critically important, it is not well understood how the recipient influences the success of conjugation. We found that the composition of phospholipids in the membranes of donors and recipients influences the success of transfer of the integrative and conjugative element ICE*Bs1* in *Bacillus subtilis*. Specifically, the presence of lysyl-phosphatidylglycerol enables relatively constant conjugation efficiencies in a range of diverse chemical environments.

Conjugation is one of several processes bacteria use to acquire new genes. During conjugation, a donor bacterium transfers DNA directly to a recipient bacterium in a contact-dependent manner. The conjugation machinery is typically encoded by a mobile genetic element, which itself is frequently transferred during conjugation. Conjugation can also deliver genes that are not directly involved in the conjugation process but that are located on the mobile genetic element or on other DNA elements that are transferred. These genes are known to confer a wide variety of phenotypes to cells, and their transfer can allow recipients to rapidly acquire new characteristics. For example, conjugative elements are widely involved in the spread of antibiotic resistances (reviewed in references 1, 2, and 3).

ICE*Bs1* is an integrative and conjugative element (ICE) found in *Bacillus subtilis* (4, 5). ICEs are widespread and found in many bacterial species (6). Unlike conjugative plasmids, ICEs integrate into the host chromosome, where they are maintained during chromosomal replication, segregation, and cell division, much like a transposon or phage lysogen (reviewed in references 3 and 7). Under certain circumstances, ICEs can excise from the chromosome, forming a plasmid intermediate that can then be transferred to recipient cells by the element-encoded conjugation machinery.

ICE*Bs1* is found integrated in the *trn-leu2* gene in the *B. subtilis* chromosome and becomes activated in response to extracellular signaling, starvation, or DNA damage (4). The regulatory genes of ICE*Bs1* involved in cell-cell signaling (*rapI* and *phrI*) have been defined (4, 8). Overexpression of RapI leads to excision of ICE*Bs1* in >90% of cells in a growing population, allowing a high frequency of experimentally induced conjugation (4, 8, 9). ICE*Bs1* encodes a type IV secretion system that transfers DNA from the donor to a recipient. Type IV secretion systems are found in other ICEs and conjugative plasmids in both Gram-positive and Gram-negative bacteria (10).

During conjugation, DNA is transferred from the cytoplasm of the donor to that of the recipient, crossing the envelope of each to generate a transconjugant. The composition of the cell envelopes of both the donor and recipient influences the success of conjugation. For example, in Gram-negative bacteria, the outer membrane protein OmpR and the lipopolysaccharide are important for formation of mating pairs (11–16). In *Enterococcus faecalis*, lipoteichoic acids may be important for mating pair formation

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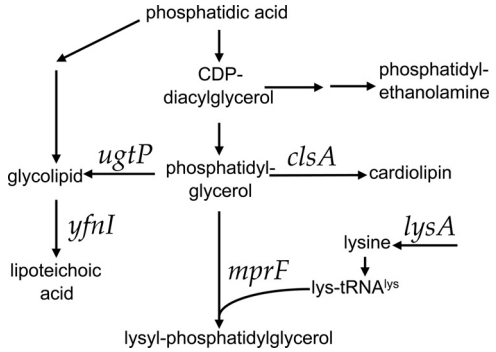

FIG 1 Pathways of phospholipid biosynthesis that affect conjugation of ICE*Bs1*. Some of the pathways involved in phospholipid biosynthesis are shown. Genes relevant to this work are indicated above the arrows.

(17–19). Recently, we found that in *B. subtilis*, the phospholipid head groups of the membrane bilayer make important contributions to conjugation (20).

The cell envelopes of *B. subtilis* and other Gram-positive bacteria contain a single lipid bilayer. The lipids of this membrane vary in the composition of their fatty acid tails and their head groups (reviewed in reference 21). The most abundant phospholipids in the membrane of *B. subtilis* are the negatively charged phosphatidylglycerol, zwitterionic phosphatidylethanolamine and neutral glycolipids, negatively charged cardiolipin, and positively charged lysyl-phosphatidylglycerol (reviewed in reference 22). The membrane of *B. subtilis* carries a net negative charge.

Although the conjugation machinery is encoded by the conjugative element, host genes, in both the donor and recipient, are also important for successful transfer of conjugative DNA. Previously, we used transposon insertions and deep sequencing (Tn-seq) to identify genes in recipients that affect the frequency of conjugation (20). We found that deletion of genes involved in the synthesis of various phospholipids has distinct effects on the ability of *B. subtilis* to act as a recipient in conjugation. Several of the mutations (in *ugtP*, *yfnI*, and *mprF*) that affect conjugation affect consumption of the phospholipid phosphatidylglycerol (Fig. 1).

Here, we analyzed these mutants to evaluate the effects of phospholipids on conjugation. We used double mutant analysis to determine epistasis between several of the phospholipid mutations. Our results indicate that lysyl-phosphatidylglycerol stimulates conjugation and that other phospholipids are also important for conjugation, independently of lysyl-phosphatidylglycerol. We also found that the phenotype caused by loss of *mprF* (needed for production of lysyl-phosphatidylglycerol) was enhanced by some environmental conditions and suppressed by others. Our results indicate that the ability of cells to function in conjugation is buffered against some chemical variations in the environment by lysyl-phosphatidylglycerol.

MATERIALS AND METHODS

Media and growth conditions. *Escherichia coli* cells were grown at 37°C in LB medium. *B. subtilis* cells were grown at 37°C in LB medium or $S7_{50}$ defined minimal medium with 0.1% glutamate and 40 μg/ml required amino acids (23). Arabinose (1%, wt/vol) was used as a carbon source. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was used to induce expression from the LacI-repressible promoter Pspank(hy) (24). Xylose (1%, wt/vol) was used to induce expression of Pxyl-*rapI*. Ampicillin was used at 100 μg/ml for *E. coli*. Antibiotics were used at the following concentrations for *B. subtilis*: spectinomycin at 100 μg/ml, kanamycin at 5 μg/ml, chloramphenicol at 5 μg/ml, and a combination of erythromycin at 0.5 μg/ml and lincomycin at 12.5 μg/ml to select for macrolide-lincosamide-streptogramin (MLS) resistance.

Strains and alleles. *B. subtilis* strains are listed in Table 1. Strains with *trp phe* alleles are derived from JH642 (*trpC2 pheA1*). *rapI* was overexpressed from the xylose-inducible fusion Pxyl-*rapI* (integrated in the chromosome at *amyE*) to activate ICE*Bs1* in donor cells. The Δ(*rapI-phrI*)*160*::*cat* allele was constructed with the same genomic boundaries as the Δ(*rapI-phrI*)*342*::*kan* allele (4). Upstream and downstream genomic DNA fragments and the chloramphenicol resistance gene *cat* were amplified by PCR and joined together by isothermal (Gibson) assembly (25). This product was used to transform naturally competent *B. subtilis* cells, a chloramphenicol-resistant isolate was selected, and the allelic exchange was verified by PCR.

The unmarked Δ*mprF459* allele was constructed by replacing *mprF* with *cat* flanked by *lox* sites to generate strain CMJ459 and then recombining out the *lox-cat* allele using Cre recombinase expressed from pDR244, as previously described (20, 26). The genomic boundaries of this allele are the same as for the Δ*mprF125*::*mls* and Δ*mprF162*::*spc* alleles.

For MprF overexpression studies, *mprF* was cloned into a plasmid that carried the IPTG-inducible promoter Pspank(hy) (24), *lacI*, and *mls* situated between genomic sequence from *lacA*. *mprF* was placed under the control of the promoter Pspank(hy). This plasmid was transformed into naturally competent *B. subtilis* cells and Pspank(hy)-*mprF*, *lacI*, and *mls* introduced by double crossover at *lacA*. Expression of *mprF* was induced by the addition of 1 mM IPTG.

Thin-layer chromatography. Lipids were extracted from cells using a modified Bligh-Dyer method (27). We grew cells in minimal medium to an optical density at 600nm (OD_{600}) of ~1, sampled 1 ml of culture, pelleted the cells, removed the supernatant, resuspended in 1 ml water, pelleted the cells, resuspended in 100 μl 1 M perchloric acid, and incubated for 30 min on ice. Lipids were extracted by adding 1 ml of 12:6:2 methanol-chloroform-water and 0.625 μg of a phosphatidylserine stan-

TABLE 1 *B. subtilis* strains used

Strain	Genotype (reference)
CAL89	*trp phe str-84 comK*::*spc* (4)
CMJ44	*trp phe* ICE*Bs1*^{0} Δ*yfnI44*::*spc* (20)
CMJ83	*trp phe* ICE*Bs1*^{0} *ugtP*::*mls amyE*::[*lacI spc*] (20)
CMJ86	*trp phe* ICE*Bs1*^{0} *clsA*::*cat amyE*::[*lacI spc*]
CMJ124	*trp phe* ICE*Bs1*^{0} Δ*mprF124*::*mls* (20)
CMJ127	*trp phe amyE*::[Pxyl-*rapI cat*] Δ(*rapI-phrI*)*342*::*kan* Δ*mprF124*::*mls* (20)
CMJ132	*trp phe* ICE*Bs1*^{0} Δ*yfnI44*::*spc* Δ*mprF124*::*mls*
CMJ161	*trp phe* ICE*Bs1*^{0} *amyE*::*spc* (20)
CMJ162	*trp phe* ICE*Bs1*^{0} Δ*mprF162*::*spc* (20)
CMJ222	*trp phe* ICE*Bs1*^{0} Δ*mprF162*::*spc lacA*::[Pspank(hy)-*mprF lacI mls*]
CMJ248	*trp phe* Δ*mprF162*::*spc lacA*::[Pspank(hy)-*mprF lacI mls*] *amyE*::[Pxyl-*rapI cat*] Δ(*rapI-phrI*)*342*::*kan*
CMJ332	*trp phe* ICE*Bs1*^{0} *clsA*::*cat* Δ*mprF162*::*spc*
CMJ333	*trp phe* ICE*Bs1*^{0} *ugtP*::*mls* Δ*mprF162*::*spc*
CMJ335	*trp phe* ICE*Bs1*^{0} Δ*lysA73*::*mls amyE*::[*lacI spc*] (20)
CMJ336	*trp phe* ICE*Bs1*^{0} Δ*lysA73*::*mls* Δ*mprF162*::*spc*
CMJ337	*trp phe* ICE*Bs1*^{0} Δ*mprF162*::*spc lacA*::[*lacI mls*]
CMJ348	*trp phe amyE*::[Pxyl-*rapI mls*] Δ(*rapI-phrI*)*160*::*cat*
CMJ459	*trp phe* ICE*Bs1*^{0} Δ*mprF459*::*lox-cat*
CMJ476	*trp phe amyE*::[Pxyl-*rapI mls*] Δ(*rapI-phrI*)*160*::*cat* Δ*mprF459* (unmarked)
HB5362	*clsA*::*cat* (27)
JMA222	*trp phe* ICE*Bs1*^{0} (4)
KM250	*trp phe amyE*::[Pxyl-*rapI cat*] Δ(*rapI-phrI*)*342*::*kan* (52)

dard (Sigma-Aldrich) to each sample and incubating at 4°C overnight on a rocking platform. Lipids were recovered by adding 300 μl H_2O and 300 μl chloroform, incubating the samples for 30 min at −20°C, and then centrifuging for 5 min at 720 × *g*. The organic (bottom) phase was recovered and dried under nitrogen, and the extracted lipids were resuspended in 12 μl of 2:1 chloroform-methanol.

The total volume of each sample was spotted on silica 60 plates (Angela) along with lysyl-phosphatidylglycerol (0.63 μg to 2.5 μg) (Avanti Polar Lipids) and phosphatidylserine (0.25 μg to 1 μg) standards and developed in a thin-layer chromatography chamber with 60:35:5 chloroform-methanol-water. The plates were dried, stained with ninhydrin (1.5 mg/ml ninhydrin in water-saturated butanol with 3% [vol/vol] acetic acid), and charred. The plates were scanned on a flat-bed scanner and analyzed with ImageJ (28). Standard curves were generated for lysyl-phosphatidylglycerol (0.16 μg to 5 μg) and phosphatidylserine (0.25 μg to 1 μg) to ensure that the amount of each phospholipid in the samples was within the linear range of the assay.

Mating assays. Mating assays were performed on filters as previously described (9, 20). Briefly, donor and recipient cells were grown separately in minimal medium with 1% arabinose as a carbon source. Donors were induced with 1% (wt/vol) xylose for 2 h to induce expression of Pxyl-*rapI*, thereby activating ICE*Bs1* gene expression. An equal number of donors and recipients was mixed, collected on a mating filter, and placed on a mating support consisting of 1.5% agar with a buffered salt solution (see below) for 90 min. Mating filters were typically placed on Spizizen's minimal salts (SMS) agar. SMS agar contains 15 mM ammonium sulfate, 80 mM dibasic potassium phosphate, 44 mM monobasic potassium phosphate, 3.4 mM trisodium citrate, 0.8 mM magnesium sulfate, and 1.5% agar at pH 7.0 (29) unless otherwise specified. TSS agar contains 37 mM ammonium chloride, 2 mM dibasic potassium phosphate, 50 mM Tris base, 1 mM magnesium sulfate, 0.004% iron(III) chloride, 0.004% trisodium citrate, and 1.5% agar at pH 7.5 (29) and was used as an alternate buffer in the mating support in some experiments. TSS was further amended in some experiments, as noted in Results. Cells were then rinsed off the filter, diluted, and spread on LB plates with selective antibiotics to determine the numbers of transconjugants, donors, and/or recipients.

RESULTS

Effects of genes involved in phospholipid biosynthesis on conjugation. Previously, we found that *mprF* and several other genes involved in the synthesis of phospholipids affect the efficiency of conjugation (20). MprF catalyzes addition of a lysyl group from Lys-$tRNA^{Lys}$ to phosphatidylglycerol to form lysyl-phosphatidylglycerol (Fig. 1) (27, 30, 31). Loss of *mprF* in donors and/or recipients causes a decrease in conjugation of ICE*Bs1* (Fig. 2) (20), indicating that elimination of lysyl-phosphatidylglycerol is detrimental for conjugation.

In contrast to the loss of *mprF*, we found that overexpression of *mprF* in recipients caused an increase in the acquisition of ICE*Bs1* via conjugation. We fused *mprF* to the LacI-repressible, IPTG-inducible promoter Pspank(hy) at an ectopic location (*amyE*) on the chromosome in a mutant missing the normal copy of *mprF*. We found that expression of Pspank(hy)-*mprF* in recipients caused an increase in mating efficiency (Fig. 2A). When *mprF* was similarly overexpressed in the donor (strain CMJ248) and mated to a wild-type recipient (CAL89), the mating efficiency was 7- to 8-fold greater than that of the wild-type donor (KM250) mated to the same recipient. Together with previous findings on the effects of loss of *mprF* on conjugation (20), our results indicate that both loss and overproduction of *mprF* affect conjugation efficiencies. Since the only known role of *mprF* in *B. subtilis* is in the production of lysyl-phosphatidylglycerol from phosphatidylglycerol and charged lysyl-tRNA, our results indicate that the amount of lysyl-phosphatidylglycerol, or of other compounds derived from phosphatidylglycerol, affects conjugation. If these effects are due to lysyl-phosphatidylglycerol, then this phospholipid appears to stimulate conjugation.

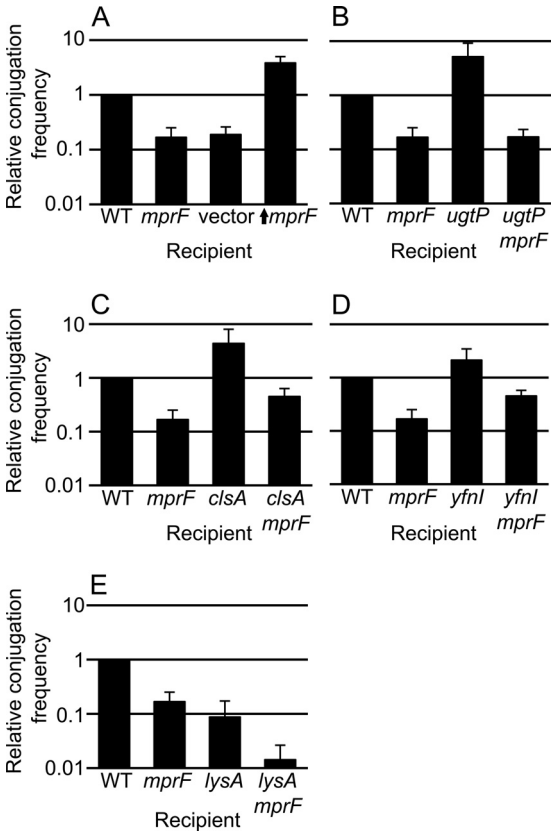

FIG 2 Effects of mutations in recipients on acquisition of ICE*Bs1*. The relative conjugation frequency (*y* axis) is shown for each of the indicated recipients (*x* axis). The same donor strain (KM250) was used for all experiments, and ICE*Bs1* was induced in the donor by overproduction of the activator RapI (see Materials and Methods). The relative conjugation frequency (*y* axis) is the number of transconjugants per donor crossed to the indicated recipient strain, normalized to that of the wild-type (WT) recipient (CMJ161) in each experiment. The wild-type conjugation efficiency was approximately 4% transconjugants per donor in these experiments. Conjugation frequencies measured with recipients that are null for *mprF*, *ugtP*, *yfnI*, and *lysA* are similar to those previously reported (20) and were included in these experiments to allow direct comparison with the appropriate double mutants. The graph shows means and standard deviation from ≥3 experiments. The conjugation efficiency for each single mutant is statistically different from that for the wild type ($P < 0.05$). Data for the wild type (CMJ161) and an *mprF* null mutant recipient (CMJ162) are included in all panels for comparison. (A) The vector (CMJ337) contains Pspank(hy) with no insert; ↑ *mprF* (CMJ222) indicates an *mprF* null mutant with Pspank(hy) driving expression of *mprF*. (B) *ugtP* (CMJ83) and *ugtP mprF* double mutant (CMJ333) ($P < 0.05$ versus *ugtP*). (C) *clsA* (CMJ86) and *clsA mprF* double mutant (CMJ332) ($P < 0.05$ versus *clsA* and *mprF*). (D) *yfnI* (CMJ44) and *yfnI mprF* double mutant (CMJ132) ($P < 0.05$ versus *yfnI* and *mprF*). (E) *lysA* (CMJ335) and *lysA mprF* double mutant (CMJ336) ($P < 0.05$ versus *lysA* and *mprF*). These strains were grown with 40 μg/ml lysine.

Other genes affecting phospholipid biosynthesis that were previously identified as having an effect on conjugation include *lysA*, *ugtP*, and *yfnI* (Fig. 1) (20). Similar to loss of *mprF*, loss of *lysA* in either the donor or the recipient inhibits conjugation (Fig. 2E) (20). *lysA* encodes diaminopimelate decarboxylase, which catalyzes synthesis of L-lysine from *meso*-diaminopimelate (32). *lysA* is

essential for synthesis of lysine, used in the production of lysyl-phosphatidylglycerol, so *lysA* mutations might affect conjugation by altering lysyl-phosphatidylglycerol production. In contrast, loss of *ugtP* or *yfnI* enhances the ability of cells to act as recipients in conjugation (Fig. 2) (20). *ugtP* is involved in synthesis of glycolipid, a component of the membrane that also acts as a precursor in the synthesis of lipoteichoic acids (Fig. 1) (33). *yfnI* is one of four genes with overlapping roles in lipoteichoic acid synthesis in *B. subtilis* (34). Like MprF, the products of *ugtP* and *yfnI* consume phosphatidylglycerol.

Based on the functions of the genes described above and their consumption of phosphatidylglycerol, we decided to test the effects of *clsA* on conjugation. The *clsA* gene product, cardiolipin synthetase, consumes phosphatidylglycerol during the synthesis of cardiolipin, another phospholipid of the membrane bilayer. *clsA* was not identified previously in our mutant hunt because the apparent effect on conjugation was below the cutoff used to identify candidate genes (20).

We found that loss of *clsA* in recipients caused an increase in the acquisition of ICE*Bs1* via conjugation (Fig. 2C). This increase was similar to that caused by a *ugtP* null mutation. Together, these results indicate that phosphatidylglycerol or derivatives of phosphatidylglycerol can stimulate and/or inhibit the efficiency of conjugation.

Double mutant analysis of phospholipid biosynthesis mutants. Deletion of individual genes encoding phospholipid synthetases that consume phosphatidylglycerol (Fig. 1) resulted in opposite affects on conjugation efficiency, depending on which gene was deleted. For example, deletion of *mprF* caused a decrease in conjugation, and deletion of *ugtP*, *yfnI*, or *clsA* caused an increase in conjugation. There are two simple models to explain these effects. (i) Lysyl-phosphatidylglycerol might enhance conjugation. In this model, loss of *mprF* (which is needed to make lysyl-phosphatidylglycerol) causes a decrease in conjugation because of loss of lysyl-phosphatidylglycerol. In addition, loss of *ugtP*, *yfnI*, and *clsA* might cause an increase in phosphatidylglycerol (substrate for MprF) and a subsequent increase in lysyl-phosphatidylglycerol, thereby causing an increase in conjugation. (ii) Alternatively (or in addition), cardiolipin, glycolipids, and lipoteichoic acids might act individually or together to inhibit conjugation. For example, phospholipids and teichoic acids can interfere with hydrolase activity (35–40) and might inhibit the cell wall hydrolase CwlT, which is encoded by and needed for transfer of ICE*Bs1*. Loss of *clsA* (cardiolipin) and *yfnI* (lipoteichoic acids), and perhaps *ugtP* (glycolipids), relieves this inhibition, causing an increase in conjugation. In this model, loss of *mprF* leads to an increase in phosphatidylglycerol and a possible increase in the inhibitory molecule(s) and thus a decrease in conjugation. To test these models, we generated strains in which multiple phospholipid synthetases were inactivated and tested them as recipients in conjugation experiments (Fig. 2). The results described below indicate that lysyl-phosphatidylglycerol enhances conjugation.

We found that the decrease in conjugation frequency caused by loss of *mprF* was epistatic to the increase in conjugation frequency due to loss of *ugtP* (Fig. 2B). We measured the conjugation efficiencies using standard mating assays between a wild-type donor (KM250) and recipients carrying the mutation(s) of interest. An *mprF ugtP* double mutant recipient had essentially the same phenotype as the *mprF* single mutant recipient (Fig. 2B). This result indicates that *mprF* is needed for the increase in conjugation caused by loss of *ugtP* and that the *ugtP* phenotype is likely due to an increase in the level of lysyl-phosphatidylglycerol.

We also made double mutants between *mprF* and *clsA* (CMJ332), *yfnI* (CMJ132), and *lysA* (CMJ336). We used the double mutants as recipients in conjugation experiments and directly compared the results to those for the single mutants. The efficiency of conjugation of ICE*Bs1* into the *mprF clsA* double mutant was about half (0.45) of that into wild-type recipients. This appeared to be partly (mostly) additive between the conjugation efficiencies of the single mutants: an approximately 6-fold reduction (0.17) and an approximately 4-fold increase (4.4) for *mprF* and *clsA*, respectively (expect: $0.17 \times 4.4 = 0.73$) (Fig. 2C). The conjugation efficiency of the *mprF yfnI* double mutant was also about half (0.45) that of wild-type recipients, indicative of additive effects of the 6-fold decrease (0.17) and 2-fold increase (2.1) in the *mprF* and *yfnI* single mutants (expected, $0.17 \times 2.1 = 0.36$) (Fig. 2D). The conjugation efficiency of the *mprF lysA* double mutant was decreased 70-fold (0.014) and appeared to be fully additive between the effects of each of the single mutants, i.e., 6-fold (0.17) and ~11-fold (0.087) decrease of the *mprF* and *lysA* mutants (expected, $0.17 \times 0.087 = 0.014$) (Fig. 2E). Although it is difficult to determine if the phenotypes of the double mutants are precisely additive, the data clearly indicate that *mprF* is epistatic to *ugtP* and not to *clsA*, *yfnI*, and *lysA*.

Together, the results of the double mutant analyses indicate that (i) loss of *ugtP* and *mprF* likely affects conjugation by affecting levels of lysyl-phosphatidylglycerol and (ii) loss of *clsA*, *yfnI*, and *lysA* probably does not affect levels of lysyl-phosphatidylglycerol, and their effects on conjugation are likely by altering other components of the cell membrane.

Analysis of lysyl-phosphatidylglycerol levels in mutant cells. To test the inferences from the genetic analyses described above, we measured the amount of lysyl-phosphatidylglycerol in each of the different phospholipid synthesis mutants (Fig. 3). We grew cells in defined minimal medium, extracted phospholipids, and used thin-layer chromatography to measure lysyl-phosphatidylglycerol (Fig. 3). As expected (27), there was no detectable lysyl-phosphatidylglycerol in the *mprF* mutant (Fig. 3). In contrast, overproduction of MprF caused an increase in the amount of lysyl-phosphatidylglycerol above that found in otherwise wild-type cells (Fig. 3). We found that the *ugtP* null mutation, and to a lesser extent the *clsA* null mutation, also caused an increase in the amount of lysyl-phosphatidylglycerol (Fig. 3). The simplest interpretation of these results is that the increase in lysyl-phosphatidylglycerol in the *ugtP* mutant, and perhaps the *clsA* mutant, likely causes the increase in conjugation efficiency. However, the double mutant analysis described above demonstrated that *mprF* was epistatic to *ugtP* and apparently additive with *clsA*. The smaller effect of *clsA* than of *ugtP* on the level of lysyl-phosphatidylglycerol and the double mutant phenotypes indicates that the conjugation phenotype of *ugtP*, but not that of *clsA*, was due to an increase in lysyl-phosphatidylglycerol.

In contrast to the mutations that affected levels of lysyl-phosphatidylglycerol, *yfnI* or *lysA* null mutations caused no detectable change in levels of lysyl-phosphatidylglycerol (Fig. 3). The results of the conjugation and thin-layer chromatography experiments are summarized in Table 2. Together with the analysis of double mutants (Fig. 2; Table 2), these results indicate that the conjugation phenotypes caused by mutations in *mprF* and *ugtP* are likely due to changes in levels of lysyl-phosphatidylglycerol and that the

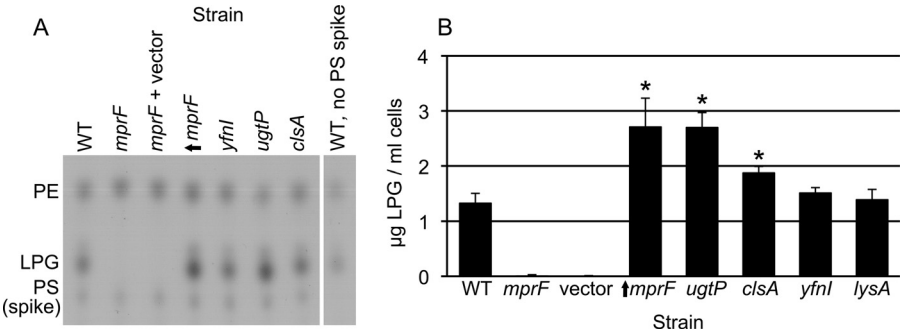

FIG 3 Effects of mutations on the level of lysyl-phosphatidylglycerol. The amount of lysyl-phosphatidylglycerol (LPG) recovered from a 1-ml culture of cells at an OD_{600} of 1 was determined for the indicated strains: CMJ161 (wild type [WT]), CMJ162 (*mprF*), CMJ337 (*mprF* plus vector), CMJ222 {↑ *mprF* [*mprF* null with Pspank(hy) driving expression of *mprF*]}, CMJ44 (*yfnI*), CMJ83 (*ugtP*), CMJ86 (*clsA*), and CMJ335 (*lysA*) grown with 40 μg/ml lysine (in panel B only). (A) LPG was extracted from cell membranes and examined using thin-layer chromatography (see Materials and Methods). LPG and phosphatidylethanolamine (PE) standards were used to identify the LPG and PE bands. Phosphatidylserine (PS) was added to samples as an internal standard. The locations of the LPG, PE, and PS bands are indicated. The last part of the panel shows the wild-type sample with no added PS. (B) The LPG content of each strain was quantified from ≥3 experiments. Asterisks indicate a significant difference in the amount of LPG recovered compared to that from the wild-type strain ($P < 0.05$, *t* test).

conjugation phenotypes caused by mutations in *clsA*, *yfnI*, and *lysA* are most likely not due to changes in levels of lysyl-phosphatidylglycerol.

The *lysA* mutant requires addition of exogenous lysine to the medium in order to grow. This lysine is evidently enough to support wild-type levels of lysyl-phosphatidylglycerol production but not wild-type levels of conjugation. We suspect that the effects of loss of *lysA* on conjugation are complex. Synthesis and enzymatic activity of aspartokinase II (the *lysC* gene product) are regulated by lysine. Aspartokinase II synthesizes L-aspartate 4-phosphate, which is a precursor for synthesis of cell wall peptidoglycan as well as the amino acids lysine, methionine, isoleucine, and threonine (reviewed in reference 41). Deleting *lysA* and providing exogenous lysine might perturb this regulatory feedback pathway and alter several cellular processes, including peptidoglycan synthesis.

The mating defect of *mprF* mutants is affected by the chemical environment. During the course of our investigations, we noticed that the composition of the agar surface on which the filter paper for mating was placed (the mating support) influenced the magnitude of the conjugation phenotype caused by loss of *mprF*. Specifically, loss of *mprF* from both donors (CMJ476) and recipients (CMJ162) caused a pronounced conjugation defect (0.031, ~30-fold) compared to a cross between wild-type donors (CMJ348) and recipients (CMJ161), similar to previously reported results (20). This drop in conjugation was observed when matings were performed on SMS agar.

In contrast to the ~30-fold decrease in conjugation between *mprF* mutants on SMS agar, there was a much smaller effect when matings were done on agar containing Spizizen's salts and Tris (TSS agar) (Fig. 4). TSS agar, compared to SMS agar, is buffered with Tris instead of potassium phosphate to pH 7.5 instead of 7.0 and contains a lower total concentration of salts (29). Under these conditions, the conjugation frequency of *mprF* mutant cells was reduced by approximately 3-fold (0.37) compared to that of wild-type donors and recipients. We ruled out the possibility that production of lysyl-phosphatidylglycerol was restored in the *mprF* mutant on the TSS agar support; there was no detectable lysyl-phosphatidylglycerol under these conditions in the mutant. These findings indicate that there is something about TSS that suppresses or something about SMS that exacerbates the conjugation defect of the *mprF* mutant.

We investigated what aspect of the different mating supports accounted for the magnitude of the *mprF* mutant phenotype. Since mating in the *mprF* mutants was much lower in SMS than TSS, we postulated that the lower pH and/or some of the additional ions in SMS were inhibiting conjugation of *mprF* mutants.

TABLE 2 Summary of mutations affecting conjugation and phospholipid synthesis

Mutation[a]	Phospholipid biosynthesis[b]	Mating[c]	LPG[d]	Phenotype with *mprF*[e]
mprF	Lysyl-phosphatidylglycerol	Decreased	None	
↑ *mprF*	Lysyl-phosphatidylglycerol	Increased	Increased	
ugtP	Glycolipid	Increased	Increased	Epistatic
clsA	Cardiolipin	Increased	(Increased)	Additive
yfnI	Lipoteichoic acid	Increased	WT	Additive
lysA	Lysyl-phosphatidylglycerol	Decreased	WT	Additive

[a] All are null mutations except ↑ *mprF*, which indicates overexpression of *mprF*.
[b] Phospholipid whose synthesis depends on the indicated gene (Fig. 1).
[c] Effect of the mutation on conjugation.
[d] Amount of lysyl-phosphatidylglycerol (LPG) produced in cells with the indicated mutation relative to the amount produced cells with the indicated mutation relative to the amount produced in wild-type (WT) cells. "None" indicates that there was no detectable LPG. Parentheses indicates a possible effect but on the edge of statistical significance (Fig. 3).
[e] Phenotype of the double mutant (with *mprF*) with respect to conjugation. Epistatic indicates that the phenotype of the double mutant is the same as that of the *mprF* single mutant (Fig. 2).

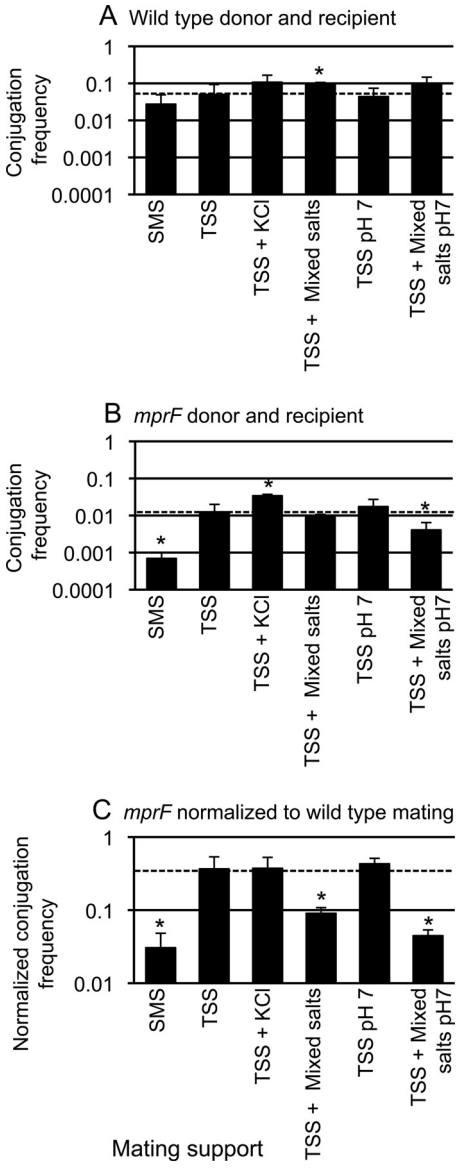

FIG 4 The chemical composition of the mating support affects conjugation. Standard filter matings were performed on supports with different chemical compositions. Donor and recipient cells were mixed in equal numbers and then collected on a filter that was placed on a mating support with the indicated composition. KCl was added to 125 mM. Mixed salts contained 106 mM sodium phosphate, 14 mM sodium sulfate, and 3 mM trisodium citrate. The dashed horizontal line in each panel marks the value for mating on TSS. The mean and standard deviation from ≥3 experiments for each condition are shown. Asterisks indicate that the difference in conjugation frequency on the given support compared to conjugation frequency on TSS is statistically significant ($P < 0.05$, *t* test). (A and B) The conjugation frequency is shown as transconjugants per donor for a wild-type donor (CMJ348) and recipient (CMJ161) (A) and for an *mprF* null mutant donor (CMJ476) and recipient (CMJ162) (B). (C) The conjugation frequencies obtained from panels A and B are directly compared. The ratio of the conjugation frequencies of the *mprF* mutant (B) and the wild-type strain (A) under each of the indicated conditions is shown.

There are several differences between TSS and SMS. Notably, SMS contains a higher total concentration of different salts than TSS and a lower pH (7 versus 7.5). SMS has higher concentrations of potassium (204 mM versus 4 mM), phosphate (124 mM versus 2 mM), sulfate (16 mM versus 1 mM), and citrate (3 mM versus 0.1 mM).

We measured mating efficiencies on TSS agar as the base support with additions to make it more closely resemble SMS. Addition of potassium chloride (125 mM) or mixed salts (106 mM sodium phosphate, 14 mM sodium sulfate, and 3 mM trisodium citrate) increased the conjugation frequency in matings between wild-type cells (Fig. 4A). Adjustment of the pH to 7.0 (without other changes) had little or no detectable effect and had no additional effect in the presence of mixed salts (Fig. 4A).

As with wild-type cells, the addition of potassium chloride also increased the conjugation frequency in matings between *mprF* mutant cells (Fig. 4B), and adjustment of the pH to 7.0 had little or no effect (Fig. 4B). However, unlike the effect on wild-type cells, addition of mixed salts did not cause an increase in the conjugation efficiency in matings between *mprF* mutants at either pH (Fig. 4B).

Direct comparison of the conjugation frequencies for wild-type cells (Fig. 4A) and *mprF* cells (Fig. 4B) showed that *mprF* caused a more severe phenotype when matings were performed on TSS with mixed salts (at pH 7.5 and pH 7) than when they were performed on TSS (Fig. 4C). Based on these results, we conclude that the salts found in SMS contributed to the defect in mating caused by loss of *mprF*, particularly at pH 7.

Ion-specific effects on conjugation and effects of *mprF*. Based on the above results, we wondered if other salts might affect wild-type and *mprF* mutant strains differently. To test this, we used TSS agar as our base medium and supplemented it with a 125 mM concentration of different salts, including sodium fluoride, trisodium citrate, magnesium chloride, sodium sulfate, dibasic sodium phosphate titrated with monobasic sodium phosphate to give a pH of 7.5 and a phosphate concentration of 125 mM, sodium iodide, sodium nitrate, or sodium chloride. We measured the mating efficiencies of wild-type cells (Fig. 5A) and *mprF* mutants (Fig. 5B) and then directly compared *mprF* to wild-type cells (Fig. 5C).

We found that addition of sodium fluoride or sodium citrate to TSS caused a decrease in the mating efficiency of wild-type cells (Fig. 5A). There was also a decrease in the mating efficiency of *mprF* mutants (Fig. 5B). With sodium fluoride, this decrease was somewhat less for the *mprF* mutants than for wild-type cells (Fig. 5C). With sodium citrate, the decrease was about the same for *mprF* and wild-type cells (Fig. 5C).

In contrast, we found that addition of magnesium chloride to TSS caused an increase of 16-fold in the mating efficiency of wild-type cells (Fig. 5A). Likewise, there was a similar or somewhat greater increase in the mating efficiency of *mprF* mutants (33-fold) (Fig. 5B and C). These results indicate that the use of TSS supplemented with magnesium chloride as a solid support for filter matings allows for highly efficient conjugation. We do not know how magnesium chloride stimulates the conjugation efficiency. It might act by affecting the activity of a cell surface component involved in conjugation or stabilizing mating pairs.

Addition of several other salts, including sodium sulfate, sodium phosphate, sodium iodide, sodium nitrate, and sodium chloride, to TSS either stimulated or had relatively little effect on the mating efficiency of wild-type cells (Fig. 5A). The stimulatory effects were less than that of magnesium chloride. The same salts had little or no effect or caused a small increase in the mating efficiency of *mprF* mutants (Fig. 5B). The stimulatory effects on

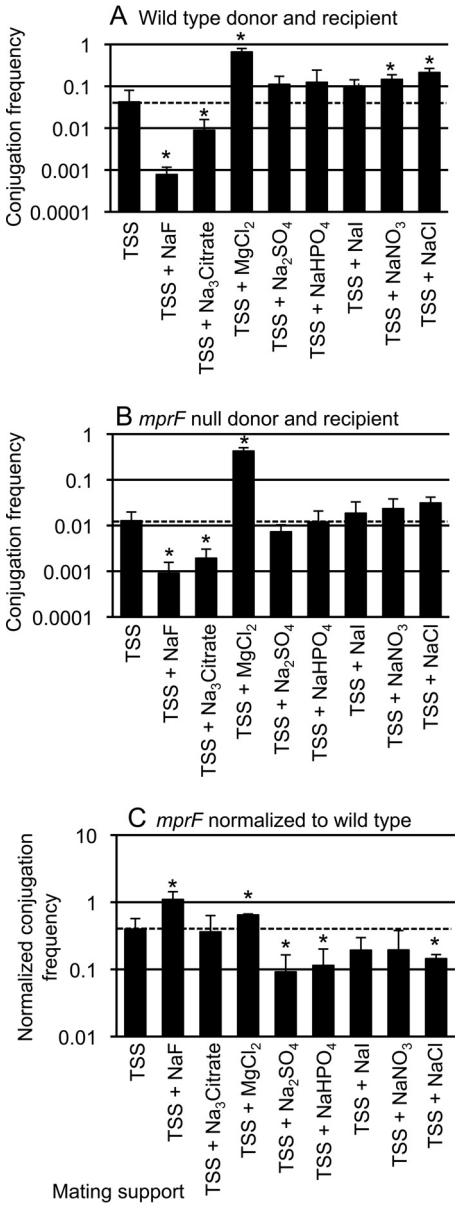

FIG 5 Some salts enhance conjugation of wild-type but not *mprF* cells. Filter matings were performed as described in Materials and Methods. Equal numbers of donor and recipient cells were mixed, collected on a filter, and placed on a mating support with the indicated composition. Chemical supplements were added at 125 mM. The samples tested with TSS plus $NaHPO_4$ also contain dibasic sodium phosphate titrated with monobasic sodium phosphate to give a pH of 7.5. The dashed horizontal line in each panel indicates the conjugation frequency on TSS. The mean and standard deviation from ≥3 experiments are shown for each condition. Asterisks indicate that the difference in conjugation frequency on the given support compared to conjugation frequency on TSS is statistically significant ($P < 0.05$, *t* test). (A and B) The conjugation frequency (transconjugants per donor) is shown for wild-type donor (CMJ348) and wild-type recipient (CMJ161) (A) and for an *mprF* null mutant donor (CMJ476) and an *mprF* null mutant recipient (CMJ162) (B). (C) The conjugation frequencies obtained from panels A and B are directly compared and plotted as the ratio of the conjugation frequencies of the *mprF* mutant (B) and the wild-type strains (A) under each of the indicated conditions.

wild-type cells were larger than the effects on *mprF* mutants, and this is most easily seen in the ratio of the mating efficiencies of *mprF* and wild-type cells (Fig. 5C). These ratios are <0.37, the ratio of efficiencies when mating is done on TSS without any modifications.

Together, our results (Fig. 4 and 5) indicate that mating efficiencies are affected by the external ionic environment and that several salts that enhance conjugation of wild-type cells do not have the same stimulatory effect on *mprF* mutants. Since *mprF* mutants do not produce lysyl-phosphatidylglycerol, we infer that the different effects of salts are due to the presence or absence of this phospholipid. The presence of *mprF* and hence lysyl-phosphatidylglycerol enables cells to have efficient conjugation under a variety of different ionic conditions.

DISCUSSION

Our findings indicate that lysyl-phosphatidylglycerol plays a role in stimulating conjugation. Preventing or reducing lysyl-phosphatidylglycerol synthesis in either the donor or the recipient reduces conjugation. Overproduction of lysyl-phosphatidylglycerol in either partner enhances conjugation. Accumulation of lysyl-phosphatidylglycerol was eliminated in *mprF* null mutants and increased in *ugtP* mutants or upon overexpression of *mprF*. Our results also indicate that alterations in phospholipid content that do not detectably affect lysyl-phosphatidylglycerol also alter conjugation efficiencies.

***mprF* and *ugtP*.** We found that *mprF* is epistatic to *ugtP* for the conjugation phenotype. That is, the *mprF ugtP* double mutant had the same phenotype as the *mprF* single mutant. This is consistent with the interpretation that the conjugation phenotypes of *ugtP* and *mprF* mutants are due to alterations in lysyl-phosphatidylglycerol and that loss of *ugtP* causes an increase in phosphatidylglycerol, which then leads to an increase in lysyl-phosphatidylglycerol (Fig. 1). *mprF* is epistatic because it is needed to make lysyl-phosphatidylglycerol.

Loss of *ugtP* caused an increase in the amount of lysyl-phosphatidylglycerol, indicating that UgtP normally plays a role limiting the amount of lysyl-phosphatidylglycerol in the cell. *ugtP* is also known to affect cell division (42), primarily by directly interacting with and inhibiting the cell division protein FtsZ (42, 43). The effects of *ugtP* on cell division and conjugation are most likely not related. We infer this mainly because the effects on cell division appear to be direct and the effects on conjugation are likely through *mprF*.

ugtP mutants also appear to have many alterations in gene expression in rich medium (27). The effects of *mprF* mutations on gene expression are not known, but based on analyses of an *mprF pssA ywnE* (*clsA*) triple mutant, there are fewer effects than in a *ugtP* single mutant (27). It is possible that the effects of *mprF* and *ugtP* on conjugation are due to alterations in gene expression. However, the simplest model is that these genes affect conjugation due to alterations in lysyl-phosphatidylglycerol and that the composition of the cell envelope directly affects activity of the conjugation machinery (see below).

***mprF* and lysyl-phosphatidylglycerol enable efficient conjugation under various ionic conditions.** Our results demonstrate that the effects of lysyl-phosphatidylglycerol on conjugation are dependent on the environmental conditions. That is, the ratio of mating efficiencies of *mprF* mutants and wild-type cells was affected by the ionic conditions used for mating. For example, the

mprF mutants had a much more severe mating defect on SMS agar (~30-fold) than of TSS agar (~3-fold). Together with analysis of the differences between SMS and TSS, our results indicate that *mprF* and lysyl-phosphatidylglycerol normally facilitate efficient mating under a variety of external ionic conditions. We suggest that the presence of lysyl-phosphatidylglycerol buffers conjugation against the some of the otherwise inhibitory effects of different salts and enhances conjugation in the presence of others, allowing the conjugation machinery to function reasonably well under a range of different ionic conditions.

mprF homologs, and by extension lysyl-phosphatidylglycerol, affect cell surface properties of other organisms. For example, *mprF* in *Staphylococcus aureus* acts as a virulence factor and potentiates resistance to several cationic antimicrobials, including those produced by potential human hosts (reviewed in reference 22). *mprF* homologs impact the ability of *Enterococcus faecium* and *Listeria monocytogenes* to adapt to different environmental conditions (44, 45). We suggest that in Gram-positive bacteria, *mprF* and lysyl-phosphatidylglycerol ensure that the cell envelope is buffered from some of the variations in the chemistry of the environment and enable the cell to perform physiological functions in a regular manner under different environmental conditions.

A model for how membrane phospholipids affect conjugation efficiencies. We suspect that alterations in the phospholipid content of the recipient (and donor) might affect the function of the conjugation machinery. This could be through changes to the physical properties of the membrane (e.g., fluidity) that might affect assembly of the machinery. This could also be through inhibition of a component of the machinery. Transfer of DNA through the ICE*Bs1*-encoded conjugation machinery depends on CwlT (46), a secreted cell wall hydrolase encoded by ICE*Bs1* (46, 47). Components of the cell envelope, including lipoteichoic acids (35, 36), wall teichoic acids (37, 38), and the phospholipids cardiolipin (36), phosphatidylglycerol (39), and lysyl-phosphatidylglycerol (36, 40), can modulate the function of at least some cell wall hydrolases. Cell wall teichoic acids inhibit hydrolase activity, at least in part, by preventing hydrolase binding to the peptidoglycan of the cell wall (37, 38). Phospholipids can stimulate or inhibit the function of particular hydrolases; for example, phosphatidylglycerol can either enhance or inhibit the *N*-acetylmuramoyl-L-alanine amidase of *E. coli*, depending on concentration, but has no effect on the major autolysin of *Clostridium acetobutylicum* under the conditions tested (39, 40). Altering the phospholipid content of the donor and/or recipient may affect a postulated interaction between the cell wall hydrolase CwlT and the cell envelope, either enhancing or inhibiting the ability of the conjugation machinery to deliver DNA. This interaction could be binding of the conjugation machinery to the recipient cell envelope and/or digestion of the donor and recipient cell wall. If this model is correct, it strongly predicts that the cell wall hydrolase acts on both donor and recipient cells.

Cell wall hydrolases are encoded by many conjugative elements (10, 48–51). Where tested, they have been found to be critical for efficient conjugation. Based on this conservation, it seems likely that the composition of the cell wall affects the efficiencies of many different conjugative elements. Perhaps the cell wall hydrolases have evolved in ways that help determine the host range of the cognate element.

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REFERENCES

1. **Garriss G, Waldor MK, Burrus V.** 2009. Mobile antibiotic resistance encoding elements promote their own diversity. PLoS Genet **5:**e1000775. http://dx.doi.org/10.1371/journal.pgen.1000775.
2. **Toleman MA, Walsh TR.** 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiol Rev **35:**912–935. http://dx.doi.org/10.1111/j.1574-6976.2011.00294.x.
3. **Johnson CM, Grossman AD.** 2015. Integrative and conjugative elements (ICEs): what they do and how they work. Annu Rev Genet **49:**577–601. http://dx.doi.org/10.1146/annurev-genet-112414-055018.
4. **Auchtung JM, Lee CA, 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 U S A **102:**12554–12559. http://dx.doi.org/10.1073/pnas.0505835102.
5. **Burrus V, Pavlovic G, Decaris B, Guedon G.** 2002. The ICE*St1* 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. http://dx.doi.org/10.1016/S0147-619X(02)00102-6.
6. **Guglielmini J, Quintais L, Garcillan-Barcia MP, de la Cruz F, Rocha EP.** 2011. The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. PLoS Genet **7:**e1002222. http://dx.doi.org/10.1371/journal.pgen.1002222.
7. **Wozniak RA, Waldor MK.** 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol **8:**552–563. http://dx.doi.org/10.1038/nrmicro2382.
8. **Auchtung JM, Lee CA, Garrison KL, Grossman AD.** 2007. Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICE*Bs1* of *Bacillus subtilis*. Mol Microbiol **64:**1515–1528. http://dx.doi.org/10.1111/j.1365-2958.2007.05748.x.
9. **Lee CA, Auchtung JM, Monson RE, Grossman AD.** 2007. Identification and characterization of *int* (integrase), *xis* (excisionase) and chromosomal attachment sites of the integrative and conjugative element ICE*Bs1* of *Bacillus subtilis*. Mol Microbiol **66:**1356–1369.
10. **Alvarez-Martinez CE, Christie PJ.** 2009. Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev **73:**775–808. http://dx.doi.org/10.1128/MMBR.00023-09.
11. **Perez-Mendoza D, de la Cruz F.** 2009. *Escherichia coli* genes affecting recipient ability in plasmid conjugation: are there any? BMC Genomics **10:**71. http://dx.doi.org/10.1186/1471-2164-10-71.
12. **Watanabe T, Arai T, Hattori T.** 1970. Effects of cell wall polysaccharide on the mating ability of *Salmonella typhimurium*. Nature **225:**70–71. http://dx.doi.org/10.1038/225070a0.
13. **Skurray RA, Hancock RE, Reeves P.** 1974. Con− mutants: class of mutants in *Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J Bacteriol **119:**726–735.
14. **Havekes L, Tommassen J, Hoekstra W, Lugtenberg B.** 1977. Isolation and characterization of *Escherichia coli* K-12 F− mutants defective in conjugation with an I-type donor. J Bacteriol **129:**1–8.
15. **Sanderson KE, Janzer J, Head J.** 1981. Influence of lipopolysaccharide and protein in the cell envelope on recipient capacity in conjugation of *Salmonella typhimurium*. J Bacteriol **148:**283–293.
16. **Ishiwa A, Komano T.** 2004. PilV adhesins of plasmid R64 thin pili specifically bind to the lipopolysaccharides of recipient cells. J Mol Biol **343:**615–625. http://dx.doi.org/10.1016/j.jmb.2004.08.059.

17. **Trotter KM, Dunny GM.** 1990. Mutants of *Enterococcus faecalis* deficient as recipients in mating with donors carrying pheromone-inducible plasmids. Plasmid **24:**57–67. http://dx.doi.org/10.1016/0147-619X(90)90025-8.
18. **Bensing BA, Dunny GM.** 1993. Cloning and molecular analysis of genes affecting expression of binding substance, the recipient-encoded receptor(s) mediating mating aggregate formation in *Enterococcus faecalis*. J Bacteriol **175:**7421–7429.
19. **Ehrenfeld EE, Kessler RE, Clewell DB.** 1986. Identification of pheromone-induced surface proteins in *Streptococcus faecalis* and evidence of a role for lipoteichoic acid in formation of mating aggregates. J Bacteriol **168:**6–12.
20. **Johnson CM, Grossman AD.** 2014. Identification of host genes that affect acquisition of an integrative and conjugative element in *Bacillus subtilis*. Mol Microbiol **93:**1284–1301. http://dx.doi.org/10.1111/mmi.12736.
21. **Parsons JB, Rock CO.** 2013. Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res **52:**249–276. http://dx.doi.org/10.1016/j.plipres.2013.02.002.
22. **Ernst CM, Peschel A.** 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Mol Microbiol **80:**290–299. http://dx.doi.org/10.1111/j.1365-2958.2011.07576.x.
23. **Jaacks KJ, Healy J, Losick R, Grossman AD.** 1989. Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. J Bacteriol **171:**4121–4129.
24. **Britton RA, Eichenberger P, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, Grossman AD.** 2002. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. J Bacteriol **184:**4881–4890. http://dx.doi.org/10.1128/JB.184.17.4881-4890.2002.
25. **Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, III, Smith HO.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods **6:**343–345. http://dx.doi.org/10.1038/nmeth.1318.
26. **Meisner J, Montero Llopis P, Sham LT, Garner E, Bernhardt TG, Rudner DZ.** 2013. FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in *Bacillus subtilis*. Mol Microbiol **89:**1069–1083. http://dx.doi.org/10.1111/mmi.12330.
27. **Salzberg LI, Helmann JD.** 2008. Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition. J Bacteriol **190:**7797–7807. http://dx.doi.org/10.1128/JB.00720-08.
28. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods **9:**671–675. http://dx.doi.org/10.1038/nmeth.2089.
29. **Harwood CR, Cutting SM.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, United Kingdom.
30. **Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KP, van Strijp JA.** 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J Exp Med **193:**1067–1076. http://dx.doi.org/10.1084/jem.193.9.1067.
31. **Oku Y, Kurokawa K, Ichihashi N, Sekimizu K.** 2004. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology **150:**45–51. http://dx.doi.org/10.1099/mic.0.26706-0.
32. **Yamamoto J, Shimizu M, Yamane K.** 1991. Molecular cloning and analysis of nucleotide sequence of the *Bacillus subtilis lysA* gene region using *B. subtilis* phage vectors and a multi-copy plasmid, pUB110. Agric Biol Chem **55:**1615–1626. http://dx.doi.org/10.1271/bbb1961.55.1615.
33. **Jorasch P, Wolter FP, Zahringer U, Heinz E.** 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. Mol Microbiol **29:**419–430. http://dx.doi.org/10.1046/j.1365-2958.1998.00930.x.
34. **Wormann ME, Corrigan RM, Simpson PJ, Matthews SJ, Grundling A.** 2011. Enzymatic activities and functional interdependencies of *Bacillus subtilis* lipoteichoic acid synthesis enzymes. Mol Microbiol **79:**566–583. http://dx.doi.org/10.1111/j.1365-2958.2010.07472.x.
35. **Holtje JV, Tomasz A.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in *Pneumococcus*. Proc Natl Acad Sci U S A **72:**1690–1694. http://dx.doi.org/10.1073/pnas.72.5.1690.
36. **Cleveland RF, Wicken AJ, Daneo-Moore L, Shockman GD.** 1976. Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J Bacteriol **126:**192–197.
37. **Yamamoto H, Miyake Y, Hisaoka M, Kurosawa S, Sekiguchi J.** 2008. The major and minor wall teichoic acids prevent the sidewall localization of vegetative DL-endopeptidase LytF in *Bacillus subtilis*. Mol Microbiol **70:**297–310. http://dx.doi.org/10.1111/j.1365-2958.2008.06397.x.
38. **Schlag M, Biswas R, Krismer B, Kohler T, Zoll S, Yu W, Schwarz H, Peschel A, Gotz F.** 2010. Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. Mol Microbiol **75:**864–873. http://dx.doi.org/10.1111/j.1365-2958.2009.07007.x.
39. **Vanderwinkel E, De Vlieghere M.** 1985. Modulation of *Escherichia coli* N-acetylmuramoyl-L-alanine amidase activity by phosphatidylglycerol. Biochim Biophys Acta **838:**54–59. http://dx.doi.org/10.1016/0304-4165(85)90249-1.
40. **Croux C, Canard B, Goma G, Soucaille P.** 1992. Autolysis of *Clostridium acetobutylicum* ATCC 824. J Gen Microbiol **138:**861–869. http://dx.doi.org/10.1099/00221287-138-5-861.
41. **Hutton CA, Perugini MA, Gerrard JA.** 2007. Inhibition of lysine biosynthesis: an evolving antibiotic strategy. Mol Biosyst **3:**458–465. http://dx.doi.org/10.1039/b705624a.
42. **Weart RB, Lee AH, Chien A-C, Haeusser DP, Hill NS, Levin PA.** 2007. A metabolic sensor governing cell size in bacteria. Cell **130:**335–347. http://dx.doi.org/10.1016/j.cell.2007.05.043.
43. **Chien AC, Zareh SK, Wang YM, Levin PA.** 2012. Changes in the oligomerization potential of the division inhibitor UgtP co-ordinate *Bacillus subtilis* cell size with nutrient availability. Mol Microbiol **86:**594–610. http://dx.doi.org/10.1111/mmi.12007.
44. **Smith AM, Harrison JS, Sprague KM, Roy H.** 2013. A conserved hydrolase responsible for the cleavage of aminoacylphosphatidylglycerol in the membrane of *Enterococcus faecium*. J Biol Chem **288:**22768–22776. http://dx.doi.org/10.1074/jbc.M113.484402.
45. **Dare K, Shepherd J, Roy H, Seveau S, Ibba M.** 2014. LysPGS formation in *Listeria monocytogenes* has broad roles in maintaining membrane integrity beyond antimicrobial peptide resistance. Virulence **5:**534–546. http://dx.doi.org/10.4161/viru.28359.
46. **DeWitt T, Grossman AD.** 2014. The bifunctional cell wall hydrolase CwlT is needed for conjugation of the integrative and conjugative element ICE*Bs1* in *Bacillus subtilis* and *B. anthracis*. J Bacteriol **196:**1588–1596. http://dx.doi.org/10.1128/JB.00012-14.
47. **Fukushima T, Kitajima T, Yamaguchi H, Ouyang Q, Furuhata K, Yamamoto H, Shida T, Sekiguchi J.** 2008. Identification and characterization of novel cell wall hydrolase CwlT: a two-domain autolysin exhibiting n-acetylmuramidase and DL-endopeptidase activities. J Biol Chem **283:**11117–11125. http://dx.doi.org/10.1074/jbc.M706626200.
48. **Koraimann G.** 2003. Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. Cell Mol Life Sci **60:**2371–2388. http://dx.doi.org/10.1007/s00018-003-3056-1.
49. **Scheurwater EM, Burrows LL.** 2011. Maintaining network security: how macromolecular structures cross the peptidoglycan layer. FEMS Microbiol Lett **318:**1–9. http://dx.doi.org/10.1111/j.1574-6968.2011.02228.x.
50. **Zahrl D, Wagner M, Bischof K, Bayer M, Zavecz B, Beranek A, Ruckenstuhl C, Zarfel GE, Koraimann G.** 2005. Peptidoglycan degradation by specialized lytic transglycosylases associated with type III and type IV secretion systems. Microbiology **151:**3455–3467. http://dx.doi.org/10.1099/mic.0.28141-0.
51. **Abajy MY, Kopec J, Schiwon K, Burzynski M, Doring M, Bohn C, Grohmann E.** 2007. A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria. J Bacteriol **189:**2487–2496. http://dx.doi.org/10.1128/JB.01491-06.
52. **Menard KL, Grossman AD.** 2013. Selective pressures to maintain attachment site specificity of integrative and conjugative elements. PLoS Genet **9:**e1003623. http://dx.doi.org/10.1371/journal.pgen.1003623.