The composition of the cell envelope affects conjugation in *Bacillus subtilis*

Christopher M. Johnson and Alan D. Grossman*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

running head: Effects of membrane phospholipids on conjugation

*Corresponding author:

Department of Biology

Building 68-530

Massachusetts Institute of Technology

Cambridge, MA 02139

phone: 617-253-1515

E-mail: adg@mit.edu

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 membrane 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. The presence of certain salts altered conjugation, and wild type cells were generally more responsive to conditions that enhanced conjugation whereas *mprF* mutant cells were more sensitive to some conditions that inhibited conjugation. Our results indicate that *mprF* and lysyl-phosphatidylglycerol allow cells to maintain relatively consistent conjugation efficiencies in 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 membrane 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.

53 **Introduction**

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

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

71 ICE*Bs1* is found integrated in the *trn-leu2* gene in the *B. subtilis* chromosome and
72 becomes activated in response to extracellular signaling, starvation, or DNA damage
73 (4). The regulatory genes of ICE*Bs1* involved in cell-cell signaling (*rapI*, *phrI*) have been
74 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, both in 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 envelope of both the donor and recipient influences the success 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 (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 envelope of *B. subtilis* and other Gram positive bacteria contains a single lipid bilayer. The lipids of this membrane vary in the composition of their fatty acid tails and their head groups (reviewed in 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 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 Tn-seq to identify mutations that increased or

decreased the ability of cells to act as recipients in conjugation (20). We found that deletion of genes involved in the synthesis of various phospholipids have distinct effects on the ability of *B. subtilis* to act as a recipient in conjugation. Several of the mutations (*ugtP*, *yfnI*, *mprF*) that affect conjugation affect consumption of the phospholipid phosphatidylglycerol (Fig. 1).

Here, we analyze 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 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% w/v) was used as a carbon source. Isopropyl-ß-D-thiogalactopyranoside (IPTG, 1 µg/ml) was used to induce expression of

Pspank(hy)-*mprF*. Xylose (1% w/v) 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 (100 µg/ml) kanamycin (5 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.

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 (24). This product was used to transform naturally competent *B. subtilis* cells, a chloramphenicol resistant isolate was selected and the allelic exchange verified by PCR.

The unmarked Δ*mprF459* allele was constructed by replacing *mprF* with *cat* flanked by *lox* sites to generate strain CMJ459, then recombining out the *lox-cat* allele using Cre recombinase expressed from pDR244, as previously described (20, 25). 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 Pspank(hy), *lacI* and *mls* situated between genomic sequence from *lacA*. *mprF* was

141 placed under control of the promoter Pspank(hy). This plasmid was transformed into
142 naturally competent *B. subtilis* cells and Pspank(hy)-*mprF*, *lacI* and *mls* introduced by
143 double cross over at *lacA*. Expression of *mprF* was induced by the addition of 1 mM
144 IPTG.

145 **Thin layer chromatography**

146 Lipids were extracted from cells using a modified Bligh-Dyer method (26). We grew
147 cells in minimal medium to an OD600 of ~1, sampled 1 ml of culture and pelleted the
148 cells, removed the supernatant, resuspended in 1 ml water, pelleted the cells and
149 resuspended in 100 µl 1 M perchloric acid and incubated for 30 minutes on ice. Lipids
150 were extracted by adding 1 ml 2:6:2 methanol:chloroform:water and 0.625 µg of a
151 phosphatidylserine standard (Sigma Aldrich) to each sample and incubating at 4°C
152 overnight on a rocking platform. Lipids were recovered by adding 300 µl H_2O and 300
153 µl chloroform and incubating the samples for 30 min at -20°C, then centrifuging for 5
154 min at 720x g. The organic (bottom) phase was recovered, dried under nitrogen and the
155 extracted lipids resuspended in 12 µl 2:1 chloroform methanol.

156 The total volume of each sample was spotted on silica 60 plates (Angela) along with
157 lysyl-phosphatidylglycerol (0.63 µg - 2.5 µg) (Avanti Polar Lipids) and
158 phosphatidylserine (0.25 µg - 1 µg) standards and developed in a thin layer
159 chromatography chamber with 60:35:5 chloroform:methanol:water. The plates were
160 dried, stained with ninhydrin (1.5 mg/ml ninhydrin in water-saturated butanol with
161 3% v/v acetic acid) and charred. The plates were scanned on a flat bed scanner and
162 analyzed with ImageJ (27). Standard curves were generated for lysyl-

phosphatidylglycerol (0.16 µg - 5 µg) and phosphatidylserine (0.25 µg - 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% (w/v) xylose for 2 hours 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 minutes. Mating filters were typically placed on SMS agar (28) unless otherwise specified. TSS agar (28) was used as an alternate buffer in the mating support in some experiments. TSS was further amended in some experiments, as noted in the text. 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-

185 phosphatidylglycerol (Fig. 1) (26, 29, 30). Loss of *mprF* in donors and-or recipients
186 causes a decrease in conjugation of ICE*Bs1* (Fig. 2) (20), indicating that elimination of
187 lysyl-phosphatidylglycerol is detrimental for conjugation.

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

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

from meso-diaminopimelate (31). *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 and reference 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) (32). *yfnI* is one of four genes with overlapping roles in lipoteichoic acid synthesis in *B. subtilis* (33). 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

229 decrease and deletion of *ugtP*, *yfnI*, or *clsA* caused an increase in conjugation. There are
230 two simple models to explain these effects. 1) Lysyl-phosphatidylglycerol might
231 enhance conjugation. In this model, loss of *mprF* (needed to make lysyl-
232 phosphatidylglycerol) causes a decrease in conjugation because of loss of lysyl-
233 phosphatidylglycerol. In addition, loss of *ugtP*, *yfnI*, and *clsA* might cause an increase
234 in phosphatidylglycerol (substrate for MprF) and a subsequent increase in lysyl-
235 phosphatidylglycerol, thereby causing an increase in conjugation. 2) Alternatively (or
236 in addition), cardiolipin, glycolipids and lipoteichoic acids might act individually or
237 together to inhibit conjugation. For example, phospholipids and teichoic acids can
238 interfere with hydrolase activity (34-39) and might inhibit the cell wall hydrolase CwlT
239 that is encoded by and needed for transfer of ICE*Bs1*. Loss of *clsA* (cardiolipin) and *yfnI*
240 (lipoteichoic acids), and perhaps *ugtP* (glycolipids), relieves this inhibition causing an
241 increase in conjugation. In this model, loss of *mprF* leads to an increase in
242 phosphatidylglycerol and a possible increase in the inhibitory molecule(s) and thus a
243 decrease in conjugation. To test these models we generated strains in which multiple
244 phospholipid synthetases were inactivated and tested them as recipients in conjugation
245 experiments (Fig. 2). Results described below indicate that lysyl-phosphatidylglycerol
246 enhances conjugation.

247 We found that the decrease in conjugation frequency caused by loss of *mprF* was
248 epistatic to the increase in conjugation frequency due to loss of *ugtP* (Fig. 2B). We
249 measured the conjugation efficiencies using standard mating assays between a wild
250 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 that of the single mutants. The conjugation efficiency 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 x 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 (expect: 0.17 x 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 6-fold (0.17) and ~11 fold (0.087) decrease of the *mprF* and *lysA* mutants (expect: 0.17 x 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: 1) loss of *ugtP* and *mprF* likely affect conjugation by affecting levels of lysyl-phosphatidylglycerol; 2) loss

of *clsA*, *yfnI*, and *lysA* probably do 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 describe 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, LPG). As expected (26), 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, is likely causing 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* compared to *ugtP* on the level of lysyl-phosphatidylglycerol and the double mutant phenotypes indicate 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 conjugation phenotypes caused by mutations in *clsA*, *yfnI*, and *lysA* are most likely not due to changes in levels of lysyl-phosphatidylglycerol.

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 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 (28).

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) (Fig. 4). TSS agar is buffered with Tris instead of potassium phosphate and contains 37 mM ammonium chloride, 2 mM dibasic potassium phosphate, 50 mM Tris base, 1 mM magnesium sulfate, 0.004% iron(III)

317 chloride, 0.004% trisodium citrate, 1.5% agar, pH 7.5 (28)). Under these conditions, the
318 conjugation frequency of *mprF* mutant cells was reduced by approximately 3-fold (0.37)
319 compared to that of wild type donors and recipients. We ruled out the possibility that
320 production of lysyl-phosphatidylglycerol was restored in the *mprF* mutant on the TSS
321 agar support; there was no detectable lysyl-phosphatidylglycerol under these
322 conditions in the mutant. These findings indicate that there is something about TSS that
323 suppresses, or something about SMS that exacerbates the conjugation defect of the *mprF*
324 mutant.

325 We investigated what aspect of the different mating supports accounted for the
326 magnitude of the *mprF* mutant phenotype. Since mating in the *mprF* mutants was much
327 lower in SMS than TSS, we postulated that the lower pH and-or some of the additional
328 ions in SMS were inhibiting conjugation of *mprF* mutants. There are several differences
329 between TSS and SMS. Notably, SMS contains a higher total concentration of different
330 salts compared to TSS and a lower pH (7 versus 7.5). SMS has higher concentrations of
331 potassium (204 mM vs 4 mM), phosphate (124 mM vs 2 mM), sulfate (16 mM vs 1 mM)
332 and citrate (3 mM vs 0.1 mM).

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

Based on the above results, we wondered if other salts might affect wild type and *mprF* mutants differently. To test this, we used TSS agar as our base medium and supplemented it with 125 mM 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 mating efficiencies of wild type cells (Fig. 5A), *mprF* mutants (Fig. 5B) and then directly compared *mprF* to wild type (Fig. 5C).

We found that addition of sodium fluoride or sodium citrate to TSS caused a decrease in 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 (Fig. 5C).

In contrast, we found that addition of magnesium chloride to TSS caused an increase of 16-fold in 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, 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.

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

Together, our results (Figs. 4, 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 is due to the presence or absence of this phospholipid. The presence of *mprF* and hence lysyl-

phosphatidylglycerol enables cells to have efficient conjugation in 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.

403 Loss of *ugtP* caused an increase in the amount of lysyl-phosphatidylglycerol,
404 indicating that UgtP normally plays a role limiting the amount of lysyl-
405 phosphotidylglycerol in the cell. *ugtP* is also known to affect cell division (40),
406 primarily by directly interacting with and inhibiting the cell division protein FtsZ (40,
407 41). The effects of *ugtP* on cell division and conjugation are most likely not related. We
408 infer this mainly because the effects on cell division appear to be direct and the effects
409 on conjugation are likely through *mprF*.
410 *ugtP* mutants also appear to have many alterations in gene expression in rich
411 medium (26). The effects of *mprF* mutations on gene expression are not known, but
412 based on analyses of an *mprF pssA ywnE* (*clsA*) triple mutant, there are fewer effects
413 than in a *ugtP* single mutant (26). It is possible that the effects of *mprF* and *ugtP* on
414 conjugation are due to alterations in gene expression. However, the simplest model is
415 that these genes affect conjugation due to alterations in lysyl-phosphotidylglycerol and
416 that the composition of the cell envelope directly affects activity of the conjugation
417 machinery (see below).
418 ***mprF* and lysyl-phosphatidylglycerol enable efficient conjugation in various ionic**
419 **conditions**
420 Our results demonstrate that the effects of lysyl-phosphatidylglycerol on
421 conjugation are dependent on the environmental conditions. That is, the ratio of mating
422 efficiencies comparing *mprF* mutants and wild type cells was affected by the ionic
423 conditions used for mating. For example, the *mprF* mutants had a much more severe
424 mating defect (~30-fold) on SMS agar compared to the modest defect (~3-fold) on TSS

425 agar. Together with analysis of the differences between SMS and TSS, our results
426 indicate that *mprF* and lysyl-phosphotidylglycerol normally facilitate efficient mating
427 under a variety of external ionic conditions. We suggest that the presence of lysyl-
428 phosphatidylglycerol buffers conjugation against the some of the otherwise inhibitory
429 effects of different salts and enhances conjugation in the presence of others, allowing
430 the conjugation machinery to function reasonably well in a range of different ionic
431 conditions.

432 *mprF* homologs, and by extension lysyl-phosphatidylglycerol, affect cell surface
433 properties of other organisms. For example, *mprF* in *Staphylococcus aureus* acts as a
434 virulence factor and potentiates resistance to several cationic antimicrobials, including
435 those produced by potential human hosts (reviewed in 22). *mprF* homologs impact the
436 ability of *Enterococcus faecium* and *Listeria monocytogenes* to adapt to different
437 environmental conditions (42, 43). We suggest that in Gram positive bacteria, *mprF* and
438 lysyl-phosphatidylglycerol ensures that the cell envelope is buffered from some of the
439 variations in the chemistry of the environment, and enables the cell to perform
440 physiological functions in a regular manner under different environmental conditions.

441 **A model for how membrane phospholipids affect conjugation efficiencies**

442 We suspect that alterations in the phospholipid content of the recipient (and donor)
443 might affect the function of the conjugation machinery. This could be through changes
444 to the physical properties of the membrane (e.g., fluidity) that might affect assembly of
445 the machinery. This could also be through inhibition of a component of the machinery.
446 Transfer of DNA through the ICE*Bs1*-encoded conjugation machinery depends on CwlT

447 (44), a secreted cell wall hydrolase encoded by ICE*Bs1* (44, 45). Components of the cell
448 envelope, including lipoteichoic acids (34, 35), wall teichoic acids (36, 37) and the
449 phospholipids cardiolipin (35), phosphatidylglycerol (38) and lysyl-
450 phosphatidylglycerol (35, 39), can modulate the function of at least some cell wall
451 hydrolases. Cell wall teichoic acids inhibit hydrolase activity, at least in part, by
452 preventing hydrolase binding to the peptidoglycan of the cell wall (36, 37).
453 Phospholipids can stimulate or inhibit the function of particular hydrolases; for
454 example, phosphatidylglycerol can either enhance or inhibit the N-acetylmuramoyl-L-
455 alanine amidase of *E. coli*, depending on concentration, but has no effect on the major
456 autolysin of *Clostridium acetobutylicum* under the conditions tested (38, 39). Altering the
457 phospholipid content of the donor and-or recipient may affect a postulated interaction
458 between the cell wall hydrolase CwlT and the cell envelope, either enhancing or
459 inhibiting the ability of conjugation machinery to deliver DNA. This interaction could
460 be binding of the conjugation machinery to the recipient cell envelope and-or digestion
461 of the donor and recipient cell wall. If this model is correct, it strongly predicts that the
462 cell wall hydrolase acts on both donor and recipient cells.

463 Cell wall hydrolases are encoded by many conjugative elements (10, 46-49). Where
464 tested, they are critical for efficient conjugation. Based on this conservation, it seems
465 likely that the composition of the cell wall affects the efficiencies of many different
466 conjugative elements. Perhaps the cell wall hydrolases have evolved in ways that help
467 determine the host range of the cognate element.

468

Acknowledgments

We thank Tony DeBono (Anthony Sinsky lab) for help with thin layer chromatography, John Helmann for strains, Suzanne Walker, Bernhardt Trout, Barbara Imperialli, and Thomas Bernhardt for helpful conversations and Laurel Wright and Monika Avello for comments on the manuscript. Research reported here is based upon work supported, in part, by the National Institute of General Medical Sciences of the National Institutes of Health under award number R01GM050895 to ADG. Any opinions, findings, and conclusions or recommendations expressed in this report are those of the authors and do not necessarily reflect the views of the National Institutes of Health.

References

1. **Garriss Gv, Waldor MK, Burrus V.** 2009. Mobile antibiotic resistance encoding elements promote their own diversity. PLoS Genetics **5:**e1000775.
2. **Toleman MA, Walsh TR.** 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiology Reviews **35:**912-935.
3. **Johnson CM, Grossman AD.** 2015. Integrative and Conjugative Elements (ICEs): What They Do and How They Work. Annu Rev Genet **49:**577-601.
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.
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.
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.
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.

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.
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.
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.
12. **Watanabe T, Arai T, Hattori T.** 1970. Effects of cell wall polysaccharide on the mating ability of *Salmonella typhimurium*. Nature **225:**70-71.
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.
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.
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.
21. **Parsons JB, Rock CO.** 2013. Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res **52:**249-276.
22. **Ernst CM, Peschel A.** 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Mol Microbiol **80:**290-299.

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. **Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods **6:**343-345.
25. **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.
26. **Salzberg LI, Helmann JD.** 2008. Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition. J Bacteriol **190:**7797-7807.
27. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods **9:**671-675.
28. **Harwood CR, Cutting SM.** 1990. Molecular Biological Methods for *Bacillus*. John Wiley & Sons, Chichester.
29. **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.
30. **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.
31. **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.
32. **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.
33. **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.
34. **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.
35. **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.
36. **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.

583 37. **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.
586 38. **Vanderwinkel E, De Vlieghere M.** 1985. Modulation of *Escherichia coli* N-acetylmuramoyl-L-alanine amidase activity by phosphatidylglycerol. Biochim Biophys Acta **838:**54-59.
589 39. **Croux C, Canard B, Goma G, Soucaille P.** 1992. Autolysis of *Clostridium acetobutylicum* ATCC 824. J Gen Microbiol **138:**861-869.
591 40. **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.
593 41. **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.
596 42. **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.
599 43. **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.
602 44. **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.
605 45. **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.
609 46. **Koraimann G.** 2003. Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. Cell Mol Life Sci **60:**2371-2388.
611 47. **Scheurwater EM, Burrows LL.** 2011. Maintaining network security: how macromolecular structures cross the peptidoglycan layer. FEMS Microbiol Lett **318:**1-9.
614 48. **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.
618 49. **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.
622 50. **Menard KL, Grossman AD.** 2013. Selective pressures to maintain attachment site specificity of integrative and conjugative elements. PLoS Genet **9:**e1003623.

624

625 **Table 1. *B. subtilis* strains used.**

626

Strain	Genotype (reference)
CAL89	*trp phe str84*, *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* (26)
JMA222	*trp phe* ICE*Bs1*^{0} (4)
KM250	*trp phe amyE*::{Pxyl-*rapI cat*}, Δ(*rapI-phrI*)*342*::*kan* (50)

627

628

629

630 **Table 2. Summary of mutations affecting conjugation and phospholipid synthesis.**

631

mutant[1]	phospholipid biosynthesis[2]	mating[3]	LPG[4]	phenotype with *mprF*[5]
mprF	lysyl-phosphatidylglycerol	decreased	none	
↑*mprF*	lysyl-phosphatidylglycerol	increased	increased	
ugtP	glycolipid	increased	increased	*mprF* is epistatic
clsA	cardiolipin	increased	(increased)	additive
yfnI	lipoteichoic acid	increased	wt	additive
lysA	lysyl-phosphatidylglycerol	decreased	wt	additive

632

633 [1]All are null mutations except ↑*mprF*, which indicates overexpression of *mprF*.

634 [2]The phospholipid whose synthesis depends on the indicated gene (Fig. 1) is indicated.

635 [3]The effect of the mutation on conjugation is indicated.

636 [4]The relative amount of lysyl-phosphatidylglycerol (LPG) produced in the indicated
637 mutant compared to wild type (wt) cells. "None" indicates that there was no
638 detectable LPG. Parentheses around increased (increased) indicates a possible effect,
639 but on the edge of statistical significance (Fig. 3). ND = not determined

640 [5]The phenotype of the double mutant (with *mprF*) with respect to conjugation. *mprF* is
641 epistatic indicates that the phenotype of the double mutant is the same as the *mprF*
642 single mutant (Fig. 2)

643

644 **Figure Legends**

645

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

649

650 **Figure 2. Effects of mutations in recipients on acquisition of ICE*Bs1*.** The relative
651 conjugation frequency (y-axis) is shown for each of the indicated recipients (x-axis). The
652 same donor strain (KM250) was used for all experiments and ICE*Bs1* was induced in the
653 donor by overproduction of the activator RapI (Materials and Methods). The relative
654 conjugation frequency (y-axis) is the number of transconjugants per donor crossed to
655 the indicated recipient strain, normalized to that of the wild type recipient (CMJ161) in
656 each experiment. The wild type conjugation efficiency was approximately 4%
657 transconjugants per donor in these experiments. Conjugation frequencies measured
658 with recipients that are null for *mprF*, *ugtP*, *yfnI* and *lysA* are similar to those previously
659 reported (20) and were included in these experiments to allow direct comparison with
660 the appropriate double mutants. The graph shows means and standard deviation from
661 ≥3 experiments. The conjugation efficiency for each single mutant is statistically
662 different from that of wild type (p <0.05). Data for wild type (CMJ161) and an *mprF* null
663 mutant recipient (CMJ162) are included in all panels for comparison.
664 **A.** vector (CMJ337) contains Pspank(hy) with no insert; ↑*mprF* (CMJ222) is an *mprF*
665 null mutant with Pspank(hy) driving expression of *mprF*.
666 **B.** *ugtP* (CMJ83); *ugtP mprF* double mutant (CMJ333 p<0.05 vs. *ugtP*).
667 **C.** *clsA* (CMJ86); *clsA mprF* double mutant (CMJ332, p<0.05 vs. *clsA* and *mprF*).
668 **D.** *yfnI* (CMJ44); *yfnI mprF* double mutant (CMJ132, p<0.05 vs. *yfnI* and *mprF*).
669 **E.** *lysA* (CMJ335); *lysA mprF* double mutant (CMJ336, p<0.05 vs. *lysA* and *mprF*).
670 These strains were grown with 40 µg/ml lysine.
671

Figure 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 OD600 of 1 was determined from the indicated strains: WT, wild type (CMJ161); *mprF* (CMJ162); *mprF* + vector (CMJ337); ↑*mprF* (CMJ222) is *mprF* null with Pspank(hy) driving expression of *mprF*; *yfnI* (CMJ44); *ugtP* (CMJ83); *clsA* (CMJ86); *lysA* (CMJ335) grown with 40 μg/ml lysine (in panel B only).

A. LPG was extracted from cell membranes and examined using thin-layer chromatography (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).

Figure 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, 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, B. The conjugation frequency is shown as transconjugants per donor for **A)** a wild type donor (CMJ348) and recipient (CMJ161); **B)** an *mprF* null mutant donor (CMJ476) and recipient (CMJ162).

C. Conjugation frequencies obtained in A and B are directly compared. The ratio of the conjugation frequencies of the *mprF* mutant (B) to that of the wild type strain (A) under each of the indicated conditions is shown.

Figure 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. Chemicals supplements were added at 125 mM. The samples tested with TSS + $NaHPO_4$ also contains 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 is 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, B. The conjugation frequency (transconjugants per donor) is shown for **A)** wild type donor (CMJ348) and wild type recipient (CMJ161); **B)** an *mprF* null mutant donor (CMJ476) and an *mprF* null mutant recipient (CMJ162).

C. The conjugation frequencies obtained in A and B are directly compared and plotted as the ratio of the *mprF* mutants (B) to that of wild type strains (A) under each of the indicated conditions.

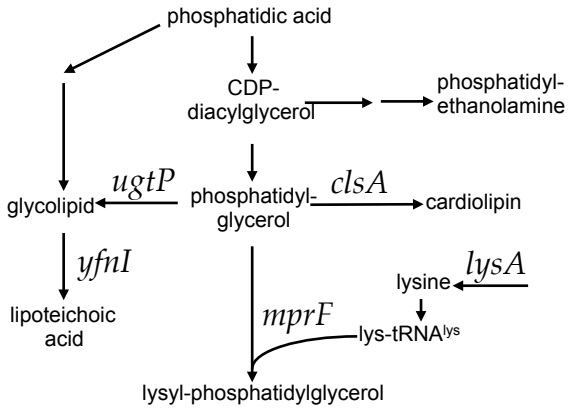

Figure 1. Pathways of phospholipid biosynthesis that affect conjugation of ICE*Bs1*. Some of the pathways involved in phospholipid biosynthesis are shown. Genes relevant to this work are indicated above the arrows.

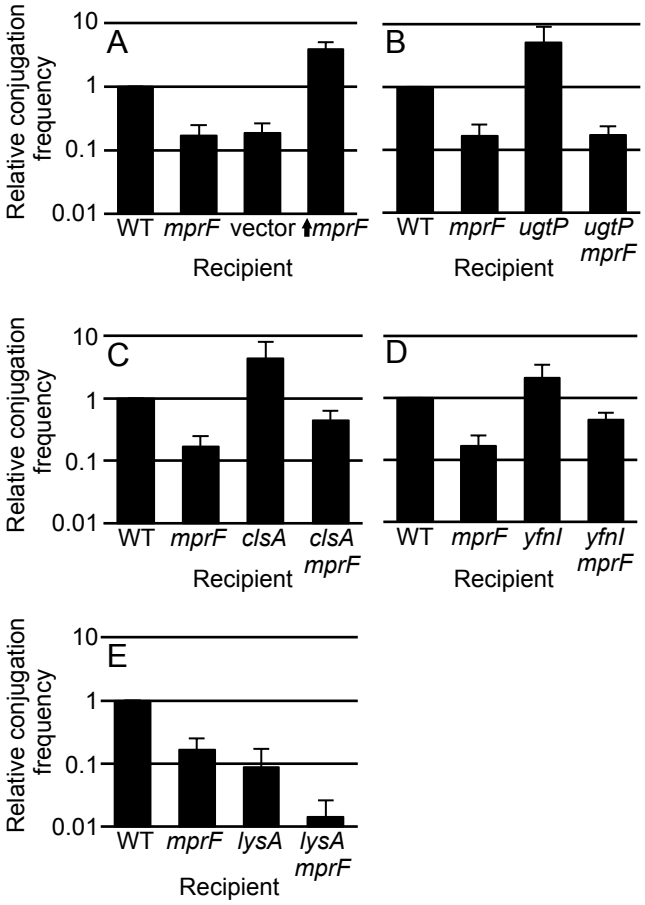

Figure 2. Effects of mutations in recipients on acquisition of ICE*Bs1*. The relative conjugation frequency (y-axis) is shown for each of the indicated recipients (x-axis). The same donor strain (KM250) was used for all experiments and ICE*Bs1* was induced in the donor by overproduction of the activator RapI (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 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 [Johnson, 2014, tn-seq] 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 of wild type ($p < 0.05$). Data for wild type (CMJ161) and an *mprF* null mutant recipient (CMJ162) are included in all panels for comparison.

A. vector (CMJ337) contains Pspank(hy) with no insert; ↑*mprF* (CMJ222) is an *mprF* null mutant with Pspank(hy) driving expression of *mprF*.

B. *ugtP* (CMJ83); *ugtP mprF* double mutant (CMJ333 $p<0.05$ vs. *ugtP*).

C. *clsA* (CMJ86); *clsA mprF* double mutant (CMJ332, $p<0.05$ vs. *clsA* and *mprF*).

D. *yfnI* (CMJ44); *yfnI mprF* double mutant (CMJ132, $p<0.05$ vs. *yfnI* and *mprF*).

E. *lysA* (CMJ335); *lysA mprF* double mutant (CMJ336, $p<0.05$ vs. *lysA* and *mprF*). These strains were grown with 40 μg/ml lysine.

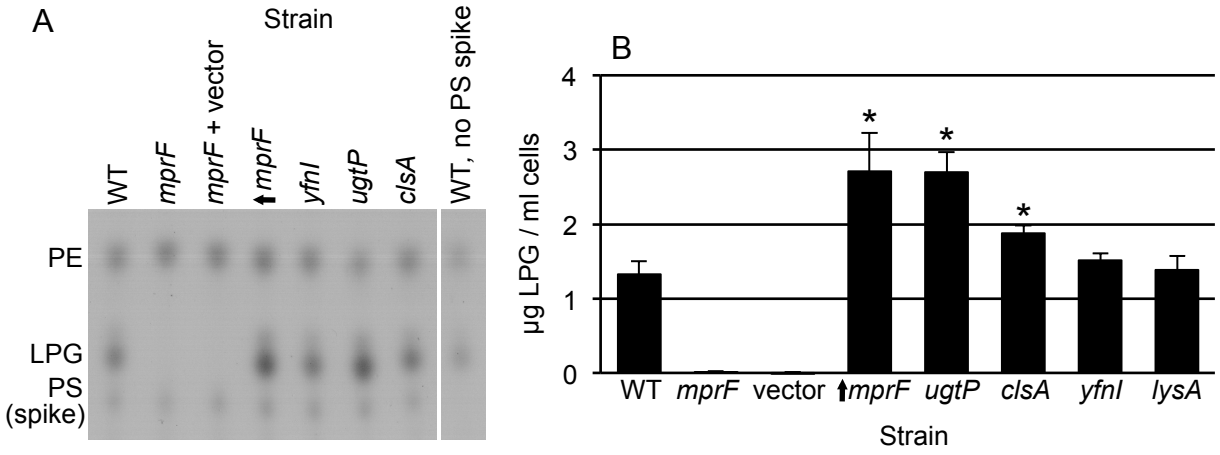

Figure 3. Effects of mutations on the level of lysyl-phosphatidylglycerol. The amount of lysyl-phosphatidylglycerol (LPG) recovered from a 1 ml culture of cells at an OD600 of 1 was determined from the indicated strains: WT, wild type (CMJ161); *mprF* (CMJ162); *mprF* + vector (CMJ337); ↑*mprF* (CMJ222) is *mprF* null with Pspank(hy) driving expression of *mprF*; *yfnI* (CMJ44); *ugtP* (CMJ83); *clsA* (CMJ86); *lysA* (CMJ335) grown with 40 µg/ml lysine (in panel B only).

A. LPG was extracted from cell membranes and examined using thin-layer chromatography (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).

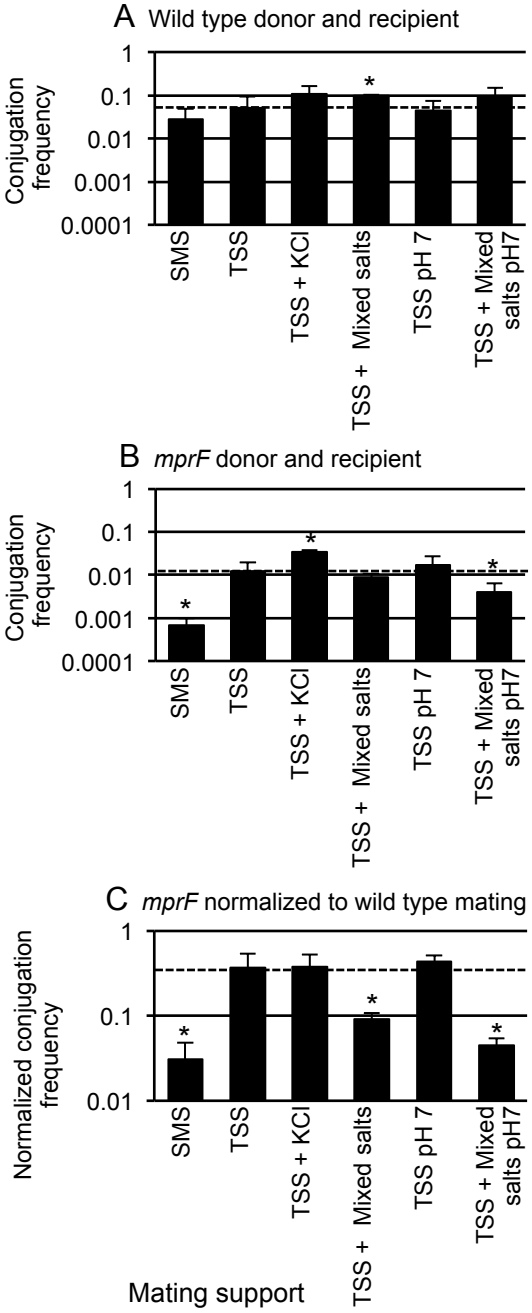

Figure 4. The chemical composition of the mating support affects conjugation. Standard filter matings were performed on supports with different chemical compositions. Donor and recipient cells were mixed in equal numbers, then collected on a filter that was placed on a mating support with the indicated 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, B. The conjugation frequency is shown as transconjugants per donor for **A)** a wild type donor (CMJ348) and recipient (CMJ161); **B)** an *mprF* null mutant donor (CMJ476) and recipient (CMJ162).
C. Conjugation frequencies obtained in A and B are directly compared. The ratio of the conjugation frequencies of the *mprF* mutant (B) to that of the wild type strain (A) under each of the indicated conditions is shown.

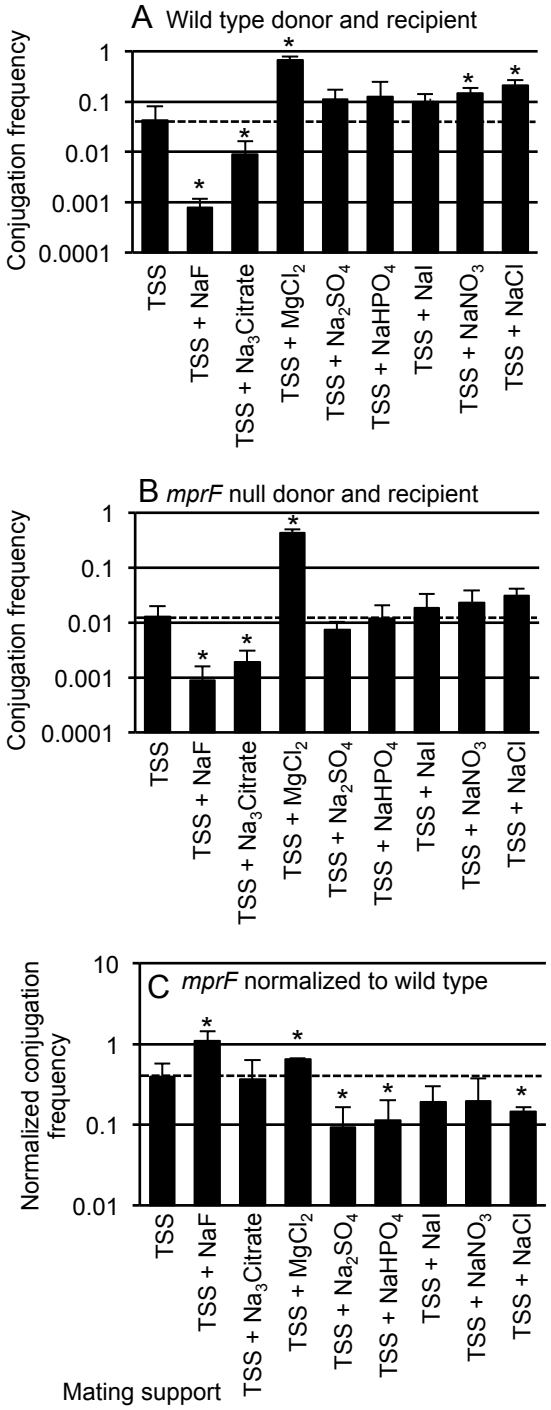

Figure 5. Some salts enhance conjugation of wild type, but not *mprF* cells. Filter matings were performed as described in Materials and Methods. Equal numbers of donor and recipient cells were mixed, collected on a filter and placed on a mating support with the indicated composition. Chemicals supplements were added at 125 mM. The samples tested with TSS + $NaHPO_4$ also contains 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 is 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, B. The conjugation frequency (transconjugants per donor) is shown for **A)** wild type donor (CMJ348) and wild type recipient (CMJ161); **B)** an *mprF* null mutant donor (CMJ476) and an *mprF* null mutant recipient (CMJ162).

C. The conjugation frequencies obtained in A and B are directly compared and plotted as the ratio of the *mprF* mutants (B) to that of wild type strains (A) under each of the indicated conditions.