

# *Bacillus subtilis* MurJ and Amj Lipid II flippases are not essential for growth

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**ABSTRACT** Identification of the protein that mediates transbilayer transport of the undecaprenyl-pyrophosphate (Und-PP) linked peptidoglycan precursor Lipid II has long been a subject of investigation. Candidates belonging to both the MOP (multidrug/oligosaccharidyl-lipid/polysaccharide) and SEDS (shape, elongation, division and sporulation) families of transmembrane proteins have been proposed, exhibiting characteristics consistent with mediating this process, including genetic essentiality and biochemical activity. While MOP family proteins including MurJ are widely considered to be the primary Lipid II transporter, questions still remain including a role for the SEDS proteins in this process. We and others previously showed that a *Bacillus subtilis* strain lacking all four MurJ homologs is viable, thereby implicating a separate mode of Lipid II transport across the membrane. However, a subsequent report of synthetic essentiality between *B. subtilis* MurJ and the flippase Amj suggested that they are necessary and sufficient. Here, we show that this effect is alleviated by excess synthesis of the enzyme responsible for Und-PP production. Thus, the inviability of a *murJ-amj* double mutant strain is not due to the essentiality of these enzymes for flipping Lipid II but is instead most likely a consequence of a reduction of free Und-PP levels. This result is consistent with a non-MOP-dependent pathway for Lipid II transport across the cytoplasmic membrane to enable cell wall peptidoglycan synthesis.

**IMPORTANCE** The assembly of peptidoglycan (PG), the typically essential polymer that provides structural integrity to bacterial cells, begins with the synthesis of the Lipid II monomer in the cytoplasm and along the cytoplasmic face of the inner membrane. Lipid II is then translocated across the membrane to the extracellular site of polymerization. The mechanistic basis for this process remains unclear, with genetic and/or biochemical evidence pointing to two different families of conserved membrane proteins. Here, we present genetic evidence that only one of these two families is essential in *Bacillus subtilis*.

**KEYWORDS** peptidoglycan

The mesh-like extracellular peptidoglycan (PG) sacculus provides structural integrity to bacterial cells. As such, PG is typically essential for growth, although under special conditions, bacteria lacking PG can survive. PG is a glycopeptide polymer composed of units of repeating GlcNAc–MurNAc disaccharides covalently attached to stem peptides. The monomeric units are synthesized initially in the cytoplasm and in the final steps, along the cytoplasmic face of the membrane, as a molecule known as Lipid II that contains the glycopeptide attached to a 55-carbon isoprenoid, undecaprenol-pyrophosphate (Und-PP). Lipid II is then transported to the outside of the cell membrane, the site of the transglycosylation (TG) and transpeptidation (TP) activities that assemble the PG polymer (1). These reactions are mediated by two classes of enzymes, the bifunctional class A PBPs, which exhibit both TG and TP activities, and the class B PBPs, which

**Editor** George O'Toole, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA

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The authors declare no conflict of interest.

**Received** 27 February 2025

**Accepted** 7 March 2025

**Published** 4 April 2025

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exhibit only TP activity, working in tandem with a SEDS (shape, elongation, division and sporulation) protein with TG activity (2).

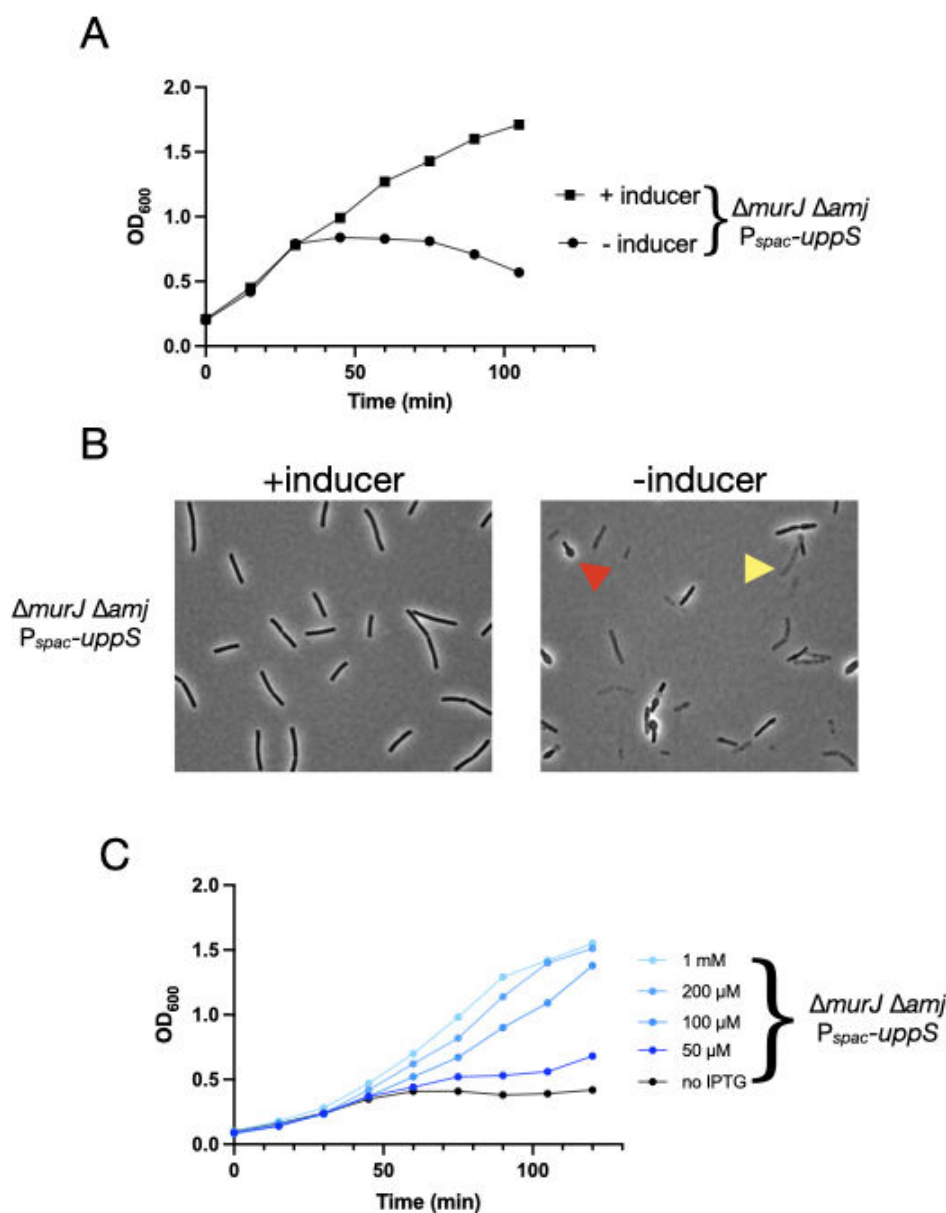
The molecular mechanism underlying Lipid II transport remained uncharacterized long after the identification of the enzymes responsible for Lipid II synthesis and polymerization. Integral membrane proteins of the SEDS family are long-hypothesized candidates. The well-studied essential cell division protein SEDS protein FtsW exhibits activity in a fluorescence-based proteoliposome assay consistent with a role in Lipid II transport (3, 4). A second candidate is an essential *Escherichia coli* protein initially known as MviN (5) and subsequently renamed MurJ (6), a member of the MOP (multi-drug/oligosaccharidyl-lipid/polysaccharide) family. Experiments using an *in vivo* assay based on the accessibility of translocated Lipid II to cleavage by a degradative enzyme are consistent with a function for MurJ in Lipid II transport in *E. coli* (7). However, *B. subtilis* MurJ and its three homologs are not essential either separately or together (8, 9), raising the question of whether the essential function of MurJ as the Lipid II flippase was phylogenetically conserved. A synthetic lethal screen identified a gene (*amj*) that was synthetically lethal with *B. subtilis murJ* (10). However, Amj has no sequence or predicted structural homology to MurJ (10), so the question remains as to why *B. subtilis* MurJ, unlike *E. coli* MurJ, is not essential. Here, we set out to investigate the origin of this difference.

## RESULTS

The original characterization of *E. coli* MurJ (MviN) reported that the growth defect of a *mviN* temperature-sensitive mutation at the non-permissive temperature was suppressed by overexpression of the undecaprenyl pyrophosphate synthase (UppS) (5). This observation suggests that MurJ-associated phenotypes in *B. subtilis* might also be subject to suppression by UppS overexpression. To examine this possibility, we determined if additional Und-PP would affect the phenotype of the *murJ/amj* depletion strain. First, we introduced an inducible allele of *uppS* [(*Physpank-uppS*) (11)] into a strain lacking *murJ* alone [ $\Delta murJ::spc$ , as derived from the published strain (10)]. Of note, this strain is wildtype with respect to endogenous *uppS*, so induced *uppS* results in levels of Und-PP that are supplemental. We made this strain competent and introduced the *amj* deletion allele ( $\Delta amj::mIs$ ), plating transformants on a selective medium in the presence of an inducer (IPTG). We obtained transformants at a normal transformation frequency and confirmed the presence of the correct *murJ* and *amj* alleles by genome sequencing and backcrossing. Consistent with a requirement for *uppS* expression, colonies were not recovered in the absence of inducer. We then grew one of these transformants (JDB4540; *murJ::spec amyE::Physpank-uppS (cat) amj::mIs*) in the presence or absence of an inducer, observing that growth was dependent on the presence of an inducer (Fig. 1A). Consistent with the restoration of growth, cells exhibited wild-type morphology in the presence of an inducer including the absence of lysed (yellow) and bulging (red) cells seen without inducer (Fig. 1B). The effect of UppS overexpression on growth is quantitatively dependent on inducer concentration (Fig. 1C), consistent with increased availability of Und-PP mediating the restoration of growth.

We then examined whether the effect of additional UppS expression on the viability of cells lacking MurJ and Amj would be observed under another condition where MurJ homologs are essential for PG synthesis. We investigated the synthesis of the spore cortex PG where the sporulation-specific MurJ homolog *spoVB* is required, and a *spoVB* mutant strain is completely asporogenous (12). We sporulated *spoVB* mutant cells containing an inducible *uppS* allele in the presence or absence of an inducer and monitored sporulation efficiency. As expected, a severe reduction in sporulation was observed in the absence of an inducer. However, the presence of an inducer did not attenuate this defect (Table 1).

Why is Und-PP supplementation successful in rescuing MurJ homolog function during growth but not during sporulation? Of potential relevance to this question is the strict requirement for class A PBP (aPBP) function in sporulation (13) but not *B. subtilis*



**FIG 1** Overexpression of *uppS* restores viability of double mutant  $\Delta murJ \Delta amj$  strain. (A) Strain JDB4540 ( $\Delta murJ \Delta amj$  *amyE::P<sub>hyspam</sub>-uppS*) was grown in LB in the presence (squares) or absence of 1 mM IPTG (circles) and OD<sub>600</sub> was recorded. Shown is an example of one of three biological replicates. (B) Samples of the cultures in A obtained at time point  $t = 90$  min were examined by microscopy as described. Lysed (yellow arrow) and morphologically distorted (red arrow) cells are noted. (C) Dose-response rescue of  $\Delta murJ \Delta amj$  strain by *uppS* overexpression. Strain JDB4540 was grown in LB in the absence (black) or the presence of a range of IPTG concentrations (blue hues) and OD<sub>600</sub> was determined. Shown is one example, and biological replicates exhibited similar patterns of growth.

growth (14, 15). Thus, if aBPB essentiality determines the effectiveness of Und-PP complementation of  $\Delta murJ$  homologs, then aBPBs and MurJ may be functionally related. If so, the absence of aBPB would affect  $\Delta murJ$  phenotypes. To test this hypothesis, we generated a strain lacking all aBPBs [*ponA::kan*;  $\Delta pbpDFG$  (16)] and carrying deletions of both *murJ* and *amj* and an inducible *amj* allele. In the presence of an inducer, both this strain and a strain wildtype for aBPBs grew similarly (Fig. 2A). In the absence of an inducer, both strains exhibited a pronounced decrease in growth rate within 60 min. However, examination of the two strains by light microscopy revealed that the absence of aBPBs substantially mitigated the aberrant morphology of the *murJ/amj* strain

**TABLE 1** Effect of UppS overexpression on the *spoVB* sporulation phenotype<sup>a</sup>

Strain	Sporulation
<i>trpC2</i>	100%
$\Delta spoVB$	$<10^{-5}$
$\Delta spoVB$ <i>Pspac-uppS</i> (-IPTG)	$<10^{-5}$
$\Delta spoVB$ <i>Pspac-uppS</i> (+IPTG)	$<10^{-5}$

<sup>a</sup>Shown is one example of three biological replicates that all exhibit similar results.

observed in the absence of inducer (Fig. 2B). Thus, aBPB activity is functionally associated with *murJ* and *amj*.

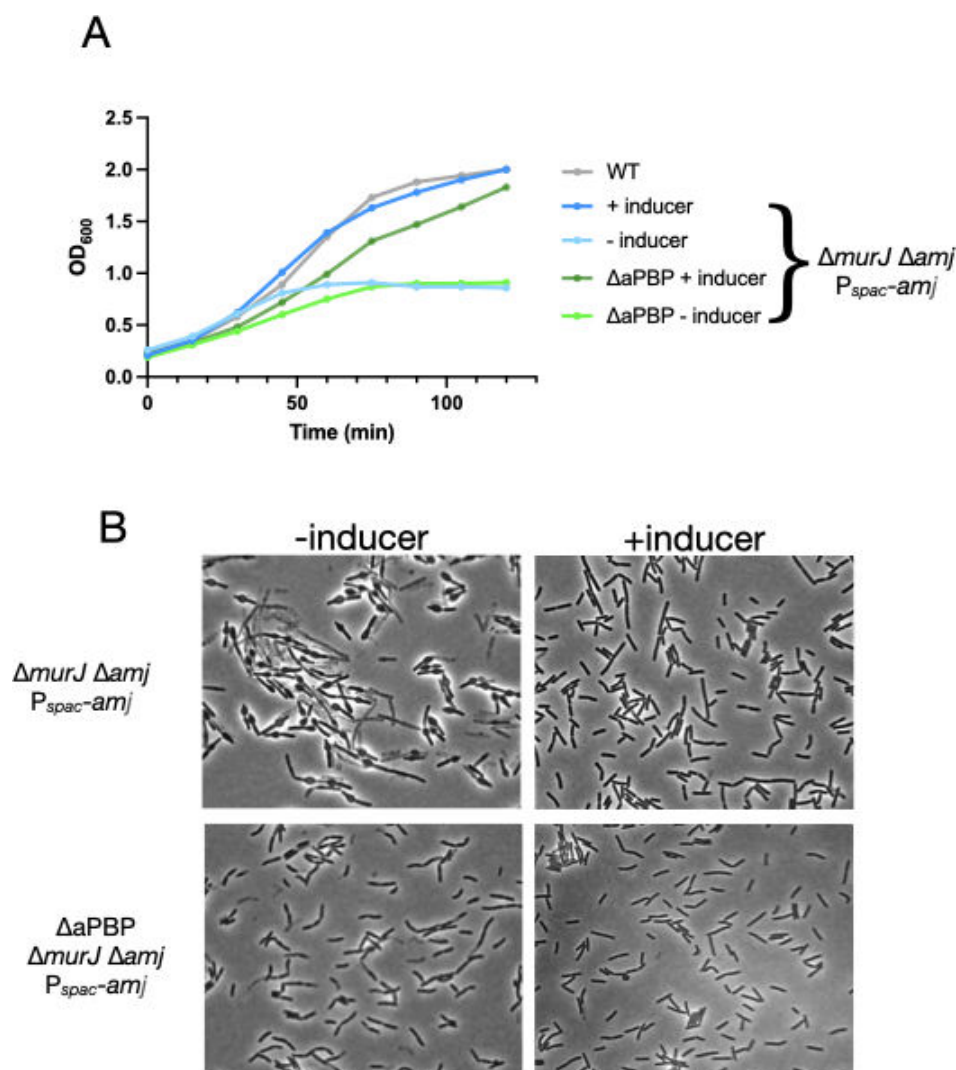
## DISCUSSION

We find that the previously reported inviability of a *B. subtilis* strain containing mutations in both *murJ* and *amj* (10) is not due to their synergistic essentiality. We show that supplemental expression of UppS, the cytoplasmic enzyme responsible for the synthesis of Und-PP, allows this strain to grow despite the absence of MurJ and Amj, consistent with a separate system for Lipid II export. Thus, these mutations likely lead to inviability because together they reduce the levels of free Und-PP below that needed for PG synthesis during growth, possibly as a consequence of Und-PP sequestration. This result implies that MurJ and Amj cannot be the sole enzymes mediating the process of Lipid II membrane translocation necessary for PG synthesis during growth in *B. subtilis*.

We further demonstrate that the elimination of aBPB function partially suppresses the phenotype of a *murJ/amj* double mutant. This effect suggests that the non-essentiality of *B. subtilis* MurJ is due to the non-essentiality of aBPB function for *B. subtilis* growth. While the loss of aBPB function does alleviate the dramatic morphological pathologies exhibited by cells depleted for *murJ/amj*, it does not allow for growth and division. Why this suppression is only partial is not clear, although since a strain lacking aBPBs is viable but phenotypically distinct from the wild-type parent (17), there could be a synergistic interaction between the two classes of mutations. In addition, we observe that UppS supplementation does not suppress the sporulation deficiency of a strain lacking the sporulation-specific MurJ homolog SpoVB. Since PG synthesis in sporulation requires aBPB function (13), UppS-dependent suppression may, therefore, only occur in contexts where aBPB-mediated PG synthesis is not essential. It is formally possible that increased levels of Und-PP affect the activity and/or expression of the other non-sporulation-specific MurJ homolog YabM.

The suppression of morphological defects of the *murJ/amj* mutant strain observed in the absence of aBPBs suggests that MurJ (and possibly Amj) are involved in aBPB-dependent PG synthesis. MurJ is a Lipid II binding protein (18), so it could mediate the transport of cytosolically produced Lipid II used by the aBPBs (Fig. 3, left). Since growing evidence indicates that aBPB enzymes function separately from the SEDS-family protein complexes containing bBPBs (2, 19), MurJ would then functionally associate with aBPB-containing PG synthetic complexes. This association is likely not mediated by specific protein-protein interactions, as *B. subtilis* Amj can functionally substitute for *E. coli* MurJ, despite the fact that they are not homologs (10).

Our observations could help resolve the apparent paradox of two different proteins—MurJ and FtsW—being identified as Lipid II flippases. *E. coli* MurJ is essential (5, 6) and *murJ* mutations reduce Lipid II accessibility on the extracellular surface of the cytoplasmic membrane (7). *E. coli* FtsW is also essential, and experiments directly monitoring the movement of fluorescent Lipid II across model membranes observed an FtsW-dependence (3, 4). MurJ (20) and FtsW (21) (and its SEDS homolog RodA [22–24]) enzymes have been investigated structurally, but to date, a flippase mechanism has not been definitively established. While MurJ and SEDS proteins could function together in translocating Lipid II (1), an alternative model consistent with the data presented here for *B. subtilis* is that they work with separate PG synthesizing pathways, one containing a SEDS protein

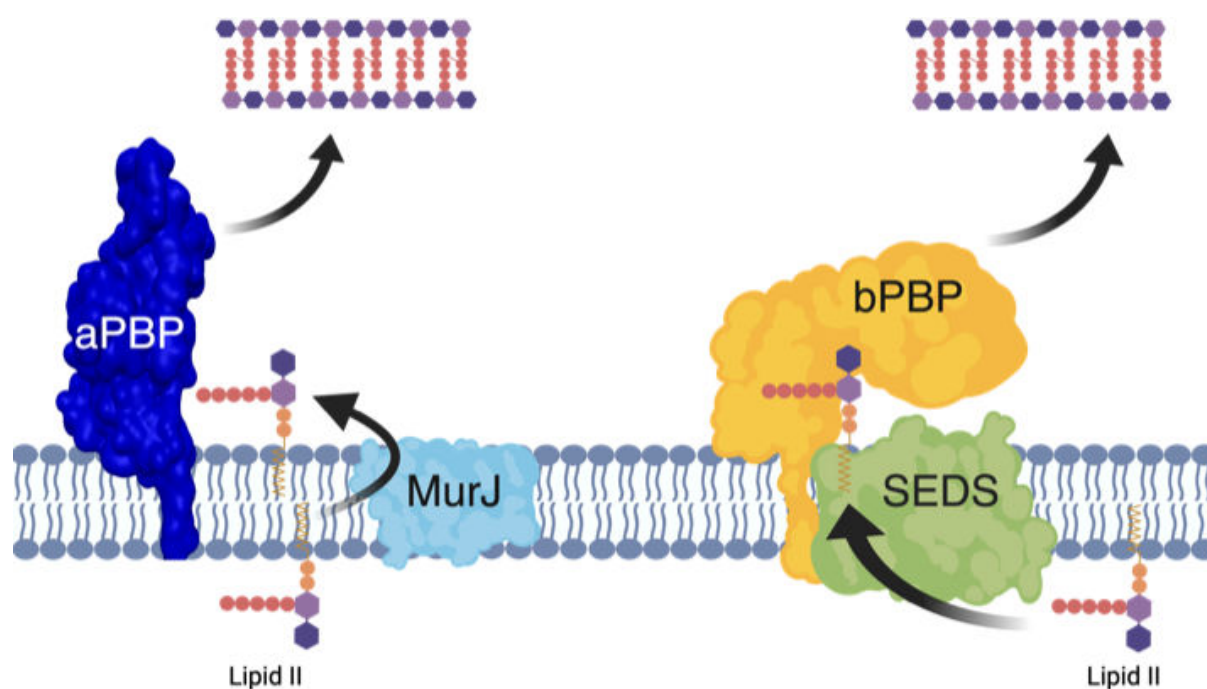


**FIG 2** Absence of aPBPs partially suppresses the phenotype of the  $\Delta murJ \Delta amj$  strain. (A) Strain JDB4181 (blue;  $\Delta murJ \Delta amj$   $P_{spac^*} amj$ ) and JDB4493 (green;  $\Delta pbpDFG \Delta ponA \Delta murJ \Delta amj$   $P_{spac^*} amj$ ) were grown in LB in the presence or absence of inducer (1 mM IPTG), and OD<sub>600</sub> was determined. (B) Samples of the cultures in A were examined by microscopy at time point  $t = 120$  min as described. Shown is one example, and biological replicates exhibited similar patterns of growth.

(either FtsW or RodA) that flips Lipid II in association with its cognate bBPB and the other including MurJ that flips Lipid II used by an aBPB (Fig. 3).

SEDS proteins have essential TG activity (25), proposed to be coupled to flippase activity (1). Given the intimate nature of the interaction between the TG activity of the SEDS protein and the TP activity of the bBPB (22, 24), it is not clear how Lipid II flipped by a SEDS protein would be made available to aPBPs that are not part of the SEDS-bBPB complex (2, 26). In addition, SEDS proteins likely function as part of a large biosynthetic complex that, as proposed, includes enzymes of the Lipid II synthesis pathway (27, 28). For example, the *E. coli* Lipid II synthase MurG interacts with PBP3, the partner of FtsW (29), and MurG forms a complex with the Lipid I synthase MraY (29) as well as the MurE and MurF enzymes (30). Thus, an appealing aspect of the proposed mechanism is that it would facilitate Lipid II availability for aBPB-dependent PG biosynthesis pathways. That is, MurJ-dependent translocation of Lipid II produced by Mur enzymes functioning independently of the SEDS-bBPB complex would allow both aBPB and bBPB pathways to simultaneously synthesize PG.





**FIG 3** Model of MurJ/Amj function during PG synthesis. Lipid II synthesized on the cytoplasmic face of the membrane is flipped either by MurJ for use by an aPBP (dual TG/TP) or by RodA for use by RodA(TG) and bPBP(TP).

The observation reported here regarding *murJ/amj* inviability has parallels in prior work addressing the apparent essentiality of other genes later found to be non-essential. For example, strains carrying knockouts in various teichoic acid biosynthetic genes encoding enzymes active in the later steps of the pathway are not viable, suggesting that teichoic acid is essential (31). However, the deletion of *tagO* that encodes the enzyme mediating the initial step in teichoic acid biosynthesis, the attachment of UDP-GlcNAc to Und-PP, allows for the deletion of later genes in the pathway (32), suggesting that the inviability of *tag* mutant strains is a consequence of Und-PP sequestration away from PG synthesis. In the present context, the alleviation of *murJ/amj* synthetic lethality by UppS expression would also result from Und-PP sequestration. While the mechanistic basis of this sequestration is unclear, the phenomenon of Und-PP pools being subject to competition by different cellular processes with PG synthesis is well characterized. For example, enterobacterial O-antigen synthesis also requires Und-PP, and disrupting O-antigen synthesis results in cell shape deformities suppressed by supplemental Und-PP expression (33). Again, the specific mechanism underlying Und-PP sequestration in this context is unclear. Mutation of an enzyme mediating enterobacterial common antigen (ECA) biosynthesis caused defects in cell shape that were suppressed by increasing Und-PP levels. In this case, sequestration was attributed to the accumulation of the Und-PP-1-linked intermediate ECA-lipid II (34).

Tight regulation of Und-PP pools is evolutionarily conserved (35, 36). This may be a consequence of the phenomenon that an overabundance of membrane-associated polyisoprenols, particularly an extended C-55 polyprenol like Und-PP, can disrupt the phospholipid bilayer architecture (37), thereby restricting the size of Und-PP pools. Given this constraint, any Und-PP-consuming pathway must function so as to not interfere with the Und-PP pool required for optimal PG synthesis, a rate-limiting step in bacterial growth (38). Recent work demonstrated the key role of the extracellular sigma factor SigM in maintaining this homeostasis (39). Such metabolic coordination is presumably characteristic of normal physiological contexts and may become particularly relevant to a more pathological context such as a mutation in one of these pathways (34). The observations presented here suggest that mutations affecting a non-essential PG

biosynthetic pathway such as that mediated by aPBPs in *B. subtilis* can similarly affect essential bPBP-dependent PG synthesis.

In summary, this work is most consistent with a model for *B. subtilis* PG biosynthesis in which the MurJ and/or Amj Lipid II flippases support normal growth but are not absolutely essential, and SEDS Lipid II flippases participate in essential elongasome and divisome function. Thus, the MOP and SEDS systems may provide a dual route for Lipid II export to the outer face of the cytoplasmic membrane, as postulated (28).

## MATERIALS AND METHODS

### Bacterial growth and sporulation

Strains were grown in Lysogeny Broth (LB) with shaking at 37°C. For measurement of growth curves, an overnight culture from a single freshly streaked colony in the mid-log phase (OD<sub>600</sub> ~0.3) was diluted 1:10 in 10 mL LB and grown to mid-log. The culture was centrifuged (5', 3,000 rpm), the pellet was washed in pre-warmed LB, and resuspended in 2 mL LB. One mL was added to a flask containing 9 mL LB and one mL to a flask with 9 mL LB with IPTG as noted. For sporulation, strains were grown in Difco Sporulation Media (DSM) for 24 h at 37°C on a roller-drum, with 1 mM IPTG as noted. Sporulation efficiency was determined by measuring colony-forming units on LB plates before and after heating to 80°C for 15 min and is calculated relative to the efficiency of the wild-type parent.

### Strain construction

Strains were derived from *B. subtilis* 168 *trpC2* and are listed in Table 2. Strains were constructed by transformation using conventional methodology, and where necessary, media was supplemented with 100 µg/mL spectinomycin, 10 µg/mL kanamycin, 5 µg/mL chloramphenicol, and 1× MLS or 20 mM MgCl<sub>2</sub> (for strains carrying aPBP mutations). JDB4540 was constructed by transforming JDB4005 gDNA into a *murJ::spc* strain (derived from JDB4181), selecting for cm<sup>R</sup>. The resulting strain was transformed with JDB4181 gDNA, selecting for mls<sup>R</sup> in the presence of 1 mM IPTG. JDB4493 was constructed by transforming JDB4181 gDNA into JDB4482, selecting for spec<sup>R</sup>. This strain was transformed with JDB4181 gDNA selecting for cm<sup>R</sup>. The resulting strain was transformed with JDB4181 gDNA, selecting for mls<sup>R</sup> in the presence of IPTG. Finally, JDB4443 gDNA was transformed into this strain, selecting for kan<sup>R</sup> in the presence of IPTG. JDB4547 was constructed by transforming JDB4006 gDNA into JDB1097, selecting for cm<sup>R</sup>.

### Microscopy

Cells were immobilized on 1% agar pads in PBS. Phase contrast microscopy was performed using a Nikon Eclipse 90i microscope and a CFI Plan Apo 100×, NA 1.45 oil objective. Images were taken with a Hamamatsu Orca ER-AG camera and processed with ImageJ.

TABLE 2 Strains

Strain	Genotype	Source
JDB1772	<i>trpC2</i>	Lab collection
JDB1097	<i>spoVBA::tet</i>	Lab collection
JDB4005	<i>W168 kan::uppS1 amyE::Pspac(hy)-uppS-FLAG (cat)</i>	(11)
JDB4181	<i>ycgO::Pspank*-amj(cat) murJ::spec amj::mls sacA::Pveg-mcherry(tet)</i>	(10)
JDB4540	<i>murJ::spec amyE::Physpank-uppS (cat) amj::mls</i>	This work
JDB4443	<i>ponA::kan</i>	<i>Bacillus subtilis</i> Genetic Stock Center
JDB4482	<i>ΔpbpDFG</i>	Helmann lab
JDB4493	<i>ΔpbpDFG ytgP::spec ycgO::Pspank*-ydaH(cat) ydaH::mls ΔponA::kan</i>	This work
JDB4547	<i>spoVBA::tet amyE::Pspac(hy)-uppS (cat)</i>	This work

## ACKNOWLEDGMENTS

K.E. and J.D. were supported by NIH R35GM141953. We thank David Roper and members of our laboratory for their comments on the manuscript. Figure 3 was made with the assistance of Biorender. Strains were generously provided by John Helmann and David Rudner.

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## DIRECT CONTRIBUTION

This article was submitted via the Active Contributor Track (ACT). Jonathan Dworkin, the ACT-eligible author, secured reviews from David L. Popham, Virginia Polytechnic Institute and State University, and Dirk-Jan Scheffers, University of Groningen.

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