Bacillus subtilis Homologs of MviN (MurJ), the Putative *Escherichia coli* Lipid II Flippase, Are Not Essential for Growth[▽][†]

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Although peptidoglycan synthesis is one of the best-studied metabolic pathways in bacteria, the mechanism underlying the membrane translocation of lipid II, the undecaprenyl-disaccharide pentapeptide peptidoglycan precursor, remains mysterious. Recently, it was proposed that the essential *Escherichia coli mviN* gene encodes the lipid II flippase. *Bacillus subtilis* contains four proteins that are putatively homologous to MviN, including SpoVB, previously reported to be necessary for spore cortex peptidoglycan synthesis during sporulation. MviN complemented the sporulation defect of a Δ*spoVB* mutation, and SpoVB and another of the *B. subtilis* homologs, YtgP, complemented the growth defect of an *E. coli* strain depleted for MviN. Thus, these *B. subtilis* proteins are likely to be MviN homologs. However, *B. subtilis* strains lacking these four proteins have no defects in growth, indicating that they likely do not serve as lipid II flippases in this organism.

Peptidoglycan synthesis is vital for cell growth and maintenance of cell shape in both gram-positive and gram-negative bacteria. This polymer of glycan chains that are cross-linked by peptide bridges forms an extracellular shell which provides protection against osmotic stresses as well as a sturdy scaffolding for extracellular appendages. The enzymes responsible for peptidoglycan synthesis are highly conserved in all bacteria with a cell wall. In the cytoplasm, the enzymes MurA to MurE synthesize the soluble MurNAc-pentapeptide starting with UDP-GlcNAc. MraY links this molecule to an isoprenoid chain, forming the membrane-associated lipid I precursor. MurG then adds UDP-GlcNAc to make lipid II, which is subsequently flipped across the cytoplasmic membrane and attached by penicillin-binding proteins via transglycosylation and transpeptidation reactions to the mature peptidoglycan.

While these cytoplasmic and extracellular steps are well characterized, comparatively little is known about the mechanism of membrane translocation. Fluorescently tagged lipid II does not spontaneously flip in protein-free liposomes (31), as would be expected given its large hydrophilic carbohydrate and protein groups. This observation suggests that that flipping is a protein-mediated process, and, consistent with this prediction, fluorescent lipid II molecules were translocated across vesicles made from *Escherichia coli* membranes. Genetic data have pointed to proteins belonging to the SEDS family as potential lipid II flippases (14). These proteins are highly conserved and contain multiple membrane-spanning domains (generally 10 to 12 transmembrane helices). Since they are in most cases essential for viability, it has been problematic to demonstrate their function. However, depletion or temperature-sensitive

mutations result in phenotypes consistent with a block in peptidoglycan synthesis. A nonessential SEDS protein, *Bacillus subtilis* SpoVE, is necessary for the formation of peptidoglycan during a later step in spore development (13), and point mutations in SpoVE block peptidoglycan synthesis without disturbing protein production or localization (24).

Recently, the integral membrane protein MviN, encoded by an essential *E. coli* gene, was proposed to be the lipid II flippase (26). Strains carrying a temperature-sensitive mutation in MviN underwent lysis following incubation at the nonpermissive temperature and showed a twofold increase in lipid II accumulation (16). While the operon that includes *mviN* is essential in the gram-negative bacteria *Sinorhizobium meliloti* and *Burkholderia pseudomallei* (20, 25), *mviN* mutations in *Rhizobium tropici*, *Salmonella enterica* serovar Typhimurium, and *Bdellovibrio bacteriovorus* have not been fully characterized, and therefore the essentiality of MviN in these species remains to be demonstrated (4, 19, 21). Due to the high degree of conservation of other proteins involved in peptidoglycan synthesis between gram-positive and gram-negative bacteria and the essential nature of peptidoglycan synthesis, the protein(s) necessary for flipping of lipid II should also be essential and conserved in a gram-positive organism. We therefore set out to identify and examine the MviN (MurJ) homologs of *B. subtilis*.

MATERIALS AND METHODS

Standard procedures were used to prepare and handle recombinant DNA and to transform *E. coli*. Plasmids and details of their construction are described in Table S1 in the supplemental material. Oligonucleotides used are described in Table S2 in the supplemental material. *B. subtilis* strains were derivatives of PY79 (Table 1) unless otherwise noted (34), and the *E. coli* strain used was DH5α. The inducible *mviN* allele was moved to MG1665 from NR1154 via P1 transduction (29). *B. subtilis* was transformed using competent cells made by the two-step method (12), and antibiotic selection used either macrolides-lincosamides-streptogramin B (MLS) (1 μg/ml erythromycin and 250 μg/ml lincomycin), 5 μg/ml chloramphenicol, 100 μg/ml spectinomycin, or 10 μg/ml tetracycline. Sporulation used either Difco sporulation medium (DSM) (12) or CH medium for growth and A+B medium for resuspension (30). Heat resistance of spores was assayed

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
B. subtilis		
PY79	Wild type	Lab stock
JDB1097	*spoVBΔ::tet*	9
JDB1395	*spoIIIAG-HΩkan*	3
JDB1752	*ΔspoVE::tet*	24
JDB2327	*ΔytgP::mls*	This work
JDB2354	*ΔytgP::mls::spc*	This work
JDB2330	*ΔykvU::mls*	This work
JDB2355	*ΔykvU::mls::spc*	This work
JDB2386	*yabM::cm*	This work
JDB2347	*spoVBΔ::tet ΔytgP::mls*	This work
JDB2361	*spoVBΔ::tet ΔytgP::mls ΔykvU::mls::spc*	This work
JDB2398	*spoVBΔ::tet ΔytgP::mls ΔykvU::mls::spc yabM::cm*	This work
JDB2329	*spoVB-gfp spc*	This work
JDB2356	*ytgP-gfp spc*	This work
JDB2358	*ykvU-gfp spc*	This work
JDB2357	*yabM-gfp spc*	This work
JDB2416	*spoVB-gfp spc spoIIIAG-HΩkan*	This work
JDB2418	*spoVB-gfp spc ΔspoVE::tet*	This work
JDB2377	*ykvU-gfp spc spoIIIAG-HΩkan*	This work
JDB2378	*ykvU-gfp spc ΔspoVE::tet*	This work
JDB2402	*amyE::P$_{spank}$-uppS lacI spc*	This work
JDB2403	*amyE::P$_{spank}$-uppS-flag lacI spc*	This work
JDB2404	*spoVBΔ::tet amyE::P$_{spank}$-uppS lacI spc*	This work
JDB2405	*spoVBΔ::tet amyE::P$_{spank}$-uppS-flag lacI spc*	This work
JDB2437	*spoVBΔ::tet amyE::Pxyl-spoVB-gfp xylR spc*	This work
JDB2504	*spoVBΔ::tet amyE::Pxyl-mviN xylR spc*	This work
JDB2506	*amyE::Pxyl-mviN xylR spc*	This work
E. coli		
NR1154	*lysA::kan murJ Ω(−14::bla araC P$_{BAD}$)*	26
MG1665	Wild type	Lab stock
JDE1118	*murJ Ω(−14::bla araC P$_{BAD}$)*	This work
JDE1140	*murJ Ω(−14::bla araC P$_{BAD}$) pAF374 (P$_{tac}$-ytgP-gfp cm)*	This work
JDE1147	*murJ Ω(−14::bla araC P$_{BAD}$) pAF375 (P$_{tac}$-spoVB-gfp cm)*	This work
JDE1148	*murJ Ω(−14::bla araC P$_{BAD}$) pMMB207(P$_{tac}$ cm)*	This work

TABLE 2. BLAST results for MviN and peptidoglycan biosynthetic homologs[a]

Query	Subtilist best hit	e value
E. coli proteins		
MviN	YtgP	9.00E-07
MraY	MraY	4.00E-59
FtsW	SpoVE	1.00E-60
MurG	MurG	4.00E-41
PBP1a (MrcA)	PonA (PBP1)	4.00E-53
B. subtilis YtgP	YtgP	0.0
	YabM	1.00E-38
	SpoVB	5.00E-29
	YkvU	1.00E-15

[a] BLAST results utilizing Subtilist (http://genolist.pasteur.fr/SubtiList/) and Colibri (http://genolist.pasteur.fr/Colibri/) databases. The top portion shows e values of best hits from BLAST analysis of MviN and conserved proteins involved in peptidoglycan synthesis from *E. coli* against *B. subtilis* genome (Subtilist). The bottom portion shows e values of MviN best hit from *B. subtilis*, YtgP, against *B. subtilis* genome (Subtilist).

Confirmation of the *yabM* deletion used assays based on product length, pattern of restriction enzyme digestion, and sequencing of a PCR amplified allele.

A strain carrying mutations in all four MviN homologs was constructed by transforming JDB1097 (*spoVBΔ::tet*) with genomic DNA from JDB2327 (*ΔytgP::mls*), selecting for MLS resistance, and screening for tetracycline resistance. This strain, JDB2347 (*spoVBΔ::tet ΔytgP::mls*), was then transformed with genomic DNA from JDB2355 (*ΔykvU::mls::spc*), selected for spectinomycin resistance, and screened for tetracycline resistance and MLS resistance. The resulting strain, JDB2361 (*spoVBΔ::tet ΔytgP::mls ΔykvU::mls::spc*), was transformed with genomic DNA from JDB2386 (*ΔyabM::cm*) and selected for chloramphenicol resistance (and screened for MLS, tetracycline, and spectinomycin resistance) to construct the quadruple mutant strain JDB2361. Strains used are listed in Table 1 and were verified by PCR for individual deletions.

All original mutations made were transformed into PY79. Transformation efficiencies were indistinguishable from those of a control genomic DNA (*amyE::cm*). The transformation efficiency for construction of a double mutant strain (*ΔytgP::mls ΔyabM::cm*) was also indistinguishable from that for construction of a control strain (*ΔytgP::mls amyE::cm*).

Fluorescence microscopy. Samples (100 μl) of cells were taken at designated times during vegetative growth in CH medium or following resuspension in A+B medium. To each sample, 0.5 ml of FM4-64 (Invitrogen; 100 μg/ml) was added just before the cells were collected by centrifugation. The pellet was resuspended in 10 ml phosphate-buffered saline and added to a poly-L-lysine pretreated coverslip. All microscopy was performed on a Nikon Eclipse 90i with a 100× objective using phase contrast and captured by a Hamamatsu Orca-ER camera using Nikon Elements BR software. Exposure for fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate images was 400 ms. ImageJ (National Institutes of Health [NIH]) was used for analysis.

following sporulation by exhaustion in DSM in 2-ml cultures for 24 h. For induction, 0.5 mM IPTG or 0.5% xylose was added 6 h after starting DSM cultures. Serial dilutions were plated before and after the cells were heated to 80°C 20 min, and the ratio of CFU obtained before and after heat treatment was determined.

MviN homolog deletions. Strains carrying single *ΔytgP* and *ΔykvU* mutations were generated with a modified long flanking homology protocol using PY79 genomic DNA as the template (33). For *ytgP*, the upstream flank was amplified with AFO606 and AFO607 and the downstream flank was amplified with AFO608 and AFO609. For *ykvU*, the upstream flank was amplified with AFO610 and AFO611 and the downstream flank was amplified with AFO612 and AFO613. Each of these PCR products was gel purified and used in the second-round reaction with linearized pDG1730, which was used as the source of the erythromycin cassette. For *ytgP*, AFO606 and AFO609 were added to the second-round reaction mixture, and for *ykvU*, AFO610 and AFO613 were added to the second-round reaction mixture. Following verification of the PCR product, it was transformed into PY79. MLS-resistant transformants were confirmed to contain an appropriate deletion by assays based on product length, pattern of restriction enzyme digestion, and sequencing of a PCR-amplified allele. The spectinomycin-resistant allele of *ykvU* (JDB2355) was generated by transformation of JDB2330 with pEr:Sp, selecting for spectinomycin resistance, and screening for MLS resistance.

The *yabM* disruption strain was generated by transformation of PY79 with pAF344 (Cm^r). Transformants were selected for chloramphenicol resistance and tested for sensitivity to spectinomycin (indicating a double-crossover event).

RESULTS

Identification of *B. subtilis* MviN homologs using BLAST and topology analysis. BLAST analysis demonstrated that *E. coli* MviN is conserved among gram-negative bacteria; however, a similar analysis in gram-positive bacteria was not reported (26). Since *E. coli* and *B. subtilis* both utilize *m*-diaminopimelic acid-containing lipid II, the proteins involved in the synthesis, translocation, and polymerization of this molecule are likely to be highly conserved. This is true for enzymes in the pathway leading to lipid II synthesis (MurA to MurG and MraY) and to its polymerization (*E. coli* PBP2/3 and *B. subtilis* PBP2B/3) that all have e values of $>10^{-20}$ (Table 2). Using BLASTp and the sequence of *E. coli* MviN as the query, the *B. subtilis* genome yielded YtgP as the top hit (e = 10^{-7} [Table 2]). Using the sequence of YtgP as the query against the *E. coli*

genome yielded MviN as the top hit (e $= 8 \times 10^{-10}$). Thus, while YtgP and MviN were not nearly as highly conserved as any other proteins in the peptidoglycan pathway, the reciprocity of the BLASTp searches indicated that YtgP was the most likely candidate for an MviN homolog in *B. subtilis*. We further analyzed the presence of MviN homologs in the genomes of 11 gram-positive organisms, including representatives from the *Bacillus*, *Streptococcus*, and *Staphylococcus* species. YtgP homologs were the top hits, with BLASTp scores similar to those seen with *B. subtilis* (data not shown).

To explore the possibility that YtgP was not the sole MviN homolog in *B. subtilis*, the YtgP sequence was used as a BLASTp query against the *B. subtilis* genome. This analysis resulted in the identification of SpoVB, YkvU, and YabM, which were more homologous to each other and to YtgP than to MviN (Fig. 1; Table 2). Consistent with the proposed role of MviN as the lipid II flippase, strains lacking SpoVB are defective in the synthesis of spore cortex peptidoglycan and accumulate peptidoglycan precursors (32).

While YtgP was the best hit, two additional proteins, YvbV and YtxB, had BLASTp scores greater than those of SpoVB, YkvU, and YabM when MviN was used as the query. Reciprocal BLASTp analysis of YvbV or YtxB against the *E. coli* genome did not return MviN as a top hit, as did YtgP. YvbV did not return MviN as a significant hit, and YtxB returned MviN as the fifth significant hit (e = 0.042). Both YvbV and YtxB are not predicted to be essential in *B. subtilis* (17). Since MviN was predicted to contain 14 transmembrane helices by TMHMM (18), true MviN homologs would be expected to have a similar number. YvbV and YtxB contain fewer than 10 predicted transmembrane helices. YvbV and YtxB were therefore excluded from further analysis. We focused on YtgP, SpoVB, and YabM, each with 14 predicted transmembrane helices, and on YkvU, with 12 predicted transmembrane helices. Also, as predicted for MviN, all four proteins have N and C termini predicted to be located in the cytoplasm.

Generation of deletions in genes encoding putative MviN homologs. Since lipid II translocation is necessary for peptidoglycan biosynthesis, the protein(s) responsible for this process are assumed to be essential. While *E. coli* MviN is essential (26), the genes encoding the most homologous *B. subtilis* protein, YtgP, or any of the other putative homologs were not identified during a genome-wide screen of essential *B. subtilis* genes (17). However, this analysis provided only a limited characterization of these genes and would not identify a gene that has a paralog providing a redundant function. The only *B. subtilis* MviN homolog that has been closely examined to date is SpoVB, and although a strain carrying a *spoVBΔ::tet* mutation is blocked in stage V of sporulation and results in the accumulation of peptidoglycan precursors, this strain exhibits no vegetative growth defects (23). However, if SpoVB functioned only during sporulation, one or more of the identified MviN homologs may be necessary for vegetative growth. As a precedent, the SpoVD and SpoVE sporulation specific proteins necessary for peptidoglycan synthesis of the spore cortex have no vegetative phenotypes (5, 13), but their homologs (FtsW/PBP2B and RodA/PBP3) function during growth and are essential.

We examined whether the three additional candidate MviN homologs were essential either singly or in combination by generating chromosomal deletions of their genes. Insertions in *ykvU* have been reported but not a full deletion (1), suggesting that *ykvU* was not essential. Thus, for *ykvU* and *ytgP*, the ribosome binding site and the entire coding region were replaced with an erythromycin resistance gene (33). These strains (and their isogenic relatives that had a spectinomycin resistance gene inserted in place of the erythromycin resistance gene) had normal growth rates (Fig. 2A) and cell morphology (Fig. 2B). Since *yabM* coding sequences partially overlap with the gene directly downstream, *yabN*, the last 300 bp of *yabM* was maintained. Again, this strain exhibited no growth defect in log phase (Fig. 2A), no change in cell morphology (Fig. 2B), and no sporulation defect (see Table 4).

While none of the four single mutations produced vegetative growth defects that would be expected for a putative lipid II flippase (Fig. 2), it is possible that they could compensate for the loss of each other. A similar redundancy is seen with the *B. subtilis* class A PBPs, where single mutations lead to very mild growth defects (7, 22). To investigate this possible redundancy, we generated a quadruple mutant strain (JDB2361) that contained null mutations in *ytgP*, *ykvU*, *yabM*, and *spoVB*. Transformation efficiencies for the movement of each single mutant allele for the construction of the quadruple mutant strain did not vary significantly from that of control allele markers (Table 3). This strain showed a stage V sporulation defect, consistent with the presence of the *spoVB* mutation; however, no growth defect or change in cell morphology was seen, as would be expected if the homologs acted redundantly (Fig. 2). Thus, the viability of the quadruple mutant suggests that none of the MviN homologs acts as the putative lipid II flippase.

Cellular localization of MviN homologs. Given the lack of an observable phenotype of strains carrying mutations in the MviN homologs, GFP fusions were generated to further examine their physiological roles. We investigated whether any of the putative homologs were recruited to sites of peptidoglycan synthesis by constructing C-terminal GFP fusions based on the predicted location of both N and C termini of all four homologs inside the cytoplasm. When these fusions were placed at their endogenous loci, the resulting strains did not show any defect in growth or sporulation, including the strain expressing SpoVB-GFP, indicating that this fusion was fully functional for spore cortex formation (Table 4). Since the other three homologs did not have any phenotype when mutated, it was not possible to assess whether the GFP fusions complemented for function.

All fusions with the exception of YabM-GFP produced detectable GFP signals. SpoVB-GFP was seen only during sporulation (Fig. 3B), consistent with the sporulation defect observed in a *spoVBΔ::tet* mutant (23) and with the observation that it is under σ^E control (32). Recruitment to the outer forespore membrane was scored by taking the ratio of average maximum forespore fluorescence to average maximum mother cell fluorescence (24). SpoVB-GFP is likely at least partially recruited to the outer forespore membrane, although the ratio obtained (2.50 ± 0.57) was lower than that reported for sporulation proteins such as SpoVE (24). YtgP-GFP was observed at the septum during vegetative growth (Fig. 3A), suggesting that it has a role in division. This localization was not unexpected despite a lack of a phenotype for $\Delta ytgP$, as *E. coli* MviN was implicated in growth and septation. YkvU-GFP accumulated

	20	40	60

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SpoVB     ------MAKQ TFLKGTLILI AAGMVTRMLG FVNRVVIARF IGE--EGVGL YMMAAPTFFL  52
YkvU      --------MN RFVKGIILLS IAAFFAECLE FVVNMILARE LGE--HGMGL YMSILPTIFL  50
YtgP      -------MSS KLLRGTFVLT LGTYISRILG MVYLIPFSIM VGA--TGGAL FQYGYNQYTL  51
YabM      MDDSIGVKRH WIWQGAFVLI LAGVITKILS AVYRVPFQNI VGD--VGFYI YQQVYPFLGI  58
MviN      ---------M NLLKSLAAVS SMTMFSRVLG FARDAIVARI FGAGMATDAF FVAFKLPNLL  51
Consensus ------XXXX XXXXGXXXLX XVXXXXXXXX XGX--XGXXX XXXXXXXXXL

                        80        100        120
SpoVB     ATTLTQFGLP VAISKLVAEA SARGDHQKTK NILVMSLTIT GVLSLIFTPL FLFFAPVMAE 112
YkvU      IIVIASLELP ISISKFIAES NPKLHESMLR HAFRMTAIFT -AFSTAAASI ALPFIPVFDT 109
YtgP      FLNIATMGFP AAVSKFVSKY NSKGDYETSR KMLKAGMSVM LVTGMIAFFI LYLSAPMFAE 111
YabM      AVMLSTSGFP VIISKLMNDY SEKNHHTILK ISALFLSLIG ILLFLCLYLG AVPIALFMGD 118
MviN      RRIFAEGAFS QAFVPILAEY KSKQGEDATR VFVSYVSGLL TLALAVVTVA GMLAAPWVIM 111
Consensus XXXXXXXXXP XXXSKXXXXX XXKXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXAPXXXX

                       140        160        180
SpoVB     TMLTDKRT-- ------LYPL LAITPVVPII AISSVLRGYF QGKQNMNPLA MSQVLEQVVR 164
YkvU      YHPFIKG--- ---------- -IVIGLIPVV AFTSIARGYF MGVQKMGKIA IANVLKKIIQ 155
YtgP      ISLGGKDNNG LTIDHVVYVI RMVSLALLVV PIMSLVRGFF QGHQMMGPTA VSQVVEQIVR 171
YabM      SHLAVLIQ-- ---------- -VAAYAFLLF PFVALLRGGF QGRHEMLPSA LSQMTEQFLR 165
MviN      VTAPGFADTA DKFALTSQLL KITFPYILLI SLASLVGAIL NTWNRFSIPA FAPTLLNISM 171
Consensus XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXSXXRGXF XGXXXMXXXA XXXXXXXXXX

                       200        220        240
SpoVB     ISLVAVCTTI FLP---YGIE YAAAGAMLSS VAGELASLLY LFVCFKYKKT ---IKIRKHF 218
YkvU      LLCLFLFFQW YS---FELD MAVLISLFVL VVSDVVVLVY LYSQFIMARR ---ALSGQQH 208
YtgP      IIFLLSATFL ILKVFNGGLV IAVGYATFAA LIGAFGGLVV LYIYWNKRKG SLLAMMPNTG 231
YabM      VAVLLGLSFW LVKKG-ASLY TAGAAAASGS LAGSLVALII LGFFWFKTKR ---DNQTDRQ 221
MviN      IGFALFAAPY FNP------- -PVLALAWAV TVGGVLQLVY QLPHLKKIGM LVLPRINFHD 223
Consensus XXXXXXXXXX XXXXX-XXXX XAXXXXXXXX XXGXXXLXX LXXXXXXXXX XXLXXXXXXX

                       260        280        300
SpoVB     LQSIKNGKQT FTQLMSVSLP TTGSRFIGNL SWFFEPIVVA QSLAIAGVAT VAATKQYGEL 278
YkvU      IHLR--GKDV RKRLLAVSIP TTGLRIFHAV VNAIEPFLVK GALLAAGVAG TAAIDQYGML 266
YtgP      PTANLSYKKM FFELFSYAAP YVFVGLAIPL YNYIDTNTFN KAMIEAGHQA IS-QDMLAIL 290
YabM      NENVITTKEL TKKLLLYSVT ICVSSLLLLF IQLVD--ALN LYALLSGGEA SEEAKCLKGI 279
MviN      AGAMRVVKQM GPAILGVSVS QISLIINTIF ASFLASGSVS WMYYADR--- ---------- 270
Consensus XXXXXXXXXX XXXLXXXSXX XXXXXXXXXX XXXXXXXXXX XXXXXXGXXX XXXXXXXXXX

                       320        340        360
SpoVB     TGFAMTLLTL PS-FITYSLS TALVPAISEG MEQKKLQVVE YRLEQAMRLC LLSGGISVVI 337
YkvU      AGVAVTIGSF PA-FIAHSLM VVMIPSISEA YALSQYDIVL KRLKQSIFIT LGYGIPAVWV 325
YtgP      TLYVQKLVMI PV-SLATAFG LTLIPTITES FTSGNYKLLN QQINQTMQTI LFLIIPAVVG 349
YabM      YDRGQPLLQL GS-VFAVSIA TSLVPYISMA VKNKELKIMK EKITSSLKLC LVLGTGASAG 338
MviN      ------LMEF PSGVLGVALG TILLPSLSKS FASGNHDEYN RLMDWGLRLC FLLALPSAVA 324
Consensus XXXXXXLXXX PX-XXXXXXX XXLXPXISXX XXXXXXXXXX XXXXXXXXXX LXXXXXXXXX

                       380        400        420
SpoVB     LFVFADELMR VMYGSS---- ----GAAVFI KVMAPFFLLY YFQGPLQAVL QALNLAGAAM 389
YkvU      MFQFAGPLTH LFFHSP---- ----EAQYYL QLLWPYFLFH LFVMPLQACL IGMGFVKEAF 377
YtgP      ISLLSGPTYT FFYGSESL-- -HPELGANIL LWYSPVAILF SLFTVNAAIL QGINKQKFAV 406
YabM      LICILKPVNI MLFQNG---- ----EGTGAL QVFSCSILFA SLAVTAAAVL QGAGYTVFPA 390
MviN      LGILSGPLTV SLFQYGKFTA FDALMTQRAL IAYSVGLIGL IVVKVLAPGF YSRQDIKTPV 384
Consensus XXXXXXPXXX XXXXXXXX-- -XXXXXXXL XXXXXXXXXX XXXXXXXAXL XXXXXXXXXX

                       440        460        480
SpoVB     MNSLIGALVK TGLIFVLATR PSLG------ IMGAALAIVT GMVLVTLLHA ATVSKVLPIS 443
YkvU      YHNVWSHIVA LSMMYVLGSM ENLQ------ MLGIILGMNT GMILLTSLHY ATICKALKVS 431
YtgP      VSLVIGIVIK LVLNVPLIKL MQADGAILAT ALGYIASLLY GFIMIKRHAG YSYKILVKRT 466
YabM      IAVGAGVAVK WVLNTLLVPR YGIEG--ASL ATAASFAAVA GLNLYQLRQK EWLDKLRGVL 448
MviN      KIAIVTLILT QLMNLAFIGP LKHAG--LSL SIGLAACLNA SLLYWQLRKQ KIFTPQPGWM 442
Consensus XXXXXXXXXX XXXXXXLXXX XXXXG--XXX XXGXXXXXXX GXXXXXXXXX XXXXXXXXXX

                       500        520        540
SpoVB     IKIKEYALSF AVIV-ICGFI SSAIKQYISF GASEAVNLAG WIAAS-AAIY MILLLVFRLI 501
YkvU      VFLTGGTRTP RIEG------ ---------- ---------- ---------- ---------- 445
YtgP      VLMLVLSAIM GIAVKIVQWV LGFFISYQDG QMQAAIVVVI AAAVG-GAVY LYCGYRLGFL 525
YabM      IPIIGSALLM SAVLLAYTRL WTFLFP-ATG RGAAVIESLS AVAIG-GAVF IYCMMRLGIF 506
MviN      AFLLRLVVAV LVMSGVLLGM LHIMPEWSLG TMPWRLLRLM AVVLAGIAAY FAALAVLGFK 502
Consensus XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXX-XXXX XXXXXXXXXX

                       560
SpoVB     KKDELRRIPI ------IGRL IIR--- 518
YkvU      ---------- ---------- ------ 445
YtgP      QKILGRRLPG ------FFRK GRHAG- 544
YabM      TDEELNSVPF GSKLSKFMRR REQNGG 532
MviN      VKEFARRTV- ---------- ------ 511
Consensus XXXXXXXXXX ------XXRX XXXXG-
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FIG. 1. Alignment of *B. subtilis* MviN homologs. A ClustalW alignment of *E. coli* MviN and *B. subtilis* SpoVD, YtgP, YabM, and YkvU is shown. The alignment was generated after a BLASTp query of the *B. subtilis* genome with the *E. coli* MviN sequence followed by a second BLASTp query using YtgP, the highest-scoring sequence.
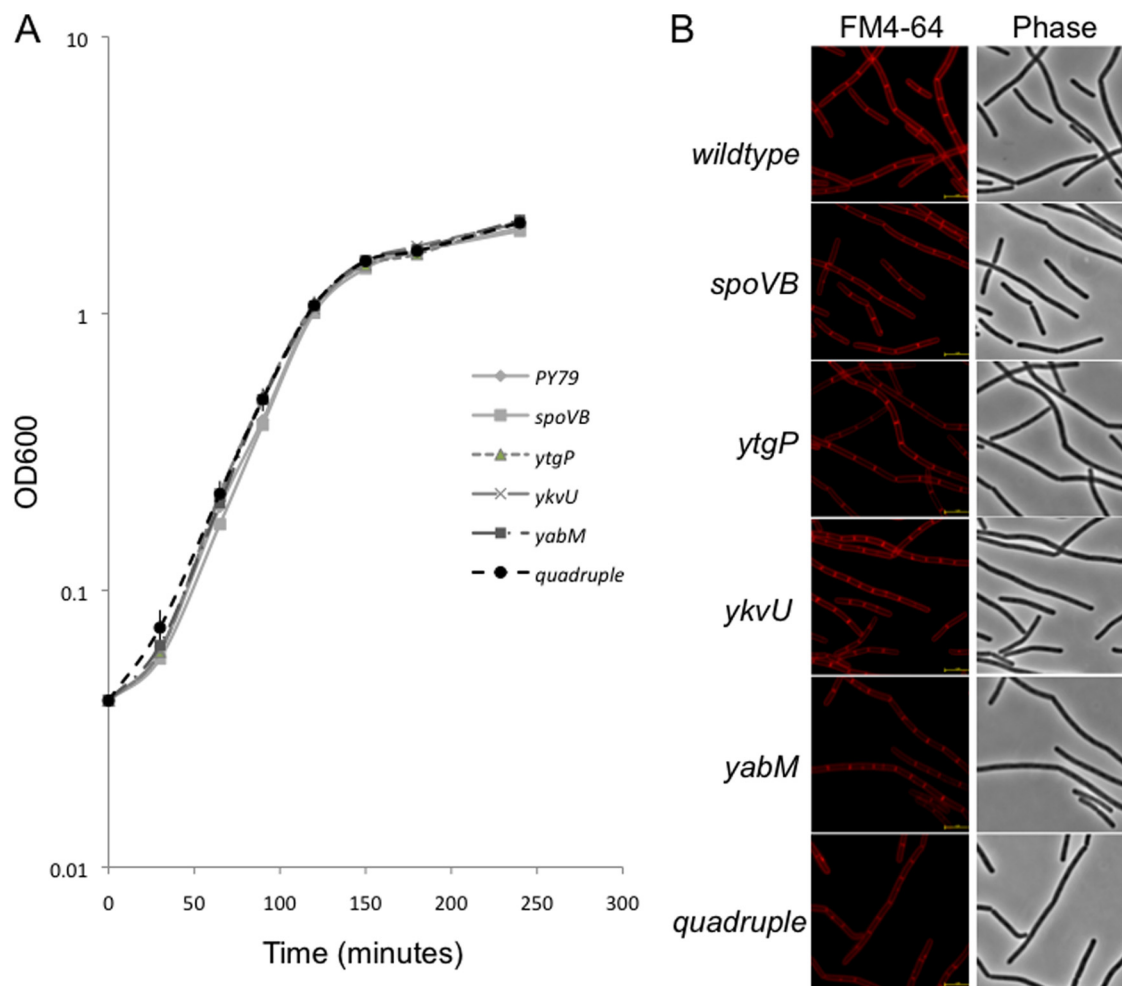
FIG. 2. Growth of *B. subtilis* strains carrying mutations in putative MviN homologs. (A) Strains carrying the single mutation Δ*ytgP*::*mls* (JDB2327), Δ*ykvU*::*mls* (JDB2330), Δ*yabM*::*cm* (JDB2386), or *spoVB*Δ::*tet* (JDB1097) or all four mutations (JDB2361) were grown in LB at 37°C following dilution to an optical density at 600 nm (OD$_{600}$) of 0.04 from an overnight culture grown in LB. OD$_{600}$ was measured at a series of time points for each of the strains. (B) *B. subtilis* strains carrying the mutations in single MviN homologs or mutations in all four homologs were grown in LB to an OD$_{600}$ of 0.9. After collection, cells were resuspended in phosphate-buffered saline with 1 mg/ml FM4-64 and imaged. Bar, 5 mm.

TABLE 3. Transformation efficiencies for construction of mutant strains[a]

Donor and/or selected mutation	Recipient	CFU/μg genomic DNA
amyE::*cm*	PY79	415
JDB1097 (Δ*spoVB*::*tet*)	PY79	435
JDB2327 (Δ*ytgP*::*mls*)	PY79	489
JDB2330 (Δ*ykvU*::*mls*)	PY79	420
JDB2386 (*yabM*::*cm*)	PY79	438
sacA::*mls*	JDB1097	280
JDB2327 (Δ*ytgP*::*mls*)	JDB1097	227
amyE::*spc*	JDB2347	354
JDB2355 (Δ*ykvU*::*mls*:*spc*)	JDB2347	380
amyE::*cm*	JDB2361	335
JDB2386 (*yabM*::*cm*)	JDB2361	322

[a] Competent cells were transformed with 1 μg of donor genomic DNA and selected for the marker listed. No single MviN homolog mutation differed significantly from the control donor genomic DNA. Introduction of the *yabM*::*cm* allele into the triple mutant strain carrying the *ytgP*::*mls* allele resulted in DNA that did not differ significantly from the control donor DNA. The similar transformation efficiencies obtained indicated that second-site suppressors were probably not generated.

at the outer membrane of an early engulfing forespore (Fig. 3B) as previously described (10), and no vegetative signal was detected. While YkvU-GFP was recruited to the outer forespore membrane (3.62 ± 0.64), it does not seem to play a vital role in spore cortex formation, since a strain carrying a Δ*ykvU* mutation produced wild-type levels of heat-resistant spores (Table 4).

MviN homologs require additional sporulation proteins for localization. Since the targeting of proteins to the outer forespore membrane during sporulation is the subject of much interest (3), we investigated whether other proteins were necessary for the recruitment of YkvU and SpoVB. For example, SpoVD, like SpoVB, is required for the spore cortex synthesis, and its localization to this membrane is dependent on SpoVE (A. Fay and J. Dworkin, unpublished data). However, both YkvU-GFP and SpoVB-GFP were recruited appropriately in the absence of SpoVE. We examined their localization in a Δ*spoIIIAH* background, since SpoIIIAH is necessary for the proper recruitment of SpoIVFA to the forespore (3, 8). SpoVB-GFP was recruited to the outer forespore membrane

TABLE 4. Sporulation of *B. subtilis* strains carrying an inducible MviN allele

Strain	Genotype	CFU[a]		% Sporulation (±SD)
		Preheat	Postheat	
PY79	Wild type	5.5×10^8	5.0×10^8	91 ± 5.7
JDB2327	Δ*ytgP*::*mls*	3.5×10^8	3.3×10^8	90 ± 2.6
JDB2330	Δ*ykvU*::*mls*	4.7×10^8	4.5×10^8	89 ± 4.7
JDB2386	*yabM*::*cm*	3.7×10^8	3.5×10^8	91 ± 2.7
JDB1097	*spoVB*Δ::*tet*	1.6×10^8	0	0
JDB2329	*spoVB-gfp spc*	4.8×10^8	4.4×10^8	90 ± 2.4
JDB2437	*spoVB*Δ::*tet amyE*::P$_{sweet}$-*spoVB-gfp spc*	3.3×10^8	3.0×10^8	91 ± 4.9
JDB2506	*amyE*::P$_{sweet}$-*mviN spc*	3.7×10^8	3.5×10^8	92 ± 4.4
JDB2504	*spoVB*Δ::*tet amyE*::P$_{sweet}$-*mviN spc*	3.9×10^8	2.4×10^8	60 ± 5.8

[a] Strains were sporulated in DSM for 24h at 37°C. Cultures were diluted, and CFU were counted before and after exposure to 80°C for 20 min.

in a SpoIIIAH-dependent manner, since the ratio was significantly lower in a *spoIIIAH* mutant strain ($P < 0.001$) than in the wild type (Fig. 4A). Furthermore, SpoVE-GFP was recruited to the outer forespore membrane in a *spoVB*Δ::*tet* background (data not shown), suggesting that these proteins localize independently of each other and that defects in spore cortex formation are not due merely to a localization effect on each other. YkvU-GFP followed a similar pattern, in which it failed to be recruited in the absence of SpoIIIAH, with a ratio

of 2.09 ± 0.38 ($P < 0.001$), but was recruited in the absence of SpoVE, with a ratio of 3.71 ± 0.54 (Fig. 4B).

A *spoVB*Δ::*tet* mutant is not rescued by overexpression of UppS. A plasmid-borne copy of *uppS*, encoding the undecaprenyl pyrophosphate synthase, rescues the growth defect at the nonpermissive temperature of an *E. coli* strain carrying an *mviN* temperature-sensitive mutation (16). This mutation lies in the *mviN* promoter region and likely leads to a decrease in MviN levels. Since the undecaprenyl chain is used to form a
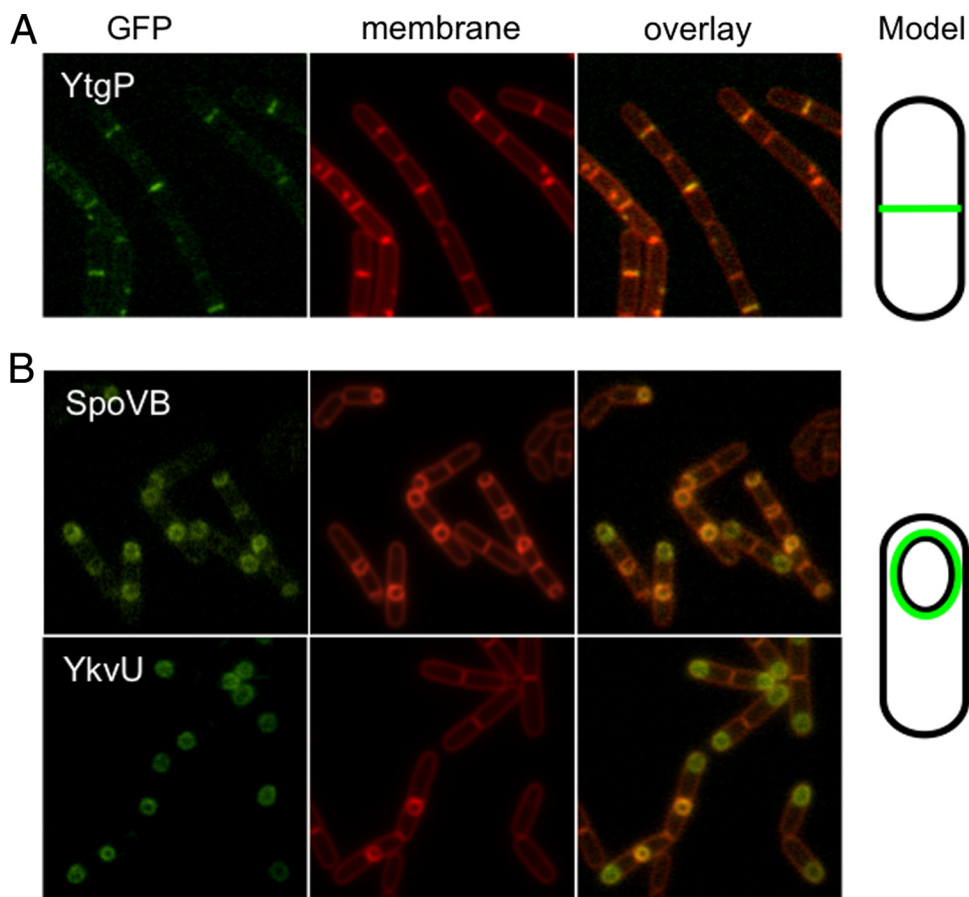

FIG. 3. Localization of GFP fusions. (A) A strain expressing YtgP-GFP (JDB2356) was grown in CH growth medium, cells were collected at an OD$_{600}$ of 0.7, and the membranes were stained with FM4-64. (B) Strains expressing SpoVB-GFP (JDB2329) and YkvU-GFP (JDB2358) were sporulated by resuspension in A+B medium, cells were collected at T3 of sporulation, and membranes were stained with FM4-64.
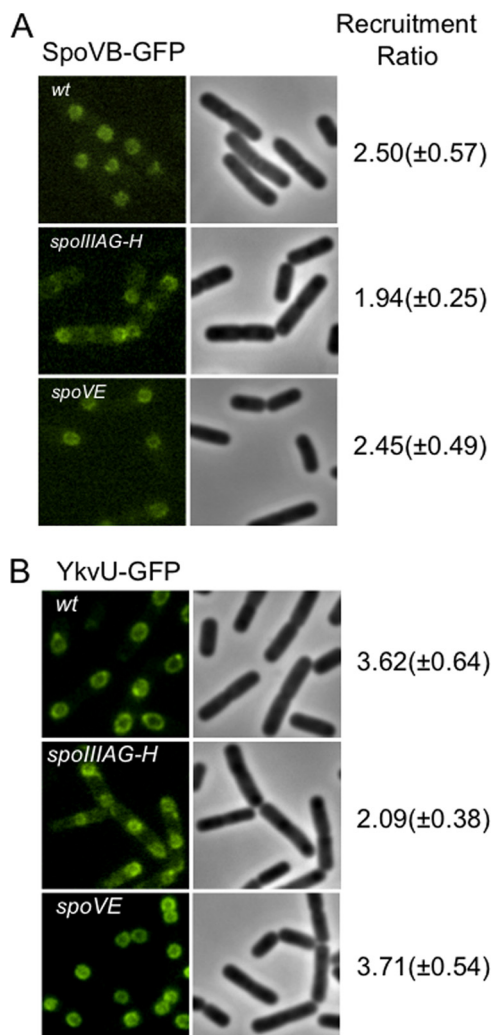
A

SpoVB-GFP

Recruitment Ratio

wt   2.50(±0.57)

spoIIIAG-H   1.94(±0.25)

spoVE   2.45(±0.49)

B

YkvU-GFP

wt   3.62(±0.64)

spoIIIAG-H   2.09(±0.38)

spoVE   3.71(±0.54)

FIG. 4. SpoVB-GFP and YkvU-GFP targeting requires additional sporulation-specific proteins. (A) SpoVB-GFP-expressing strains JDB2329 (wt), JDB2416 (*spoIIIAG-H*::*kan*), and JDB2418 (*spoVE*::*tet*) were sporulated by resuspension, and images were taken at 3 h. (B) YkvU-GFP-expressing strains JDB2358 (wt), JDB2377 (*spoIIIAG-H*::*kan*), and JDB2378 (*spoVE*::*tet*) were sporulated by resuspension, and images were taken at T3. Recruitment ratios are the ratios of the average maximum forespore fluorescence to the average maximum mother cell fluorescence.

number of metabolic intermediates, including lipid I (the immediate precursor of lipid II), this result suggests that MviN is involved in lipid II synthesis. We therefore investigated whether a *spoVB*Δ::*tet* mutation could be similarly rescued by constructing a merodiploid strain that carried an additional, inducible copy of *uppS*. However, the sporulation defect of the *spoVB*Δ::*tet* background was not rescued by overexpression of UppS or UppS-FLAG (data not shown). We confirmed that UppS was overexpressed by introducing a FLAG tag into the construct (data not shown). While the inability of UppS to rescue the *spoVB*Δ::*tet* mutation may indicate that SpoVB and MviN are not true homologs, this may be due to unrelated reasons. The lack of a detectable phenotype of mutations in any of the other candidate MviN homologs excludes examining their possible suppression by UppS.
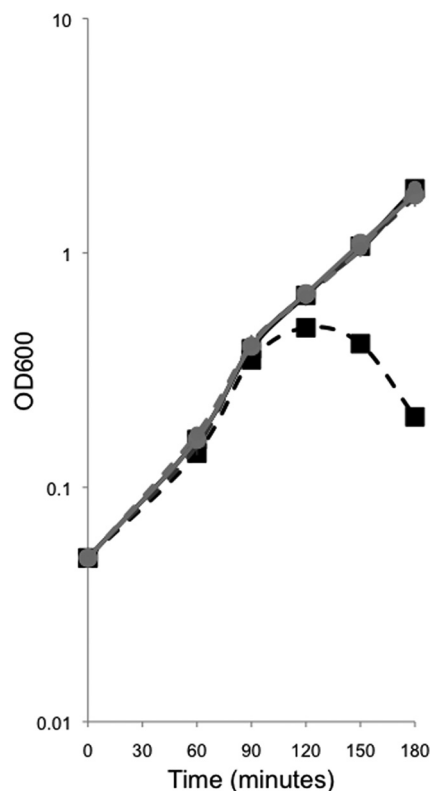

FIG. 5. SpoVB-GFP and YtgP-GFP rescue the *mviN* depletion mutant. JDE1148 (pMMB207) (■), JDE1140 (pAF374) (+), and JDE1147 (pAF375) (●) were depleted for *mviN* by growth in LB in the absence (dashed) or presence (solid) of the inducer arabinose. The presence of pMMB207 did not affect *mviN* depletion, as the strain required arabinose for growth and IPTG had no effect. Leaky expression of YtgP-GFP or SpoVB-GFP rescued the *mviN* mutation, as seen by growth without arabinose.

MviN complements SpoVB function during sporulation. SpoVB is essential for spore formation, since a strain carrying a *spoVB* deletion does not produce any heat-resistant spores (23) (Table 4). This phenotype is complemented by expression of a SpoVB-GFP fusion (Table 4). We demonstrated that MviN (MurJ) was a homolog of SpoVB by placing MviN under inducible (P_{sweet}) control in a Δ*spoVB* strain. In the absence of inducer, no spores were observed in this strain, similar to what was observed with the Δ*spoVB* parental strain (Table 4). However, addition of inducer resulted in spore production by this strain that was only slightly reduced compared to that of the wild type (Table 4). This complementation indicates that MviN (MurJ) and SpoVB are homologs.

YtgP or SpoVB complements the growth defect of an *E. coli* MviN depletion strain. An *E. coli* strain carrying MviN under inducible (P_{BAD}) control is dependent on arabinose for growth (16, 26). To determine whether the growth defect in the absence of arabinose could be complemented by a *B. subtilis* homolog, we introduced into this strain a plasmid containing a C-terminal GFP fusion of one of these homologs, YtgP, under the control of P_{tac}. This strain was now able to grow in the absence of arabinose, unlike the parent strain, indicating that YtgP complemented this growth defect (Fig. 5). We also introduced into the MviN depletion strain a plasmid containing

a C-terminal GFP fusion to a second homolog, SpoVB, under P*tac* control. As with YtgP, this strain was able to grow in the absence of arabinose, unlike the parent strain, indicating that SpoVB also complemented this growth defect (Fig. 5). Lethality was noted when YtgP-GFP or SpoVB-GFP expression was induced in parental strain MG1665 and the MviN depletion strain; however, low levels of leaky expression were not. All complementation analysis was therefore performed without addition of the inducer IPTG to avoid this toxicity (data not shown). Thus, two of the *B. subtilis* homologs identified by BLAST (Fig. 1; Table 2) complemented the lethality resulting from MviN depletion, even at the low levels of induction resulting from read-through transcription. Taken together, these genetic complementation experiments demonstrate that the *B. subtilis* proteins we identified by bioinformatics are likely to be true MviN homologs.

DISCUSSION

Very recently, it was proposed that the essential MviN protein of *E. coli* is the lipid II flippase in this organism (26). Consistent with this hypothesis, an *mviN* temperature-sensitive mutation results in the accumulation of peptidoglycan precursors and cell lysis, similarly to depletion of MviN (16). If MviN is the lipid II flippase, then MviN homologs in organisms that also utilize lipid II are likely to be essential as well. We examined this possibility in *B. subtilis*, a gram-positive organism that, like *E. coli*, uses diaminopimelic acid-containing lipid II, by identifying putative MviN homologs using BLASTp-based analyses. Four proteins showed significant homology either to MviN or to each other and were predicted to contain a number of transmembrane sequences similar to that in MviN (Fig. 1). We confirmed that MviN complemented the sporulation defect of a *B. subtilis* strain carrying a null mutation in SpoVB, one of these homologs (Table 4). In addition, two of these homologs, YtgP and SpoVB, complemented the growth defect of an *E. coli* strain carrying an inducible allele of MviN (Fig. 5). Thus, these four proteins are likely to be real MviN (MurJ) homologs. Recently, an essential YtgP homolog from *Streptococcus pyogenes*, Spy_0390, was shown to complement MviN depletion strains of *E. coli* (27), although its physiological role in *S. pyogenes* is not well understood.

Strains carrying mutations in each or all of these homologs did not exhibit any shape or growth rate defects (Fig. 2). This result is inconsistent with the expected requirement for lipid II flipping for growth and suggests that other proteins mediate this function. It is formally possible, however, that an additional unidentified gene that lacks significant sequence homology to MviN could be functionally redundant for lipid II flipping.

If these MviN homologs are not lipid II flippases, what role do they play in *B. subtilis* physiology? SpoVB is necessary for spore cortex formation, and a Δ*spoVB* mutation results in the accumulation of peptidoglycan precursors (32). Further, the localization of SpoVB-GFP to the outer forespore membrane (Fig. 3) is consistent with a role in spore peptidoglycan synthesis. However, this localization does not depend on SpoVE, another protein essential for spore peptidoglycan synthesis (Fig. 4). This lack of dependence is different from that observed with another protein, SpoVD, that is also essential for spore peptidoglycan synthesis, suggesting that SpoVB and SpoVE/SpoVD are present in different complexes and therefore may mediate different functions.

The recruitment of YtgP-GFP to septa (Fig. 3) indicates a possible role in division, although it is not essential and vegetative cells lacking YtgP divide and grow normally (Fig. 2). Mutagenesis of *Listeria monocytogenes* identified YtgP as a virulence factor in a mouse model (2). This particular screen identified several other genes involved in peptidoglycan modifications, suggesting that YtgP may play a role in such processes. YkvU localizes to the outer forespore membrane (Fig. 3), and its dependence on SpoIIIAH for this targeting (Fig. 4) suggests that it may play a role in late spore formation and/or germination (1, 15), although it is not required for the production of heat-resistant spores. Our failure to observe any specific localization of YabM-GFP and the lack of any observable phenotype associated with a Δ*yabM* mutation make it difficult to identify a physiological role for this protein.

Our data do not rule out the possibility that these MviN homologs play an accessory but nonessential role in lipid II translocation. Recently, it was suggested that the yeast protein Rft1, a member of the family that includes MviN, may not act as a flippase directly but rather may facilitate glycolipid flipping (11), since in vitro flippase activity appeared to result from a protein or proteins other than Rft1 (28). By analogy, *E. coli* MviN and the *B. subtilis* MviN homologs we have characterized would have a role in peptidoglycan synthesis that is essential in *E. coli* but that is not essential in *B. subtilis*.

Alternatively, MviN could be involved in a cell wall modification that utilizes the isoprenoid chain that is also a component of lipid II. Thus, cell lysis would result from an indirect defect in the recycling of the isoprenoid chain and not from a direct defect in peptidoglycan synthesis. A similar scenario occurs in the teichoic acid synthesis pathway in *B. subtilis*, which was long thought to be essential. However, strains lacking TagO, the first enzyme in the pathway, are viable, and mutations of enzymes later in the pathway are probably lethal due to their sequestration of the isoprenoid chain (6). It is therefore possible that the suppression of the *mviN* temperature-sensitive mutant by *uppS*, the gene encoding the undecaprenyl pyrophosphate synthetase, is similarly the result of sequestration of the isoprenoid chain component of lipid II. This suggestion is reinforced by the ability of either YtgP or SpoVB to complement the growth defect of a strain carrying an inducible allele of MviN but only in the absence of induction. This lethality suggests that the overexpression of YtgP or SpoVB depletes the cell of an essential substrate, such as the isoprenoid chain. Regardless of the biochemical activity of these proteins, the observation that they are not required for growth indicates that they are not likely to mediate the essential process of lipid II flipping.

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