

# Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*

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**Peptidoglycan is a cell-wall glycopeptide polymer that protects bacteria from osmotic lysis. Whereas in Gram-positive bacteria it also serves as scaffold for many virulence factors, in Gram-negative bacteria, peptidoglycan is an anchor for the outer membrane. For years, we have known the enzymes required for the biosynthesis of peptidoglycan; what was missing was the flippase that translocates the lipid-anchored precursors across the cytoplasmic membrane before their polymerization into mature peptidoglycan. Using a reductionist bioinformatics approach, I have identified the essential inner-membrane protein MviN (renamed MurJ) as a likely candidate for the peptidoglycan flippase in *Escherichia coli*. Here, I present genetic and biochemical data that confirm the requirement of MurJ for peptidoglycan biosynthesis and that are in agreement with a role of MurJ as a flippase. Because of its essential nature, MurJ could serve as a target in the continuing search for antimicrobial compounds.**

endosymbiont | genome | lysis | murein | reductionist

**A**lmost all bacteria possess an extracytoplasmic glycopeptide polymer known as peptidoglycan (or murein) that is composed of glycan chains connected by peptide bridges (1–3). This mesh-like rigid structure protects bacteria from lysis caused by osmotic pressure and determines their shape. In addition, peptidoglycan serves as an anchor for virulence factors and cellular structures (4). For these reasons, and the fact that peptidoglycan is absent in humans but present in most bacteria, its biogenesis has been one of the preferred targets for antibiotics (5–7).

Biogenesis of peptidoglycan begins in the cytoplasm and culminates in the extracytoplasmic environment, where assembly and cross-linking of glycan chains occurs. Therefore, this process requires the transport of intermediates across the inner (or cytoplasmic) membrane (IM). In *Escherichia coli*, peptidoglycan biogenesis starts with the synthesis of the nucleotide precursors in the cytoplasm (Fig. 1) (8). The GlmSMU enzymes synthesize UDP-N-acetylglucosamine (UDP-GlcNAc), which is then transformed in step-wise fashion to UDP-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide) by MurABCDE. At the IM, MraY transfers the MurNAc-pentapeptide to the C<sub>55</sub>-lipid carrier undecaprenyl phosphate to form the undecaprenyl pyrophosphoryl-MurNAc-pentapeptide known as lipid I (1, 2). GlcNAc is next added to lipid I by the IM-associated MurG enzyme, resulting in lipid II (1, 2). Lipid II is then flipped across the IM by an unknown mechanism that involves an unidentified protein(s) (2, 9). Once at the periplasmic side of the IM, the penicillin-binding proteins (PBPs) use lipid II to catalyze the maturation of peptidoglycan into a mesh-like three-dimensional structure (3, 10).

Maturation of peptidoglycan involves the assembly and elongation of the glycan chains by glycosyltransferases, cross-linking of their peptide chains by transpeptidases, and removal of the terminal D-Ala from the stem pentapeptide by DD-carboxypeptidases (Fig. 1) (3, 10). The peptidoglycan glycosyltransferases use lipid II to assemble and elongate the glycan chains. During this processive chain-elongation step, undecaprenyl pyrophosphate is released from the growing glycan chain as new lipid II precursors are added (11); the lipid carrier can then be recycled

in a process that is not well understood but that is known to involve dephosphorylation by multiple phosphatases and transport across the IM (1, 12). In *E. coli*, recycled and newly synthesized undecaprenyl phosphate can be used in new rounds of peptidoglycan biosynthesis as well as in the transport across the IM of other cell envelope polysaccharides, such as lipopolysaccharides (LPS) and enterobacterial common antigen (ECA) (13, 14). Thus, although we do not understand how undecaprenyl pyrophosphate is flipped back across the IM for recycling, the only unknown factor required for an essential step unique to peptidoglycan biogenesis is the flippase that transports lipid II across the IM.

For decades, a large body of research has elucidated the steps of peptidoglycan biogenesis and the enzymes that catalyze them, except for how lipid II is translocated across the IM. Lipid II flippase is, therefore, the last essential player in peptidoglycan biogenesis that remains to be identified (2, 9). Because the search for the lipid II flippase using standard genetic and biochemical approaches has proven technically difficult and unsuccessful (2), I performed a reductionist bioinformatics search that led me to propose that MviN (renamed MurJ) is the putative lipid II flippase in *E. coli*.

## Results

**Bioinformatics Search for the *E. coli* Lipid II Flippase.** Recently, together with the T. J. Silhavy and D. Kahne laboratories, I searched for essential outer membrane (OM) biogenesis factors in *E. coli* using a reductionist bioinformatics approach that reduced the number of possible candidates from ≈2,000 proteins of unknown function to 3 (15). All three candidates have been shown to be the missing components of the machinery that transports LPS from the IM to the OM (15, 16). A key step of this approach was based on the realization that a factor that is essential for OM biogenesis in *E. coli* would be conserved in Gram-negative endosymbiotic bacteria despite these organisms having very small genomes. Even though evolution of endosymbionts is marked by extensive gene loss, their genomes should still encode all of the machinery required for building an OM (15).

Here, I applied a similar reductionist approach to identify the lipid II flippase of *E. coli*. If *E. coli* has a protein that is required for flipping peptidoglycan lipid II across the IM, this factor would be: (i) an IM protein, (ii) conserved among Gram-negative endosymbiotic bacteria that possess peptidoglycan, (iii) absent in bacteria that lack peptidoglycan, and (iv) essential in *E. coli*, unless there are two or more proteins that can perform this function. To conduct this search, I obtained the list of 963 predicted IM proteins (integral, bitopic, and lipoproteins) from

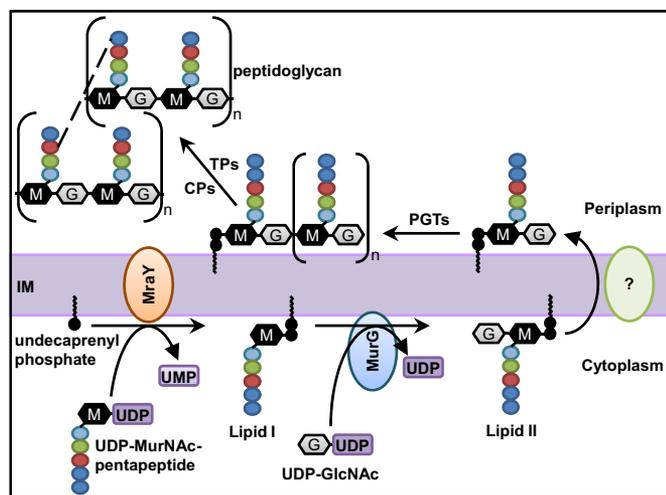
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The author declares no conflict of interest.

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**Fig. 1.** Biogenesis of peptidoglycan in *E. coli*. After being synthesized by MurABCDEF in the cytoplasm, UDP-MurNAc-pentapeptide is transferred to undecaprenyl phosphate by MraY, generating lipid I. Next, MurG synthesizes lipid II by adding GlcNAc (from UDP-GlcNAc) to lipid I. Lipid II is translocated across the IM by an unknown flippase. At the periplasmic side of the IM, glycosyltransferases (PGTs) assemble and elongate glycan chains, and transpeptidases (TPs) cross-link their peptide chains (dashed lines). DD-carboxypeptidases (CPs) remove terminal D-Ala from the stem pentapeptide in mature peptidoglycan.

EchoLOCATION (17). Next, I determined which of these 963 proteins are present in the proteomes of the two endosymbiotic bacteria *Blochmannia floridanus* and *Buchnera aphidicola* str. APS. I chose these endosymbionts because both are closely related to *E. coli* but their proteomes are  $\approx 86\%$  smaller than *E. coli*'s; nevertheless, proteomes of both endosymbionts contain enzymes required for peptidoglycan biosynthesis, such as MraY and MurG. However, the cell envelopes of these endosymbionts are not identical, because the proteome of *Bl. floridanus* contains the proteins required for LPS synthesis and transport, whereas that of *B. aphidicola* str. APS does not (15, 18).

Using GeneVenn (19), I found that 854 of the 963 predicted IM proteins of *E. coli* are absent in both endosymbionts. Reflective of the intracellular lifestyle of these endosymbionts, this large subset of IM proteins include many known nutrient transporters, proteins involved in sensing the environment, such as histidine kinases, and factors involved in the biogenesis of surface polysaccharides, such as the *O*-antigen of LPS and ECA (data not shown).

This analysis also revealed that only 39 *E. coli* IM proteins are

present in both *Bl. floridanus* and *B. aphidicola* str. APS. As expected, these 39 proteins include factors required for essential processes such as the insertion of IM proteins into the IM, protein transport across the IM, processing of signal sequences, transport of lipoproteins from the IM to the OM, peptidoglycan biosynthesis, and cell division [supporting information (SI) Table S1]. The essential IM protease FtsH and its regulators HfICK are also conserved as well as proteins involved in generating energy, phosphate transport, and cardiolipin biosynthesis (Table S1). More importantly, there are six proteins of unknown function (MviN, YajR, YhgN, YibN, YoaE, and YtfN) among the 39 conserved proteins that could be candidates for the peptidoglycan lipid II flippase (Table S1). I must clarify that the function of MviN is unknown; *mviN* (or *mviS*) was misnamed as a gene encoding a factor required for mouse virulence, but it was later found that the mutation responsible for the virulence phenotype maps to nearby genes responsible for flagellum biogenesis (20). Therefore, because the Mvi nomenclature is a misnomer and for reasons described below, I propose to rename this factor MurJ.

Because a lipid II flippase should be absent in bacteria that lack peptidoglycan, I searched for conservation of these six proteins of unknown function among bacteria that do not produce peptidoglycan. I conducted Genomic BLAST (21) searches of MurJ (MviN), YajR, YhgN, YibN, YoaE, and YtfN in the proteomes of *Mollicutes*, *Dehalococcoides*, *Candidatus Carsonella ruddii* PV, and *B. aphidicola* str. Cc, bacteria known to lack peptidoglycan (22–24). Notably, *B. aphidicola* str. Cc belongs to the same species as the peptidoglycan producer *B. aphidicola* str. APS, and both are members of the *Enterobacteriaceae* family to which *E. coli* belongs. As controls for conservation among peptidoglycan producers, I included in the Genomic BLAST searches proteomes of two endosymbionts that belong to the  $\gamma$ -Proteobacteria class, *B. aphidicola* str. APS and *Candidatus Vesicomysocius okutanii* HA.

These Genomic BLAST analyses revealed that only MurJ was conserved among the two peptidoglycan-producing endosymbionts but absent in those aforementioned bacteria that lack peptidoglycan (Table 1). Interestingly, the cell division protein FtsW, a RodA homolog, has been postulated as a lipid II flippase candidate (25). However, Genomic BLAST searches revealed that the peptidoglycan producer *V. okutanii* lacks FtsW (but has RodA), whereas the peptidoglycan-less *Mollicute Eubacterium dolichum* DSM 3991 has two FtsW homologs, one of which is likely RodA (data not shown). Together, these data suggest that MurJ is the likely candidate for lipid II flippase in *E. coli*.

**Functional Predictions Support MurJ (MviN) as the *E. coli* Lipid II Flippase Candidate.** The bioinformatics search described above identified MurJ as the only putative lipid II flippase in *E. coli*.

**Table 1.** Conservation of proteins of unknown function in proteomes of bacteria that either lack or produce peptidoglycan (PG)

Protein	Lack PG				Produce PG	
	<i>Mollicutes</i> *	<i>D. ethenogenes</i>	<i>B. aphidicola</i> str. Cc	<i>C. ruddii</i> PV	<i>B. aphidicola</i> str. APS	<i>V. okutanii</i> HA
MurJ	–†	–	–	–	5.00e <sup>–126</sup>	4.00e <sup>–94</sup>
YajR	–	3.00e <sup>–05</sup>	–	–	1.00e <sup>–79</sup>	2.00e <sup>–73</sup>
YhgN	–	4.00e <sup>–16</sup>	–	–	9.00e <sup>–61</sup>	–
YibN	3.00e <sup>–04</sup>	1.00e <sup>–05</sup>	1.00e <sup>–03</sup>	–	1.00e <sup>–19</sup>	3.00e <sup>–07</sup>
YoaE	6.00e <sup>–14</sup>	5.00e <sup>–17</sup>	1.00e <sup>–73</sup>	1.00e <sup>–73</sup>	1.00e <sup>–173</sup>	3.00e <sup>–17</sup>
YtfN	–	–	–	–	1.00e <sup>–39</sup>	–

\**Mollicutes* refers to: *Aster yellows witches'-broom phytoplasma* AYW, *Eubacterium dolichum* DSM 3991, *Mesoplasma florum* L1, *Mycoplasma agalactiae* PG2, *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343, *Mycoplasma gallisepticum* R, *Mycoplasma genitalium* G37, *Mycoplasma hyopneumoniae* 232, *Mycoplasma hyopneumoniae* 7448, *Mycoplasma hyopneumoniae* J, *Mycoplasma mobile* 163K, *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1, *Mycoplasma penetrans* HF-2, *Mycoplasma pneumoniae* M129, *Mycoplasma pulmonis* UAB CTIP, *Mycoplasma synoviae* 53, *Onion yellows phytoplasma* OY-M, and *Ureaplasma parvum* serovar 3 str. ATCC 700970.

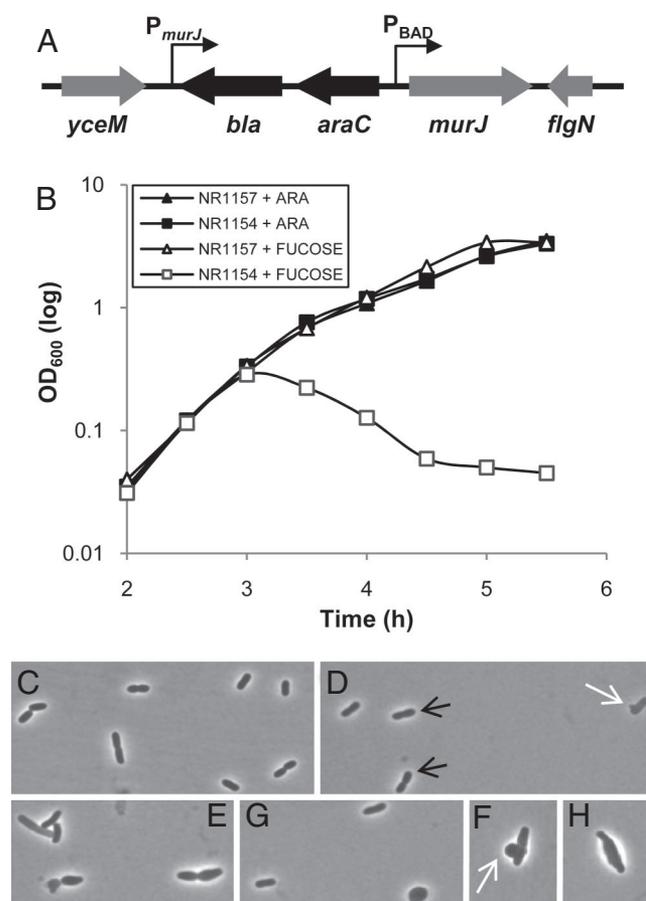
†Numbers refers to E (expectation) values in Genomic BLAST searches; "–" indicates that no homologs were found with E values  $\leq 0.1$ .

Additional findings support this hypothesis. First, searches for predicted functional partners of MurJ, YajR, YhgN, YibN, YoaE, and YtfN using the STRING database (26) revealed that MurJ is the only of these six proteins predicted to be a functional partner of several proteins involved in peptidoglycan biosynthesis (Table S2). Second, if MurJ was the only lipid II flippase candidate in *E. coli*, it should be essential; indeed, attempts to delete *murJ* in *E. coli*, *Burkholderia pseudomallei*, and *Sinorhizobium meliloti* have failed (27–29). Third, by using ParAlign (30), MurJ homologs can be found in the archaeal *Methanobacteriales* and *Methanosarcinales*, which produce peptidoglycan-like cell-wall polymers whose biogenesis includes undecaprenyl-linked lipid intermediates (31). Likewise, MurJ homologs are also present in the plastid of the amoeba *Paulinella chromatophora* and in the moss *Physcomitrella patens*, both of which produce peptidoglycan (32, 33). Finally, MurJ belongs to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (34). MurJ is the single member of the mouse virulence family (MVF) family, one of the four families that compose the MOP exporter superfamily (34). The MVF family has the greatest sequence similarity to members of the polysaccharide transporter (PST) family, which include WzxE, the flippase of the undecaprenyl-phosphate-linked saccharide precursor of ECA and WxB (or RfbX), the LPS *O*-antigen flippase (34–36). In addition, the MOP exporter superfamily also encompasses the oligosaccharidyl-lipid flippase (OLF) family, which includes Rtf1p, a protein that was proposed to flip dolichyl diphosphate-GlcNAc<sub>2</sub>Man<sub>5</sub> across the endoplasmic reticulum (37). Consequently, it had been proposed that members of the MVF family might export complex carbohydrates across the IM (34). Independently, Pfam classifies MurJ in the MviN\_MATE clan (CL0222), which consists of four protein families that correspond to those members of the MOP exporter superfamily (38).

**MurJ Is Essential in *E. coli*.** If MurJ is the peptidoglycan lipid II flippase in *E. coli*, as the compelling bioinformatic evidence provided above indicates, then two predictions can be made. MurJ should be an essential protein, and abolishing MurJ function should inhibit peptidoglycan biogenesis. Because attempts to delete *murJ* had failed (27), I built a MurJ-depletion strain where *murJ* expression is under the control of an inducible promoter. Specifically, I inserted the arabinose-inducible P<sub>BAD</sub> promoter 14 bp upstream of the *murJ* start codon, decoupling *murJ* from its own promoter (Fig. 2A). The growth of such a MurJ-depletion strain is dependent on the presence of the inducer arabinose in the growth medium (Fig. 2B), demonstrating that MurJ is essential in *E. coli*.

**MurJ Is Required for Peptidoglycan Biogenesis in *E. coli*.** Peptidoglycan protects bacteria from lysis caused by osmotic pressure and determines their shape (1–3). Thus, if MurJ was required for peptidoglycan biogenesis, MurJ depletion would cause cell-shape defects and eventually cell lysis. Indeed, in the absence of arabinose, cell density of the MurJ-depletion strain decreases (Fig. 2B), indicative of cell lysis. In addition, during MurJ depletion, cell morphology becomes heterogeneous (Fig. 2C–H). Cells can be irregularly shaped and larger, and they form blebs large enough to be observed by light microscopy (Fig. 2D–H). Eventually, cells lyse, as evidenced by the appearance of irregularly shaped bacterial ghost envelopes (data not shown). However, because *E. coli* cells cannot be synchronized, cultures of this strain contain cells at various stages of depletion at any given time, so the severity of these phenotypes varies from cell to cell (Fig. 2D–H).

When sucrose is present in the growth medium as an osmotic stabilizer to prevent cell lysis during depletion, MurJ-depleted cells become round, and they can reach higher cell densities than in the absence of sucrose (data not shown). If sucrose is removed from such cultures, the round MurJ-depleted cells immediately



**Fig. 2.** MurJ is essential. (A) Chromosomal organization of the MurJ-depletion strain. The P<sub>BAD</sub> promoter, *araC* and *bla* were inserted 14 bp upstream of the *murJ* start codon so that *murJ* expression is under the control of P<sub>BAD</sub> and decoupled from its native P<sub>murJ</sub> promoter. (B) Growth (measured by OD<sub>600</sub>) of depletion strain NR1154 (MC4100 *ara*<sup>+</sup>  $\Delta$ *lysA::kan murJ*  $\Omega$ (–14::*bla araC* P<sub>BAD</sub>) depends on the presence of arabinose in the medium. Addition of D-fucose, a nonmetabolizable analog of L-arabinose, causes cell lysis of NR1154 but not the MurJ<sup>+</sup> parent strain NR1157 (MC4100 *ara*<sup>+</sup>  $\Delta$ *lysA::kan*). (C–H) Cell morphology (under magnification  $\times 100$  phase objective) of wild-type (NR1157, C) and MurJ-depletion (NR1154, D–H) strains grown in the presence of fucose. During MurJ depletion, whereas some cells appear wild type (black arrows), many are larger, irregularly shaped, and form large blebs (white arrows).

lyse (data not shown). Moreover, although sucrose can prevent lysis, the MurJ-depletion strain cannot form colonies on plates lacking arabinose even if sucrose is provided. Thus, as MurJ is gradually depleted, cell lysis can be prevented by adding sucrose to the growing media, and growth can be maintained temporarily. However, there is a threshold of MurJ level below which growth cannot be maintained even in the presence of sucrose. This is consistent with the recent finding that L-form-like *E. coli* induced by antibiotics requires low levels of peptidoglycan synthesis for growth (39).

To further demonstrate that MurJ depletion inhibits peptidoglycan biogenesis, I compared the amount of incorporation of <sup>3</sup>H-diaminopimelic acid (DAP) into mature peptidoglycan (i.e., sacculus) in equivalent amount of cells from wild-type and MurJ-depleted cultures grown in the absence of arabinose. After four generations of growth in the absence of arabinose, the levels of <sup>3</sup>H-DAP incorporation into mature peptidoglycan in the MurJ-depleted cultures were similar (112.0  $\pm$  7.6%) to those in the wild-type strain. However, two to three generations later,

**Table 2. Distribution of <sup>3</sup>H-DAP in wild-type and MurJ -depletion strains**

Species	Wild type*	MurJ depletion
PG	85.2 ± 3.8 (12,054 ± 905)	60.5 ± 5.5 (3,589 ± 582)
Nucleotides	12.3 ± 3.9 (1,745 ± 451)	31.3 ± 1.6 (1,838 ± 132)
Lipids	2.4 ± 0.2 (351 ± 36)	8.1 ± 3.9 (460 ± 132)

\*Data are presented as percentages, where 100% corresponds to the sum of <sup>3</sup>H-DAP incorporated into mature peptidoglycan (PG) and nucleotide and lipid precursors. Cpm values are shown in parentheses. Data are presented as the average ± standard deviation of three independent experiments.

even before cell density declined, the MurJ-depletion strain incorporated 70.3 ± 3.9% less <sup>3</sup>H-DAP into mature peptidoglycan than wild type, demonstrating that MurJ is required for peptidoglycan biosynthesis.

As indicated above, MurJ-depleted cultures synthesize ≈30% of the amount of mature peptidoglycan that wild-type cultures do. Next, I analyzed the distribution of <sup>3</sup>H-DAP incorporated among mature peptidoglycan and its nucleotide and lipid precursors in wild-type and MurJ-depleted cultures. These analyses revealed that depletion of MurJ causes not only the decrease in incorporation into mature peptidoglycan but also the accumulation of both nucleotide and lipid precursors (Table 2). Inhibition of the lipid II synthase MurG (Fig. 1) leads to both the accumulation of peptidoglycan nucleotide precursors and only a limited accumulation of the lipid precursor lipid I, likely because of the fact that *MraY* is believed to be reversible and because of the low availability of the undecaprenyl-phosphate lipid carrier (40). Because the lipid carrier is thought to be limiting in the cell (40), it is possible that inhibition of lipid II translocation across the IM could also result in the accumulation of nucleotide precursors, as reported here. Together, the data presented here show that MurJ catalyzes a step in peptidoglycan biosynthesis before polymerization but downstream, at least, of lipid I synthesis. Because the only missing step between lipid I synthesis and peptidoglycan polymerization is the translocation of lipid II across the IM, these data are consistent with a role for MurJ as the lipid II flippase in *E. coli*.

## Discussion

The reactions necessary for the biogenesis of peptidoglycan have been known for decades. The enzymes required for these reactions have all been identified. What has been missing is the factor that translocates the lipid II intermediate across the IM. Van Dam *et al.* (9) determined that this translocation step requires a protein(s) whose identity has long remained elusive. Using a reductionist bioinformatics approach, I accumulated compelling bioinformatics evidence suggesting that MurJ is the missing lipid II flippase. Genetic and biochemical data demonstrate that MurJ is indeed an essential *E. coli* protein required for peptidoglycan biosynthesis. Although additional biochemical studies are needed to demonstrate its lipid II flippase activity, the data presented here are in agreement with MurJ's performing such a function.

The reductionist bioinformatics approach of using a comparative analysis between the proteomes of *E. coli* and closely related endosymbionts has successfully identified four proteins that are essential for the biogenesis of the cell envelope of *E. coli* (15). The great diversity among bacteria and the increasing number of sequenced genomes offer an opportunity to conduct similar comparative analyses to identify missing components of known pathways. However, although bioinformatics has been instrumental in the identification of MurJ as the potential lipid II flippase, it does not provide insight into how MurJ might act as a flippase. For example, it does not provide information as to what energy source might drive MurJ activity or whether export

of lipid II might be coupled to the import of any molecule across the IM. The fact that depletion of MurJ only results in a low level of accumulation of lipid intermediates could be the result of MurJ's also being involved in the recycling of undecaprenyl phosphate.

To understand MurJ activity, biochemical assays are needed. Because those available for flippase activity are difficult and controversial (37, 41), the identification of MurJ will hopefully aid in the development of better methods to measure flippase activity. This is an especially important goal for MurJ, because it represents an undeveloped target for broad-spectrum antibacterial agents.

## Materials and Methods

**Bacterial Strains.** Strains were derived from NR754, an *araD*<sup>+</sup> revertant of MC4100 (15, 42). Alleles were transferred by using P1 transduction (43). A MurJ-depletion strain was constructed by recombinering as follows. Primers 5mviNPbad (5'-CATAATCCAGGTAGAC TATTCGCTCTTCAGCGCCTGCTTG-CAGGCGTTTTGCCGTTGGTCTGTATATATGAGTAACTTGGTCTG) and 3mviNPbad (5'-CACGCGAAAACATGGTCATCGAGCTGA CGGCGGCCAGCGATTTA-ATAAATTCATCGGTGTTCTAATCCTCCTTAGAGCTCGAATTCCC) were used to amplify the *bla-araC-P<sub>BAD</sub>* region of pKD46 (44). The ends of the resulting PCR product are identical (italicized portion of primers) to the chromosomal region upstream of *murJ*. This PCR product was used for recombinering to insert *bla-araC-P<sub>BAD</sub>* in the chromosome of DY378 (45) 14 bp upstream of the *murJ* start codon. The resulting strain (NR1151) was selected at 30°C on media containing ampicillin and arabinose and confirmed by PCR analysis. The *murJ* Ω(-14::*bla-araC-P<sub>BAD</sub>*) insertion of NR1151 was introduced into NR574 to construct NR1152 by using P1 transduction (43) by selecting ampicillin-resistant transductants in the presence of arabinose. To label with <sup>3</sup>H-DAP, NR1157 and NR1154 were constructed by introducing the Δ*lysA*::*kan* allele from the Keio collection (27) into NR754 and NR1152, respectively. The presence or absence of *lysA* did not affect any of the MurJ-dependent phenotypes.

**Growth Conditions.** LB broth and agar were prepared as described (43). When appropriate, ampicillin (25 μg/ml), kanamycin (25 μg/ml), L-arabinose (0.2% wt/vol), D-fucose (0.05% wt/vol), and sucrose (0.23 M) were added. For labeling experiments, 100 μg/ml methionine, lysine, and threonine were added to growth media to reduce the internal pool of DAP (46). For data shown in Fig. 2B, 1 ml of overnight cultures grown with arabinose was pelleted, washed once with LB broth, and resuspended in 1 ml of LB broth. Fresh LB broth with either arabinose or fucose was inoculated with a 1:5,000 dilution of washed cultures and grown under aeration at 37°C. Growth was monitored by optical density at OD<sub>600</sub>.

**Microscopy.** Cells grown in the presence of either arabinose or fucose were placed on a pad of 1% agarose (wt/vol) in M63 minimal medium (43) on a microscope slide and covered with a coverslip. Samples were examined under a Nikon 90i fluorescence microscope (magnification ×100 objective), and images were captured with a Rolera XR Fast 1394 camera (Qimaging).

**Labeling with <sup>3</sup>H-DAP.** Cells (NR1157 and NR1154) grown overnight in LB broth containing arabinose were washed as indicated above. Fresh LB broth containing fucose, methionine, lysine, and threonine was inoculated with a 1:100 dilution of washed cultures and grown under aeration at 37°C for 2 h to OD<sub>600</sub> ≈ 0.6 (approximately four generations). Then, 1 ml of each culture was labeled as indicated below. At the same time, cells were diluted to OD<sub>600</sub> ≈ 0.1 into a fresh culture containing fucose, methionine, lysine, and threonine and grown until OD<sub>600</sub> ≈ 0.6. At that time, 1 ml of each culture was labeled for 15 min at 37°C with 5 μCi of <sup>3</sup>H-DAP (60 Ci/mmol; American Radiolabeled Chemicals). At the end of the labeling period, cells were placed on ice and pelleted at 4°C for 5 min at 16,100 × g. Pellets were resuspended in 7 μl of ice-cold water and immediately frozen in dry ice until they were subjected to paper chromatography.

**Cellular Distribution of <sup>3</sup>H-DAP.** Labeled cells prepared as above were spotted onto Whatman 3MM paper, and labeled peptidoglycan precursors were separated by ascending chromatography by development with isobutyric acid:1 M NH<sub>4</sub>OH (5:3; vol/vol) for ≈16 h (47). Paper was dried and cut into 1-cm squares that were counted in vials containing scintillation mixture (Ready Safe; Beckman Coulter). Mature peptidoglycan remained at the origin, whereas nucleotide and lipid precursors ran at R<sub>f</sub> values of 0.15–0.25 and 0.8–0.9, respectively (48). For each strain, counts corresponding to those R<sub>f</sub> values were added, and their sum represented 100% of incorporated label.

The percentage corresponding to each species (mature peptidoglycan, nucleotides, and lipid precursors) was calculated accordingly for each experiment, and data from three independent experiments were used to calculate the average and standard deviation. To compare the mature peptidoglycan synthesis in wild-type and MurJ-depleted cultures, cpm values at the origin were used. Comparison of incorporation between strains was done by calculating the percentage of incorporation in the MurJ-depletion strain with respect to wild type. Data from three independent experiments were used to calculate percentage average and standard deviation.

**Proteome-Based Analysis.** For Genomic BLAST searches (21), genome sequences were obtained from GenBank for the following organisms: *Blochmannia floridanus* (BX248583); *Buchnera aphidicola* str. APS (BA000003); *Aster yellows witches'-broom phytoplasma* AYWB (CP000061); *Eubacterium dolichum* DSM 3991 (ABAW00000000); *Mesoplasma florum* L 1 (AE017263); *Mycoplasma agalactiae* PG2 (CU179680); *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343 (CP000123); *Mycoplasma gallisepticum* R (AE015450); *Mycoplasma genitalium* G37 (L43967); *Mycoplasma hyopneumoniae* 232 (AE017332); *Mycoplasma hyopneumoniae* 7448 (AE017244); *Mycoplasma*

*hyopneumoniae* J (AE017243); *Mycoplasma mobile* 163K (AE017308); *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1 (BX293980); *Mycoplasma penetrans* HF-2 (BA000026); *Mycoplasma pneumoniae* M129 (U00089); *Mycoplasma pulmonis* UAB CTIP (AL445566); *Mycoplasma synoviae* 53 (AE017245); *Onion yellows phytoplasma* OY-M (AP006628); *Ureaplasma parvum* serovar 3 str. ATCC 700970 (AF222894); *Dehalococcoides ethenogenes* 195 (CP000027); *Buchnera aphidicola* str. Cc (Cinara cedri) (CP000263); *Candidatus Carsonella ruddii* PV (AP009180); *Candidatus Vesicomysocius okutanii* HA (AP009247). Genomic BLAST searches were conducted by using the BLASTP 2.2.18 program (49). In all searches, protein sequences for MurJ (MviN), YajR, YhgN, YibN, YoaE, and YtfN were obtained from EcoCyc (50).

**Note Added in Proof.** Inoue *et al.* have recently reported that MviN (MurJ) is involved in peptidoglycan synthesis (51).

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