

A *Burkholderia cenocepacia* MurJ (MviN) homolog is essential for cell wall peptidoglycan synthesis and bacterial viability

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The cell wall peptidoglycan (PG) of *Burkholderia cenocepacia*, an opportunistic pathogen, has not yet been characterized. However, the *B. cenocepacia* genome contains homologs of genes encoding PG biosynthetic functions in other bacteria. PG biosynthesis involves the formation of the undecaprenyl-pyrophosphate-linked N-acetyl glucosamine-N-acetyl muramic acid-pentapeptide, known as lipid II, which is built on the cytosolic face of the cell membrane. Lipid II is then translocated across the membrane and its glycopeptide moiety becomes incorporated into the growing cell wall mesh; this translocation step is critical to PG synthesis. We have investigated candidate flippase homologs of the MurJ family in *B. cenocepacia*. Our results show that BCAL2764, herein referred to as *murJ_{Bc}*, is indispensable for viability. Viable *B. cenocepacia* could only be obtained through a conditional mutagenesis strategy by placing *murJ_{Bc}* under the control of a rhamnose-inducible promoter. Under rhamnose depletion, the conditional strain stopped growing and individual cells displayed morphological abnormalities consistent with a defect in PG synthesis. Bacterial cells unable to express MurJ_{Bc} underwent cell lysis, while partial MurJ_{Bc} depletion sensitized the mutant to the action of β -lactam antibiotics. Depletion of MurJ_{Bc} caused accumulation of PG precursors consistent with the notion that this protein plays a role in lipid II flipping to the periplasmic compartment. Reciprocal complementation experiments of conditional *murJ* mutants in *B. cenocepacia* and *Escherichia coli* with plasmids expressing MurJ from each strain indicated that MurJ_{Bc} and MurJ_{Ec} are functional homologs. Together, our results are consistent with the notion that MurJ_{Bc} is a PG lipid II flippase in *B. cenocepacia*.

Keywords: cell wall / cystic fibrosis / essential gene / flippase / undecaprenylphosphate

Introduction

Burkholderia cenocepacia, a metabolically diverse Gram-negative β -proteobacterium, is widespread in the rhizosphere. This bacterium often causes opportunistic, chronic lung infections in patients with cystic fibrosis (Vandamme et al. 1997). Environmental bacteria are usually exposed to dramatic changes in growth conditions, which include variations in temperature, pH, concentration of nutrients and salinity, exposure to toxic metals and biocides and encounters with predator and host organisms. We speculate that the ability of these bacteria to adapt to and withstand these changes relies in part on the synthesis and remodeling of the cell wall peptidoglycan (PG), which is key to cell division, bacterial shape and survival under high internal turgor pressure. PG surrounds bacterial cells forming a mesh-like glycopeptide polymer made of repeating units of N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc). The carboxy group of the MurNAc residues is substituted by a pentapeptide that consists of L-alanine, L-glutamic acid, meso-diaminopimelic acid (or L-lysine) and D-alanyl-D-alanine. The neighboring glycan chains are cross-linked by peptide linkages, resulting in rigid mesh-like macromolecular structure (van Heijenoort 2001).

To our knowledge, the biosynthesis of PG in the *Burkholderia* genus has not yet been studied. However, the *B. cenocepacia* genome contains homologs of the genes encoding all the enzymes required for the synthesis of PG precursors in other bacteria, suggesting that the PG biosynthesis pathway is conserved. PG biosynthesis starts in the cytoplasm with the synthesis of the UDP-MurNAc-pentapeptide, which is catalyzed by the Mur ligases (Barreteau et al. 2008) MurC (BCAL3641), MurD (BCAL3464), MurE (BCAL3467) and MurF (BCAL3466). This molecule is the substrate of the membrane protein MraY (BCAL3465) that catalyzes the formation of a phosphoanhydride bond with the lipid carrier, undecaprenyl phosphate (Und-P), and gives rise to Und-PP-MurNAc-pentapeptide also known as lipid I. The GlcNAc moiety from UDP-GlcNAc is transferred to lipid I, resulting in lipid II, a reaction catalyzed by the inner membrane-associated transferase MurG (BCAL3462) (Bouhss et al. 2008). Lipid II is translocated across the cytoplasmic membrane to incorporate the PG subunits into the growing cell wall mesh. At the periplasmic side of the inner membrane, various penicillin-binding proteins catalyze the maturation of the growing PG chain. Glycosyltransferases assemble lipid II to the existing PG releasing Und-PP, which is recycled by an as-yet-unclear mechanism (Valvano 2008). The peptide chains are cross-linked by transpeptidases and terminal D-Ala is removed from the pentapeptide by D-D-carboxypeptidases (van Dam et al. 2007).

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The transport of lipid II across the inner membrane is a key step in the synthesis of PG, but its mechanism is not completely elucidated. Studies using a fluorescence lipid II analog have revealed that the transbilayer movement of lipid II is independent of ATP hydrolysis or proton motive force, but it appears to be coupled to transglycosylation activity on the periplasmic side of the inner membrane (van Dam et al. 2007). The *Escherichia coli* MurJ (Ruiz 2008), also known as MviN (Inoue et al. 2008), is an integral membrane protein with multiple predicted transmembrane helices that belongs to the multi-antimicrobial extrusion-like superfamily. Members of this family include proteins like Wzx, which are implicated in the membrane flipping of lipid-linked oligosaccharide and polysaccharide precursors of lipopolysaccharide O-antigen and capsules (Islam and Lam 2013; Valvano and Hanuszkiewicz 2012). Studies in *E. coli* demonstrated that MviN is essential for viability. Cells from conditional mutants under non-permissive conditions burst and accumulate lipid II (Inoue et al. 2008; Ruiz 2008), suggesting that this protein is involved in PG biosynthesis and functions as the lipid II flippase, being renamed MurJ (Ruiz 2008). Other studies have shown that MurJ (MviN) is essential in *Burkholderia pseudomallei* (Ling et al. 2006) and *Sinorhizobium meliloti* (Rudnick et al. 2001). In contrast, the simultaneous inactivation of four *murJ* homologs in *Bacillus subtilis* did not compromise bacterial viability, casting doubts on the functional assignment of this protein as a flippase (Fay and Dworkin 2009). It has been proposed that in *E. coli*, other membrane proteins such as RodA and FtsW function as lipid II flippases (Ehlert and Holtje 1996; Mohammadi et al. 2011). RodA is required for the synthesis of lateral PG, while FtsW is involved in septal PG synthesis (Ishino et al. 1986, 1989; Ikeda et al. 1989; Khattar et al. 1994; Henriques et al. 1998; Mercer and Weiss 2002; Tamaki et al. 1980). A biochemical study using *E. coli* membrane vesicles demonstrated that transport of lipid II requires FtsW, but not MurJ, to mediate the transbilayer movement of lipid II in model membranes (Mohammadi et al. 2011). However, this study did not rule out nonspecific flipping, a common occurrence in isolated vesicles and reconstituted in vitro systems (Kol et al. 2001, 2003). More recently, a three-dimensional model of the *E. coli* MurJ, together with topological studies and functional analyses of specific charged residues, strongly suggest that MurJ is a member of a large family of flippase proteins involved in O-antigen and capsule biosynthesis (Butler et al. 2013).

In this study, we investigated candidate homologs of the MurJ (MviN) family in *B. cenocepacia*. We show that BCAL2764, herein designated *murJ_{Bc}*, encodes a membrane protein that is indispensable for the viability of *B. cenocepacia* and also required for PG synthesis, while the other flippase homologs were not required for bacterial survival under laboratory conditions. Furthermore, *MurJ_{Bc}* shared predicted structural features with its *E. coli* counterpart, and complementation experiments of conditional *murJ* mutants in *B. cenocepacia* and *E. coli* with plasmids expressing MurJ from each strain indicated that *MurJ_{Bc}* and *MurJ_{Ec}* are functional homologs.

Results and discussion

B. cenocepacia MurJ is an essential gene and a functional homolog of the E. coli murJ

The initial aim of this study was to identify and elucidate the function of predicted flippases in *B. cenocepacia*. The

annotated genome of *B. cenocepacia* J2315 was manually inspected for genes encoding proteins with 12 or more transmembrane helices, which were further examined by HHPRED using the Bioinformatics Toolkit from the Max-Planck Institute for Developmental Biology (<http://toolkit.tuebingen.mpg.de/hhpred>; Remmert et al. 2011) to identify hypothetical proteins with predicted flippase function based on homologies with domains commonly found in these proteins (MviN, RfbX and Rft1 domains). This analysis identified the following genes: BCAL1907, BCAL2764 (herein designated *murJ_{Bc}*) and BCAL3114 in chromosome 1; BCAM0204 and BCAM1007 in chromosome 2. No flippase homologs were noted in chromosome 3 genes. The gene context was also taken into account. BCAL1907 is part of a potential two-gene operon that includes BCAL1906, a predicted glycosyltransferase. *murJ_{Bc}* is also part of a potential two-gene operon including BCAL2763, encoding a protein of unknown function. BCAL3114 was previously identified as *wzx* and is located on a transcriptional region immediately adjacent to the O-antigen gene cluster (Ortega et al. 2005). Deletion of this gene does not affect O-antigen production in *B. cenocepacia* K56-2 (X. Ortega and M. A. Valvano, unpublished), since the O-antigen in this strain is exported by an ABC transporter encoded by *wzt* and *wzm*, both of which are located within the O-antigen gene cluster (Ortega et al. 2005). BCAM0204 and BCAM1007 are part of the capsule polysaccharide cluster. Therefore, BCAL3114, BCAM0204 and BCAM1007 were not investigated further. *B. cenocepacia* has RodA and FtsW homologs (BCAL0478 and BCAL3463, respectively), which were also not investigated in this study.

To elucidate the possible function of BCAL1907 and *murJ_{Bc}*, we constructed unmarked deletion mutants as described in Materials and Methods. Whereas BCAL1907 was readily deleted without any apparent functional consequences to the bacterial cells, attempts to delete *murJ_{Bc}* were consistently unsuccessful, as colonies carrying the deleted gene were never obtained. We suspected from these results that *murJ_{Bc}* could be essential for *B. cenocepacia* viability. Therefore, we constructed a conditional mutant derivative of strain K56-2 by placing *murJ_{Bc}* under the control of the rhamnose-inducible promoter (*P_{rha}*). The resulting *P_{rha}::murJ_{Bc}* strain (YFM1, Table I) grew on solid medium in the presence of rhamnose (permissive conditions), but failed to grow in the presence of glucose (non-permissive conditions), as expected for a mutant with an essential gene under the control of *P_{rha}* (Figure. 1A). Loss of viability in the absence of rhamnose was comparable with that found with the positive control strain XOA11, which has the essential gene *arnT* gene under the control of rhamnose (*P_{rha}::arnT*) (Ortega et al. 2007) (Figure. 1A). In contrast, the negative control strain XOA10 (*P_{rha}::BCAL1928*) grew at the same rate with either rhamnose or glucose, as expected (Ortega et al. 2007). Growth without added rhamnose was restored upon complementation of the *P_{rha}::murJ_{Bc}* conditional mutant by introduction of the replicative plasmid pDA12 expressing *murJ_{Bc}* from a constitutive promoter (Figure. 1B). This confirmed that the growth defect was due to lack of *murJ_{Bc}* expression and demonstrated that this gene encodes an essential function supporting bacterial viability.

We also assessed whether *murJ_{Bc}* is functionally analogous to its *E. coli* ortholog. For these experiments, we employed the *E. coli* strain NR1152, in which *murJ_{Ec}* expression is under the control of the arabinose inducible promoter (*P_{ara}::murJ_{Ec}*)

Table I. Bacterial strains and plasmids

| Strain or plasmid | Characteristics | Source or reference |
|-----------------------|--|------------------------------|
| <i>E. coli</i> | | |
| DH5 α | F ⁻ 80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K m _K ⁺) <i>supE44 thi-1 ΔgyrA96 (ΔlacZYA-argF)U169 <i>relA1</i></i> | Invitrogen |
| GT115 | F ⁻ <i>mcrA Δ(<i>mrr-hsdRMS-mcrBC</i>) 80lacZ</i> Δ M15 <i>ΔlacX74 recA1 rpsL endA1 Δdcm uidA(ΔMlu)::pir-116 ΔsbC-sbcD</i> | Lab stock |
| NR1152 | NR754; <i>araD</i> ⁺ revertant of MC4100; <i>murJ</i> Ω (-14:: <i>bla araC</i> P _{BAD} (Ruiz 2008) | |
| W3110 | IN (<i>rrnD-rrnE</i>)1 <i>rph-1</i> | Lab stock |
| <i>B. cenocepacia</i> | | |
| K56-2 | Clinical isolate, ET12 clone related to J2315 | BCRRC |
| YFM1 | K56-2; <i>P_{rha}::murJ_{Bc}</i> (BCAL2764); Tp ^R | This study |
| YFM2 | YFM11; <i>P_{rha}::murJ_{Bc}</i> (BCAL2764); Tp ^R | This study |
| YFM11 | K56-2; Δ lysA (BCAM2076) | This study |
| XOA10 | K56-2; <i>P_{rha}::BCAL1928</i> | Ortega et al. (2007) |
| XOA11 | K56-2; <i>P_{rha}::arnT</i> (BCAL1929) | Ortega et al. (2007) |
| <i>Plasmids</i> | | |
| pDA12 | Cloning vector, <i>ori_{pBBR1}</i> , Tet ^R , <i>mob</i> ⁺ , P _{dhfr} | Aubert et al. (2008) |
| pDAI-SceI-SacB | <i>ori_{pBBR1}</i> , Tet ^R , P _{dhfr} , <i>mob</i> ⁺ , <i>sacB</i> ⁺ | Hamad et al. (2010) |
| pEXT21 | Low copy number cloning vector, <i>ori_{IncW-pSF6}</i> , Sp ^R , P _{tac} | Dykxhoorn et al. (1996) |
| pGPISce-I | <i>ori_{R6K}</i> , <i>mob</i> ⁺ , Ω Tp ^R , ISce-I restriction site | Flannagan et al. (2008) |
| pRK2013 | <i>ori_{colE1}</i> , RK2 derivative, Kan ^R , <i>mob</i> ⁺ , <i>tra</i> ⁺ | Figurski and Helinski (1979) |
| pSC200 | Cloning vector, <i>ori_{pBBR1}</i> <i>rhaR</i> , <i>rhaS</i> , <i>PrhaB</i> , Tp ^R | Cardona and Valvano (2005) |
| pYM1 | pSC200- <i>murJ_{Bc}</i> | This study |
| pYM3 | pGPI-SceI with fragments flanking BCAM2076 | This study |
| pYM21 | pDA12- <i>murJ_{Bc}</i> , Tet ^R | This study |
| pYM28 | pDA12- <i>murJ_{Bc}</i> , Tet ^R | This study |
| pYM29 | pDA12- <i>FLAGmurJ_{Bc}</i> , N-terminal FLAG-Tag, Tet ^R | This study |
| pYM30 | pEXT21- <i>FLAGmurJ_{Bc}</i> , N-terminal FLAG-Tag, Sp ^R | This study |
| pYM31 | pDA12- <i>FLAGmurJ_{Bc}</i> -R18A, N-terminal FLAG-Tag, Tet ^R | This study |
| pYM32 | pDA12- <i>FLAGmurJ_{Bc}</i> -R24A, N-terminal FLAG-Tag, Tet ^R | This study |
| pYM33 | pDA12- <i>FLAGmurJ_{Bc}</i> -D39A, N-terminal FLAG-Tag, Tet ^R | This study |
| pYM34 | pDA12- <i>FLAGmurJ_{Bc}</i> -R52A, N-terminal FLAG-Tag, Tet ^R | This study |
| pYM35 | pDA12- <i>FLAGmurJ_{Bc}</i> -R274A, N-terminal FLAG-Tag, Tet ^R | This study |

^aTp^R, trimethoprim resistance; Tet^R, tetracycline resistance; Kan^R, kanamycin resistance; Sp^R, spectinomycin resistance.

^bBCRRC, *B. cenocepacia* Research and Referral Repository for Canadian CF Clinics.

(Ruiz 2008). Introduction of either the constitutive pDA12 or IPTG-inducible pEXT21 plasmids encoding *murJ_{Bc}* into NR1152 supported growth of this strain in the absence of arabinose, while the strain containing the empty vectors was nonviable (Figure. 1C). In the converse experiment, pDA12-*murJ_{Bc}* was able to support viability of the strain carrying the *P_{rha}::murJ_{Bc}* construct (Figure. 1B). Together, these reciprocal complementation experiments demonstrated that the MurJ proteins of *B. cenocepacia* and *E. coli* are functionally interchangeable.

Suppression of *MurJ_{Bc}* expression causes growth arrest, dramatic morphological changes and cell lysis

To better characterize the phenotype of the *P_{rha}::murJ_{Bc}* strain under non-permissive conditions, we performed rhamnose depletion experiments in liquid medium (Ortega et al. 2007). Figure. 2 shows that after serial passage in medium with glucose, the turbidity of the *P_{rha}::murJ_{Bc}* strain culture rapidly reached a plateau compared with wild-type strain and strain *P_{rha}::murJ_{Bc}* grown in the presence of rhamnose. Bacterial cells at 6-h post-depletion were examined for morphological changes by phase-contrast microscopy. The majority of the *P_{rha}::murJ_{Bc}* cells appeared swollen, forming blebs, and also many cells had burst forming “ghosts” (Figure. 3). Ultrastructural analysis by transmission electron microscopy revealed that cells had extremely

irregular shapes, suggesting a dramatic defect in the rigidity of cell wall (Figure. 4). Using fluorescent microscopy and the LIVE/DEAD kit, we noticed that under rhamnose depletion bacterial cells became permeable to propidium iodide (Figure. 5). Also, red fluorescent mesh-like aggregates appeared suggesting binding of propidium iodide to released DNA upon cell lysis (Figure. 5). None of these changes in morphology or viability was apparent in *P_{rha}::murJ_{Bc}* cells that were not subjected to rhamnose depletion. Together, these results demonstrate that under non-permissive conditions, *P_{rha}::murJ_{Bc}* bacteria were not only unable to grow, but also lost their normal shape, becoming osmotically fragile and bursting, which suggests a major defect in cell wall PG synthesis.

More conclusive evidence of cell lysis was obtained by examining leakage of intracellular components into the culture supernatants of *P_{rha}::murJ_{Bc}* cells placed under permissive and non-permissive conditions. The results show that the supernatant from bacteria exposed to non-permissive conditions has much more protein content than that of control cells (Figure. 6A). An immunoblot of the same gel using anti-RNA-polymerase α -subunit antibody showed the leakage of this large protein into the supernatant (Figure. 6B). RNA polymerase is normally localized in the cytoplasmic compartment and cannot be released by active secretion in cells containing an intact cell envelope. This experiment confirms that the supernatant of cells under non-

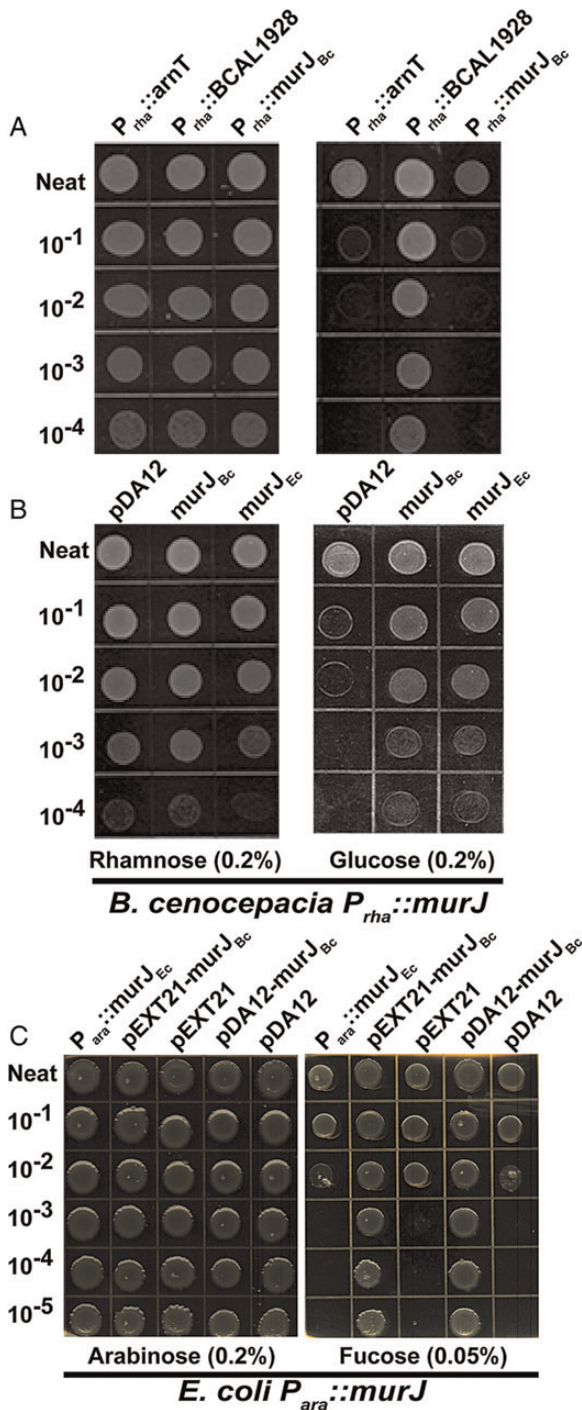


Fig. 1. (A) Conditional lethal phenotype of strain YFM1 (*P_{rha}::murJ_{Bc}*) on LB agar plates supplemented with 0.2% rhamnose or 0.2% glucose. XOA10 (*P_{rha}::BCAL1928*) and XOA11 (*P_{rha}::arnT*) are rhamnose-independent and -dependent control mutants (Ortega et al. 2007). (B) Complementation of YFM1 (*P_{rha}::murJ_{Bc}*) with pDA12-BCAL2764 (*murJ_{Bc}*) and pDA12-*murJ_{Ec}* compared with vector control in the presence and absence of rhamnose. (C) Complementation of *E. coli* NR1152 (*P_{ara}::murJ_{Ec}*) with pEXT21-*murJ_{Bc}* and pDA12-*murJ_{Bc}* compared with the corresponding vector controls in the presence and absence of arabinose.

permissive conditions contains released cytoplasmic content, supporting the conclusion that cells unable to express *murJ_{Bc}* undergo cell lysis.

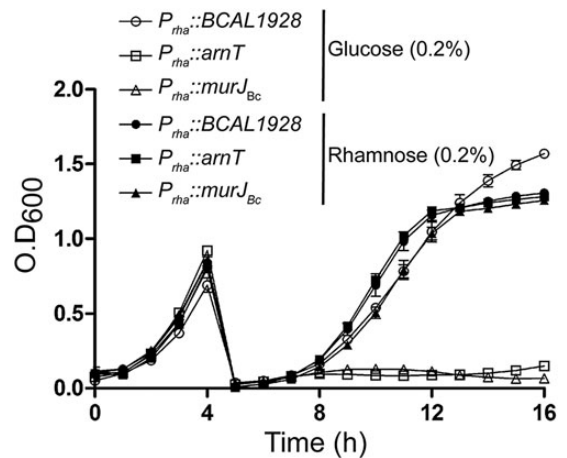


Fig. 2. Depletion experiment in liquid medium using YFM1 (*P_{rha}::murJ_{Bc}*), and the control strains XOA10 (*P_{rha}::BCAL1928*) and XOA11 (*P_{rha}::arnT*) (Ortega et al. 2007). The solid symbols indicate permissive conditions, whereas the empty ones correspond to non-permissive conditions. Growth was monitored every hour using a Bioscreen system. The figure is representative of three independent biological repeats with similar results. Bars indicate the standard errors of triplicate observations at each time point.

Partial suppression of *MurJ_{Bc}* expression sensitizes cells to the action of β -lactam antibiotics

We reasoned that if *MurJ_{Bc}* functions in PG synthesis, bacterial growth of the conditional mutant under limiting amounts of rhamnose would be further reduced in the presence of cell wall-acting antibiotics. We used ceftazidime and imipenem for these experiments. Ceftazidime is a semisynthetic aminothiazolyl cephalosporin that inhibits penicillin-binding proteins, mainly penicillin-binding protein-3, and induces rapid cell lysis (Hayes and Orr 1983). Imipenem is a carbapenem antibiotic that like all other β -lactams inhibits bacterial cell wall synthesis by binding to and inactivating penicillin-binding proteins (Neu 1983) and also induces formation of spherical cells and subsequent cell lysis (Rodloff et al. 2006). Bacteria were grown in either 0.2 or 0.05% rhamnose. The high-rhamnose concentration is the normal inducing concentration for optimal gene expression, as empirically determined by the absence of any growth and morphology phenotypes, as described above. The lower concentration was selected to reach a state of partial depletion. At this concentration, the growth rate of the conditional mutant was not affected, as minimal amounts of rhamnose can induce the expression of *MurJ_{Bc}* and rescue the mutant from cell death (Supplementary data, Figure S1). However, under this minimal amount of rhamnose, defects in PG start to appear which are still not enough to compromise viability. Under the rhamnose-rich concentration, *P_{rha}::murJ_{Bc}* had the same MIC values for ceftazidime and imipenem as the XOA10 (*P_{rha}::BCAL1928*) control strain (Table II). However, the *P_{rha}::murJ_{Bc}* bacteria grown under rhamnose-limiting conditions had 6-fold and more than 32-fold decrease in the MIC to ceftazidime and imipenem, respectively. In contrast, the MIC value for chloramphenicol, a non-cell wall-acting antibiotic, did not change under the same conditions (Table II). In liquid growth experiments, a pronounced killing effect of ceftazidime and imipenem was also observed in cells grown under rhamnose-

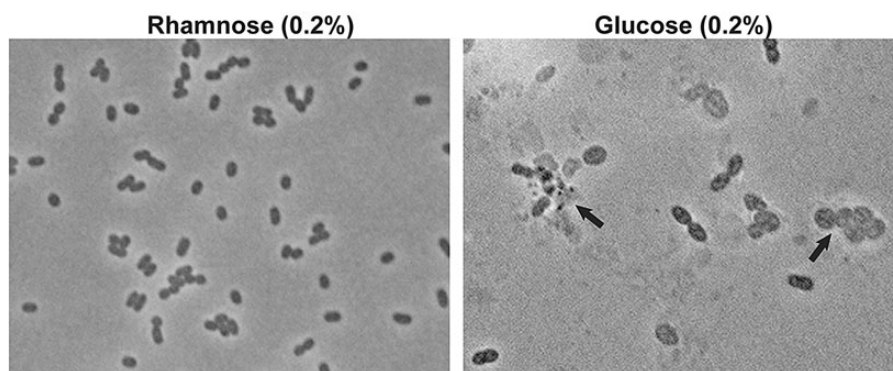


Fig. 3. Phase-contrast microscopy of YFM1 (*P_{rha}::murJ_{Bc}*) bacteria at 6-h following growth under permissive (0.2% rhamnose) and non-permissive (0.2% glucose) conditions using oil-immersion objective lens in a Zeiss Axioscope II microscope (magnification: $\times 1000$). Arrows indicate ghost cells.

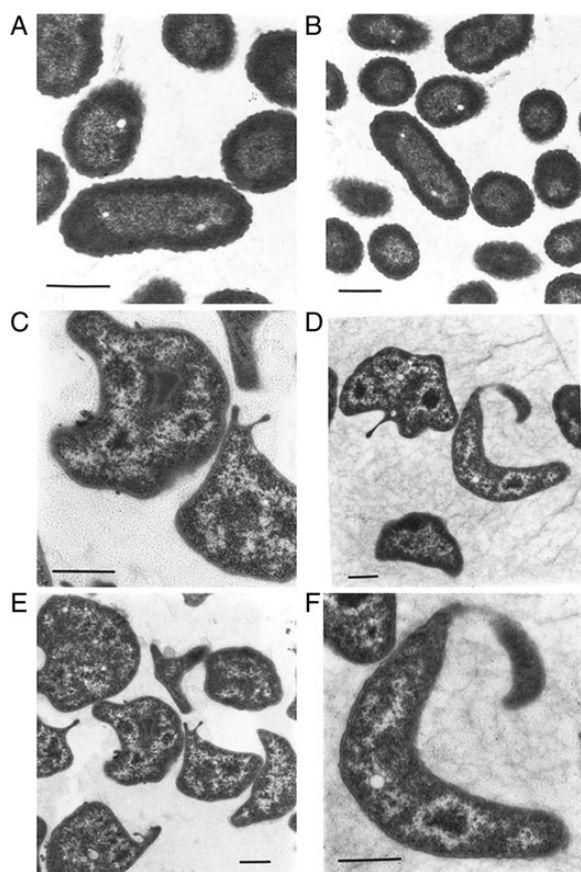


Fig. 4. Transmission electron microscopy of YFM1 (*P_{rha}::murJ_{Bc}*) bacteria under permissive (A and B) and non-permissive (C–F) conditions. Bars: 0.5 μm .

limiting conditions (Supplementary data, Figure S1), while growth rate was comparable under rhamnose-rich and -limiting concentrations in the absence of the antibiotics. Furthermore, growth rate did not vary in the presence of chloramphenicol (Supplementary data, Figure S1). We conclude from these experiments that conditions leading to *murJ_{Bc}* reduced expression result in higher bacterial

susceptibility to cell wall-acting antibiotics, supporting the notion of a defect in PG synthesis.

Depletion of MurJ_{Bc} causes accumulation of [³H]-DAP

The *E. coli murJ* has been proposed to encode the lipid II flip-pase required for PG synthesis (Ruiz 2008). Therefore, to further understand the function of *murJ_{Bc}* in *B. cenocepacia* PG synthesis, we determined the level of accumulation of PG cell wall precursors under rhamnose depletion in a *P_{rha}::murJ_{Bc}* derivative carrying a *lysA* (BCAM 2076) deletion (strain YFM2, Table III). To achieve this, we radiolabeled lipid II with [³H]-DAP, which is incorporated into the MurNAc-pentapeptide chain. We tested the cellular distribution of [³H]-DAP into the mature PG from an equivalent amount of cells under permissive and non-permissive conditions. Using ascending paper chromatography, we observed that rhamnose-depleted bacterial cells labeled with [³H]-DAP accumulated a radioactive species with an average *R_f* value of 0.47 (Figure. 7A). Also, the amount of incorporation of the radioactive label under non-permissive conditions decreased to half as compared with permissive conditions. Lipids were extracted from the conditional mutant cells after labeling under permissive and non-permissive conditions with a modified Bligh and Dyer two-phase system (Guan et al. 2005). Depending on the selective solubility of lipid II at different pH values, this molecule partitions in the aqueous phase at neutral pH and in the organic phase at acidic pH (Guan et al. 2005). Our results showed a higher level of [³H]-DAP accumulation in the aqueous phase at neutral pH for the cell extracts under non-permissive conditions in comparison with the level of [³H]-DAP in the presence of rhamnose (2.25 ± 0.45 ratio) as shown in Figure. 7B. Separation of the aqueous phase by ascending chromatography revealed peaks of [³H]-DAP accumulation at comparable *R_f* value as obtained before from ascending chromatography of the cell pellets (Figure. 7A). The PG is expected to remain at the origin in the paper chromatography and the lipid precursors migrate to a further distance by the effect of the lipophilic solvent. Ruiz showed that the nucleotide and lipid precursors accumulated in MurJ-depleted mutant in *E. coli* at *R_f* values of 0.25–0.35 and 0.8–0.9, respectively (Ruiz 2008). However, it was reported that a mixture of lipid I and lipid II could migrate to an *R_f* value of 0.6 in *E. coli* (El Ghachi et al. 2006). We

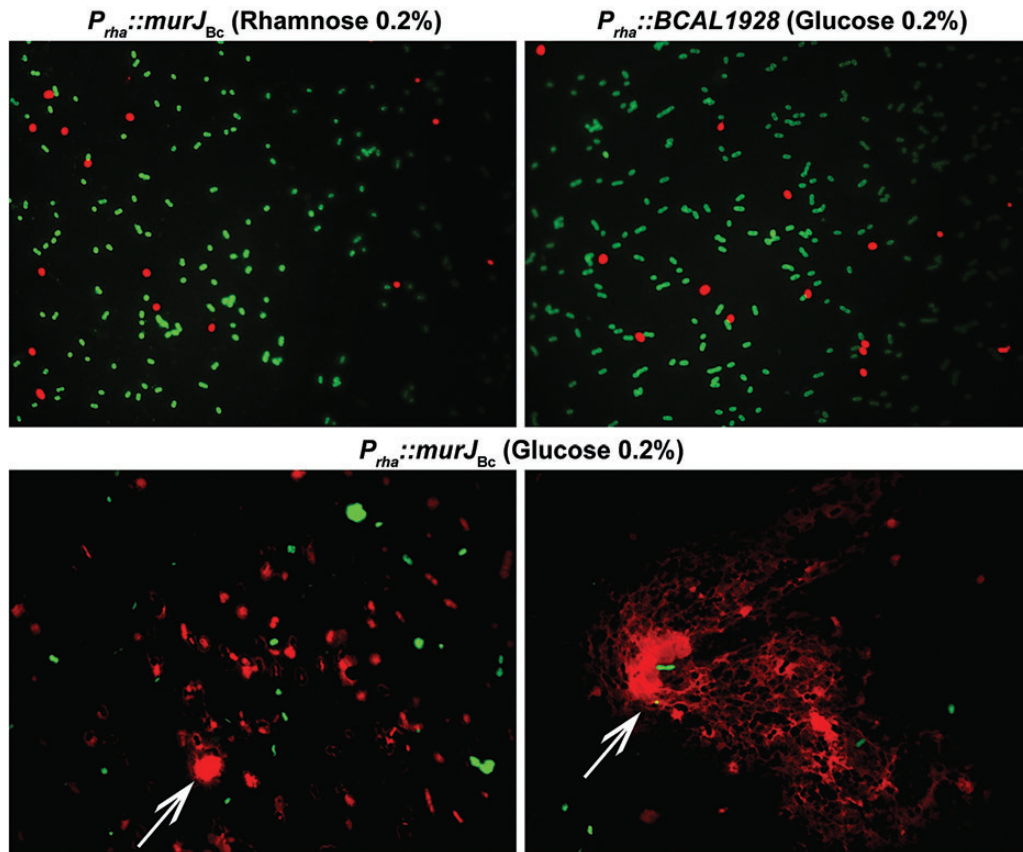


Fig. 5. LIVE/DEAD staining of YFM1 ($P_{rha}::murJ_{Bc}$) under permissive (0.2% rhamnose) and non-permissive (0.2% glucose) conditions compared with rhamnose-independent control strain XOA10 ($P_{rha}::BCAL1928$). Live bacteria appear fluorescent green, while dead bacteria and bacteria with compromised membranes appear fluorescent red. Arrow indicates a red fluorescent mesh consistent with extracellular DNA.

confirmed the identity of lipid II by MS analysis using MALDI-TOF in negative ion mode and comparing the Mass spectra of lipid II from *E. coli* W3110 and *B. cenocepacia* K56-2 (Figure. 7C and D). The lipid II from *E. coli* showed two prominent peaks at m/z 956.39 and 925.4 (monoisotopic peaks) (Figure. 7C), which are very similar to those previously reported (Guan et al. 2005). These peaks correspond to molecular weights of 1919 and 1851, which match the expected masses of lipid II with 11 and 10 isoprene units, respectively (Guan et al. 2005). The *B. cenocepacia* lipid II preparation also revealed two peaks in the region of the spectrum expected for lipid II (Figure. 7D). The 956.39 peak of the *B. cenocepacia* lipid II was identical to the corresponding peak of the *E. coli* lipid II (Figure. 7C). However, the *B. cenocepacia* lipid II had a novel peak at an m/z value of 933.29 (Figure. 7D). Compared with the corresponding peak in *E. coli* lipid II, the difference in m/z value indicates a difference in molecular weight of ~ 15.78 , which could correspond to either an extra methyl or amino group in lipid II of *B. cenocepacia*. The nature of this difference will be investigated in a separate study, but it can account for a change that can also be reflected as a difference in the lipid II migration by paper chromatography and hence an R_f value different from that reported for *E. coli* (Ruiz 2008).

Murj proteins from *B. cenocepacia* and *E. coli* share a similar predicted fold and criticszal amino acid residues

In a recent study (Butler et al. 2013), a structural model of MurJ_{Ec} was proposed predicting a central, solvent-exposed cavity containing charged residues that are essential for function. Essential amino acid residues in the central cavity of MurJ_{Ec} were identified by a combination of cysteine and alanine replacements (Butler et al. 2013). This work demonstrated that the charged residues located in trans-membrane helix 1 (R18 and R24), periplasmic loop 1 (D39), trans-membrane helix 2 (R52) and trans-membrane helix (R270) of MurJ_{Ec} are accessible from the periplasmic space and required for function, as determined by complementation (Butler et al. 2013). By CLUSTAL analysis, MurJ from *E. coli* and *B. cenocepacia* share 50% similarity (data not shown). As with the *E. coli* homolog, an *in silico* structural model of MurJ_{Bc} was generated by I-Tasser (Zhang 2008; Roy et al. 2010), and the resulting 3D structure was aligned to that of MurJ_{Ec}. Both structures were nearly superimposable at most positions (Figure. 8A) and display a characteristic central cavity exposed to the periplasmic space (Figure. 8B). The alignment of the 3D predicted models for the *E. coli* and *B. cenocepacia* MurJ proteins revealed that the MurJ_{Bc} residues R18, R24, D39 and R52 are localized in the same regions as the corresponding residues in MurJ_{Ec}, while MurJ_{Bc} R274 corresponds to

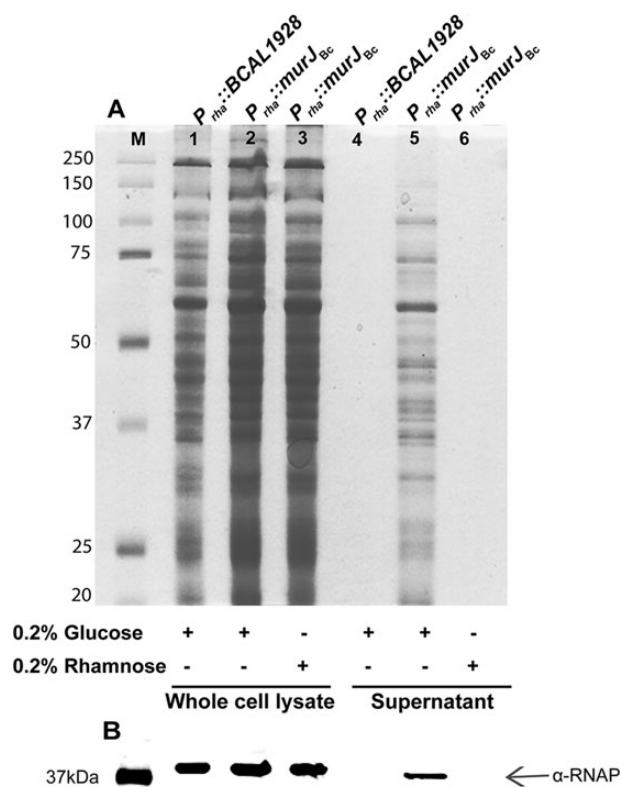


Fig. 6. Loss of cell membrane integrity by determining release of cytoplasmic content to the culture supernatant. Whole-cell lysates and culture supernatants of YFM1 (*P_{rha}::murJ_{Bc}*) and the control strain XOA10 (*P_{rha}::BCAL1928*) were prepared as described in *Material and methods* after 6 h of growth in non-permissive (Lanes 1, 2, 4 and 5) and -permissive (Lanes 3 and 6) conditions (Lanes 3 and 6). Ten to Fifteen micrograms of protein were loaded in each lane. (A) Coomassie blue staining. (B) Western blot developed using anti-RNA polymerase antibodies. Only the section of the blot with the detected protein bands is shown. The arrow indicates the presence of α subunit of the RNA polymerase (α -RNAP). M, Precision Plus™ Dual Color Protein Standards (a mixture of 10 recombinant proteins of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa).

Table II. MIC of ceftazidime and imipenem

| Antibiotic | MIC (μ g/ml) | | | |
|-----------------|-------------------|-------|----------------|-------|
| | Rhamnose 0.2% | | Rhamnose 0.05% | |
| | YFM1 | XOA10 | YFM1 | XOA10 |
| Ceftazidime | 0.75 | 0.75 | 0.125 | 0.75 |
| Imipenem | >32 | >32 | 1 | >32 |
| Chloramphenicol | 32 | 32 | 32 | 32 |

MurJ_{EC} R270 (Figure. 8C). MurJ_{Bc} derivatives containing alanine replacements of these residues were constructed and tested for their ability to restore the viability of the *P_{rha}::murJ_{Bc}* mutant under non-permissive conditions. Alanine replacement of R18, R24, and R274 in MurJ_{Bc} rendered the protein not functional, while the replacements in D39 and R52 did not compromise function (Figure. 9A and B). These results were not due to

lack of protein expression, as all the mutant proteins were expressed at levels similar to that of the parental MurJ_{Bc} (Figure. 9C). In MurJ_{EC}, the D39 residue was also not required for function, although it might be required only for structural stability (Butler et al. 2013), which could explain why the alanine replacement of the MurJ_{Bc} D39 did not impair function. Another difference between the *E. coli* and *B. cenocepacia* MurJ proteins is that MurJ_{EC} completely loses function when R52 is replaced by cysteine, and has a partial defect when replaced by alanine (Butler et al. 2013). Remarkably, the structural alignment of the predicted MurJ_{Bc} and MurJ_{EC} structures also revealed that the side chains of the residues investigated by alanine replacement mutagenesis were also superimposable spatially, except for R270 and R274. These differences could be attributed to the nature of the modeling approach that is not based on raw structural data but rather from an *in silico* approach. Regardless, both R270 and R274 were located at the same site and face of the predicted α -helix in both models and were also required for function. Together, these results show that despite some differences, MurJ_{Bc} and MurJ_{EC} belong to the same family of putative lipid II flippases.

Concluding remarks

The role of MurJ as the lipid II flippase has been disputed in part due to the reported absence of a lethal phenotype of a *B. subtilis* quadruple mutant in the *murJ* homologs *ytgP*, *spoVB*, *ykvU* and *yabM* (Fay and Dworkin 2009; Vasudevan et al. 2009). However, *B. subtilis* has two other genes, *epsK* and *tuaB*, which encode membrane proteins that share homologies with MurJ/MVIN/Wzx/Rft1 PFAM motifs (data not shown). Although EpsK and TuaB are respectively required for the synthesis of extracellular matrix and teichuronic acid, it may also be possible that any of these proteins provides functional redundancy involving lipid II flipping. This is conceivable, taking into account that the related Wzx family flippases, which are involved in the export of lipid-linked O-antigen intermediates, have relaxed specificity for their substrates (Feldman et al. 1999; Marolda et al. 2004; Alaimo et al. 2006). In our study, we demonstrate that the *murJ* homolog in *B. cenocepacia* K56-2 is indispensable for viability. A viable mutant in this gene could only be obtained using a conditional expression system. Under non-permissive conditions the mutant rapidly ceased growing and individual bacterial cells lost their bacillary shape and showed indications of osmotic fragility, suggesting a cell wall defect. Biochemically, the mutant accumulates radiolabeled PG intermediates, which is consistent with the flipping function attributed to this family of proteins. We also demonstrated that MurJ proteins from both *B. cenocepacia* and *E. coli* are functional homologs, since each of these proteins could restore viability in conditional mutant strains of both species. Furthermore, despite being only 50% related in their primary amino acid sequencing, the *E. coli* and *B. cenocepacia* MurJ proteins afforded similar predicted three-dimensional structures that were virtually superimposable including the predicted spatial location of residues that are critical to function. Therefore, our results with *B. cenocepacia* MurJ bring additional support to the notion that MurJ functions as lipid II flippase, as initially proposed for *E. coli* (Inoue et al. 2008; Ruiz 2008).

Table III. Primers

| Primer number | Oligonucleotide sequence, 5'–3' | Restriction enzyme |
|---------------|--|--------------------|
| 6122 | AAAAAACATATGAATCTATCCGAGCCCTGCT | NdeI |
| 6123 | AAAAAATCTAGATCTTGAACCTCGGCGAGGAT | XbaI |
| 6290 | AAAACCTCGAGGACGTTTCAATTCGCCTTCT | XhoI |
| 6291 | AAAACCTCGAGGCGTTCGATGTCGTCGAACATA | XhoI |
| 6347 | AAAATCTAGACGTTTCGAGCCCCACATAC | XbaI |
| 6348 | AAAAGAATTCGTTCTTCGGCCTCCTGCT | EcoRI |
| 6491 | TTTTTTCATATGGCGCAACGTCCGTATAATA | NdeI |
| 6388 | TTTTTCTAGATCACTTGGCGCGCCTT | XbaI |
| 6578 | GATCGATCATATGAATTTATTAATAATCGCTGGCCGCCGTCAGCTCG | NdeI |
| 6579 | CCGCACCTCTAGATTACACCGTCCGGCGGGCAAATCTTTAACTTTG | XbaI |
| Q294 | TTTTTTCATATGgactacaaggacgacgacgacgacaagGCGCAACGTCCGTATAATA | NdeI |
| Q369 | TTTTTTGAATTCgactacaaggacgacgacgacgacaagGCGCAACGTCCGTATAATA | EcoRI |
| Q295 | TTCACGCTGCTGTCGGCGGTGACCCGACTGGCC | |
| Q296 | GGCCAGTCCGGTACCCGCGGACAGCAGCGTGAA | |
| Q297 | GTGACCCGACTGGCCGCGGAGACGCTGATCGCC | |
| Q298 | GGCGATCAGCGTCTCCGCGGCCAGTCCGGTCAC | |
| Q299 | CCAGTCAATACACCGCGGCGTTCACGTGCC | |
| Q300 | GGCGACGTAGAACCCTCGCGGTGATTGACTGG | |
| Q301 | ATTCCGAACCTGCTGGCGCGCTGTCTGCCGAA | |
| Q302 | TTCGGCAGACAGGCGCGCCAGCAGGTTCCGGAAT | |
| Q303 | ATCAATTACGCCGACGCGCTGATGGAGTTCCCG | |
| Q304 | CGGGAACCTCATCAGCGCGTCGGCGTAATTGAT | |

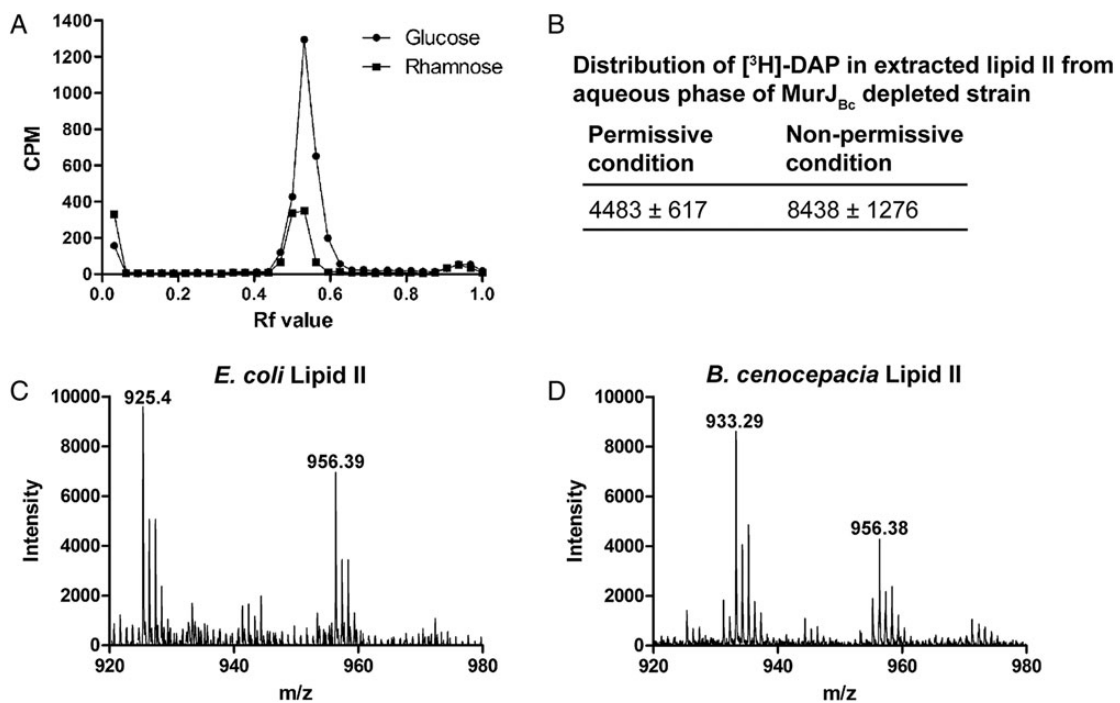


Fig. 7. Characterization of lipid II in *B. cenocepacia*. (A) Ascending paper chromatography of cell pellets of the strain YFM2 (*P_{rha}::murJ_{Bc}, ΔlysA*) under permissive (rhamnose) and non-permissive (glucose) conditions showing accumulation of cell wall precursors under non-permissive conditions. This experiment is representative of three independent experiments showing accumulation of lipid II at average R_f value of 0.47. (B) Accumulation of extracted lipid II from aqueous phase of the strain YFM2 under non-permissive conditions. Data correspond to the average of CPM ± SD of three independent experiments. $n = 3$. P -value = 0.0247. (C) MALDI-TOF MS of lipid II of *E. coli* W3110. D. MALDI-TOF MS of lipid II of *B. cenocepacia* K56-2. (C) and (D) are representative spectra of three independent preparations of lipid II.

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table I. Bacteria were grown at 37°C in Luria-Bertani (LB) medium,

SOB medium (2% tryptone, 0.5% yeast extract, 0.05% sodium chloride, 0.24% magnesium sulfate, 0.0186% potassium chloride) or M9 minimal medium, as required. Antibiotics were added to reach final concentrations as follows: 50 µg

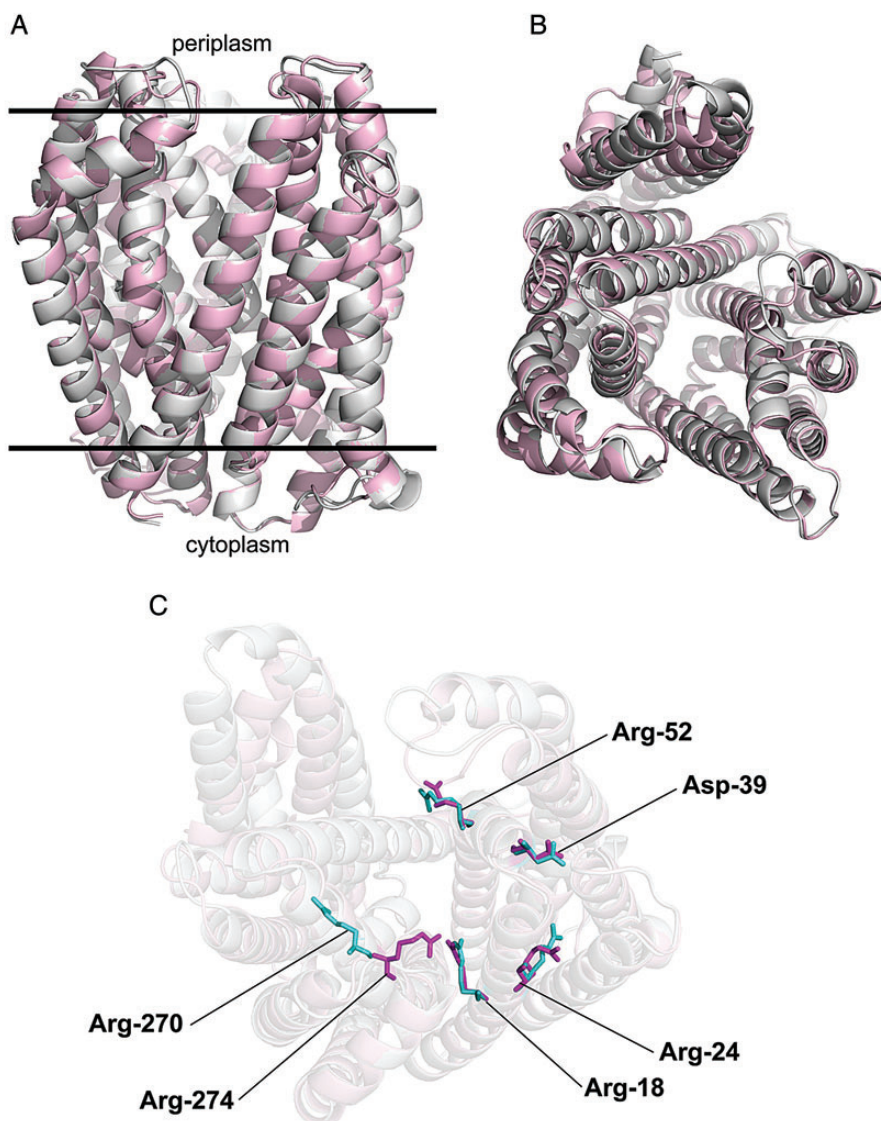


Fig. 8. Structural model of MurJ proteins from *B. cenocepacia* (gray) and *E. coli* (pink). (A) Front view, (B) top view from the periplasmic side and (C) position of conserved functional residues.

trimethoprim mL^{-1} for *E. coli* and $100 \mu\text{g mL}^{-1}$ for *B. cenocepacia* and $40 \mu\text{g}$ kanamycin mL^{-1} for *E. coli*. Ampicillin at $200 \mu\text{g mL}^{-1}$ and polymyxin at $20 \mu\text{g mL}^{-1}$ were used for tri-parental mating to select against donor and helper *E. coli* strains. Rhamnose and glucose were added to final concentrations of 0.2% (w/v) as needed. Antibiotics and chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Recombinant DNA methods

The primers used in this study are listed in Table III. The construction of pSC200 was previously reported (Cardona and Valvano 2005). DNA ligations, restriction endonuclease digestions and agarose gel electrophoresis were performed according to standard techniques (Sambrook et al. 1990). Restriction enzymes, T4 DNA polymerase and T4 DNA ligase were purchased from Roche Diagnostics, Dorval, Quebec, Canada. PCR

amplifications were carried out using the HotStar HiFidelity polymerase (Qiagen). Colony-PCR was performed with Taq polymerase (Qiagen). Amplifications were done according to the manufacturer's instructions and optimized for each primer pair. DNA sequencing was performed at the sequencing facility in York University (Toronto, Canada).

Construction of a *murJ_{Bc}* conditional mutant

A 300-bp fragment of the upstream region of the *murJ_{Bc}* gene (BCAL2764) was amplified using primers 6122 and 6123 and the amplicon cloned into pSC200 behind the plasmid-borne rhamnose promoter. Transformation of the ligation mixture was carried out in *E. coli* GT115 competent cells by the calcium chloride method (Cohen et al. 1972). Transformants were selected on LB agar plates containing $50 \mu\text{g}$ trimethoprim mL^{-1} . Resistant colonies were screened by colony-PCR.

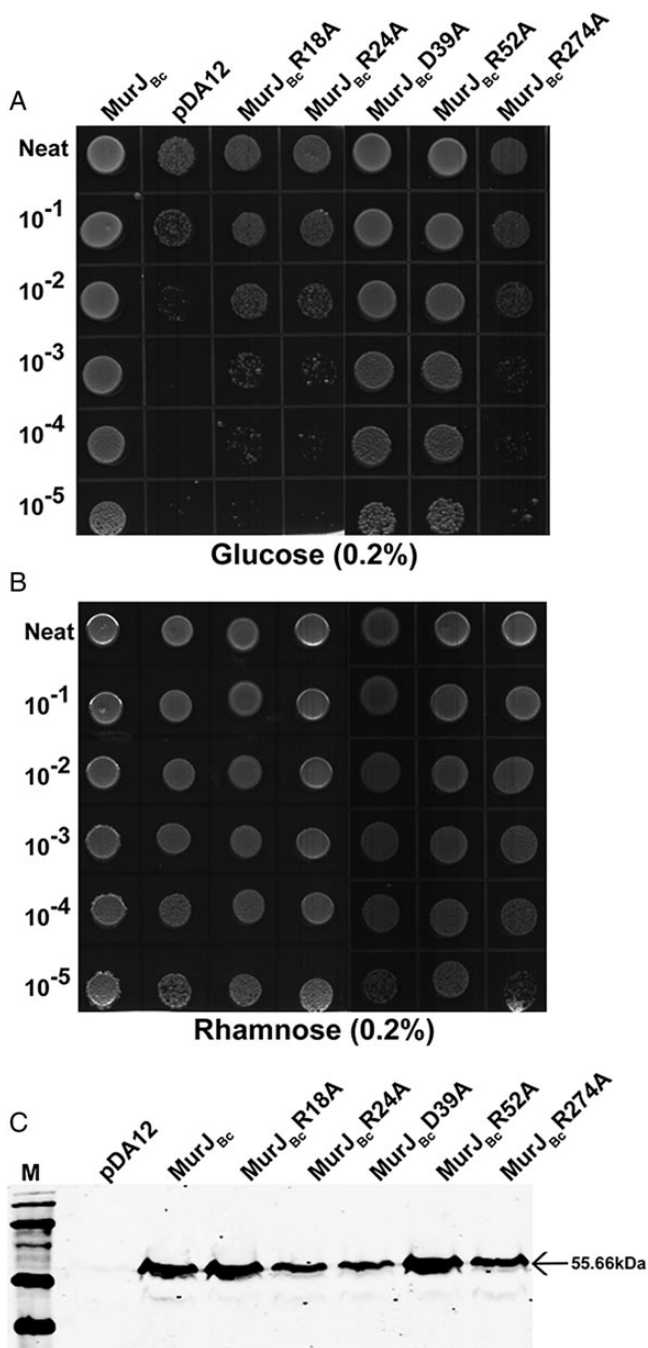


Fig. 9. Growth of YFM1 (*P_{rha}::murJ_{Bc}*) using MurJ_{Bc} and its derivatives carrying alanine replacements in the following charged residues: arginine-18 (R18A), arginine-24 (R24A), aspartic acid-39 (D39A), arginine-52 (R52A) and arginine-274 (R274A). Bacteria were grown under non-permissive (A) and permissive conditions (B). (C). Western blot demonstrating that all MurJ_{Bc} parental and mutant derivatives (expressed as N-terminal FLAG fusions) are correctly expressed (arrow) in the (*P_{rha}::murJ_{Bc}*) strain background. M, molecular mass ladder.

Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (Craig et al. 1989) using the pRK2013 helper plasmid (Figurski and Helinski 1979) on SOB plates containing 0.2% (w/v) rhamnose. Exconjugants were then

isolated by plating on LB agar plates supplemented with 100 µg trimethoprim mL⁻¹, 200 µg ampicillin mL⁻¹, 20 µg polymyxin mL⁻¹ and 0.2% rhamnose (Ortega et al. 2007). This experiment resulted in the isolation of the conditional mutant strain YFM1 (*P_{rha}::murJ_{Bc}*), in which the *murJ* gene is only expressed in the presence of rhamnose (see Results and discussion).

Complementation experiments

A plasmid constitutively expressing MurJ_{Bc} was constructed by amplifying the gene with primers 6491 and 6388. Amplicons were digested with NdeI–XbaI and cloned into a similarly digested pDA12, resulting in pDA12-*murJ_{Bc}*. We also constructed another plasmid, pEXT21-*murJ_{Bc}*, in which *murJ* gene expression was placed under the control of the *lac* promoter using the primers Q369 and 6388. The *murJ* homolog from *E. coli* K-12 was amplified with primers 6578 and 6579 and also cloned into pDA12. For complementation in *B. cenocepacia*, pDA12-*murJ_{Bc}* or pDA12-*murJ_{Ec}* was introduced into the conditional mutant strain YFM1 by triparental mating as described above. For complementation in *E. coli*, pDA12-*murJ_{Bc}* was introduced by electroporation into the *E. coli* mutant NR1152 (*P_{ara}::murJ_{Ec}*) (Ruiz 2008) and transformants selected on 25 µg ampicillin mL⁻¹, 30 µg tetracycline mL⁻¹ and arabinose 0.2%. Also, pEXT21-*murJ_{Bc}* was introduced by electroporation into NR1152 and selection was made on plates containing 80 µg spectinomycin mL⁻¹. For induction of MurJ_{Bc} expression, an overnight culture of NR1152 (pEXT21-*murJ_{Bc}*) was diluted to an OD₆₀₀ of 0.2 in the presence of the appropriate antibiotics, incubated at 37°C for 2 h up to an OD₆₀₀ of 0.6 at which time IPTG (isopropyl-β-D-1-thiogalactopyranoside) was added to a final concentration of 0.1 mM, and the culture was incubated for further 3 h.

Conditional viability by rhamnose depletion

The conditional viability of mutant YFM1 (*P_{rha}::murJ_{Bc}*) was assessed after an overnight growth at 37°C in M9 minimal medium supplemented with 0.5% yeast extract, 1 µM CaCl₂, 1 µM MgSO₄, 0.2% rhamnose and 100 µg trimethoprim mL⁻¹. An aliquot of the overnight culture was centrifuged and the bacterial pellet was washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an OD₆₀₀ of 1. Ten 10 µL drops of the undiluted suspension and 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ dilutions were deposited onto M9 agar plates supplemented with 0.2% glucose or 0.2% rhamnose and incubated at 37°C for 24 h. The same procedure was done to test the complementation of NR1152 by *murJ_{Bc}* except that the M9 agar plates were supplemented with either 0.2% arabinose or 0.05% fucose (Ruiz 2008). The essentiality of *murJ_{Bc}* was further demonstrated by monitoring growth in liquid medium in a rhamnose depletion assay (Ortega et al. 2007). Washed, rhamnose-depleted cells were diluted to an OD₆₀₀ of 0.15 in M9 medium supplemented with 0.5% yeast extract and 0.2% glucose, and incubated at 37°C for 4 h with shaking. Growing cells were aliquoted to 100-well plates containing fresh medium with 0.2% glucose to give an OD₆₀₀ of 0.05 and monitored for growth for an additional 12 h in a Bioscreen C automated growth curve reader (MTX Lab Systems, Inc.,

Vienna, VA) at 37°C with constant, low shaking with OD₆₀₀ readings taken every 30 min.

Microscopy

Samples of depleted cultures grown in the absence of rhamnose were taken after 6 h, placed on 0.8% agarose slides, covered by a coverslip and examined by phase-contrast microscopy. Other samples from the same cells were fluorescently stained with Syto 9 and propidium iodide (LIVE/DEAD BacLight bacterial viability kit; Molecular Probes, Invitrogen Detection Technologies, Eugene, OR). Samples were also examined by electron microscopy after fixing with 2.5% glutaraldehyde and staining with 2% uranyl acetate and lead citrate, as previously described (Schmerk et al. 2011). Grids were visualized with a Philips 410 transmission electron microscope at 60 kV.

Killing curves using β -lactam antibiotics and E-tests

Washed rhamnose-depleted cells were diluted to an OD₆₀₀ of 0.15 in M9 minimal medium supplemented with either 0.2 or 0.05% rhamnose and allowed to grow for 4 h. Then, cultures were diluted to $\sim 2 \times 10^5$ cfu mL⁻¹ in M9 minimal media and dispensed in 100-well plates in 300 μ L volumes. Either ceftazidime, imipenem or chloramphenicol was previously added to the 100-well plate to give final concentrations ranging from 2 to 128 μ g mL⁻¹ in two-fold increments. Plates were incubated in a Bioscreen C automated growth curve reader for 24 h with OD₆₀₀ readings taken every 30 min. For E-tests, depleted cells were washed and the OD₆₀₀ adjusted to 0.5 McFarland opacity standard. A sterile cotton swab was dipped into the bacterial suspension and streaked all over the surface of the Müller-Hinton agar plates in three directions to obtain an even distribution of the inoculum. The E-test strips (BioMérieux Clinical Diagnostics) for ceftazidime and imipenem were deposited onto the inoculated plates by means of a sterile forceps and the plates were incubated at 37°C for 48 h.

Detection of cytoplasmic content leakage using anti-RNA polymerase antibody

One-milliliter volumes of rhamnose-depleted cultures at an OD₆₀₀ of 1 were centrifuged for 1 min at $\times 13,000g$; cells were resuspended in 20 μ L distilled water and 10 μ L of 3 \times loading dye, and boiled for 5 min to prepare whole-cell lysates. To examine the culture supernatants, 35 mL of depleted culture was centrifuged for 15 min at $7000 \times g$ at 4°C. Supernatant was collected, filter-sterilized and proteins were precipitated overnight at 4°C in 10% trichloroacetic acid and protease inhibitor. Then, the mixture was centrifuged at $10,000 \times g$ for 30 min at 4°C. The pellet was washed with 5 mL acetone and centrifuged for another 30 min. Acetone was removed and pellet was suspended in 150 μ L sodium phosphate buffer 0.1 M (pH 7). The resulting suspension was boiled for 5 min. Five-microliter aliquots of boiled samples for both the whole-cell lysate and the supernatant were separated by gel electrophoresis using 14% (w/v) SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was rinsed with Tris-buffered saline (TBS), pH 8, and then blocked overnight with 10% (v/v) western

blocking reagent (Roche) in TBS at 4°C. The membrane was then incubated with monoclonal antibody to *E. coli* RNA-polymerase α -subunit (Neoclone) diluted 1:10,000 for 1.5 h at room temperature. The membrane was washed with TBS and then incubated at room temperature for 20 min with anti-mouse IgG antibody conjugated to Alexa Fluor 680 (Molecular Probes) diluted 1:20,000. Proteins were visualized using a Licor Infrared Imaging System with the Odyssey software. A separate gel was also prepared for Coomassie blue staining.

Detection of accumulation of PG precursors using [³H]-DAP

To increase the incorporation of [³H]-diaminopimelic acid (DAP) into growing cells, we constructed a strain with a *lysA* (BCAM2076) deletion using the method of Flannagan et al. (2008) for the construction of unmarked, non-polar deletions in *B. cenocepacia*. *lysA* is responsible for decarboxylation of DAP into lysine, and its deletion would result in the direct incorporation of exogenously added DAP to the PG (Wientjes et al. 1985). A *murJ* conditional mutant was constructed in the Δ *lysA* background strain. The resulting strain, YFM2 (*P_{rha}::murJ* Δ *lysA*), was grown overnight in M9 minimal medium containing rhamnose and 100 μ g trimethoprim mL⁻¹. Cells were washed as indicated above, diluted 1:100 into fresh M9 minimal medium containing glucose, methionine, lysine and threonine and grown under aeration at 37°C for 4 h to OD₆₀₀ of 0.6 (approximately four generations). Cells were diluted to an OD₆₀₀ of 0.1 in fresh medium containing glucose, methionine, lysine and threonine and labeled for 2 h at 37°C with 5 μ Ci of [³H]-DAP (30 Ci/mmol; American Radiolabeled Chemicals) (Wientjes et al. 1985). After 2 h, the cells were exposed again to 5 μ Ci of [³H]-DAP for another 2 h. At the end of the labeling period, cells were placed on ice and pelleted at 4°C for 5 min at $13,000 \times g$. Pellets were resuspended in 10 μ L of ice-cold water and immediately frozen until they were subjected to paper chromatography (Ramey and Ishiguro 1978; Ruiz 2008). Labeled cells were spotted onto Whatman 3MM paper (57 \times 49 cm), and labeled PG precursors were separated by ascending chromatography by development with isobutyric acid:1M NH₄OH(5:3; v/v) for 36 h. Paper was dried and cut into 1-cm squares that were counted in vials containing scintillation mixture (Ready Safe; Beckman Coulter).

Lipid II extraction of radiolabeled cells

Radiolabeled cells were resuspended in 100 μ L of PBS (pH 7.4), followed by the addition of 125 μ L of chloroform and 250 μ L of methanol. After periodic Vortex mixing for 10 min at room temperature, the homogenates were centrifuged at $4,000 \times g$ for 10 min and then the supernatants were converted to a two-phase Bligh–Dyer system by adding 125 μ L chloroform and 125 μ L PBS (Bligh and Dyer 1959; Guan et al. 2005). Since Lipid II partitions into the upper aqueous phase of a two-phase Bligh–Dyer system at the neutral pH of PBS, this aqueous layer was separated and counted in vials containing scintillation mixture (Bligh and Dyer 1959; Guan et al. 2005). To confirm the presence of [³H]-DAP-labeled lipid II in the aqueous phase, this layer was also spotted onto Whatman 3MM paper for separation of lipid II by ascending chromatography. The identity of lipid II was confirmed by mass spectrometry (MS) analysis using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).

Lipid II was extracted as previously described (Guan et al. 2005) and dissolved in 100 μ L of chloroform/methanol (2:1, v/v). One-microliter of this mixture was loaded on the target and covered by 1 μ L of the 5-chloro-2-mercaptobenzothiazole (20 mg/mL) matrix. The target was inserted in a Bruker Autoflex MALDI-TOF spectrometer. Data acquisition and analysis were performed using the Flex Analysis software.

Site-directed mutagenesis

murJ_{Bc} was PCR amplified from genomic *B. cenocepacia* using the primers Q294 (introducing a Flag-tag at the N-terminal) and 6388. The PCR product was cut with NdeI and XbaI and ligated to pDA12 cut with the same restriction enzymes to create pYM29 (pDA12-FLAG*murJ_{Bc}*), which is used as a template. Five point mutations were created individually in pDA12-FLAG*murJ_{Bc}* as previously described (Hamad et al. 2012). Complementary primers were designed to contain the desired mutation (Ala), flanked by unmodified sequence to anneal to the same sequence on opposite strands of the template plasmid. Primers from Q295 to Q304 (Table III) were used in pairs to create Ala point mutations in five selected residues, R18, R24, D39, R52 and R274 which correspond to similar residues in the *E. coli murJ* (Butler et al. 2013) to create the plasmids pYM31–pYM35, respectively. The plasmids were introduced into the strain YFM1 (*P_{rha}::murJ_{Bc}*) by triparental mating and tested for their ability to complement YFM1 under non-permissive conditions. MurJ protein expression was detected on total membranes that were prepared as described before (Patel et al. 2012) with the only modification that the overnight culture was diluted to an OD₆₀₀ of 0.2 then incubated at 37°C to grow for about 6 h to reach an OD₆₀₀ of 0.8–1. Protein concentration was measured using a nanodrop (Nanovue Plus, GE Healthcare). Flag-tagged MurJ was revealed by immunoblotting using a 1:10,000 dilution of the mouse anti-Flag monoclonal antibody. Proteins were visualized using a Licor Infrared Imaging System with the Odyssey software.

Supplementary Data

Supplementary data for this article are available online at <http://glycob.oxfordjournals.org/>.

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Conflict of interest

None declared.

Abbreviations

DAP, [³H]-diaminopimelic acid; GlcNAc, *N*-acetyl glucosamine; LB, Luria-Bertani; PBS, phosphate-buffered saline; PG, peptidoglycan; MurNAc, *N*-acetyl muramic acid; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; TBS, Tris-buffered saline.

References

- Alaimo C, Catrein I, Morf L, Marolda CL, Callewaert N, Valvano MA, Feldman MF, Aebi M. 2006. Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. *Embo J.* 25:967–976.
- Aubert DF, Flannagan RS, Valvano MA. 2008. A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. *Infect Immun.* 76:1979–1991.
- Barreteau H, Kovac A, Boniface A, Sova M, Gobec S, Blanot D. 2008. Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol Rev.* 32:168–207.
- Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 37:911–917.
- Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreux D. 2008. The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol Rev.* 32:208–233.
- Butler EK, Davis RM, Bari V, Nicholson PA, Ruiz N. 2013. Structure-function analysis of MurJ reveals a solvent-exposed cavity containing residues essential for peptidoglycan biogenesis in *Escherichia coli*. *J Bacteriol.* 195:4639–4649.
- Cardona ST, Valvano MA. 2005. An expression vector containing a rhamnase-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid.* 54:219–228.
- Cohen SN, Chang AC, Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc Natl Acad Sci USA.* 69:2110–2114.
- Craig FF, Coote JG, Parton R, Freer JH, Gilmour NJ. 1989. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. *J Gen Microbiol.* 135:2885–2890.
- Dykxhoorn DM, St Pierre R, Linn T. 1996. A set of compatible *tac* promoter expression vectors. *Gene.* 177:133–136.
- Ehlert K, Holtje JV. 1996. Role of precursor translocation in coordination of murein and phospholipid synthesis in *Escherichia coli*. *J Bacteriol.* 178:6766–6771.
- El Ghachi M, Bouhss A, Barreteau H, Touze T, Auger G, Blanot D, Mengin-Lecreux D. 2006. Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. *J Biol Chem.* 281:22761–22772.
- Fay A, Dworkin J. 2009. *Bacillus subtilis* homologs of MviN (MurJ), the putative *Escherichia coli* lipid II flippase, are not essential for growth. *J Bacteriol.* 191:6020–6028.
- Feldman MF, Marolda CL, Monteiro MA, Perry MB, Parodi AJ, Valvano MA. 1999. The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. *J Biol Chem.* 274:35129–35138.
- Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA.* 76:1648–1652.
- Flannagan RS, Linn T, Valvano MA. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ Microbiol.* 10:1652–1660.
- Guan Z, Breazeale SD, Raetz CR. 2005. Extraction and identification by mass spectrometry of undecaprenyl diphosphate-MurNAc-pentapeptide-GlcNAc from *Escherichia coli*. *Anal Biochem.* 345:336–339.
- Hamad MA, Di Lorenzo F, Molinaro A, Valvano MA. 2012. Aminoarabinose is essential for lipopolysaccharide export and intrinsic antimicrobial peptide resistance in *Burkholderia cenocepacia*. *Mol Microbiol.* 85:962–974.
- Hamad MA, Skeldon AM, Valvano MA. 2010. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in

- studies of intracellular bacteria with the gentamicin protection assay. *Appl Environ Microbiol.* 76:3170–3176.
- Hayes MV, Orr DC. 1983. Mode of action of ceftazidime: Affinity of the penicillin-binding proteins of *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *J Antimicrob Chemother.* 12:119–126.
- Henriques AO, Glaser P, Piggot PJ, Moran CP, Jr. 1998. Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. *Mol Microbiol.* 28:235–247.
- Ikeda M, Sato T, Wachi M, Jung HK, Ishino F, Kobayashi Y, Matsuhashi M. 1989. Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. *J Bacteriol.* 171:6375–6378.
- Inoue A, Murata Y, Takahashi H, Tsuji N, Fujisaki S, Kato J. 2008. Involvement of an essential gene, *mviN*, in murein synthesis in *Escherichia coli*. *J Bacteriol.* 190:7298–7301.
- Ishino F, Jung HK, Ikeda M, Doi M, Wachi M, Matsuhashi M. 1989. New mutations *fts-36*, *fts-33*, and *ftsW* clustered in the *mra* region of the *Escherichia coli* chromosome induce thermosensitive cell growth and division. *J Bacteriol.* 171:5523–5530.
- Ishino F, Park W, Tomioka S, Tamaki S, Takase I, Kunugita K, Matsuzawa H, Asoh S, Ohta T, Spratt BG, et al. 1986. Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and RodA protein. *J Biol Chem.* 261:7024–7031.
- Islam ST, Lam JS. 2013. Wzx flippase-mediated membrane translocation of sugar polymer precursors in bacteria. *Environ Microbiol.* 15:1001–1015.
- Khattar MM, Begg KJ, Donachie WD. 1994. Identification of FtsW and characterization of a new *ftsW* division mutant of *Escherichia coli*. *J Bacteriol.* 176:7140–7147.
- Kol MA, de Kroon AI, Rijkers DT, Killian JA, de Kruijff B. 2001. Membrane-spanning peptides induce phospholipid flop: A model for phospholipid translocation across the inner membrane of *E. coli*. *Biochemistry.* 40:10500–10506.
- Kol MA, van Laak AN, Rijkers DT, Killian JA, de Kroon AI, de Kruijff B. 2003. Phospholipid flop induced by transmembrane peptides in model membranes is modulated by lipid composition. *Biochemistry.* 42:231–237.
- Ling JM, Moore RA, Surette MG, Woods DE. 2006. The *mviN* homolog in *Burkholderia pseudomallei* is essential for viability and virulence. *Can J Microbiol.* 52:831–842.
- Marolda CL, Vicarioli J, Valvano MA. 2004. Wzx proteins involved in biosynthesis of O antigen function in association with the first sugar of the O-specific lipopolysaccharide subunit. *Microbiology.* 150:4095–4105.
- Mercer KL, Weiss DS. 2002. The *Escherichia coli* cell division protein FtsW is required to recruit its cognate transpeptidase, FtsI (PBP3), to the division site. *J Bacteriol.* 184:904–912.
- Mohammadi T, van Dam V, Sijbrandi R, Vernet T, Zapun A, Bouhss A, Diepeveen-de Bruin M, Nguyen-Disteche M, de Kruijff B, Breukink E. 2011. Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *Embo J.* 30:1425–1432.
- Neu HC. 1983. Clinical perspectives on imipenem. *J Antimicrob Chemother.* 12(Suppl. D):149–153.
- Ortega X, Hunt TA, Loutet S, Vinion-Dubiel AD, Datta A, Choudhury B, Goldberg JB, Carlson R, Valvano MA. 2005. Reconstitution of O-specific lipopolysaccharide expression in *Burkholderia cenocepacia* strain J2315, which is associated with transmissible infections in patients with cystic fibrosis. *J Bacteriol.* 187:1324–1333.
- Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ, Govan JR, Valvano MA. 2007. A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. *J Bacteriol.* 189:3639–3644.
- Patel KB, Toh E, Fernandez XB, Hanuszkiewicz A, Hardy GG, Brun YV, Bernards MA, Valvano MA. 2012. Functional characterization of UDP-glucose:Undecaprenyl-phosphate glucose-1-phosphate transferases of *Escherichia coli* and *Caulobacter crescentus*. *J Bacteriol.* 194:2646–2657.
- Ramey WD, Ishiguro EE. 1978. Site of inhibition of peptidoglycan biosynthesis during the stringent response in *Escherichia coli*. *J Bacteriol.* 135:71–77.
- Remmert M, Biegert A, Hauser A, Soding J. 2011. HHblits: Lightning-fast iterative protein sequence searching by HMM-HMM alignment. *Nat Methods.* 9:173–175.
- Rodloff AC, Goldstein EJ, Torres A. 2006. Two decades of imipenem therapy. *J Antimicrob Chemother.* 58:916–929.
- Roy A, Kucukural A, Zhang Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc.* 5:725–738.
- Rudnick PA, Arcondeguy T, Kennedy CK, Kahn D. 2001. *glnD* and *mviN* are genes of an essential operon in *Sinorhizobium meliloti*. *J Bacteriol.* 183:2682–2685.
- Ruiz N. 2008. Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proc Natl Acad Sci USA.* 105:15553–15557.
- Sambrook J, Fritsch EF, Maniatis T. 1990. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmerk CL, Bernards MA, Valvano MA. 2011. Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in *Burkholderia cenocepacia*. *J Bacteriol.* 193:6712–6723.
- Tamaki S, Matsuzawa H, Matsuhashi M. 1980. Cluster of *mrdA* and *mrdB* genes responsible for the rod shape and mecillinam sensitivity of *Escherichia coli*. *J Bacteriol.* 141:52–57.
- Valvano MA. 2008. Undecaprenyl phosphate recycling comes out of age. *Mol Microbiol.* 67:232–235.
- Valvano MA, Hanuszkiewicz A. 2012. Proteins involved in the membrane translocation of O-antigen lipopolysaccharide. In: Andrade M, editor. An overview on the chemistry and biochemistry of carbohydrates. Mini-Reviews in Carbohydrate Chemistry. Bussum, The Netherlands: Bentham Science Publishers Ltd. p. 261–269.
- van Dam V, Sijbrandi R, Kol M, Swiezewska E, de Kruijff B, Breukink E. 2007. Transmembrane transport of peptidoglycan precursors across model and bacterial membranes. *Mol Microbiol.* 64:1105–1114.
- van Heijenoort J. 2001. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. *Glycobiology.* 11:25R–36R.
- Vandamme P, Holmes B, Vancanneyt M, Coenye T, Hoste B, Coopman R, Revets H, Lauwers S, Gillis M, Kersters K, et al. 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol.* 47:1188–1200.
- Vasudevan P, McElligott J, Attkisson C, Betteken M, Popham DL. 2009. Homologues of the *Bacillus subtilis* SpoVB protein are involved in cell wall metabolism. *J Bacteriol.* 191:6012–6019.
- Wientjes FB, Pas E, Taschner PE, Woldringh CL. 1985. Kinetics of uptake and incorporation of meso-diaminopimelic acid in different *Escherichia coli* strains. *J Bacteriol.* 164:331–337.
- Zhang Y. 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics.* 9:40.