# Supplementary Materials for

## MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis

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**This PDF file includes:**

Materials and Methods
Figs. S1 to S9
Tables S1 to S2

## Materials and Methods

Bacterial strains and growth conditions

Strains are listed in Table S1. Lysogeny broth (LB), glucose M63 and M9 minimal media were prepared as described previously (*14, 15*). All liquid cultures were grown under aeration at 37°C unless otherwise noted. Growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). When appropriate, kanamycin (25 µg/ml), ampicillin (25 µg/ml), chloramphenicol (20 µg/ml), tetracyclin (5 µg/ml), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal; 20 µg/ml), and arabinose (0.2% v/v) were added. The Δ*fhuA::kan* and Δ*lysA::kan* alleles were obtained from Keio collection (*16*) and introduced into the appropriate strains by P1*vir* transduction (*14*). Kanamycin-resistance cassettes flanked by FLP recombinase sites FRT were excised using pCP20 (*17*).

Plasmid pCS37 (*cat araC* $P_{ara}$::$RBS_{ftsW}$-*ftsW*) was constructed in several steps. A fragment encoding *ftsW* with its native ribosome-binding site (RBS) was synthesized by PCR using KOD DNA polymerase, and primers CS70 and CS4. The resulting product was digested with XbaI and HindIII, and ligated into plasmid pBAD33 (*cat araC* $P_{ara}$) digested with the same enzymes. The cloned fragment was confirmed by PCR and sequencing.

Plasmid pHC808 (*cat* $P_{tac}$::*uppS*) was constructed by first amplifying the *uppS* gene by PCR using primers NdeI-uppS and HindIII-uppS. The resulting product was digested with NdeI and HindIII and ligated into plasmid pHC788 (*cat* $P_{ara}$). The *uppS* open reading frame along with the strong RBS encoded by pHC788 was excised by digestion with XbaI and HindIII, followed by ligation into plasmid pHC800 (*cat* $P_{tac}$) digested with the same enzymes. The cloned fragment was confirmed by sequencing. Both pHC788 and pHC800 are medium copy vectors related to pBAD33 with a p15A (pACYC) origin of replication.

Strain CS20 (Δ*rodA*::$kan^R$ Δ*ftsW*::*frt*) harboring plasmids pCS37 (*cat araC* $P_{ara}$::$RBS_{ftsW}$-*ftsW*) and pTB63 (*ftsQAZ*) was constructed in several steps. The Δ*ftsW*:: $kan^R$ allele was made by replacing nucleotides 19 through 2245 of the *ftsW* open reading frame with a $kan^R$ cassette as described previously (*18*). To avoid potential polar effects of the insertion, the $kan^R$ cassette was amplified from pKD4 using primers CS81 and CS82 such that, once recombined, the synthetic RBS from pKD4 would be inserted 8 bp upstream of the start codon of the downstream *murG* reading frame. The PCR product with the $kan^R$ cassette was then purified and electroporated into TB10/pCS37 (*cat araC* $P_{ara}$::$RBS_{ftsW}$-*ftsW*) as described (*18*). Recombinants were then selected on M9 agar supplemented with 25 µg/mL kanamycin and 0.2% arabinose at 30°C. The resulting recombinants were then purified on M9 agar with or without 0.2% arabinose to screen for isolates that require arabinose for growth. From one such isolate, the Δ*ftsW*::$kan^R$ allele was transferred to strain NR2590/pCS37 by P1 transduction, followed by excision of $kan^R$ cassette by pCP20 to construct strain CS16 (NR2590 Δ*ftsW*::*frt* /pCS37). Excision was confirmed by PCR and kanamycin sensitivity. Plasmid pTB63 carrying *ftsQAZ* (*19*) was used to allow growth of Δ*rodA* strains (*20*). It was transformed into strain CS16 to construct strain CS17 (NR2590 Δ*ftsW*::*frt* /pCS37 /pTB63). The Δ*rodA*:: $kan^R$ allele was obtained from strain FB22(λCH221) (Δ*rodA*:: $kan^R$ $P_{lac}$::$gfp_{mut2}$-T7-*rodA*) (*21*), and was transduced into MG1655/pTB63 to obtain strain TU231/pTB63. Finally, the Δ*rodA*:: $kan^R$ allele from strain TU231/pTB63 was transduced into CS17 to produce CS20. Transductants were selected on M9 agar supplemented with 25 µg/mL kanamycin and 0.2% arabinose at 30°C for 2 days. The presence of Δ*rodA*::$kan^R$ was further confirmed by PCR and sequencing.

Cys substitutions in *flag-murJΔcys* were generated in pFLAGMurJΔCys by site-directed mutagenesis (SDM) using the *Pfu* Turbo polymerase (Agilent Technologies) and primers listed in Table S2, and the functionality of the resulting alleles was assessed as described previously (*8*).

Screen for MTSES sensitivity

In order to screen for sensitivity to MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES; Santa Cruz Biotechnology), overnight cultures of haploid strains expressing MurJ variants grown in glucose M63 broth were diluted in duplicate to an $OD_{600}$ of ~0.1. Once $OD_{600}$ reached 0.5, MTSES (in water) was added to one of the dilutions of each strain at a final concentration of 0.1 mM. All assays were done in triplicate and performed in glucose M63 broth unless indicated otherwise. For cultures grown in LB, the final concentration of MTSES was increased to 0.5 mM to account for higher levels of *flag-murJΔcys* expression we have observed in this medium.

Overexpression and purification of colicin M

Plasmid pMLD237 ($His_6$-ColM) was a generous gift from Dominique Mengin-Lecreulx (*22*). Production of colicin M is essentially the same as described (*22*) with slight modification. Briefly, BL21(DE3)/pMLD237 ($P_{T7}$::$His_6$-ColM) was grown overnight in 5 mL of LB medium supplemented with 25 µg/ml kanamycin and 0.2% (w/v) glucose. The resulting culture was diluted 1:100 in 1 L of LB medium with kanamycin and grown at 30°C to $OD_{600}$ 0.4-0.6. IPTG was then added to a final concentration of 0.5 mM and growth was continued for 3 h at 30°C. Cells were collected by centrifugation at 8,000 x g for 10 min at room temperature (RT), and resuspended in 25 ml of buffer A (20mM Tris-HCl pH 7.4, 0.5M NaCl, 20mM imidazole). After the addition of 100 µl of protease inhibitor cocktail III (EMD Millipore), cells were lysed by passage through a french pressure cell (twice at ≈15,000 psi). Remaining intact cells were removed by centrifugation at 17,100 x g for 10 min at 4°C, and the supernatant was mixed with 1.5 ml of Ni-NTA agarose (QIAGEN). The mixture was incubated at 4°C for 1 h with gentle shaking. The suspension was then transferred to a gravity flow column (Biorad) and the resin was washed twice with 4 ml of buffer A. Protein was eluted by adding 0.5 ml of buffer B (20mM Tris-HCl pH 7.4, 0.5M NaCl, 300 mM imidazole) four times. Eluate fractions containing $His_6$-ColM were pooled and dialyzed against 1X PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4), 10% (w/v) glycerol, and stored at -80°C. Protein concentration was measured by NI protein assay kit (G bioscience) using bovine serum albumin (BSA) as a standard.

Detecting lipid II flippase activity with colicin M

Strains NR2592 and NR2593 were grown overnight in M9 medium supplemented with 0.2% glucose and 100 µg/mL of methionine, threonine and lysine. The resulting cultures were diluted 1:100 into 10 ml of the same medium and growth was continued at 37°C. At an $OD_{600}$ 0.2 - 0.4, [$^3$H] m-DAP (American Radiolabeled Chemicals) was added to a final concentration of 1.5 µCi/ml, and growth was continued. After 15 min, 20 µl of 0.1 M MTSES (in DMSO) and 10µl of 50 µg/ml ColM were added to cultures. After growth for an additional 10 min, cells were collected by centrifugation at 8,000 x g for 2 min at 4°C. The resulting pellet was resuspended in 1 ml of preheated (95°C) water, and incubated for 30 min at 100°C. The hot water extract was then centrifuged at 100,000 x g for 20 min at 4°C. The resulting supernatant fraction, which contains water soluble UDP-precursors and ColM degradation products, was frozen on dry ice

and lyophilized overnight. The lyophilized sample was resuspended in 300 µl of buffer C (50 mM triethylammonium formate pH4.6, 6% (v/v) methanol) and analyzed by RP-HPLC as described (*11*). Specifically, 150µl of sample was injected onto a Nucleosil C18 column (A0119250X046 Agilent or 00G-0323-E0 Phenomenex), and eluted with a isocratic flow of buffer C at 0.6 ml/min at 25$^{o}$C for 70 min. Radiolabeled compounds were detected using an inline radioflow detector (LB513, Berthold) according to the manufacturer and Ultima-Flo M scintillation fluid (6013759, Perkin Elmer) at a flow rate of 1.5 ml/min. Peaks were identified using purified m-DAP and UDP-MurNAc-pep5 (gift from Ted Park) as standards. The amount of ColM product was measured as the area under the peak using Chromeleon software (Dionex).

Lipid I and lipid II were extracted from hot water-insoluble material (*23*). Briefly, the pellet fraction was resuspended in 1 mL of water and boiled for 3 min to remove residual water-soluble radiolabeled compounds. The PG/lipid fraction was then collected by centrifugation at 21,130 x g for 5 min at RT. The resulting pellet fraction was resuspended in 100 µl of 10 mM Tris-HCl pH7.4, followed by the addition of 100 µl of 6M pyridinium acetate/1-butanol (1:2 v/v). The mixture was vortexed for 30 sec, and centrifuged at 16,100 x g for 30 sec at RT. The top organic fraction was collected and carefully transferred to a clean microcentrifuge tube. The extraction was repeated three times and the organic fractions were pooled. Butanol-saturated water (100 µl) was added to the pooled organic fractions and the samples were vortexed for 30 sec, centrifuged, and 100 µl of the top layer was transferred to a scintillation vial containing 10 ml of Ecolite scintillation fluid (882475, MP biochemicals) and subjected to scintillation counting.

Lipid I/lipid II degradation by colicin M in spheroplasts produced from cells deprived of MurJ activity

Strain CS12 and CS13 were grown in M9 0.2% glucose with 100 µg/mL of lysine, methionine and threonine. Overnight cultures were diluted 100-fold in 10 ml of the same medium, and grown to $OD_{600}$ 0.3. [$^{3}$H] m-DAP (American Radiolabeled Chemicals) was added to a final concentration of 1.5 µCi/ml and growth was continued for 15 min. Then, 40 µL of 0.1 M MTSES (in DMSO) was added and the mixture was incubated for an additional 10 min. The cultures were rapidly chilled on ice and cells were harvested by centrifugation at 6,000 rpm for 2 min at 4$^{o}$C (Fiberlite F13-14x50cy rotor, Thermo Scientific). Spheroplasts were prepared from the cell pellets by resuspending them in 250 µl of SP buffer [30 mM Tris-HCl pH8, 25% (w/v) sucrose] and 250 µl of 0.5 M Tris-HCl pH8, followed by the addition of 10 µl of 0.25 M EDTA and 10 µl of 10 mg/ml lysozyme (*24*). The mixture was incubated at RT for 5 min, and the formation of spheroplasts was assessed to be complete in 3 min using phase contrast microscopy. Spheroplasts were collected by centrifugation for 1 min at 16,100 x g at RT and resuspended in 400 µl of colicin M reaction buffer (18.8 mM Tris-HCl pH 8, 15.6% (w/v) sucrose, 2.5 mM $CaCl_2$, 1.8 mM $MgCl_2$). Alternatively, spheroplasts were ruptured by resuspension in colicin M reaction buffer lacking sucrose. Colicin M and MTSES were added to a final concentration of 0.25 mg/ml and 0.8 mM, respectively, and the spheroplasts were incubated at 37$^{o}$C for 15 min. Spheroplasts were then collected by centrifugation at 16,100 x g for 1 min at RT and the supernatant fraction was discarded. Colicin M activity in the pellet fraction was inactivated by heating at 100$^{o}$C for 5 min. For osmotically lysed spheroplasts, membrane fragments and right-side out vesicles were collected by centrifugation at 100,000 x g for 20 min at 4$^{o}$C after colicin M activity was heat inactivated. The pellet fraction was resuspended in 1 mL of preheated water, and the suspension was boiled for 30 min. The water insoluble fraction was collected by

centrifugation at 100,000 x g for 20min at 4°C and washed once by resuspension in 1 ml of water and boiling for 3 min to remove residual UDP-containing precursors. The pellet was collected again by centrifugation and the lipid precursors were extracted and quantified as described above for whole cell experiments.

LC/MS analysis

The identity of the major products in the radiolabeled experiments was confirmed by LC/MS analysis of analogous, non-radiolabeled, samples from UppS-overproducing *E. coli* treated with ColM. To overexpress UppS, strain T8640 was grown in M9 medium supplemented with 0.2% glucose, 25μM IPTG and 100 μg/mL of methionine, threonine and lysine to an $OD_{600}$ of approximately 0.2. Colicin M treatment and hot water extraction of soluble metabolites then performed exactly as described above. The reaction mixture was lyophilized and dissolved in 50 μL $H_2O$. A 10-μL sample of this solution was subjected to LC/MS analysis, conducted with ESI-MS operating in negative mode (*25*). The instrument was equipped with a Waters Symmetry Shield RP18 column (5 μm, 3.9X150 mm) with matching column guard. The fragments were separated using the following method: 0.5 mL/min $H_2O$ (0.1% formic acid) for 5 min followed by a gradient of 0% ACN (0.1% formic acid)/$H_2O$ (0.1% formic acid) to 20% ACN (0.1% formic acid)/$H_2O$ (0.1% formic acid) over 40 min. Molecular ions (M-H) and (M-2H)/2 corresponding to expected disaccharide fragments were extracted from the chromatograms (Fig. S4). **a**: 1192.3 + 595.7; **b**: 1098.3 + 548.7; **c**: 1018.4 + 508.7; PP-Mpep5: 966.3 + 482.7 (not observed). Products in the LC/MS assay eluted ~3.5 min later than in the compatible radiolabeled assay (see Fig. S3) due to dead volume differences in the instruments.

Microscopy

At the indicated time post-MTSES addition, 3 μl of culture was spotted on an agarose pad (1% agarose in 1X PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1.46 mM $KH_2PO_4$). Cells were visualized on a Nikon Eclipse Ti-E by phase contrast using a 100X oil objective lens.

Detection of FLAG-MurJ by immunoblotting

Assays for MTSES binding were performed as described previously (*8*) with the exception that the MTSES blocking step was performed in LB broth for 5 min with 0.1 mM MTSES. For detection of FLAG-MurJ A29 variants, overnight cultures were diluted to an $OD_{600}$ ~0.1 in LB with chloramphenicol and grown until $OD_{600}$ 1.5. Cells were resuspended in 1X AB Buffer (3.42 mM $Na_2HPO_4$, 1.58 mM $NaH_2PO_4$, 25 mM Tris-HCl, pH 6.8, 3 M urea, 0.5% β-mercaptoethanol, 1.5% SDS, 5% glycerol, 0.05% bromophenol blue) and incubated for 30 min at 45°C prior to loading onto a 12% SDS-polyacrylamide gel for separation, transfer, and probing as previously described (*8*). The signal was developed with Clarity Western ECL substrate kit (BioRad) and detected using a ChemiDoc XRS+ system (Bio-Rad). Image acquisition and densitometry was performed using the Image Lab software (BioRad). Immunoblots were done in triplicate with representative results shown.

*In silico* modeling

Renderings of the MurJ structural model (*8*) were generated using the PyMOL molecular graphics system, version 1.5.0.5 (Schrodinger, LLC, Portland, OR).

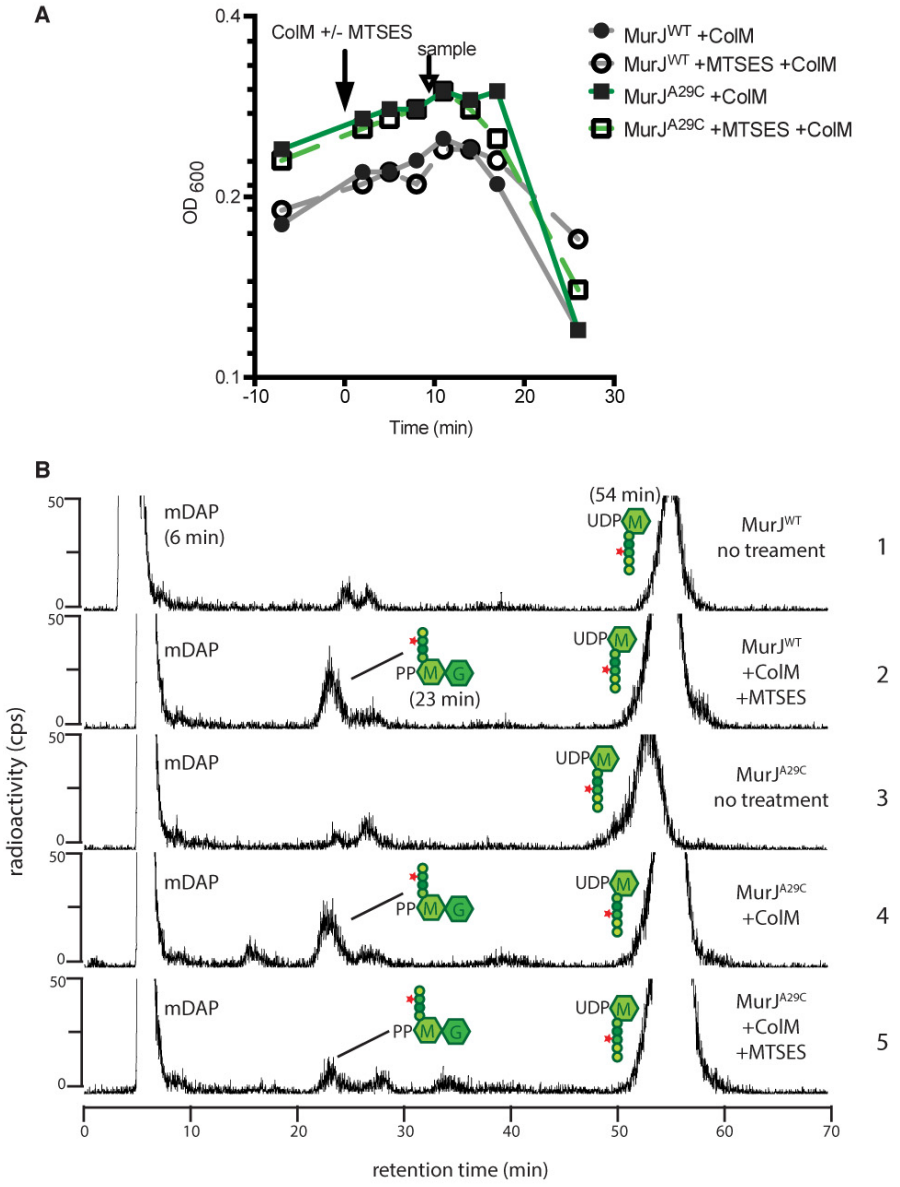


**Fig. S1. Growth data and HPLC chromatograms for the detection of lipid-II cleavage by ColM.** (**A**) Growth of cells used for labeling experiments described in fig. 1 was monitored by following culture $OD_{600}$. Times of ColM and/or MTSES addition, and sample preparation are indicated by the arrows. Samples were collected before lysis caused by ColM was observed. Addition of [$^3$H]-mDAP was at t= -15min. (**B**) Labeled PG precursors and ColM cleavage products in the hot water extracts from the labeled cells in (**A**) were separated by reverse-phase (RP) HPLC using isocratic elution with 50mM triethylammonium formate pH 4.6 in 6% (v/v) methanol, and detected with an inline radiodetector (Berthold). The MurJ variant being produced and culture treatments are indicated to the right of the representative chromatograms.

Retention times of the labeled peaks are given in parentheses. The mDAP and UDP-$Mpep_5$ peaks were identified using authentic standards. The identity of the ColM product was confirmed based on the results presented in fig. S2-S4. For the bar graph in Fig. 1B, the ColM product was quantified by determining the area under the peak.

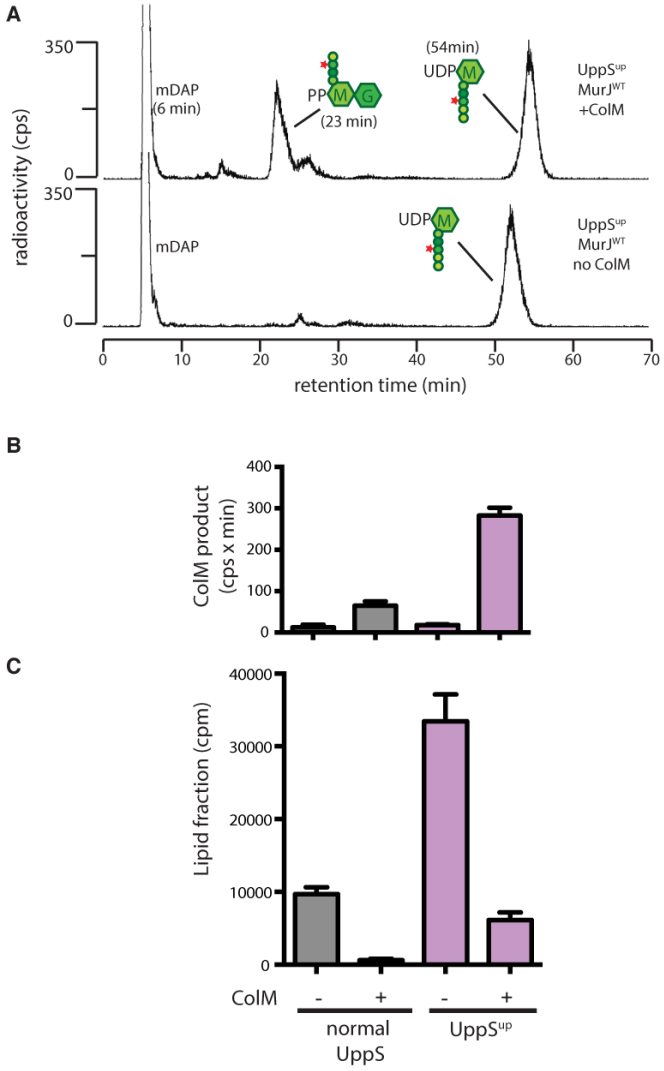


**Fig. S2. Enhanced accumulation of lipid-precursors and the ColM cleavage product upon UppS overproduction.** (**A**) Cells of NR2590 containing pHC808 [$P_{tac}$::*uppS*] and producing $MurJ^{WT}$ were grown in labeling medium containing 25 µM IPTG. Labeling and ColM treatment as indicated was performed as described in the legend for fig. 1. Labeled PG precursors and ColM cleavage products were analyzed as in fig. S1B. Note the large increase in the ColM-dependent product. (**B-C**) Levels of ColM cleavage product and lipid intermediates were quantified as described in the legend for Fig. 1. The mean ± SD of duplicate measurements is shown. Data from fig. 1B and 1C from samples without overproduction of the undecaprenol-PP synthase UppS (*2*) were added for comparison.

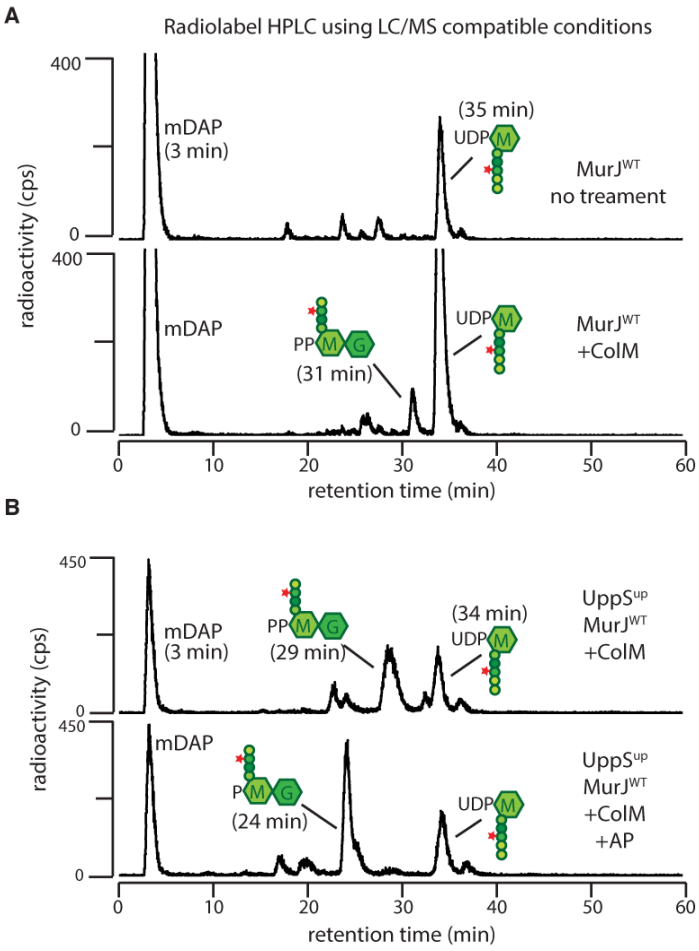


**Fig. S3. Detection of ColM-dependent cleavage product using an LC/MS compatible HPLC separation.** (**A**) Cells of NR2592 producing MurJ$^{WT}$ were grown in labeling medium. Labeling and ColM treatment as indicated was performed as described in fig. 1. For this experiment, labeled material in the hot water extracts were separated by RP-HPLC using a linear gradient of 0-20% (v/v) acetonitrile in 0.1% (v/v) formic acid so that equivalent unlabeled extracts could be analyzed in parallel by LC/MS (see fig. S4). As with the previous RP-HPLC conditions, a ColM-dependent peak was observed and well separated from the UDP-Mpep$_5$ peak. (**B**) Cells were grown and labeled as in (**A**), except that they also contained plasmid pHC808 [$P_{tac}$::*uppS*] and 25 µM IPTG was included in the medium to induce UppS overproduction. Hot water extracts were again separated by the modified RP-HPLC procedure described in A. Note the large increase in the ColM-dependent peak relative to that of UDP-Mpep$_5$. In the lower panel, the extract was treated with alkaline phosphatase (AP), which caused a shift in the ColM-dependent peak, consistent with the product having an accessible phosphate. Corresponding unlabeled extracts analyzed in parallel by LC/MS definitively identified PP-Mpep$_4$-G as the compound appearing at the retention time of the ColM-dependent peak in the labeling experiment, and P-Mpep$_4$-G as the compound resulting from AP treatment. See fig. S4 for LC/MS data.

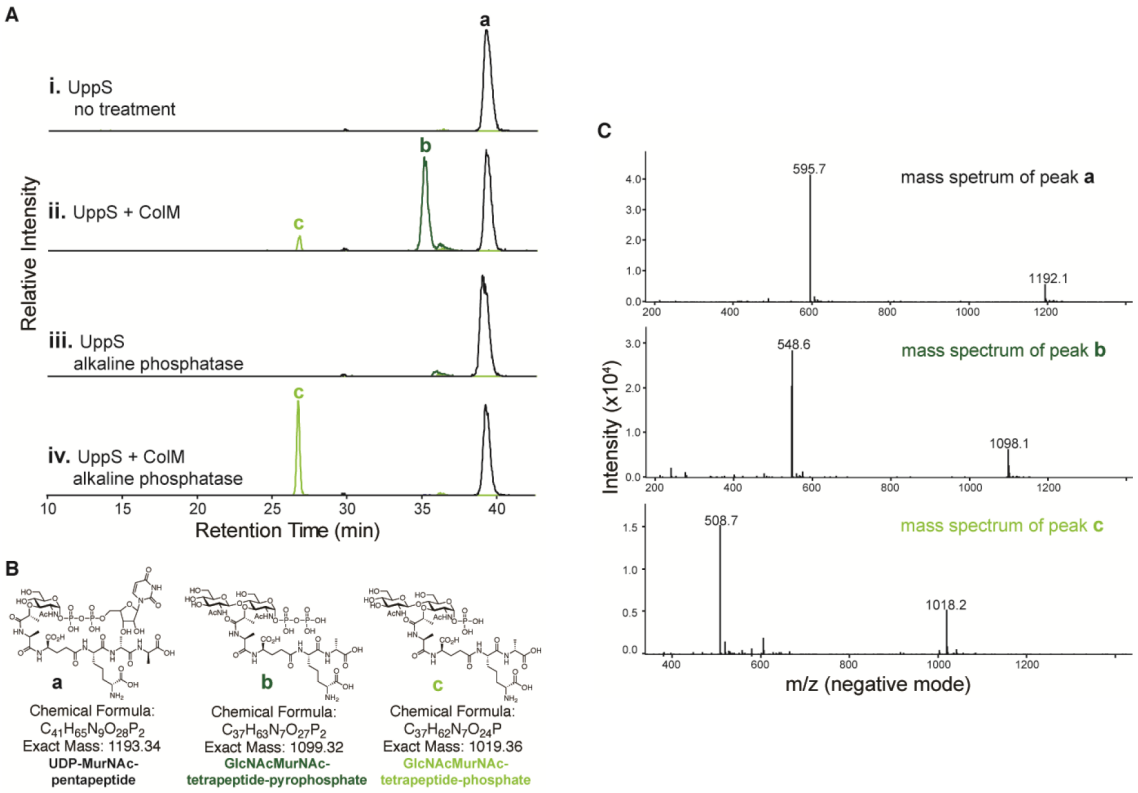


**Fig. S4. The major product of ColM cleavage in vivo is GlcNAc-MurNAc(tetrapeptide)-PP.** (**A**) To identify major products in the radiolabeled experiments (Fig. S2), analogous, non-radiolabeled hot water extracts prepared from cells overproducing UppS and either untreated (**i** and **iii**) or treated (**ii** and **iv**) with ColM were analyzed via LC/MS in negative-ion mode. (**B**) Chemical structures and formulas of identified products in peaks a-c in (**A**): UDP-$M_{pep5}$ (**a**); PP-$M_{pep4}$-G (**b**); and P-$M_{pep4}$-G (**c**). (**C**) Extracted ion chromatograms corresponding to M-H and (M-2H)/2 for UDP-$M_{pep5}$ (**a**), 38.5 min; PP-$M_{pep4}$-G (**b**), 34.8 min; and P-$M_{pep4}$-G (**c**). The expected cleavage fragment, PP-$M_{pep5}$-G, based on a previous report (*11*) was not observed. Rather, our analysis indicates that the major product of ColM cleavage in vivo is PP-$M_{pep4}$-G. As expected, this product is converted to P-Mpep$_4$-G (**c**, 26.8 min) upon treatment with alkaline phosphatase.

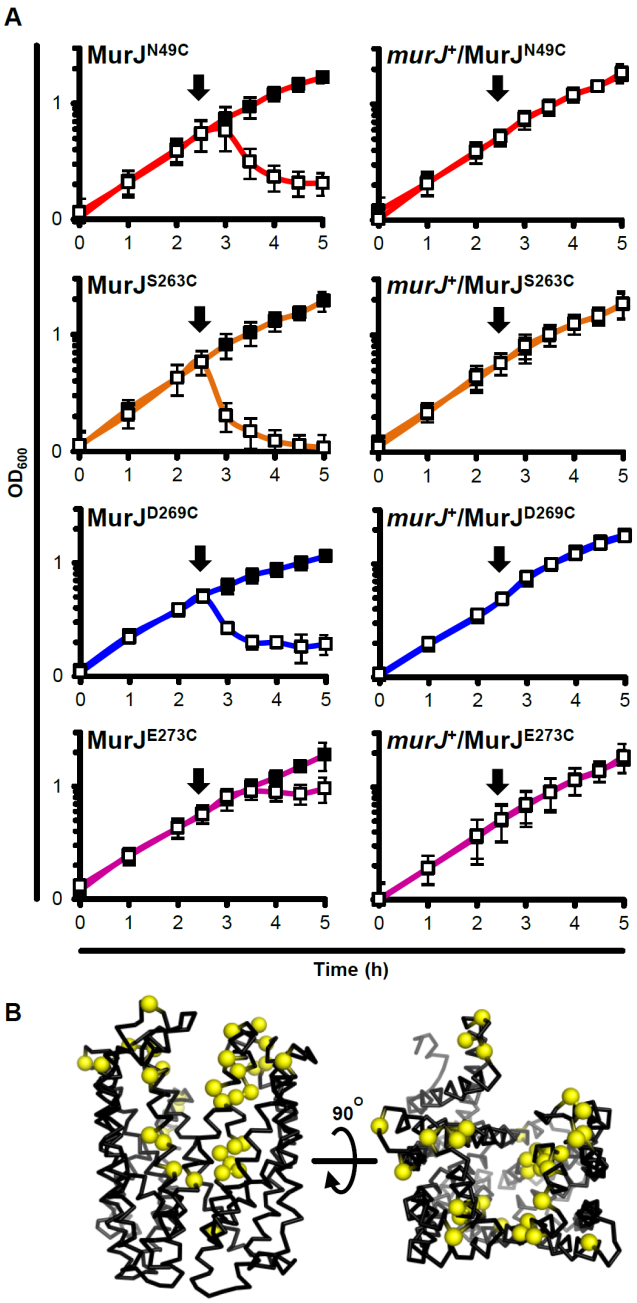


**Fig. S5. MTSES specifically inhibits the function of a subset of MurJ variants** (**A**) Effect of MTSES on the growth of haploid cells producing MurJ variant (left) in glucose M63 medium. As seen with MurJ$^{A29C}$ (see text), cells expressing MurJ$^{N49C}$, MurJ$^{S263C}$, or MurJ$^{D269C}$ rapidly lyse following MTSES addition. Addition of MTSES results in growth inhibition of haploid MurJ$^{E273C}$ cells, which we interpret to indicate a partial loss of MurJ$^{E273C}$ function. The MTSES sensitive phenotypes of MurJ variant cells is suppressed by the presence of a wild-type *murJ* allele (right). Arrows indicate time of MTSES addition; marker, no MTSES; empty marker, MTSES treated. Data are shown as the mean ± SD; $N = 3$. (**B**) Structural model of MurJ (*8*). Yellow spheres mark positions where substituted Cys residues can be modified by MTSES (*8*) without affecting cell growth.

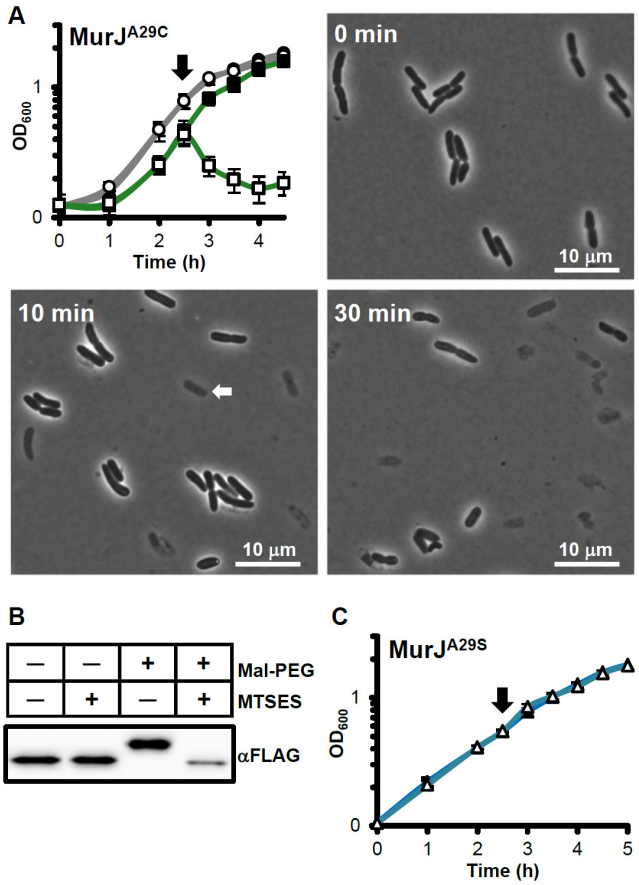


**Fig. S6. Rapid induction of lysis by MTSES.** (**A**) In LB broth, MTSES treatment of haploid cells producing MurJ$^{A29C}$ results in lysis, while growth of MurJ$^{WT}$ is unaffected. Growth curves are shown at top left. The untreated MurJ$^{A29C}$ variant has a decreased growth rate in LB broth compared to MurJ$^{WT}$ (● : MurJ$^{WT}$; ○ : MurJ$^{WT}$ + MTSES; ■ : MurJ$^{A29C}$; □ : MurJ$^{A29C}$ + MTSES). Data are shown as the mean ± SD; $N = 3$. Phase contrast images of untreated (0 min) and MTSES-treated (10 min, 30 min post-addition) MurJ$^{A29C}$ cells growing in LB broth are shown in the top right and bottom panels. Lysed cells (e.g. white arrow) are observed within 10 min after the addition of MTSES in cultures growing in LB. Scale bar represents 10 µm. (**B**) Anti-FLAG immunoblot detection of MurJ$^{A29C}$ reveals that a 5-min treatment with MTSES in LB broth is sufficient to block labeling with Mal-PEG of Cys 29. The decreased detection seen in the MTSES- and Mal-PEG- treated condition has been observed previously in a subset of single-Cys variants and occurs for unknown reasons. (**C**) Haploid cells producing MurJ$^{A29S}$ are not sensitive to MTSES treatment, suggesting that lysis of treated cells producing MurJ$^{A29C}$ results from the modification of the thiol group of Cys 29 by MTSES and not exposure of an alternate MTSES-binding site (*26*). Arrows indicate time of MTSES addition; filled triangles, no MTSES; empty triangles, MTSES treated. Data are shown as the mean ± SD; $N = 3$.

A

| AA change | Complementation of Δ*murJ* | Relative protein levels |
|---|---|---|
| none | + | 1.00 |
| A29C | + | 0.80 ± 0.16 |
| A29Q | — | 0.67 ± 0.12 |
| A29E | — | 0.83 ± 0.20 |
| A29K | — | 0.32 ± 0.20 |

B

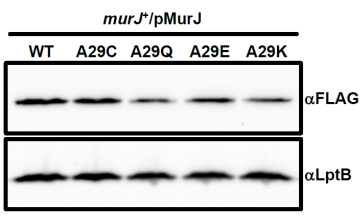


**Fig. S7. Substitution of A29 with large, polar amino acids results in total loss of MurJ function**. (**A**) Functionality of $MurJ^{WT}$ and $MurJ^{A29}$ variants was determined by their ability to complement the deletion of chromosomal *murJ*. Haploid strains expressing functional alleles are viable (+), while those expressing total loss-of-function alleles are not (-). Anti-FLAG immunoblot was used to determine relative levels of MurJ variants with respect to $MurJ^{WT}$ (value set to 1) in merodiploid strains carrying a $murJ^{+}$ chromosomal allele. Data are shown as the mean ± SD from three independent experiments and a representative anti-FLAG immunoblot is shown in (**B**, top). Anti-LptB immunoblotting was performed to control for sample loading (**B**, bottom).

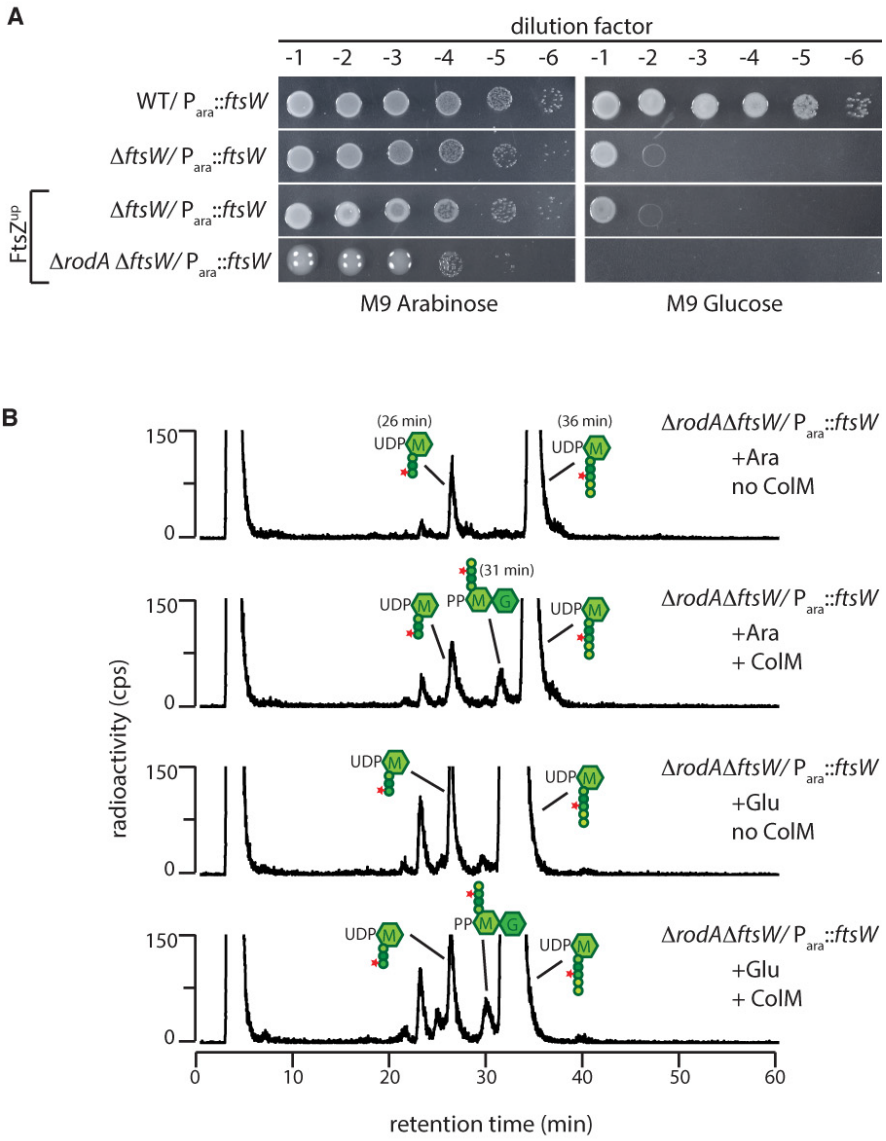


**Fig. S8. Properties of the SEDS depletion strain and representative chromatograms from flippase activity measurements.** (**A**) Cells of NR2590/pCS37 [Δ*lysA*/ $P_{ara}$::*ftsW*], CS16/pCS37 [Δ*ftsW* Δl*ysA*/ $P_{ara}$::*ftsW*], CS16/pCS37/pTB63 [Δ*ftsW* Δ*lysA*/ $P_{ara}$::*ftsW* / *ftsQAZ*], and CS20/pCS37/pTB63 [Δ*rodA* Δ*ftsW* Δ*lysA*/ $P_{ara}$::*ftsW* / *ftsQAZ*] were grown overnight in LB supplemented with 0.2% arabinose. The resulting cultures were normalized for $OD_{600}$, serial dilutions were prepared in LB, and 5 µl of each dilution was spotted on the indicated medium. Plates were incubated overnight at 30°C, or for two nights in the case of the CS20 strain, and photographed. (**B**) Shown are representative chromatographs from the labeling experiments described in fig. S9C-D. The LC/MS compatible RP-HPLC conditions were used, and peak identities were confirmed with unlabeled reactions run in parallel (data not shown).

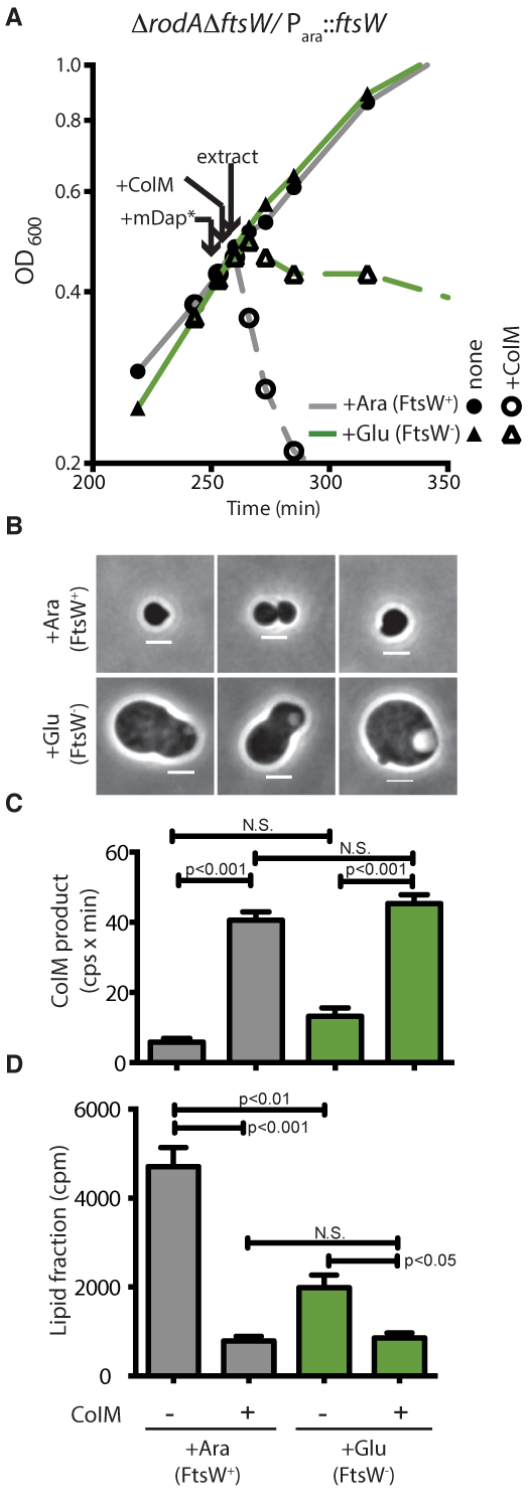


**Fig. S9. Lipid-II cleavage by ColM does not require the activity of SEDS proteins.** (**A**) Cultures of CS20 [Δ*rodA* Δ*ftsW* Δ*lysA*/ $P_{ara}$::*ftsW* / *ftsQAZ*] were grown overnight in labeling medium supplemented with 0.2% arabinose. Cells were washed in M9 salts and diluted 1:100 into labeling medium supplemented with 0.2% arabinose ($FtsW^+$) or glucose ($FtsW^-$) as indicated and growth was monitored by following culture $OD_{600}$. Cells were labeled with [$^3$H]-mDAP and

treated with ColM as described in the legend for fig. 1. (**B**) Because RodA is required for rod shape and FtsW is an essential division protein (*3, 5*), depletion of FtsW in Δ*rodA* cells results in the formation of large spherical cells. Shown are representative phase-contrast micrographs of cells showing their morphology just prior to [$^3$H]-mDAP addition. Bar equals 2 μm. (**C-D**). ColM cleavage products and lipid intermediates, respectively, were quantified as described in fig. 1. Even in the giant spheres resulting from depletion of FtsW in medium containing glucose, flippase activity remains as robust as in dividing FtsW-replete cells in medium containing arabinose. Shown are the mean ± SD; $N = 3$. A Student's *t* test was used to compute the *P* values shown. N.S., not significant. Representative chromatograms are shown in fig. S8B.

**Table S1. Strains used in this study.**

| STRAIN | RELEVANT GENOTYPE | REFERENCE |
|---|---|---|
| DH5α | *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Gibco BRL |
| BL21(DE3) | *ompT⁻ rb⁻ mb⁻* ($P_{lac}$UV5::T7gene1) | Novagen |
| T7266 | BL21(DE3)/pMLD237 ($P_{T7}$::$His_6$-ColM) | This study |
| TB10 | *rph1 ilvG rfb-50* λΔ*cro-bio nad*::Tn*10* | (*27*) |
| MC4100 | F⁻ λ⁻ *e14⁻ araD139* Δ(*argF-lac*)*U169 rspL150*(*strR*) *relA1 flhD5301* Δ(*fruK-yeiR*)725(*fruA*25) *rbsR*22 *deoC*1 Δ(*fimB-fimE*)632(::IS1) | (*28*) |
| FB22(λCH221) | *rph1 ilvG rfb*-50 Δ*lacIZYA<>frt* Δ*rodA*::$kan^R$ ($P_{lac}$::$gfp_{mut2}$-T7-*rodA*) | (*21*) |
| TU231 | *rph1 ilvG rfb-50* Δ*rodA*::$kan^R$ (pTB63) | This study |
| NR754 | *araD*⁺ revertant of MC4100 | (*29*) |
| NR1648 | NR754 Δ*murJ*::*kan* (pRC7MurJ) | |
| NR2066 | NR754 Δ*murJ*::FRT (pRC7MurJ) | |
| NR2590 | NR754 Δ*lysA*::FRT | This study |
| NR2591 | NR754 Δ*murJ*::FRT Δ*lysA*::*kan* (pRC7MurJ) | This study |
| NR2592 | NR754 Δ*murJ*::FRT Δ*lysA*::*kan* (pFLAGMurJΔCys) | This study |
| NR2593 | NR754 Δ*murJ*::FRT Δ*lysA*::*kan* (pFLAGMurJΔCys/A29C) | This study |
| T8640 | NR2590 (pHC808) | This study |
| CS12 | NR2592 Δ*fhuA*::*kan* | This study |
| CS13 | NR2593 Δ*fhuA*::*kan* | This study |
| CS15 | TB10 Δ*ftsW*::*kan* (pCS37) | This study |
| CS16 | NR2590 Δ*ftsW*::*kan* (pCS37) | This study |
| CS17 | NR2590 Δ*ftsW*::FRT (pCS37/pTB63) | This study |
| CS20 | CS17 Δ*rodA*::*kan* (pCS37/pTB63) | This study |
| NR2117 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pRC7MurJ) | (*8*) |
| NR2131 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys) | (*8*) |
| NR2449 | NR754 (pFLAGMurJΔCys) | (*8*) |
| NR2186 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A29C) | (*8*) |
| NR2303 | NR754 (pFLAGMurJΔCys/A29C) | This study |
| NR2891 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A29S) | This study |
| NR2829 | NR754 (pFLAGMurJΔCys/A29Q) | This study |
| NR2827 | NR754 (pFLAGMurJΔCys/A29E) | This study |
| NR2825 | NR754 (pFLAGMurJΔCys/A29K) | This study |
| NR2184 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/T14C) | (*8*) |
| NR2297 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/D25C) | (*8*) |
| NR2185 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A26C) | (*8*) |
| NR2298 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/R30C) | (*8*) |
| NR2214 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A37C) | (*8*) |
| NR2279 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A44C) | (*8*) |

| | | |
|---|---|---|
| NR2299 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/K46C) | (*8*) |
| NR2503 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/L47C) | (*8*) |
| NR2205 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/N49C) | (*8*) |
| NR2421 | NR754 (pFLAGMurJΔCys/N49C) | This study |
| NR2300 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/R53C) | (*8*) |
| NR2504 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/I54C) | (*8*) |
| NR2505 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/F55C) | (*8*) |
| NR2206 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A56C) | (*8*) |
| NR2207 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/E57C) | (*8*) |
| NR2416 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/G58C) | (*8*) |
| NR2187 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/M111C) | (*8*) |
| NR2188 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/V112C) | (*8*) |
| NR2442 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/D119C) | (*8*) |
| NR2427 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A121C) | (*8*) |
| NR2224 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A179C) | (*8*) |
| NR2463 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/R228C) | (*8*) |
| NR2283 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/T251C) | (*8*) |
| NR2479 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A254C) | (*8*) |
| NR2480 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/F256C) | (*8*) |
| NR2144 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/S259C) | (*8*) |
| NR2419 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/S263C) | (*8*) |
| NR2422 | NR754 (pFLAGMurJΔCys/S263C) | This study |
| NR2481 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A268C) | (*8*) |
| NR2465 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/D269C) | (*8*) |
| NR2545 | NR754 (pFLAGMurJΔCys/D269C) | This study |
| NR2358 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/S276C) | (*8*) |
| NR2359 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/E273C) | (*8*) |
| NR2423 | NR754 (pFLAGMurJΔCys/E273C) | This study |
| NR2485 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/G277C) | (*8*) |
| NR2285 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/V281C) | (*8*) |
| NR2226 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A344C) | (*8*) |
| NR2228 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/G403C) | (*8*) |
| NR2535 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/A408C) | (*8*) |
| NR2361 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/I465C) | (*8*) |
| NR2211 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/S470C) | (*8*) |
| NR2456 | NR754 Δ*murJ*::FRT Δ*hsdR*::*kan* (pFLAGMurJΔCys/T473C) | (*8*) |

**Table S2. Primers used in this study.**

| PRIMER NAME | SEQUENCE 5'-3' |
|---|---|
| 5murJA29S | cacgagacgcaattgtctctagaatctttggcgcag |
| 3murJA29S | ctgcgccaaagattctagagacaattgcgtctcgtg |
| 5murJA29Q | gcacgagacgcaattgtccagagaatctttggcgcaggg |
| 3murJA29Q | ccctgcgccaaagattctctggacaattgcgtctcgtgc |
| 5murJA29E | acgagacgcaattgtcgagagaatctttggcgcagg |
| 3murJA29E | cctgcgccaaagattctctcgacaattgcgtctcgt |
| 5murJA29K | gcacgagacgcaattgtcaagagaatctttggcgcaggg |
| 3murJA29K | ccctgcgccaaagattctcttgacaattgcgtctcgtgc |
| CS4 | gctaaagctttcatcgtgaacctcgtacaaacgcc |
| CS70 | gctatctagaaatgagtttgcccgtctggcgaaggagttaggttg |
| CS81 | tctggcgaaggagttaggttgatgcgtttatctctccctcgtgtaggctggagctgcttc |
| CS82 | gttccgcctgccatcaccattaatcgctttccttgaccactcatatgaatatcctcctta |
| NdeI-uppS | gctacatatgatgttgtctgctactc |
| HindIII-uppS | gctaaagctttcaggctgtttcatcacc |