SUPPORTING INFORMATION FOR:

Title: Evidence for the coupling of substrate recognition with transporter opening in MOP-family flippases

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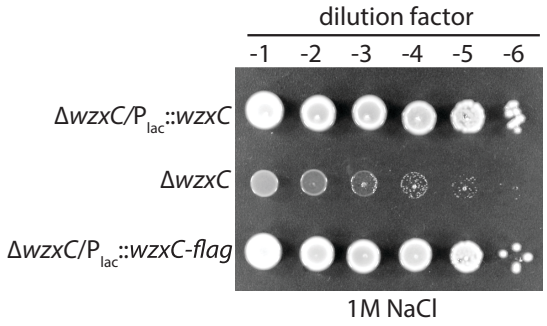

Figure S1. WzxC-FLAG is functional. Cells of CS38 [Δ*wzxC*], CS38/pCS123 [Δ*wzxC*/P$_{lac}$::*wzxC*], or CS38/pCS124 [Δ*wzxC*/P$_{lac}$::*wzxC*-FLAG] were grown overnight in LB medium (CS38) or LB medium with 25μg/ml chloramphenicol (CS38/pCS123 and CS38/pCS124) at 37^{o}C with aeration. Cultures were then serial diluted and 5μl of the culture was spotted on LB plates supplemented with 1M NaCl. On this medium, cells inactivated for WzxC display a strong growth defect. This defect was corrected by production of WzxC-FLAG as well as untagged WzxC indicating that the fusion is functional.

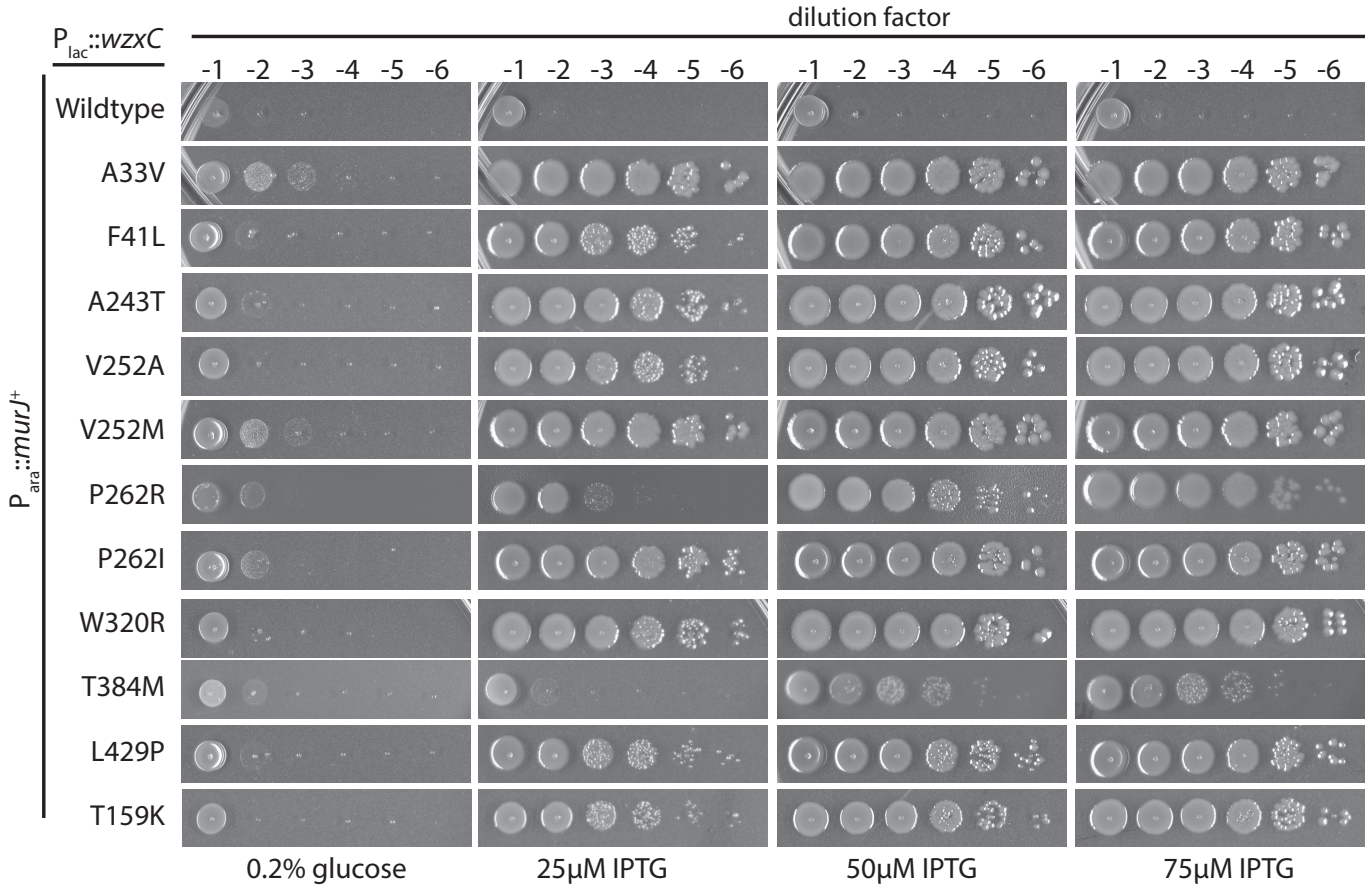

Figure S2. WzxC variants can substitute for MurJ. Cells of CS7 [P_{ara}::*murJ*] harboring plasmids encoding C-terminally FLAG-tagged WzxC (WT) or the indicated derivatives were grown in LB medium with arabinose overnight. Following normalization for culture density, serial dilutions (10^{-1} to 10^{-6}) were prepared and 5 μl of each were spotted onto LB plates supplemented with either glucose or the indicated IPTG concentration. Plates were photographed after incubation at 37°C for ≈16 hours. All of the strains grew similarly on plates supplemented with arabinose under this condition.

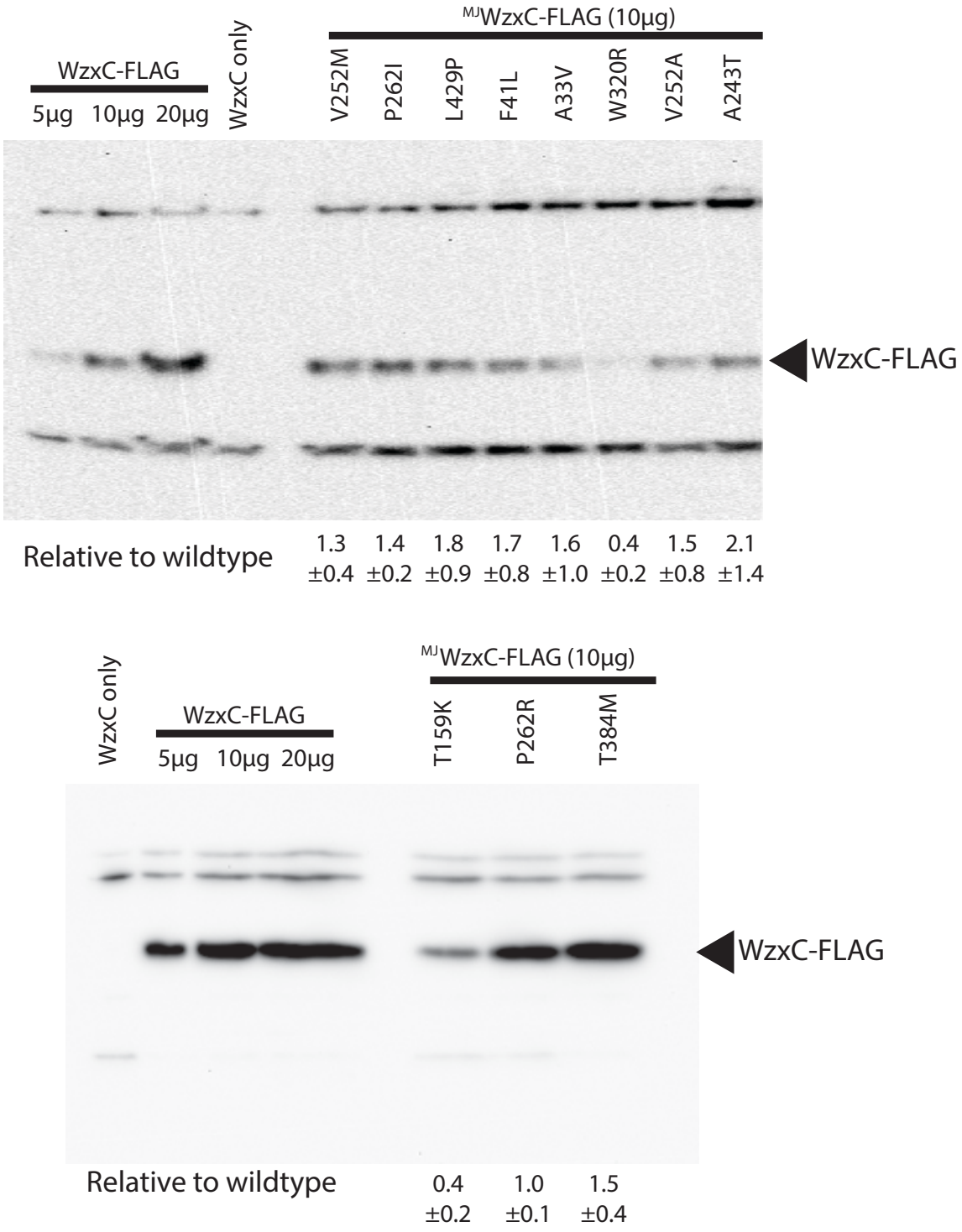

Figure S3. WzxC-FLAG variants accumulate to levels similar to wild-type WzxC-FLAG. Cells of CS7 [P_{ara}::*murJ*] harboring plasmids encoding C-terminally FLAG-tagged WzxC (WT) or the indicated derivatives were grown in LB medium with chloramphenicol, 0.2% (w/v) arabinose, and 500 μM IPTG at 37^{o}C with aeration. When cultures reached an OD_{600} of 0.4, cells were harvested and resuspended in sample buffer followed by heating at 50^{o}C. The protein concentration in each extract was determined and the indicated amount of total protein was subjected to SDS-PAGE and immunoblotting for the detection of the FLAG epitope. Shown are representative blots from three independent experiments. The relative amount of ^{MJ}WzxC-FLAG compared to wildtype WzxC-FLAG and the standard deviation between replicates is shown at the bottom of the blot. As a negative control, strain CS7 harboring plasmid encoding the untagged WzxC was included.

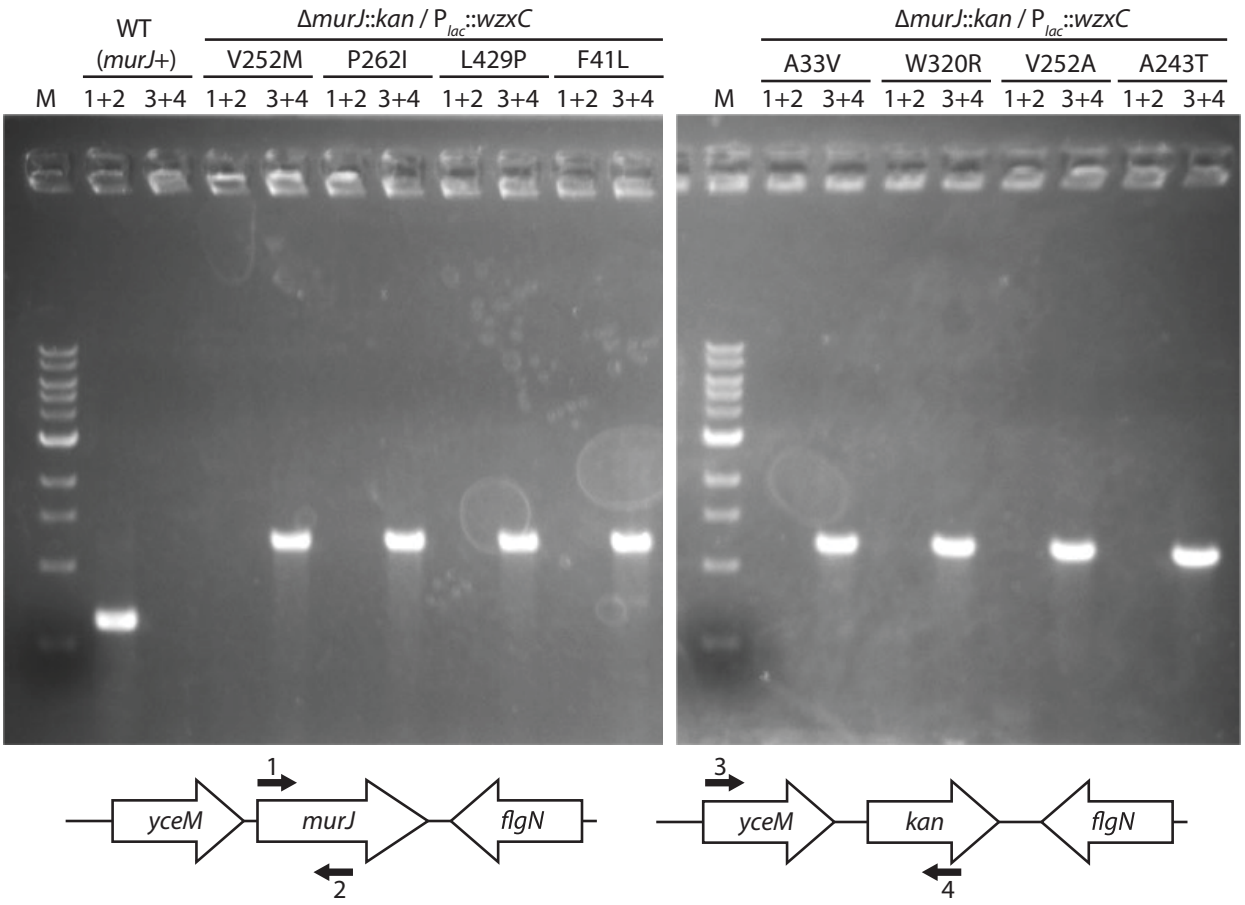

Figure S4. WzxC variants can compensate for the deletion of chromosomal *murJ*. Cells harboring plasmids encoding the indicated *wzxC-flag* variants were used as recipients in a P1 transduction from a Δ*murJ*::*kan*^{R} donor. Transducatants were selected on LB kanamycin plates supplemented with IPTG. Successful replacement of the chromosomal *murJ* gene was confirmed by diagnostic PCR using primers 1 and 2 for detection of the *murJ* gene (628bp product) or primers 3 and 4 to detect the *kan*^{R} cassette at the *murJ* locus (1.2 kb product). As shown in the agarose gel, the native *murJ* gene was detected in the TB28 [WT] parent but not the transductants. Conversely, the *kan*^{R} cassette was detected in the *murJ* locus in the transductants but not the WT strain. Thus, all of the WzxC variants tested were able to support growth upon the complete loss of MurJ.

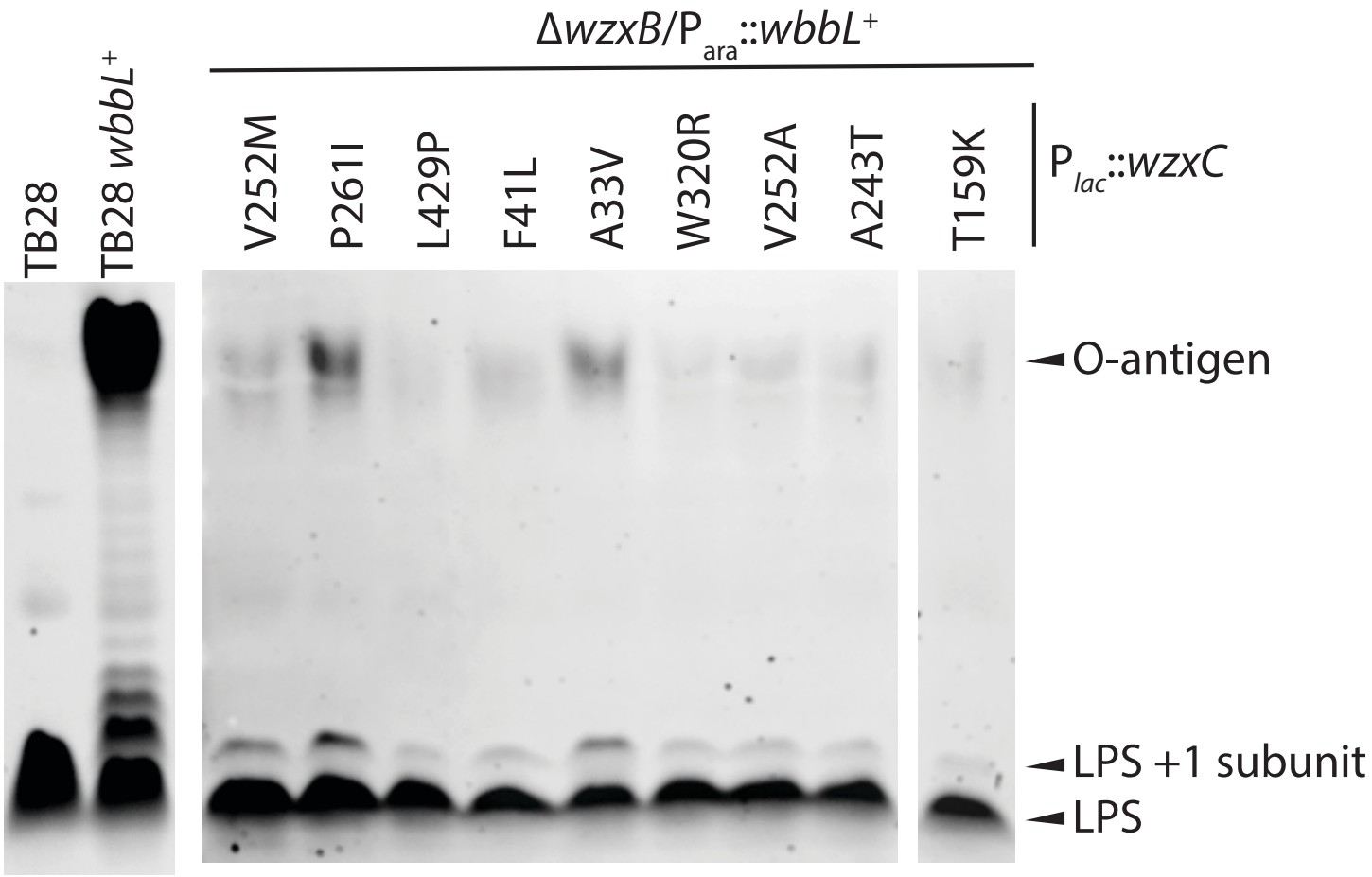

Figure S5. ^{MJ}WzxC variants can substitute for WzxB in O-antigen production. Cells of TB28 [*wbbL*$^-$], AAY1 [TB28 *wbbL*$^+$], or CS39/pCS160 [Δ*wzxB*/P$_{ara}$::*wbbL*] harboring plasmids encoding the indicated ^{MJ}WzxC variants were grown in LB medium with 0.2% (w/v) arabinose and 100μM IPTG. The medium for plasmid containing cells also included spectinomycin and chloramphenicol. When the cultures reached an OD_{600} of 0.4, cells were then harvested, lysed by SDS, and treated with proteinase K. O-antigen and LPS was detected by ProQ Emerald staining after resolution on a 4/13% Tris-Tricine gel. Loading of each lane was normalized such that they included an equivalent amount of total protein as assessed prior to proteinase K treatment. This experiment was performed three time with similar results. Note that CS39/pCS160 [Δ*wzxB*/P$_{ara}$::*wbbL*] cells harboring plasmids encoding WzxC(WT), WzxC(T384M), and WzxC(P262R) failed to grow when *wbbL* was induced for O-antigen synthesis and thus were not included in this assay.

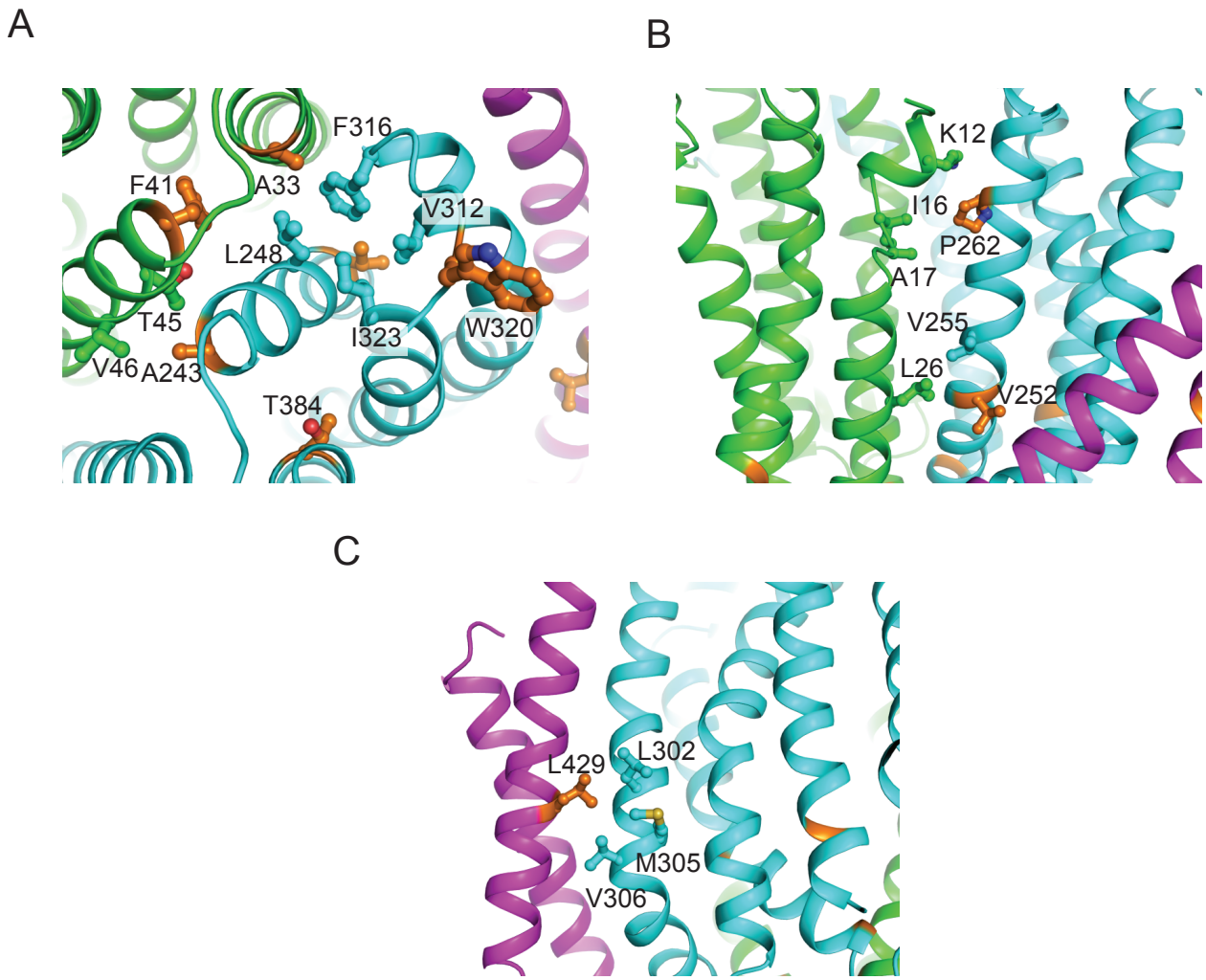

Figure S6. Structural environment of mutated residues.
Loss of specificity mutations in WzxC are mapped onto a homology model of the protein (orange sticks). Residues near these positions are also shown as sticks, and are colored according to their position within the protein: the N-lobe is shown in green, the C-lobe in cyan, and the TM13/14 region in violet. Mutations are primarily located in the cytosolic gate, at the interface between the N- and C-lobes of the protein (A), while other mutations are found in the central cavity (B), or at the interface between TM13/14 and the C-lobe (C).

Table S1. WzxC variants isolated that can substitute for MurJ

Mutant[a]	Amino acid change(s) identified in WzxC
D2	**V252M**
D3	L57I **A243T**
D4	**P262I**
D6	**W320R**
D7	**L429P**
D8	**F41L** T117R A163V V292I
D9	V46M **F422S**
D10	**A235V** V312L
D11	**A243V** P257T
D12	**L429P**
D13	**F41L**
D15	**A33V**
D16	F187L **L327P**
D17	**A33V**
D18	F190Y **A235V V252L**
D19	**V252M**
D21	**W320R**
D22	**V252A**
D24	S14W **F41I** L471V
D25	**F41L**
D27	**P262S** G379D
D28	**A243T**
D29	**A243V** Q376L
D30	**A33V**
NF3	**T159K**
NF12	**K143E L327P**
NF15	**T384M L389P**

[a] The amino acid changes highlighted in bold are predicted to be the causative and were further studied.

Table S1. Strains used in this study

Strain	Genotype[a]	Source/Reference[b]
DH5α	*F⁻ hsdR17 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 ϕ80dlacZΔM15*	Gibco BRL
MG1655	*rph-1 ilvG rfb-50*	[1]
NR2528	*F- araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flB5301 deoC1 ptsF25 rbsR wbbL*⁺::*kan*	[2]
AAY1	TB28 *wbbL*⁺::*kan*	P1(NR2528) x TB28
TB10	MG1655 *λΔcro-bio nad::Tn10*	[3]
TB28	MG1655 *ΔlacIZYA*::*frt*	[4]
JW2022	BW25113 *ΔwzxB::kan*	[5]
JW3758	BW25113 *ΔwecA::kan*	[5]
CS7	TB28 *bla-araC*-P_{ara}::*murJ*	[6]
CS31	TB28 Δ*murJ*::*kan*	[7]
CS38	TB28 *ΔwzxC::kan*	P1(λRed allele) x TB28
CS39	TB28 *ΔwzxB::kan*	P1(JW2022) x TB28

[a] The Kan^R cassette is flanked by *frt* sites for removal by FLP recombinase. An *frt* scar remains following removal of the cassette using FLP recombinase expressed from pCP20.

[b] Strain constructions by P1 transduction are described using the shorthand: P1(donor) x recipient. Transductants were selected on LB Kan, LB Tet, or LB Cm plates where appropriate. λRed indicates strains constructed by recombineering (see Experimental Procedures for details). Strains resulting from the removal of a drug resistance cassette using pCP20 are indicated as: Parental strain/pCP20.

Table S2. Plasmids used in this study

Plasmid	Genotype[a]	Origin	Source or Reference
pBAD43	*spec araC* P_{ara}	pSC101	[8]
pCP20	*bla cat* cI875 *repA(*Ts*)* P_R*::flp*	pSC101	[9]
pCS123	*cat lacI* P_{lac}*::wzxC*$^{+}$*-flag*	p15A	This study
pCS124	*cat lacI* P_{lac}*::wzxC*$^{+}$*-flag*	p15A	This study
pCS126	*cat lacI* P_{lac}*::murJ*$^{+}$*-flag*	p15A	This study
pCS133	*cat lacI* P_{lac}*::wzxE*$^{+}$*-flag*	p15A	This study
pCS134	*amp araC* P_{ara}*::wecA*$^{+}$	pBR	This study
pCS158	*cat lacI* P_{lac}*::wzxB*$^{+}$*-flag*	p15A	This study
pCS160	*spec araC* P_{ara}*::wbbL*$^{+}$	pSC101	This study
pCS161	*cat lacI* P_{lac}*::wzxC*^{K143E}*-flag*	p15A	This study
pCS162	*cat lacI* P_{lac}*::wzxC*^{T159R}*-flag*	p15A	This study
pCS163	*cat lacI* P_{lac}*::wzxC*^{P262R}*-flag*	p15A	This study
pCS164	*cat lacI* P_{lac}*::wzxC*^{P262S}*-flag*	p15A	This study
pCS165	*cat lacI* P_{lac}*::wzxC*^{S308L}*-flag*	p15A	This study
pCS166	*cat lacI* P_{lac}*::wzxC*^{L327P}*-flag*	p15A	This study
pCS167	*cat lacI* P_{lac}*::wzxC*^{T384M}*-flag*	p15A	This study
pCS168	*cat lacI* P_{lac}*::wzxC*^{L389P}*-flag*	p15A	This study
pCS169	*cat lacI* P_{lac}*::wzxC*^{F422S}*-flag*	p15A	This study
pCS170	*cat lacI* P_{lac}*::wzxC*^{F422C}*-flag*	p15A	This study
pD2	*cat lacI* P_{lac}*::wzxC*^{V252M}	p15A	This study
pD4	*cat lacI* P_{lac}*::wzxC*^{P262I}	p15A	This study
pD12	*cat lacI* P_{lac}*::wzxC*^{L429P}	p15A	This study
pD13	*cat lacI* P_{lac}*::wzxC*^{F41L}	p15A	This study
pD15	*cat lacI* P_{lac}*::wzxC*^{A33V}	p15A	This study
pD21	*cat lacI* P_{lac}*::wzxC*^{W320R}	p15A	This study
pD22	*cat lacI* P_{lac}*::wzxC*^{V252A}	p15A	This study
pD28	*cat lacI* P_{lac}*::wzxC*^{A243T}	p15A	This study

Plasmid	Genotype[a]	Origin	Source or Reference
pDF2	*cat lacI* P_{lac}*::wzxC*^{V252M}*-flag*	p15A	This study
pDF4	*cat lacI* P_{lac}*::wzxC*^{P262I}*-flag*	p15A	This study
pDF12	*cat lacI* P_{lac}*::wzxC*^{L429P}*-flag*	p15A	This study
pDF13	*cat lacI* P_{lac}*::wzxC*^{F41L}*-flag*	p15A	This study
pDF15	*cat lacI* P_{lac}*::wzxC*^{A33V}*-flag*	p15A	This study
pDF21	*cat lacI* P_{lac}*::wzxC*^{W320R}*-flag*	p15A	This study
pDF22	*cat lacI* P_{lac}*::wzxC*^{V252A}*-flag*	p15A	This study
pDF28	*cat lacI* P_{lac}*::wzxC*^{A243T}*-flag*	p15A	This study
pNF3	*cat lacI* P_{lac}*::wzxC*^{T159K}	p15A	This study
pNFF3	*cat lacI* P_{lac}*::wzxC*^{T159K}*-flag*	p15A	This study
pNF12	*cat lacI* P_{lac}*::wzxC*$^{K143E\ L327P}$	p15A	This study
pNF15	*cat lacI* P_{lac}*::wzxC*$^{T384M\ L389P}$	p15A	This study
pMT77	*cat lacI* P_{lac}	p15A	This study

[a] P_{ara}, $P_{\lambda R}$, and P_{lac} indicate the arabinose, λR, and lactose promoters, respectively.

Table S4. Oligonucleotides used in this study

Name	Sequence (5' to 3')
CS163	CGTCTGACTGGACGTTACAAG
CS165	GAACACCGATGAATTTATTAAAATCGC
CS166	TGCGGTAGCTGATACACCAGC
CS201	TTCCCCTCTAGATTTAAGAAGGAGATATACATATGAATTTATTAAAA TCGCTGGCCGCCG
CS203	CCCCTCTAGATTTAAGAAGGAGATATACATATGAGCTTACGTGAA AAAACCATCAGC
CS204	TACCAAGCTTTCACCCCGCCCGTAAAAGC
CS233	TACCAAGCTTTCATTTATCATCATCATCTTTATAATCCTCGAGCCC CGCCCGTAAAAGCA
CS237	TACCAAGCTTTCATTTATCATCATCATCTTTATAATCCTCGAGCAC CGTCCGGCGGGCAA
CS249	TCCCCTCTAGATTGCGACTTTGTTGAACTACTTTTCCTG
CS250	TAATCCTCGAGTGCCCGCCTACGCCAGAGTAAAAAC
CS302	ATATACATATGAATACGAATAAATTATCTTTAAGAAGAAACG
CS303	ATAATCCTCGAGTCAGCAAACCAGTAATTTATTATTTCTTAC
CS308	ATATACATATGGTATATATAATAATCGTTTCCCA
CS309	CAGCCAAGCTTTTACGGGTGAAAAACTGATGAAATTC
CS319	CATGCCGATTTCGTTGAACTCCAGCTCTTTTTGC
CS320	ATCGAAACCAGCGCGGTGCTGGC
CS321	GCTAACCACGCTACAAGTGAAGCCCGCCA
CS322	GCCCATTTCTGGCCGCTGGC
CS323	TGATGATGCGGTTCAGCTTCATCGGTGGC
CS324	CCCGCGTGTTGTTTCCGGCATTC
CS325	GGGTGATGATAGAGTTCAGCTTCATCGGTGGC
CS326	GCGTGTTGTTTCCGGCATTCG
CS327	TACAAAGTTATTCAGCACCACCATTAGCCC

Name	Sequence (5’ to 3’)
CS328	CCGCTGGTCTTTGGTGAGAAGTG
CS329	GCAATTGCGGCACCGGAATAATGCTGTTCC
CS330	TGTGTGTGGTGGGTCTGCTGCGC
CS331	AAGCCAAGCATGACGCCGATCGCGCCCG
CS332	CCTGCTGGTGCAAATTATCAACACCA
CS333	ATAATTTGCACCGGCAGGAAGCCAAGCGTGACG
CS334	CAACACCATTCTGAGTTACTTCGTG
CS335	GCGAGAGATAGCTCGGCAGCCATAAACTCAGG
CS336	TGCCGACGCTGGTGGTCAGTTATGCGC
CS337	GCGAGAGATAGCACGGCAGCCATAAACTCAGG
CS338	TGCCGACGCTGGTGGTCAGTTATG
CS365	TCCCCTCTAGATTTAAGAAGGAGATATACATATGAATACGAATAAA TTATCTTTAAGAAG
kan5‘extout	CACTGCAAGCTACCTGCTTTCTC
p15AR	CGCCGGTGATGCCGGCCACGATGCGTCCGG
pMLB5’a	TTAGGCACCCCAGGCTTTACAC

SUPPLEMENTAL MATERIALS AND METHODS

Growth conditions, bacterial strains and plasmids.

Strains used in this study are listed in **Table S2**. Unless other specified, *E. coli* cells were grown in lysogeny broth (LB) under aeration at 37^{o}C. Where indicated, arabinose and glucose were added to a final concentrations of 0.2% (w/v). When indicated, ampicillin, chloramphenicol, and kanamycin were used at a final concentration of 25 μg/ml. Spectinomycin was used at a final concentration of 40 μg/ml. Plasmids and oligonucleotides used in this study are listed in **Table S3** and **Table S4**, respectively. Unless otherwise indicated, genomic DNA from strain MG1655 was used as a template for PCR.

Strain construction

Strain CS38 [TB28 Δ*wzxC*::*kan*] was constructed by recombineering. First, a PCR amplicon was generated using primers CS231 and CS232, and Phusion DNA polymerase (NEB). After PCR purification, the product was electroporated into strain TB28/pKD46. The recombinants were plated on LB supplemented with kanamycin, and incubated at 37^{o}C overnight. After confirming the presence of Δ*wzxC*::*kan* at the native locus by PCR, a P1 lysate was prepared on the recombinant and used to transduce the Δ*wzxC*::*kan*^{R} allele into strain TB28 [WT] to generate CS38.

Plasmid construction

For pCS123 [*cat lacI* P_{lac}::*wzxC*], the *wzxC* gene was amplified with primers CS203 and CS204. The PCR amplicon was digested with XbaI and HindIII, and ligated to pMT77 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS124 [*cat lacI* P_{lac}::*wzxC-flag*], the *wzxC* open reading frame was amplified with primers CS203 and CS333. The PCR amplicon was digested with XbaI and HindIII, and ligated to pMT77 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS126 [*cat lacI* P_{lac}::*murJ-flag*], the *murJ* open reading frame was amplified with primers CS237 and CS201. The PCR amplicon was digested with XbaI and XhoI, and ligated to pMT77 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS133 [*cat lacI* P_{lac}::*wzxE-flag*], the *wzxE* gene was amplified with primers CS249 and CS250. The PCR amplicon was digested with XbaI and XhoI, and ligated to pCS124 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS158 [*cat lacI* P_{lac}::*wzxB-flag*], the *wzxB* gene was amplified with primers CS302 and CS303. The PCR amplicon was digested with NdeI and XhoI, and ligated to pCS124 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS160 [*spec araC* P_{ara}::*wbbL*], the *wbbL* gene was amplified with primers CS308 and CS309 using genomic DNA template from strain AAY1 (TB28 *wbbL*$^{+}$ kan). The PCR product

was digested with NdeI and HindIII, and ligated to pBAD43 digested with the same restriction enzymes. The cloned fragment was confirmed by PCR and sequencing.

For pCS161 [*cat lacI* P_{lac}::*wzxC*(K143E)-*flag*], site directed mutagenesis was performed on pCS124 using phosphorylated primers CS319 and CS320 and amplification with Phusion polymerase (NEB). To phosphorylate CS319 and CS320, 0.5 μl of a 100 μM primer stock was mixed with 1 μl of T4 ligase buffer (NEB), 7.5 μl of water, and 0.5 μl of T4 PNK (NEB). The reaction was performed at 37^{o}C for one hour, chilled on ice, and mixed with 2 μl of purified pCS124, 0.5 μl of 10mM dNTPs, 5 μl of 10x Phusion polymerase buffer, 7.25 μl of water and 0.25 μl of Phusion polymerase. After PCR purification (QIAGEN), the amplified linearized plasmids were ligated using T4 ligase and transformed into DH5α(λpir) competent cells. The presence of the desired mutation was confirmed by sequencing. Plasmids pCS162 (P_{lac}::*wzxC*(T159R)-*flag*), pCS163 (P_{lac}::*wzxC*(P262R)-*flag*), pCS164 (P_{lac}::*wzxC*(P262S)-*flag*), pCS165 (P_{lac}::*wzxC*(S308L)-*flag*), pCS166 (P_{lac}::*wzxC*(L327P)-*flag*), pCS167 (P_{lac}::*wzxC*(T384M)-*flag*), pCS168 (P_{lac}::*wzxC*(L389P)-*flag*), pCS169 (P_{lac}::*wzxC*(F422S)-*flag*), and pCS170 (P_{lac}::*wzxC*(F422C)-*flag*) were generated using the same approach. The oligonucleotides used were CS321 and CS322 (T159R), CS323 and CS324 (P262R), CS325 and CS326 (P262S), CS327 and CS328 (S308L), CS329 and CS330 (L327P), CS331 and CS332 (T384M), CS333 and CS334 (L389P), CS335 and CS336 (F422S), and CS337 and CS338 (F422C). The cloned fragments were confirmed by PCR and sequencing.

For plasmids pDF2, pDF4, pDF12, pDF13, pDF15, pDF21, pDF22, and pDF28, the inserts containing the *wzxC* variants were amplified by Phusion polymerase using primers CS233 and CS203, and DNA templates from the corresponding clones (pD2, pD4, pD12, pD13, pD15,

pD21, pD22, and pD28). The amplicons were PCR purified and digested with XbaI and HindIII, and ligated into the vector backbone of pCS124 digested with the same enzymes. The cloned fragments were confirmed by PCR and sequencing.

Construction of a plasmid library of mutagenized *wzxC*.

The *wzxC* gene was amplified from pCS124 by PCR using primers pMLB5'a and p15AR and Q5 polymerase. Error-prone PCR was then performed using purified Pfu(D473G), 750 ng of the purified PCR product, 2μl of 10 μM dNTPs, 4 μL of DMSO, 2 μl of 10 μM primers pMLB5'a and p15AR, 77.2 μl of water, 10 μl of 10x buffer (200mM Tris-HCl pH 8, 100mM KCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% (v/v) Triton X-100, 1mg/ml BSA), and 0.1 μl of 1 mg/ml Pfu(D473G). PCR cycling conditions were: 95^{o}C for 5 minutes, 20 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 2 minutes, and finally 72^{o}C for 10 minutes. After purification, the mutagenized PCR product (≈2μg) and pMT77 (≈1μg) were digested with 50U of XbaI and HindIII overnight at 30^{o}C. The digested DNA was further purified using AMPure beads (Agencourt). First, 25 μl of AMPure XP beads were added to 50 μl of digestion product. The mixture was incubated at room temperature for 5 minutes. Beads were absorbed with a magnetic stand and were washed twice with freshly prepared 80% (v/v) ethanol. Bound DNA was eluted by adding 50 μl of warm water and incubation for 5 minutes at room temperature. Eluted PCR products and pMT77 were ligated overnight with T4 ligase, purified using DNA Clean & Concentrator (Zymo research), and further concentrated by vacuum centrifugation (SpeedVac). The ligated plasmid was transformed into NEB5α cells (NEB) according to the manufacturer's protocol and plated on LB supplemented with chloramphenicol. This procedure yielded approximately 1 million transformant colonies. At least half of the colonies contained the desired insert as determined by diagnostic PCR using GoTaq polymerase (Promega) and

pMLB5'a and p15R primers. Transformants were scraped using a cell scraper (Falcon 353085) and transferred into 5ml of LB medium. Plates were further washed with 1ml of LB per plate. The resulting cell suspension was diluted to OD_{600} ≈3. Glycerol was then added to a final concentration of 15% and the library stored at -80°C. Plasmid was purified from 10 ml of the diluted cell suspension using the Zippy plasmid Miniprep kit (Zymo research).

Selection of WzxC variants that can suppress MurJ essentiality

Strain CS7 [P_{ara}::*murJ*] was transformed with mutagenized *wzxC* plasmid library. Briefly, CS7 was grown in 35 ml of LB medium supplemented with 0.2% (w/v) arabinose to OD_{600} ≈0.3. Cells were chilled for 15 minutes, collected by centrifugation, and washed 1 time with 35 ml ice-cold water followed by 5 times with 1ml of ice-cold 10% glycerol. The washed cells were resuspended in 350 µl of cold 10% glycerol. Then, 100 µl of cells were mixed with 2 µl of the purified plasmid library and electroporated at 1.7kV using a Biorad MicroPulser electroporator. The mixture was immediately resuspended in 1ml of SOB medium supplemented with 0.2 % (w/v) arabinose and 10 mM $MgCl_2$, and incubated at 37°C for 1 hour. Cells were plated on LB medium supplemented with 0.2 % arabinose and chloramphenicol and incubated overnight at 37°C. Cells from the plates were scraped using a cell scraper into 5ml of LB medium, residual cells were harvested by washing with 1ml of LB per plate, and the cell suspension was diluted to OD_{600} ≈3. The cell suspension was serial diluted and plated on LB with chloramphenicol supplemented with either 0.2% glucose, 0.2% arabinose, 100µM or 500µM IPTG. Plating efficiencies under the different conditions were estimated by counting colonies. Only cells harboring mutagenized *wzxC* plasmid showed a significant increase in plating efficiency on LB with 100 µM IPTG over LB with 0.2% glucose. Isolates that required induction of the *wzxC* plasmid for growth in the absence of *murJ* expression were selected for sequencing.

LPS extraction and O-antigen staining

To measure O-antigen production, cells of CS39/pCS160 [Δ*wzxB*::*kan*/P_{ara}::*wbbL*] harboring plasmids encoding *wzxC* variants were grown in 5ml of LB medium with chloramphenicol, spectinomycin, 500μM of IPTG, and 0.2% (w/v) glucose overnight at 37^{o}C with aeration. Cultures were then diluted to OD of 0.01 in 3ml of the same medium, except glucose was replaced by arabinose and the level of IPTG was reduced to 100μM. When the culture OD_{600} reached 0.4 and 0.6, cells were collected by centrifugation at 16,100 x*g* for 30 seconds at room temperature, washed once with 1ml of LB, and resuspended in 100 μl of resuspension buffer (30mM Tris-HCl pH 8, 10mM EDTA, 1% SDS). The suspension was boiled for 5 minutes and cooled to room temperature. The amount of protein was quantitated using the NI protein assay (Gbioscience) and used to normalize loading. Two microliters of proteinase K (NEB P8107S, 1.6U) was added and the mixture was incubated at 60^{o}C for 1 hour. An equal volume of LPS sample buffer (50mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 0.01% (w/v) bromophenol blue) was then added, and the mixture was boiled again for 5 minutes. Crude LPS extracts were resolved on a 4/13% Tris-tricine gel. The gel was first run at 60V for 1 hour, then 150V until dye front reach the bottom, approximately for 40 minutes. LPS on the gel was visualized by the Pro-Q LPS staining kit (Life technology P20495). Briefly, the gel was washed with water for 10 minutes. Destaining solution (40% methanol, 10% acetic acid) was added followed by incubation for 45 minutes. The gel was then washed twice with 3% (v/v) acetic acid for 10 minutes each, and oxidizing reagent was added. The gel was incubated for 30 minutes with agitation, and washed two times with 3% (v/v) acetic acid for 10 minutes each. The gel was then soaked in the staining solution, which was prepared by mixing 500 μl of the provided dye and 25 ml of Pro-Q reagent buffer. After 30 minutes of staining, the gel was washed twice

with 3% (v/v) acetic acid for 5 minutes and visualize using a FluorChem R FR0162 using UV excitation (280nm) and green emission (530nm).

Detection of WzxC-FLAG by Immunoblotting

To quantitate the amount of WzxC-FLAG produced, cells of CS7 [P_{ara}::*murJ*] harboring plamids encoding WzxC(WT) or ^{MJ}WzxC variants were grown in LB medium supplemented with chloramphenicol and arabinose overnight at 37^{o}C. Overnight cultures were diluted 1:100 in 3ml of LB with chloramphenicol, arabinose, and 500μM of IPTG. When the culture OD_{600} reached 0.4 to 0.6, an equivalent of 0.4 OD_{600} units of cells were collected by centrifugation at 16100 x*g* for 30 seconds at room temperature. Spent medium was discarded and pellets were resuspended in 100 μl of 2x buffer A+B (10mM sodium phosphate pH 7.2, 1% (v/v) beta-mercaptoethanol, 3% (w/v) SDS, 36% (w/v) urea, 10% (v/v) glycerol, 50mM Tris-HCl pH 6.8). The mixture was incubated at 45^{o}C for 15 minutes. To reduce the viscosity, samples were pipetted up and down vigorously, then incubated again at 45^{o}C for 15 minutes. The amount of protein in each sample was determined using the NI protein assay (Gbioscience). The amount of protein specified (5, 10, or 20 μg) was loaded on a precast Any kD gel (Biorad) or a 4/12% SDS-PAGE gel, and transferred to a PVDF membrane. Immunoblotting was performed essentially as described (6), using 1:1000 dilutions of the anti-FLAG M2 antibodies (Sigma) and a 1:1000 dilution of the Trublot anti-mouse HRP antibodies (Rockland) respectively. followed by detection with Pico Western reagent (Thermofisher) according to the manufacturer's protocol.

Detection of lipid II flippase activity using Colicin M

The Colicin M assay was performed essentially as described previously (6). Briefly, cells of CAM290/pCS124 [*murJ*(A29C)/P_{lac}::*wzxC*] and CAM290/pDF2 [*murJ*(A29C)/ P_{lac}::wzxC(V252M)] were grown in LB medium with chloramphenicol overnight. Cultures were diluted 100 fold in 40ml of the labeling medium with 100μM IPTG (M9 supplemented with 0.1% (w/v) casamino acids, 0.2% (w/v) maltose, 0.1mg/ml of lysine, threonine and methionine) and grown at 37^{o}C with aeration. When the culture OD_{600} reached 0.2, 15μl of 1.5 μCi/μl of ^{3}H-mDAP (ARC) was added to 10ml of the culture and incubated for 15 minutes at 37^{o}C. When indicated, colicin M and MTSES were added to a final concentration of 500 ng/ml and 0.4 mM, respectively. The cultures were then incubated for 10 minutes and chilled immediately on ice. Cells were collected by centrifugation at 8,000 x *g* for 2 minutes at 4^{o}C and resuspended in 1ml of preheated water. Samples were then boiled for 30 minutes. Colicin M product in the water soluble fractions were separated from the PG/lipid fractions by centrifugation at 100,000 x*g* for 20 minutes at 4^{o}C. Supernatant fractions collected were lyophilized and reconstituted in 300 μl of running buffer (50mM triethylammonium formate pH 4.6, 6% (v/v) methanol). Particulates were removed by centrifugation and 100μl of the samples were subjected to RP-HPLC with a C18 column (Nucleosil A0119250X046 Agilent or 00G-0323-E0 Phenomenex) at a flow rate of 0.5 ml/min at 25^{o}C. Radioactivity was detected by an inline radioflow detector (Berthold) and the colicin M product was quantitated by measuring the area under the peak using the Chromelion software (Dionex). To extract radiolabeled lipid-linked PG precursors from the PG/lipid fractions, pellet fractions after ultracentrifugation were boiled again in 1ml of water for 3 minutes and centrifuged at 100,000 x*g* for 20 minutes at 4^{o}C. Supernatant fractions were discarded and the pellets were resuspended in 100 μl of 10mM Tris-HCl pH 8, followed by addition of 100 μl of 6M pyridinium acetate/1-butanol (1:2 v/v). The mixture was vortexed for 30 seconds and centrifuged at 3000 x*g* for 30 seconds at room temperature. The supernatant

fractions were collected carefully and transferred to a new centrifuge tube containing 100 μl of butanol saturated water and vortexed. The butanol extraction process was repeated once and the butanol fractions were pooled. After centrifugation at 3,000 x*g* for 30 seconds at room temperature, 100μl of the top butanol layer mixed with 10ml of Ecolite scintillation fluid, and radioactivity quantified using a scintillation counter.

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