Supporting Information for:

An ATP-binding cassette transporter-like complex governs cell wall hydrolysis at the bacterial cytokinetic ring

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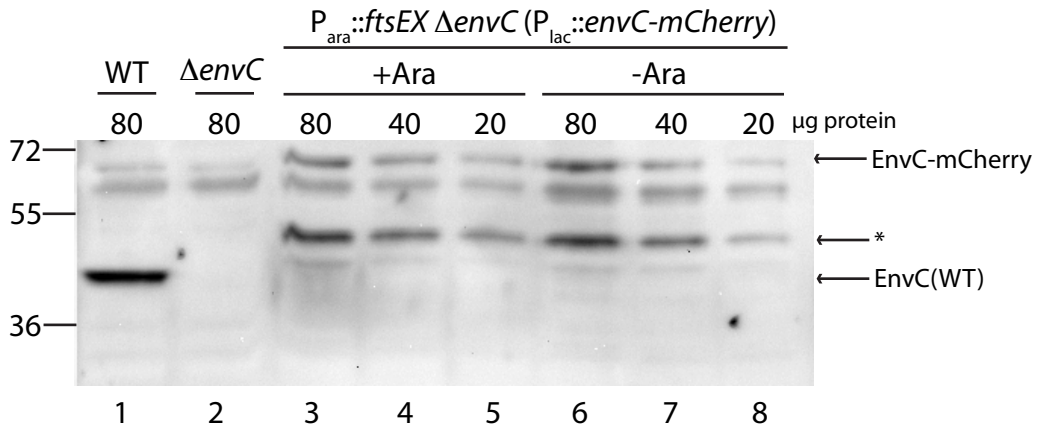
P ara ::ftsEX ΔenvC (P lac ::envC-mCherry)
WT
ΔenvC
+Ara
-Ara
80
80
80
40
20
80
40
20
µg protein
72
55
36
EnvC-mCherry
*
EnvC(WT)
1
2
3
4
5
6
7
8

Figure S1

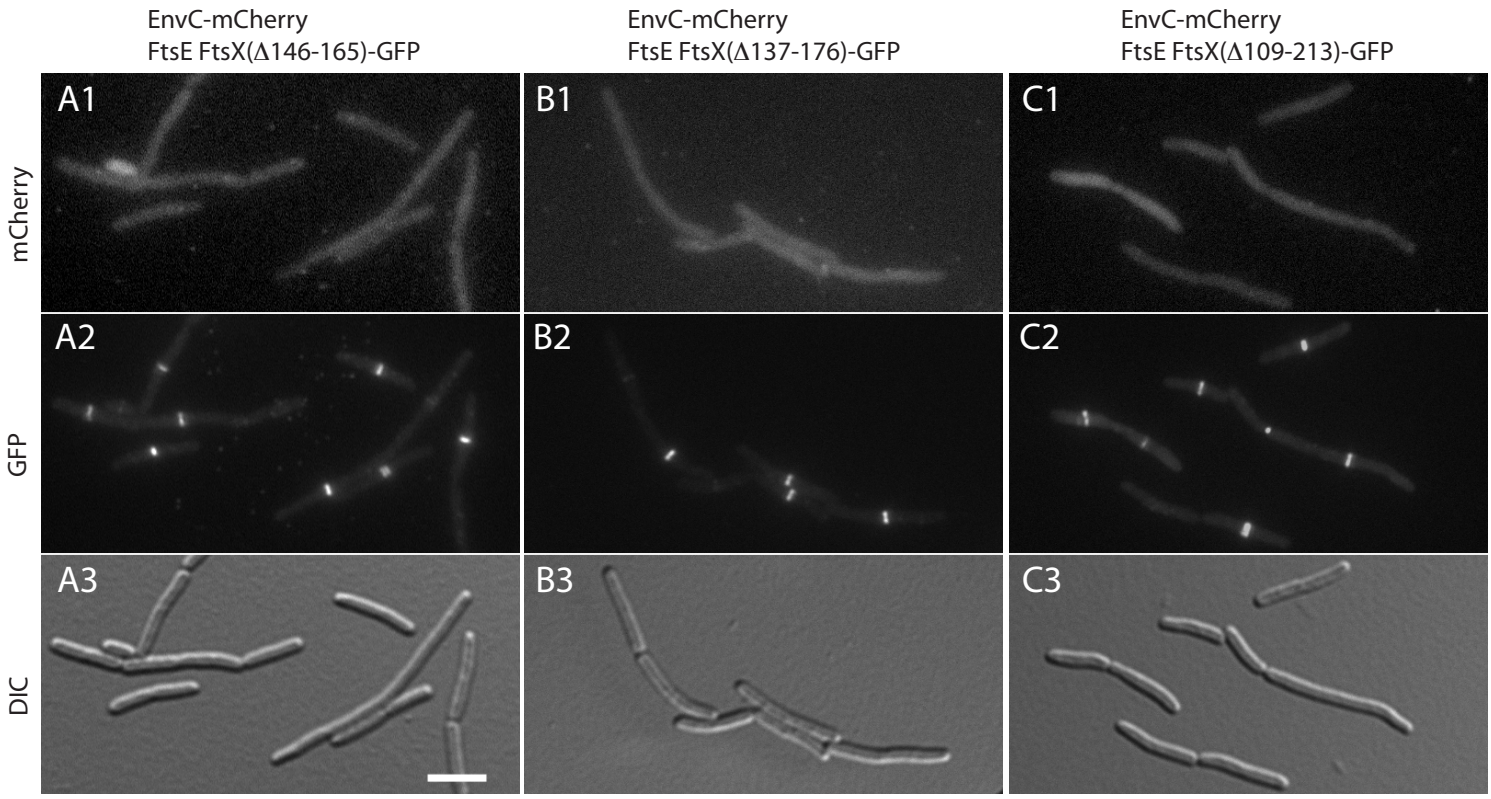
EnvC-mCherry
FtsE FtsX(Δ146-165)-GFP
EnvC-mCherry
FtsE FtsX(Δ137-176)-GFP
EnvC-mCherry
FtsE FtsX(Δ109-213)-GFP
mCherry
GFP
DIC
A1
B1
C1
A2
B2
C2
A3
B3
C3

Figure S2

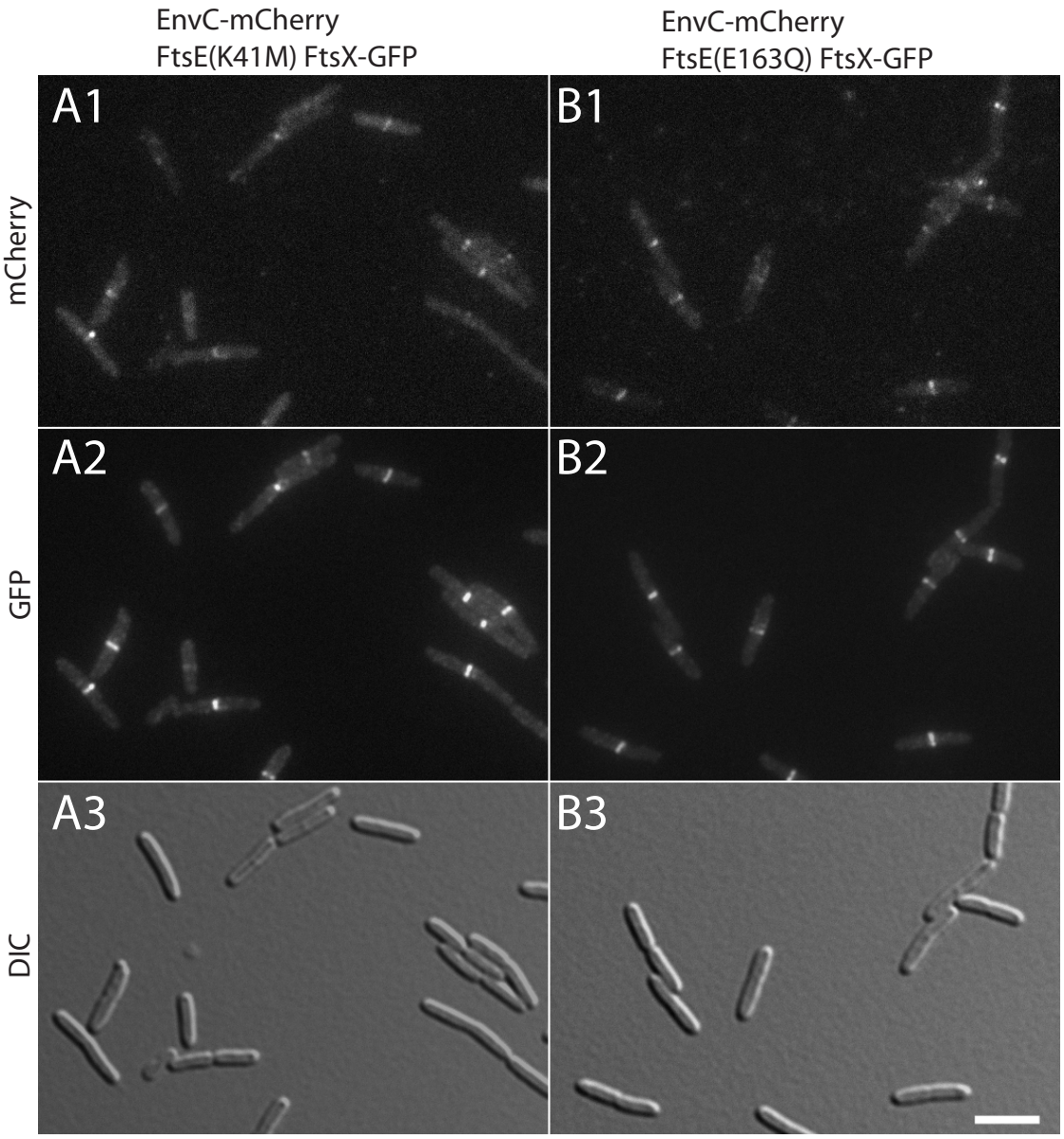
EnvC-mCherry
FtsE(K41M) FtsX-GFP
EnvC-mCherry
FtsE(E163Q) FtsX-GFP
mCherry
GFP
DIC
A1
B1
A2
B2
A3
B3

Figure S3

Figure Legends

Figure S1. EnvC-mCherry accumulation in FtsEX$^+$ and FtsEX$^-$ cells. Cells of TB28 [WT], TB140 [Δ*envC*], and NP69(attλTU188)(attHKTB316) [Δ*ftsEX* Δ*envC zapA-gfp* (P_{ara}::*ftsEX*) (P_{lac}::*envC-mCherry*)] were grown as described in the legend for Figure 5. When the cultures reached an OD_{600} of 0.4-0.6, cells were harvested for whole-cell extract preparation. Proteins in the resulting extracts were separated by SDS-PAGE, transferred to PVDF, and EnvC was detected with affinity purified anti-EnvC antibodies. Relevant genotypes and growth conditions are indicated above the corresponding lanes as well as the amount of total protein (μg) loaded per lane. Asterisk indicates an EnvC-mCherry breakdown product observed in both FtsEX$^+$ and FtsEX$^-$ cells.

Figure S2. EnvC localization in cells producing ^{Loop1}FtsX deletions. Cells of DY18 (*att*λTD80) [Δ*ftsEX* Δ*envC* (P_{lac}::*envC-mCherry*)] harboring the integrated expression constructs (*att*HKDY162) [P_{lac}::*ftsEX$^{(Δ146-165)}$-GFP*] (A), (*att*HKDY163) [P_{lac}::*ftsEX$^{(Δ137-176)}$-GFP*] (B), or (*att*HKDY165) [P_{lac}::*ftsEX$^{(Δ109-213)}$-GFP*] (C) were grown and visualized as described in the legend for **Figure 7**.

Figure S3. EnvC localization in cells producing FtsE* variants. Cells of DY18(*att*λTD80) [Δ*ftsEX* Δ*envC* (P_{lac}::*envC-mCherry*)] harboring the integrated expression constructs (*att*HKDY166) [P_{lac}::*ftsE(K41M)X-GFP*] (A) or (*att*HKDY168) [P_{lac}::*ftsE(E163Q)X-GFP*] (B) were grown and visualized as described in the legend for **Figure 7**.

Table S1. Cell separation phenotypes of FtsE* NlpD$^-$ cells.

strain	genotype	#cells[a]	# cell units[b]	total L (μm)[c]	avg. L (μm)[c]	total septa	L/septum	septa/cell
KP7	Δ*ftsEX* Δ*nlpD*	4	225	388	97	218	1.8	55
KP7 (attHKDY156)	Δ*ftsEX* Δ*nlpD* (P_{lac}::*ftsEX-GFP*)	144	200	475	3.3	56	8.5	0.4
KP7 (attHKDY166)	Δ*ftsEX* Δ*nlpD* (P_{lac}::*ftsE(K41M)X-GFP*)	31	202	491	15.8	171	2.9	6
KP7 (attHKDY167)	Δ*ftsEX* Δ*nlpD* (P_{lac}::*ftsE(D162N)X-GFP*)	9	214	434	48.2	208	2.1	23
KP7 (attHKDY168)	Δ*ftsEX* Δ*nlpD* (P_{lac}::*ftsE(E163Q)X-GFP*)	74	200	618	8	127	4.9	2

[a]Cell chains were considered as a single cell regardless of the number of segments they contained.
[b]Indicates the number of cell compartments in chaining cells.
[c]Length measurements represent a minimum because many of the cell chains extended beyond the field of view.

Table S2. Length and localization measurements of FtsE* cells producing FtsX-GFP and EnvC-mCherry.

Strain	genotype	#cells	total L (μm)	avg. L (μm)	# of FtsX-rings	# of EnvC-rings	% co-localization of FtsX and EnvC*	L/FtsX-ring (μm)	L/EnvC-ring (μm)	L/septum (μm)
DY18 (attHKDY156) (attλTD80)	Δ*ftsEX* Δ*envC* (P_{lac}::*ftsEX-GFP*) (P_{lac}::*envC-mCherry*)	125	457	3.7	115	93	81	4.0	4.9	9.3
DY18 (attHKDY166) (attλTD80)	Δ*ftsEX* Δ*envC* (P_{lac}::*ftsE(K41M)X-GFP*) (P_{lac}::*envC-mCherry*)	125	692	5.5	124	95	77	5.5	9.0	13.6
DY18 (attHKDY167) (attλTD80)	Δ*ftsEX* Δ*envC* (P_{lac}::*ftsE(D162N)X-GFP*) (P_{lac}::*envC-mCherry*)	125	1096	8.8	131	109	83	8.4	13.2	12.2
DY18 (attHKDY168) (attλTD80)	Δ*ftsEX* Δ*envC* (P_{lac}::*ftsE(E163Q)X-GFP*) (P_{lac}::*envC-mCherry*)	125	655	5.2	124	91	73	7.2	9.0	12.1

*Reflects the number of FtsX-GFP rings clearly associated with a ring of EnvC-mCherry.

Table S3. Bacterial strains used in this study.

Strain[a]	Genotype[b]	Source/Reference[c]
DH5α	F⁻ *hsdR*17 *deoR recA*1 *endA*1 *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 Δ(*lacZYA-argF*)U169 ϕ80d*lacZ*ΔM15	Gibco BRL
BL21(λDE3)	*ompT* rB– mB– (P_{lac}UV5::T7*gene1*)	Novagen
BTH101	F⁻, *glnV*44(AS), *recA*1, *endA*1, *gyrA*96, *thi*1, *hsdR*17, *spoT*1, *rfbD*1 *cya*	(20)
BW25113	Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-3) *rph*-1 Δ(*rhaD-rhaB*)568 *hsdR*514	(13)
JW5646	BW25113 Δ*envC*::Kan^R	(13)
JW2985	BW25113 Δ*ftsP*(*sufI*)::Kan^R	(13)
MG1655	*rph*1 *ilvG rfb*-50	(2)
MM9	TB28 (Kan^R *araC* P_{ara})::*ponB*	(5)
MM11	TB28 (*frt araC* P_{ara})::*ponB*	(5)
TB10	*rph*1 *ilvG rfb*-50 λΔ*cro-bio nad*::Tn10	(21)
TB28	MG1655 Δ*lacIZYA*::*frt*	(16)
TB140	TB28 Δ*envC*::*frt*	(7)
TB145	TB28 Δ*nlpD*747::*frt*	(7)
TB172	TB28 Δ*amiA*::*frt* Δ*amiB*::*frt* (Kan^R *araC* P_{ara})::*ponB*	P1(MM9) x TU207
TU122	TB28 Δ*ponB*::frt	(5)
TU189	TB10 Δ*ftsEX*::Kan^R	λRec
TU190**	TB28 Δ*ftsEX*::Kan^R	P1(TU189) x TB28(*att*λTU188)
TU191	TB28 Δ*ftsEX*::frt	TU190/pCP20
TU195**	TB28 Δ*ftsEX*::frt Δ*envC*::Kan^R	P1(JW5646) x TU191(*att*λTU188)
TU196**	TB28 Δ*ftsEX*::frt Δ*ftsP(sufI)*::Kan^R	P1(JW2985) x TU191(*att*λTU188)
TU205***	TB28 Δ*envC*::frt Δ*ftsP(sufI)*::Kan^R	P1(JW2985) x TB140(*att*λTD25)
TU207	TB28 Δ*amiA*::*frt* Δ*amiB*::*frt*	(12)
HC260	TB10 *zapA-gfp* Cam^R	λRec
HC261	TB28 *zapA-gfp* Cam^R	P1(HC260) x TB28
HC262	TB28 Δ*envC::frt zapA-gfp* Cam^R	P1(HC261) × TB140

NP32	TB28 Δ*envC::frt zapA-gfp frt*	HC262/pCP20
NP65*	TB28 (*frt araC* P_{ara})::*ponB* Δ*envC*::Kan^R	P1(JW5646) x MM11/pTB63
NP66*	TB28 (*frt araC* P_{ara})::*ponB* Δ*ftsEX*::Kan^R	P1(TU190) x MM11/pTB63
NP69	TB28 Δ*envC::frt zapA-gfp frt* Δ*ftsEX*::Kan^R	P1(TU190) x NP32
KP4*	TB28 Δ*envC*::Kan^R	P1(JW5646) x TB28/pTB63
KP5*	TB28 Δ*ftsEX*::Kan^R	P1(TU190) x TB28/pTB63
KP6*	TB28 Δ*nlpD*747::*frt* Δ*envC*::Kan^R	P1(JW5646) x TB145/pTB63
KP7*	TB28 Δ*nlpD*747::*frt* Δ*ftsEX*::Kan^R	P1(TU190) x TB145/pTB63
DY18	TB28 Δ*envC::frt* Δ*ftsEX*::Kan^R	P1(TU190) x TB140

[a] Stains marked with a single asterisk were made by transducing Kan^R cassettes into a recipient harboring plasmid pTB63. Stains marked with a double or triple asterisk were made by transducing Kan^R cassettes into recipients harboring *att*λTU188 or *att*λTD25, respectively.

[b] 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 expressed from pCP20.

[c] Strain constructions by P1 transduction are described using the shorthand: P1(donor) x recipient. Transductants were selected on LB Kan or Cam plates where appropriate. Strains resulting from the removal of a Drug^R cassette using pCP20 are indicated as: Parental strain/pCP20. λRec indicates strains were constructed by recombineering (see Experimental Procedures for details).

Table S4. Plasmids used in this study[a].

Plasmid	Genotype	Origin	Source or reference
pCP20	*bla cat* cI875 *repA*(Ts) P_R::*flp*	pSC101	(15)
pInt-ts	*bla* cI875 *repA*(Ts) P_R::int^{λ}	pSC101	(3)
pKNT25	*aph Plac::T25*	p15A	(20)
pCH363	*bla* $lacI^q$ P_{lac}::*T18*	pBR/colE1	(8)
pDY133	*bla* $lacI^q$ P_{T7}::^{10}*H-*^{loop1}*ftsX*	pBR/colE1	This study
pDY138	*bla* $lacI^q$ P_{lac}:: ^{loop1}*ftsX-T18*	pBR/colE1	This study
pDY151	*bla* $lacI^q$ P_{T7}::*H-SUMO-envC(35-276)*	pBR/colE1	This study
pDY156	*att*HK *bla* P_{lac}::*ftsEX-GFP*	R6K	This study
pDY161	*att*HK *bla* P_{lac}::*ftsEX*$^{(\Delta 152-161)}$*-GFP*	R6K	This study
pDY162	*att*HK *bla* P_{lac}::*ftsEX* $^{(\Delta 146-165)}$*-GFP*	R6K	This study
pDY163	*att*HK *bla* P_{lac}::*ftsEX* $^{(\Delta 137-176)}$*-GFP*	R6K	This study
pDY164	*att*HK *bla* P_{lac}::*ftsEX* $^{(\Delta 109-188)}$*-GFP*	R6K	This study
pDY165	*att*HK *bla* P_{lac}::*ftsEX* $^{(\Delta 109-213)}$*-GFP*	R6K	This study
pDY166	*att*HK *bla* P_{lac}::*ftsE(K41M)X-GFP*	R6K	This study
pDY167	*att*HK *bla* P_{lac}::*ftsE(D162N)X-GFP*	R6K	This study
pDY168	*att*HK *bla* P_{lac}::*ftsE(E163Q)X-GFP*	R6K	This study
pTB63	*tetAR ftsQAZ*	pSC101	(16)
pTB102	*cat* cI875 *repA*(Ts) P_R::int^{HK022}	pSC101	(4)
pTB316	*attHK022 bla* $lacI^q$ P_{lac}::*envC-mCherry*	R6K	(7)
pTB332	*aph* P_{lac}*::envC(277-419)-T25*	p15A	This study
pTB333	*aph* P_{lac}*::envC(34-419)-T25*	p15A	This study
pTD25	*att*λ *cat* P_{ara}::*envC*	R6K	(7)
pTD80	*att*λ *cat* P_{lac}::*envC-mCherry*	R6K	This study
pTU110	*cat* $lacI^q$ P_{lac}::*ponB lacZ*	F	(5)
pTU188	*att*λ *cat* P_{ara}::*ftsEX*	R6K	This study

[a] P_R, P_{lac}, and P_{ara} indicate the phage λR, lactose, and arabinose promoters, respectively. P_{syn135} is a synthetic *lac* promoter with a consensus -35 element and no operators. Numbers in parenthesis indicate the codons included in the relevant clones. The GFP allele used in plasmid constructs was superfolder GFP (22) and mCherry was

from Shaner and co-workers (23). The linker LEGPAGL was present between the fusion proteins and the protein of interest.

Supporting Methods and Materials

Media, bacterial strains, and plasmids

Cells were grown in LB [1% tryptone, 0.5% yeast extract, (0.5%-1.5% NaCl as indicated)] or minimal M9 medium (1) supplemented with 0.2% casamino acids and 0.2% sugar (glucose, maltose, or arabinose as indicated). Unless otherwise indicated, antibiotics were used at 5, 10, 15, 20, or 50 μg/ml for tetracycline (Tet), chloramphenicol (Cam), ampicillin (Amp), kanamycin (Kan), or spectinomycin (Spec), respectively.

The bacterial strains used in this study are listed in Table S1. All strains used in the reported experiments are derivatives of MG1655 (2). Plasmids used in this study are listed in Table S2. Vectors with R6K origins are all derivatives of the CRIM plasmids developed by Wanner and co-workers (3). They were either maintained in the cloning strain DH5α(λ*pir*) where they replicate as plasmids, or they were integrated into phage attachment sites (HK022 or λ) using the helper vectors pTB102 (4) or pInt-ts (3), respectively, as described previously (3). Single copy integrants were identified using diagnostic PCR (3). Integrated vectors were transferred between strains by P1-mediated transduction. In all cases, PCR was performed using KOD polymerase (Novagen) according to the instructions. Unless otherwise indicated, MG1655 chromosomal DNA was used as a template. Restriction sites for use in plasmid constructions are italicized and underlined in the primer sequences given below. Plasmid DNA and PCR fragments were purified using the Qiaprep spin miniprep kit (Qiagen) or the Qiaquick PCR purification kit (Qiagen), respectively.

pTU188

To construct pTU188 [*att*λ *cat* P_{ara}::*ftsE-ftsX*], a fragment containing *ftsE* and *ftsX* was amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTCA*AAGCTT*TTATTATTCAGGCGTAAAGTGGCG-3', digested with XbaI and HindIII, and inserted into the corresponding XbaI-HindIII sites of pTB285 (5).

pTB332-333

For pTB332 [*aph Plac::envC(277-419)-T25*], *envC(278-419)* was amplified with the primers 5'-GTAC*AAGCTT*GACCGAAAGCGAAAAATCGCTGATG-3' and 5'-GTCA*GGATCC*TCTCTTCCCAACCACGGCTGTGG-3'. The resulting fragment was digested and ligated with HindIII and BamHI digested pKNT25 (6). pTB333 [*aph* P_{lac}*::envC(34-419)-T25*] was constructed in the same manner except that *envC(34-419)* was amplified with the primers 5'-GTAC*AAGCTT*GGATGAGCGTGACCAACTCAAATCTATTC-3' and 5'-GTCA*GGATCC*TCTCTTCCCAACCACGGCTGTGG-3'.

pTD80

To construct pTD80 [*att*λ *cat lacI*^{q} P_{lac}::*envC-mCherry*], the P_{lac}::*envC-mCherry* containing EcoRI-HindIII fragment of pTB316 [*att*HK022 *bla lacI*^{q} P_{lac}::*envC-mCherry*] (7) was used to replace the corresponding P_{ara}::*envC* fragment of pTD25 [*att*λ *cat* P_{ara}::*envC*] (7).

pMT1

pMT1 [*cat* P_{ara}::*ftsEX*] was constructed in multiple steps. First, pTU170 [*att*HK022 *bla* P_{syn135}::*gfp-zapA*] was made by ligating the *EcoR*I-*Sal*I digested fragment from pEZ4 (8) into

the *EcoR*I-*Sal*I digested backbone of pTB263 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-sfgfp*] (7). Then pTU176 [*att*HK022 *bla* P_{syn135}::^{ss}*dsbA-sfgfp*] was made by ligating the ^{ss}*dsbA-sfgfp*-containing *Xba*I-*Hind*III fragment from pTB263 (7) with *Xba*I-*Hind*III digested pTU170 to generate pTU176. The ^{ss}*dsbA-sfgfp*-containing *Xba*I-*Hind*III fragment from pTU176 was ligated with *Xba*I-*Hind*III digested pBAD18-cat (9) to generate pTU214 [*cat* P_{ara}::^{ss}*dsbA-sfgfp*]. Using pTU188 [*att*λ *cat* P_{ara}::*ftsEX*] as a template, *RBSwk2-ftsEX* was amplified with primers 5'-GTCA*TCTAGA*AAAAAGGAAAAATGATTCGCTTTGAACATGTCAGCAAGG-3' and 5'-GTCA*AAGCTT*TTATTATTCAGGCGTAAAGTGGCG-3'. The resulting fragment was digested with *Hind*III and *Xba*I and ligated with pTU214 digested with the same enzymes to generate pMT1.

pDY133

pDY133 [*bla* P_{lac}::^{loop1}*ftsX-T18*] was constructed in two steps. First, the *amiA* gene was amplified using the primers 5'-GTCA*TCTAGA*AGGATCCGCCAAAGACGAACTTTTAAAAACCAGC-3' and 5'-GTCA*GAGCTC*GGCTCGAGTCGCTTTTTCGAATGTGCTTTCTGGTTG-3'. The resulting fragment was digested with *Xba*I and *Sac*I and ligated with pCH363 [*bla* P_{lac}::*lacZ-T18*] (8) digested with the same enzymes to generate pTU236 [*bla* P_{lac}::*amiA-T18*]. Second, ^{loop1}*ftsX* was amplified from pMT1 [*cat* P_{ara}::*RBSwk2-ftsEX*] using the primers 5'-GTCATT*GGATCC*GTGTACAAAAACGTTAACCAGGCGGCG-3' and 5'-AGTTAAGCTTATTA*CTCGAG*GCGCCCGACCAGCCCGGTCAACGCC-3'. The resulting fragment was digested with *BamH*I and *Xho*I and ligated with pTU236 [*bla* P_{lac}::*amiA-T18*] digested with the same enzymes to generate pDY133.

pDY138

pDY138 [*bla* P_{T7}*::His10-ftsXloop1*] was constructed in several steps. pDY42 [*bla* P_{T7}::*His10-mcs*] was made by using the synthetic oligonucleotides 5'-CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGCGTGGTTCTCACCACCATCACCACCATCACCACCATCATGCTAGCG-3'and 5'-GATCCGCTAGCATGATGGTGGTGATGGTGGTGATGGTGGTGAGAACCACGCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT-3'. These oligonucleotides were annealed, resulting in the formation of dsDNA with overhangs complementary to *BamH*I and *Xho*I restriction sites. pTD68 [*bla* P_{lac}::^{ss}*dsbA-linker-*^{sf}*gfp*] (10) was digested with *BamH*I and *Xho*I, and the resulting plasmid backbone was ligated with the complementary synthetic dsDNA. Finally, the ^{loop1}*ftsX* was amplified from pMT1 [*cat* P_{ara}::*ftsEX*] using the primers 5'-GTCATT*GGATCC*GTGTACAAAAACGTTAACCAGGCGGCG-3' and 5'-AGTTAAGCTTATTA*CTCGAG*GCGCCCGACCAGCCCGGTCAACGCC-3'. The resulting fragment was digested with *BamH*I and *Xho*I and ligated with pDY42 digested with the same enzymes to generate pDY138.

pDY151

pDY151 [*bla* P_{T7}::*H-SUMO-*^{cc}*envC*] was constructed in several steps. First, pTU138 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-envC*(35-419)-^{sf}*gfp*] was made by amplifying ^{fl}*envC* using the primers 5'-GTCA*GGATCC*GGTGATGAGCGTGACCAACTCAAATCTATTC-3' and 5'-GTCA*CTCGAG*TCTTCCCAACCACGGCTGTGG-3'. The resulting fragment was digested with *BamH*I and *Xho*I and ligated with pTB282 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-*^{sf}*gfp*] (11) digested with

the same enzymes. Using pTB138 as a template, ^{cc}*envC* was amplified with the primers 5'-GTCA*GGATCC*GGTGATGAGCGTGACCAACTCAAATCTATTC-3' and 5'-GTCA*CTCGAG*CGGTTTGTAGGTGGTGCCTTTGC-3'. The resulting fragment was digested with *BamH*I and *Xho*I and ligated with pTB282 (11) digested with the same enzymes to generate pTU150 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-*^{cc}*envC-*^{sf}*gfp*]. Finally, the ^{cc}*envC*-containing *BamH*I-*Xho*I fragment from pTU150 was ligated with *BamH*I-*Xho*I digested pTD68 [*bla* P_{T7}::*H-SUMO-mcs*] (12) to generate pDY151.

pDY156

pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] was constructed as follows. *ftsEX* was amplified from MG1655 with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-*^{sf}*gfp*] (12) digested with the same enzymes.

pDY158-160

pDY158 [*attλ cat* P_{ara}::*ftsE(K41M)X*] was made by site-directed mutagenesis of pTU188 [*attλ cat* P_{ara}::*ftsEX*] using the QuickChange method (Stratagene) and the primer 5'-GATCAGCTTCAGGAGGGTACTCATCCCTGCGCCGGAATGACCGGT-3'.

pDY159 [*attλ cat* P_{ara}::*ftsE(D162N)X*] was constructed by site-directed mutagenesis of pTU188 [*attλ cat* P_{ara}::*ftsEX*] using the QuickChange method (Stratagene) and the primer 5'-GTCCAGGTTACCAGTCGGTTCGTTCGCCAGCAGTACCGCGGGCTT-3'.

pDY160 [*att*λ *cat* P_{ara}::*ftsE(E163Q)X*] was constructed by site-directed mutagenesis of pTU188 [*att*λ *cat* P_{ara}::*ftsEX*] using the QuickChange method (Stratagene) and the primer 5'-GTCGTCCAGGTTACCAGTCGGTTGGTCCGCCAGCAGTACCGCGGG-3'.

pDY161-pDY165

pDY161 [*att*HK022 *bla* P_{lac}::*ftsEX(152-161)-sfgfp*] was constructed as follows. To make the 10aa *ftsX* internal deletion *ftsEX(152-161)*, two overlap extension PCR fragments were amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTTTTCTTCCAGCATATCCAGCTCACCCAGTGCGTCTTCACG-3' as well as 5'-CGTGAAGACGCACTGGGTGAGCTGGATATGCTGGAAGAAAAC-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] as the DNA template. The resulting PCR fragments were purified and used together as the final DNA template to amplify the *ftsEX(152-161)* fragment using the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY162

pDY162 [*att*HK022 *bla* P_{lac}::*ftsEX(146-165)-sfgfp*] was constructed as follows. To make the 20aa *ftsX* internal deletion *ftsEX(146-165)*, two overlap extension PCR fragments were amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-

TGCCGGAAGCGGGTTTTCTTCACGAGAAAGATAGTTCACTTT-3' as well as 5'-AAAGTGAACTATCTTTCTCGTGAAGAAAACCCGCTTCCGGCA-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] as the DNA template. The resulting PCR fragments were purified and used together as the final DNA template to amplify the *ftsEX(146-165)* fragment using the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY163

pDY163 [*att*HK022 *bla* P_{lac}::*ftsEX(137-176)-sfgfp*] was constructed as follows. To make the 40aa *ftsX* internal deletion *ftsEX(137-176)*, two overlap extension PCR fragments were amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-CTGGAAATCGAGTTTCGGGATGCCTTGCTCGGCCTGCAACTG-3' as well as 5'-CAGTTGCAGGCCGAGCAAGGCATCCCGAAACTCGATTTCCAG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] as the DNA template. The resulting PCR fragments were purified and used together as the final DNA template to amplify the *ftsEX(137-176)* fragment using the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY164

pDY164 [*att*HK022 *bla* P_{lac}::*ftsEX(109-188)-sfgfp*] was constructed as follows. To make the 80aa *ftsX* internal deletion *ftsEX(109-188)*, two overlap extension PCR fragments were amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GATACGATCACGCAGCGTATTTGACGGATAATACTGCGTCGC-3' as well as 5'-GCGACGCAGTATTATCCGTCAAATACGCTGCGTGATCGTATC-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] as the DNA template. The resulting PCR fragments were purified and used together as the final DNA template to amplify the *ftsEX(109-188)* fragment using the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY165

pDY165 [*att*HK022 *bla* P_{lac}::*ftsEX(109-213)-sfgfp*] was constructed as follows. To make the 105aa *ftsX* internal deletion *ftsEX(109-213)*, two overlap extension PCR fragments were amplified with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-CAGCCCGGTCAACGCCGCCAGTGACGGATAATACTGCGTCGC-3' as well as 5'-GCGACGCAGTATTATCCGTCACTGGCGGCGTTGACCGGGCTG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY156 [*att*HK022 *bla* P_{lac}::*ftsEX-sfgfp*] as the DNA template. The resulting PCR fragments were purified and used

together as the final DNA template to amplify the *ftsEX(109-213)* fragment using the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3'. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY166-pDY168

pDY166 [*att*HK022 *bla* P_{lac}::*ftsE(K41M)X-sfgfp*] was constructed by amplifying *ftsE(K41M)X* with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY158 [*attλ cat* P_{ara}::*ftsE (K41M)X*] as the DNA template. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY167

pDY167 [*att*HK022 *bla* P_{lac}::*ftsE(D162N)X-sfgfp*] was constructed by amplifying *ftsE(D162N)X* with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY159 [*attλ cat* P_{ara}::*ftsE (D162N)X*] as the DNA template. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

pDY168

pDY168 [*att*HK022 *bla* P_{lac}::*ftsE(E163Q)X-sfgfp*] was constructed by amplifying *ftsE(E163Q)X* with the primers 5'-GTCA*TCTAGA*TTTGCCCGAGAGGATTAACAATG-3' and 5'-GTCA*CTCGAG*TTCAGGCGTAAAGTGGCGTAAATG-3' using pDY160 [*att*λ *cat* P_{ara}::*ftsE (E163Q)X*] as the DNA template. The resulting fragment was digested with *Xba*I and *Xho*I and ligated with pTB311 [*att*HK022 *bla* P_{lac}::^{ss}*dsbA-amiB(23-445)-sfgfp*] (12) digested with the same enzymes.

Recombineering

The Δ*ftsEX*::Kan^R allele was constructed by replacing the region between the 2nd codon of *ftsE* and the 7th codon from the stop codon of *ftsX* with a Kan^R cassette as described previously (13, 14). The Kan^R cassette was amplified from pKD13 (15) using the primers 5'-ACTTTATAGAGGCACTTTTTGCCCGAGAGGATTAACAATGATTCCGGGGATCCGTCGACC-3' and 5'-AGAGTATAACACGCTTTTATTATTCAGGCGTAAAGTGGCGTGTAGGCTGGAGCTGCTTCG-3'. The resulting product was electroporated into strain TB10 as described previously (16), and the recombinants were selected at 30˚C on an LB plate containing 1% NaCl and 20 μg/ml kanamycin to generate the chromosomal deletion.

To generate a marker for the Z-ring, a *zapA-gfp* fusion was created at its native chromosomal locus by λ recombineering (14). *gfp-mut2* (17) coding sequence and a linked *cat* cassette flanked by *zapA* 3' end sequence and sequence downstream of *zapA* was amplified using pTB24 (8) as a template and the primers 5'-ACAAGGTCGCATCACCGAAAAAACTAACCAAAACTTTGAAGATCCCCCCGCTGAATTCATG

-3’ and 5’-

TTGTCTTCACGGTTACTCTACCACAGTAAACCGAAAAGTGGTGTAGGCTGGAGCTGCTTCG

-3’. The resulting fragment was used for recombineering in strain TB10 as described previously (16). The *zapA-gfp* fusion linked to the *cat* cassette was transferred between strains by P1-transduction.

Synthetic lethal screen

The screen for mutants with a Slb phenotype was performed as previously described (5). Briefly, TU122/pTU110 [*ΔlacIZYA ΔponB*/P_{lac}::*ponB lacZ*] was mutagenized with the EzTn-Kan2 transposome (Epicenter) as previously described (16). Mutants were selected for Kan resistance at room temperature, yielding a library of 75,000 independent transposon insertions. This mutant library was plated on LB agar supplemented with 50 *μ*M IPTG and X-gal (40 *μ*g/ml) at 30˚C and room temperature to identify mutants with a Slb phenotype. In addition to transposon the insertions in *ponA* and *lpoA* described previously (5), we also isolated mutants with insertions in *envC* (between codon 74 and 75) and *ftsX* (within codon 59).

Fluorescence microscopy

Fluorescence microscopy was performed as described previously (7). See figure legends for specific growth conditions employed for each experiment. Cell fixation and membrane staining was performed as described previously (7).

Protein Purification, pull-up assays, and two-hybrid analysis

^{FL}EnvC and ^{Lyt}EnvC were purified as described previously (12). ^{CC}EnvC was overexpressed and purified with a 6xHis-SUMO (H-SUMO) tag fused to its N-terminus. H-SUMO-^{CC}EnvC was purified from BL21(λDE3)/pDY151 and a 10xHis-tagged version of ^{Loop1}FtsX [H-^{Loop1}FtsX] was purified from BL21(λDE3)/pDY138. Overnight cultures were grown at 37°C in LB supplemented with ampicillin (50 μg/ml) and glucose (0.2%). The cultures were diluted 1:100 into 0.1 L of LB supplemented with ampicillin (50 μg/ml) and glucose (0.04%), and cells were grown at 30°C to an OD_{600} of 0.81 and 0.86 respectively. IPTG was added to 1 mM and the cultures were grown for an additional 3 hrs at 30°C. Cells were harvested by centrifugation and the cell pellets were resuspended in 3 ml of buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol) with 20 mM imidazole and stored at -80°C. Cells were thawed, disrupted by sonication, and cell debris was pelleted by centrifugation at 20,000 x g for 20 min at 4°C. The supernatants were passed through 0.2 μM syringe filters and loaded onto ProPur IMAC mini spin columns (Nunc) equilibrated in buffer A with 20 mM imidazole. Columns were washed 3x using buffer A with 50 mM imidazole and eluted using buffer A with 300 mM imidazole. For H-SUMO-^{CC}EnvC, the H-SUMO tag was cleaved with 6xHis-tagged SUMO protease (H-SP) as previously described (12). The cleavage reaction was passed through Ni-NTA resin (ProPur IMAC midi spin column) to remove free H-SUMO and H-SP, yielding a pure preparation of untagged ^{CC}EnvC. Amicon Centrifugal Filter Units (MWCO 10 kDa) were used to concentrate both protein preparations and exchange the buffer to buffer A without imidazole. Protein preparations were stored at -80°C in buffer A. The BACTH assay and Ni-NTA “pull-up” assays are described in the legend to **Figure 6**.

EnvC antisera and affinity purification of anti-EnvC antibodies

Polyclonal rabbit antisera was raised agains purified ^{FL}EnvC by Covance according to their standard protocol. The resulting anti-EnvC antibodies were affinity purified using ^{FL}EnvC coupled to AminoLink resin (Pierce) as described previously for SlmA antibody purification (11).

Cell fractionation and immunoblotting

Whole-cell extracts for **Fig. 4** were prepared as described previously (18). The protein concentration in each extract was determined using the non-interfering protein assay (Genotech) according to the manufacturers instructions. Protein concentrations were normalized between extracts and 20, 10, or 5 μg of total protein from each extract was separated on a 12% SDS-PAGE gel. Proteins were transferred to a PVDF membrane (Whatman) and the membrane was blocked with Rapid-Block (Genotech) for 5 minutes. The membrane was incubated with anti-EnvC antibodies (1:5000 in Rapid-Block) for 1 hour at room temperature. The membrane was washed three times with 25 ml TBST (10 mM Tris-HCl pH7.5, 100 mM NaCl, 0.1% Tween-20) for 10 minutes. Following the wash, the membrane was incubated with goat anti-rabbit antibodies conjugated with HRP (Rockland) (1:20,000 in Rapid-Block) for 1 hour at room temperature. Finally, the membrane was washed an additional four times as above and developed using the Pierce Super-Signal West-Pico reagents. Chemiluminescence was detected using a BioRad Chemidoc system. Cell fractionations and immunoblotting for **Fig. 4B** were performed as described previously (19), except that 500 mM NaCl was added to the spheroplasts after they were formed and EnvC was detected as described above. NaCl addition was required to promote the complete release of EnvC to the periplasmic fraction in spheroplasts from FtsEX$^-$ cells.

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