Supporting Information

Narita et al. 10.1073/pnas.1312012110

SI Materials and Methods

Bacterial Strains. SN1145 was constructed by removing the kanamycin-resistance cassette from GC187 by using pCP20. Gene disruption of AD16, MC4100, and SN1145 was conducted by transferring the kanamycin-resistance cassette from a corresponding strain of the Keio collection by P1-mediated transduction (1). Multiple-gene disruptants were constructed by successive transduction combined with removal of the kanamycin-resistance cassette by using pCP20 as described previously (2). SN147, SN150, and SN909 were constructed by transducing the Δ*bamB*::*tet* allele from SM4106 to AD16, SN56, and SN896, respectively, by P1 transduction. All gene disruptions were verified by PCR with appropriate primers.

Plasmids. For construction of pCA24NΔNot, which was used as an empty vector control for ASKA plasmids, a NotI fragment was deleted from pCA24N (3) by digestion with NotI followed by self-ligation. pUC-bepA-his_6 was constructed as follows. A DNA fragment encoding $BepA_{His6}$ was amplified by PCR from the genomic DNA of *Escherichia coli* BW25113 (4) using a pair of primers, yfgC-1 and yfgC-2 (Table S4). The amplified DNA fragment was digested with EcoRI and KpnI and then cloned into the same sites of pUC18. pTTQ-bepA-his_6 was constructed essentially as described for pUC-bepA-his_6 except that the DNA fragment was amplified with yfgC-3 instead of yfgC-1 and cloned into pTTQ18. pUC-bepA-his_{10} was constructed by self-ligation of a DNA fragment that was PCR-amplified from pUC-bepA-his_6 using a pair of primes, yfgC-his10-1 and yfgC-his10-2, after treatment with T4 polynucleotide kinase (TaKaRa Bio). pTH-bepA-his_{10} was constructed by subcloning the EcoRI–SalI fragment derived from pUC-bepA-his_{10} into the same sites of pTH18cr. For construction of pCDF-bepA-his_{10}, a DNA fragment was PCR-amplified from pUC-bepA-his_{10} using a pair of primers, yfgC-4 and M4C, digested with NcoI and SalI, and cloned into the same sites of pCDFDuet-1. For construction of pUC-bepA, a DNA fragment was PCR-amplified from pTH-bepA-his_{10} using a pair of primers, yfgC-1 and yfgC-Pst-2, and cloned into the EcoRI–SmaI site of pUC18 after digestion with EcoRI. Plasmids encoding the H136R mutant form of BepA were constructed by site-directed mutagenesis using a pair of complementary primers, yfgC_H136R and yfgC_H136R-r. Plasmids encoding the E137Q mutant form of BepA were constructed in the same way using primers yfgC_E137X and yfgC_E137X-r. pUC-bepA(Q428Amber) was also constructed in the same way using primers TPR4-2 and TPR4-2-r. pMAN-lptE was constructed as follows. The *lptE* coding region was PCR-amplified from the genomic DNA of BW25113 using a pair of primers, lptE-1 and lptE-2. The amplified DNA fragment was digested with EcoRI and HindIII and then cloned into the same sites of pMAN885EH. To construct pMAN-lptD-his_6, a DNA fragment encoding $LptD_{His6}$ was amplified by PCR from the genomic DNA of BW25113 using a pair of primers, lptD-1 and lptD-2, digested with XbaI, and cloned into the SmaI–XbaI site of pMAN885EH. pMAN-lptD-his_6 derivatives encoding $LptD_{His6}$ with Cys to Ser substitutions were constructed by site-directed mutagenesis. To construct pMAN-bamB-his_6, a DNA fragment encoding $BamB_{His6}$ was amplified by PCR from the genomic DNA of DH5α using a pair of primers, yfgL-1 and yfgL-2, digested with EcoRI and HindIII, and cloned into the same sites of pMAN885EH.

Purification of BepA and in Vitro Proteolytic Activity Assay. KRX cells were transformed with pCDF-bepA-his_{10} or pCDF-bepA (E137Q)-his_{10} encoding His_{10}-tagged wild-type BepA or BepA (E137Q) and grown in L medium supplemented with 0.1% rhamnose at 37 °C. When the culture OD (at 600 nm) reached 1.0, cells were harvested by centrifugation at 5,000 × *g* for 10 min and then resuspended in 20 mM Tris·HCl (pH 7.5) containing 1 μg/mL DNase I. Cells were disrupted by a single passage through a French Press cell at 10,000 psi. After removal of unbroken cells by centrifugation at 10,000 × *g* for 5 min, membranes were removed by centrifugation at 100,000 × *g* for 1 h. The supernatant was applied to TALON metal affinity resin (Clontech), and the column was successively washed with buffer A [20 mM Tris·HCl (pH 7.5), 50 mM NaCl] supplemented with 0, 5, or 20 mM imidazole, and finally eluted with buffer A containing 50 mM imidazole.

For protease activity assay, a purified preparation of $BepA_{His10}$ or $BepA(E137Q)_{His10}$ (200 μg/mL) was mixed with α-casein (400 μg/mL; Sigma) and incubated at 37 °C in buffer containing 20 mM Tris·HCl (pH 7.5), 0.05% *n*-dodecyl-β-D-maltopyranoside (DDM), and 10 μM $ZnCl_2$. Where specified, 1,10-phenanthroline or EDTA instead of $ZnCl_2$ was added at 250 μM. Samples were mixed with a quarter volume of 5× SDS sample buffer, boiled for 5 min, and subjected to SDS/PAGE. Proteins were visualized by staining with Coomassie brilliant blue R-250 (CBB).

Pull-Down Assay. Cells of AD16 transformed with pMAN885EH and pUC-bepA(E137Q), and SN147 transformed with pMAN-bamB-his_6 and pUC-bepA(E137Q), were grown in L medium supplemented with 0.01% arabinose and 0.1 mM IPTG at 30 °C. When the culture OD reached 1.0, cells were harvested by centrifugation at 5,000 × *g* for 10 min, and resuspended in 20 mM Tris·HCl (pH 7.5) containing 1 μg/mL DNase I. Cells were disrupted by a single passage through a French Press cell at 10,000 psi. After removal of unbroken cells by centrifugation at 10,000 × *g* for 5 min, the membrane fraction was recovered by centrifugation at 100,000 × *g* for 1 h. The membranes were resuspended in 20 mM Tris·HCl (pH 7.5) containing 300 mM NaCl and 2% (wt/vol) DDM, and incubated at 4 °C for 30 min for solubilization. After removal of insoluble materials by centrifugation at 100,000 × *g* for 30 min, the supernatant was diluted 10-fold with 20 mM Tris·HCl (pH 7.5) containing 300 mM NaCl and applied to Dynabeads His-tag Isolation and Pulldown (Invitrogen). The beads were successively washed with buffer B [20 mM Tris·HCl (pH 7.5), 300 mM NaCl, 0.05% DDM], supplemented with 0, 5, 20, or 50 mM imidazole, and eluted with buffer B containing 250 mM imidazole.

Mass Spectrometry. The membrane fraction of bis(sulfosuccinimidyl) suberate-treated spheroplasts of MC4100 transformed with pTTQ-bepA(E137Q)-his_6 was prepared as described above and solubilized in 50 mM Tris·HCl (pH 7.5) containing 0.5% SDS at 37 °C for 5 min. After 10-fold dilution with 20 mM Tris·HCl (pH 7.5) containing 2% (wt/vol) Triton X-100, insoluble materials were removed by ultracentrifugation at 100,000 × *g* for 30 min, and the supernatant was mixed with Ni-NTA agarose resin (Qiagen) for 1 h at 4 °C. Resin was washed with 20 mM Tris·HCl (pH 7.5) containing 300 mM NaCl, 0.2% Triton X-100, and either 0, 5, or 20 mM imidazole, and bound $BepA_{His6}$ was eluted with 20 mM Tris·HCl (pH 7.5) containing 300 mM NaCl and 250 mM imidazole. The cross-linked product containing BepA was visualized by staining with CBB after SDS/PAGE. An excised CBB-stained gel band was destained with 50% (vol/vol)

acetonitrile in 10 mM Tris·HCl (pH 8.0) and then dried in vacuo. The band was incubated with 0.01 μg of trypsin, TPCK-treated (Worthington Biochemical Corporation) in 10 mM Tris·HCl (pH 8.0) at 37 °C for 12 h. An aliquot of the digest was analyzed by nano LC-MS/MS using an LCQ Deca XP instrument (Thermo Fisher Scientific). The peptides were separated using a nano ESI spray column (100 μm i.d. × 375 μm o.d.) packed with a reversed-phase material (Inertsil ODS-3, 3 μm; GL Science) at a flow rate of 200 nL/min. The mass spectrometer was operated in the positive-ion mode, and the spectra were acquired in a data-dependent MS/MS mode. The MS/MS spectra were searched against the NCBInr 20111107 database (15,916,306 sequences; 5,467,648,827 residues) using an in-house MASCOT server (version: 2.2.1; Matrix Science).

Alkylation of $LptD_{His6}$. Cells of AD16 transformed with pMAN885EH derivatives encoding wild-type $LptD_{His6}$, $LptD(SSCC)_{His6}$, or $LptD(CCSS)_{His6}$ were grown overnight in L medium at 30 °C. They were harvested, resuspended in 100 mM Tris·HCl (pH 8.0), and incubated in the absence or presence of 100 mM DTT at room temperature for 30 min. Total cellular proteins were precipitated by trichloroacetic acid treatment and solubilized with 100 mM Tris·HCl (pH 8.0) containing 1% SDS by vortexing at room temperature for 30 min. Samples were incubated in the absence or presence of 10 mM EZ-Link Maleinide-PEG_2-Biotin (Thermo Fisher Scientific) at 37 °C for 30 min. Proteins were separated by SDS/PAGE, and $LptD_{His6}$ was detected by immunoblotting with anti-His antibodies.

Cell Fractionation. Subcellular localization of LptD was determined as follows. Cells of AD16 and SN56 (Δ*bepA*) were grown overnight in L medium at 30 °C. They were harvested, resuspended in 20 mM Tris·HCl (pH 7.5) containing 1 mM Pefabloc (Merck) and 100 μg/mL lysozyme, and subjected to freezing and thawing. Then, 10 μg/mL DNase I was added to the samples, and cells were disrupted by sonication. After removal of unbroken cells, membranes were recovered by centrifugation at 100,000 × *g* for 1 h at 4 °C and suspended in 20 mM Tris·HCl (pH 7.5) containing 1 mM EDTA and 1 mM Pefabloc. The supernatant was saved as the soluble fraction. Total membranes thus obtained were layered on a 30–55% (wt/wt) stepwise sucrose gradient and centrifuged at 60,000 × *g* for 12 h at 4 °C. The sucrose gradient was fractionated into portions and analyzed by SDS/PAGE and immunoblotting.

Growth of LptE-Deficient Strains. Cells of GC187 and their derivatives were grown overnight in M9 medium supplemented with 19 amino acids other than methionine, 2 μg/mL thiamine, 0.2% arabinose, and 0.2% glucose or maltose. Cells from 1-mL aliquot cultures were pelleted, washed three times with equal volumes of the same medium without arabinose, and inoculated into the same medium without arabinose to an initial OD_{600} of ∼0.001. Cells were subsequently grown at 30 °C to an early log phase.

Other Techniques. The minimum inhibitory concentrations (MICs) of antibiotics were determined by the agar dilution method using L agar. SDS/PAGE was carried out as described (5), unless otherwise specified. WIDE RANGE Gel Preparation buffer (Nacalai Tesque) was used in some experiments to improve separation of proteins. To raise anti-BepA antibodies, $BepA_{His6}$ was purified from SN150 carrying pTTQ-bepA-his_6 by metal affinity chromatography using TALON resin (Clontech) and used to immunize rabbits. A Penta-His HRP Conjugate Kit (Qiagen) or anti–His-tag polyclonal antibodies (MBL) were used to probe polyhistidine-tagged proteins. Anti-BamA, -BamB, -BamD, -LptD, and -LptE antisera were provided by Shin-ichi Matsuyama (Rikkyo University, Tokyo, Japan). Anti-BamC and -LamB antisera were provided by Thomas J. Silhavy (Princeton University, Princeton, NJ). Anti-OmpA antiserum was provided by Hajime Tokuda (University of Morioka, Iwate, Japan). Anti-MBP antiserum was purchased from MBL. Immunoblotting was carried out essentially as described previously (6), and proteins were visualized using enhanced chemiluminescence substrate (ECL or ECL Prime; GE Healthcare), followed by detection with a lumino-image analyzer (LAS-3000mini; Fujifilm).

1. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
2. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158(1):9–14.
3. Kitagawa M, et al. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): Unique resources for biological research. *DNA Res* 12(5):291–299.
4. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* 2:2006.0008.
5. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685.
6. Shimoike T, et al. (1995) Product of a new gene, *syd*, functionally interacts with SecY when overproduced in *Escherichia coli*. *J Biol Chem* 270(10):5519–5526.

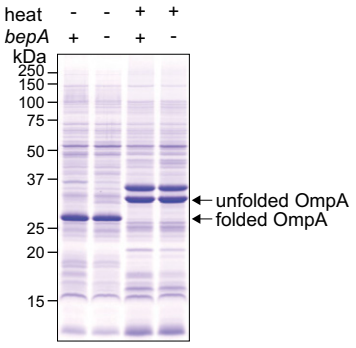

Fig. S1. Membrane protein profiles of the wild-type and Δ*bepA* strains. Crude membranes prepared from overnight cultures of wild-type (+) or Δ*bepA* cells (–) were solubilized in an SDS/PAGE sample buffer with (+) or without (–) heating at 99 °C for 5 min and were analyzed by the WIDE RANGE PAGE (10% acrylamide) followed by CBB staining.

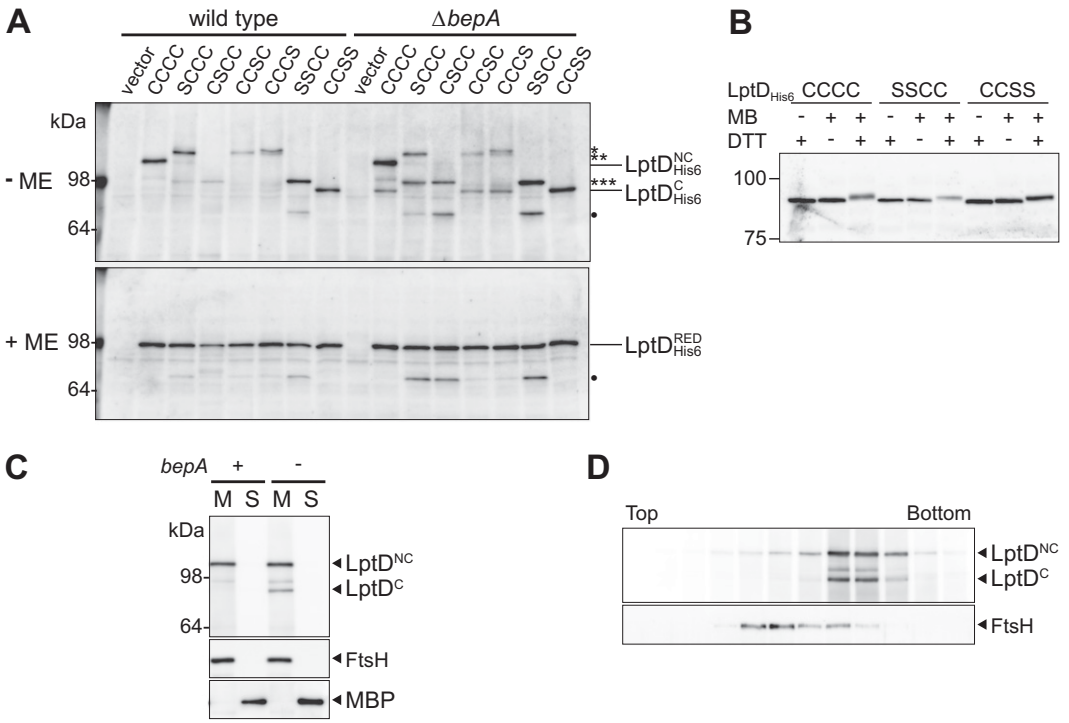

Fig. S2. Connectivity of disulfide bonds and subcellular localization of LptD^{C}. (*A*) Cells of wild-type or Δ*bepA* strains were transformed with plasmids encoding C-terminally His_6-tagged LptD (LptD$_{His6}$) with Cys to Ser substitutions. Total cellular proteins were acid precipitated and analyzed by nonreducing (−ME) or reducing (+ME) SDS/PAGE and immunoblotting with anti–His-tag antibodies. Asterisks indicate LptD$_{His6}$ with a disulfide bond of C_{31}–C_{724} (*), C_{173}–C_{724}, or C_{173}–C_{725} (**), or C_{724}–C_{725} (***) whereas bullets indicate a degradation product of LptD$_{His6}$. (*B*) Sulfhydryl modification. Wild-type cells transformed with a plasmid encoding wild-type LptD$_{His6}$ (denoted as CCCC) or LptD$_{His6}$ with $C_{31}S/C_{173}S$ substitutions (SSCC) or $C_{724}S/C_{725}S$ substitutions (CCSS) were incubated in the presence or absence of DTT. Acid-denatured cellular proteins were labeled with maleimide-PEG_2-biotin (MB) and analyzed by reducing SDS/PAGE followed by immunoblotting with anti-His antibodies. (*C*) Subcellular localization of LptD^{C}. Wild-type (+) or Δ*bepA* (–) cells were fractionated into membrane (M) and soluble (S) fractions. Anti-LptD, anti-MBP, or anti-FtsH immunoblotting was carried out after nonreducing SDS/PAGE. FtsH (1) and MBP were detected by immunoblotting as markers for membrane and soluble fractions, respectively. (*D*) Membranes of Δ*bepA* cells were fractionated into the IM and OM by sucrose gradient centrifugation. Proteins of each fraction were analyzed by nonreducing SDS/PAGE and immunoblotting with anti-LptD antiserum. FtsH was used as a marker for the IM.

1. Kihara A, Akiyama Y, Ito K (1996) A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J* 15(22):6122–6131.

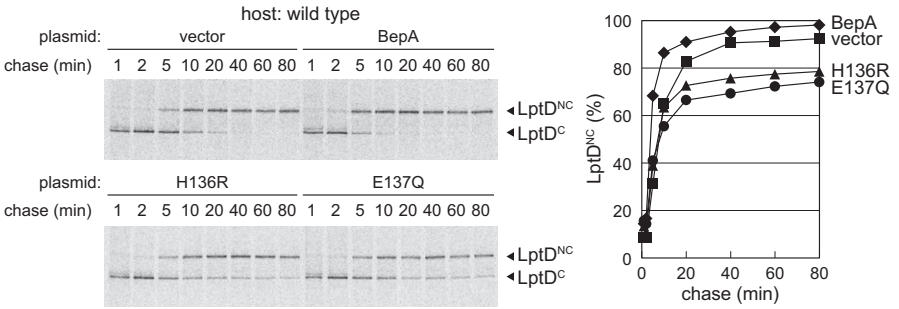

Fig. S3. Dominant-negative effect of the BepA protease active-site mutants on disulfide isomerization of LptD. Oxidative folding of LptD was monitored using wild-type cells transformed with either of the plasmids encoding C-terminally His_{10}-tagged wild-type BepA or its H136R or E137Q derivative.

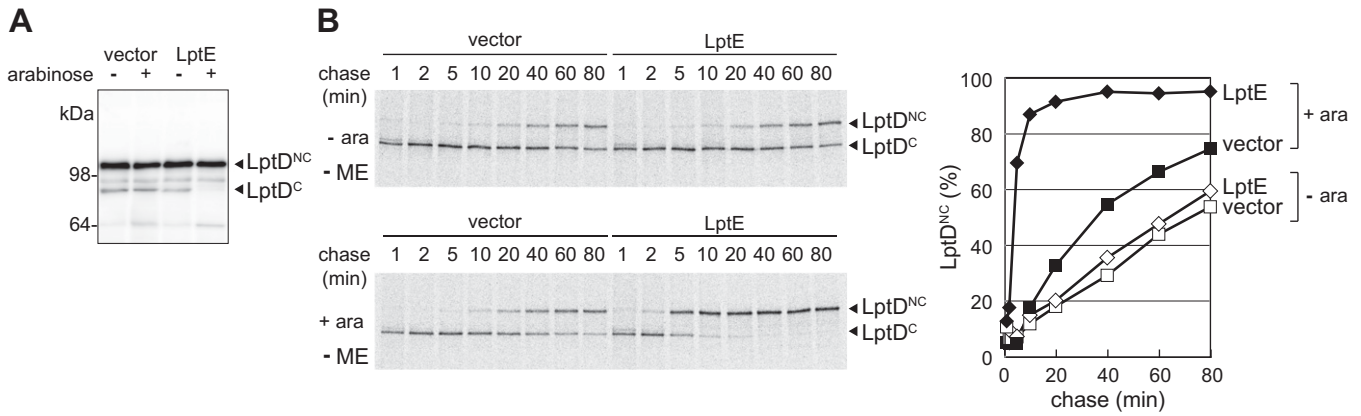

Fig. S4. Overexpression of LptE facilitates disulfide isomerization in LptD. (*A*) Immunoblotting analysis. Δ*bepA* cells were transformed with an empty vector or pMAN-lptE carrying *lptE* under the control of the *araBAD* promoter and grown in the absence (–) or presence (+) of 0.2% arabinose. Immunoblotting of LptD was carried out after nonreducing SDS/PAGE of total cellular proteins. (*B*) Pulse–chase analysis. Oxidative folding of LptD was monitored as in Fig. 4 using Δ*bepA* cells transformed with an empty vector or pMAN-lptE grown in M9 medium supplemented with 0.2% glucose (– ara) or 0.2% maltose plus 0.2% arabinose (+ ara).

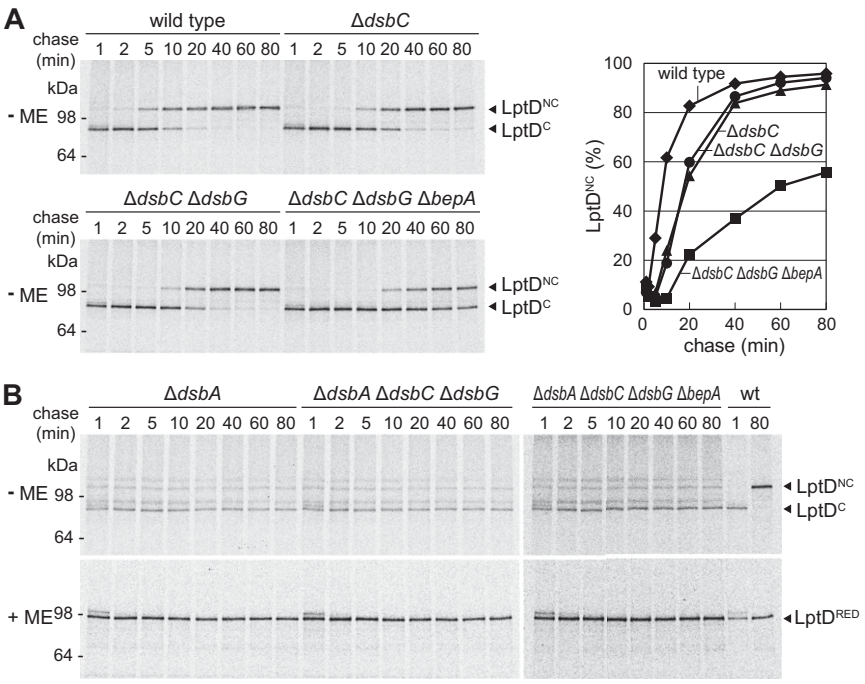

Fig. S5. Disulfide isomerization of LptD in the cells lacking periplasmic oxidoreductases. (*A*) Effects of the *dsbC* and *dsbD* disruption. Oxidative folding of LptD was monitored as in Fig. 4 using *dsbC* and/or *dsbG* knockout cells carrying wild-type *bepA* or its disruptant. (*B*) Effects of the *dsbA* disruption. *dsbA* knockout cells additionally lacking *dsbC*, *dsbG*, and/or *bepA* as indicated were analyzed as in *A* with nonreducing (–ME) or reducing (+ME) SDS/PAGE.

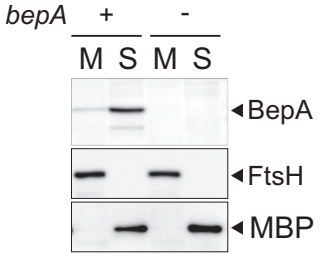

Fig. S6. Subcellular localization of BepA. Wild-type (+) or Δ*bepA* (–) cells were disrupted by sonication and fractionated into membrane (M) and soluble (S) fractions by ultracentrifugation. Each protein was detected by SDS/PAGE and immunoblotting with antiserum against respective proteins.

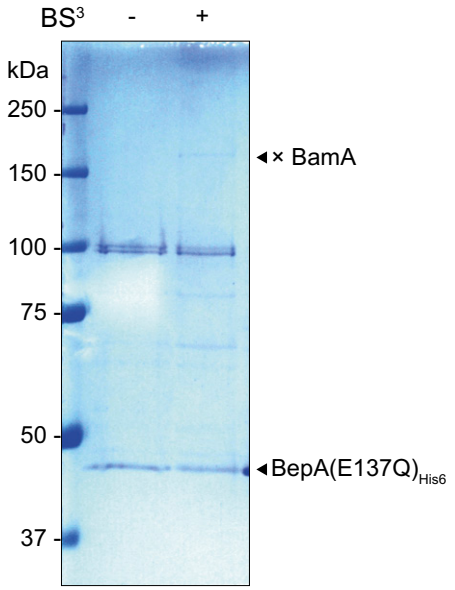

Fig. S7. Purification of the BS^3-mediated cross-linked product. Spheroplasts of wild-type cells transformed with a plasmid encoding C-terminally His_6-tagged BepA with E137Q substitution were treated with BS^3. $BepA(E137Q)_{His6}$ and its cross-linked products were affinity purified as described in *Materials and Methods* and visualized by staining with CBB after SDS/PAGE. The cross-linked product (× BamA) that was subsequently identified as the BamA–BepA adduct by mass spectrometry is indicated.

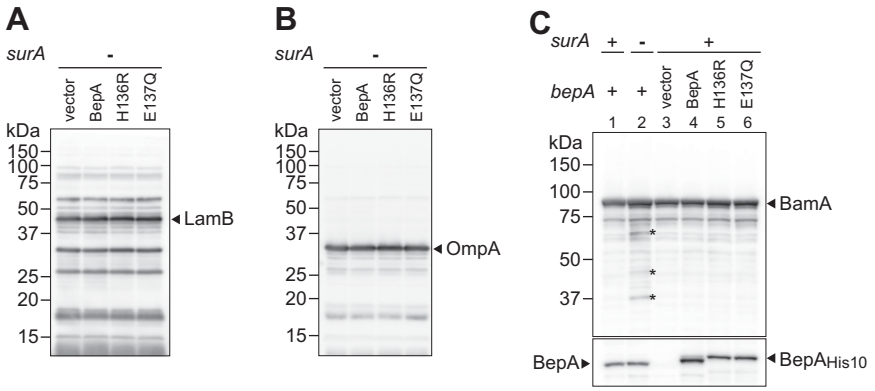

Fig. S8. Effect of the *surA* disruption or BepA overproduction on the OM protein profiles. (*A* and *B*) Effect on LamB and OmpA. Total cellular protein was prepared from Δ*surA*/Δ*bepA* cells harboring empty vector or either of the plasmids encoding C-terminally His_{10}-tagged wild-type BepA or its H136R or E137Q derivative and was subjected to SDS/PAGE followed by immunoblotting with anti-LamB (*A*) or anti-OmpA (*B*) antisera. (*C*) Effect on BamA. Overexpression of BepA does not cause the degradation of BamA in the $surA^+$ cells. Total cellular proteins were prepared from wild-type (lanes 1 and 3–6) or the Δ*surA* cells (lane 2) without plasmid (lanes 1 and 2) or with either empty vector or a plasmid encoding C-terminally His_{10}-tagged wild-type BepA or its H136R or E137Q derivative (lanes 3–6) and was subjected to SDS/PAGE followed by immunoblotting with anti-BamA or anti-BepA antisera. Putative BepA degradation products of BamA are indicated by asterisks.

Table S1. List of ASKA plasmids that suppressed erythromycin sensitivity of the Δ*bepA* Δ*bamE* strain

			MIC, μg/mL			
			Erythromycin		Vancomycin	
Plasmid*	Gene product	Growth on SDS†	−IPTG	+ IPTG	− IPTG	+ IPTG
pTH-bepA-his$_{10}$	BepA	+	12.5	25	50	100
pCA24NΔNot	Empty vector	—	6.25	3.13	25	25
fhuF	Ferric iron reductase	+	25	12.5	25	50
mppA	Murein tripeptide transporter	+	6.25	12.5	25	50
stfQ	Predicted side tail fiber assembly protein	+	6.25	12.5	25	50
yaiU	Conserved protein	+	6.25	12.5	25	50
ydfW	Pseudogene, integrase fragment, Qin prophage	+	25	25	25	50
yeaH	Conserved protein	+	6.25	12.5	25	25
ychP	Predicted invasin	+	6.25	12.5	25	50
yneO	AidA homolog, autotransporter	+	6.25	12.5	25	25
ynjE	Predicted thiosulfate sulfur transferase	+	6.25	12.5	25	50
appY	Global transcription regulator	+	6.25	12.5	25	25
gadW	Transcriptional activator of *gadA* and *gadBC*	+	6.25	12.5	25	50
soxS	DNA-binding transcriptional dual regulator	+	6.25	12.5	25	25
ydeO	Transcriptional activator for *mdtEF*	+	6.25	25	25	50
rluE	23S rRNA U2457 pseudouridine synthase	+	6.25	12.5	25	25
lptE	LPS assembly OM complex	±	12.5	12.5	50	50
pagP	Palmitoyl transferase for Lipid A	±	6.25	12.5	25	50
ybiI	DksA-type zinc finger protein	±	6.25	6.25	25	25
bcsC	Cellulose synthase subunit	±	6.25	12.5	25	25
rrmJ	23S rRNA U2552 ribose 2′-O-methyltransferase	±	6.25	12.5	12.5	25
rplO	50S ribosomal subunit protein L15	—	6.25	6.25	25	25
rpsM	30S ribosomal subunit protein S13	—	6.25	6.25	25	25
ykgO	Predicted ribosomal protein	—	6.25	6.25	25	25
marA	Transcriptional activator of multiple antibiotic resistance	—	6.25	6.25	12.5	6.25
yaiT	Conserved protein	—	6.25	12.5	25	50
yhfG	Predicted protein	—	6.25	6.25	12.5	25

+, normal growth; ±, weak growth; −, no growth.
*Cloned genes in ASKA plasmids are shown.
†Growth phenotype on L agar containing 0.5% SDS is shown.

Table S2. Strains used in this study

E. coli strains	Genotype	Source
AD16	Δ*pro-lac thi* [F′ *lacI*^{q} *Z*M15 *Y*$^+$ *pro*$^+$]	1
SN56	AD16 Δ*bepA*	This study
SN147	AD16 Δ*bamB*::*tet*	This study
SN150	AD16 Δ*bamB*::*tet* Δ*bepA*	This study
SN305	AD16 Δ*surA*::*kan*	This study
SN259	AD16 Δ*surA*::*kan* Δ*bepA*	This study
SN531	AD16 Δ*bamC*::*kan*	This study
SN533	AD16 Δ*bamC*::*kan* Δ*bepA*	This study
SN535	AD16 Δ*bamE*::*kan*	This study
SN537	AD16 Δ*bamE*::*kan* Δ*bepA*	This study
SN547	AD16 Δ*dsbA*::*kan*	This study
SN548	AD16 Δ*dsbA*::*kan* Δ*bepA*	This study
SN807	AD16 Δ*dsbC*	This study
SN773	AD16 Δ*dsbC* Δ*bepA*	This study
SN834	AD16 Δ*dsbC* Δ*dsbG*	This study
SN835	AD16 Δ*dsbC* Δ*dsbG* Δ*bepA*	This study
SN836	AD16 Δ*dsbA*::*kan* Δ*dsbC* Δ*dsbG*	This study
SN838	AD16 Δ*dsbA*::*kan* Δ*dsbC* Δ*dsbG* Δ*bepA*	This study
MC4100	*araD*139 Δ(*argF-lac*)*U*169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR*	2
SN896	MC4100 Δ*bepA*	This study
SN909	MC4100 Δ*bamB*::*tet* Δ*bepA*	This study
SN910	MC4100 Δ*bamC*::*kan* Δ*bepA*	This study
SN911	MC4100 Δ*bamE*::*kan* Δ*bepA*	This study
AM604	MC4100 *ara*$^+$	3
GC187	AM604 Δ*lptE2*::*kan* Δ(λ_{att}-*lom*)::*bla*-P_{ara}*lptE*(atg)-*araC*	Gift from T. Silhavy
SN1145	GC187 Km^S	This study
SN1159	SN1145 Δ*bepA*::*kan*	This study
SN1194	SN1145 Δ*bepA*	This study
KRX	Δ*pro-lac thi* [F′ *traD*36 Δ*ompP lacI*^{q} *Z*M15 *pro*$^+$] Δ*ompT endA*1 *recA*1 *gyrA*96 *hsdR*17 e14$^-$ *relA*1 *supE*44 Δ*rhaBAD*::T7 RNA polymerase	Promega

1. Kihara A, Akiyama Y, Ito K (1996) A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J* 15(22):6122–6131.
2. Casadaban MJ (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* 104(3):541–555.
3. Wu T, et al. (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci USA* 103(31):11754–11759.

Table S3. Plasmids used in this study

Plasmids	Description	Source
pCA24NΔNot	Expression vector for ASKA clones; $P_{T5\text{-}lac}$ *cat*	This study
pCP20	pSC101 derivative; *Rep*(Ts) *bla cat* λcI857 λP_R *FLP*$^+$	1
pUC18	Expression vector; P_{lac} *bla*	2
pUC-bepA	pUC18 derivative encoding BepA	This study
pUC-bepA(H136R)	pUC18 derivative encoding BepA(H136R)	This study
pUC-bepA(E137Q)	pUC18 derivative encoding BepA(E137Q)	This study
pUC-bepA(Q428Amber)	pUC18 derivative encoding BepA(Q428Amber)	This study
pUC-bepA-his_6	pUC18 derivative encoding $BepA_{His6}$	This study
pUC-bepA-his_{10}	pUC18 derivative encoding $BepA_{His10}$	This study
pUC-bepA(H136R)-his_{10}	pUC18 derivative encoding $BepA(H136R)_{His10}$	This study
pUC-bepA(E137Q)-his_{10}	pUC18 derivative encoding $BepA(E137Q)_{His10}$	This study
pTTQ18	Expression vector; P_{tac} *bla*	3
pTTQ-bepA-his_6	pTTQ18 derivative encoding $BepA_{His6}$	This study
pTTQ-bepA(E137Q)-his_6	pTTQ18 derivative encoding $BepA(E137Q)_{His6}$	This study
pTH18cr	Expression vector; P_{lac} *cat*	4
pTH-bepA-his_{10}	pTH18cr derivative encoding $BepA_{His10}$	This study
pTH-bepA(H136R)-his_{10}	pTH18cr derivative encoding $BepA(H136R)_{His10}$	This study
pTH-bepA(E137Q)-his_{10}	pTH18cr derivative encoding $BepA(E137Q)_{His10}$	This study
pCDFDuet-1	Expression vector; P_{T7} *aadA*	Novagen
pCDF-bepA-his_{10}	pCDFDuet-1 derivative encoding $BepA_{His10}$	This study
pCDF-bepA(E137Q)-his_{10}	pCDFDuet-1 derivative encoding $BepA(E137Q)_{His10}$	This study
pEVOL-pBpF	p15A derivative encoding an evolved *M. jannaschii* aminoacyl-tRNA synthetase/suppressor tRNA pair for incorporation of pBPA at the amber site	5
pMAN885EH	Expression vector, P_{BAD} *cat*	6
pMAN-lptD-his_6	pMAN885EH derivative encoding $LptD_{His}$	This study
pMAN-lptD(C31S)-his_6	pMAN885EH derivative encoding $LptD(SCCC)_{His6}$	This study
pMAN-lptD(C173S)-his_6	pMAN885EH derivative encoding $LptD(CSCC)_{His6}$	This study
pMAN-lptD(C724S)-his_6	pMAN885EH derivative encoding $LptD(CCSC)_{His6}$	This study
pMAN-lptD(C725S)-his_6	pMAN885EH derivative encoding $LptD(CCCS)_{His6}$	This study
pMAN-lptD(C31S/C173S)-his_6	pMAN885EH derivative encoding $LptD(SSCC)_{His6}$	This study
pMAN-lptD(C724S/C725S)-his_6	pMAN885EH derivative encoding $LptD(CCSS)_{His6}$	This study
pMAN-lptE	pMAN885EH derivative encoding LptE	This study
pMAN-bamB-his_6	pMAN885EH derivative encoding $BamB_{His6}$	This study

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Table S4. Primers used in this study

Name	Sequence* (5′–3′)
yfgC-1	GATGAATTCCAGAAATACAGGATAGAG
yfgC-2	GGGGTACCTAATGATGATGATGATGATGCTCGAGCATCTTGGTATAAGGCTTAAAG
yfgC-3	ATGAATTCCAGGCAGTTGAAAAAAAACC
yfgC-4	CATGCCATGGTCAGGCAGTTGAAAAAAAACCTGG
M13 Primer M4	GTTTTCCCAGTCACGAC
yfgC-his10-1	CATCATCATCATTAAGTACCCG
yfgC-his10-2	ATGATGATGATGATGATGCTCG
yfgC_PstI-2	TTACATCTTGGTATAAGGCTTAAAGCGTTCCTGCA
yfgC_H136R	CTGGCTTCAGTTATGGCGCGCGAAATCTCCCACGTCACC
yfgC_H136R-r	GGTGACGTGGGAGATTTCGCGCGCCATAACTGAAGCCAG
yfgC_E137X	GGCTTCAGTTATGGCGCACYAGATCTCCCACGTCACCCAACG
yfgC_E137X-r	CGTTGGGTGACGTGGGAGATCTRGTGCGCCATAACTGAAGCC
TPR4-2	GCTAAATAACCGCGATTAGGAGCTGGCTGCGCG
TPR4-2-r	CGCGCAGCCAGCTCCTAATCGCGGTTATTTAGC
lptE-1	GATGAATTCTGGTCGTTGGCTAAGCGC
lptE-2	CAATTCAAGCTTCATTGAGCTGCGCGCGG
lptD-1	AACGTTACCGATGATGGAAC
lptD-2	CAATTCTCTAGATTAGTGATGGTGATGGTGATGCTCCAAAGTGTTTTGATACGGCAG
lptD_C31S	GCCGACCTCGCCTCACAGTCAATGTTGGGCGTGCCAAGC
lptD_C31S-r	GCTTGGCACGCCCAACATTGACTGTGAGGCGAGGTCGGC
lptD_C173S	CGGTAGCTTTACCTCCTCACTGCCGGGTTCTGACACCTG
lptD_C173S-r	CAGGTGTCAGAACCCGGCAGTGAGGAGGTAAAGCTACCG
lptD_C724S	GCAATACAGCTCCTCATGCTATGCAATTCGC
lptD_C724S-r	GCGAATTGCATAGCATGAGGAGCTGTATTGC
lptD_C725S	GTGCAATACAGCTCCTGCTCATATGCAATTCGCGTCGGTTAC
lptD_C725S-r	GTAACCGACGCGAATTGCATATGAGCAGGAGCTGTATTGCAC
lptD_C724S/C725S	GTGCAATACAGCTCCTCATCATATGCAATTCGCGTCGGTTAC
lptD_C724S/C725S-r	GTAACCGACGCGAATTGCATATGATGAGGAGCTGTATTGCAC
yfgL-1	GATGAATTCGGAGGTTTAAATTTATGCAATTGCGTAAATTACTGC
yfgL-2	CAATTCAAGCTTAGTGATGGTGATGGTGATGCTCCAGACGTGTAATAGAGTACACGGTTC

*The following IUB codes are used to indicate base mixtures: Y= C + T; R = A + G.