Supplementary Information

A Supercomplex Spanning the Inner and Outer Membranes Mediates the Biogenesis of β-barrel Outer Membrane Proteins in Bacteria

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Extended Experimental Procedures

Semi-native SDS-PAGE and immunoblotting

Semi-native and denaturing SDS-PAGE were respectively performed with the loading samples being not boiled or boiled for 10 minutes. The immunoblotting was performed according to standard protocols(1-2), but the gels from the semi-native SDS-PAGE were steamed for 10 minutes (for denaturing the lipid-surrounded β-barrel OMPs) before the proteins were transferred to the PVDF membrane.

Mass Spectrometry analysis of proteins

The gel around the 400 kD band on the Blue-Native gel was excised, dehydrated in acetonitrile, incubated in 50 mM ammonium bicarbonate containing 10 mM DTT at 56 °C for 40 min, then incubated in 50 mM ammonium biocarbonate containing 55 mM iodoacetamide at ambient temperature for 1 hr in the dark before dehydrated again. The proteins in the treated gel was subsequently digested with sequencing grade trypsin (1 ng/μL, in 50 mM ammonium bicarbonate) at 37 °C overnight, with the resulting peptides being extracted twice with 50% acetonitrile containing 5% formic acid before dried by vacuum centrifugation.

For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the samples were desolved in 0.2% formic acid and then loaded onto a 100 μm x 2 cm pre-column and separated on a 75 μm × 15 cm capillary column with laser-pulled sprayer. Both columns were packed in-house with 4 μm C18 bulk material (InnosepBio, P.R.China). An Easy nLC 1000 system (Thermo Scientific, USA) was used to deliver the following HPLC gradients: First, 5-30% of solution A (0.1% formic acid in acetonitrile) in 40 min, then 30-75% of solution A in 4 min before being held at 75% of A for 20 min (all being mixed with 0.1% formic acid in water). The eluted peptides were directly sprayed into a Velos Pro Orbitrap Elite mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray ionization (ESI) source. The mass spectrometer was operated in the data-dependent mode with a full MS scan in FT mode at a resolution of 120,000 followed by CID (Collision Induced Dissociation) MS/MS scans on the 15 most abundant ions in the initial MS scan.

The raw data files were converted to mascot generic format (".mgf") using the MSConvert software before submitted for database search. Mascot (version 2.3.02) carried out all database search with the following parameters: Carbamidomethyl (Cys) as fixed modification and Oxidation (Met) as variable modification; +/- 5 ppm for peptide pass tolerance and +/- 0.6 Da for fragment mass tolerance; Max Missed Cleavages 2.

Table S1. ***E. coli*** **strains used in this study**

Strain	Description	Sources
BW25113	Wild type cells	Keio collection, Japan.
ΔompF	*ompF* gene deleted	Keio collection, Japan.
ΔlamB	*lamB* gene deleted	Keio collection, Japan.

ΔsurA	*surA* gene deleted	Keio collection, Japan.
LY928	Modified BW25113 strain whose chromosome was inserted with genes encoding the orthogonal Bpa-tRNA synthase and $tRNA^{Bpa}$	Laboratory storage
BamA-K135Bpa	Modified LY928 strain whose wild type *bamA* gene was replaced by a gene encoding BamA-K135Bpa	Laboratory storage

Table S2. Plasmids used in this study

Plasmids	**Description**	**Sources**
pSUP-Bpa-RS-6TRA	Encoding both the $tRNA^{Bpa}$ and the Bpa-tRNA synthetase; carrying chloramphenicol resistant gene	(3)
pACE; pDOC	For λ Red recombination-based gene-doctoring	Laboratory storage; (4)
pYLC	A modified low-copy-number pDOC containing a mutation in the replication origin	Laboratory storage
pYLC-BamA-135K	For replacing the wild type *bamA* gene with the gene encoding BamA-K135Bpa at the genome level	This study
pBAD-OmpF-A1Bpa-His_6, pBAD-OmpF-V11Bpa-His_6, pBAD-OmpF-Y32Bpa-His_6, pBAD-OmpF-L43Bpa-His_6, pBAD-OmpF-Q66Bpa-His_6, pBAD-OmpF-S95Bpa-His_6, pBAD-OmpF-E117Bpa-His_6, pBAD-OmpF-Y157Bpa-His_6, pBAD-OmpF-S169Bpa-His_6, pBAD-OmpF-E183Bpa-His_6, pBAD-OmpF-N198Bpa-His_6, pBAD-OmpF-N252Bpa-His_6, pBAD-OmpF-V289Bpa-His_6, pBAD-OmpF-Y313Bpa-His_6, pBAD-OmpF-D319Bpa-His_6, pBAD-OmpF-G325Bpa-His_6, pBAD-OmpF-T331Bpa-His_6,	Encoding the Bpa-incorporated variants of His-tagged OmpF; carrying ampicillin resistant gene	This study
pBAD-LamB-His_6	Encoding the His-tagged LamB; carrying ampicillin resistant gene	This study
pBAD-LamB-G5Bpa-His_6, pBAD-LamB-G12Bpa-His_6, pBAD-LamB-Q20Bpa-His_6, pBAD-LamB-G27Bpa-His_6, pBAD-LamB-L34Bpa-His_6, pBAD-LamB-N116Bpa-His_6, pBAD-LamB-S146Bpa-His_6, pBAD-LamB-L176Bpa-His_6, pBAD-LamB-N206Bpa-His_6, pBAD-LamB-T236Bpa-His_6, pBAD-LamB-N266Bpa-His_6,	Encoding the Bpa-incorporated variants of His-tagged LamB; carrying ampicillin resistant gene	This study

pBAD-LamB-N296Bpa-His_6, pBAD-LamB-I326Bpa-His_6, pBAD-LamB-S356Bpa-His_6, pBAD-LamB-Y378Bpa-His_6, pBAD-LamB-N386Bpa-His_6, pBAD-LamB-A393Bpa-His_6, pBAD-LamB-G400Bpa-His_6, pBAD-LamB-S408Bpa-His_6,		
pBAD-SurA-His_6	Encoding the His-tagged SurA; carrying ampicillin resistant gene	This study
pBAD-SurA-A1Bpa-His_6, pBAD-SurA-D6Bpa-His_6, pBAD-SurA-V17Bpa-His_6, pBAD-SurA-V22Bpa-His_6, pBAD-SurA-S28Bpa-His_6, pBAD-SurA-Q35Bpa-His_6, pBAD-SurA-L40Bpa-His_6, pBAD-SurA-R47Bpa-His_6, pBAD-SurA-E52Bpa-His_6, pBAD-SurA-G64Bpa-His_6, pBAD-SurA-I71Bpa-His_6, pBAD-SurA-D77Bpa-His_6, pBAD-SurA-N82Bpa-His_6, pBAD-SurA-N88Bpa-His_6, pBAD-SurA-M94Bpa-His_6, pBAD-SurA-Y100Bpa-His_6, pBAD-SurA-Y108Bpa-His_6, pBAD-SurA-E120Bpa-His_6, pBAD-SurA-E125Bpa-His_6, pBAD-SurA-I130Bpa-His_6, pBAD-SurA-Q135Bpa-His_6, pBAD-SurA-Q142Bpa-His_6, pBAD-SurA-D149Bpa-His_6, pBAD-SurA-L156Bpa-His_6, pBAD-SurA-P162Bpa-His_6, pBAD-SurA-D170Bpa-His_6, pBAD-SurA-Q178Bpa-His_6, pBAD-SurA-Q185Bpa-His_6, pBAD-SurA-F192Bpa-His_6, pBAD-SurA-H199Bpa-His_6, pBAD-SurA-N207Bpa-His_6, pBAD-SurA-R215Bpa-His_6, pBAD-SurA-I222Bpa-His_6, pBAD-SurA-T229Bpa-His_6, pBAD-SurA-V236Bpa-His_6, pBAD-SurA-G244Bpa-His_6, pBAD-SurA-D252Bpa-His_6, pBAD-SurA-I260Bpa-His_6, pBAD-SurA-R268Bpa-His_6, pBAD-SurA-P276Bpa-His_6, pBAD-SurA-R284Bpa-His_6, pBAD-SurA-S291Bpa-His_6, pBAD-SurA-G297Bpa-His_6, pBAD-SurA-A305Bpa-His_6,	Encoding the Bpa-incorporated variants of His-tagged SurA; carrying ampicillin resistant gene	This study

pBAD-SurA-N311Bpa-His_6, pBAD-SurA-N316Bpa-His_6, pBAD-SurA-L321Bpa-His_6, pBAD-SurA-N327Bpa-His_6, pBAD-SurA-F333Bpa-His_6, pBAD-SurA-N341Bpa-His_6, pBAD-SurA-V349Bpa-His_6, pBAD-SurA-A357Bpa-His_6, pBAD-SurA-N365Bpa-His_6, pBAD-SurA-Y378Bpa-His_6, pBAD-SurA-F386Bpa-His_6, pBAD-SurA-S392Bpa-His_6, pBAD-SurA-Q397Bpa-His_6, pBAD-SurA-V403Bpa-His_6,		

Table S3. Antibodies used in this study

Antibodies	**Animal immunized**	**Type**	**Working dilution**	**Sources**
Anti-His tag	Mouse	Monoclonal	1/2,000	TransGen Biotech
Anti-SurA	Rabbit	Polyclonal	1/10,000	Our lab
Anti-BamA	Rabbit	Polyclonal	1/10,000	Our lab
Anti-OmpF	Mouse	Polyclonal	1/10,000	Our lab
Anti-LamB	Goat	Polyclonal	1/10,000	Silhavy lab
Anti-SecY	Rabbit	Polyclonal	1/10,000	Tokuda lab and Nishiyama lab
Anti-SecE	Rabbit	Polyclonal	1/10,000	Tokuda lab and Nishiyama lab
Anti-BamB	Rabbit	Polyclonal	1/10,000	Our lab
Anti-SecA	Rabbit	Polyclonal	1/10,000	Our lab
Anti-PpiD	Rabbit	Polyclonal	1/10,000	Our lab
Anti-ATPase-alpha subunit	Rabbit	Polyclonal	1/5,000	Our lab
Anti-MalE	Rabbit	Polyclonal	1/5,000	Our lab
Anti-DegP	Rabbit	Polyclonal	1/5,000	Our lab
Anti-GroEL	Mouse	Polyclonal	1/5,000	Our lab
AP-conjugated anti-mouse IgG	Rabbit	Polyclonal	1/5,000	TransGen Biotech
AP-conjugated anti-rabbit IgG	Goat	Polyclonal	1/5,000	TransGen Biotech
AP-conjugated anti-goat IgG	Rabbit	Polyclonal	1/5,000	TransGen Biotech

Table S4. Proteins identified by Mass spectrometry analysis from the 400 kD band resolved from the inner membrane fraction of the wild type cells.

NAME	DESCRIBTION	SCORE
MCP1	Methyl-accepting chemotaxis protein I	345
ASPG2	L-asparaginase 2	291
YAET（BamA）	Outer membrane protein assembly factor yaeT	285
MCP2	Methyl-accepting chemotaxis protein II	240
OMPF	Outer membrane protein F	176
LPP	Major outer membrane lipoprotein	133
OMPC	Outer membrane protein C	122
SECA	Protein translocase subunit secA	86
MCP3	Methyl-accepting chemotaxis protein III	71
PTTBC	PTS system trehalose-specific EIIBC component	66
RLPA	Rare lipoprotein A	63
TOLC	Outer membrane protein tolC	60
METQ	D-methionine-binding lipoprotein metQ	56
TSX	Nucleoside-specific channel-forming protein tsx	53
PPID	Peptidyl-prolyl cis-trans isomerase D	53

Table S5. Proteins identified by mass spectrometry analysis from the 400 kD band region resolved from the inner membrane fraction of the BamA-depleted cells.

NAME	DESCRIBTION	SCORE
OPGG	Glucans biosynthesis protein G	762
FDOG	Formate dehydrogenase-O major subunit	526
OMPA	Outer membrane protein A	401
UBID	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	306
ATPG	ATP synthase gamma chain	285
MSBA	Lipid A export ATP-binding/permease protein msbA	225
MIND	Septum site-determining protein minD	128
CORA	Magnesium transport protein corA	104
YADG	Uncharacterized ABC transporter ATP-binding protein yadG	86
CYOA	Ubiquinol oxidase subunit 2	73
PTND	Mannose permease IID component	54

References

1. Ge, X., et al. (2014) DegP primarily functions as a protease for the biogenesis of beta-barrel outer membrane proteins in the Gram-negative bacterium Escherichia coli. *FEBS J* **281**, 1226-40.
2. Wimley, W.C. (2002) Toward genomic identification of beta-barrel membrane proteins: composition and architecture of known structures. *Protein Sci* **11**, 301-12.
3. Chin, J.W., Martin, A.B., King, D.S., Wang, L., and Schultz, P.G. (2002) Addition of a photocrosslinking amino acid to the genetic code of Escherichiacoli. *Proc Natl Acad Sci U S A* **99**, 11020-4.
4. Lee, D.J., et al. (2009) Gene doctoring: a method for recombineering in laboratory and

pathogenic Escherichia coli strains. *BMC Microbiol* **9**, 252.

Supplemental Figure Legends

Figure S1 Immunoblotting results of the *in vivo* photo-crosslinking products of the 58 Bpa variants of SurA, resolved by SDS-PAGE and probed with antibody against OmpF. Indicated on the right are the positions of the mature OmpF (mOmpF), OmpF precursor (pre-OmpF) and the SurA-OmpF crosslinked product. Indicated on the left are the positions of the molecular weight markers.

Figure S2. Immunoblotting results of the *in vivo* photo-crosslinking products of the 58 Bpa variants of SurA, resolved by SDS-PAGE and probed with antibody against LamB,

Figure S3. Immunoblotting results of the *in vivo* photo-crosslinking products of the 58 Bpa variants of SurA, resolved by SDS-PAGE and probed with antibody against BamA.

Figure S4. Immunoblotting results of the *in vivo* photo-crosslinking products of the 58 Bpa variants of SurA, resolved by SDS-PAGE and probed with antibody against the His-tag linked to SurA.

Figure S5. Functional complementation of the 58 Bpa variants of SurA in Δ*surA* cells cultured under the envelope-disrupting stress condition
The Δ*surA* cells expressing each of the 58 Bpa variants of SurA were cultured at 37°C in LB medium to an OD_{600} of about 1.0 before diluted and plated on LB dishes (with 0.005% arabinose added for inducing the expression of SurA variant proteins) under normal condition (left panel) or the envelope-disrupting stress condition (with 0.5% (w/v) SDS and 1 mM EDTA; right panel). The *ΔsurA* cells, that are unable to grow under the stress condition, and the SurA-containing wild type cells, that are able to grow under the stress condition, were respectively analyzed as negative and positive controls (displayed on the top part).

Figure S6. Analyzing the folding capacity of the Bpa variants of OmpF with the Bpa residue placed in the N- and C-terminal region, and the nascent nature of the OmpF bound to SurA.
(A, B) Immunoblotting results of the *in vivo* photo-crosslinking products of the 17 Bpa variants of OmpF or the 19 Bpa variants of LamB, resolved by SDS-PAGE and probed with antibody against the His-tag linked to OmpF or LamB,
(**C**) Immunoblotting results to detect the folding/assembling status of the Bpa variants of His-tagged OmpF, resolved by semi-native SDS-PAGE and probed with antibody against the His-tag. The His-tagged OmpF with no Bpa incorporation was analyzed as the negative control.
(D) Immunoblotting analysis results of the photo-crosslinking products of the OmpF-Y32Bpa variant probed with the antibody against SurA, the cells were treated with chloramphenicol (a protein synthesis inhibitor) for the indicated length of time before UV irradiation.

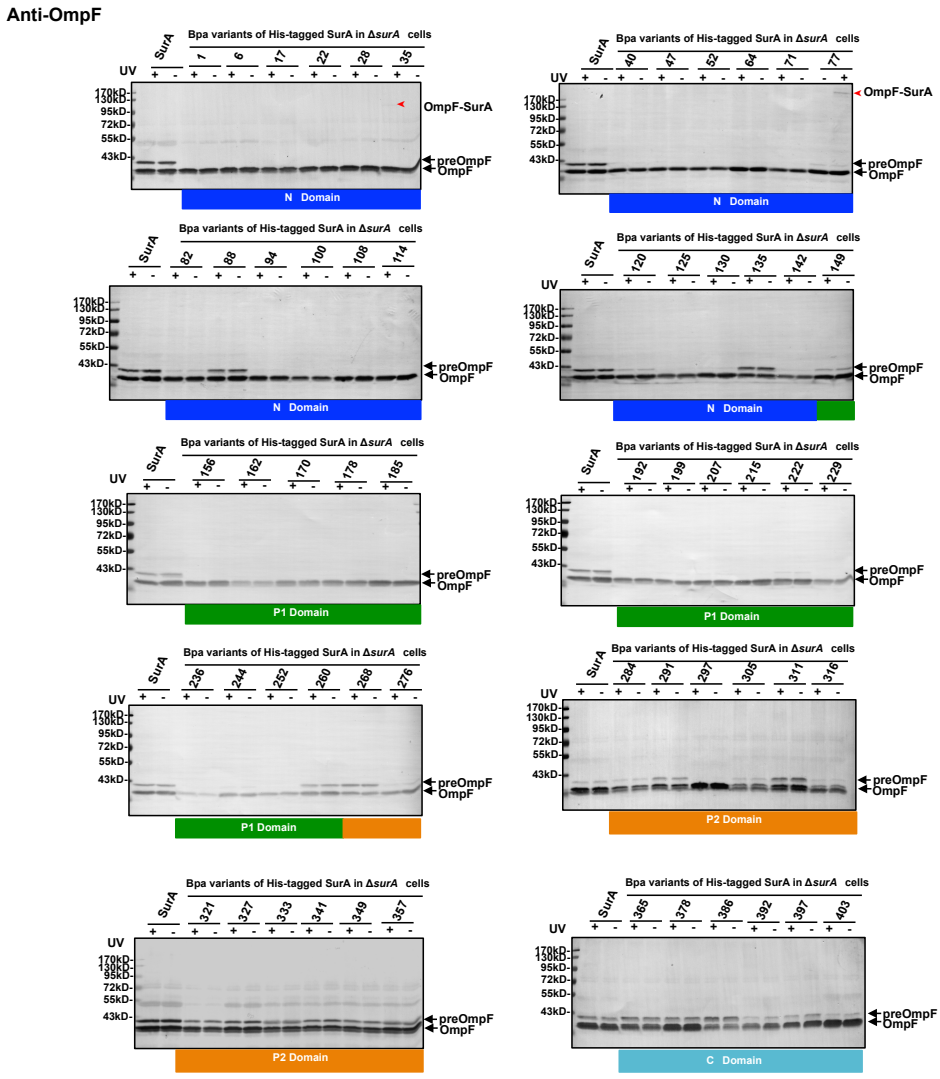
Figure S7 Immunoblotting analysis of the photo-crosslinked SurA-PpiD in the inner and outer membrane fractions that were separated by sucrose density gradient centrifugation. The α-subunit of ATP synthase and the OmpF were respectively analyzed as markers of the inner and outer membranes..

Figure S8. Immunoblotting results of the *in vivo* photo-crosslinking products of the 58 Bpa variants of SurA resolved by SDS-PAGE and probed with antibody against PpiD.

Figure S9. Immunoblotting analysis of the inner and outer membrane fractions of the wild-type cells treated with chloramphenicol.
Membrane fractions were separated by sucrose density gradient centrifugation, resolved by SDS-PAGE and probed with antibodies against SurA, BamA, SecY, SecE, PpiD, SecA or BamB. The relative amounts of the probed protein in each fraction were quantified (mean ± SEM; n = 3) from the immunoblotting results and displayed in the right panel. The α-subunit of ATP synthase and the OmpF were respectively analyzed as markers of the inner and outer membranes.

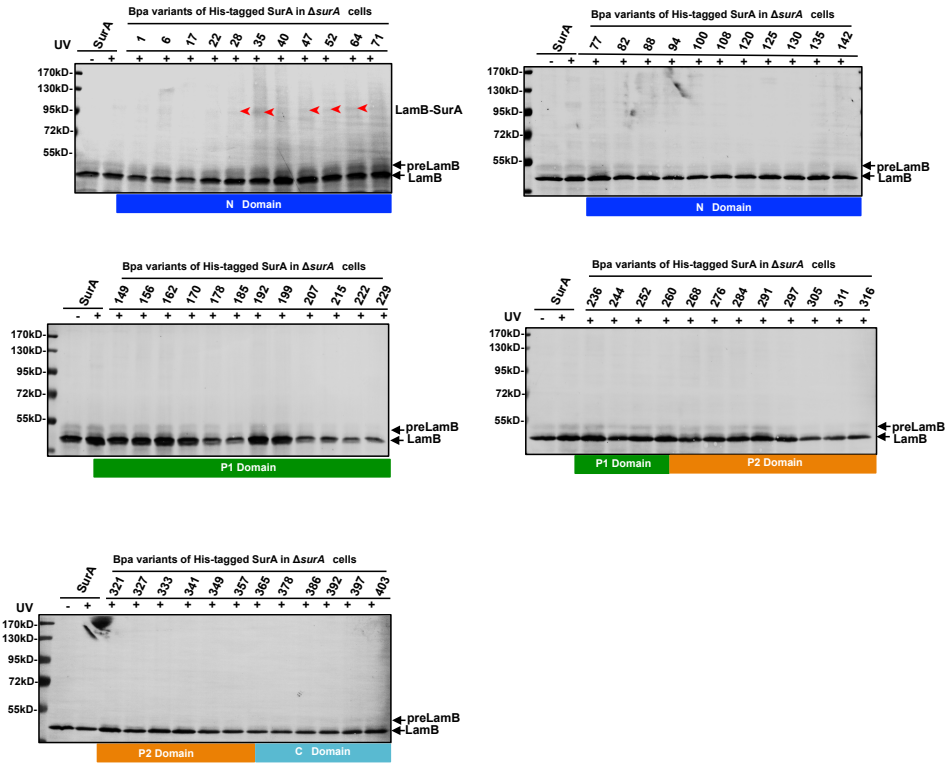
Figures

Supplemental Figure 1



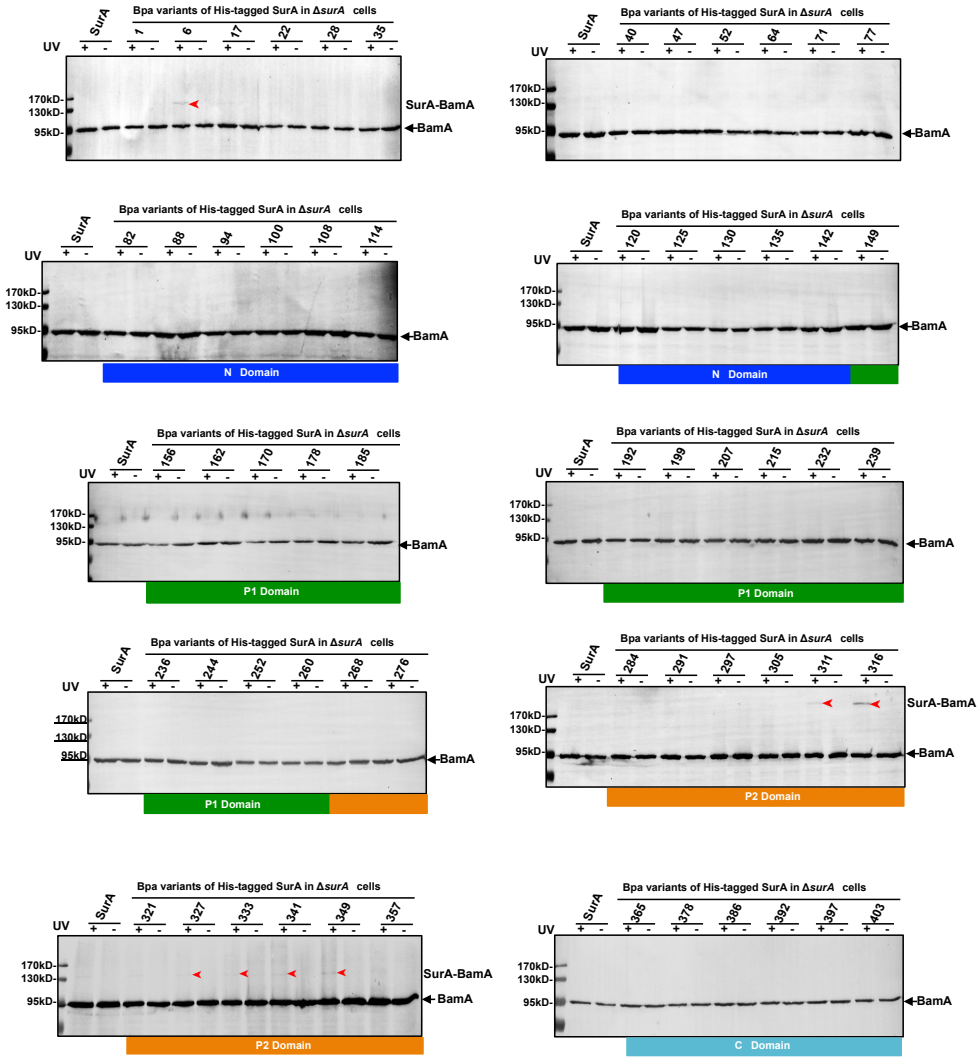
Supplemental Figure 2

Anti-LamB



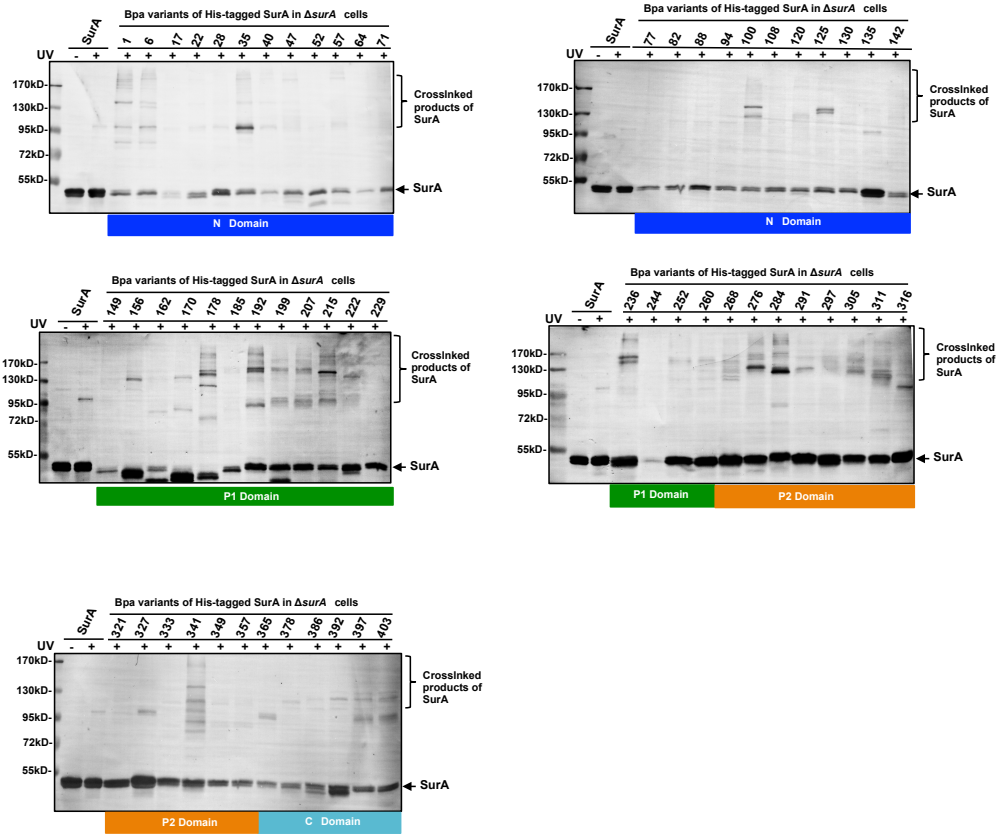
Supplemental Figure 3

Anti-BamA

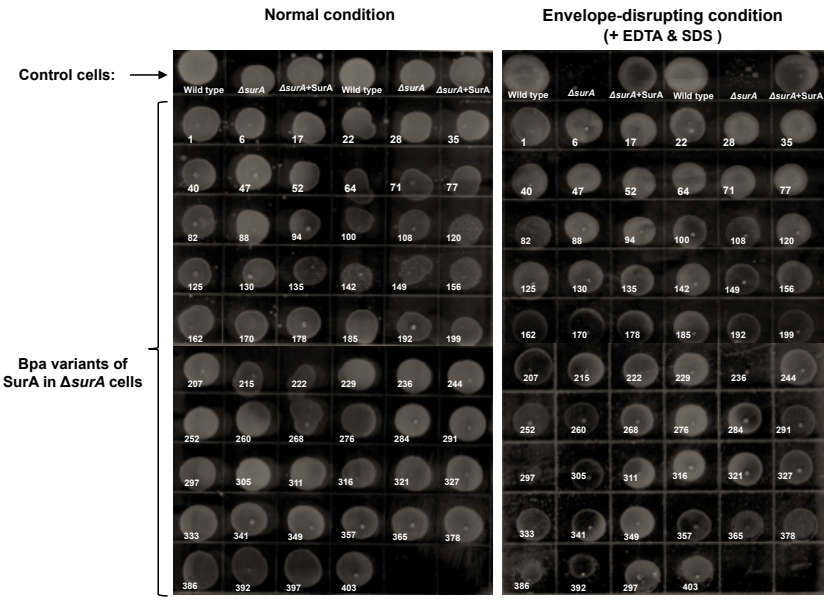


Supplemental Figure 4

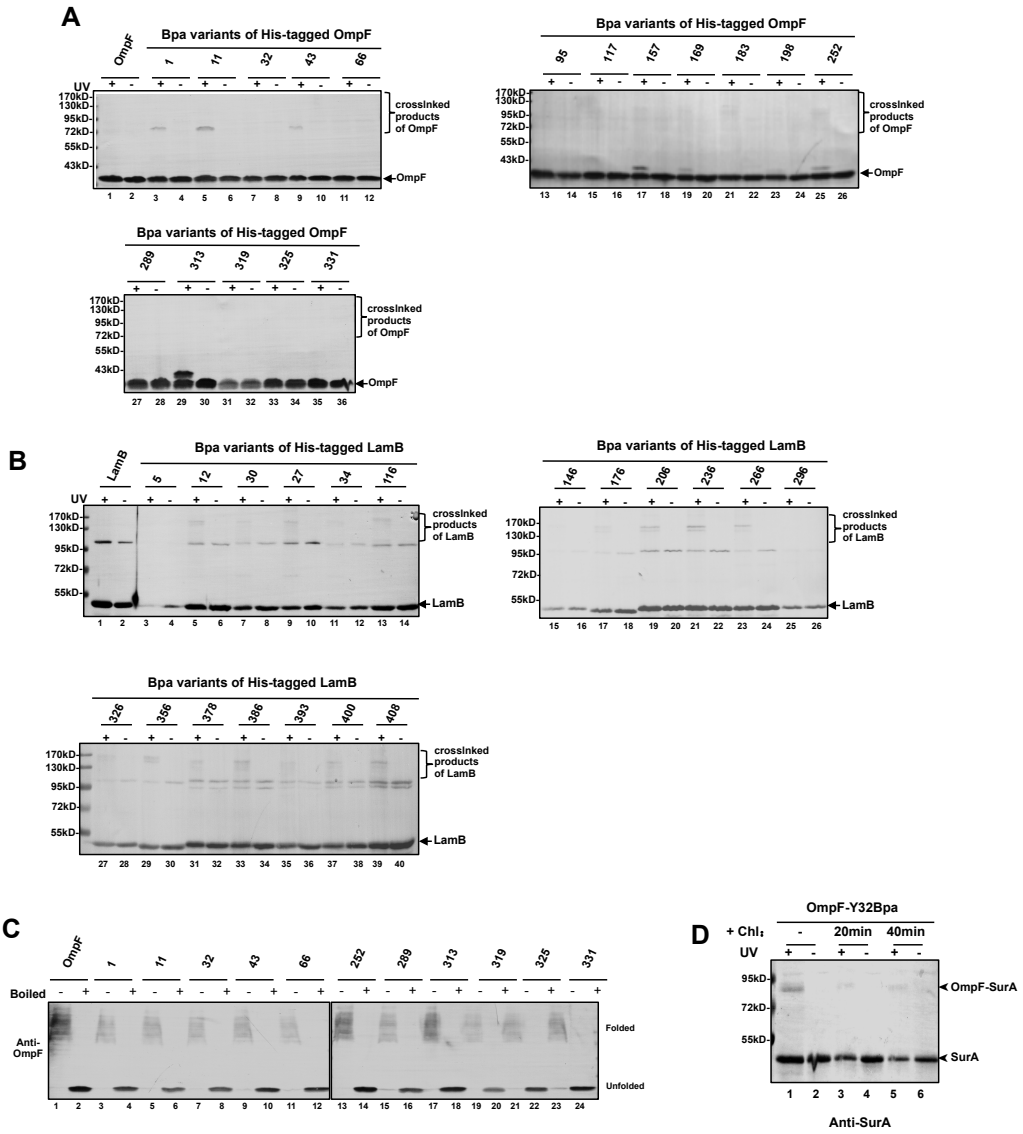
Anti-His



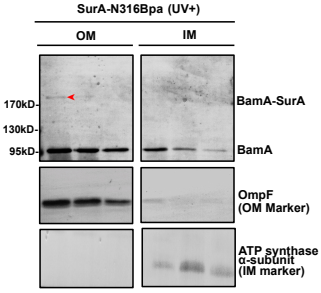
Supplemental Figure 5



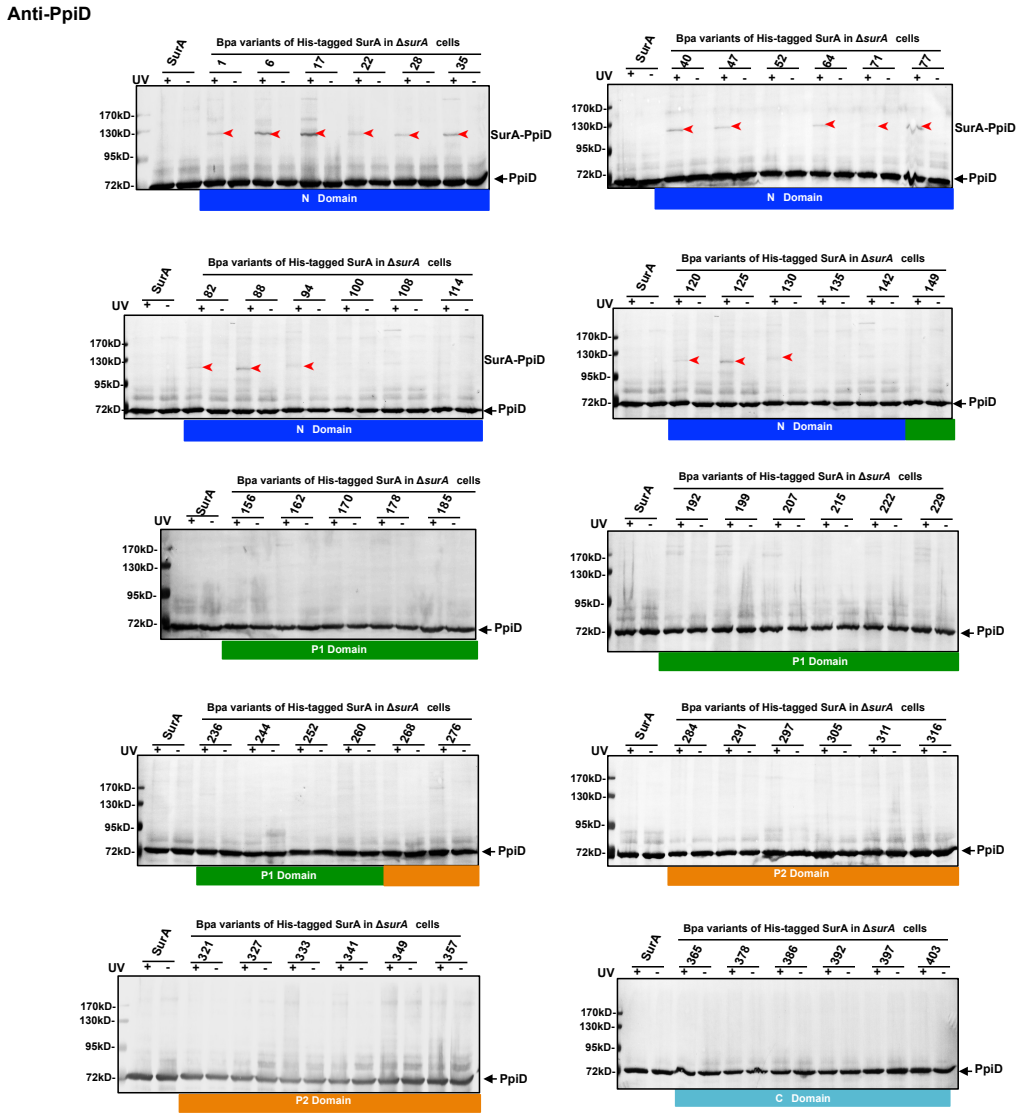
Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9