Supporting Information

Title: Characterization of interactions and phospholipid transfer between substrate binding proteins of the OmpC-Mla system

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Materials and Methods.

Growth conditions. Luria-Bertani (LB) broth and agar were prepared as previously described.[1] Unless otherwise noted, ampicillin (Amp) was used at a concentration of 200 μg/mL and chloroamphenicol (Cam) at 15 μg/mL. *para*-benzoyl-L-phenylalanine (*p*Bpa) (Alfa Aesar) was dissolved in 1 M NaOH and used at a concentration of 1 mM.

Bacterial strains and plasmid construction. All bacterial strains and plasmids used in this study are summarized in Table S1 and S2, respectively. Strains MC4100 [*F*- *araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 ptsF25 thi*][2] and BL21(λDE3) [*F*– *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)] (Novagen) were used for *in vivo* and protein overexpression experiments, respectively. For overexpression of MlaC, the plasmid pET22/42[3] was used to construct pET22/42*mlaC-His*. DNA fragment encoding full length MlaC was amplified by PCR using MC4100 genomic DNA template and primers listed in Table S3. The amplified DNA fragments were digested with XhoI and NdeI enzymes (New England Biolabs) and ligated into the same restriction sites of pET22/42. The plasmid pET23/42[4] was used to construct pET23/42*mlaC-His* and pET23/42*mlaD-His* with the same strategy. For in vivo UV-crosslinking experiments, amber mutants of MlaC and MlaD were constructed by site-directed mutagenesis using the primers listed in Table S3 and pET23/42*mlaC-His* and pET23/42*mlaD-His* as templates, respectively. For in vitro UV-crosslinking, MlaC$_{V171pBpa}$ and MlaC$_{V60pBpa}$ were constructed by using pET22/42*mlaC-His* as template. NovaBlue cells were transformed with the ligation products and selected on LB plates

containing respective antibiotics. All constructs were verified by DNA sequencing (Axil Scientific, Singapore).

In vivo UV-crosslinking experiments with whole cell lysates. The reported protocol[5] was modified for all in vivo UV-crosslinking experiments. For MlaC crosslinking, MC4100 Δ*mlaC::kan* background cells harbouring two plasmids, pSupBpaRS-6TRN and pET23/42*mlaC-His* with the amber stop codon (TAG) introduced at selected positions, were used. For validation purposes, the same two plasmids were introduced into Δ*mlaA::kan* and/or Δ*mlaD::kan* background cells. For MlaD crosslinking, MC4100, MC4100 Δ*mlaC::kan* and MC4100 Δ*mlaD::kan* background cells harbouring two plasmids, pSupBpaRS-6TRN and pET23/42*mlaD-His* with the amber stop codon (TAG) introduced at selected positions, were used. For each experiment, an overnight 3-mL culture was grown in LB with antibiotics from a single colony at 37 °C. Overnight cultures were diluted 1:100 into 5 mL of the same media containing 1 mM *p*Bpa and grown until OD_{600} ~0.6-0.7. The cultures were pelleted and resuspended in 2 mL ice cold TBS (20 mM Tris pH 8.0, 150 mM NaCl). Samples were either used directly or irradiated with UV light (analytikjena) at 365 nm for 15 min at room temperature. The samples were pelleted again and resuspended in 2X Laemmli buffer (normalized by their respective optical densities), boiled for 10 min, and centrifuged for five min at 13,000 x *g* in a microcentrifuge at room temperature; 15 µL of each sample were subjected to SDS-PAGE, using 4-12% Tris.HCl stacking gels[6], and immunoblot analyses.

In vivo UV-crosslinking and subsequent pull-down experiments and and mass spectrometry analyses. Affinity purification experiments were carried out in MC4100 Δ*mlaC::kan* and

Δ*mlaD::kan* background strains containing two plasmids, pSupBpaRS-6TRN and pET23/42*mlaC*$_{V171pBpa}$-*His*. A 1.5-L culture (inoculated from an overnight culture at 1:100 dilution), supplemented with antibiotics and 1mM *p*Bpa, was grown at 37 °C until OD_{600} of ~0.6. Cells were pelleted by centrifugation at 4, 700 x *g* for 20 min and subsequently resuspended in 40 mL TBS (20 mM Tris-HCl pH 8.0, 300 mM NaCl). The cells were either used directly or irradiated with UV light (analytikjena) at 365 nm for 30 min at room temperature in a petri dish. The samples were then supplemented with 1 mM PMSF (Calbiochem), 50 µg/mL DNase I (Sigma-Aldrich) and 100 µg/mL lysozyme (Calbiochem) before being passed twice through a high pressure French Press (French Press G-M, Glen Mills) homogenizer at 20,000 psi. Cell debris was removed by centrifugation at 4,700 x *g* for 10 min at 4 °C. Subsequently, the supernatant was subjected to ultra-centrifugation (Model Optima L-100K, Beckman Coulter) at 145,000 x *g* for 1 h at 4 °C to separate membrane and soluble fractions. The soluble fraction was incubated with 2 mL TALON cobalt resin (Clontech) and rocked for 30 min on ice. The membrane fraction was extracted with 5 ml TBS (20 mM Tris-HCl pH 8.0, 300 mM NaCl) containing 5 mM imidazole, 5 mM $MgCl_2$, 10% glycerol, 1% n-Dodecyl β-D-maltoside (DDM) and rocked for 1 h on ice before being subjected to ultra-centrifugation (Model Optima L-100K, Beckman Coulter) at 145,000 x *g* for 1 h at 4 °C. The supernatant from this membrane fraction was then incubated with 1 mL TALON cobalt resin (Clontech) and rocked for 30 min on ice. The resin mixtures were later loaded onto gravity columns. The filtrates were collected, and the columns were washed with either 8 x 10 mL TBS (20 mM Tris-HCl pH 8.0, 300 mM NaCl) containing 20 mM imidazole or 10 x 2 mL TBS (20 mM Tris-HCl pH 8.0, 300 mM NaCl) containing 20 mM imidazole, 5 mM $MgCl_2$, 10% glycerol and 0.05% DDM for the soluble and membrane fraction, respectively. Proteins from the soluble fraction were eluted from the

columns with 8 mL of 20 mM Tris.HCl pH 8.0, 150 mM NaCl, 200 mM imidazole while proteins from the membrane fraction were eluted from the columns with 2.5 mL of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 200 mM imidazole, 5 mM $MgCl_2$, 10% glycerol and 0.05% DDM. The eluates were concentrated in a 10 kDa cut-off ultra-filtration device (Amicon Ultra, Merck Millipore) by centrifugation at 4,000 x *g* to ~100 µL. The concentrated sample was mixed with equal amounts of 2X Laemmli buffer, boiled at 100 °C for 10 min, and subjected to SDS-PAGE, using 4-12% Tris.HCl stacking gels[6], immunoblot analyses and silver staining (Silver Quest, Invitrogen). The desired protein bands together with their respective controls were carefully excised with a surgical scalpel from a silver stained gel. Samples for tandem MS were kept in sterile 1.5-mL centrifuge tubes before submission for analyses at Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, USA.

OM permeability studies. SDS/EDTA sensitivity of cells expressing only MlaC/$D_{p\text{Bpa}}$ variants was evaluated by efficiency-of-plating (EOP) analyses on LB agar plates containing appropriate antibiotics and indicated concentrations of *p*Bpa and SDS/EDTA. 5-mL cultures (inoculated from an overnight culture at 1:100 dilution), were grown in LB broth at 37 °C until OD_{600} of ~0.6. The various cultures were normalized by optical density, first diluted to OD_{600} = 0.5 (~$0.5x10^8$ cells) and then serially diluted (5-fold) in LB broth using 96-well microtiter plates. 2 µL of the diluted cultures were manually spotted onto the plates, dried, and incubated overnight at 37 °C. Plate images were visualized by G:Box Chemi-XT4 (Genesys version 1.4.3.0, Syngene).

Overexpression and purification of MlaC-His, MlaC$_{V171pBpa}$-His, MlaC$_{V60pBpa}$-His. MlaC-His was over-expressed and purified from BL21(λDE3) harboring pET22/42*mlaC-His*. MlaC$_{V171pBpa}$-His or MlaC$_{V60pBpa}$-His was overexpressed and purified from BL21(λDE3) harboring pSupBpaRS-6TRN together with pET22/42*mlaC$_{V171pBpa}$-His* or pET22/42*mlaC$_{V60pBpa}$-His*, respectively. A 5-mL culture was grown from a single colony in LB broth supplemented with antibiotics at 37 °C until OD_{600} ~ 0.6. The cell culture was then used to inoculate into 500 mL LB broth (containing 1 mM *p*Bpa for amber mutants) and grown at the same temperature until OD_{600} ~0.6-0.7. At this time, for WT MlaC-His and MlaC$_{V171pBpa}$-His, 1 mM IPTG was added to the cultures and grown at 37 °C for 3 h. For MlaC$_{V60pBpa}$-His, 0.1 mM IPTG was added to the cultures and grown at 18 °C for overnight. Cells were pelleted by centrifugation at 4,700 x *g* for 20 min and then resuspended in 20 mL TBS pH 8 (20 mM Tris.HCl pH 8, 300 mM NaCl, 5 mM imidazole). Buffers were supplemented with 1 mM PMSF (Calbiochem), 50 µg/mL DNase I (Sigma-Aldrich) and 100 µg/mL lysozyme (Calbiochem). Resuspended cells were passed twice through a high-pressure French Press (French Press G-M, Glen Mills) homogenizer at 20,000 psi. Cell debris was removed by centrifugation at 4,700 x *g* for 10 min at 4 °C. Subsequently, supernatant was subjected to ultra-centrifugation (Model Optima L-100K, Beckman Coulter) at 145,000 x *g* for 1 h at 4 °C to separate membrane and soluble fractions. Soluble fraction was incubated with 2 mL TALON cobalt resin (Clontech) and rocked for 30 min on ice. The resin mixtures were later loaded onto gravity column. The filtrates were collected, and the columns were washed with 4 x 20 mL TBS pH 8.0 containing 20 mM imidazole. Proteins were eluted from columns with 8 mL of 20 mM Tris.HCl pH 8.0, 150 mM NaCl, 200 mM imidazole. The eluates were concentrated in a 10 kDa cut-off ultra-filtration device (Amicon Ultra, Merck Millipore) by centrifugation at 4,000 x *g* to ~500 µL. Proteins were further purified by SEC

system (AKTA, GE Healthcare) at 4 °C on a prepacked Superdex 200 increase 10/300 GL column, using 20 mM Tris.HCl pH 8.0, 150 mM NaCl as the eluents. sMlaD-His and dLolB-His were over-expressed and purified as previously described[3].

In vitro UV-crosslinking of MlaC$_{V171pBpa}$-His, MlaC$_{V60pBpa}$-His and sMlaD-His. Purified sMlaD-His (0.03 mM, 0.38 mg) was incubated for 30 min with either MlaC$_{V171pBpa}$-His (0.21 mM, 0.5 mg) or MlaC$_{V60pBpa}$-His (0.21 μM, 0.5 mg) in 100 μL of TBS (20 mM Tris-HCl pH 8.0, 150 mM NaCl) on ice. The mixtures were either used directly or irradiated with UV light for 20 min as described for the in vivo crosslinking experiments. 200 uL 2X Laemmli buffer were then added to the samples. Samples were heated for 10 min at 100 °C and centrifuged for 5 min at 13,000 x *g* in a microcentrifuge at room temperature; 15 μL of each sample were subjected to SDS-PAGE, using 4-12% Tris.HCl stacking gels[6], and Coomassie Blue staining (Sigma-Aldrich).

Purification of apo MlaC-His and apo sMlaD-His. 500-mL cell culture pellet was resuspended in 20 mL of lysis buffer (20 mM Tris.HCl pH 8, 300 mM NaCl, 5 mM imidazole, 8 M urea). Resuspended cells were rocked for 1.5 h at room temperature. Cell debris was removed by centrifugation at 4,700 x *g* for 10 min at 4 °C. Subsequently, the supernatant was subjected to ultra-centrifugation (Model Optima L-100K, Beckman Coulter) at 145,000 x *g* for 1 h at 4 °C to separate membrane and soluble fractions. The supernatant was incubated with 2 mL TALON cobalt resin (Clontech), by rocking for 2 h at room temperature. The resin mixture was later loaded onto a column allowed to drain by gravity. The column was washed with 2 x 20 mL of wash buffer 1 (20 mM Tris.HCl pH 8, 300 mM NaCl, 20 mM SDS, 8 M urea) and 2 x 20 mL of wash buffer 2 (20 mM Tris.HCl pH 8, 300 mM NaCl, 20 mM imidazole, 8 M urea) and eluted

with 8 mL of elution buffer (20 mM Tris.HCl pH 8, 150 mM NaCl, 200 mM imidazole, 8 M urea). The eluate was pipetted into Spectra/Por 3, 3.5 kDa MWCO, 18 mm flat width dialysis membranes and dialyzed against 20 mM Tris.HCl pH 8, 150 mM NaCl for 2 h and then overnight. Next morning, the protein solution was transferred to a 10 kDa cut-off ultra-filtration device (Amicon Ultra, Merck Millipore) and concentrated by centrifugation at 4,000 x *g* to ~500 µL and further purified by SEC system (AKTA, GE Healthcare) at 4 °C on a prepacked Superdex 200 increase 10/300 GL column using 20 mM Tris.HCl pH 8, 150 mM NaCl, as the running buffer.

Overexpression of MlaC with a cleavable His-tag for antibody generation. The vector pETMHis-MlaC expressing MlaC with a thrombin cleavable N-terminal His-tag was introduced into BL21(λDE3). Cell culture growth and affinity purification were performed according to the same protocol as MlaC-His. The eluate was dialyzed to remove imidazole in the elution buffer. Dialyzed solution was concentrated in a 10 kDa cut-off ultra-filtration device (Amicon Ultra, Merck Millipore) by centrifugation at 4,000 x *g* to ~250 µL. Tagless MlaC was prepared by cleaving the N-terminal His-tag with the addition of thrombin (1 U/0.3 mg His-MlaC) following Co-TALON affinity chromatography. After overnight digestion, protease activity was stopped by adding 2 mM protease inhibitor, PMSF. Liberated proteins were further purified by SEC system (AKTA, GE Healthcare) at 4 °C on a prepacked Superdex 200 10/300 GL column using 20 mM Tris.HCl pH 8, 150 mM NaCl, as the running buffer. Fractions containing pure proteins were pooled together and concentrated. The proteins were sent to Pacific Immunology for raising polyclonal antibodies in New Zealand rabbits.

Lipid extraction and thin layer chromatography. 1 mg/100 uL of purified proteins were used for the PL extraction according to the Bligh-Dyer method.[8] Purified protein solutions were mixed with 3.75 volumes of chloroform:methanol (1:2 vol/vol). The mixtures were vortexed and sonicated sequentially for 30 seconds for three times. The mixtures were centrifuged at 21,000 x *g* for 5 min, and the supernatant was recovered. 1.25 volumes of chloroform and 1.25 volumes of TBS were added to the supernatants. The mixtures were then centrifuged at 4000 x *g* for 5 min to separate organic and aqueous phases. The organic phase was gently removed to another vial, and the organic solvent was evaporated under N_2 gas. Remaining dried lipids were dissolved in 10 uL chloroform:methanol (4:1 vol/vol) and loaded onto a TLC Silica gel 60 F_{254} plate (Merck). The plate was developed by chloroform:methanol:water (65:25:4) solvent system, left to dry at room temperature and then stained with iodine vapor for 15 min.

In vitro PL transfer assay. 3 mg purified native or apo sMlaD-His was incubated with 4.07 mg apo or native MlaC-His in a 500 uL reaction mixture for 1 h on ice. This gives 1:6 sMlaD-His hexamers:MlaC-His molar ratio. Next, the mixture was separated by SEC (AKTA, GE Healthcare) at 4 °C on a prepacked Superdex 200 increase 10/300 GL column. The fractions belonging to the two proteins were collected and concentrated with 10 kDa and 50 kDa cut-off ultra-filtration device (Amicon Ultra, Merck Millipore) for MlaC-His (holo or apo) and sMlaD-His (holo or apo), respectively. Lipids were extracted from the respective proteins as described above.

^{31}P Nuclear magnetic resonance (NMR) analysis for extracted phospholipid species from MlaC-His. To find out the selectivity of MlaC-His for different PL head groups, we performed

^{31}P NMR analysis with Bruker AV500 (500 MHz) instrument as previously reported.[3] Commercial *E. coli* phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Avanti Polar Lipids) were used as standards. Lipids from MlaC-His (from 4 mg of purified protein) were extracted as described above. Organic solvents from samples were evaporated under N_2 gas and remaining dried lipids were resuspended in 1 mL water containing 5 % Triton X-100 (w/v) and 10 % D_2O (v/v), and subsequently sonicated for 1 h at room temperature. NMR analysis was performed at room temperature and acquisition times for all samples were 10 h (40,000 number of scans (NS)). Fourier transformation of FID files and integration of phosphate peak areas were done in MestReNova 9.0 software.

SDS-PAGE, immunoblotting and silver staining. All samples subjected to SDS-PAGE were mixed with equal amounts of 2X Laemmli reducing buffer. The samples were subsequently either kept at room temperature (- heat) or subjected to boiling at 100°C for 10 min (+ heat). Equal volumes of the samples were loaded onto the gels. Unless otherwise stated, SDS-PAGE was performed according to Laemmli using the 4-20% Tris.HCl gradient gels.[6] After SDS-PAGE, gels were visualized by either Coomassie Blue staining (Sigma-Aldrich), silver staining (Life Technologies) or subjecting to immunoblotting.

Immunoblotting was performed by transferring protein bands from the gels onto polyvinylidene fluoride (PVDF) membranes (Immun-Blot® 0.2 μm, Bio-Rad) using semi-dry electroblotting system (Trans-Blot® Turbo™ Transfer System, Bio-Rad). Membranes were blocked by 1X casein blocking buffer (Sigma-Aldrich). α-His antibody (pentahistidine) conjugated to the horseradish peroxidase (HRP) (Qiagen) was used at a dilution of 1:5,000. Mouse α-MlaA was used at a dilution of 1:3000. Sheep α-Mouse conjugated to HRP (GE

Healthcare) was used at a dilution of 1:5000. Rabbit α-MlaC was used at a dilution of 1:500. Donkey α-Rabbit conjugated to HRP (GE Healthcare) was used at a dilution of 1:5000. Luminata Forte Western HRP Substrate (Merck Milipore) was used to develop the membranes and chemiluminescence signals were visualized by G:Box Chemi-XX 6 (Genesys version 1.4.3.0, Syngene).

Visualization of channels in the sMlaD crystal structure. The CAVER PyMOL plugin v3.0[11] was used to detect and visualize possible cavities in static sMlaD crystal structure (PDB: 5UW2)[12]. Default settings were used.

Table S1. Bacteria strains used in this study.

Strains	Relevant genotypes or characteristics	Reference
MC4100	*F⁻ araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 ptsF25 thi*	Lab collection
Novablue	*endA1 hsdR17* (r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′ [$proA^{+}B^{+}lacI^{q}Z\Delta M15$::Tn*10*]	Novagen
BL21(λDE3)	F^{-} *ompT gal dcm lon hsdS*$_B$($r_B^{-}m_B^{-}$) *λ(DE3* [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) [$malB^{+}$]$_{K-12}$(λ^{S})	Novagen
CZS009	MC4100 Δ*mlaA::kan*	7
CZS011	MC4100 Δ*mlaC::kan*	This study
CZS012	MC4100 Δ*mlaD::kan*	3

Table S2. Plasmids used in this study.

Plasmids	Relevant genotypes or characteristics	References
pET23/42	pT7 inducible expression vector, contains multiple cloning site of pET42a(+) in pET23a(+) backbone; Amp^R	7
pET22/42	pT7*lac* inducible expression vector, contains multiple cloning site of pET42a(+) in pET22b(+) backbone; Amp^R	3
pSup-BpaRS-6TRN	Encodes an orthogonal tRNA and aminoacyl-tRNA synthetase permitting ribosomal incorporation of *p*Bpa at TAG stop codons	10
pET23/42*mlaC-His*	Encodes full length MlaC with C-terminal His_8 tag; Amp^R	This study
pET23/42*mlaD-His*	Encodes full length MlaD with C-terminal His_8 tag; Amp^R	This study
pET22/42*mlaC-His*	Encodes full length MlaC with C-terminal His_6 tag; Amp^R	This study
pET22/42*smlaD-His*	Encodes soluble domain of MlaD with C-terminal His_6 tag; Amp^R	3
pETM*His-mlaC*	Encodes MlaC without its signal sequences with N-terminal His_6 tag with a thrombin cleavage site; Amp^R.	This study

Table S3. Primers used in this study.

Primers	**Sequence (5' to 3')*/****
MlaC-C-XhoI	ATA CCT CGA GTT TTT TCT CTT CCA GAG
MlaC-N-NdeI	GAG CCA TAT GTT TAA ACG TTT AAT GAT GG
MlaC-T25*p*Bpa-N FWD	GCG GCA GAC CAG **TAG** AAT CCG TAT AAG
MlaC-T25*p*Bpa-C REV	CTT ATA CGG ATT **CTA** CTG GTC TGC CGC
MlaC-Y28*p*Bpa-N FWD	CAG ACC AAT CCG **TAG** AAG CTG ATG GAC
MlaC-Y28*p*Bpa-C REV	GTC CAT CAG CTT **CTA** CGG ATT GGT CTG
MlaC-F39*p*Bpa-N FWD	GCG CAG AAA ACG **TAG** GAT CGC CTG AAG
MlaC-F39 *p*Bpa-C REV	CTT CAG GCG ATC **CTA** CGT TTT CTG CGC
MlaC-R41*p*Bpa-N FWD	GCG CAG AAA ACG TTC GAT **TAG** CTG AAG AAT GAG C
MlaC-R41*p*Bpa-C REV	G CTC ATT CTT CAG **CTA** ATC GAA CGT TTT CTG CGC
MlaC-E45*p*Bpa-N FWD	CGC CTG AAG AAT **TAG** CAA CCG CAA A
MlaC-E45*p*Bpa-C REV	T TTG CGG TTG **CTA** ATT CTT CAG GCG
MlaC-Q46*p*Bpa-N FWD	CTG AAG AAT GAG **TAG** CCG CAA ATT CGG
MlaC-Q46*p*Bpa-C REV	CCG AAT TTG CGG **CTA** CTC ATT CTT CAG
MlaC-R50*p*Bpa-N FWD	CAA CCG CAA ATT **TAG** CGG AAC CCG G
MlaC-R50*p*Bpa-C REV	C CGG GTT CCG **CTA** AAT TTG CGG TTG
MlaC-Y55*p*Bpa-N FWD	GCC AAC CCG GAT **TAG** CTG CGT ACC ATT
MlaC-Y55*p*Bpa-C REV	AAT GGT ACG CAG **CTA** ATC CGG GTT GGC
MlaC-Q62*p*Bpa-N FWD	ACC ATT GTT GAT **TAG** GAA CTG CTG CCA
MlaC-Q62*p*Bpa-C REV	TGG CAG CAG TTC **CTA** ATC AAC AAT GGT
MlaC-Y67*p*Bpa-N FWD	GAA CTG CTG CCA **TAG** GTA CAG GTG AAA
MlaC-Y67*p*Bpa-C REV	TTT CAC CTG TAC **CTA** TGG CAG CAG TTC
MlaC-Y72*p*Bpa-N FWD	GTA CAG GTG AAA **TAG** GCC GGT GCG CTG
MlaC-Y72*p*Bpa-C REV	CAG CGC ACC GGC **CTA** TTT CAC CTG TAC
MlaC-L76*p*Bpa-N FWD	AAA TAC GCC GGT GCG **TAG** GTG CTG GGC CAG TAT
MlaC-L76*p*Bpa-C REV	ATA CTG GCC CAG CAC **CTA** CGC ACC GGC GTA TTT
MlaC-Y81*p*Bpa-N FWD	TG CTG GGC CAG **TAG** TAC AAG AGT GC
MlaC-Y81*p*Bpa-C REV	GC ACT CTT GTA **CTA** CTG GCC CAG CA
MlaC-F94*p*Bpa-N FWD	GT GAA GCC TAC **TAG** GCC GCT TTC CGT
MlaC-F94*p*Bpa-C REV	ACG GAA AGC GGC **CTA** GTA GGC TTC AC
MlaC-A95*p*Bpa-N FWD	GAA GCC TAC TTT **TAG** GCT TTC CGT GAG
MlaC-A95*p*Bpa-C REV	CTC ACG GAA AGC **CTA** AAA GTA GGC TTC
MlaC-Y100*p*Bpa-N FWD	GCT TTC CGT GAG **TAG** CTG AAG CAG GCT
MlaC-Y100*p*Bpa-C REV	AGC CTG CTT CAG **CTA** CTC ACG GAA AGC
MlaC-K102*p*Bpa-N FWD	CGT GAG TAC CTG **TAG** CAG GCT TAC GGT
MlaC-K102*p*Bpa-C REV	ACC GTA AGC CTG **CTA** CAG GTA CTC ACG
MlaC-M111*p*Bpa-N FWD	G GCG CTG GCG **TAG** TAT CAC GGT CAA
MlaC-M111*p*Bpa-C REV	TTG ACC GTG ATA **CTA** CGC CAG CGC C
MlaC-Y112*p*Bpa-N FWD	CG CTG GCG ATG **TAG** CAC GGT CAA A
MlaC-Y112*p*Bpa-C REV	T TTG ACC GTG **CTA** CAT CGC CAG CG
MlaC-H113*p*Bpa-N FWD	CTG GCG ATG TAT **TAG** GGT CAA ACC TAT
MlaC-H113*p*Bpa-C REV	ATA GGT TTG ACC **CTA** ATA CAT CGC CAG

MlaC-Q115*p*Bpa-N FWD	CG ATG TAT CAC GGT **TAG** ACC TAT CAG ATT GC
MlaC-Q115*p*Bpa-C REV	GC AAT CTG ATA GGT **CTA** ACC GTG ATA CAT CG
MlaC-Y117*p*Bpa-N FWD	CAC GGT CAA ACC **TAG** CAG ATT GCG CCA
MlaC-Y117*p*Bpa-C REV	TGG CGC AAT CTG **CTA** GGT TTG ACC GTG
MlaC-E122*p*Bpa-N FWD	G ATT GCG CCA **TAG** CAG CCG CTG G
MlaC-E122*p*Bpa-C REV	C CAG CGG CTG **CTA** TGG CGC AAT C
MlaC-L125*p*Bpa-N FWD	CCA GAA CAG CCG **TAG** GGC GAT AAA ACC
MlaC-L125*p*Bpa-C REV	GGT TTT ATC GCC **CTA** CGG CTG TTC TGG
MlaC-T136*p*Bpa-N FWD	CCT ATT CGC GTT **TAG** ATT ATT GAC CCG
MlaC-T136*p*Bpa-C REV	CGG GTC AAT AAT **CTA** AAC GCG AAT AGG
MlaC-I138*p*Bpa-N FWD	CGC GTT ACC ATT **TAG** GAC CCG AAT GGC
MlaC-I138*p*Bpa-C REV	GCC ATT CGG GTC **CTA** AAT GGT AAC GCG
MlaC-R147*p*Bpa-N FWD	CGT CCG CCG GTG **TAG** CTG GAC TTC CAG
MlaC-R147*p*Bpa-C REV	CTG GAA GTC CAG **CTA** CAC CGG CGG ACG
MlaC-D149*p*Bpa-N FWD	CCG GTG CGT CTG **TAG** TTC CAG TGG CG
MlaC-D149*p*Bpa-C REV	ACG CCA CTG GAA **CTA** CAG ACG CAC CGG
MlaC-N155*p*Bpa-N FWD	TTC CAG TGG CGT AAA **TAG** TCC CAG ACG GGC AAT
MlaC-N155*p*Bpa-C REV	ATT GCC CGT CTG GGA **CTA** TTT ACG CCA CTG GAA
MlaC-W161*p*Bpa-N FWD	CAG ACG GGC AAT **TAG** CAG GCT TAC GAC
MlaC-W161*p*Bpa-C REV	GTC GTA AGC CTG **CTA** ATT GCC CGT CTG
MlaC-Y164*p*Bpa-N FWD	AAT TGG CAG GCT **TAG** GAC ATG ATT GCT
MlaC-Y164*p*Bpa-C REV	AGC AAT CAT GTC **CTA** AGC CTG CCA ATT
MlaC-V171*p*Bpa-N FWD	ATT GCT GAA GGC **TAG** AGT ATG ATC ACC
MlaC-V171*p*Bpa-C REV	GGT GAT CAT ACT **CTA** GCC TTC AGC AAT
MlaC-Q178*p*Bpa-N FWD	ATC ACC ACC AAA **TAG** AAC GAG TGG GGA
MlaC-Q178*p*Bpa-C REV	TCC CCA CTC GTT **CTA** TTT GGT GGT GAT
MlaC-T183*p*Bpa-N FWD	AAC GAG TGG GGA **TAG** CTG CTG CGT ACC
MlaC-T183*p*Bpa-C REV	GGT ACG CAG CAG **CTA** TCC CCA CTC GTT
MlaC-L184*p*Bpa-N FWD	GAG TGG GGA ACG **TAG** CTG CGT ACC AAA
MlaC-L184*p*Bpa-C REV	TTT GGT ACG CAG **CTA** CGT TCC CCA CTC
MlaC-Q196*p*Bpa-N FWD	GGC CTG ACT GCG **TAG** CTG AAA TCG A
MlaC-Q196*p*Bpa-C REV	T CGA TTT CAG **CTA** CGC AGT CAG GCC
MlaC-K198*p*Bpa-N FWD	G ACT GCG CAA CTG **TAG** TCG ATT TCT C
MlaC-K198*p*Bpa-C REV	G AGA AAT CGA **CTA** CAG TTG CGC AGT C
MlaC-F97*p*Bpa-N FWD	TAC TTT GCC GCT **TAG** CGT GAG TAC CTG
MlaC-F97*p*Bpa-C REV	CAG GTA CTC ACG **CTA** AGC GGC AAA GTA
MlaC-P124*p*Bpa-N FWD	GCG CCA GAA CAG **TAG** CTG GGC GAT AAA
MlaC-P124*p*Bpa-C REV	TTT ATC GCC CAG **CTA** CTG TTC TGG CGC
MlaC-K128*p*Bpa-N FWD	CCG CTG GGC GAT **TAG** ACC ATT GTG CCT
MlaC-K128*p*Bpa-C REV	AGG CAC AAT GGT **CTA** ATC GCC CAG CGG
MlaC-T129*p*Bpa-N FWD	CTG GGC GAT AAA **TAG** ATT GTG CCT ATT
MlaC-T129*p*Bpa-C REV	AAT AGG CAC AAT **CTA** TTT ATC GCC CAG
MlaC-P132*p*Bpa-N FWD	AAA ACC ATT GTG **TAG** ATT CGC GTT ACC
MlaC-P132*p*Bpa-C REV	GGT AAC GCG AAT **CTA** CAC AAT GGT TTT
MlaC-N141*p*Bpa-N FWD	ATT ATT GAC CCG **TAG** GGC CGT CCG CCG

MlaC-N141*p*Bpa-C REV	CGG CGG ACG GCC **CTA** CGG GTC AAT AAT
MlaC-R147*p*Bpa-N FWD	CGT CCG CCG GTG **TAG** CTG GAC TTC CAG
MlaC-R147*p*Bpa-C REV	CTG GAA GTC CAG **CTA** CAC CGG CGG ACG
MlaC-D149*p*Bpa-N FWD	CCG GTG CGT CTG **TAG** TTC CAG TGG CG
MlaC-D149*p*Bpa-C REV	ACG CCA CTG GAA **CTA** CAG ACG CAC CGG
MlaC-Q151*p*Bpa-N FWD	CGT CTG GAC TTC **TAG** TGG CGT AAA AAC
MlaC-Q151*p*Bpa-C REV	GTT TTT ACG CCA **CTA** GAA GTC CAG ACG
MlaC-I167*p*Bpa-N FWD	GCT TAC GAC ATG **TAG** GCT GAA GGC GTC
MlaC-I167*p*Bpa-C REV	GAC GCC TTC AGC **CTA** CAT GTC GTA AGC
MlaC-E169*p*Bpa-N FWD	GAC ATG ATT GCT **TAG** GGC GTC AGT ATG
MlaC-E169*p*Bpa-C REV	CAT ACT GAC GCC **CTA** AGC AAT CAT GTC
MlaC-S172*p*Bpa-N FWD	GCT GAA GGC GTC **TAG** ATG ATC ACC ACC
MlaC-S172*p*Bpa-C REV	GGT GGT GAT CAT **CTA** GAC GCC TTC AGC
MlaC-M173*p*Bpa-N FWD	GAA GGC GTC AGT **TAG** ATC ACC ACC AAA
MlaC-M173*p*Bpa-C REV	TTT GGT GGT GAT **CTA** ACT GAC GCC TTC
MlaC-I174*p*Bpa-N FWD	GGC GTC AGT ATG **TAG** ACC ACC AAA CAA
MlaC-I174*p*Bpa-C REV	TTG TTT GGT GGT **CTA** CAT ACT GAC GCC
MlaC-T175*p*Bpa-N FWD	GC GTC AGT ATG ATC **TAG** ACC AAA CAA AAC G
MlaC-T175*p*Bpa-C REV	C GTT TTG TTT GGT **CTA** GAT CAT ACT GAC GC
MlaC-T176*p*Bpa-N FWD	AGT ATG ATC ACC **TAG** AAA CAA AAC GAG
MlaC-T176*p*Bpa-C REV	CTC GTT TTG TTT **CTA** GGT GAT CAT ACT
MlaC-E180*p*Bpa-N FWD	ACC AAA CAA AAC **TAG** TGG GGA ACG CTG
MlaC-E180*p*Bpa-C REV	CAG CGT TCC CCA **CTA** GTT TTG TTT GGT
MlaC-V60*p*Bpa-N FWD	CTG CGT ACC ATT **TAG** GAT CAG GAA CTG C
MlaC-V60*p*Bpa-C REV	G CAG TTC CTG ATC **CTA** AAT GGT ACG CAG
MlaD-60*p*Bpa-N FWD	TCT CCG GTC AGT **TAG** GGT GGC GTT GTT
MlaD-I60*p*Bpa-C REV	AAC AAC GCC ACC **CTA** ACT GAC CGG AGA
MlaD-V63*p*Bpa-N FWD	AGT ATT GGT GGC **TAG** GTT GTG GGT CGG
MlaD-V63*p*Bpa-C REV	CCG ACC CAC AAC **CTA** GCC ACC AAT ACT
MlaD-R67*p*Bpa-N FWD	GTT GTT GTG GGT **TAG** GTG GCG GAT ATT
MlaD-R67*p*Bpa-C REV	AAT ATC CGC CAC **CTA** ACC CAC AAC AAC
MlaD-I71*p*Bpa-N FWD	CGG GTG GCG GAT **TAG** ACG CTG GAC CCG AAA
MlaD-I71*p*Bpa-C REV	TTT CGG GTC CAG CGT **CTA** ATC CGC CAC CCG
MlaD-L73*p*Bpa-N FWD	GCG GAT ATT ACG **TAG** GAC CCG AAA ACC
MlaD-L73*p*Bpa-C REV	GGT TTT CGG GTC **CTA** CGT AAT ATC CGC
MlaD-K76*p*Bpa-N FWD	ATT ACG CTG GAC CCG **TAG** ACC TAT CTG CCG
MlaD-K76*p*Bpa-C REV	CGG CAG ATA GGT **CTA** CGG GTC CAG CGT AAT
MlaD-L79*p*Bpa-N FWD	CCG AAA ACC TAT **TAG** CCG CGC GTA ACG
MlaD-L79*p*Bpa-C REV	CGT TAC GCG CGG **CTA** ATA GGT TTT CGG
MlaD-H92*p*Bpa-N FWD	GAA CAA CGT TAT AAC **TAG** ATT CCA GAT ACC AGT
MlaD-H92*p*Bpa-C REV	ACT GGT ATC TGG AAT **CTA** GTT ATA ACG TTG TTC
MlaD-T96*p*Bpa-N FWD	CAC ATT CCA GAT **TAG** AGT TCG CTG AGC
MlaD-T96*p*Bpa-C REV	GCT CAG CGA ACT **CTA** ATC TGG AAT GTG
MlaD-S100*p*Bpa-N FWD	ACC AGT TCG CTG **TAG** ATT CGT ACT TCC
MlaD-S100*p*Bpa-C REV	GGA AGT ACG AAT **CTA** CAG CGA ACT GGT

MlaD-L106*p*Bpa-N FWD	CGT ACT TCC GGC **TAG** CTG GGG GAA CAA
MlaD-L106*p*Bpa-C REV	TTG TTC CCC CAG **CTA** GCC GGA AGT ACG
MlaD-L107*p*Bpa-N FWD	ACT TCC GGC CTG **TAG** GGG GAA CAA TAT
MlaD-L107*p*Bpa-C REV	ATA TTG TTC CCC **CTA** CAG GCC GGA AGT
MlaD-N115*p*Bpa-N FWD	CAA TAT CTG GCA TTA **TAG** GTC GGT TTT GAA GAC
MlaD-N115*p*Bpa-C REV	GTC TTC AAA ACC GAC **CTA** TAA TGC CAG ATA TTG
MlaD-F118*p*Bpa-N FWD	TTA AAC GTC GGT **TAG** GAA GAC CCG GAA
MlaD-F118*p*Bpa-C REV	TTC CGG GTC TTC **CTA** ACC GAC GTT TAA
MlaD-Q135*p*Bpa-N FWD	GAT GGC GAT ACA ATT **TAG** GAC ACT AAG TCT GCG
MlaD-Q135*p*Bpa-C REV	CGC AGA CTT AGT GTC **CTA** AAT TGT ATC GCC ATC
MlaD-K138*p*Bpa-N FWD	ATT CAG GAC ACT **TAG** TCT GCG ATG GTG
MlaD-K138*p*Bpa-C REV	CAC CAT CGC AGA **CTA** AGT GTC CTG AAT
MlaD-M141*p*Bpa-N FWD	ACT AAG TCT GCG **TAG** GTG CTG GAA GAT
MlaD-M141*p*Bpa-C REV	ATC TTC CAG CAC **CTA** CGC AGA CTT AGT
MlaD-Q149*p*Bpa-N FWD	GAT CTC ATT GGT **TAG** TTC CTT TAC GGT
MlaD-Q149*p*Bpa-C REV	ACC GTA AAG GAA **CTA** ACC AAT GAG ATC
MlaD-F150*p*Bpa-N FWD	CTC ATT GGT CAG **TAG** CTT TAC GGT AGT
MlaD-F150*p*Bpa-C REV	ACT ACC GTA AAG **CTA** CTG ACC AAT GAG
MlaD-Y152*p*Bpa-N FWD	GGT CAG TTC CTT **TAG** GGT AGT AAA GGC
MlaD-Y152*p*Bpa-C REV	GCC TTT ACT ACC **CTA** AAG GAA CTG ACC
MlaD-S154*p*Bpa-N FWD	TTC CTT TAC GGT **TAG** AAA GGC GAT GAC
MlaD-S154*p*Bpa-C REV	GTC ATC GCC TTT **CTA** ACC GTA AAG GAA
MlaD-N161*p*Bpa-N FWD	GAT GAC AAT AAG **TAG** AGT GGC GAT GCG
MlaD-N161*p*Bpa-C REV	CGC ATC GCC ACT **CTA** CTT ATT GTC ATC
MlaD-T175*p*Bpa-N FWD	CCA GGT AAT AAT GAA **TAG** ACT GAA CCT GTG GGT
MlaD-T175*p*Bpa-C REV	ACC CAC AGG TTC AGT **CTA** TTC ATT ATT ACC TGG

* Relevant restriction sites are underlined.

**Relevant sites for mutagenesis are bolded.

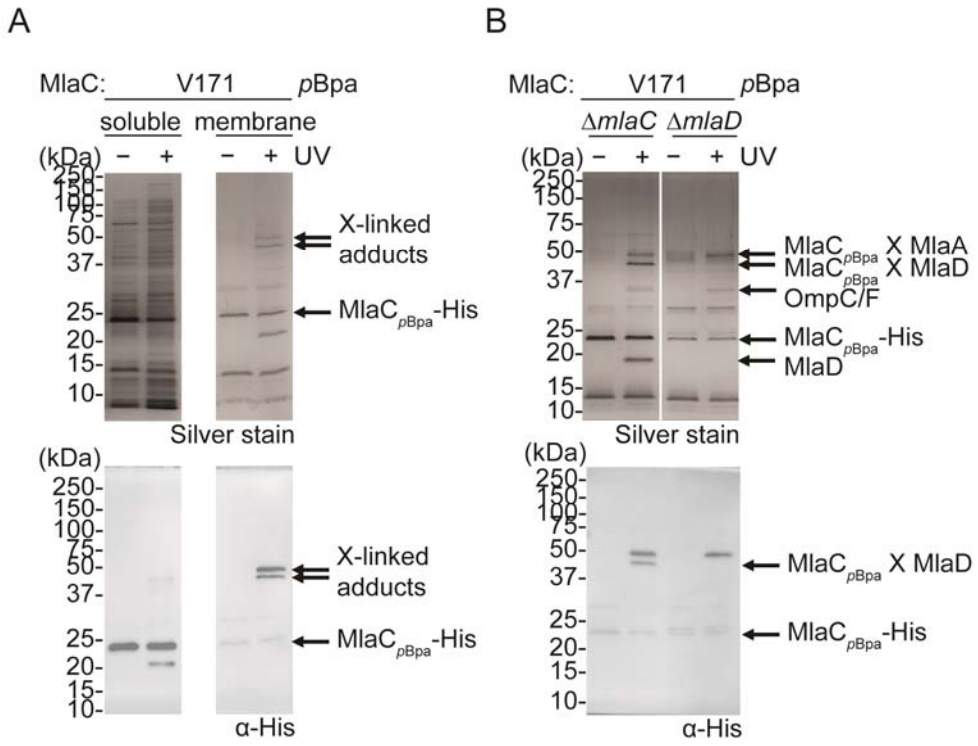

Figure S1. MlaC$_{V171pBpa}$-His interacts with membrane proteins, including MlaD, in vivo. (A) Co-TALON affinity purification of the crosslinked adducts of MlaC$_{V171pBpa}$-His in Δ*mlaC* cells from both the soluble and membrane fractions. (B) SDS-PAGE and immunoblot analyses showing the presence or absence of UV-crosslinked and monomeric MlaD bands formed in Δ*mlaC* or Δ*mlaD* cells after Co-TALON affinity purification of the membrane fractions. Samples were heated before SDS-PAGE analysis and visualized by silver staining and/or immunoblots using α-His antibodies.

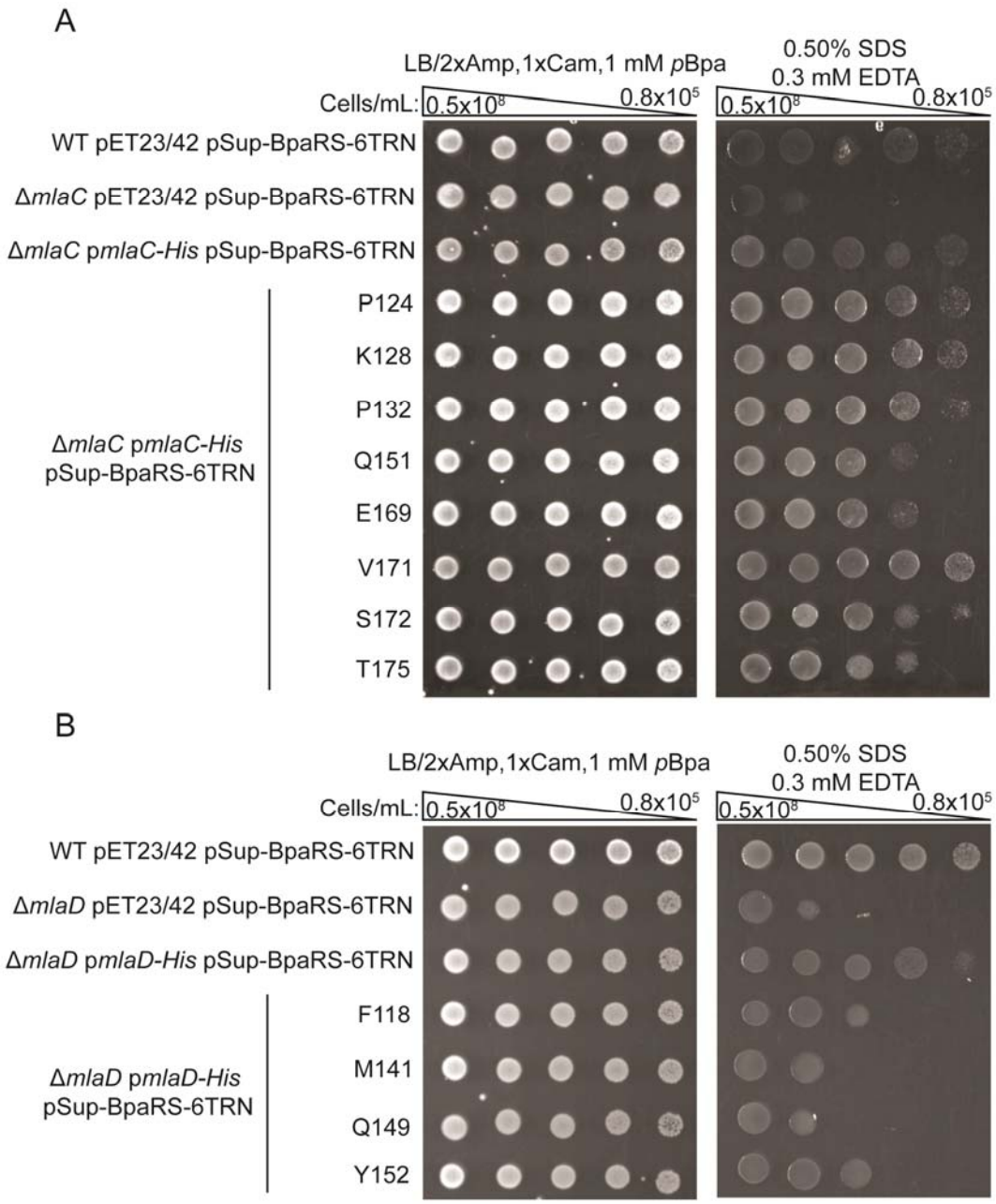

Figure S2. His-tagged MlaC$_{p\text{Bpa}}$ and MlaD$_{p\text{Bpa}}$ variants are able to partially complement the loss of chromosomally-encoded MlaC or MlaD, respectively. Analysis of SDS/EDTA sensitivity of (A) Δ*mlaC* or (B) Δ*mlaD* strains harboring pSup-BpaRS-6TRN[5] and producing the indicated His-tagged MlaC/D$_{p\text{Bpa}}$ variants at low levels from the pET23/42 vector. Serial dilutions of respective cultures were spotted on LB agar plates containing Amp, Cam, 1mM *p*Bpa,

supplemented with or without 0.5% SDS and 0.3 mM EDTA, as indicated, and incubated overnight at 37 °C. *mla* mutants are typically sensitive to 0.5% SDS only at high concentrations of EDTA (0.7-1.0 mM) but the presence of amber suppression in the strains here somehow made them sensitive even at 0.3 mM EDTA.

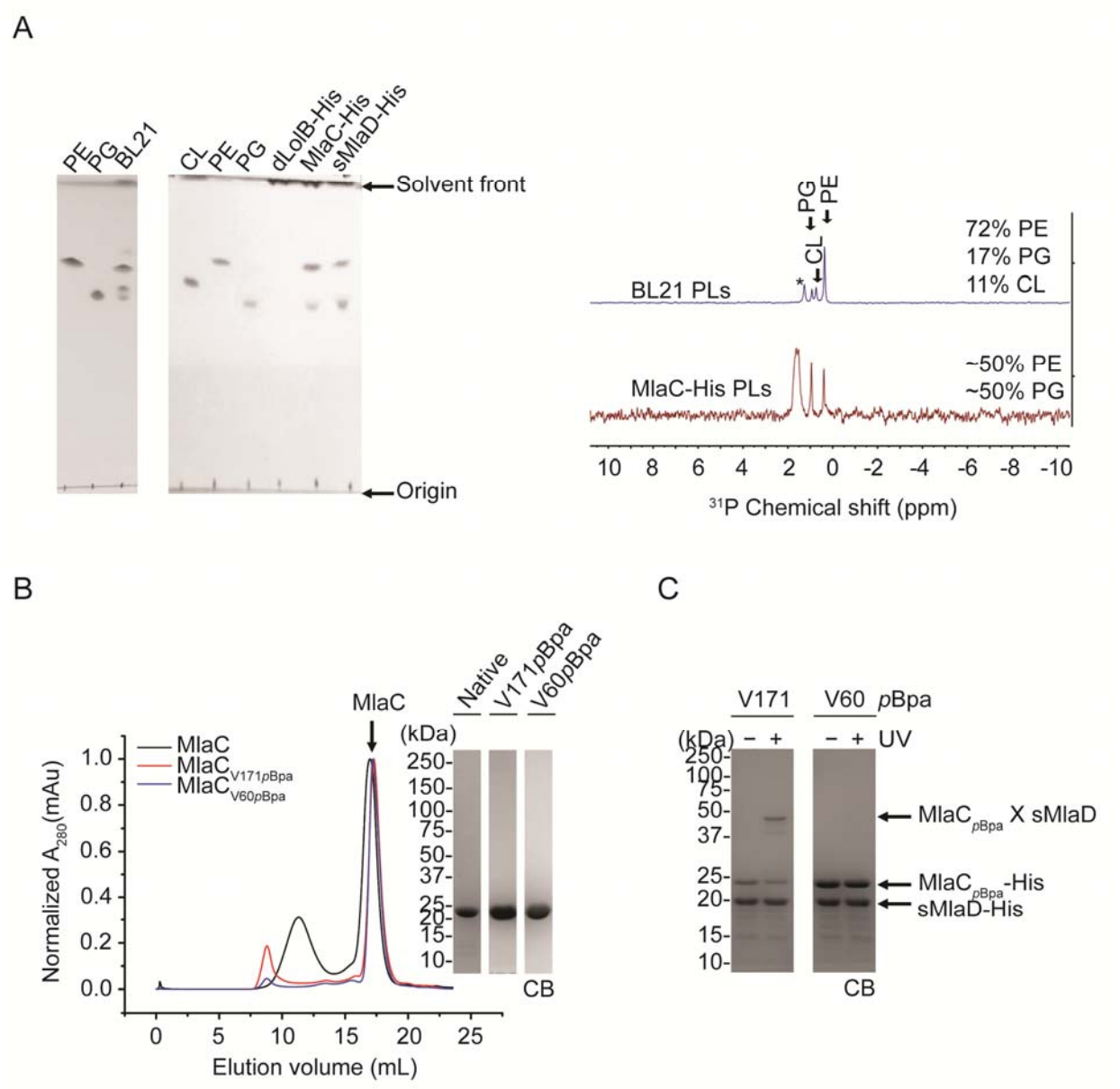

Figure S3. MlaC-His copurifies with PLs and interacts with the periplasmic soluble domain of MlaD in vitro. (A) TLC analysis of PLs extracted from BL21(λDE3) cells, purified soluble domain of LolB (dLolB-His), MlaC-His and sMlaD-His. dLolB-His was used as a negative control; LolB is the OM receptor for lipoproteins and has a hydrophobic cavity that does not bind PLs.[9] ^{31}P NMR analysis of PLs extracted from BL21(λDE3) cells and purified MlaC-His in 5% Triton X-100. Peaks are assigned based on comparison with known PL standards.[3] Unknown peaks that cannot be assigned to any PL species in *E. coli* are annotated with asterisks (*).

Compositions of bound PLs were obtained via integration of peak areas and normalized to the number of phosphorus atoms per PL molecule (i.e. one for PE/PG and two for CL). CL; cardiolipin, PE; phosphatidylethanolamine, PG; phosphatidylglycerol. (B) SEC profiles of WT MlaC-His, MlaC$_{V171pBpa}$-His and MlaC$_{V60pBpa}$-His purified from cells overexpressing each MlaC variant. Samples from the peak fraction were heated before SDS-PAGE analysis and visualized by Coomassie Blue (CB) staining. (C) CB-stained gel showing the presence or absence of UV-crosslinked adduct (MlaC$_{pBpa}$ X sMlaD) formed between purified sMlaD and MlaC$_{V171pBpa}$-His or MlaC$_{V60pBpa}$-His, respectively.

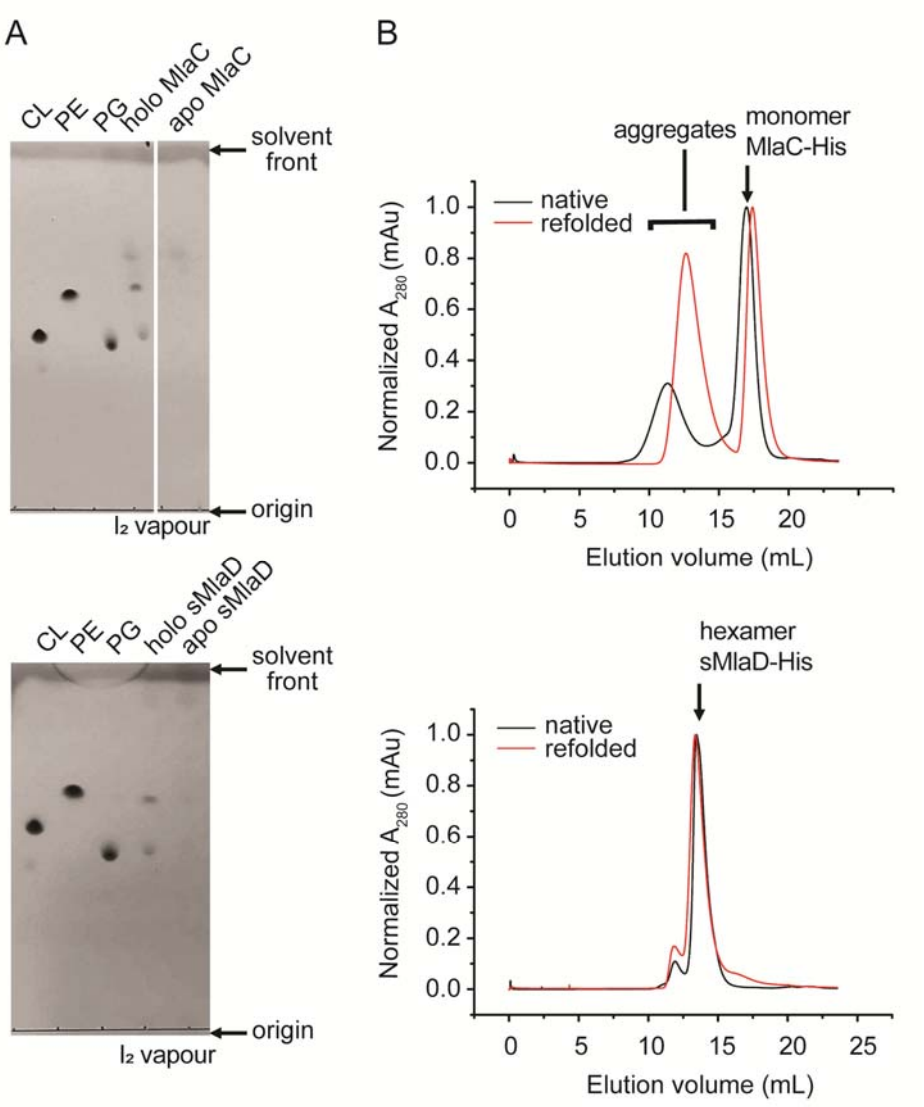

Figure S4. Refolded apo-MlaC and apo-sMlaD exhibit similar SEC profiles to their holo forms. (A) TLC analyses of the organic extracts from purified and refolded apo-MlaC-His and apo-sMlaD-His showing the absence of PLs, in contrast to their holo forms. (B) SEC profiles of both refolded apo-MlaC-His and apo-sMlaD-His compared to holo proteins. Some aggregation of apo-MlaC-His was observed.

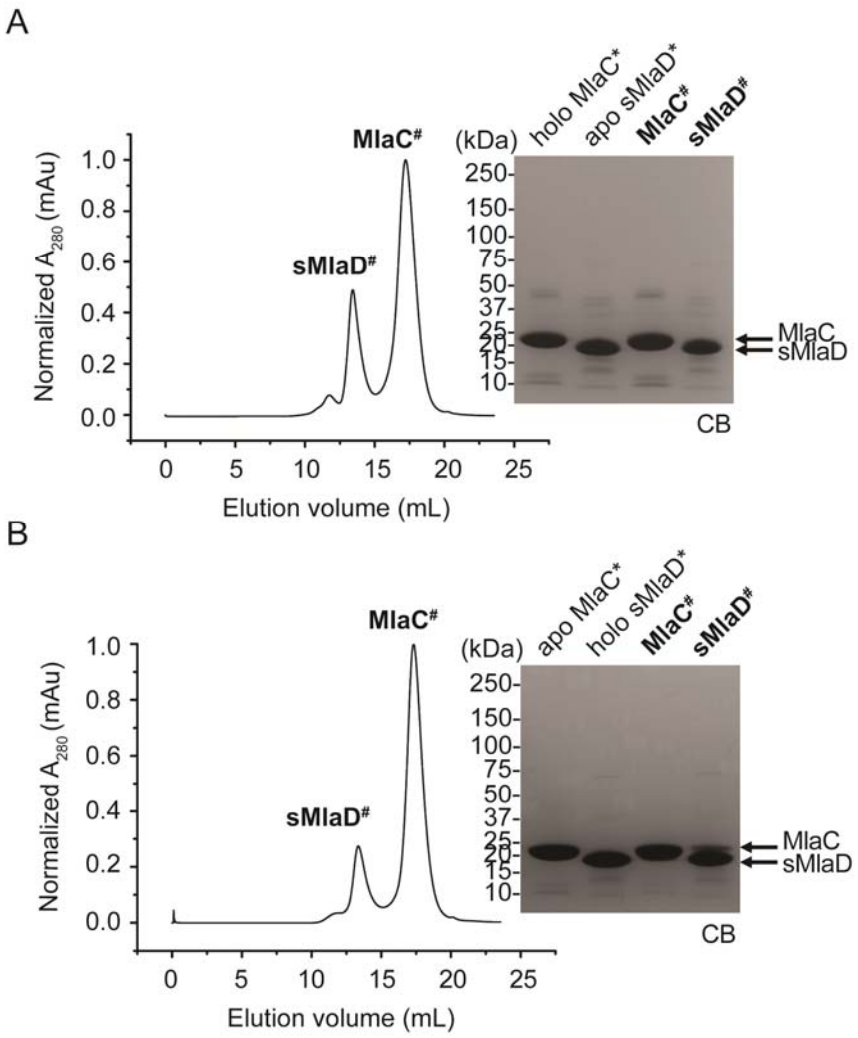

Figure S5. MlaC and sMlaD can be separated by SEC with minimal cross-contamination after incubation with each other. SEC profiles of sMlaD-His and MlaC-His separated after incubation between (A) holo-MlaC-His and apo-sMlaD-His and (B) holo-sMlaD-His and apo-MlaC-His. Samples from the respective peak fraction of the separated proteins prior to (marked by asterisks) and after incubation with each other (bolded/marked by hashes) were heated before SDS-PAGE and visualized by Coomassie Blue (CB) staining. PLs extracted from these proteins were analyzed by TLC and shown in Figure 3.

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