Supplementary Material & Methods

Bacterial Strains and Growth

All strains and plasmids used in this study are listed in Table S8. All *A. baumannii*, *E. coli*, and *S. aureus* strains were grown from freezer stocks initially on Luria-Bertani (LB) agar at 37° C. For selection, 100 μg/ml of ampicillin, 7.5 μg/ml of kanamycin, or 10 μg/ml of colistin were used when appropriate. Strains that harbored the pABBRKn or pMMB67Kn plasmid for complementation or overexpression were grown in 30 μg/ml of kanamycin.

Construction of mutant and complementation *A. baumannii* strains

Primers used in this study are listed in Table S9. All *A. baumannii* mutations were isolated as previously described (1). Briefly, *A. baumannii* ATCC 17978 pMMB67EH carrying the REC_{Ab} coding sequences (pAT04) were inoculated from an overnight culture at OD_{600}= 0.05 and grown for 45 m. Expression of REC_{Ab} (pAT03) was induced by addition of 2 mM IPTG and cells were grown at 37° C until they reached mid-exponential growth phase (OD_{600} = 0.4). Cells were washed in 10% glycerol and 10^{10} cells were electroporated in a 2 mM cuvette at 1.8 mV with 5 μg of a recombineering linear PCR product. For recovery, cells were grown for 4 h in 4 ml of LB broth with 2mM IPTG. Cells were collected via centrifugation and plated. PCR and Sanger sequencing verified all genetic mutations.

Removal of the pMMB67EH::REC_{Ab} Tet^R plasmid following isolation of mutants was performed as previously described (2). pMMBR67EH carrying the FLP recombinase was electroporated into cured mutants. Cells were recovered for 1h in 4 ml of LB and plated on agar containing 2 mM IPTG to induce expression of the FLP recombinase. PCR and Sanger sequencing confirmed excision of the kanamycin cassette. To complement *A. baumannii* mutants, the coding sequence from *ponA* encoding PBP1A (ATCC 17978), *lpxC* encoding LpxC (ATCC 19606), and *lpxA* encoding LpxA (ATCC 17978) were cloned into the XhoI and KpnI sites in pABBRKn. These plasmids were transformed into the respective mutant for complementation. To overexpress *A. baumannii* lipoproteins, the coding sequence from *HMPREF0010_01944*, *HMPREF0010_01945*, and *HMPREF0010_02739*, were cloned into the KpnI and SalI sites in pMMB67EHKn. The IPTG-inducible constructs were transformed into wild type ATCC 19606 *A. baumannii* and grown in 0.5 mM IPTG to induce lipoprotein expression.

Isolation of LOS-deficient *A. baumannii* and determination of mutation frequency

To isolate LOS-deficient colonies, an OD_{600} of 1.0 (~10^9 CFU) of *A. baumannii* was plated on LB agar containing 10 μg/ml of colistin. Isolated colonies were picked and replica plated on LB vancomycin (10 μg/ml) and LB colistin (10 μg/ml). Colonies sensitive to vancomycin, but resistant to colistin were deemed LOS-deficient. We made this assertion after a pilot experiment where we examined ten isolates from three distinct *A. baumannii* strains. We stained each for the presence or absence of LOS using the ProQ Emerald 300 Lipopolysaccharide gel stain (Thermo Fisher Scientific). All colonies (100%) were defective in LOS biosynthesis so we designated these selective parameters as indicative of the absence of LOS.

To determine the frequency of lipid A inactivation (LOS-deficiency) in the various *A. baumannii* strains, OD_{600} of 1.0 (~10^9 CFU) of each strain was plated on LB agar containing 10 μg/ml of colistin in triplicate. Colonies were replica plated on LB vancomycin (10 μg/ml) and LB colistin (10 μg/ml). Variants sensitive to vancomycin, but resistant to colistin were defined as lacking lipid A (LOS-deficient). The mutation rate was calculated for three biological replicates and one representative set was reported.

Isolation of Lipid A, Phospholipids, and LOS staining

Isolation of *A. baumannii* lipid A for TLC analysis involved ^{32}P-radiolabelling of whole cells as previously described (2, 3). Lipid A extraction was carried out by mild-acid hydrolysis as previously described (4), while phospholipids were isolated using Bligh/Dyer extractions (5). Radiolabelled lipids were used for TLC, while unlabeled lipids were used for MS analysis.

For LOS staining whole cells were grown to OD_{600} of 1.0 (~10^9 CFU) and then 1 ml was collected washed and suspended in 1X loading buffer. The cells were treated with proteinase K followed by separation using SDS-PAGE. Staining was performed using the ProQ Emerald 300 Lipopolysaccharide gel stain (Thermo Fisher Scientific) as described by the manufacture to visualize LOS.

Mass Spectrometry

Lipid A was analyzed using a MALDI-TOF (ABI 4700 Proteomics Analyzer) mass spectrometer in the positive or negative mode as previously described (2, 3).

Phospholipids analysis was performed using normal phase LC on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 mm, 25 cm x 2.1 mm (Sigma-Aldrich, St. Louis, MO). Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v); mobile phase C

consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 ml/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: IS= -4500 V; CUR= 20 psi; GSI= 20 psi; DP= -55 V; and FP= -150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham, MA).

TLR-4 Signaling Assays

The HEK-Blue human TLR4 (hTLR-4), cell line was maintained according to the manufacturer specifications (InvivoGen). Bacterial strains were serial diluted for assays and used as previously described (6). Two biological replicates were done in triplicate and one representative set was reported.

Nucleic Acid Extraction

Total RNA was extracted using the Direct-Zol RNA MiniPrep Kit (Zymo Research) from *A. baumannii* grown to a final OD_{600} of 0.6. Isolated RNA was treated with DNA-free DNA removal kit (Thermo-Fisher Scientific) to eliminate genomic DNA contamination. DNase-depleted RNA was used for qRT-PCR and RNA-seq.

Chromosomal DNA was extracted using the Easy-DNA gDNA Purification kit (Thermo Fisher Scientific) from cultures grown to a final OD_{600} of 0.6. Isolated DNA sonicated to an average DNA fragment size of ~300bp with a spread of 50-800bp.

Quantitative RT-PCR

Relative-abundance quantitative PCR (qPCR) was performed as previously described (7). In brief, the Sybr Fast One-Step qRT-PCR kit (Kapa Biosystems) was used with 16S rDNA as the internal reference. For gene expression analysis, relative expression reverse-transcription quantitative PCR was performed with Applied Systems RNA-Ct one-step system. Relative expression levels were calculated using the ΔΔCt method (8), with normalization of gene targets to16S rDNA signals.

RNA-sequencing

RNA-sequencing was performed as previously described (9). Briefly, DNA-depleted RNA was processed for Illumina sequencing using the NEB Next Ultra Directional RNA Library Prep kit for Illumina as described by the manufacturer (NEB). Sequencing was performed using Illumina HiSeq. Sequencing data was aligned to the respective *A. baumannii* published genome annotations using CLC genomic workbench software (Qiagen) and RPKM expression values were determined. The weighted proportions fold change of expression values between samples was determined and a Baggerley's test on proportions was used to generate a false discovery rate adjusted P-value. We then used a cut-off of 3-fold weighted proportions absolute change with a false-discovery rate adjusted P-value of ≤ 0.05 to identify significantly differentially regulated genes between samples. Differentially regulated pathway analysis was performed using a hypergeometric test on annotations from the KEGG pathway database and with manual annotation of the putative lipoproteins. All significantly altered pathways had a cut-off of P-value ≤ 0.05. Venn diagrams were constructed using the website http://bioinformatics.psb.ugent.be/webtools/Venn/.

Genomic-sequencing

Genomic sequences were processed for Illumina sequencing using the NEB Next Ultra DNA Library Prep kit as described by the manufacturer (NEB). Sequencing was performed using Illumina HiSeq. Reads were aligned to the respective *A. baumannii* published genome annotations using CLC genomic workbench software (Qiagen) using 90% length fraction and 90% similarities parameters. Mapped reads were locally realigned and fixed ploidy detection identified low and high frequency variants. *A. baumannii* wild type and LOS-deficient variant tracks were compared to identify mutations. Mutations in the LOS-deficient strain not present in the parent strain were called if 85% of aligned reads contained the variant. Of note, our *Ab* AYE strain did not have any reads map to plasmid NC_010404, suggesting that it may have lost the plasmid.

Generation of PBP1A and NADH-specific polyclonal antibody

PBP1A and NADH dehydrogenase chain L-specific polyclonal antibody was generated (Thermo Fisher Scientific). Briefly, a seventeen amino acid peptide from PBP1A (RIEDAYGKVIYEAKPEY) and an eighteen amino acid peptide from NADH dehydrogenase chain L (KGGNSFTSSRQTGSLREY) predicted to be solvent-exposed was selected from the primary sequence of ATCC 17978 *A. baumannii* and used to generate each specific antibody from rabbits.

Whole cell labeling with Sulfo-NHS-LC-LC-Biotin

Labeling of intact whole cells or lysates with EZ-link Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific) was performed as previously described with slight modifications (5, 10). Briefly, *E. coli* and *A. baumannii* were grown to OD_{600} of 0.6 and intact whole cells or lysates were labeled with 0.2mM final concentration of Sulfo-NHS-LC-LC-Biotin in 1ml of phosphate buffered saline. After 90 s Tris HCl (pH 7.5) was added to a final concentration of 250 mM to quench the reaction. Whole cells were washed after labeling, resuspended in 1X loading buffer, boiled for 5 min, and proteins were separated on SDS-PAGE and transferred to nitrocellulose for Western blotting. For fractionations, cells were lysed either before or after labeling using a needle tip sonicator (Qsonica) by pulsing for 3 X 30 s at 60% power on ice. Unlysed cells were removed by centrifugation at 10,000 X g for 10 min. The soluble and membrane fractions were separated by ultracentrifugation at 160,000 X g for 1h. Isolated fractions were loaded at equal protein concentrations and total proteins were separated using SDS-PAGE. Proteins were then transferred to nitrocellulose and subjected to Western blotting.

Western blotting

Western blot analysis was carried out via gel transfer to nitrocellulose with a 0.45 μM pore size (Thermo Fisher Scientific). All blots were blocked in 5% milk for 2 h. Antibodies were diluted in 5% milk and were used at specific concentrations. Streptavidin-HRP (Thermo Scientific) was used at 1:7500 overnight. The primary antibodies α-PBP1A and α-NADH chain L were used at 1:2000 and 1:500, respectively followed by secondary an α rabbit antibody at 1:10,000 (GE). Amersham ECL Prime Western Blotting Detection Reagent (GE) was used to measure relative protein concentrations.

Determination of MICs

Minimal inhibitory concentrations (MICs) were determined using E-strips (Biomerieux). Briefly, *A. baumannii* strains were spread on plates before a sterile E-strip was added to the plate. Inoculated plates were incubated overnight at 37° and MICs were assigned as the concentration where bacterial growth was inhibited.

Peptidoglycan analysis

A. baumannii strains were grown to a final OD_{600} of 0.6. Cells were collected and suspended in 6 mL chilled PBS and lysed by drop wise addition to 6 mL boiling 8% SDS. PG was prepared from cell lysate as previously described (11). Briefly, muropeptides were released from PG by the muramidase Cellosyl (Hoechst, Frankfurt am Main, Germany), reduced by sodium borohydride, and separated on a 250 × 4.6 mm 3 μm Prontosil 120-3-

C18 AQ reversed phase column (Bischoff, Leonberg, Germany). The eluted muropeptides were detected by their absorbance at 205 nm.

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Figure S1: Characterization of lipid A and phospholipid content in wild type and LOS-deficient *A. baumannii*. (A) MALDI-TOF MS analysis of wild type (ATCC 19606 and ATCC 17978), LOS-deficient, and complemented *A. baumannii*. Major species are indicated at *m/z* 1729.12 and *m/z* 1911.29 (B) ^{32}P-radiolabelled phospholipids were isolated from ATCC 19606, ATCC 17978, 5075, AYE parent *A. baumannii* strains and their LOS-deficient progeny and separated based on hydrophobicity using thin layer chromatography. Phosphatidylethanolmine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and Lyso-phosphatidylethanolamine (Lyso-PE) are indicated.

Figure S2: TLR-4/MD-2 activation of multidrug resistant *A. baumannii* strains. Stimulation of human TLR-4/MD-2 following incubation of increasing concentrations (0, 10^2, 10^3, 10^4, 10^5, 10^6, CFU/ml) of bacterial cells with HEK blue cells expressing TLR-4, MD2, and CD14 is depicted. Detection of a secreted reporter indicates differential activation of TLR-4 in response to lipid A.

Figure S3: Antibiotic resistance in wild type and LOS-deficient ATCC 19606 *A. baumannii*. ATCC 19606 *A. baumannii* minimal inhibitory concentrations (MICs) to Polymyxin B, Ciprofloxacin, Tobramycin, and Tigecycline in wild type (top) and LOS-deficient (bottom) isolates.

Fig S4: Analysis of wild type and LOS-deficient phospholipids. (A) Normal phase MS of $[M-H]^-$ ions of PG isolated from wild type or LOS-deficient *A. baumannii* (strain ATCC 17978 and 19606). MS/MS was performed on major peaks and the acyl chains associated with each peak are indicated in red. (B) Normal phase MS of $[M-H]^-$ ions of CL isolated from wild type or LOS-deficient *A. baumannii* (strain ATCC 17978 or 19606).

Fig S5: Analysis of wild type and LOS-deficient phospholipids. (A) Normal phase MS of $[M-H]^-$ ions of PE isolated from wild type or LOS-deficient *A. baumannii* (strain ATCC 17978 or 19606). MS/MS was performed on major peaks and the acyl chains associated with each peak are indicated in red. (B) Normal phase MS of $[M-H]^-$ ions of Lyso-PE and PA from wild type or LOS-deficient *A. baumannii* (strain ATCC 17978 or 19606). MS/MS was performed on major Lyso-PE peaks and the acyl chain associated with each peak are indicated in red.

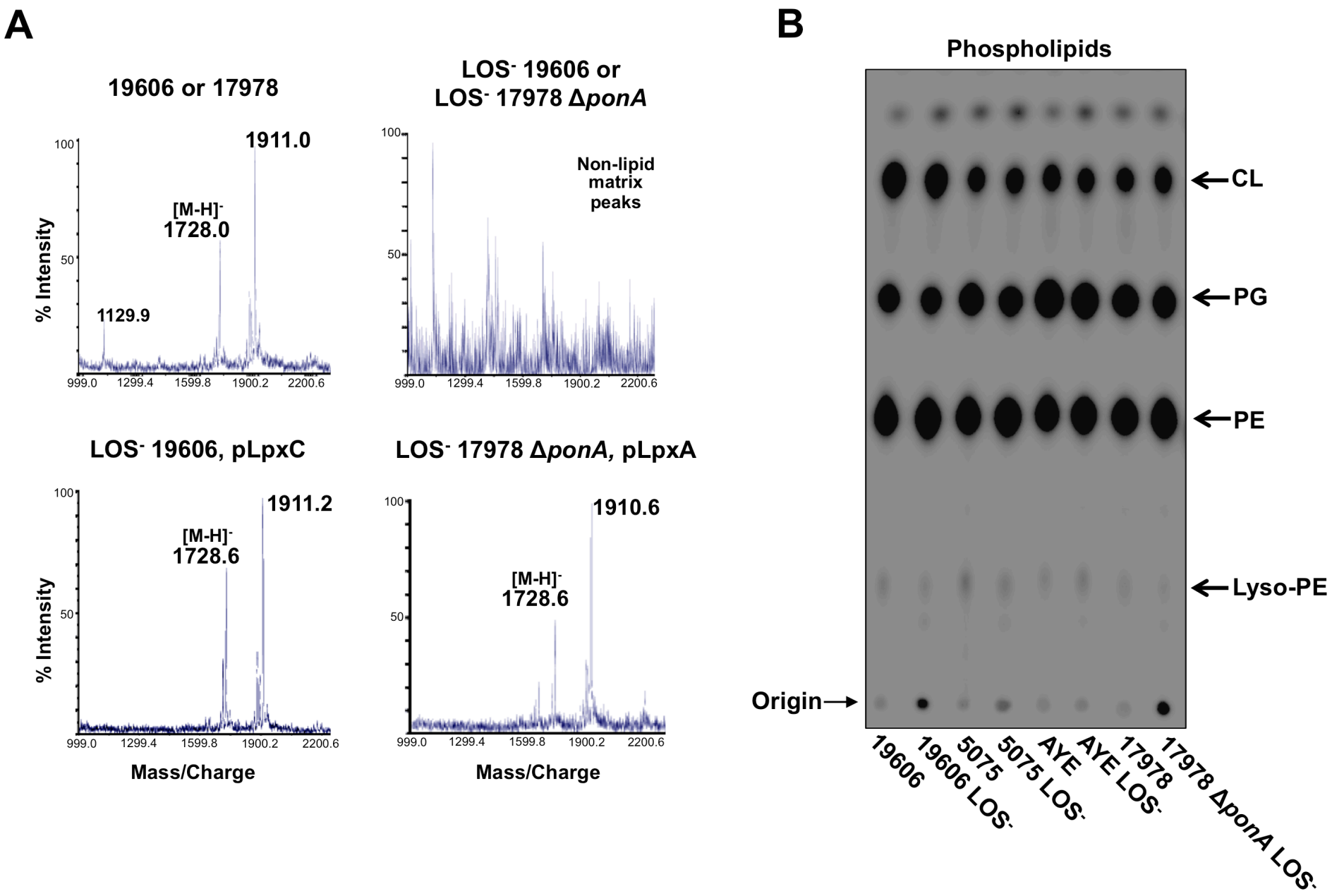
Figure S6: Characterization of the ATCC 17978 *A. baumannii* transposon (Tn) mutants. (A) ^{32}P-radiolabelled lipid A was isolated from *E. coli*, parent *A. baumannii* strains, and the ATCC 17978 *ponA::Tn* mutants that have inactivated LOS biosynthesis. Lipid A was separated based on hydrophobicity using thin layer chromatography. (B) Wild type ATCC 17978 *A. baumannii* and the *ponA::Tn* LOS-deficient progeny

minimal inhibitory concentrations (MICs) to colistin (polymyxin E) and vancomycin. (C) Stimulation of human TLR-4/MD-2 following incubation of increasing concentrations (0, 10^2, 10^3, 10^4, 10^5, 10^6, CFU/ml) of bacterial cells with HEK blue cells expressing TLR-4, MD2, and CD14 is depicted. Detection of a secreted reporter indicates differential activation of TLR-4/MD-2 in response to lipid A.

Figure S7: PBP1A is a penicillin binding protein with two highly conserved domains. Sequence alignments of the glycosyltransferase and transpeptidase domains of *A. baumannii* and other relevant strains illustrate that PBP1A (encoded by *ponA*) is highly conserved among gammaproteobacteria. The conserved residues are highlighted (yellow). Inactivation of the glycosyltransferase catalytic domain in *A. baumannii* PBP1A was accomplished by replacing the Glutamic acid (E) at position 92 with glutamine (Q), while inactivation of the transpeptidase domain involved replacement of the serine (S) at position 459 with alanine (A). The mutated residues are highlighted (red) in strains ATCC 17978 and ATCC 19606 *A. baumannii*.

Figure S8: A1S_3196 and A1S_3197 are one consecutive gene (*ponA*) that encodes PBP1A. The published PBP1A (encoded by *ponA*) annotation of ATCC 19606 and 17978 beginning at tryptophan (W) 490. A guanine (highlighted) insertion in the ATCC 17978 annotation causes a frameshift resulting in Serine (S) 495 to Valine (V) followed by a stop codon. This predicted guanine insertion truncates the predicted *ponA* coding sequence into A1S_3196 and A1S_3197. However, Sanger sequencing indicates that the guanine insertion in ATCC 17978 is an artifact. A full length PBP1A protein is produced in *A. baumannii* ATCC 17978 as indicated in the immunoblot using PBP1A polyclonal antiserum.

Figure S9: Characterization of ATCC 17978 *ponA* mutants. (A) Minimal inhibitory concentrations (MICs) of wild type and Δ*ponA* LOS-deficient ATCC 17978 *A. baumannii* to colistin (polymyxin E) and vancomycin. (B) ^{32}P-radiolabelled lipid A was isolated from parent ATCC 17978 *A. baumannii*, the Δ*ponA* mutant, and mutants complemented with either a native copy of PBP1A or with an inactivated glycosyltransferase domain (E92Q) or transpeptidase domain (S459A). Lipid A was separated based on hydrophobicity using thin layer chromatography. (C) Proteinase K treated whole-cell lysates were separated and stained with Pro-Q emerald 300 Lipopolysaccharide gel stain kit to visualize the LPS (*Salmonella*) or LOS (*E. coli* and *A. baumannii* strains).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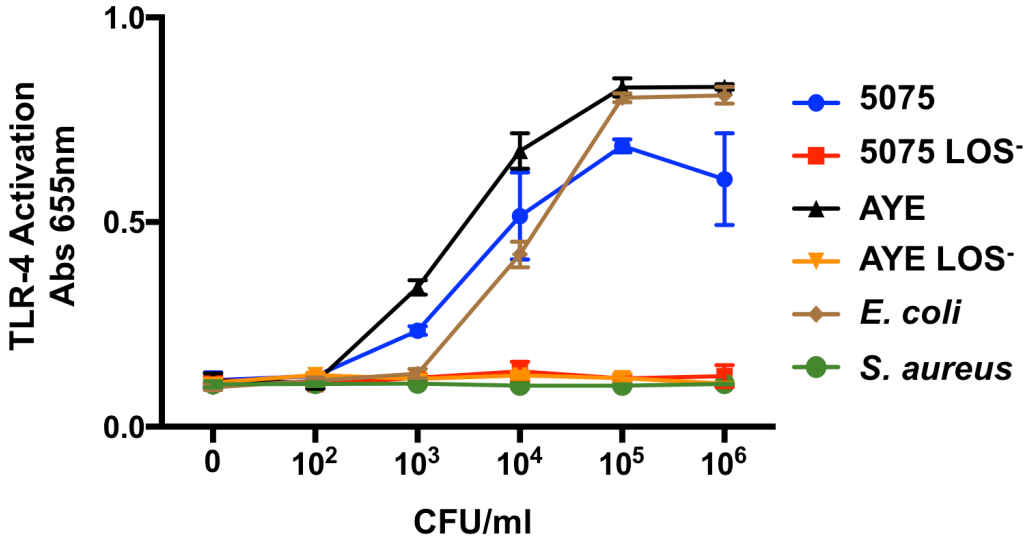
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AYE LOS-
E. coli
S. aureus

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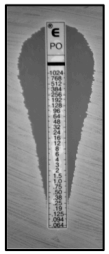
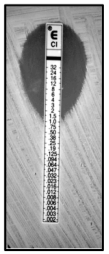
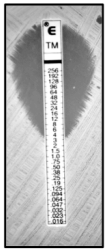
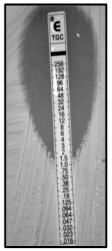
19606				
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19606 LOS$^-$				
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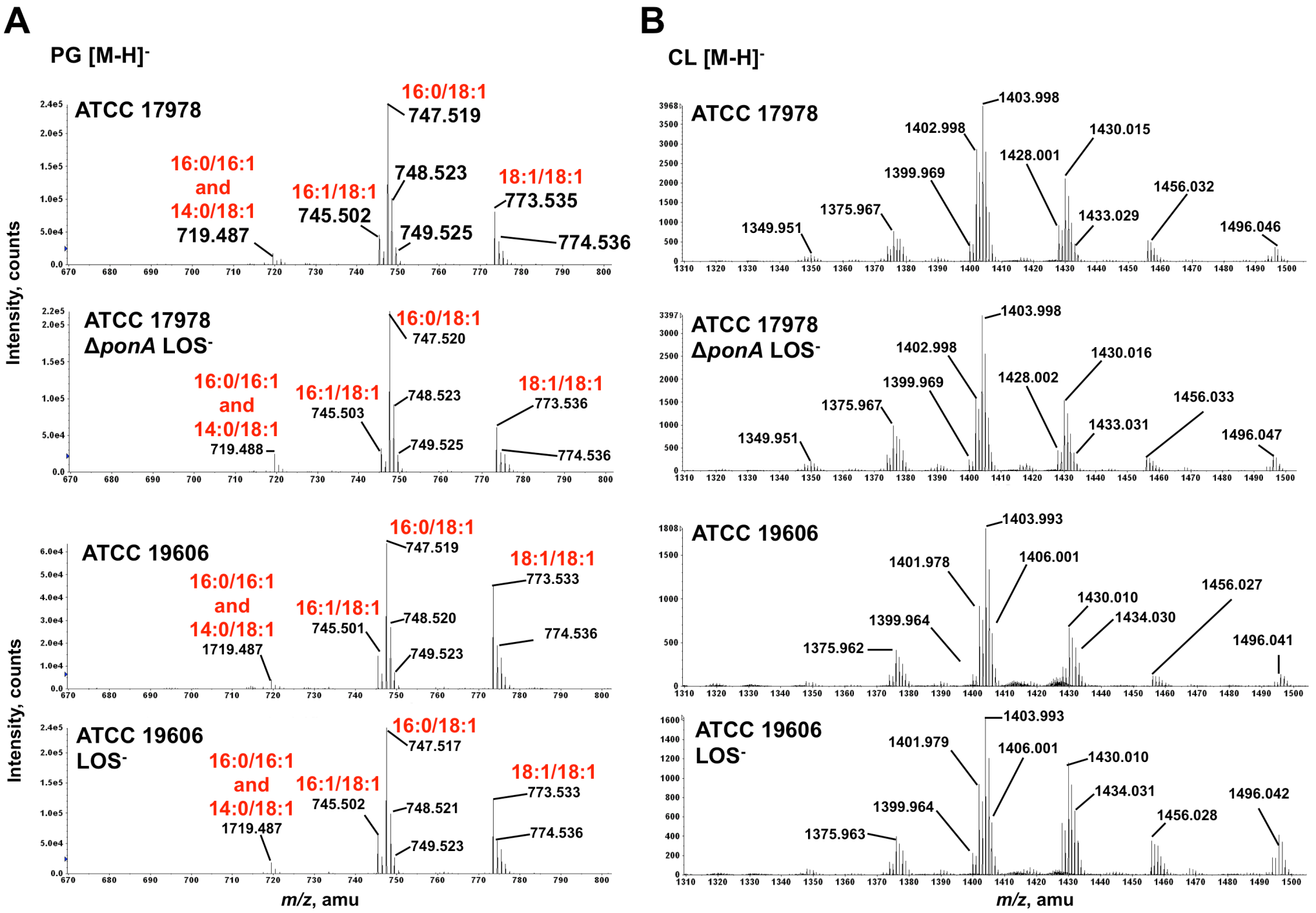
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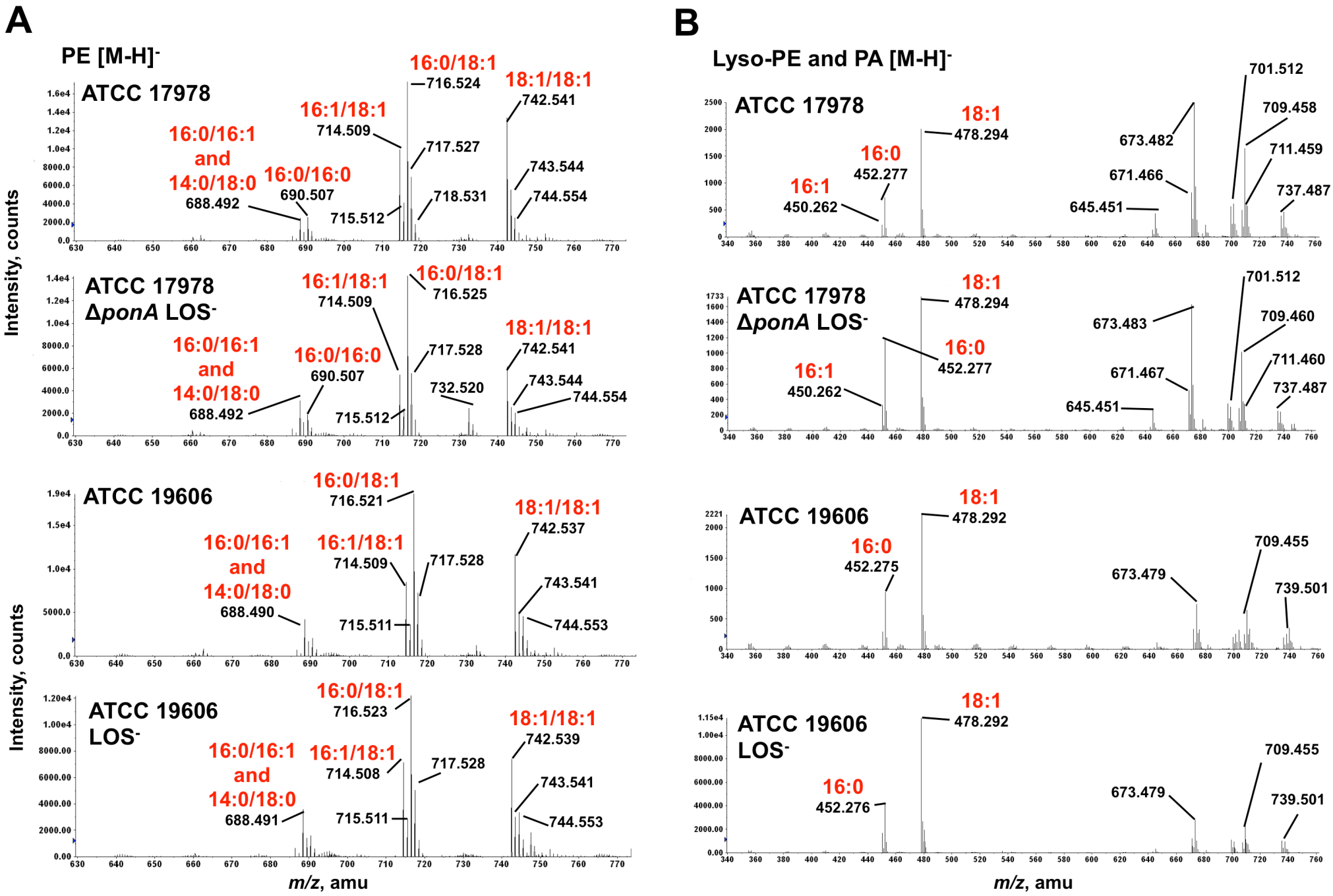
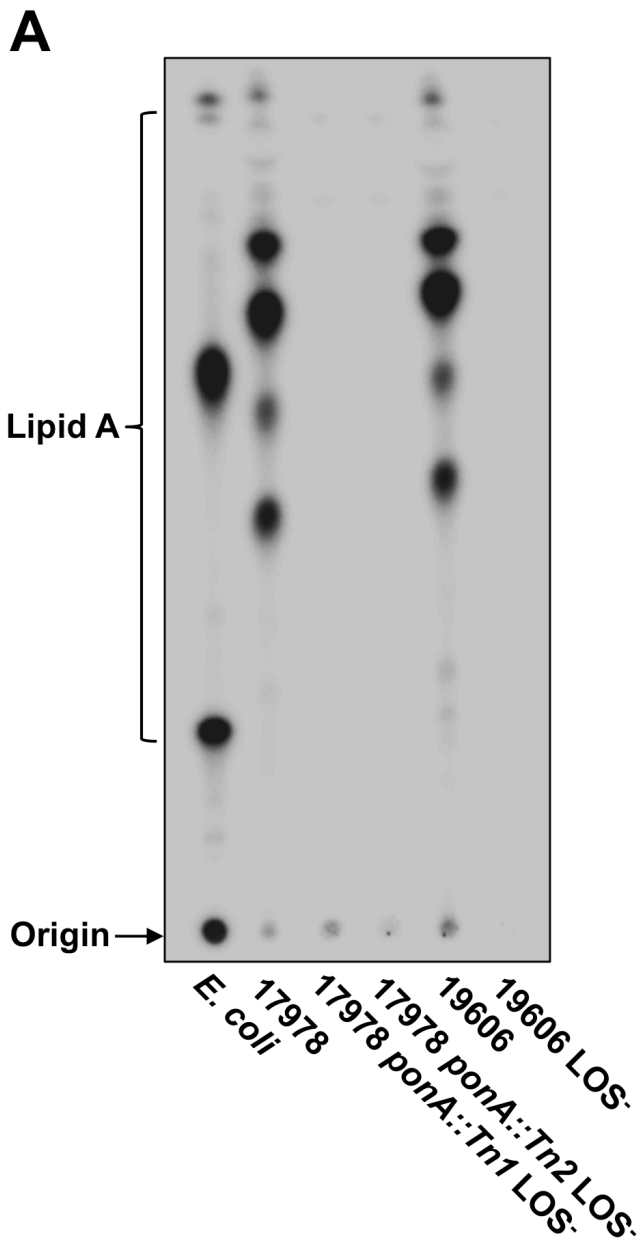
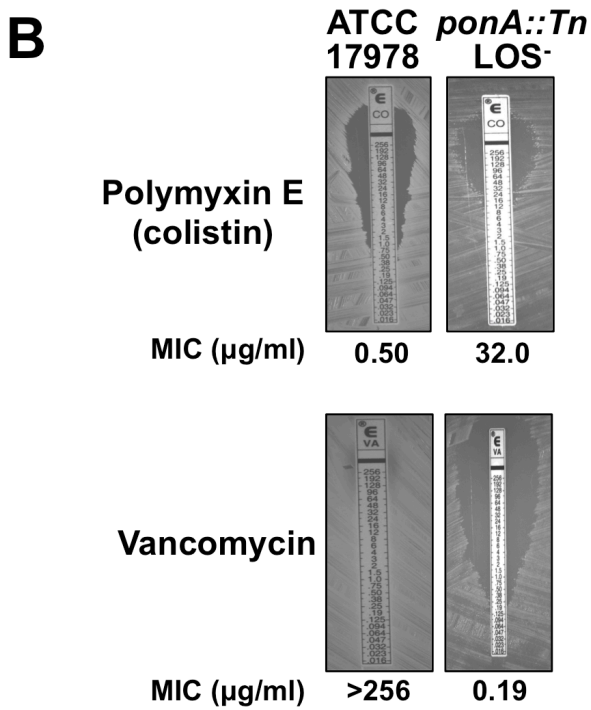
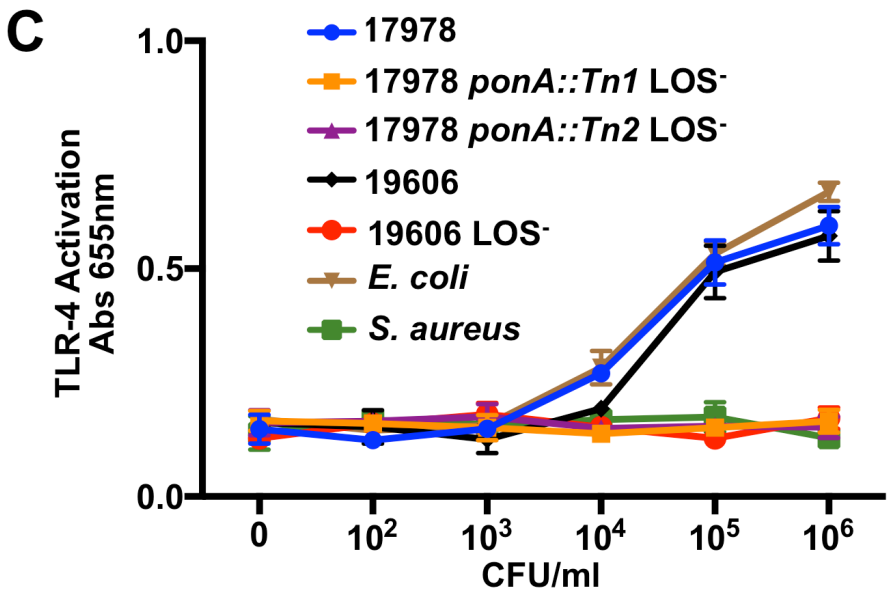
1375.963
m/z, amu


A
PE [M-H]-
ATCC 17978
16:0/18:1
716.524
18:1/18:1
742.541
16:1/18:1
714.509
16:0/16:1
and
14:0/18:0
688.492
16:0/16:0
690.507
717.527
718.531
715.512
743.544
744.554
ATCC 17978
ΔponA LOS-
16:1/18:1
714.509
16:0/18:1
716.525
18:1/18:1
742.541
16:0/16:1
and
14:0/18:0
688.492
16:0/16:0
690.507
717.528
732.520
715.512
743.544
744.554
ATCC 19606
16:0/18:1
716.521
18:1/18:1
742.537
16:0/16:1
and
14:0/18:0
688.490
16:1/18:1
714.509
717.528
715.511
743.541
744.553
ATCC 19606
LOS-
16:0/18:1
716.523
18:1/18:1
742.539
16:0/16:1
and
14:0/18:0
688.491
16:1/18:1
714.508
717.528
715.511
743.541
744.553
Intensity, counts
Intensity, counts
m/z, amu
B
Lyso-PE and PA [M-H]-
ATCC 17978
18:1
478.294
16:0
452.277
16:1
450.262
673.482
701.512
709.458
671.466
711.459
645.451
737.487
ATCC 17978
ΔponA LOS-
18:1
478.294
16:0
452.277
16:1
450.262
673.483
701.512
709.460
671.467
711.460
645.451
737.487
ATCC 19606
18:1
478.292
16:0
452.275
673.479
709.455
739.501
ATCC 19606
LOS-
18:1
478.292
16:0
452.276
673.479
709.455
739.501
m/z, amu

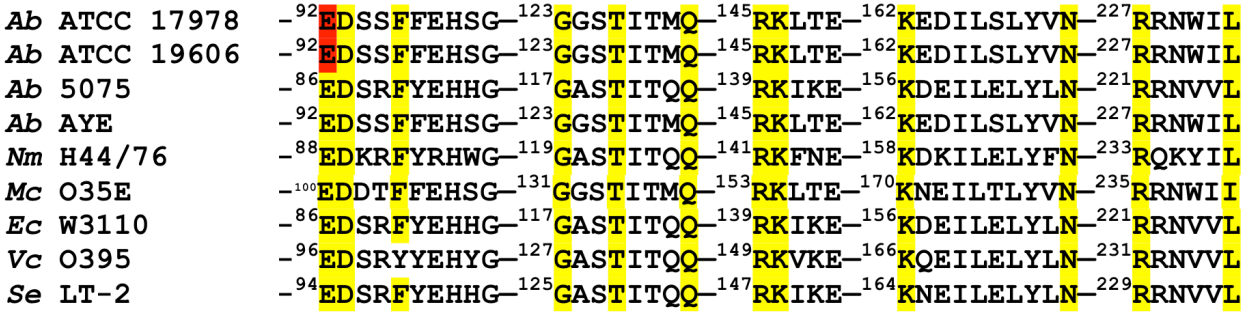

A
Lipid A
Origin
E. coli
17978
17978 ponA::Tn1 LOS-
17978 ponA::Tn2 LOS-
19606
19606 LOS-


B
ATCC 17978
ponA::Tn LOS-
Polymyxin E (colistin)
MIC (µg/ml)
0.50
32.0
Vancomycin
MIC (µg/ml)
>256
0.19

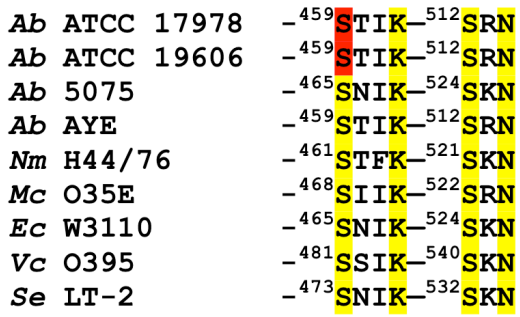

C
17978
17978 ponA::Tn1 LOS-
17978 ponA::Tn2 LOS-
19606
19606 LOS-
E. coli
S. aureus
TLR-4 Activation Abs 655nm
1.0
0.5
0.0
0
10^2
10^3
10^4
10^5
10^6
CFU/ml

PBP1A Domain alignments

PBP1A Glycosyltransferase Domain



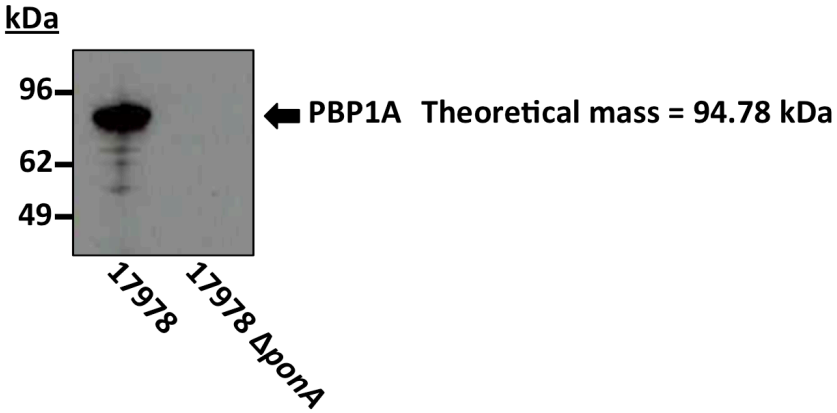
PBP1A Transpeptidase Domain



Published Annotations

ATCC 19606- TGG ACA CCG AAG AAC TCT GAT GGT CGC TAC CTA GGC ATG ATC CCG TTA CGC
W T P K N S D G R Y L G M I P L R

ATCC 17978- TGG ACA CCG AAG AAC GTC TGA TGG TCG CTA CCT AGG C ATG ATC CCG TTA CGC
W T P K N V * M I P L R



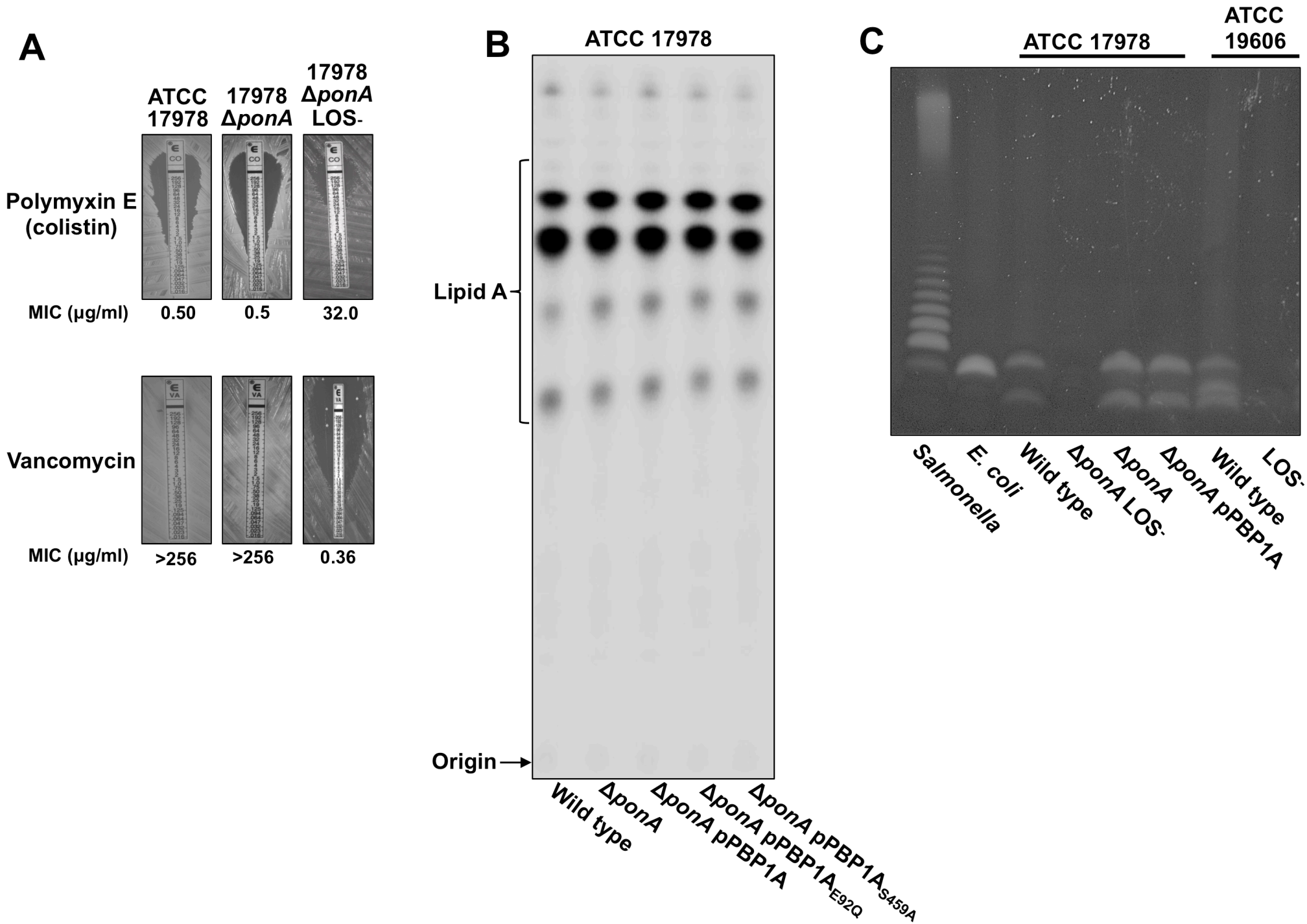

A
ATCC 17978
17978 ΔponA
17978 ΔponA LOS-
Polymyxin E (colistin)
MIC (µg/ml)
0.50
0.5
32.0
Vancomycin
MIC (µg/ml)
>256
>256
0.36
B
ATCC 17978
Lipid A
Origin
Wild type
ΔponA
ΔponA pPBP1A
ΔponA pPBP1A E92Q
ΔponA pPBP1A S459A
C
ATCC 17978
ATCC 19606
Salmonella
E. coli
Wild type
ΔponA LOS-
ΔponA
ΔponA pPBP1A
Wild type
LOS-

Table S1: Genomic Mutations in LOS-deficient *A. baumannii* strains

***Ab* strain**	**Lpx- gene mutation**	**Protein product**	**Mutation**	**Position**	**Protein Mutation**	**Confidence**	**Count**	**Coverage**	**Frequency**	**Other mutations**
ATCC 17978	*A1S_1965*	LpxA	Insertion (- to C)	2277630	V102 to Frameshift	0.99	850	874	97.25	None
ATCC 19606	*HMPREF0010_03502*	LpxC	SNP[a] (C to T)	Contig1.9 138087	Q58 to STOP	0.99	171	175	97.71	*HMPREF0010_00426* (Transcriptional Regulator) frameshift from deletion 7% into coding sequence
AYE	*ABAYE0154*	LpxC	Insertion	168192	Insertion to Frameshift	Insertion of ≈1.5 Kb fragment at base 395 of the *lpxC* coding sequence was confirmed by Sanger sequencing				1. *ABAYE0386* (*mlaE*) frameshift from deletion 10% into coding sequence. 2. *ABAYE3076* (TetR Transcriptional Regulator) SNP (T to G) at position 3111926 (N to K mutation).
5075	*ABUW_0152 (ABUW_RS00755)*	LpxC	SNP (C to T)	168546	Q58 to STOP	0.99	77	79	97.47	None

[a]single nucleotide polymorphism

Table S2: Global Peptidoglycan Composition of *A. baumannii* strains

		Relative % of Each Muropeptide[a]									
		ATCC 17978		ATCC 17978 Δ*ponA* LOS$^-$		ATCC 17978 Δ*ponA*		ATCC 17978 Δ*ponA* pPonA		ATCC 17978 Δ*ponA* pPonA$_{E92Q}$	
Peak number	**Name**	mean of peaks	variation[b]	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation
Peak 1	**Tri**	2.36	± 0.48	3.25	± 0.77	2.49	± 0.73	3.09	± 1.89	3.48	± 0.95
Peak 2	**TetraGly4**	2.54	± 0.38	1.58	± 1.95	2.53	± 0.16	2.77	± 0.37	2.20	± 3.01
Peak 3	**Tetra**	19.81	± 0.97	23.14	± 7.52	19.71	± 1.11	19.13	± 2.76	22.88	± 6.21
Peak 4	**TetraTri**	3.32	± 0.76	4.47	± 3.21	3.98	± 1.00	4.69	± 3.15	4.39	± 1.41
Peak 5	**TetraTetra**	37.89	± 1.19	37.82	± 4.72	37.74	± 0.71	36.78	± 0.77	37.87	± 0.26
Peak 6	**TetraTetraTri**	0.90	± 0.14	1.29	± 0.28	1.03	± 0.55	1.33	± 0.39	1.38	± 0.32
Peak 8	**TetraTetraTetra**	18.74	± 0.07	12.45	± 0.14	16.86	± 4.99	15.79	± 7.87	12.22	± 0.96
Peak 9	**TetraTri(Anh) (~70%)**	3.42	± 0.47	1.20	± 0.14	2.84	± 2.24	2.42	± 2.67	1.18	± 0.38
Peak 9	**TetraTetraTetraTri (~30%)**	1.46	± 0.21	0.52	± 0.06	1.22	± 0.95	1.04	± 1.15	0.51	± 0.16
Peak 10	**TetraTetraAnh I**	1.17	± 0.15	1.71	± 0.41	1.54	± 0.71	1.06	± 0.14	1.27	± 0.78
Peak 11	**TetraTetraAnhII**	0.67	± 0.21	1.67	± 0.64	0.91	± 0.95	0.81	± 0.04	1.29	± 0.33
Peak 11B	**TetraTetraTetraAnh**	2.00	± 0.23	1.92	± 1.76	2.30	± 0.39	1.91	± 0.35	2.06	± 0.97
Sum of known peaks		94.25	± 0.10	91.21	± 4.42	93.00	± 2.00	90.90	± 3.80	90.64	± 0.73
Monomers		26.21	± 0.14	30.68	± 3.64	26.57	± 2.43	27.59	± 6.76	31.47	± 2.18
Dimers		49.29	± 0.07	51.51	± 0.67	50.49	± 2.40	50.42	± 2.14	50.71	± 1.50
Trimers		22.95	± 0.00	17.25	± 3.00	21.65	± 3.83	20.86	± 7.70	17.26	± 0.50
Tetramers		1.56	± 0.21	0.57	± 0.03	1.30	± 1.00	1.13	± 1.21	0.56	± 0.18
% peptides in crosslinkage		73.79	± 0.14	69.32	± 3.64	73.44	± 2.43	72.41	± 6.76	68.53	± 2.18
Average chain length		30.47	± 2.64	34.30	± 4.70	29.40	± 1.41	38.29	± 18.70	39.92	± 10.83

		Relative % of Each Muropeptide[a]											
		ATCC 19606		ATCC 19606 LOS$^-$		5075		5075 LOS$^-$		AYE		AYE LOS$^-$	
Peak number	**Name**	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation	mean of peaks	variation
Peak 1	**Tri**	1.28	± 0.31	1.93	± 0.79	1.75	± 0.30	1.48	± 0.17	2.03	± 0.94	1.62	± 0.21
Peak 2	**TetraGly4**	1.49	± 0.60	1.47	± 2.07	2.23	± 0.48	3.98	± 4.61	2.86	± 0.98	0.79	± 0.08
Peak 3	**Tetra**	19.42	± 0.10	22.73	± 3.67	19.89	± 0.30	23.49	± 4	19.77	± 2.27	25.58	± 4.70
Peak 4	**TetraTri**	1.94	± 0.53	3.01	± 1.20	3.66	± 1.07	3.30	± 2.24	4.06	± 0.91	2.63	± 0.72
Peak 5	**TetraTetra**	43.73	± 1.82	38.81	± 0.23	40.43	± 1.94	37.19	± 2.85	39.28	± 2.04	40.98	± 3.03
Peak 6	**TetraTetraTri**	0.46	± 0.04	0.79	± 0.28	1.00	± 0.31	1.05	± 0.82	1.18	± 0.71	0.91	± 0.66
Peak 8	**TetraTetraTetra**	19.27	± 0.62	16.50	± 4.03	17.60	± 0.30	14.70	± 2.26	17.20	± 0.48	15.68	± 0.81
Peak 9	**TetraTri(Anh) (~70%)**	2.95	± 0.21	2.63	± 1.12	3.04	± 0.38	2.13	± 0.44	2.77	± 0.28	2.31	± 0.21
Peak 9	**TetraTetraTetraTri(~30%)**	1.26	± 0.09	1.13	± 0.48	1.30	± 0.16	0.91	± 0.19	1.19	± 0.12	0.99	± 0.09
Peak 10	**TetraTetraAnh I**	2.35	± 0.22	1.73	± 1.21	0.96	± 0.35	0.93	± 0.26	1.37	± 0.76	1.02	± 0.16
Peak 11	**TetraTetraAnhII**	0.88	± 0.16	1.10	± 1.31	0.98	± 0.91	0.79	± 0.27	0.81	± 0.04	1.04	± 1.08
Peak 11B	**TetraTetraTetraAnh**	3.17	± 0.32	1.65	± 0.39	1.19	± 0.45	1.32	± 0.85	1.67	± 1.33	0.77	± 0.05
Sum of known peaks		98.16	± 0.31	93.45	± 4.42	96.84	± 0.32	96.88	± 4.76	96.36	± 3.28	94.31	± 4.09
Monomers		22.60	± 1.09	27.98	± 2.19	25.39	± 0.34	31.61	± 7.51	26.19	± 0.43	29.64	± 3.39
Dimers		52.80	± 0.89	50.61	± 2.36	52.18	± 1.48	48.61	± 1.91	51.28	± 0.09	50.89	± 0.95
Trimers		23.33	± 0.29	20.22	± 4.11	21.04	± 0.98	18.77	± 5.53	21.28	± 0.51	18.43	± 2.31
Tetramers		1.29	± 0.09	1.20	± 0.46	1.39	± 0.17	1.00	± 0.26	1.27	± 0.17	1.05	± 0.14
% peptides in crosslinkage		77.41	± 1.09	72.03	± 2.19	74.61	± 0.34	68.39	± 7.51	73.82	± 0.43	70.36	± 3.39
Average chain length		24.16	± 1.43	30.78	± 5.35	34.74	± 0.71	42.48	± 4.24	33.40	± 7.31	41.47	± 9.25

[a]Percentages calculated as per (Glauner *et al*).

[b]variation of two independent experiments

Table S3: Strains and Plasmids used in this study

Strain or Plasmid	Genotype or Description	Reference
Strain		
E. coli W3110	Wild type, F- λ- *rph-1 IN(rrnD, rrnE)1*	*E. coli* Genetic Stock Center (Yale) (12)
E. coli MFDpir	MG1655 *RP4-2-Tc::[ΔMu1::aac(3)IV-ΔaphA-Δnic35-ΔMu2::zeo] ΔdapA::(erm-pir) ΔrecA*	(22)
Staphylococcus aureus strain Newman	Wild type	ATCC (13)
A. baumannii ATCC 17978	Wild type	ATCC (14)
A. baumannii ATCC 19606	Wild type	ATCC (15)
A. baumannii 5075	Wild type	(16)
A. baumannii AYE	Wild type	ATCC (17)
A. baumannii SDF	Wild type	ATCC (18)
A. baumannii ACICU	Wild type	(19)
A. baumannii Recent Clinical #1	Wild type	This study
A. baumannii Recent Clinical #2	Wild type	This study
A. baumannii Recent Clinical #3	Wild type	This study
A. baumannii Recent Clinical #4	Wild type	This study
A. baumannii Recent Clinical #5	Wild type	This study
A. baumannii Recent Clinical #6	Wild type	This study
A. baumannii Recent Clinical #7	Wild type	This study
A. baumannii Recent Clinical #8	Wild type	This study
A. baumannii Recent Clinical #9	Wild type	This study
A. baumannii ATCC 17978, LOS^-	Δ*ponA*, *lpxA* SNP	This study
A. baumannii ATCC 19606, LOS^-	*lpxC* SNP	This study
A. baumannii 5075, LOS^-	*lpxC* SNP	This study
A. baumannii AYE, LOS^-	*lpxC* insertion	This study
A. baumannii ATCC 17978	Δ*ponA*	This study
A. baumannii ATCC 17978	Δ*ponA* / pPonA	This study
A. baumannii ATCC 17978	Δ*ponA* / $pPonA_{E92Q}$	This study
A. baumannii ATCC 17978	Δ*ponA* / $pPonA_{S459A}$	This study
A. baumannii ATCC 17978	Δ*ponB*	This study
A. baumannii ATCC 17978, LOS^-	Δ*ponA*, *lpxA* SNP (LOS^-) / pLpxA	This study
A. baumannii ATCC 19606, LOS^-	l*pxC* SNP (LOS^-) / pLpxC	This study
A. baumannii ATCC 19606	*HMPREF0010_01944-His_6*	This study
A. baumannii ATCC 19606, LOS^-	*HMPREF0010_01944-His_6*	This study
A. baumannii ATCC 19606	Δ*HMPREF0010_01944-His_6*	This study
A. baumannii ATCC 19606, LOS^-	Δ*HMPREF0010_01944-His_6*	This study
A. baumannii ATCC 19606	p1944-His_6-tag	This study
A. baumannii ATCC 19606	*HMPREF0010_01945-His_6*	This study
A. baumannii ATCC 19606, LOS^-	*HMPREF0010_01945--His_6*	This study
A. baumannii ATCC 19606	Δ*HMPREF0010_01945-His_6*	This study
A. baumannii ATCC 19606, LOS^-	Δ*HMPREF0010_01945-His_6*	This study
A. baumannii ATCC 19606	p1945-His_6-tag	This study
A. baumannii ATCC 19606	*HMPREF0010_02739-His_6*	This study
A. baumannii ATCC 19606, LOS^-	*HMPREF0010_02739-His_6*	This study
A. baumannii ATCC 19606	Δ*HMPREF0010_02739-His_6*	This study
A. baumannii ATCC 19606, LOS^-	Δ*HMPREF0010_02739-His_6*	This study
A. baumannii ATCC 19606	p2739-His_6-tag	This study
Plasmid		
pMMB67EH	Amp^R	ATCC (20)
pABBR	Amp^R	(1)
pABBRKn	pABBR_MCS with the *Kan^R* gene from pKD4	This study

	inserted into the PvuI site, Kn^R	
pMMB67EHKn	pMMB67EH with the *Kan*^{R} gene from pKD4 inserted into the PvuI site, Kn^R	This study
p1944-His_6-tag	pMMB67EHKn with *HMPREF0010_01944* (ATCC 19606) cloned into the SalI and KpnI sites, Kn^R	This study
p1945-His_6-tag	pMMB67EHKn with *HMPREF0010_01945* (ATCC 19606) cloned into the SalI and KpnI, Kn^R	This study
p2739-His_6-tag	pMMB67EHKn with *HMPREF0010_02739* (ATCC 19606) cloned into the SalI and KpnI, Kn^R	This study
pAT03	pMMB67EH with FLP recombinase, Amp^R	(1)
pAT04	pMMB67EH with REC_{Ab} system, Tet^R	(1)
pKD4	Kan^R	(21)
pJNW684	Tn Vector, Am^R, Kan^R	(23)

Table S4: Primers used in this study

Primers	
Deletion Primers	
Ab 17978 Δ*ponA Kan-FRT* Fwd	5'-actgaccacaattagtttgtattcgatcttattaaagcttacttatcatgaaaaagctatccagtttgggcttcgtgcgtccaatttttttgatcattattattatcttagtctcacttccgatgagcgattgtgtaggctggagctgcttcg -3'
Ab 17978 Δ*ponA Kan-FRT* Rev	5'-gcatctccagaacgtcgtatgcaattagaacagaaagcacacttacaagtgaccgcatcttttcaaatttatctttttcaaaagcgtaataaaaaagccatctaacgatggctttttttatttttaatatcctccttagttcctattccg-3'
Ab 17978 Δ*ponB Kan-FRT* Fwd	5'-cataacaacaagatactgtaaaattcaatctaagttttccatttattcacagttttatcattgcaaattgatgacaagctatcgcatcattaacgcattgtttagggttttatctcatttatatgagcgattgtgtaggctggagctgcttcg -3'
Ab 17978 Δ*ponB Kan-FRT* Rev	5'-agaaacaggaggtgttttctcttttggagctgaatgatctggtaaagttgtacagccgaccataagtacagcaccaatataaaaaacacctttctttaacatatcctgtttcctgttaacgcttaatatcctccttagttcctattccg -3'
Ab 19606 Δ*HMPREF0010_02739 Kan-FRT* Fwd	5'-ctcatttttactctatattttgcattgtaaccaacaacgaaaagaaaaactctccattgtttcttcatctttattatctaatctttaagtcatcttaaaacaaacattaaagggtttaaccatgagcgattgtgtaggctggagctgcttcg-3'
Ab 19606 Δ*HMPREF0010_02739 Kan-FRT* Rev	5'-gtaagtgctttgagtgtaaacacttttgatgaagtttagatgagacaaatagtcaaaatcgtaaatagttatgtataaacaaaaggcgccttctggcgccttttgttttattattttttagaattaatatcctccttagttcctattccg-3'
Ab 19606 Δ*HMPREF0010_01944-45 Kan-FRT* Fwd	5'-ggaaaaaataagccatttttacaaaaagtctattaaagcactcttaatttattacttaatgggggaaaaccctccatgaaagcatactattctaggcgcaactgagtagaggagtagctttaatgagcgattgtgtaggctggagctgcttcg-3'
Ab 19606 Δ*HMPREF0010_01944-45 Kan-FRT* Rev	5'-cgatgaatcaggtgtgagaattgctgaagtccaaaagacactttatatccgacgtaaaaaagcaaaaccttctactaataaaataagcgcctgaaggcgcttatttttcatttcacttatttttaatatcctccttagttcctattccg-3'
Complementation Primers	
Ab 17978 pPBP1A Native Promoter XhoI Fwd	5'- cgcgCTCGAGtaacctgagcacactattgtcctattatttttttatc -3'
Ab 17978 pPBP1A Native Promoter KpnI Rev	5'-cgcGGTACCcaagttattgatgctcctttagtacttgaccag-3'

Ab 17978 pLpxA Native Promoter XhoI Fwd	5'-cgcgCTCGAGccaggttcattattcctgtttgctgg-3'
Ab 17978 pLpxA Native Promoter KpnI Rev	5'-cgcGGTACCcaagtaaggcacctcgagcattgtac-3'
Ab 19606 pLpxC Native Promoter XhoI Fwd	5'- cgcgCTCGAGtgcagctgatgcagagccaag-3'
Ab 19606 pLpxC Native Promoter KpnI Rev	5'-cgcGGTACCgctctttaattattactggtgaaaatcaggcaatg-3'
Mutagenesis Primers	
Ab pPBP1A E92Q Fwd	5'-ttttattcatgcattcctggcagcaCAAgattcttctttctttgaacatagtg-3'
Ab pPBP1A E92Q Rev	5'-cactatgttcaaagaaagaagaatcTTGtgctgccaggaatgcatgaataaaa-3'
Ab pPBP1A S459A Fwd	5'-tttacaaggctggcgtcagccaggtGCTactatcaaaccattcctctatgctt-3'
Ab pPBP1A S459A Rev	5'-aagcatagaggaatggtttgatagtAGCacctggctgacgccagccttgtaaa-3'
6X-his tag Primers	
Ab 19606 Δ*HMPREF0010_02739 6X-his Kan-FRT* Fwd	5'-ttggacaaactgtcaatgttcaagttggagataaaacacttgaaggtacatgtaacctccgctttgagccaaaacgtcctcaaccaccagtcaatgcacctgctccagtagcatctcaagcaaaacaccatcaccatcaccattaaagcgattgtgtaggctggagctgcttcg-3'
Ab 19606 Δ*HMPREF0010_02739 6X-his Kan-FRT* Rev	5'-gtaagtgctttgagtgtaaacacttttgatgaagtttagatgagacaaatagtcaaaatcgtaaatagttatgtataaacaaaaggcgccttctggcgccttttgttttattattttagaaatatcctccttagttcctattccg-3'
Ab 19606 Δ*HMPREF0010_01944 6X-his Kan-FRT* Fwd	5'-ccaagagtgcgattggtgcaggtattggttcggtcgttggtaaagctatcataggtggtgatacaggtgctgcaattggtggcgcaatcggcggcggtgctggtgctgcgattgaagaaaagaaacaccatcaccatcaccattaaagcgattgtgtaggctggagctgcttcg-3'
Ab 19606 Δ*HMPREF0010_01944 6X-his Kan-FRT* Rev	5'-tttcgatgaatcaggtgtgagaattgctgaagtccaaaagacactttatatccgacgtaaaaaagcaaaaccttctactaataaaataagcgcctgaaggcgcttatttttcatttcacttatttatatcctccttagttcctattccg-3'
Ab 19606 Δ*HMPREF0010_01945 6X-his Kan-FRT* Fwd	5'-attggtgcaggtgttggggcaatcttaggtggtgcagtgattggtggtgacgctggtgctgctgcaggtggagcactcggtggtagtgctggtgcagcctatgaagagaaaaaaggtaagtaccaccatcaccatcaccattaaagcgattgtgtaggctggagctgcttcg-3'

Ab 19606 Δ*HMPREF0010_01945 6X-his Kan-FRT* Rev	5'-taatagatattttgtaaagccaatttatgtgcagtgatgagttaaatttacatctttaaaagcagataaaaaagaagctcttaatgaaataagagcttctttttttaatatagttagaggtagttatatcctccttagttcctattccg-3'
Overexpression Primers	
Ab 19606 HMPREF0010_02739 6X-his KpnI Fwd	5'-cgcGGTACCatgaaaatgacggctaaaattgcattattcagt-3'
Ab 19606 HMPREF0010_02739 6X-his SalI Rev	5'-cgcGTCGACttagtgatggtgatggtgatgttttgcttgagatgctactggagcag-3'
Ab 19606 HMPREF0010_01944 6X-his KpnI Fwd	5'-cgcGGTACCatgaataaaaaaattggtttaatatctactgtcatattatcaacag-3'
Ab 19606 HMPREF0010_01944 6X-his SalI Rev	5'-cgcGTCGACttagtgatggtgatggtgatgtttcttttcttcaatcgcagcaccagc-3'
Ab 19606 HMPREF0010_01945 6X-his KpnI Fwd	5'-cgcGGTACCatgaaaaaaatgatgatgattgcaggtgttgg-3'
Ab 19606 HMPREF0010_01945 6X-his SalI Rev	5'-cgcGTCGACttagtgatggtgatggtgatggtacttacctttttctcttcataggctgcac-3'
qPCR Primers	
ponA Fwd	5'-accgcttttaaaaccattgc-3'
ponA Rev	5'-tgccaggaatgcatgaataa-3'
lolA Fwd	5'-ctgccgattttgaacagaca-3'
lolA Rev	5'-ccggacgttcaactttcatt-3'
16S rDNA Fwd	5'-gggagaaccagctatcacca-3'
16S rDNA Rev	5'-gcaggttgaaggttgggtaa-3'