Supplemental Material

Materials and Methods

Purification of OXA-58. Two-liter cultures of *Acinetobacter baumannii* strain Ab290 (pOXA-58-2) were grown in LB broth at 37 °C for 2 h. Protein expression was induced by adding 8 μg/ml imipenem and incubating for further 4 h. Cells were harvested by centrifugation at 6,000 rpm for 30 min, and cell pellets were stored at −20 °C until further use. OXA-58 was purified by nickel-chelated immobilized metal affinity chromatography (IMAC, GE Healthcare Bio-Sciences Corp., NJ). Briefly, cell pellets were resuspended in chilled lysis buffer (50 mM Tris-HCl, 200 mM NaCl, pH 9.0). Cell suspensions were sonicated with 10 short bursts of 10 s each with 30 s of cooling between bursts, and cell debris was removed by centrifugation at 32,000 rpm at 4 ºC for 45 min. The supernatant was loaded onto a 10 ml Ni Sepharose 6 Fast Flow resin (GE Healthcare Bio-Sciences Corp) that was equilibrated with column buffer (50 mM Tris-HCl pH 9.0, 200 mM NaCl, 5 mM imidazole). After loading the supernatant, the column was washed 5 times with 10 ml of column buffer. Protein was eluted with 500 mM imidazole in column buffer. Fractions containing OXA-58 were analysed by SDS-PAGE and confirmed by Western blotting with anti-OXA-58 polyclonal antibody. Fractions containing a single protein band were pooled and dialyzed against phosphate buffered saline (PBS). Protein concentrations were

determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA) and aliquots were stored at −80 °C until further use.

Gel-assisted enzymatic digestion of outer membrane vesicles (OMVs). OMVs were mixed with an equal volume of a solution containing N,N,N′,N′-Tetramethylene diamine, 10 % ammonium persulfate, and 30 % bis-acrylamide and keep in the dark until the gel polymerized. The gel was cut into small pieces and trypsin digested in ammonium bicarbonate buffer (pH 7.8) at 37 °C overnight at an enzyme: protein w/w ratio of 1:30. The digested peptides were extracted with 50 % acetonitrile (ACN), lyophilized, and stored at -80 °C until identified by LCMS/MS (1).

LC-MS/MS analysis and data analysis. Peptides were analyzed by SYNAPT HDMS ESI-Q-TOF coupling using a nanoACQUITY UPLC system (Waters Corp., Milfors, MA). LC separation was performed using a reverse phase C18 capillary column (75 μm x 100 mm; particle size: 1.7 μm, Waters Corp.) with a trap column (180 μm x 20 mm; particle size: 5 μm, Waters Corp.). The flow rate was set at 0.3 μl/min and the gradient was 1-50 % of Mobile phase B for 30 min, 50-65 % of Mobile phase B for 20 min, 65 % Mobile phase B for another 5 min, and then back to 1 % of Mobile phase B for 10 min (Mobile phase A: 5 %ACN/0.1 % formic acid (FA);

Mobile phase B: 95 %ACN/0.1 %FA). The MS scan was set from 400-1600 m/z, and the MS/MS acquisition started from 50 m/z. The four most intense ions from the MS scan were selected for the MS/MS scans. Raw data from G1 Q-TOF were processed into peak lists by Masslynx software version 4.0 (Waters Corp.) for further protein identification using the search algorithm MASCOT (version 2.4, Matrix Science, London, UK). The *A. baumannii* database at the National Center for Biotechnology Information was used for protein identification. Search parameters included trypsin cleavage at the C-termini of lysine and arginine and two maximum missed cleavages were allowed. The mass tolerance was set at ± 0.2 Da (2).

Checkerboard microdilution. The imipenem minimum inhibitory concentration in the presence and absence of SecA inhibitors was determined using Mueller-Hinton broth (MHB; Difco, Detroit, MI) and a microdilution checkerboard procedure (3). A two-dimensional checkerboard with 2-fold dilutions of imipenem and 2-fold dilutions of SecA inhibitors was prepared. Control wells containing media only were included in each plate.

Alkaline phosphatase activity assay. To measure alkaline phosphatase activity, *A. baumannii* strain Ab290 (pOXA-58-2) was grown to logarithmic phase in LB with or

without rose bengal. Cells from 1 ml aliquots of the cultures were harvested, washed once with 1 ml Tris-HCl (pH 8.0), and resuspended in 1 ml assay buffer (1 M Tris-HCl, 0.1 mM ZnCl2, 0.1 mM CaCl2, pH 8.0). Cells were permeabilized with 50 µl of chloroform and 50 µl of 0.1 % SDS, vortexed gently, and incubated at 30 °C for 20 min prior to centrifugation. After centrifugation, 200 µl of the upper aqueous phase was transferred to the well of a microplate, 25 µl of *p*-nitrophenylphosphate (pNpp) (Sigma-Aldrich, St. Louis, MO) was added, and the mixture was incubated at 30 °C. Formation of *p*-nitrophenol was measured by absorbance at 405 nm (4).

References

1. **Lu X, Zhu H.** 2005. Tube-gel digestion: a novel proteomic approach for high throughput analysis of membrane proteins. Mol Cell Proteomics : MCP **4:**1948-1958.

2. **Chen JH, Chang YW, Yao CW, Chiueh TS, Huang SC, Chien KY, Chen A, Chang FY, Wong CH, Chen YJ.** 2004. Plasma proteome of severe acute respiratory syndrome analyzed by two-dimensional gel electrophoresis and mass spectrometry. Proc Natl Acad Sci USA **101:**17039-17044.

3. **Clinical and Laboratory Standards Institute.** 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 10th ed. Approved standard M7-A10.CLSI, Wayne. PA.

4. **Tse YM, Yu M, Tsang JS.** 2009. Topological analysis of a haloacid permease of a *Burkholderia sp.* bacterium with a PhoA-LacZ reporter. BMC microbiol **9:**233.

Table S1. Primers used in this study [a]

Primer	Sequence, 5'–3'
IS1008(XbaI)F	tctagaTCTATTTGCAACAGTGCCATTTTTCTT
XbaIIS1008	tctagaTCGGCACTTTCAAGGTGAAAT
IS1008(M-BamHI)R	ggatccCATGATATACAACTTTAAAATTTGG
IS1008(BamHI)F	CCGggatccTCTATTTGCAACAGTGCCATTTTTCTT
IS1008(NcoI)R	CTAGccatggACATGATATACAACTTTAAAATTTGGTAGTG
IS1008-ΔP2-1(XbaI)F	tctagaTTGCAACAGTGCCATTTTTCTT
IS1008-ΔP2-2(XbaI)F	CTAGtctagaAACAGTGCCATTTTTCTTTATAC
IS1008-ΔP2-3(XbaI)F	CTAGtctagaACAGTGCCATTTTTCTTTATACTAT
OXA-58(XhoI)R	ctcgagTTATAAATAATGAAAAACACC
NcoIOXA58	ccatggTTATAAATAATGAAAAACACCCA
OXA-58Δ20aaSP(BamHI)F	ggatccGAGCATAGTATGAGTCGA
OXA-58(XhoI)R	ctcgagTTATAAATAATGAAAAACACC
OXA-58-20aaSP(NcoI)R	CATGccatggCACAAGCCCCAATACTTATGC
OXA-58-CΔ5aa(XhoI)R	CCGctcagaTTAACCCAACTTATCTAGCACATCTAAA
OXA-58-CΔ10aa(XhoI)R	CCGctcagaTTACACATCTAAAGACAATTGTTTACGT
OXA-58-CΔ15aa(XhoI)R	CCGctcgagTTATTGTTTACGTAGAGCAATATCATCA
ISAba1(XbaI)F	tctagaCACGAATGCAGAAGTTG
OXA-23-like(XhoI)R	ctcgagAATAATATTCAGCTGTTTTAATG
OXA-24-like(XbaI)F	tctagaCTCTAAGCCCCAAAATTTCC
OXA-24-like(XhoI)R	ctcgagAATGATTCCAAGATTTTCTAGCG
OXA-51-like(XhoI)R	ctcgagTAAAATACCTAATTGTTCTAA

[a] Lower-case letters indicate restriction sites.

Table S2. Proteins identified in Ab290 (pOXA-58-2) OMVs by 2DE and MS/MS analysis

Accession no.	MW (Da)	pI	Protein	Score	Matched peptides	Coverage (%)
gi|425484345	42851	5.21	Translation elongation factor Tu	595	35	57
gi|425699439	37953	5.3	Outer membrane protein Omp38	389	37	31
gi|301347500	54408	5.05	Chaperonin GroEL	265	17	39
gi|183208476	100043	5.25	Aconitase A	216	13	14
gi|126641617	52602	5.57	30S ribosomal protein S1	212	14	20
gi|422942104	14435	6.64	Elongation factor Tu GTP binding domain protein	211	14	45
gi|408511889	56018	5.57	IMP cyclohydrolase	186	6	13
gi|162286755	50243	5.03	F0F1 ATP synthase subunit beta	184	11	25
gi|425496383	22878	5.56	Superoxide dismutase (Fe)	155	7	31
gi|422944122	30702	5.6	Succinate-CoA ligase, alpha	154	6	16

			subunit			
gi|425495090	78880	5.05	Translation elongation factor G	136	11	22
gi|169150075	37082	6.14	Phenylalanyl-tRNA synthetase, alpha-subunit	127	3	9
gi|400204896	57226	4.86	Chaperonin GroL	119	9	18
gi|213056438	27562	5.47	3-hydroxybutyrate dehydrogenase	95	4	30
gi|126640373	11787	5.09	50S ribosomal protein	89	3	16
gi|410396584	63475	5.69	Putative negative regulator of genetic competence ClpC/MecB	88	10	18
gi|395552217	77826	5.85	Fatty oxidation complex, alpha subunit FadB	80	4	6
gi|425492474	4995	9.66	Entericidin EcnA/B family protein	79	2	34
gi|407901835	84951	5.08	Aconitate hydratase 2	79	7	11
gi|425491061	35329	5.2	Malate dehydrogenase	79	8	26
gi|425492452	67016	5.94	Succinate dehydrogenase, flavoprotein subunit	74	4	7

gi|425489025	21174	5.31	OmpW family protein	71	3	24
gi|400203568	24143	6.3	Succinate dehydrogenase and fumarate reductase iron-sulfur protein	68	3	13
gi|425491451	41003	9.1	Outer membrane assembly lipoprotein YfgL	70	3	8
gi|169152905	26807	6.1	3-oxoacyl-[acyl-carrier-protein] reductase	67	2	11
gi|301510213	45894	4.81	Aspartate kinase	64	1	6
gi|169151156	14222	10.05	30S ribosomal protein S8	63	4	32
gi|425496343	30607	5.27	Translation elongation factor Ts	62	4	14
gi|169148062	82621	5.63	Isocitrate dehydrogenase	58	4	11
gi|301510300	33381	6.64	Dihydrolipoyllysine-residue succinyltransferase	55	8	20
gi|311350290	20094	5.77	**Oxacillinase 58, partial**	54	7	55
gi|301511454	59346	5.31	Isocitrate lyase	54	1	2
gi|425699254	46216	5.81	Glutamate dehydrogenase	51	1	11
gi|422945433	20774	5.01	Peroxiredoxin	47	4	14

gi|213155931	22774	5.26	Putative deoxyribonucleotide triphosphate pyrophosphatase	45	1	9

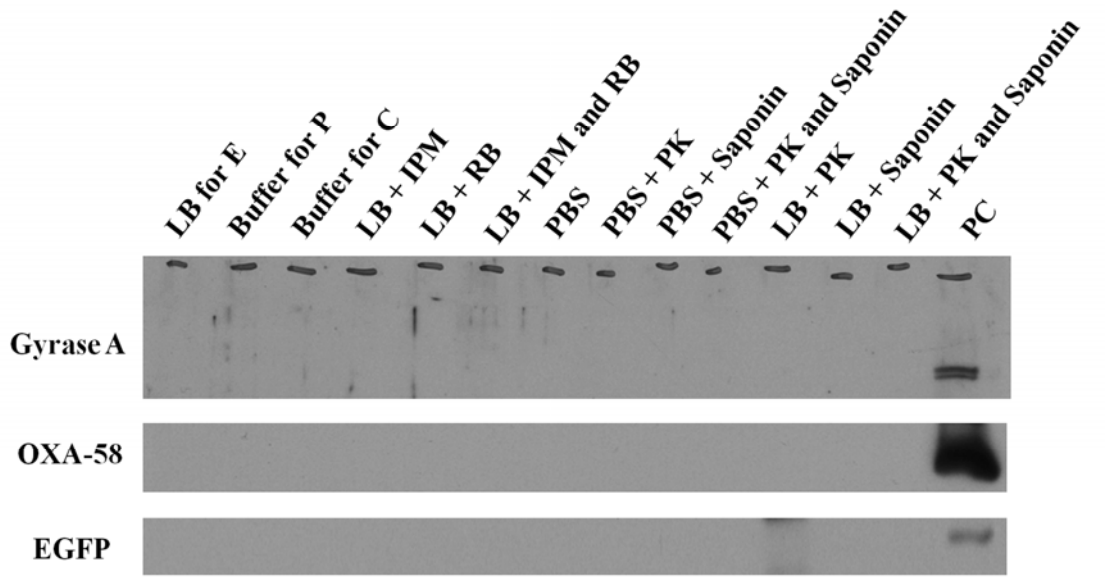
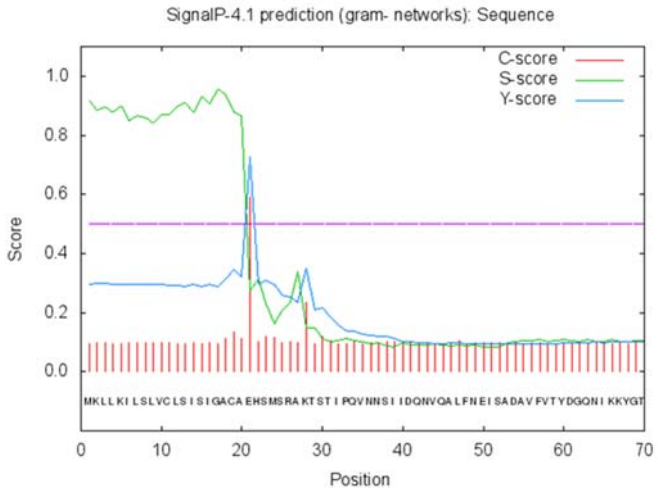


FIG S1. Sample buffers were negative for gyrase A, OXA-58, and EGFP. Samples included Luria-Bertani (LB) broth for the extracellular fraction (E), osmotic shock buffer (30 mM Tris-HCl, 20% sucrose, pH 8.1) for the periplasmic fraction (P), lysis buffer (3 mM EDTA, pH 7.3) for the cytoplasmic fraction (C), LB broth with 8 μg/ml imipenem (IPM), LB broth with 12.5 μg/ml rose bengal (RB), LB broth with 8 μg/ml IPM and 12.5 μg/ml RB, phosphate-buffered saline (PBS), PBS with 100 μg/ml proteinase K (PK), PBS with 2% saponin, and PBS with 100 μg/ml PK and 2% saponin. The positive control (PC) sample for gyrase was derived from the extracellular fraction of IPM-treated *Acinetobacter baumannii*, the PC sample for OXA-58 was purified OXA-58 protein, and the PC sample for EGFP was EGFP fused with the OXA-58 signal peptide.

A



B

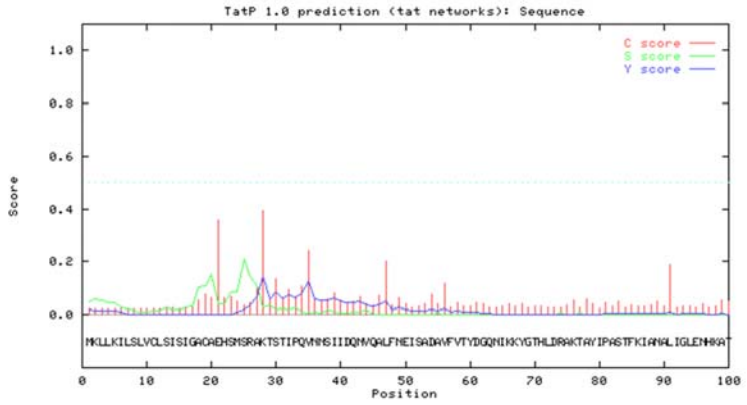


FIG S2. *In silico* analysis of the OXA-58 signal sequence predicted that OXA-58 was preferentially translocated via the Sec system. The OXA-58 signal peptide was predicted with the SignalP 4.0 (A) and Tat 1.0 (B) servers. The S score (green line) was used to predict the residues of the signal peptide. The cleavage site was determined by the C score (red lines). The better cleavage site was determined by the Y score, which combines the predictions of the S and C scores.

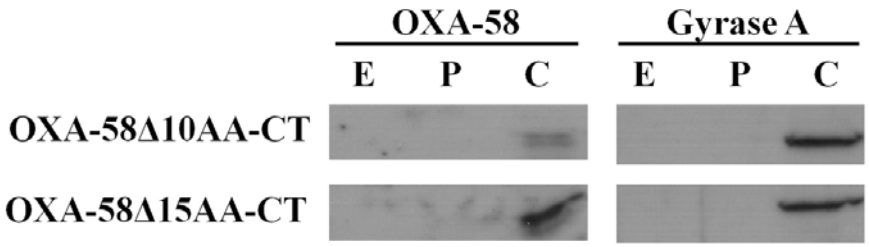


FIG S3. Extracellular release and periplasmic translocation of OXA-58 were dependent on the C-terminus. Western blot analysis of truncated OXA-58 fractions from *Acinetobacter baumannii* strains that express OXA-58 with the last 10 C-terminal amino acids deleted (OXA-58Δ10AA-CT) or OXA-58 with the last 15 C-terminal amino acids deleted (OXA-58Δ15AA-CT). E, P, and C indicate extracellular, periplasmic, and cytoplasmic fractions, respectively.

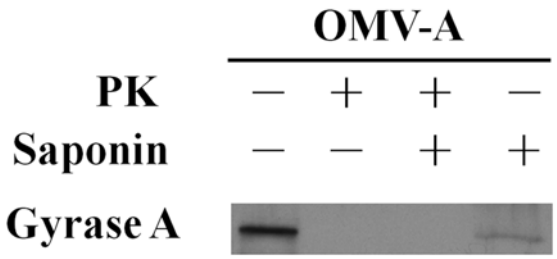


FIG S4. Extracellular gyrase from IPM-treated *A. baumannii* was susceptible to proteinase K (PK) degradation. Western blot analysis of extracellular gyrase treated with (+) or without (-) 100 μg/ml PK, 2% saponin, or both.

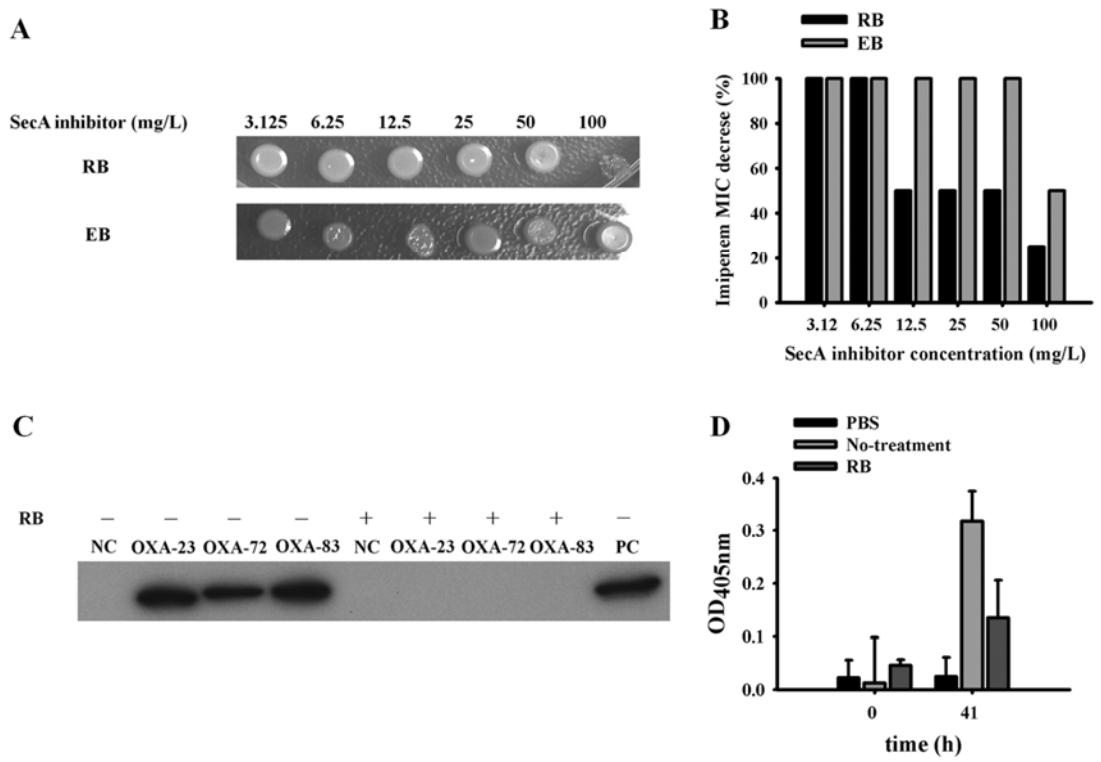


FIG S5. The effects of SecA inhibitors rose bengal and erythrosin B. (A) Addition of both SecA inhibitors up to 100 μg/ml did not inhibit the growth of *A. baumannii* strain Ab290 (pOXA-58-2). (B) The checkerboard microdilution assay showed that addition of rose bengal (RB) and erythrosin B (EB) at sublethal doses decreased the imipenem minimum inhibitory concentration (MIC) of Ab290 (pOXA-58-2) and showed that RB was more potent than EB. (C) Western blot analysis using anti-His antibody to detect His-tagged OXA-23, OXA-72, and OXA-83 in OMVs. OMVs from the strain carrying the shuttle vector pYMAb-2 were used as the negative control (NC). Purified OXA-58 was used as the positive control (PC). RB inhibited the OMV-associated release of OXAs. (D) RB inhibited another Sec-dependent periplasmic protein, alkaline phosphatase (Alk-P). Enzyme activity of Alk-P from the periplasm of strain

Ab290 (pOXA-58-2) with or without RB treatment, was determined by formation of *p*-nitrophenol from *p*-nitrophenylphosphate (pNpp), which was measured by absorbance at 405 nm. In the phosphate buffered saline (PBS) group, PBS was incubated with pNpp as a negative control.

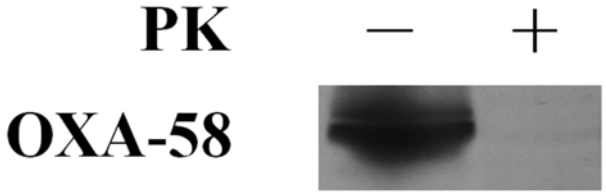


FIG S6. The extracellular OXA-58 collected after bacteria was treated with imipenem and rose bengal was susceptible to proteinase K degradation. After *A. baumannii* was treated with rose bengal and imipenem, extracellular OXA-58 were collected and concentrated with a 10 kDa MWCO Amicon Ultra centrifugal concentrator. This extracellular OXA-58 was degraded by proteinase K (PK), indicating that it was OMV independent.