Supplemental Materials and Methods

Bacterial strains and growth conditions

Table S1 describes bacterial strains used in this study and their source. Gram-negative bacteria were cultured in lysogeny broth (LB, EMD Millipore), and Gram-positive bacteria were grown in Mueller Hinton broth (Difco) at 37°C, with shaking at 200 rpm.

Gene synthesis and cloning

To facilitate preliminary screening of *Pseudomonas* lysins, pAR553, a derivate of pBAD24 containing a new MCS (EcoRI – SalI – NotI – KpnI – XbaI – PstI), was constructed by aligning primers 629_5_pBAD_MCS (5'-aattcgtcgacggggcggccg cggtacctctagactgcag), and 630_3_pBAD_MCS (5'-gtctagaggtaccgcggccgccccgtcgacg), and inserting the resulting double-stranded DNA into the EcoRI and PstI sites of pBAD24 (1). *Pseudomonas* lysins were identified in the NCBI database through BLAST search using the *Acinetobacter* lysin PlyF307 as query, yielding over 100 hits. All hits were aligned using the Lasergene MegAlign Pro software, with the MUSCLE algorithm. A candidate was selected from each group (see table S2 for protein identifiers). Nucleotide sequences for selected lysins were designed with an upstream SalI and a downstream NotI restriction sites, and were synthesized by Genewiz. Creation of plasmids for the initial screen was done by inserting the lysin sequence into the SalI and NotI sites of pAR553. Creation of a 3C-cleavable

hexahistidine-tagged versions of the lysins was done by inserting the lysin sequence into the SalI and NotI sites of a modified pET21 vector.

Purification of phage lysins

An overnight culture of *E. coli* BL21 containing a lysin cloned into a modified pET21a vector was diluted 1:100 into 1 L of LB medium containing ampicillin, and placed in an environmental shaker. Upon reaching OD_{600} 0.5, the expression of the lysin was induced with 0.2 mM IPTG for 4 h at 37°C, and the cells were then shaken overnight at 4°C. The cells were harvested and resuspended in 40 ml MCAC buffer (30 mM Tris pH 7.4, 0.5 M NaCl, 10% glycerol, 1 mM DTT), and homogenized using an Emulsiflex-C5 homogenizer (Avestin, Ottawa, Ontario, Canada). Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.22-µm filter (Millipore). The cleared lysate was loaded on a NiNTA column equilibrated with MCAC buffer, followed by washes with MCAC containing 20 mM imidazole and elution with MCAC containing 150 mM imidazole. The eluted fraction was supplemented with 10× 3C buffer for a final concentration 150 mM NaCl, 50 mM tris pH 7.6, 10 mM EDTA, 1 mM DTT, and 50 µl of 3C protease were added per 1 mg of purified protein. The mix was incubated overnight at 4°C, placed in a dialysis bag with a 3 kDa cutoff, and dialyzed for 24 h against PBS with 3 buffer changes. The protein was then concentrated using an Amicon ultrafiltration device, fitted with a 3-kDa molecular weight cutoff membrane, and the final concentration was

determined using a ND-1000 spectrophotometer (Nanodrop), according to absorbance at 280 nm.

Overlay assays

To prepare *P. aeruginosa* overlay agarose, strain PAO1 was grown overnight in 6 L of LB medium, harvested, and suspended in 3 L PBS. The cells were aliquoted into bottles containing agarose to a final concentration of 0.7%, autoclaved, and stored at 4°C until use.

E. coli strains containing a lysin gene in pAR553 (pBAD24-based) were streaked on LB + ampicillin 15 cm glass plates containing 0.2% arabinose (to induce protein expression) overnight at 37°C. The plates were exposed to chloroform vapor for 5 minutes to permeabilize the cells. Then, soft agar containing autoclaved (to destabilize the outer membrane) *P. aeruginosa* cells at 50°C was poured over the plates, covering the cells. The plates were incubated at 37°C and examined for the presence of clearing zones following 1, 2, 5, and 16 hours.

To test activity of the lysins in crude lysate, *E. coli* strains containing the gene in pAR553 were diluted 1:100 from an overnight culture into 400 ml LB + ampicillin and grown at 37°C with shaking at 200 RPM. Once the cultures reached OD_{600} 0.5, arabinose was added to a final concentration of 0.2% to induce expression of the lysin. The cells were incubated for 4 h at 37°C, and placed at 4°C with gentle agitation overnight. Cells were harvested, suspended in 40 ml PBS, and

homogenized. Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.22-μm filter (Millipore). Varying amounts of the cleared lysate was applied to a 15 cm plate containing autoclaved *P. aeruginosa* agarose. Observations for the presence of clearing zones were done following 1, 2, 5, and 16 hours.

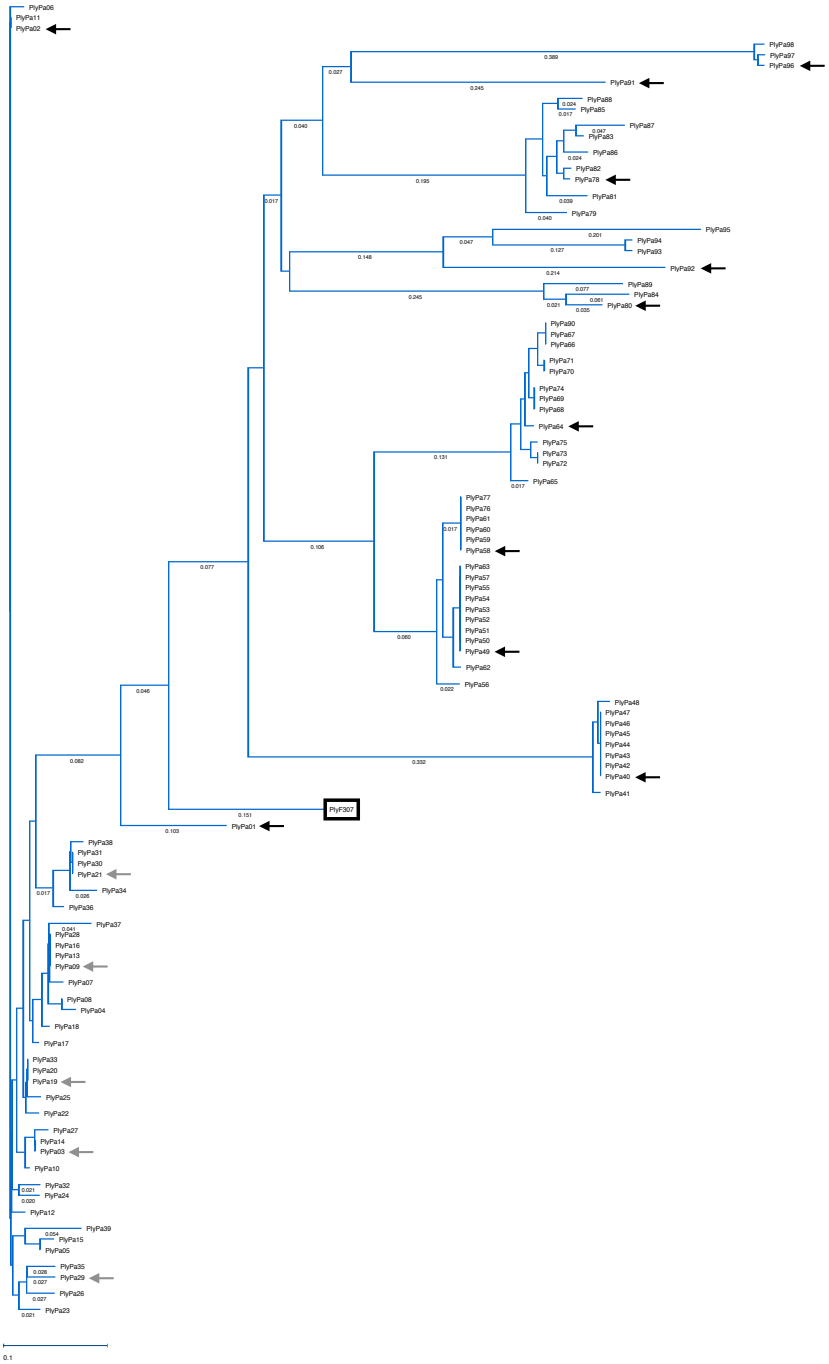

Fig. S1 – Phylogenetic tree of *P. aeruginosa* phage lysins with homology to PlyF307

Phage lysins were identified through homology search of the NCBI database using PlyF307 as query (black rectangle). Sequences were analyzed using Lasergene MegAlign Pro with the MUSCLE algorithm, producing a phylogenetic tree. Lysins chosen for the initial screen are denoted with black arrows. Lysins chosen for the second step are denoted with grey arrows.

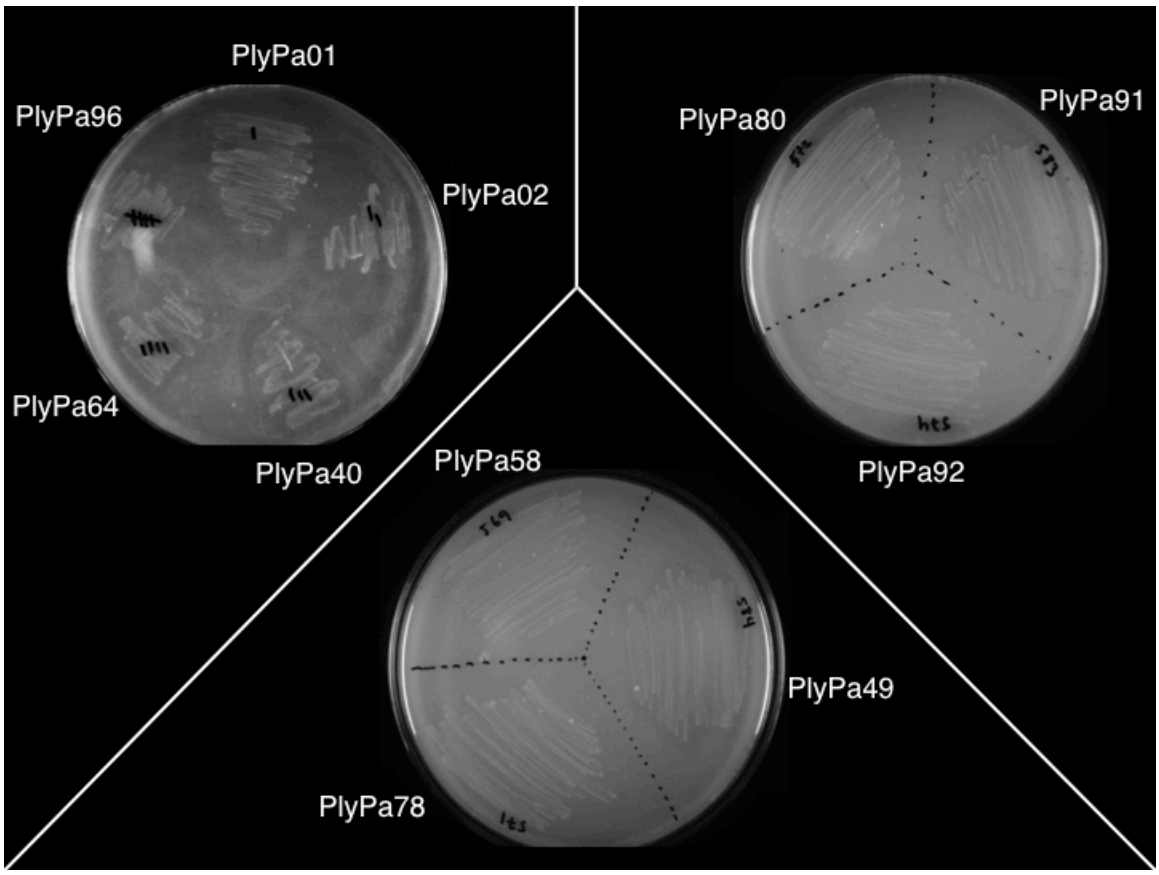

Fig. S2 – Evaluation of lysin peptidoglycan hydrolase activity using the plate overlay method. *E. coli* strains containing lysin genes in pAR553 were grown on a plate containing 0.2% arabinose to induce lysin expression. Cells were permeabilized with chloroform vapor and overlayed with soft agar containing autoclaved *P. aeruginosa* cells. Enzymatic activity was evaluated by the appearance of clearing zones.

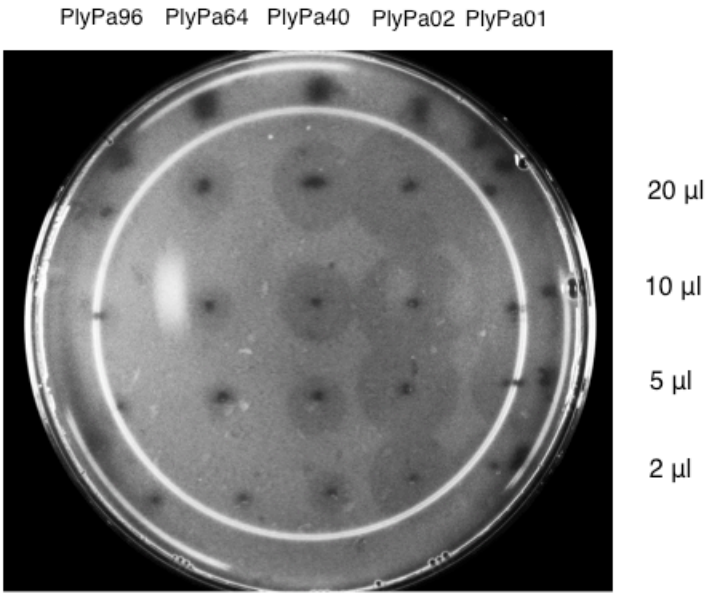

Fig. S3 – Evaluation of lysin peptidoglycan hydrolase activity in crude lysate. Induced crude lysates of *E. coli* strains harboring the lysin genes in pAR553 were spotted in different amounts on a plate containing soft agar with autoclaved *P. aeruginosa*. Enzymatic activity was evaluated by the appearance of clearing zones.

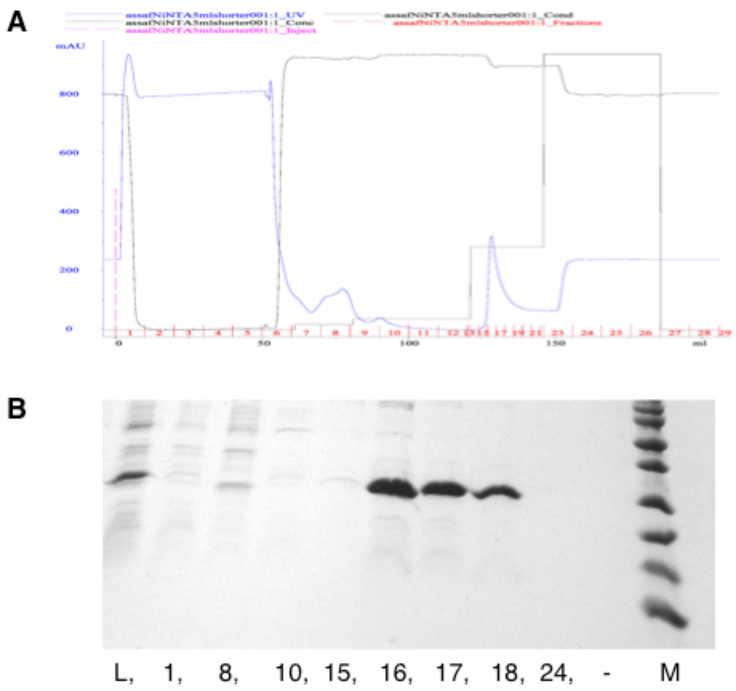

Fig. S4 – Purification of PlyPa02. A PlyPa02 fused to a 3C-cleavable hexahistidine tag was purified from an induced *E. coli* lysate by a single step metal affinity chromatography: L – Induced lysate; fractions 1-5 – load; fractions 6-15 – wash steps; fractions 16-18 – collected elution; fractions 23-29 – column regeneration. (A) AKTA Prime chromatograph of the purification process. (B) Coomassie stain of a 15% SDS-PAGE containing select fractions.

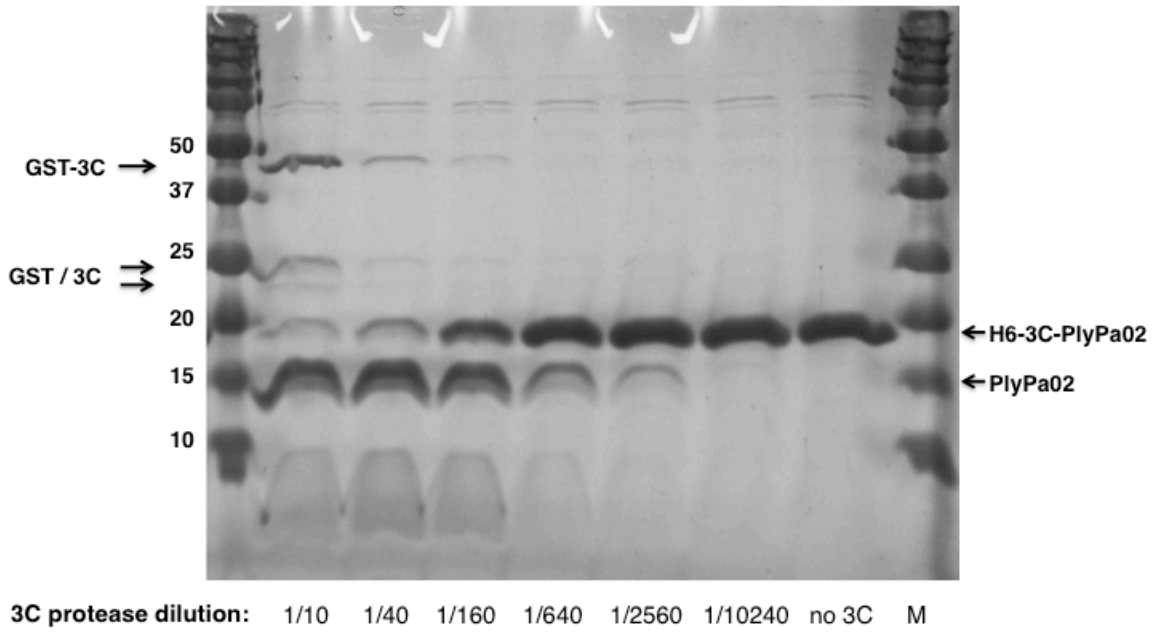

Fig. S5 – Cleavage of PlyPa02 with various doses of 3C protease.
Reaction mixtures with a total volume of 20 µl were prepared by combining 10 µg of PlyPa02, 2 µl of 4-fold serially diluted 3C protease and the following buffer composition: 150 mM NaCl; 50 mM tris; 10 mM EDTA; and 1 mM DTT, pH 7.6. Reactions were incubated at 4°C for 16 h, samples were loaded on 15% SDS-PAGE, and the gel stained with Coomassie blue.

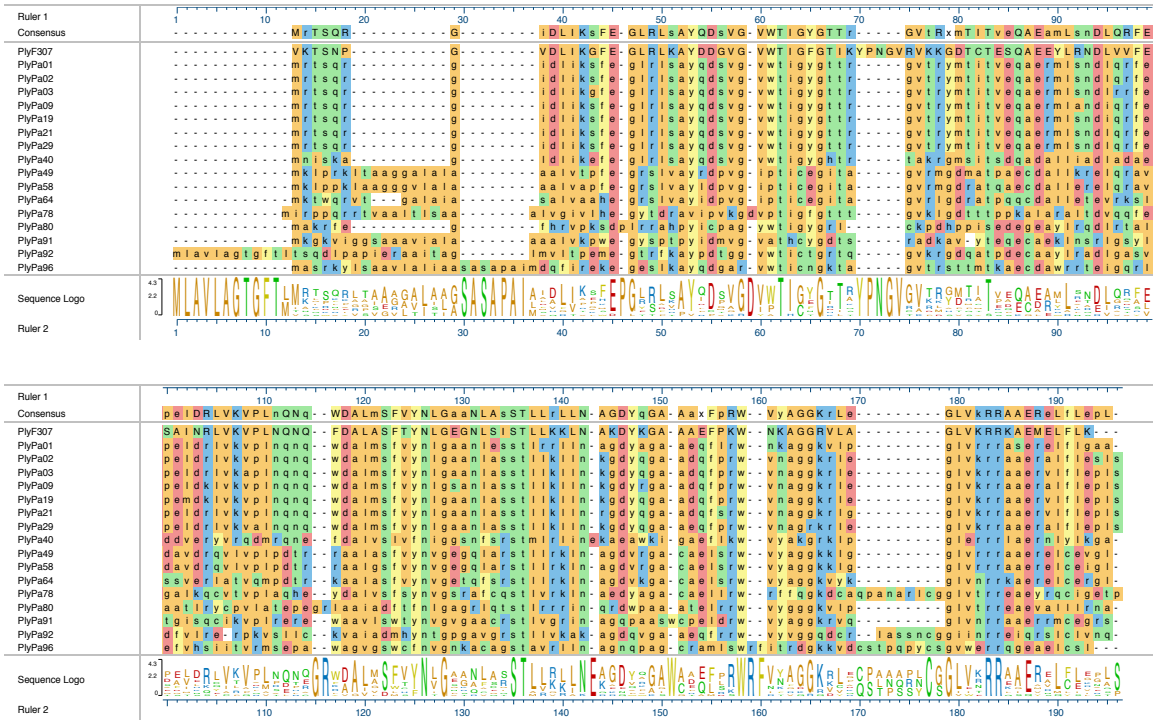

Fig. S6 – Homology analysis of chosen *P. aeruginosa* lysins. Amino acid sequences for select phage lysins were aligned using the Lasergene MegAlign Pro software with MUSCLE algorithm.

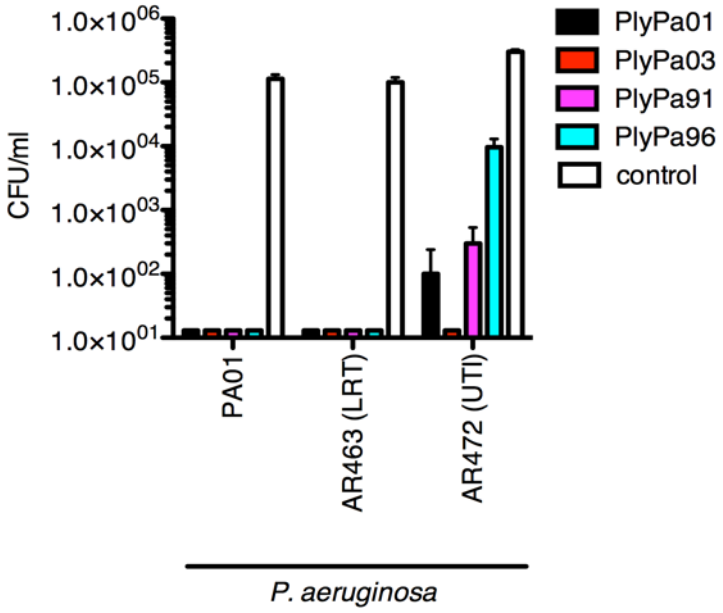

Fig. S7 – Activity of lysins against *P. aeruginosa* strains at 250 µg/ml. *P. aeruginosa* strains PAO1, AR463, and AR463 were incubated with 250 µg/ml of the lysins in 30 mM HEPES buffer pH 7.4 for 1 h at 37°C. Viable bacteria were enumerated by serial dilution and plating. Experiments were done in duplicates, error bars represent standard deviation.

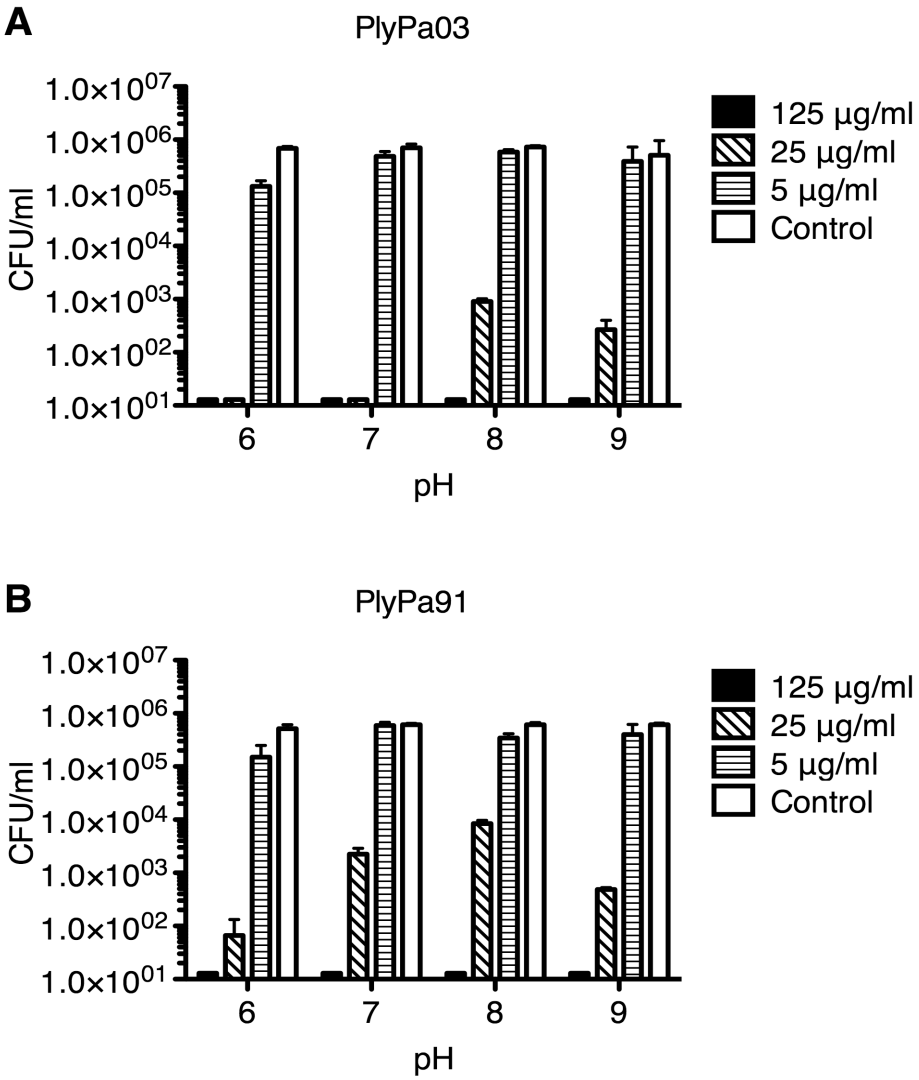

Fig. S8 – Effect of pH on the activity of PlyPa03 and PlyPa91. Log-phase *P. aeruginosa* PAO1 cells were incubated for 1 h at 37°C with various lysin concentrations in 25 mM of the following buffers: pH 6.0 - MES buffer; pH 7.0 and 8.0 - HEPES buffer; pH 9.0 - CHES buffer. Surviving bacterial CFU/ml are presented; experiments were performed in triplicates. Error bars represent standard deviation.

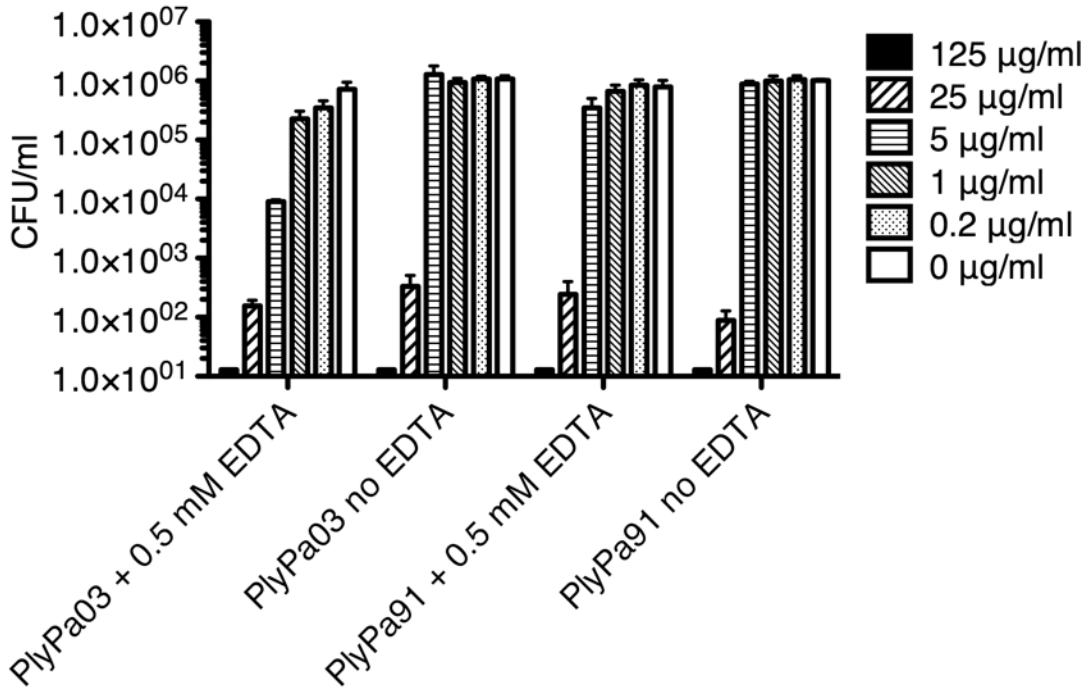

Fig. S9 – Effect of EDTA on lysin activity
Log-phase *P. aeruginosa* PAO1 cells were incubated for 1 h at 37°C with serially diluted PlyPa03 or PlyPa91 in the presence or absence of 0.5 mM EDTA. Viable bacterial CFU are presented. Experiments were done in triplicates, error bars represent standard deviation.

Table S1 - Strains used in this study

Organism	Source
A. baumannii, ATCC 17978	ATCC
A. baumannii, ATCC BAA-1791	ATCC
B. anthracis, Δ Stern	(2)
C. freundii, ATCC 8090	ATCC
E. aerogenes, NR-48555 (CRE)	BEI
E. Cloacae, NR-50391	BEI
E. Cloacae, NR-50392	BEI
E. Cloacae, NR-50393	BEI
E. coli, DH5α	Invitrogen
E. coli, AR531	NYU Hospital (UTI)
K. pneumoniae, ATCC700603	ATCC
K. pneumoniae, ATCC10031	ATCC
K. pneumoniae, ATCC700603	ATCC
K. pneumoniae, NR-15410 (bla_{KPC})	BEI
K. pneumoniae, NR-15411 (bla_{KPC})	BEI
K. pneumoniae, NR-41923	BEI (Urine)
K. pneumoniae, NR-44349	BEI (Sepsis)
P. aeruginosa, PAO1	ATCC
P. aeruginosa, AR443	Cornell Hospital
P. aeruginosa, AR444	Cornell Hospital
P. aeruginosa, AR461	NYU Hospital (LRT)
P. aeruginosa, AR463	NYU Hospital (LRT)
P. aeruginosa, AR465	NYU Hospital (LRT)
P. aeruginosa, AR468	NYU Hospital (wound)
P. aeruginosa, AR469	NYU Hospital (wound)
P. aeruginosa, AR470	NYU Hospital (stool)
P. aeruginosa, AR471	NYU Hospital (UTI)
P. aeruginosa, AR472	NYU Hospital (UTI)
P. aeruginosa, AR474	NYU Hospital (UTI)
P. mirabilis, AR397	Hunter College Collection
Salmonella spp. Serogroup D AR396	Hunter College Collection
S. marcescens, AR401	Hunter College Collection
S. flexneri, ATCC 12022	ATCC
S. sonnei, ATCC 25931	ATCC
S. aureus, Newman	(3)

Table S2 – Protein identifiers

Lysin	Protein identifier
PlyPa01	WP_058157505
PlyPa02	WP_073667504
PlyPa03	WP_070344501
PlyPa09	WP_042930029
PlyPa19	WP_034013816
PlyPa21	WP_042853300
PlyPa29	WP_058158945
PlyPa40	WP_058171189
PlyPa49	WP_058355500
PlyPa58	WP_058182687
PlyPa64	WP_033973815
PlyPa78	WP_034067975
PlyPa80	WP_057386760
PlyPa91	CRR10611
PlyPa92	WP_052160556
PlyPa96	WP_019681133

References

1. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177:4121-30.
2. Schuch R, Nelson D, Fischetti VA. 2002. A bacteriolytic agent that detects and kills *Bacillus anthracis*. Nature 418:884-9.
3. Daniel A, Euler C, Collin M, Chahales P, Gorelick KJ, Fischetti VA. 2010. Synergism between a novel chimeric lysin and oxacillin protects against infection by methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 54:1603-12.