Supplemental information

Clinical Strains of *Pseudomonas aeruginosa* secrete LasB elastase to induce hemorrhagic diffuse alveolar damage in mice

Yajie Zhu, Xiaoli Ge, Di Xie, Shangyuan Wang, Feng Chen and Shuming Pan

Supplemental figures

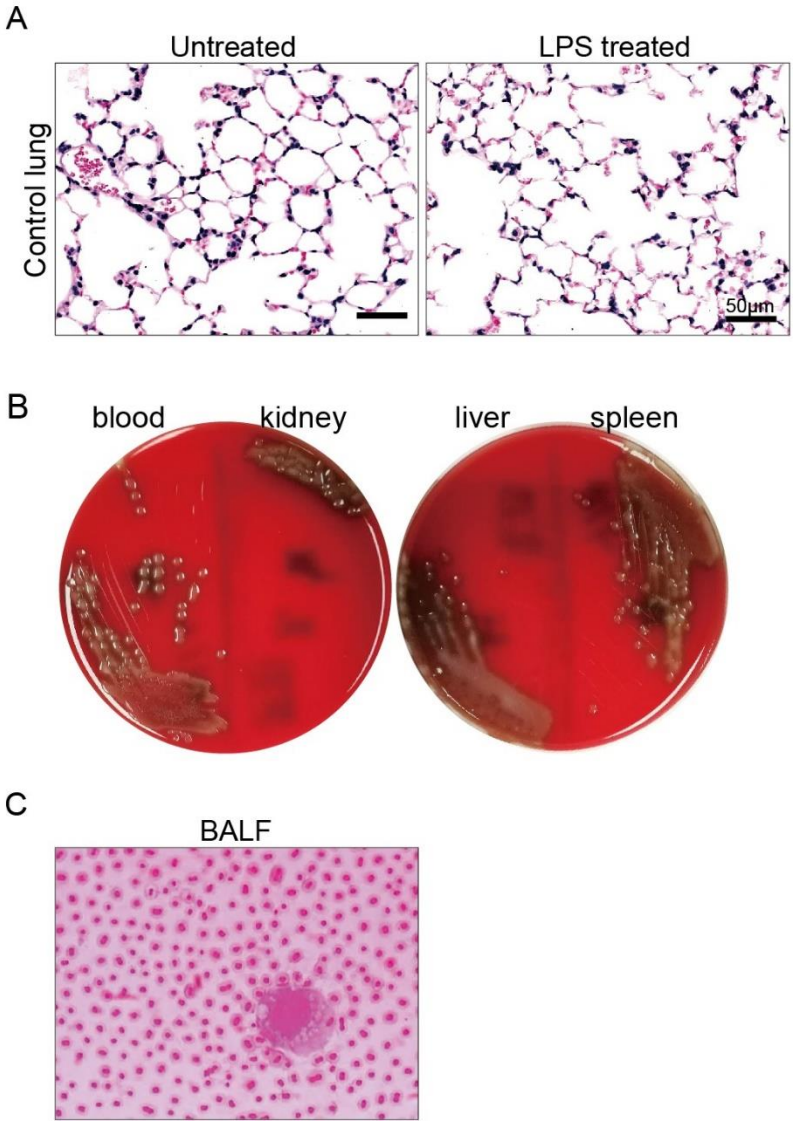

Figure S1 (A) HE stain of lung tissue from the control lung (right lung) from both normal mice (Control) and LPS unilateral instilled mice. No visible differences observed between the two left lungs. (B) Tissue culture of mice infected with hypervirulent *S. pneumoniae*. All specimen contained high load of *S. pneumoniae*. (C) Bronchoalveolar lavage fluid (BALF) smear and Gram's stain, from *K. pneumoniae* infected mice. One leukocyte found, surrounded by all thick capsular *K. pneumoniae*.

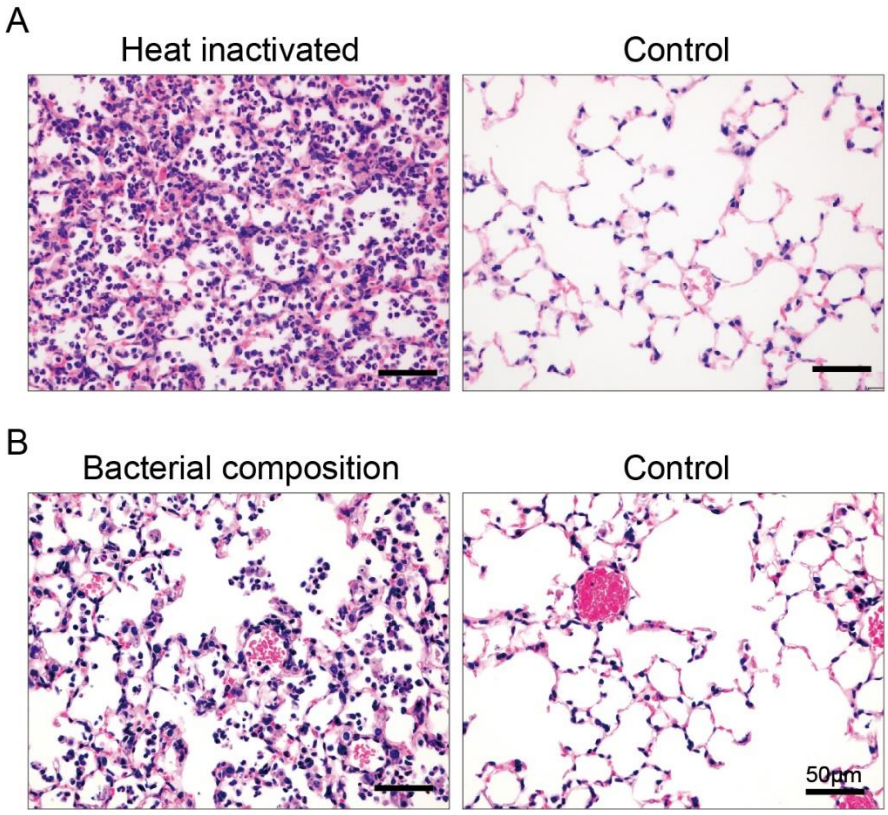

Figure S2 (A) Heat inactivated *P. aeruginosa* exoproducts (10 min, 100°C) induced neutrophil accumulation in the lung of mice, but didn't evoke hemorrhage or hyaline membrane formation. (B) *P. aeruginosa* bacterial composition induced mild neutrophil infiltration without causing hemorrhage or DAD. HE stain.

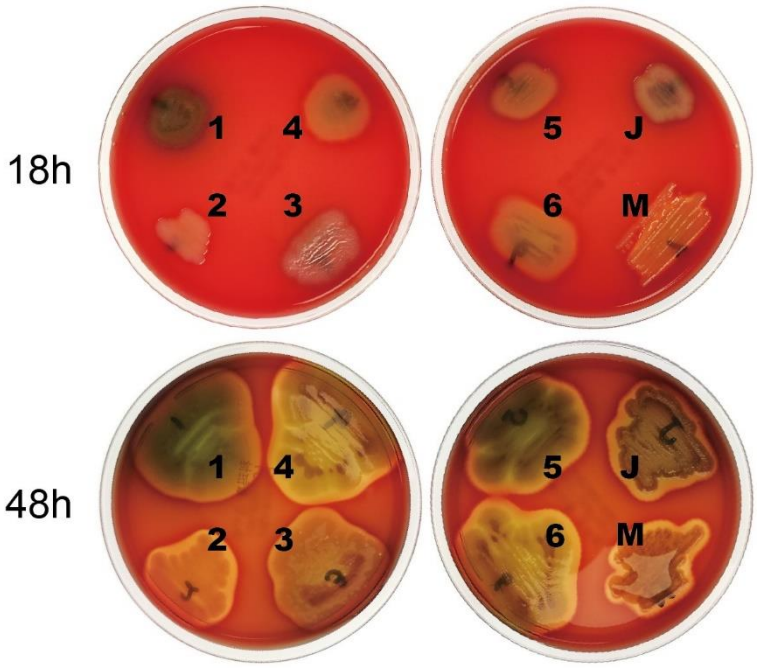

Figure S3 Morphology of 8 *P. aeruginosa* strains cultured on sheep blood agar plates for 18 h or 48 h.

Table S 1. Virulence genes analysis in clinical isolates of *Pseudomonas aeruginosa*

		PCR results							
Gene	**Protein**	Pa 1	Pa 2	Pa 3	Pa 4*	Pa 5*	Pa 6*	Pa M	Pa J
aprA	Alkaline protease	+	+	+	+	+	+	+	+
toxA	Exotoxin A	+	+	+	+	+	+	+	+
lasB	Elastase (LasB)	+	+	+	+	+	+	+	+
lasA	Elastase (LasA)	+	+	+	+	+	+	+	+
plcH	Phospholipase C	+	+	+	+	+	+	+	+
prpL	Protease IV	+	+	+	+	+	+	+	+
exoS	ExoS	+	(-)	+	+	+	+	+	+
exoU	ExoU	(-)	+	(-)	(-)	(-)	(-)	+	(-)
exoT	ExoT	+	+	+	+	+	+	+	+
exoY	ExoY	+	+	+	+	+	+	+	+
lepA	large extracellular protease	+	+	+	+	+	+	+	+
phzS	flavin-dependent hydroxylase	+	(-)	(-)	(-)	(-)	(-)	(-)	+
rhlR	QS	+	+	+	+	+	+	+	+
mucA	QS	+	+	+	+	+	+	+	+
algR	QS	+	+	+	+	+	+	+	+
fliC	flagellar filament	+	(-)	+	+	(-)	(-)	+	(-)

QS, quorum sensing.

+, positive.

(-), negative.

* Hypervirulent strains

Table S 2. Drug resistance and pigment producing of *P. aeruginosa* isolates

	Pa 1	Pa 2	Pa 3	Pa 4	Pa 5	Pa 6	Pa M	Pa J
Antimicrobial agents								
Cefoperazone-sulbactam	S	R	S	S	S	S	S	I
Meropenem	S	R	S	S	S	S	S	S
Levofloxacin	R	R	S	S	S	S	S	I
Pigment producing								
Pyocyanin	+	(-)	(-)	(-)	(-)	(-)	(-)	+

S, susceptible; I, intermediate; R, resistance.

+, positive.

(-), negative or absent.

Table S 3. Multilocus sequence typing of clinical isolates of *P. aeruginosa*

		Allele types						
	ST	acsA	aroE	guaA	mutL	nuoD	ppsA	trpE
Pa 1	3405	17	5	5	4	137	4	3
Pa 2	1076	5	4	57	62	1	1	26
Pa 3	3580*	15	5	36	11	27	189*	2
Pa 4	3118	6	5	11	11	4	4	193
Pa 5	3576*	5	4	57	62	4	1	26
Pa 6	3577*	1	178	26	3	1	4	34
Pa M	3578*	6	5	11	4	4	3	3
Pa J	871	16	3	1	5	1	55	61

ST, sequence type.

* Newly found in this research.

Table S 4. Primer sequences used in PCR for detecting virulence genes

GN		Sequence	product length (bp)
toxA	F	5’ - CTGCGCGGGTCTATGTGCC	270
	R	5’ - GATGCTGGACGGGTCGAG	
lasB	F	5’ - GCGAATTGGCCAACAGGTAG	544
	R	5’ - CCGACCAACACCTACAAGCA	
lasA	F	5’ - GCAGCACAAAAGATCCC	1075
	R	5’ - GAAATGCAGGTGCGGTC	
aprA	F	5’ - ATGTACCGGATGCGCTCAAG	506
	R	5’ - CGCCTTCTCGTTGAGGTTGA	
exoS	F	5’ - GAGAGATAGCCGTCGTCGTG	355
	R	5’ - AGAGCGAGGTCAGCAGAGTA	
exoU	F	5’ - GCTGAAGCTGTTCCAACACA	466
	R	5’ - CTGGTACGGCTGATCACTCA	
exoT	F	5’ - CAGCATGTACTCAGCGCAAG	515
	R	5’ - AACAGGGTGGTTATCGTGCC	
exoY	F	5’ - CCCTGCCATAGAATCCGTCC	525
	R	5’ - TCTCGGTGAAGGGGAAAAGC	
rhlR	F	5’ - TGCTCAGGATGATGGCGATT	507
	R	5’ - TGGGCTTCGATTACTACGCC	
mucA	F	5’ - GGAAACTCTGTCCGCTGTGA	522
	R	5’ - CTCTCTGTACCACTGACGGC	
algR	F	5’ - ACGTACTTGTGGTCGGCAAT	450
	R	5’ - ATTGGTAGGGCAACTGGACG	
lepA	F	5’ - GCGGGTGAAAGAGGAAATCG	567
	R	5’ - CTTTCCGGCTCGTATTGCAG	
phzS	F	5’ - CTGGTCGCCTATCCGATCTC	507
	R	5’ - GCTCTTCTCGGTCTTCGGTC	
plcH	F	5’ - CCAGGGCGAGATGTTTTCCT	528
	R	5’ - CTCGCCTGGTTCAGGAACTT	
fliC	F	5’ - CTGACCATCACCTCCGCTAC	443
	R	5’ - CGAGCGTTGGTAGCGTTTTC	
prpL	F	5’ - TATGGATCCGCCGGCTACCGCGACGG	733
	R	5’ - GCCTCGAGGGGCGCGAAGTAGCGGGAGA	

Supplemental experimental procedures

Unilateral intubation and instillation

To perform unilateral lung intubation, a 24G, 19mm long intravenous indwelling catheter (Introcan, BRAUN) was used as the intratracheal tube and inserted through a cervical incision. Mice were anesthetized by 1% pentobarbital sodium (50mg/Kg) intraperitoneally and put to a proper supine position. The anterior cervical hair was removed and the skin disinfected with 75% ethanol. In order to expose the trachea, a small longitudinal incision was made. The indwelling catheter was then punctured gently into the trachea between the second and third cricoid cartilage. As anatomical data shows that the mouse main trachea is approximately 10mm long, therefore the 19mm cannula full length inserted will enter the left or right bronchus. Left bronchus intubation was achieved when the insertion angle is 5-10° to the anterior median line as indicated in Figure 1A. Then, 25μl bacteria suspension or 30μl *P. aeruginosa* exoproducts were deposited into the left lung of each mouse using microliter syringes. After instillation, mice were placed on a warm blanket, resting to left recumbent position until fully awake.

Enrichment culture

For enrichment culture, non-fastidious bacteria: *Staphylococcus aureus, Streptococcus agalactiae, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* were inoculated into LB broth (Beyotime, China) incubated overnight at 36±1°C, 60rpm. Fastidious bacteria: *Streptococcus pneumoniae* was cultured on sheep blood agar plates (Yi Hua, Shanghai) overnight at 36±1°C, 5%CO2. Bacteria from exponential

growth were collected, washed twice and resuspended in sterile normal saline (NS). The turbidity was adjusted to 3 McFarland standards (MCF), which equals 9×10^8 CFU/ml, by the standard procedure for later use. Each of the 7 species was isolated and prepared as above three times randomly.

PCR and MLST

A single colony of *P. aeruginosa* was added to 1ml ddH2O, washed twice and resuspended. We extracted bacterial DNA by boiling the suspension for 10 min at 100 °C. Using pairs of specific primers (Table S4), we examined the presence or absence of the following genes among clinical isolates of *P. aeruginosa*: aprA, toxA, lasA, lasB, plcH, prpL, exoS, exoY, exoT, exoU, lepA, phzS, rhlR, mucA, algR and fliC. PCR amplifications were performed in 50μl reaction mixtures. Based on product size (Table S4), genes were identified and recorded as positive (+) or negative (-).

For multilocus sequence typing, seven house-keeping genes: acsA, aroE, guaA, mutL, nuoD, ppsA, trpE were amplified and sequenced (Sangon, Shanghai) according to standard procedures[1]. Allele type and sequence type were acquired by querying on PubMLST website[2].

Gelatin zymography

8% gel containing 0.1% gelatin was used in SDS-PAGE. *P. aeruginosa* exoproducts (2mg protein/ml) were preincubated without or with 25 mM EDTA for 30 min at room temperature. 3.5μg of each sample (measured by protein) was mixed with non-reducing loading buffer (1%

SDS added), without boiling, loaded at interval wells to avoid cross-contamination. Electrophorese was performed at 4 °C, 110 V constant voltage. The gels were washed twice before transferred to incubate within 200ml development buffer (Tris-HCl 50 mM pH 7.5, CaCl2 5mM, NaCl 0.2 M, Brij-35 0.02%) for 16–20 h at 37 °C. At last, gels were stained by Coomassie blue dye R-250 (0.25%) for 1 h, decolorized by destainer (methanol 20%, acetic acid 10%, 70%ddH_2O) until clear bands were seen, and scanned by Amersham Imager (AI680, GE).

Fibrinogenolytic and fibrinolytic assay

Fibrinogenolytic activity was assayed using bovine fibrinogen as a substrate. Exoproducts of Pa 1-6, M, J (1 mg protein/ml) were preincubated with Ilomastat at room temperature for 30 min. 100 µl of fibrinogen solution (4 mg/ml in saline) was mixed with 10 µl of each sample and incubated at 37 °C for 1 h. 5µl of the product from each sample was subjected to SDS-PAGE.

For the fibrinolytic activity, 50 µl fibrinogen (6 mg/ml in PBS) and 30 units of thrombin were mixed in a 200 µl PCR tube, and preincubated for 15 min at 37 °C to form the fibrin clot. Then, 15 µl exoproducts (1 mg protein/ml) were added to each tube and incubated for 1 h at 37 °C. The sample was gently stirred using a syringe needle to check for fibrin fibers, and the solubility of fibrin was observed.

Thrombin degradation assay

Exoproducts of Pa 1-6, M and J (1 mg protein/ml) were preincubated with 100 mM EDTA at room temperature for 30 min. 2µl of each sample was mixed with 100 µl bovine thrombin

solution (2 mg/ml) and incubated at 37 °C for 1 h. 5µl of the mixture was subjected to SDS-PAGE. The thrombin activity was also measured, by mix 5µl of each product with 50 µl fibrinogen (6 mg/ml in PBS), incubated at 37 °C for 15 min and then checked for fibrin fibers formation.

Purification of LasB

P. aeruginosa strain Pa 4 was cultivated on Columbia agar plates (90mm) supplied with 5% sheep blood at 37°C for 3 days (For each chromatography column 8 plates were used). Each plate was washed by 8-10ml 20 mM Tris-Cl buffer (pH 8.0). After centrifugated for 3200 × g, 60 minutes, the pellet (bacterial cells) was discarded. Ammonium sulphate was added to the supernatant up to 20% saturation, sediments removed by centrifugation at 3200 × g for 30 min, and continued up to 80% saturation, precipitates were collected by the same way and resuspended in 20 mM Tris-Cl buffer (pH 7.5). This crude enzyme solution was desalted using ultra centrifugal filters of 10 kDa cutoff value (Amicon Ultra, Merck) and then loaded on a 15 mm× 200 mm DEAE cellulose column pre-equilibrated with 20 mM Tris-Cl buffer (pH 7.5). After washing the column with the same buffer, elastase was eluted by applying a linear gradient of NaCl from 0 to 1000 mM at a flow rate of 0.34 ml/min. Fractions of 2.5 ml were collected. Activity of the fractions were measured using EnzCheck Elastase Assay Kit (Thermo Fisher) according to the manufacturer's instructions. Active fractions were concentrated by ultracentrifugation described as above and stored -20°C for later use.

Supplemental results

Virulence genes, drug resistance and MLST analysis of 8 *P. aeruginosa* strains

Virulence of *P. aeruginosa* is typically attributed to its type I secretion system (T1SS), type II secretion system (T2SS), type III secretion system (T3SS), quorum sensing (QS), antibiotic resistance and pyocyanin production. To find the common pathogenic mechanism of the 8 *P. aeruginosa* strains in lung, we set up PCR tests to examine the possible virulence genes. As shown in Table S1, all 8 strains tested are positive with gene of T1SS including aprA, T2SS including toxA, lasB, lasA, plcH, prpL and QS including rhlR, mucA and algR. Differences were detected in genes of T3SS (exoS, exoT), and bacterial structure genes (phzS, fliC), whereas, they were not correspondent to the bacteria virulence in lung tested earlier (Figure 2).

For drug resistance (Table S2), Pa 2 showed the strongest resistance towards all three antibiotics tested: Cefoperazone-sulbactam, Meropenem and Levofloxacin. However, the highly lung destructive strains Pa 4, 5, 6 were sensitive to all 3 antibiotics, meaning the virulence were not related to drug resistance either. Pyocyanin as the main virulence factor was also ruled out, for only Pa 1 and Pa J produced pyocyanin.

Since Pa 4, 5, and 6 were quite similar judging from morphology and injury effects (Figure S3), we performed multilocus sequence typing (MLST) to identify if they were actually the same clone. MLST results (Table S3) showed all 8 isolates have different sequence types. From them, four new sequence type of *P. aeruginosa* (Pa 3, 5, 6 and M) were identified. And a new allele type of ppsA from Pa 3 was found.

Supplemental references

1. Curran, B., et al., *Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa.* J Clin Microbiol, 2004. **42**(12): p. 5644-9.

2. Jolley, K.A., J.E. Bray, and M.C.J. Maiden, *Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications.* Wellcome Open Res, 2018. **3**: p. 124.