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The Mla pathway is critical for *Pseudomonas aeruginosa* resistance to outer membrane permeabilization and host innate immune clearance

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Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen that has become a serious problem due to increased rates of antibiotic resistance. Due to this along with a dearth in novel antibiotic development, especially against Gram-negative pathogens, new therapeutic strategies are needed to prevent a post-antibiotic era. Here, we describe the importance of the vacJ/Mla pathway in resisting bactericidal actions of the host innate immune response. *P. aeruginosa* tn5 transposon mutants in genes from the VacJ/Mla pathway showed increased susceptibility to killing by the host cathelicidin antimicrobial peptide, LL-37, when compared to the wild-type parent strain. The *P. aeruginosa vacJ⁻* mutant demonstrated increased membrane permeability upon damage as well as sensitivity to killing in the presence of the detergent sodium dodecyl sulfate and the divalent cation chelator EDTA. When exposed to human whole blood and serum complement, the *vacJ⁻* mutant was killed more rapidly when compared to the wild-type parent strain and complemented mutant. Finally, in an in vivo mouse lung infection model, infection with the *vacJ⁻* mutant resulted in reduced mortality, lower bacterial burden, and reduced lung damage when compared to the wild-type strain. This study highlights the potential in therapeutically targeting the VacJ/Mla pathway in sensitizing *P. aeruginosa* to killing by the host innate immune response.

Key messages

- The Mla pathway regulates outer membrane dynamics in human pathogen *Pseudomonas aeruginosa (PA)*.
- Disruption of Mla pathway gene *vacJ* sensitizes PA to host cathelicidin antimicrobial peptide LL-37.
- Loss of *vacJ* expression renders PA more sensitive to human whole blood and serum killing.
- Loss of *vacJ* expression reduces PA survival and virulence in a murine lung infection model.
- The Mla pathway merits exploration as a pharmacologic target to sensitize PA to host innate immunity.

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Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative facultative anaerobic bacterium that inhabits both soil and aqueous environments. A leading opportunistic human pathogen, *P. aeruginosa*, is highly adaptable and intrinsically able to resist diverse antibiotic classes posing a tremendous challenge to medical management [1]. *P. aeruginosa* preferentially colonizes immunocompromised patients thereby causing many nosocomial infections including ventilator-associated pneumonia, urinary tract infections, bacteremia, and infections of surgical sites or burn wounds [1, 2]. Individuals with cystic fibrosis are at extreme risk of chronic pulmonary infection with *P. aeruginosa*, with 47.5% of patients being colonized [3].

P. aeruginosa can also cause community-acquired infections including keratitis, otitis externa, and skin and soft tissue infections. Therapeutic options are dwindling due to a decrease in novel antibiotic development as well as an emergence of highly multidrug-resistant *P. aeruginosa* strains, placing the pathogen on the Centers for Disease Control list of "serious threats" [4]. The discovery of new targets and alternate strategies for treating *P. aeruginosa* infections is imperative.

Gram-negative bacteria such as *P. aeruginosa* are protected by their outer membrane (OM), which is highly impermeable to toxic compounds such as detergents, bile salts, and antibiotics [5]. This OM is composed of an asymmetric bilayer containing lipopolysaccharide (LPS) on the outer leaflet and phospholipids (PLs) on the inner leaflet. Perturbation of the OM can lead to accumulation of PLs on the outer leaflet, which can make the bacteria vulnerable to further OM damage. The Mla system is involved in maintaining OM lipid asymmetry and is composed of seven proteins that are distributed across the cell envelope [6, 7]. VacJ (also known as MlaA) and other components of the Mla pathway have been shown to play a role in intracellular spread of *Shigella flexneri* [8, 9], for survival of *Haemophilus influenzae* during lung infections and in serum [10, 11], resistance to surfactants in *Escherichia coli* [6, 12, 13] and *Pseudomonas putida* [14], antibiotic resistance in *P. aeruginosa* [15, 16] and *Salmonella enterica* serovar Typhimurium [17], and has a possible role in the creation of outer membrane vesicles [18]. VacJ has also been explored as a possible vaccine target in *Pasteurella multocida* [19]. Genes encoding components of the Mla pathway, including VacJ, are upregulated in response to colistin, and mutations in the pathway have been observed in colistin-resistant mutants [20–22].

Among the critical frontline effectors of host innate immune defense are endogenous cationic antimicrobial peptides (AMPs), which are produced by epithelial cells and circulating or tissue-resident leukocytes, and are known to provide protection against invasive bacterial infections [23, 24]. Cathelicidins are a family of mammalian AMPs with broadspectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as certain fungi, viruses, and protozoan parasites [25]. The human cathelicidin AMP, LL-37, is an alpha-helical, amphipathic, and anionic peptide that integrates and destabilizes bacterial cell membranes causing bacterial death [26]. LL-37 also has important immunomodulatory roles in the proper regulation of cytokine release, chemotaxis, and antigen presentation (reviewed in [27]). Animals deficient in cathelicidin are hyper-susceptible to bacterial infection in multiple organs including the skin [28], lung [29, 30], gut [31, 32], brain [33], kidney [34], and eye [35]. A bacterial pathogen producing significant clinical disease in the human host must maintain some level of functional resistance to endogenous AMPs including cathelicidin in order for infection to persist [36, 37].

In this study, we explored for the first time the role(s) of the Mla pathway in *P. aeruginosa* resistance to host innate immune defenses and animal virulence. Though failing to show a growth defect in standard bacteriological and tissue culture media conditions, *P. aeruginosa* Mla pathway mutants showed increased susceptibility to human cathelicidin antimicrobial peptide LL-37 associated with increased membrane permeability. Compared to wild-type parent strain, an isogenic *P. aeruginosa vacJ⁻* mutant showed increased susceptibility to LL-37 and the cationic peptide antibiotics colistin and polymyxin B, along with decreased survival in human whole blood and serum containing active complement; wild-type resistance to these factors was restored by complementation of the *vacJ⁻* mutant with the wild-type gene with a transposon insertion. The *P. aeruginosa vacJ⁻* mutant showed decreased virulence in a murine in vivo pneumonia model, as measured by lower bacterial burden in the lungs and blood, diminished lung injury and inflammation, and reduced mortality. Together, our results show the importance of the Mla pathway in *P. aeruginosa* resistance to the bactericidal activities of the innate immune system.

Materials and methods

Bacterial strains and reagents

P. aeruginosa PAO1 [38] along with corresponding transposon Tn5 IS50L mutants [39] disrupting the genes encoding *mlaA* (*vacJ*) and *mlaC-F* were generously provided by Professor Colin Manoil (University of Washington, Seattle, WA). Strains were propagated in Luria broth (LB) medium (Hardy Diagnostics) or on LB agar (LA) plates at 37 °C. For infection studies, bacteria were grown to mid-log phase (OD$_{600 \text{ nm}}$ = 0.4) before diluting to experimental inoculum. Complementation was performed as previously described [40]. Briefly, *P. aeruginosa vacJ* gene and its upstream region were cloned into pUC18T-mini-Tn7T-*hph* using primers PA2800-miniTn7t-F (TGCTGCAAAGCCTGCACGGA TCCACTAGTGAGCTCATGC), PA2800-miniTn7t-R (GAGGTGGAGGACGACTTCTAACCAGATAAGTG AAATCTAGTTCAAAAC), miniTn7t-PA2800-F (GCATGAGCTCACTAGTGGATCCGTGCAGGCTT TGCAGCA), and miniTn7T-PA2800-R (GTTTGGAA CTAGATTTCACTTATCTGGTTAGAAGTCGTCCTCCA CCTC). PA2800-miniTn7t-F and -R primers were used to amplify the pUC18T-mini-Tn7T-*hph* PCR product with portions of the *vacJ* gene, while primers miniTn7t-F and -R were used to amplify the *vacJ* gene with a portion of pUC18T-mini-Tn7T-*hph*. PCR products were combined 1:1 and electroporated into *E. coli* TOP10 cells, and transformants selected on LB agar plates containing 100 μg/mL Hygromycin B (Life Technologies). qPCR of wild-type,

vacJ mutant, and complemented strains was performed on RNA isolated using RNeasy mini kit (Qiagen) and following primers: PAO1_PA2800_qPCR_F (GCGTACGTCGTCCA TGTAGT), PAO1_PA2800_qPCR_R (CAGGCCAA GTTCCACAATGC), PAO1_16s_F (CCCAACATCTCACG ACACGA), and PAO1_16s_R (ACGCGAAGAACCTT ACCTGG).

Antibiotics and antimicrobial peptides

For in vitro studies, colistin sulfate (Sigma-Aldrich) stock solutions were prepared in molecular biology grade water at 10,000 mg/L and frozen at − 80 °C. LL-37 and TAMRA fluorescently tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular biology grade water at 640 and 320 μM, respectively, and stored at − 80 °C. LL-37 minimum inhibitory concentration (MIC) assays were performed in RPMI containing 5% LB with 5×10^5 cfu/mL in 96-well round bottom plates (Corning) while all others were performed in cation-adjusted Mueller Hinton broth (Hardy Diagnostics). All MICs were performed by serial 2-fold dilutions, repeated 3 times.

NPN bacterial outer membrane permeability assay

Assay was performed as previously described [41]. Briefly, overnight cultures of *P. aeruginosa* were grown in LB at 37 °C while shaking, washed twice with PBS via centrifugation at $3220 \times g$ at room temperature, and resuspended to $OD_{600\ nm}$ of 0.40 in 6 mL of RPMI + 5% LB. LL-37 was added to a final concentration of 4 μM to a subset of the bacterial cultures. Cultures were shaken at 37 °C for 1 h, spun at $3000 \times g$ at room temperature for 5 min, and resuspended in 0.5 mL of 10 mM Tris buffer pH 8.0. The concentrated 0.5 mL cultures were used to prepare 1 mL bacterial stocks at $OD_{600\ nm}$ of 0.40 in 10 mM Tris. Assays were conducted in a final volume of 200 μL in triplicate in 96-well flat bottom plates (Costar). Four conditions were tested: (1) 100 μL of bacterial stock + 50 μL of NPN (40 μM final) + 50 μL of 10 mM Tris, (2) 100 μL of bacterial stock + 50 μL of NPN + 50 μL of EDTA (10 mM final), (3) 100 μL of bacterial stock + 50 μL of EDTA + 50 μL of 10 mM Tris, and (4) 100 μL of 10 mM Tris + 50 μL of NPN + 50 μL of EDTA. As soon as all of the components were added and mixed, plates were immediately read in a fluorescent plate reader: excitation 250 nm and emission 420 nm. The NPN fluorescence signal from conditions (3) and (4), background, was subtracted from the signals measured from conditions (1) and (2). The NPN intensity from condition (1) bacteria + NPN was divided by the NPN signal measured from condition (2) bacteria + NPN + EDTA to obtain the percentage of permeability recorded in the presence of 10 mM EDTA which permeabilizes the outer membrane of Gram-negative bacteria.

Whole blood and serum killing

Heparinized blood was collected from healthy adult volunteers and placed in siliconized tubes. 1×10^6 cfu of *P. aeruginosa* in RPMI was added to whole blood to make final concentration 90% whole blood in 100 μl volume. Samples were taken at time 0, 15, 30, 45, and 60 min, serially diluted, and plated on LB agar plates for cfu enumeration. Serum was collected from healthy volunteers by collecting blood in serum separating tubes (BD) which were spun at $1300 \times g$ for 10 min to separate serum from erythrocytes and other cells. Collected serum was pooled and split; half was heat-inactivated at 56 °C for 30 min while the other half was stored at room temperature for 30 min. Serum was partitioned into wells of a 96-well round bottom plate and 1×10^6 cfu of *P. aeruginosa* wild-type, *vacJ*¯, or complemented mutant in 10 μl RPMI was added to a final serum concentration of 80% in 100 μl volume. Samples were collected at 60, 120, and 180 min, serially diluted, and plated on LB agar for enumeration. Experiments were performed in triplicate.

Murine pneumonia model

Eight-week-old female C57BL/6J mice (Jackson Labs) were used. *P. aeruginosa* cultures were grown overnight in LB at 37 °C with shaking, washed twice with PBS, diluted 1:50 in fresh LB, and grown to mid-log phase. Bacteria were washed twice with PBS via centrifugation at $3220 \times g$ at room temperature and concentrated in PBS to yield the appropriate inoculum. For histopathology, lung bacterial load, and blood bacterial load, 4×10^6 cfu per 30 μL was used to infect each animal (WT infection $n = 10$; *vacJ*¯ infection $n = 10$). Mice were anesthetized with 100 mg/kg ketamine + 10 mg/kg xylazine. Once sedated, the vocal cords were visualized using an operating otoscope (Welch Allyn) and 30 μL of bacteria or PBS instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery. After 24 h, mice were sacrificed with CO_2 and the lungs were collected for bacterial counts and histopathological analysis. To enumerate bacteria remaining in the lungs, the organs were placed in a 2 mL sterile micro tube (Sarstedt) containing 1 mL of PBS and 1 mm silica beads (Biospec). The lungs were homogenized by shaking at 6000 rpm for 30 s ($\times$ 3) using a MagNA Lyser (Roche), with the specimens placed on ice as harvested. Aliquots from each tube were serially diluted for enumeration on LB agar plates. For blood counts, mice were infected as described and sacrificed 24 h post-infection and blood obtained by cardiac puncture. For the *P. aeruginosa* survival experiment, 8.5×10^6 cfu was given in 30 μL of PBS (WT infection $n = 15$; *vacJ*¯ infection $n = 15$); animals were monitored every 12 h for 7 days. All animal studies were performed under protocols approved by the UCSD Institutional Animal Use and Care Committee with

national and local guidelines in place to maximize humane animal treatment.

Histopathologic scoring

A veterinary pathologist (IC), blinded to treatment, scored the lung lesions from five mice infected with WT *P. aeruginosa* and five mice infected with the *vacJ*⁻ strain based on severity of bacterial load, neutrophilic inflammatory cell infiltration, tissue damage/necrosis, and percent lung tissue involvement. The lungs were then ranked 1–10 from most to least severe based on a composite of lesion severity and distribution. Lesion ranks from the two groups (WT and *vacJ*⁻) were compared by Mann-Whitney test (two-tailed analysis).

Imaging

Tissue sections were imaged on a Nikon E800M microscope using a Nikon DSRi2 camera and Nikon Elements D Version 4 software. Each section was imaged using the same white balance and shading correction settings. Whole image sharpness, contrast, and brightness were uniformly adjusted with Photoshop Elements version 11 software.

Results

Loss of function *P. aeruginosa mla* mutants are hypersusceptible to antimicrobial peptides

In recent years, the roles of the Mla pathway to resist stresses to the OM have been described; it is involved in resistance to hydrophobic compounds [14, 42], ethanol [14, 42], serum [11], and certain antibiotics [15]. To determine if *P. aeruginosa* mutants containing transposon insertions in genes encoding Mla pathway proteins (Fig. 1a) were susceptible to outer membrane attack by the human cathelicidin antimicrobial peptide LL-37, a minimum inhibitory concentration (MIC) assay was performed. Wild-type *P. aeruginosa* consistently resisted the bactericidal action of LL-37 up to a concentration of 16 μM while all *mla* transposon insertion mutants revealed a consistent 2-fold reduction in MIC at 8 μM (Fig. 1c). We next complemented one of the *mla* mutants, *vacJ*⁻ with a mini-Tn7t transposon containing the *P. aeruginosa* PAO1 *vacJ* gene. Complementation restored over half (58%) of transcript levels back to the mutant (Fig. 1b). A wild-type LL-37 resistance phenotype was restored to the complemented *vacJ*⁻ mutant (Fig. 1c). Challenging the *P. aeruginosa vacJ*⁻ mutant with the murine cathelicidin related antimicrobial peptide (mCRAMP) yielded a similar 2-fold increased sensitivity compared to the wild-type and the *vacJ*⁻ complemented mutant strains (Table 1). Acceleration in LL-37 killing kinetics of *vacJ*⁻ mutant lacking a functional Mla system was observed in time course assays at

16 μM (Fig. 1d) and 8 μM (Fig. 1e) LL-37 compared to both the wild-type and the complemented strains.

The related cationic peptide antibiotics colistin and polymyxin B are increasingly being used as last-line agents for treatment of patients with highly multidrug-resistant strains of *P. aeruginosa* [43, 44]. Since these agents have similar charge characteristics and mechanisms of action to the host defense peptide LL-37, we examined the effect of the *vacJ* inactivation on sensitivity to these therapeutic agents. MIC testing revealed a 2-fold increase in susceptibility of the *vacJ*⁻ mutant *P. aeruginosa* to both polymyxin B and colistin, which was restored to wild-type resistance levels upon genetic complementation (Table 1). Sensitivity to colistin has been observed before in the *mlaE* mutant of *P. aeruginosa* showing that this is conserved [16]. These results do not match previously reported MIC values for *P. aeruginosa vacJ* mutant, but this could be due to the use of different media in antibiotic susceptibility tests than a more standard methods [15]. These results show that the Mla pathway is needed in order to effectively resist the bactericidal effects of antimicrobial peptides that target the outer membrane.

P. aeruginosa mla-deficient mutants are more susceptible to cell wall stress

To ensure that cationic peptide hypersusceptibility of the *vacJ*⁻ mutant did not represent a general growth defect, we confirmed equal logarithmic growth properties throughout 24-h incubation in standard bacteriologic media compared to the WT parent strain (Fig. 2a). Bacteria grown in RPMI supplemented with 5% LB also demonstrated no difference in growth rates between the wild-type, *vacJ*⁻ mutant, and complemented strain in a 24-h period, though they reached a lower optical density compared to when grown in LB. To probe further into OM barrier function, we grew *P. aeruginosa* wild-type, *vacJ*⁻ mutant, and complemented strains on agar media containing 0.5% sodium dodecyl sulfate (SDS) with varying amounts of the divalent cation chelator ethylenediaminetetraacetic acid (EDTA), known to destabilize LPS bonds and increase OM permeability. We saw an increase in SDS-EDTA sensitivity of the *P. aeruginosa vacJ*⁻ mutant compared to the wild-type and complemented strains with increasing EDTA concentrations (Fig. 2b). To quantify the degree of permeability engendered, we performed an assay using the small molecule n-phenyl-1-napthylamine (NPN) which fluoresces only when it is in a hydrophobic environment, in this case the inner membrane of Gram-negative bacteria, reflecting leakiness of the OM to allow small molecules to passively cross [45]. No significant difference in NPN fluorescence was observed between *P. aeruginosa* wild-type and *vacJ*⁻ mutant at baseline (Fig. 2c), but upon exposure to 4 μM LL-37, a 2-fold increase in permeability was seen in the *vacJ*⁻ mutant compared to wild-type. The complemented strain demonstrated reduced permeability to
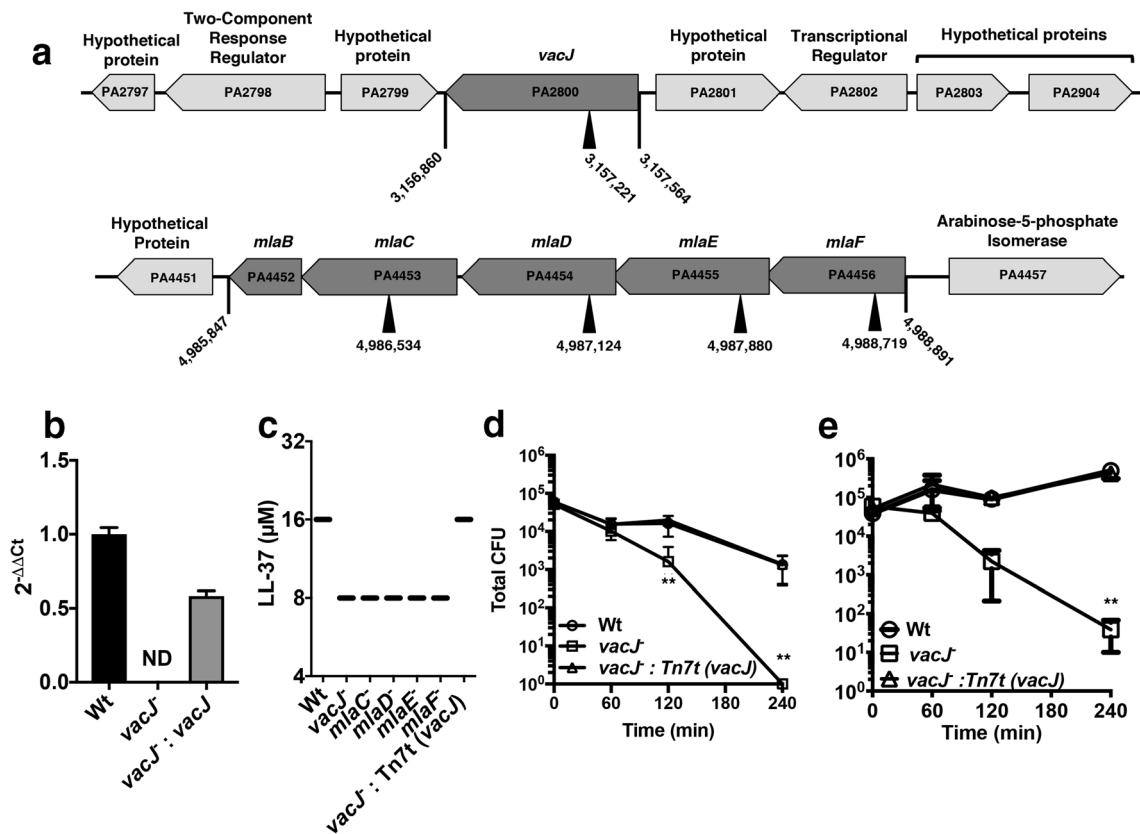
Fig. 1 *P. aeruginosa mla* deficient mutants demonstrate increased sensitivity to antimicrobial peptide LL-37. Position of transposons for all mla deficient strains are denoted by black arrows (**a**). qPCR of transcript levels for *vacJ*, normalized to 16S rRNA and Wt levels (**b**).

LL-37 MIC for *P. aeruginosa* wild-type, *mla* deficient mutants, and *vacJ⁻* complemented strains. MIC results were performed 3 times (**c**). LL-37 killing kinetics of *P. aeruginosa* WT, *vacJ⁻*, and *vacJ⁻* complemented strains at 16 μM (**d**) and 8 μM (**e**). **P < 0.01 *t* test

NPN at baseline and upon LL-37 treatment (Fig. 2c). These results are consistent with a mechanism in which sensitivity to antimicrobial peptides can be attributed to an impaired OM repair in the Mla pathway mutant.

The *P. aeruginosa vacJ⁻* mutant is susceptible to whole blood or serum killing

To further examine the *P. aeruginosa vacJ⁻* mutant's ability to withstand outer membrane attack generated in the context of the innate immune system, we used freshly isolated human whole

Table 1 Summary of antimicrobial MICs on *P. aeruginosa* wild-type, *vacJ⁻* mutant, and complemented strain

	Pseudomonas aeruginosa PAO1		
Antimicrobial	Wt	*vacJ⁻*	*vacJ⁻:vacJ*
Cathelicidin LL-37	16 μM	8 μM	16 μM
mCRAMP	> 64 μM	32 μM	> 64 μM
Colistin	1 μg/mL	0.5 μg/mL	1 μg/mL
Polymyxin B	0.5 μg/mL	0.25 μg/mL	0.5 μg/mL

blood that contains several OM active components (complement, platelet, and neutrophil-derived antimicrobial peptides and proteases, antibodies, etc.). Within 1 h of incubation in human whole blood, we observed a log-fold increase in killing of the *vacJ⁻* mutant compared to *P. aeruginosa* wild-type strain (Fig. 3a); complementation of the mutant with the *vacJ* gene restored blood survival back to wild-type levels. Likewise, when placed in 80% human serum for 1 h, the *P. aeruginosa vacJ⁻* mutant exhibited a nearly 2-log-fold reduction in survival compared to wild-type and complemented mutant strains (Fig. 3b). When the serum was heat-inactivated to degrade active complement, the survival differences between wild-type, *vacJ⁻* mutant, and complemented mutant strain were lost. This finding illustrates the importance of having an intact Mla pathway to withstand serum- and complement-dependent killing in blood, consistent with experiments performed on the pathway in the Gram-negative coccobacillus *Haemophilus influenzae* [11].

The *P. aeruginosa* Mla pathway is essential for full virulence in murine pneumonia model

To determine the essentiality of the Mla pathway during *P. aeruginosa* lung infections, we used a murine pneumonia

Fig. 2 *P. aeruginosa mla* mutants are susceptible to OM stress with no defects in growth. Growth curves of *mla* mutants in Luria broth (LB) (**a**). Serial culture dilutions spotted on to Luria broth agar (LA), LA + 0.5% SDS and 0.25 mM EDTA, and LA + 0.5% SDS and 0.5 mM EDTA (**b**). Representative graph of NPN fluorescent assay measuring outer membrane permeability in either RPMI + 5% LB or RPMI + 5% LB containing 4 μM LL-37 (**c**). **$P < 0.01$, ***$P < 0.001$ two-way ANOVA
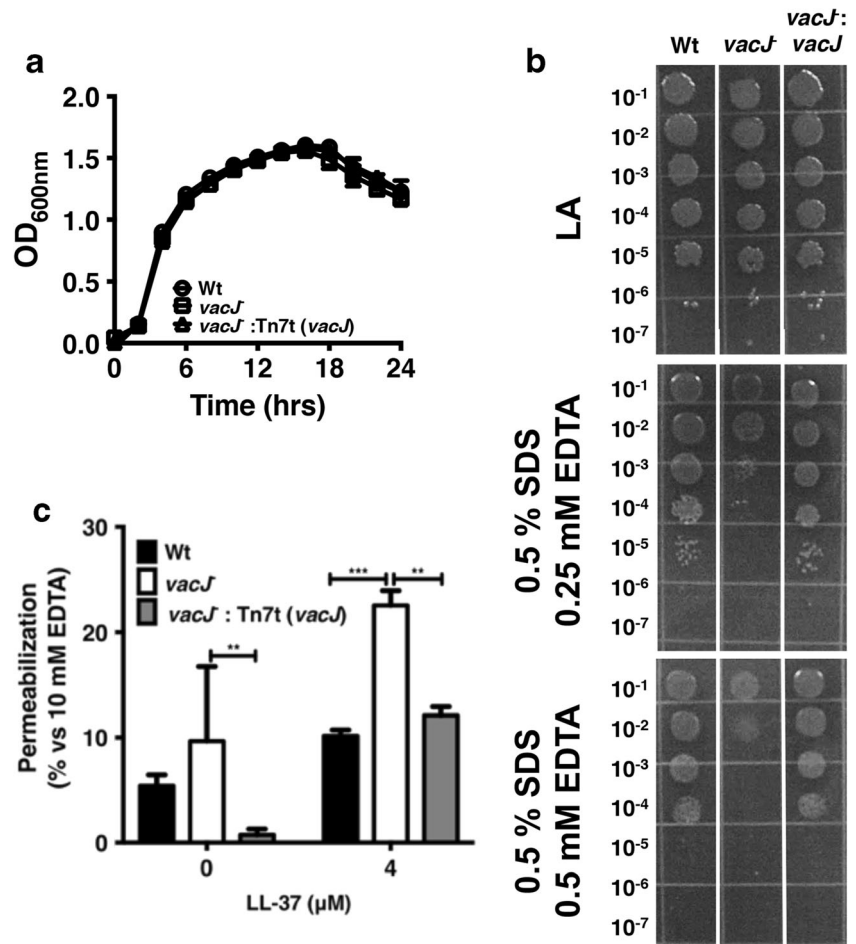
model in which 8.5×10^6 cfu of either *P. aeruginosa* wild-type or *vacJ* mutant were administered intratracheally to 8-week-old female C57BL/6 mice. Mouse survival, bacterial load, bacteremia, and histopathology of the lungs of infected mice were assessed. *P. aeruginosa* wild-type infection led to 100% mortality within 3 days while the *vacJ*-infected mice had 40% survival at the same time point and a final survival of 27% at the end of the 7-day experiment ($P < 0.05$) (Fig. 4a). In a separate cohort of mice, the lungs were harvested and

homogenized to determine the bacterial burden 24 h post-infection (hpi). A 2-fold reduction in *vacJ* mutant bacterial recovery compared to a wild-type infection was found ($p < 0.05$) (Fig. 4b). At this time point, we also observed a 3-fold reduction in bacterial cfu in the blood of mice infected with the *P. aeruginosa vacJ* mutant compared to wild-type; 4 of the 6 *vacJ*-infected mice had bacterial counts below 1×10^6 cfu/mL while 5 of 6 wild-type infected mice had bacterial counts greater than 1×10^6 cfu/mL ($P < 0.05$) (Fig. 4c). The lungs

Fig. 3 Ex vivo survival of *P. aeruginosa mla* defective mutant *vacJ*. 2.5×10^6 bacterial cfu in RPMI was placed in heparinized human whole blood to make the final concentration 90% blood in a final volume of 200 μL (**a**). 1×10^6 bacterial cfu in RPMI was placed in either serum (S) or heat-inactivated serum (HIS) so the final concentration was 80% serum in a final volume of 200 μL (**b**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ two-way ANOVA
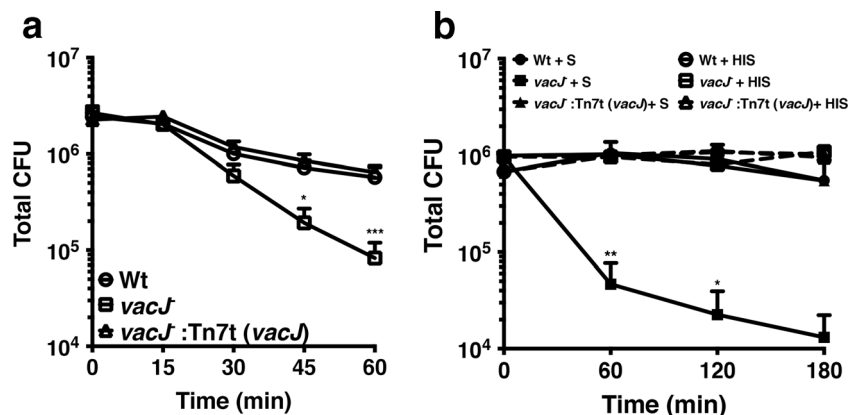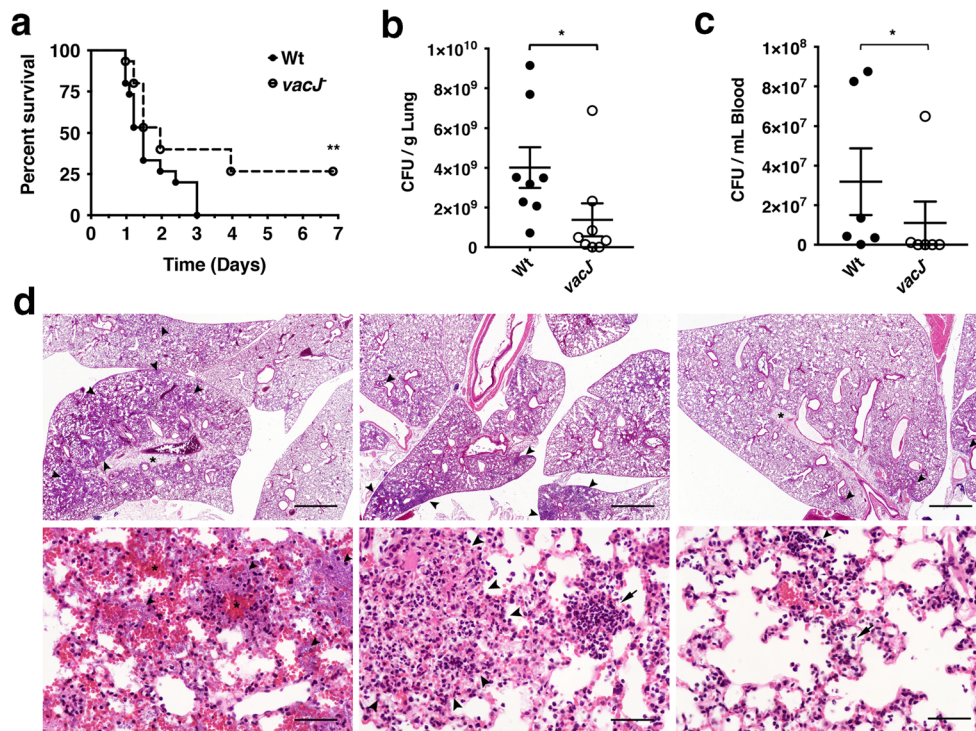
Fig. 4 Effect of *vacJ* mutation on *P. aeruginosa* virulence in murine pneumonia model. Mice (*n* = 15 per group) were infected with *P. aeruginosa* wild-type or *vacJ*⁻ (8.5 × 10⁶ cfu/30 μL PBS) intratracheally and observed for survival over a period of 7 days; *P < 0.05 log-rank (Mantel-Cox) test (**a**). Mice (*n* = 8 per group) were infected with *P. aeruginosa* wild-type or *vacJ*⁻ (4 × 10⁶ cfu/30 μL) intratracheally and sacrificed 24 h post-infection. The lungs were harvested (**b**) and blood collected (**c**) for bacterial enumeration. *P < 0.05 two-tailed Mann-Whitney test. **d** Top panels (scale bar = 1 mm). Left: Wt infected mouse 4, severity rank 1. Middle: *vacJ*⁻ infected mouse 2, severity rank 5. Right: *vacJ*⁻ infected mouse 1, severity

rank 10. Alveoli (arrowheads) contain bacteria, inflammatory exudate, and hemorrhage. The interstitium is markedly expanded by edema (asterisks). Bottom panels (scale bar = 50 μm). Left: Wt infected mouse 4, severity rank 1. The alveolar walls are coated by rod-shaped bacteria (arrowheads) and multifocal hemorrhage is present (asterisks). Middle: *vacJ*⁻ infected mouse 2, severity rank 5. The pulmonary architecture is disrupted by a rare focus of necrosis (outlined by arrowheads) and alveoli are occasionally filled with neutrophils. Right: *vacJ*⁻ infected mouse 1, severity rank 10. Small aggregates of (arrowhead) and scattered individual neutrophils (arrow) are present in alveoli

from five mice infected with either wild-type or *vacJ*⁻ *P. aeruginosa* were examined by histology (Table 2 and Fig. 4d). Lesions were significantly more severe and widely disseminated in the wild-type infected group (*p* = 0.036). Four of the five mice in the wild-type group had moderate to severe lesions affecting approximately 30–50% of the lung tissue. Notably, large numbers of rod-shaped bacteria coated the alveolar walls and occasionally formed aggregates with degenerate neutrophils within the airways. Alveolar inflammation was mild to moderate and primarily neutrophilic in nature. Multiple areas of mild to moderate hemorrhage and small foci of tissue necrosis were present in severely affected animals (Wt mouse 4 and mouse 2). The remaining mouse (Wt mouse 3) had lesions more like those of the *vacJ*⁻ group. Compared to wild-type infected mice, markedly fewer bacteria were seen in the lungs from mice in the *vacJ*⁻ group. However, the degree of neutrophilic inflammation was generally comparable. Apart from *vacJ*⁻ mouse 1, the distribution of the inflammatory lesions in the *vacJ*⁻ group was less disseminated often forming several moderately well-contained foci. Hemorrhage in the *vacJ*⁻ group was minimal, and foci of necrosis were infrequent.

Mice in both groups exhibited moderate to severe interstitial pulmonary edema. One PBS-control mouse and one uninfected

Table 2 Histopathologic scoring of lung lesions

Rank[a]	Group	Mouse	Severity/% lung involvement
1	Wt	4	Severe/50%
2	Wt	2	Severe/40%
3	Wt	1	Moderate/50%
4	Wt	5	Moderate/50%
5	*vacJ*⁻	2	Moderate/30%
6	*vacJ*⁻	4	Moderate to severe/20%
7	Wt	3	Moderate/30%
8	*vacJ*⁻	3	Moderate to severe/10%
9	*vacJ*⁻	5	Mild to moderate/15%
10	*vacJ*⁻	1	Mild/50%

[a] Lung lesions were ranked 1–10 from most to least severe based on degree of histologically evident bacterial load, neutrophilic inflammation, tissue damage, and approximate percent of the lung involved. The lesions in the wild-type group were significantly worse than the *vacJ*⁻ group (*p* = 0.036, Mann-Whitney test)

mouse were also examined. No lesions were present in the lungs from the uninfected mouse and lesions in the PBS-control mouse were minimal comprising mild alveolar infiltrates of neutrophils and macrophages in a single lung lobe. These findings demonstrate enhanced pulmonary innate immune clearance of the *P. aeruginosa vacJ*⁻ mutant in concert with decreased propensity to bloodstream dissemination.

Discussion

Nosocomial bacterial infections have become an ever-increasing public health concern, with 650,000 individuals experiencing health care-associated infections per year, at least 20% of which are characterized by antibiotic resistance [46]. Sustained selective pressure caused by the constant use of antibiotics has allowed the rise of a select group of pathogens that are commonly isolated with multidrug-resistant profiles [47]. The most challenging threats in the so-called "ESKAPE group" of pathogens are the Gram-negatives due to the dearth of novel antibiotic candidates in the pipeline to treat these infections [48]. Among Gram-negative bacterial threats, *Pseudomonas* is of particular concern due to the ubiquitous nature of the pathogen and its intrinsic ability to resist many antibiotic classes and mechanisms of action. *P. aeruginosa* is a recurring problem in older cystic fibrosis (CF) patients [3], and increasing rates of *P. aeruginosa* antibiotic resistance have been recently observed in isolates from central line-associated bloodstream infection as well as ventilator-associated pneumonia in the intensive care unit [46].

The Mla pathway plays a critical role in maintaining OM integrity in different Gram-negative infections but has not been studied in great detail in the context of infection and innate immune resistance among the leading antibiotic-resistant human pathogens. Here, we report that *P. aeruginosa* deficient in the Mla pathway and the gene *vacJ* in particular, though exhibiting normal growth characteristics in vitro, demonstrate increased sensitivity to endogenous and pharmaceutical cationic antimicrobial peptides, serum, and whole blood. A *vacJ*⁻ mutant also showed decreased virulence in a murine model of *P. aeruginosa* lung infection, with reduced bacterial burden and increased survival compared to wild-type *P. aeruginosa*-infected mice. Much like previous results seen in Gram-negatives with Mla pathway mutations, we see that *P. aeruginosa* has increased susceptibility to outer membrane damage. Across murine models of infection, this susceptibility to outer membrane damage has translated to reduced virulence across a variety of Gram-negative pathogens such as *Haemophilus* and *P. aeruginosa* [16, 49]. In the *Drosophila* feeding assay used to probe virulence, *P. aeruginosa vacJ* mutant surprisingly caused increased mortality compared to wild-type and complemented strains, suggesting that there are different

mechanisms at play in the model that do not translate into the murine infection models [15].

With the rapid rate of emerging Gram-negative bacterial resistance to modern antibiotics, it is critical to continue to explore alternative strategies and drug targets to combat these pathogens. Molecules designed to disrupt Gram-negative OM stability are being investigated, and show great promise as a therapeutic strategy [50]. In particular, targeting the Mla pathway could be an interesting pharmacological target as it is conserved among all Gram-negative bacteria [18] and therefore offers the potential for broad-spectrum activity and clinical utility. Our research suggests that inhibitors of the Mla pathway in *P. aeruginosa* would not be directly bactericidal, but would rather sensitize Gram-negative bacteria to killing by the host's endogenous defenses (e.g., antimicrobial peptides and serum complement), potentially limiting collateral damage to the host microbiome that can be seen with repeated broad-spectrum antibiotic administration. The Mla system has also been reported to be important in maintaining colistin resistance in *A. baumannii* [20, 21]; inhibitors may be able to reverse colistin resistance and render them susceptible to a last line of defense antibiotic used for Gram-negative infections.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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