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Authors

Chien, Jason
Hwang, John H
Nilaad, Sedtavut
et al.

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CELLULAR MICROBIOLOGY:
PATHOGEN-HOST CELL MOLECULAR INTERACTIONS

Cigarette Smoke Exposure Promotes Virulence of *Pseudomonas aeruginosa* and Induces Resistance to Neutrophil Killing

Jason Chien,[a,b] John H. Hwang,[a,b] Sedtavut Nilaad,[a,b] Jorge A. Masso-Silva,[a,b] Sae Jeong Ahn,[a,b] Elisa K. McEachern,[a,b] Alexander Moshensky,[a,b] Min-Kwang Byun,[a,b,c] Laura E. Crotty Alexander[a,b]

[a]Pulmonary and Critical Care Section, VA San Diego Healthcare System, La Jolla, California, USA
[b]Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University of California San Diego, La Jolla, California, USA
[c]Division of Pulmonology, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea

Jason Chien and John H. Hwang contributed equally. First authorship was determined by alphabetical order of last names. Sedtavut Nilaad and Jorge A. Masso-Silva contributed equally, and order of authorship was determined alphabetically.

ABSTRACT It is widely known that cigarette smoke damages host defenses and increases susceptibility to bacterial infections. *Pseudomonas aeruginosa*, a Gram-negative bacterium that commonly colonizes the airways of smokers and patients with chronic lung disease, can cause pneumonia and sepsis and can trigger exacerbations of lung diseases. *Pseudomonas aeruginosa* colonizing airways is consistently exposed to inhaled cigarette smoke. Here, we investigated whether cigarette smoke alters the ability of this clinically significant microbe to bypass host defenses and cause invasive disease. We found that cigarette smoke extract (CSE) exposure enhances resistance to human neutrophil killing, but this increase in pathogenicity was not due to resistance to neutrophil extracellular traps. Instead, *Pseudomonas aeruginosa* exposed to CSE (CSE-PSA) had increased resistance to oxidative stress, which correlated with increased expression of *tpx*, a gene essential for defense against oxidative stress. In addition, exposure to CSE induced enhanced biofilm formation and resistance to the antibiotic levofloxacin. Finally, CSE-PSA had increased virulence in a model of pneumonia, with 0% of mice infected with CSE-PSA alive at day 6, while 28% of controls survived. Altogether, these data show that cigarette smoke alters the phenotype of *P. aeruginosa*, increasing virulence and making it less susceptible to killing by neutrophils and more capable of causing invasive disease. These findings provide further explanation of the refractory nature of respiratory illnesses in smokers and highlight cigarette smoking as a potential driver of virulence in this important airway pathogen.

KEYWORDS cigarette smoke, *Pseudomonas aeruginosa*, neutrophil, oxidative burst, biofilm, pneumonia, bacterial virulence

Despite dramatic reductions in prevalence, cigarette smoking continues to be one of the leading causes of death, disease, and ill health. It is estimated that direct and secondhand exposure to cigarette smoke are responsible for nearly 6 million deaths a year, causing a plethora of diseases that include lung cancer, stroke, chronic obstructive pulmonary disease (COPD), and other chronic illnesses worldwide (1).

Cigarette smokers also have higher rates of respiratory tract infections and more severe presentations (2). Prior research in this area has primarily focused on the impact of cigarette smoke on host respiratory health and airway function. Cigarette smoke contains nitric oxide, acrolein, acetaldehyde, formaldehyde, and free radical elements (3–5) that lead to structural changes in humans, including disruption of airway epithe-

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Address correspondence to Laura E. Crotty Alexander, lca@ucsd.edu.

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lial cells and thus reduced mucociliary clearance of potential bacterial pathogens (6, 7). Similarly, smoking leads to a hypersecretion of mucus and impediment of epithelial elastic properties that lead to chronic inflammation, a feature that is increasingly associated with higher rates of infection (8–12).

The primary innate immune cells of host defense are also adversely impacted by cigarette smoke exposure. Neutrophils, the first cells recruited to a site of infection, are vital in our immune response against bacterial pathogens. The airways of cigarette smokers have higher numbers of neutrophils due to higher rates of recruitment (13, 14). Their arsenal of antimicrobial weapons includes phagocytosis and production of reactive oxygen species (ROS), antimicrobial peptides, and neutrophil extracellular traps (NETs). In particular, neutrophils sequester phagocytosed microorganisms in phagolysosomes for degradation with ROS. In their study, Dunn et al. found that human neutrophils exposed to smoke *ex vivo* had greatly reduced ROS production (15, 16). In conjunction with this finding, Xu et al. showed that HL60 cells differentiated into immature neutrophils had a suppressed bacterial killing capacity when exposed to cigarette smoke during development (16). Overall, cigarette smoking impedes numerous host defense mechanisms in the lungs.

Cigarette smoke affects not only host cells but also the bacteria that are present in the airways (17). Previously, we determined that cigarette smoke directly enhanced the virulence of another common human pathogen, *Staphylococcus aureus* (18), which has been corroborated by other groups (19). In addition, we found that exposure of *S. aureus* to electronic cigarettes (e-cigarettes) also alters its virulence level through a separate mechanism (20). El Ahmer et al. found that cigarette smoke enhances the binding of multiple pathogens, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *S. aureus*, to epithelial buccal cells (21). These findings were corroborated by a separate *in vivo* study in which rats chronically exposed to cigarette smoke had a greater attachment of *S. pneumoniae* bacteria to cells of the oral airway (22).

Pseudomonas aeruginosa, a common airway pathogen which plays a prominent role in the progression and exacerbation of chronic diseases (23, 24), has also been studied. Antunes et al. showed that cigarette smoke exposure promoted pseudomonal biofilm production (25), and Goldstein-Daruech et al. showed that *P. aeruginosa* isolates from smokers had higher biofilm production when challenged with cigarette smoke, relative to isolates from nonsmokers (26). Simple overgrowth of this bacterium in the airways is known to drive pulmonary disease exacerbations in COPD and bronchiectasis (27–33).

To further understand the dynamic interplay between cigarette smoking, infection, and immunity, we undertook these studies to define the effects of cigarette smoke on the virulence of *P. aeruginosa*.

RESULTS

Cigarette smoke exposure increases pseudomonal resistance to neutrophil killing. Because neutrophils are a key player in clearing *P. aeruginosa* infection, we assessed the effect of cigarette smoke on this human pathogen in terms of susceptibility to neutrophil killing. Neutrophils were isolated from the blood of healthy human subjects and infected with control *P. aeruginosa* strain PAO1 versus cigarette smoke extract-exposed *Pseudomonas aeruginosa* (CSE-PSA). Control *P. aeruginosa* succumbed to neutrophil killing by 60 minutes, with reduced bacterial counts compared with time 0 min, while CSE-PSA was resistant to neutrophil killing ($P < 0.0001$) (Fig. 1A). In addition, a single exposure to different doses of CSE prior to infection of neutrophils demonstrated a dose-dependent effect, with 75% CSE inducing more resistance to neutrophil killing than 25% CSE and 50% CSE (Fig. 1B). CSE is known to have direct cytotoxic effects on human cells (34, 35). Therefore, to exclude the possibility that residual CSE from the CSE-PSA samples could be killing the neutrophils and thus leading to the increased bacterial survival seen with CSE-PSA, we assessed neutrophil viability by propidium iodide uptake after exposure to bacterial supernatants from both control and CSE-PSA samples. Cell viability was similar across supernatant exposures

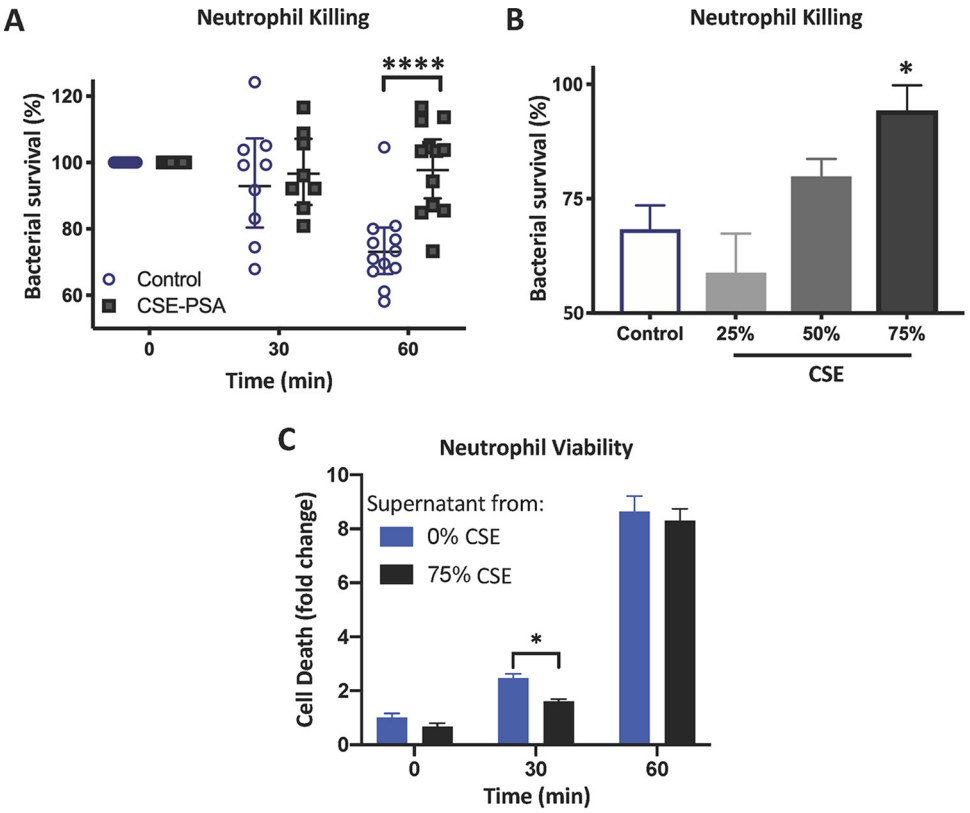

FIG 1 *Pseudomonas aeruginosa* becomes resistant to neutrophil killing after cigarette smoke exposure. (A) Neutrophil killing *in vitro* assay. *P. aeruginosa* was grown to mid-log phase in media with and without 75% CSE. *P. aeruginosa* exposed to 75% CSE for 2 hours prior to infection of human neutrophils was resistant to neutrophil killing relative to control *P. aeruginosa*. (B) CSE-mediated acquired resistance to neutrophil killing was dose dependent. Exposures were done in 12 wells each, for 60 minutes, and experiments repeated 6 times. (C) Incubation with bacterial supernatants did not affect neutrophil viability, as measured by propidium iodide uptake by flow cytometric analysis. Data represent relative change compared with that of controls. *, $P < 0.05$; ****, $P < 0.001$.

(Fig. 1C), which demonstrates that the several washes of the bacteria sufficiently removed the potential cytotoxic effect of residual CSE prior to infection of the neutrophils. These data suggest that exposure of CSE to *P. aeruginosa* confers resistance to neutrophil killing in a dose-dependent manner.

Cigarette smoke exposure slows pseudomonal growth. Pseudomonal growth is crucial during infection; hence, we assessed whether CSE can affect growth. We found that exposure to CSE dampens pseudomonal growth in a dose-dependent manner. Growth curves demonstrate normal, high growth rates of control *P. aeruginosa*, with dose-dependent slowing as CSE concentrations are increased (Fig. 2A). Thus, increased numbers of CSE-PSA in the setting of neutrophil infection was not due to overgrowth as a result of CSE exposure. These data suggest that CSE might induce alterations in *P. aeruginosa* pathogenicity, which can explain the greater survival of CSE-PSA during neutrophil infection.

Cigarette smoke exposure does not promote cationic binding to the surface of *Pseudomonas aeruginosa*. Our previous research with *S. aureus* and methicillin-resistant *S. aureus* (MRSA) demonstrated that CSE induced changes in the surface of these Gram-positive bacteria, causing the bacteria to become more positively charged, which diminished the ability of antimicrobial peptides to bind to the surface and eliminate the bacteria (18). To see if similar effects of CSE occur in *P. aeruginosa*, we assessed binding by a positively charged molecule, poly-L-lysine (PLL) labeled with fluorescein isothiocyanate (FITC). Interestingly, PLL-FITC binding to CSE-PSA was increased relative to control *P. aeruginosa* at 0.2 μg/ml but not at 2 μg/ml PLL-FITC

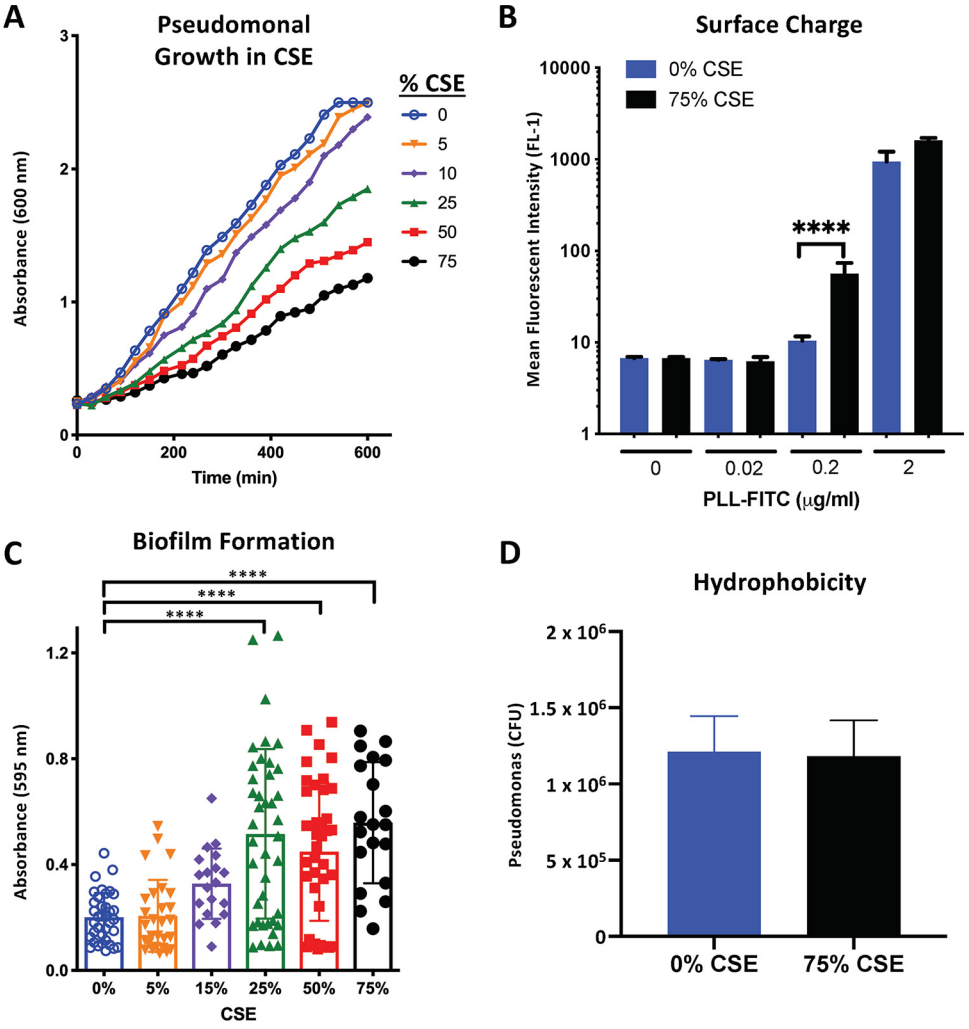

FIG 2 Cigarette smoke suppresses pseudomonal growth and promotes biofilm production. (A) CSE impairs bacterial growth. Control *P. aeruginosa* (blue) was cultured in MBM, at 37°C with shaking, and absorbance at 600 nm was measured over time. Each dose was tested in triplicate, with experiments repeated 3 times. (B) Cigarette smoke exposure for 2 hours induced mild alterations in surface charge since PLL-FITC binding was similar at 0.02 μg/ml and 2 μg/ml but different at 0.2 μg/ml. Each dose was tested in triplicate, with experiments repeated 3 times. Blue and black bars represent control *P. aeruginosa* and CSE-PSA, respectively. (C) Overnight exposure of *P. aeruginosa* to CSE significantly increased the production of extracellular biofilm relative to control counterparts at 25%, 50%, and 75% doses. Absorbance values reflecting the amount of biofilm per well were generated over 9 *in vitro* trials. (D) Cell surface hydrophobicity assessment by adhesion to n-hexadecane. The experiment was performed three times in triplicates. ****, $P < 0.0001$.

(Fig. 2B). These data show that CSE does not necessarily increase virulence by inducing *P. aeruginosa* to develop a more positively charged surface (Fig. 2B). Thus, although the surface charge of *S. aureus* strains become more positively charged in response to cigarette smoke at a concentration of 2 μg/ml PLL-FITC (18), this does not occur with the Gram-negative pathogen *P. aeruginosa*, and we found differences in surface charge only at a lower concentration of 0.2 μg/ml PLL-FITC.

Exposure to cigarette smoke increases *P. aeruginosa* biofilm formation. The production of biofilms is a key virulence factor for many microbial pathogens. As *P. aeruginosa* is a prolific maker of biofilm structural components and other researchers have demonstrated increased biofilm production in response to cigarette smoke (25, 26), we sought to determine whether exposure of *P. aeruginosa* PAO1 to CSE in our system would lead to increased biofilm production. A comparison of biofilm production by control *P. aeruginosa* versus CSE-PSA demonstrated a 1.6- to 2.8-fold increase in biofilm mass in wells containing *P. aeruginosa* exposed to 25% to 75% CSE ($P < 0.01$)

TABLE 1 Antimicrobial resistance of *Pseudomonas aeruginosa* to levofloxacin was increased by exposure to cigarette smoke[a]

Antimicrobial	MIC (μg/ml) at:		*P* value
	0% CSE	75% CSE	
Levofloxacin	3.58 ± 1.7	4.24 ± 2.16	0.018
Gentamicin	2.13 ± 0.62	2.58 ± 0.95	0.077
LL-37	44.44 ± 25.20	21.33 ± 9.23	0.21

[a]Antimicrobial resistance to gentamicin and human antimicrobial peptide LL-37 was not affected by CSE exposure. MIC assays were repeated >3 times, with biological triplicates.

(Fig. 2C). Thus, in our system, a single exposure with 25% to 75% CSE can induce biofilm formation in *P. aeruginosa*.

Cigarette smoke does not affect hydrophobicity. Previous studies have found that increased hydrophobicity can lead to enhance bacterial biofilm formation. Thus, we sought to evaluate whether CSE-mediated enhanced biofilm formation may be attributed to higher hydrophobicity. Hydrophobicity was determined by adhesion to n-hexadecane as previously described (36). We found that CSE does not induce significant changes in bacterial cell surface hydrophobicity (Fig. 2D), which suggests that other factors are involved in the CSE-mediated biofilm formation in *P. aeruginosa*.

Antibiotic resistance. It is known that biofilm formation can confer resistance to antibiotics (37). In addition, a recent study has shown that CSE can induce antibiotic resistance in *S. aureus* (19). Thus, we sought to investigate for changes in pseudomonal antibiotic resistance to two different antibiotics commonly used to treat clinical infections, namely, gentamicin (an aminoglycoside) and levofloxacin (a fluoroquinolone), and also the antimicrobial peptide LL-37. MICs were determined with *P. aeruginosa* and CSE-PSE at a wide range of levofloxacin, gentamicin, and LL-37 doses and demonstrated that cigarette smoke exposure induced resistance to levofloxacin (Table 1). We also analyzed genes of multidrug efflux pumps that have shown to be involved in antibiotic resistance, such as *mexA*, *mexX*, and *mexZ*, and found that these three genes were upregulated upon exposure to CSE (Fig. 3A to C). Altogether, these data show that CSE induced resistance to levofloxacin and upregulated expression of multidrug efflux pumps.

Cigarette smoke generates a protective response against oxidative stress. To assess how CSE exposure leads to the protection of *P. aeruginosa* from neutrophil killing, neutrophil antimicrobial mechanisms were assayed. While no differences were seen in the susceptibility of *P. aeruginosa* and CSE-PSA to neutrophil extracellular trap (NET)-based killing (data not shown), bacteria exposed to cigarette smoke exhibited resistance to reactive oxygen species (ROS), simulated via H_2O_2 exposure ($P < 0.01$)

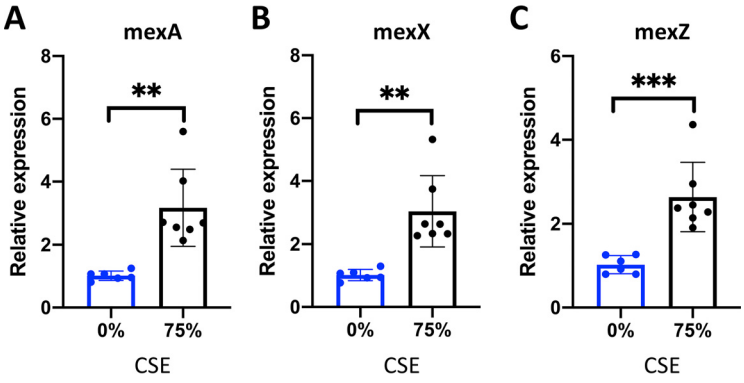

FIG 3 Cigarette smoke upregulated genes involved in multidrug efflux pumps. CSE increased the expression of *mexA* (A), *mexX* (B), and *mexZ* (C). Gene expression was analyzed using the comparative C_T method using *rspL* as a housekeeping gene. Experiments were performed three times in triplicates. **, $P < 0.01$; ***, $P < 0.005$.

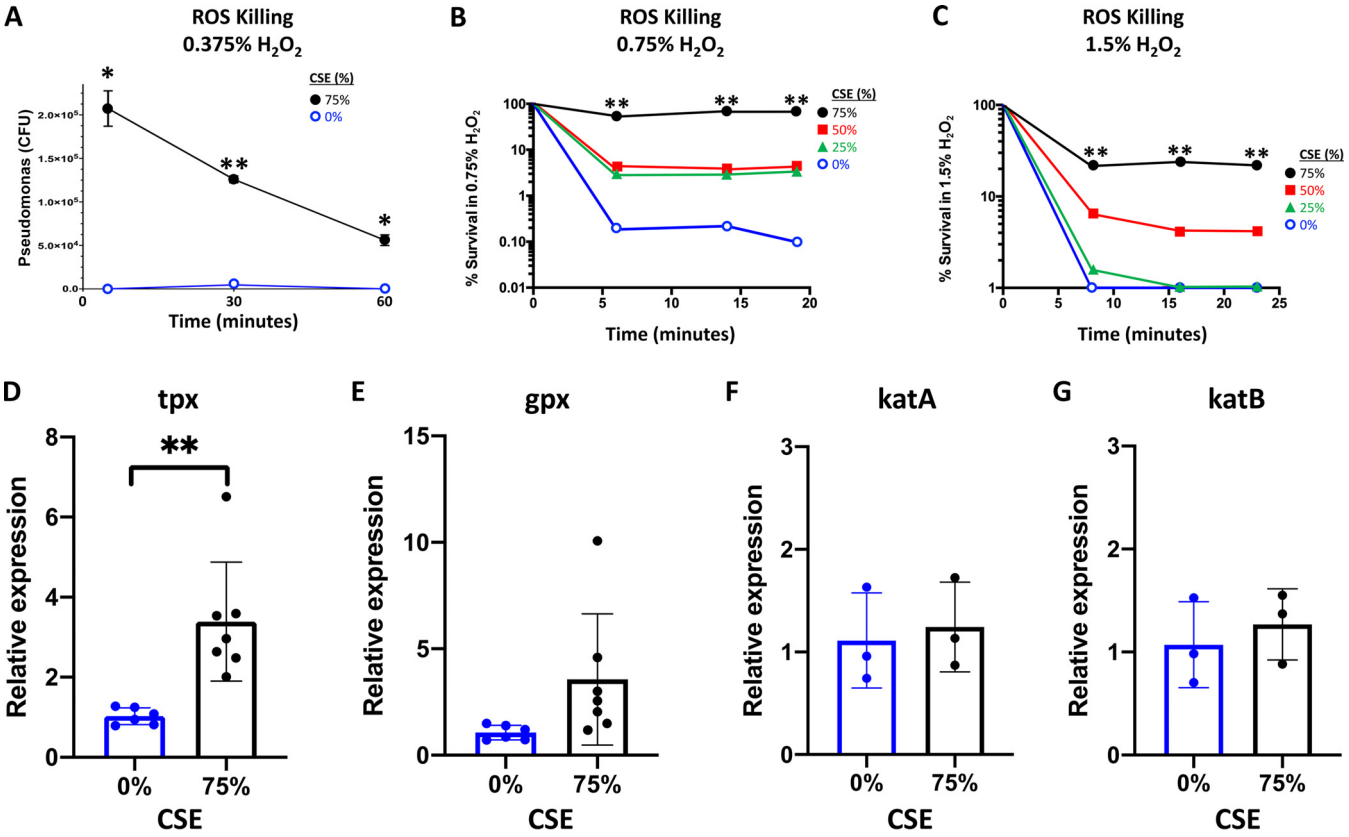

FIG 4 Exposure to cigarette smoke induced resistance to killing by reactive oxygen species (ROS). (A) CSE-PSA was resistant to be killed by exposure to 0.375% H_2O_2, compared to all control *P. aeruginosa*. CSE-mediated resistance to H_2O_2 was dose dependent, with 75% CSE conferring complete protection, 25% to 50% CSE conferring partial protection at 0.75% H_2O_2 (B), and 75% and 50% CSE conferring protection at twice the dose H_2O_2 (1.5%) (C). Experiments were repeated 6 times. In addition, the expression of genes involved in oxidative stress were evaluated and showed that thiol peroxidase (*tpx*) was upregulated (D), although glutathione peroxidase (*gpx*) (E), catalase *KatA* (G), and catalase *KatB* (G) did not change. Experiments were performed three times in triplicates.* $P < 0.05$, ** $P < 0.01$.

(Fig. 4A). Moreover, we found a CSE dose-dependent protection against neutrophil killing (Fig. 1B), which was further mirrored in H_2O_2 killing assays, where CSE exposure lead to a protective response, allowing CSE-PSA to survive, despite treatment with different doses of H_2O_2 that are lethal to control *P. aeruginosa* (Fig. 4B and C). Exposure to increasing concentrations of CSE induces resistance to H_2O_2, demonstrating a dose effect (Fig. 4B and 4C). These data suggest that exposure to CSE generates a protective response against oxidative burst, one of the primary mechanisms by which neutrophils kill pathogens within the phagolysosome (38). Finally, in order to better understand the mechanism of this CSE-mediated resistance to H_2O_2, we assessed the expression of four genes that are known to protect bacteria against oxidative insults, namely, thiol peroxidase (*tpx*) and glutathione peroxidase (*gpx*) and two catalases (*katA* and *katB*) (39, 40). We found that the acquired protective effect of CSE exposure (inducing resistance to oxidative burst) was associated with increased expression of *tpx* (Fig. 4D). Although *gpx* trended upward, it was not significantly increased in CSE-PSA compared with that of *P. aeruginosa* (Fig. 4E). Moreover, expression of the catalases *katA* and *katB* were not affected by CSE (Fig. 4F and 4G, respectively). Thus, these data suggest that the conferred resistance to neutrophil killing mediated by CSE exposure could be attributed in part through an acquired resistance to ROS, which correlates with increased gene expression of thiol peroxidase, which has been previously shown to promote bacterial resistance to oxidative stress.

Cigarette smoke increases pseudomonal virulence in a murine model of pneumonia. Finally, to assess whether the effects on *P. aeruginosa* virulence mechanisms induced by CSE *in vitro* and *ex vivo* may have physiologically relevant consequences,

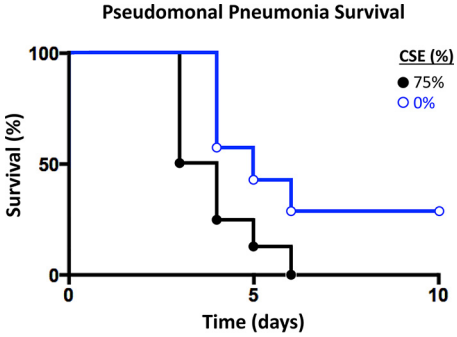

FIG 5 *Pseudomonas aeruginosa* exposed to cigarette smoke had a higher virulence in a mouse model of pneumonia. A total of 100% of mice infected with CSE-PSA died within 6 days, while 28% of mice infected with control *P. aeruginosa* survived. $n = 7$ for control group (0%), $n = 8$ for CSE group (75%). Experiments were performed twice.

CD-1 mice were infected intranasally with control *P. aeruginosa* or CSE-PSA. Within 72 hours of infection, only 50% of CSE-PSA-infected mice were surviving, while all mice in the control *P. aeruginosa* group were alive (Fig. 4). By 6 days postinfection, all CSE-PSA-infected mice succumbed to infection, while 28% of control *P. aeruginosa* mice survived ($P < 0.05$) (Fig. 5). These data demonstrate that CSE exposure increases *P. aeruginosa* virulence.

DISCUSSION

Cigarette smoking is one of the leading causes of death, disease, and ill health (1). Some of these life-threatening diseases are related to respiratory tract infections (2), and most studies have focused on the effects of CSE on host health. However, few studies have targeted the effects of CSE on the bacterial pathogens that can infect the airways (18, 19, 21, 22). Previously, we found that CSE enhanced the virulence of *Staphylococcus aureus* (18), which has been corroborated by other groups (19). Despite this, there is poor knowledge about the effects of CSE on the relevant bacterial pathogen *Pseudomonas aeruginosa*, a common airway pathogen (23, 24).

The study presented here demonstrates that cigarette smoke exposure may drive *P. aeruginosa* resistance to oxidative stress and neutrophil killing. First, we found that *P. aeruginosa* exposed to CSE was protected from neutrophil killing (Fig. 1A) in a dose-dependent manner (Fig. 1B) with no residual cytotoxic effect present in the bacterial suspension prior to infecting neutrophils, which could account for bacterial survival (Fig. 1C). Second, in exploring this observed phenotype of resistance to neutrophil killing, we found that CSE can affect *P. aeruginosa* growth *in vitro* in a dose-dependent manner (Fig. 2A). Cigarette smoke has been previously shown to inhibit bacterial growth *in vitro* (41). Furthermore, we also assessed changes in cell surface charge, which might make bacteria more susceptible or resistant to antimicrobial peptides (42), and found no major differences in *P. aeruginosa* and CSE-PSA at high concentrations of PLL-FITC (2 μg/ml). In fact, exposure at a concentration of 0.2 μg/ml of PLL-FITC shows CSE-PSA to be more susceptible to cationic antimicrobial binding (Fig. 2B). Another important virulence mechanism of *P. aeruginosa* and bacteria in general is biofilm formation. Biofilms are tenaciously attached to surfaces and impede the ability of host defense molecules and cells to penetrate them (43). We found that CSE induces biofilm formation in *P. aeruginosa* in a dose-dependent manner (Fig. 2C). As others have shown, cigarette smoke exposure is a strong driver of biofilm production in a range of pathogenic microbes, including *S. aureus* and *P. aeruginosa* (25, 26, 44). Our biofilm studies are consistent with prior studies, demonstrating that cigarette smoke induces biofilm production in this Gram-negative bacteria, potentially as a mechanism to defend the bacteria from the toxins within cigarette smoke. Related to this information, it is known that increases in cell surface hydrophobicity can enhance biofilm formation (45), so we tested whether CSE can cause changes in cell surface hydrophobicity and

found no differences (Fig. 2D). Thus, enhanced biofilm formation cannot be explained by changes in hydrophobicity and other mechanisms should be involved.

In addition, since our previous study with methicillin-resistant *Staphylococcus aureus* (MRSA) showed that cigarette smoke exposure increases resistance to human AMP LL-37 (18), we also assessed this effect in *P. aeruginosa* and found no significant differences in susceptibility to LL-37 (Table 1). We also evaluated changes in susceptibility to two different antibiotics from two different classes, namely, gentamicin (an aminoglycoside) and levofloxacin (a fluoroquinolone), finding an increase in resistance to levofloxacin alone (Table 1). An assessment of the expression of genes involved in multidrug efflux pumps (*mexA*, *mexX*, and *mexZ*) found upregulation of these three genes upon exposure to CSE (Fig. 3A to C). Both *mexA* and *mexX* have been shown previously to be involved in susceptibility to antibiotics in *P. aeruginosa* (46–48). Remarkably, previous studies have shown *mexA* and *mexX* could be critical on the virulence potential of *P. aeruginosa* for its invasiveness in the host (49, 50). In the case of *mexZ*, we observed an unexpected upregulation upon CSE exposure since *mexZ* is a repressor of the mexXY operon (51). Altogether, these data show that CSE does not induce resistance to gentamicin and LL-37 but induces resistance to levofloxacin, potentially via upregulation of genes of multidrug efflux pumps. Upregulation of these efflux pumps may additionally account for the enhanced virulence of CSE-exposed *P. aeruginosa* in our *ex vivo* system.

To further understand how *P. aeruginosa* can avoid neutrophil killing, we assessed neutrophil antimicrobial mechanisms that might be involved. Neutrophils are well known for their essential role in clearance of bacteria through different mechanisms, including NETs, antimicrobial peptides, and ROS production (52, 53). We found no difference in susceptibility to NET killing in both *P. aeruginosa* and CSE-PSA. However, our data show that CSE-exposed *P. aeruginosa* was more resistant to killing by H_2O_2 (a reactive oxygen species produced by neutrophils) in a dose-dependent manner (Fig. 4A to C), which suggests that in our system, ROS may be the primary mechanism behind the increased resistance to neutrophil killing. In light of this evidence, we believe that CSE is an environmental stressor for *P. aeruginosa*, slowing growth by forcing the bacteria to shift metabolic efforts toward defense against ROS and ultimately selecting for bacteria that are resistant to ROS. Over time, this generates a bacterial population that is resistant to oxidative stressors, such as H_2O_2. Related to this idea, only a few studies have shown that CSE can induce oxidative stress in bacteria (particularly in *S. aureus*) that might lead to adaptation and resistance to such stress (19, 44). Furthermore, seeking for an explanation of this enhanced resistance to neutrophil killing and ROS, we evaluated the expression of genes involved in protection against oxidative insults, including thiol peroxidase (*tpx*), glutathione peroxidase (*gpx*) and two catalases (*KatA* and *KatB*) (39, 40), and only tpx was significantly upregulated (Fig. 4D to H). A previous study has shown that expression of the *tpx* gene has a protective role against H_2O_2 in *P. aeruginosa* (39). Thus, these data suggest that the conferred resistance to neutrophil killing mediated by CSE exposure could be attributed in part through an acquired resistance to ROS, which correlates with increased gene expression of *tpx*.

Finally, to correlate our *ex vivo* and *in vitro* data of CSE-mediated enhanced virulence in *P. aeruginosa*, we assessed its virulence in a *in vivo* system. As a result of CSE exposure, *P. aeruginosa* became more virulent, leading to 100% of the mice succumbing to infection (Fig. 5). This finding correlated with our observation of increase expression of the genes involved in multidrug efflux systems, which have been shown to play a role in the invasiveness of *P. aeruginosa* in *in vivo* systems (49, 50). Thus, altogether, all our approaches indicate that CSE can promote the virulence of *P. aeruginosa*, which might involve resistance to oxidative stress and the expression of genes of multidrug efflux pumps.

This study has multiple limitations, including the use of high concentrations of CSE. While studies of cigarette smoke effects on mammalian cells typically use concentrations of 10% of CSE down to 0.001%, they are primarily to mimic the exposure of cells within the body to components of cigarette smoke that are absorbed into the blood-

stream and then diluted throughout the body. However, bacteria that colonize the airways are exposed to full-strength cigarette smoke during inhalation. Thus, high concentrations of CSE are more physiologically relevant in studies of airway microbes. Another limitation is that *P. aeruginosa* has a multitude of virulence factors, with more being identified every year, and these studies were limited to changes in surface charge and hydrophobicity, biofilm formation, antibiotic resistance to three antimicrobials, drug efflux pumps, and oxidative burst resistance. It remains unknown whether the increased virulence identified here in *P. aeruginosa* strain PAO1 will occur with other pseudomonal strains. Also, while we assessed the ability of CSE-exposed *P. aeruginosa* to cause more severe invasive disease (pneumonia), it is possible that CSE-driven virulence may alter how *P. aeruginosa* causes other invasive diseases, such as sepsis. Further studies are needed to define how different inhalants impact airway microbes and potentially increase the susceptibility of their hosts to invasive disease, including changing the way they interact and respond to the immune system.

Our data demonstrate that exposure to cigarette smoke generates a hardier phenotype of *P. aeruginosa* that is more difficult for host innate immune cells to kill. In the broader sense of infection and immunity, we believe that our study demonstrates that cigarette smoke exposure not only alters *P. aeruginosa* characteristics, such as growth and biofilm formation, but also drives pseudomonal virulence. We believe these findings provide a valuable explanation of the refractory nature of respiratory illnesses in cigarette smokers and highlight cigarette smoking as a potential driver of virulence in an important airway pathogen. There are a number of intracellular processes that could be affected that might explain the CSE-induced effects in *P. aeruginosa*. Follow-up investigations seeking to expand on our current findings may be beneficial to elucidate the specific mechanisms by which *P. aeruginosa* becomes resistant to oxidative stress and more virulent in general.

MATERIALS AND METHODS

CSE preparation. CSE was prepared based on prior published methods (54, 55). A total of 10 ml of appropriate control medium was drawn into a 60-ml sterile syringe. A 3R4F research cigarette with the filter removed was placed in a holder attached to tubing and a 3-way stopcock. The cigarette was lit and 40 ml of smoke (one puff over 2 seconds) was drawn into the syringe containing the 10 ml of media. The syringe was attached to a platform shaker and shaken at level 4 for 15 seconds, allowing the infusion of smoke components into the media. Smoke was "exhaled" through the stopcock and the smoking procedure repeated until less than 1 cm of the cigarette was left, an average of 12 puffs total, producing 100% CSE.

Preparation of control and CSE subcultures. Control medium was added to 100% CSE to create different percentages of CSE (vol/vol) for each assay. Subcultures of control and CSE-PSA were created each morning by inoculating 10 ml of control media and 10 ml of 75% CSE with 1:20 and 1:100 (controls) and 1:10 (75% CSE) dilutions of the overnight *P. aeruginosa* (PAO1 strain) culture. Tubes were incubated at 37°C with shaking until mid-log (optical density at 600 nm [OD_{600}] of 1.2 to 1.4) growth was reached.

Neutrophil killing of bacteria. Control and CSE-PSA subcultures were prepared by inoculating control mammalian-based media (MBM) (RPMI + 10% fetal bovine serum [FBS] + 20% LB) with overnight cultures. FBS and LB were included, as bacterial growth is stunted in their absence. After the mid-log growth phase was reached, control and CSE-PSA subcultures were transferred into separate 50-ml conical tubes, washed with phosphate-buffered saline (PBS), and then centrifuged at 3,200 rpm for 8 min in 4°C. Supernatants were discarded, and each pellet was resuspended in 300 μl of PBS. Two glass tubes were filled with 3 ml of PBS and both slurries added until an OD_{600} of 1.2 to 1.4 was reached.

Under approval from the University of California San Diego (UCSD) institutional review board (IRB), 25 ml of venous blood was collected from healthy donors using a 30-ml heparinized syringe. Blood was transferred to a 50-ml conical tube and layered on top of 20 ml of Polymorphprep, taking care not to disturb the interface between the two liquids. The blood was centrifuged at 1,600 rpm for 35 min at room temperature (22°C) with no brake. The plasma and upper mononuclear cell layer were aspirated; and the polymorphonuclear leukocytes (PMNs) were transferred to a fresh 50-ml conical tube, rinsed with 50 ml with PBS, and centrifuged at 1,600 rpm for 10 min. The supernatant was discarded, and 5 ml of molecular-grade water was added and mixed via pipetting for 30 s to lyse residual red blood cells. Cells were rinsed again with 50 ml of PBS and centrifuged at 1,600 rpm for 10 min. The pellet was resuspended in 1 ml of PBS and enumerated with trypan blue on a hemocytometer.

Cells were prepared at a concentration of 5×10^6 cells/ml. A total of 50 μl was added to each row A of a flat-bottom 96-well plate, and 50 μl of RPMI +10% FBS +20% LB was added to empty wells as a growth control. Phorbol myristate acetate (PMA; activator of PMN antimicrobial pathways, including NETs) was prepared at 2× (50 nM in RPMI + 2% FBS), and 50 μl was added to all wells to a final

concentration of 25 nm of PMA. Plates were incubated for 20 min at 37°C with 5% CO_2, or for a pure-NET killing assay, for 3 h at 37°C with 5% CO_2.

A bacterial slurry of an OD of 0.7 was prepared in 3 ml PBS as previously described. This slurry was diluted in MBM to obtain 5×10^6 CFU/ml, centrifuged at 1,600 rpm for 5 min, and resuspended in MBM at 5×10^5 CFU/ml to give a minimum of infectivity (MOI) of 0.1. Neutrophils were infected with *P. aeruginosa* in 50 μl, and plates were centrifuged at 1,600 rpm for 10 min to increase bacterium-PMN contact. Cells were incubated for 30 and 60 min at 37°C with 5% CO_2. A total of 5 μl of 0.25% Triton X-100-PBS was added to each well, and the *P. aeruginosa*:PMN mixture was serially diluted. A total of 25 μl of the bottom 3 dilutions were streaked onto LB plates and incubated at 37°C overnight prior to enumeration of surviving CFU.

Cell viability. Neutrophils were isolated as mentioned above and then treated with the supernatant of the last wash of the bacterial preparation after CSE exposure using the same volume used during the neutrophil killing assays mentioned above. Then, cells were treated with propidium iodide, and its uptake was measure using flow cytometry following the manufacturer's protocol (Invitrogen). Cell viability was calculated as relative to control time 0 for each time point.

Surface charge change. *Pseudomonas aeruginosa* was grown in 0%, 25%, 50%, and 75% CSE to an OD_{600} of 0.6 to 0.8. Bacteria were washed three times in 0.02 M HEPES (pH 7.5) and resuspended to an OD_{600} of 0.3; and poly-L-lysine (PLL)-FITC (Sigma) at 0, 0.02, 0.2, and 2 μg/ml was added. Tubes were vortexed every 5 min for 15 min in the dark. Cells were pelleted and resuspended and PLL-FITC binding was quantified via flow cytometric analysis.

Hydrophobicity. The bacterial suspension was transferred to a microcentrifuge tube, and n-hexadecane was added to a final concentration of 20%. Tubes were vortexed for 2 min and then incubated at room temperature for 30 min. Samples from the lower aqueous layers were transferred into a 96-well plate, serial dilutions were performed in 1× PBS in triplicate, and 10 μl was plated onto LB plates and incubated at 37°C overnight prior to CFU enumeration.

Biofilm assay. An overnight culture of *P. aeruginosa* was diluted 1:100 in LB (control) and CSE at 5%, 10%, 15%, 25%, 50% and 75%. A total of 100 μl of control and each CSE-PSA subculture was transferred to the middle of a 96-well round-bottom plate in replicates of 10. Outer wells of the plate were filled with PBS to minimize evaporation. The plate was incubated at 37°C with shaking for 24 hours. Supernatant, planktonic *P. aeruginosa* was aspirated, and the wells washed three times with 250 μl of sterile PBS, turning the plate each time and flicking gently to discard the PBS. The plate was turned upside down and allowed to dry for 5 min. A total of 200 μl of a 0.1% aqueous crystal violet (CV) solution was added into each well, and the plate was incubated for 15 min at room temperature. A PBS wash was performed three times to remove unbound CV. Following another 5-min drying period, the bound CV was extracted using 200 μl of an 80:20 (vol/vol) mixture of ethanol and acetone for 15 min. Absorbance was measured at 595 nm with a plate reader.

Antibiotic resistance assays. *Pseudomonas aeruginosa* and CSE-PSA subcultures were grown to mid-log phase in RPMI with 5% Mueller-Hinton broth (MHB). Cultures were spun at 3,200 rpm for 10 minutes, and bacteria were resuspended to an OD_{600} of 0.8. Bacteria were diluted 1:100 in assay media and plated at 100 μl per well. Levofloxacin and gentamicin powders were suspended in RPMI with 5% MHB at 16 μg/ml and LL-37 in RPMI with 5% LB at 64 μg/ml. Antibiotics were serially diluted 9 times. A total of 100 μl of each antibiotic dose was plated in a 96-well plate, and 100 μl of RPMI with 5% MHB or RPMI with 5% LB was plated as controls. *P. aeruginosa* and CSE-PSA cultures were pipetted into the wells in triplicate for each concentration of antibiotic and incubated in a 37°C shaker for 24 h. A total of 5 μl of each well was then plated onto Todd Hewitt agar (THA) and incubated at 37°C for 24 hours. CFUs were counted to determine MIC.

H_2O_2 sensitivity. CSE was prepared as described. Subcultures of control and CSE-PSA were created by inoculating 10 ml of control media and 10 ml of 75% CSE with 1:20 and 1:100 (controls) and 1:10 (75% CSE) dilutions of the overnight *P. aeruginosa* culture. Tubes were incubated at 37°C with shaking until mid-log (absorbance, 1.2 to 1.4) growth was reached. CSE and control *P. aeruginosa* subcultures of 0%, 25%, 50%, and 75% were created as described previously and grown to an OD_{600} of 1.2 to 1.4. Subcultures were washed with PBS in 50-ml conical tubes and spun at 3,200 rpm for 8 min. Bacterial pellets were resuspended in 300 μl of RPMI with 10% LB and 5% FBS to an OD_{600} of 0.7 to 1.0. A total of 500 μl of each bacterial subculture was added to 500 μl of 0%, 1.5%, and 3% H_2O_2 solutions and incubated with shaking at 37°C for 1 h. At approximately 0, 20, and 40 min, 10 μl was serially diluted in a 96-well round-bottom plate. Diluted bacteria were plated onto LB agar plates for enumeration of viable cells.

Murine pneumonia infection model. Totals of 100 mg/kg intraperitoneal ketamine and 10 mg/kg xylazine were used to sedate 5- to 7-month-old female CD-1 mice (Charles River). Mice were infected intranasally with 5×10^6 CFU *P. aeruginosa* in 75 μl, with the entire volume delivered into the left nare and each mouse kept upright for 1 min postinfection. Mice were recovered in the right lateral decubitus position on heating pads until they were mobile. Mice were weighed every 24 h and mortality documented. Experiments were conducted twice. All animal studies were approved on IACUC protocols s16021 (UCSD) and A14-024 (VASDHS).

Gene expression. RNA extraction was conducted by using an Qiagen RNeasy protect bacteria minikit following the manufacturer's protocol, and RNA extracts were frozen in a −80°C freezer until use. Later, RNA was quantified, and quantitative PCR (qPCR) was performed using the Bio-Rad iTaq universal one-step reverse transcriptase quantitative PCR (RT-qPCR) kit and protocol in a 96-well PCR plate and run on a Applied Biosystems StepOnePlus real-time system. The gene expression data acquired were

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)	Reference
rspL 5′	GCAAGCGCATGGTCGACAAGA	56
rspL 3′	CGCTGTGCTCTTGCAGGTTGTGA	
katA 5′	ATGCGTTTCTACACCGAGCA	57
katA 3′	ATGGTCAACTGATGCAGCGA	
katB 5′	GGTTTCGCCACCAAGTTCTA	57
katB 3′	CGTGGGAGAAGAAATCGAAG	
tpx 5′	GAAGGATCAACGCAATGG	39
tpx 3′	ACCACGGTGTTGGCCAGC	
oxyR 5′	CTCACCGAACTGCGCTACA	58
oxyR 3′	CGAGTCGGCCAGCACTT	
gpx 5′	TGCGGCTTACCCCGCAGTA	59
gpx 3′	ACTTGGTGAAGTTCCACTT	
mexA 5′	ACCTACGAGGCCGACTACCAGA	60
mexA 3′	GTTGGTCACCAGGGCGCCTTC	
mexX 5′	TGAAGGCGGCCCTGGACATCAGC	56
mexX 3′	GATCTGCTCGACGCGGGTCAGCG	
mexZ 5′	GCATGGGCTTTCTCCGCCAGTGC	56
mexZ 3′	GCGTCCGCCAGCAACAGGTAGGG	

analyzed using the comparative threshold cycle (C_T) method using *rspL* as housekeeping gene. Primers used are shown in Table 2.

Statistical analyses. All *in vitro* studies are representative of at least three replicate experiments, of which each was performed in triplicate. All averages, significance values (*P* values), *t* tests, and other parameters were analyzed using GraphPad Prism. One-way analysis of variance (ANOVA) was used to analyze neutrophil killing. Growth curves were analyzed with the Friedman test. H_2O_2 resistance was analyzed with the Mann-Whitney test. Antibiotic resistance was analyzed by the ratio-paired two-tailed *t* test. Mouse pneumonia studies were conducted once, and survival analysis was performed via a Kaplan-Meier survival curve.

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L.E.C.A., J.C., J.H.H., J.A.M.-S., S.J.A., and E.K.M. designed the studies. J.C., J.H.H., J.A.M.-S., S.J.A., E.K.M., and S.N. ran the experiments and acquired the data. J.H.H., J.A.M.-S., S.A., M.-K.B., and L.E.C.A. analyzed the data. All authors drafted and edited the manuscript.

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