Peroxiredoxin AhpC1 protects *Pseudomonas aeruginosa* against the inflammatory oxidative burst and confers virulence

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A B S T R A C T

*Pseudomonas aeruginosa* is an opportunistic bacterium in patients with cystic fibrosis and hospital acquired infections. It presents a plethora of virulence factors and antioxidant enzymes that help to subvert the immune system. In this study, we identified the 2-Cys peroxiredoxin, alkyl-hydroperoxide reductase C1 (AhpC1), as a relevant scavenger of oxidants generated during inflammatory oxidative burst and a mechanism of *P. aeruginosa* (PA14) escaping from killing. Deletion of AhpC1 led to a higher sensitivity to hypochlorous acid (HOCl, IC50 3.2 ± 0.3 versus 19.1 ± 0.2 μM), hydrogen peroxide (IC50 91.2 ± 0.3 versus 496.5 ± 6.4 μM) and the organic peroxide urate hydroperoxide. ΔahpC1 strain was more sensitive to the killing by isolated neutrophils and less virulent in a mice model of infection. All mice intranasally instilled with ΔahpC1 survived as long as they were monitored (15 days), whereas 100% wild-type and ΔahpC1 complemented with ahpC1 gene (ΔahpC1 ΔahpC1) died within 3 days. A significantly lower number of colonies was detected in the lung and spleen of ΔahpC1-infected mice. Total leukocytes, neutrophils, myeloperoxidase activity, pro-inflammatory cytokines, nitrite production and lipid peroxidation were much lower in lungs or bronchoalveolar liquid of mice infected with ΔahpC1. Purified AhpC1 neutralized the inflammatory organic peroxide, urate hydroperoxide, at a rate constant of 2.3 ± 0.1 × 10⁶ M⁻¹ s⁻¹, and only the ΔahpC1 strain was sensitive to this oxidant. Incubation of neutrophils with uric acid, the urate hydroperoxide precursor, impaired neutrophil killing of wild-type but improved the killing of ΔahpC1. Hyperuricemic mice presented higher levels of serum cytokines and succumbed much faster to PA14 infection when compared to normouricemic mice. In summary, ΔahpC1 PA14 presented a lower virulence, which was attributed to a poorer ability to neutralize the oxidants generated by inflammatory oxidative burst, leading to a more efficient killing by the host. The enzyme is particularly relevant in detoxifying the newly reported inflammatory organic peroxide, urate hydroperoxide.

1. Introduction

The microbial ability to subvert host defense and to develop antibiotic resistance are the main causes of persistent infection and septicemia. Inhibition of phagocytosis, evasion from phagosome, production of resisting antimicrobial peptides, degradation of neutrophil extracellular traps (NETs) and detoxification of the oxidants produced during inflammatory oxidative burst are well recognized mechanisms...
for subverting neutrophil killing [11]–[6]. Therefore, bacteria survival will be proportional to the ability of combining the above mechanisms.

These subversion mechanisms are intimately associated with virulence and, in the case of *Pseudomonas aeruginosa*, it is less relevant in healthy individuals though a major cause of acute, hospital-acquired infections, microbial keratitis, as well as chronic lung infections in cystic fibrosis patients [7]–[9]. Indeed, *P. aeruginosa* is responsible for ∼10% of all hospital-acquired infections worldwide leading to a high mortality rate [10,11]. The bacteria present a plethora of virulence factors including the secretion of cytotoxic and adherence molecules. The effector proteins ExoU, ExoS and ExoT, which are ejected through type III secretion system (T3SS) into the host cell cytosol after phagocytosis, suppress neutrophil response [5,12–14]. ExoS and ExoT suppress immune response mainly through ADP-ribosylation of host proteins. For instance, ExoS ADP-ribosylates Ras, disrupting PI3K signaling, decreasing superoxide production by NADPH oxidase [5,14].

In addition to inhibit superoxide production by the host, *P. aeruginosa* possesses an extraordinary number of antioxidant enzymes. The PA14 strain contains two superoxide dismutase (SodM and SodB); five heme-peroxidases (chloroperoxidase, cytochrome c peroxidase and the catalases, KatA, KatB and KatE); eleven thiol peroxidases (thiol peroxidase - Tpx, cytoplasmic glutathione peroxidase - GPx, thiol-peroxidases in the virulence of different microorganisms [19,23] and a bunch of studies have investigated the role of thiol-peroxidases - Tpx, LsfA and Ohr in the neutralization of hydroperoxides and perox...
detector UV SPD-20A, system controller CBM-20A connected with a computer with LC solution software. The stationary phase was a preparative TSK-Gel Amide column (10 μm; 21.5 mm × 30 cm, Tosoh Bioscience, Tokyo, Japan). The mobile phase consisted of 40% 10 mM ammonium acetate (pH 6.8) and 60% acetonitrile, with a constant flux of 4 mL/min in an isocratic mode for 30 min. Excess of acetonitrile from urate hydroperoxide sample was evaporated with inert gas. To guarantee the maximal removal of the organic solvent, a system equipped with a kitsasato flask connected to a vacuum bomb was used. The remaining acetonitrile was quantified by gas chromatography and showed a maximum of 0.07%. Vehicle samples were performed in the presence of mobile phase submitted to acetonitrile evaporation or ammonium acetate. All solutions were stirred with Chelex® 100 Resin (Sigma-Aldrich) for at least 1 h to remove any trace metals. The concentration of urate hydroperoxide was measured by its absorbance at 308 nm (E260nm = 6540 M⁻¹ cm⁻¹) and compared with the concentration determined by Ferrous Oxidation Xylenol Orange (FOX) as previously described [40].

2.2. Bacterial strain and culture

P. aeruginosa UCBB-P14 (PA14) wild-type (WT) [41] and ΔlsFA [17] and MAR2xT7 mutants, ΔahpC1, ΔahpC2, Δohr, Δgpx, and ΔomegaR [42] were kindly provided by Prof Dr Regina Baldini, Institute of Chemistry, University of São Paulo, São Paulo, Brazil. Otherwise stated, all strains were grown in Luria-Bertani medium (LB) under shaking at 350 rpm and 37 ºC. After ~18 h, cells were diluted in fresh LB to OD₆₅₀ = 0.1 and incubated until reaching the OD₆₅₀=1.2 (1 × 10⁶ CFU/ml) for large exponential growth phase. Bacterial suspension was then centrifuged at 1700 g for 10 min and suspended in an equal volume of either minimal medium or PBS-glucose according to the experiment.

For a single copy ahpC1 complementation, the fragment of 1172 bp corresponding to ahpC1 with its native promoter was PCR amplified from genomic DNA of PA14 strain using primers ahpC1-compl_F – ATGGGATCTTACGGTCCGTCGCTACAC and ahpC1-compl_R ATGCCAAGCTTGAATCCGGGAACGTATC, cloned into BamHI/HindII restriction sites of integrative mini-CTX2 vector and sequenced, originating mini-CTX2-ahpC construct. Integration at the attB site of P. aeruginosa PA14 ahpC:MAR2xT7 strain was done by conjugation with E. coli BL21(DE3) strain was transformed by heat shock method to allow expression of pET15b-ahpC and cultured overnight at 37 ºC for 18 h and bactericidal activity was determined by Ferrous Oxidation Xylenol Orange (FOX) as previously described [28].

2.3. Bactericidal activity of oxidants

PA14 strains (1 × 10⁶ CFU/ml) were incubated with HOCI (5–50 μM), H₂O₂ (0.01–4 mM) or urate hydroperoxide (5–100 μM) in minimal medium at 37 ºC. After 30 min, bacterial suspension was 200-fold diluted in LB, and 10 μL were plated onto Muller Hinton (MH) agar plates. Plates were maintained at 30 ºC for 18–20 h and bactericidal activity was estimated by counting CFU [28]. The IC₅₀ values were calculated using GraphPad Prism. The dose-response curve using the logarithmic concentrations of the oxidants was fitted to a sigmoid.

2.4. Purification of human neutrophils

Human neutrophils were isolated from fresh peripheral blood of healthy donors (25 ± 5 years old) by gradient centrifugation [44] with minor modifications. Blood was harvested in a sterile sodium heparin vacutainer, and homogenized by slow inversion. Next, 3 mL of Histopaque 1119 was placed in a 15 mL conical tube, carefully overlaid with 2 mL of Histopaque 1077, and 4 mL of blood. After 30 min centrifugation at 700g, room temperature, neutrophil layer was obtained from the interface between both gradients. Erythrocytes were discarded by hypotonic hemolysis in ice-cold ultrapure water with 10 min centrifugation at 300g. The resulting pellet was suspended in 2 mL PBS-glucose, cells were counted in a Neubauer chamber (Sigma-Aldrich, MA, EUA) using trypan blue (0.2%) exclusion method [45]. The protocol was approved by Human Research Ethics Committee from Universidade Estadual do Sudoeste da Bahia (#46133515.4.0000.0055).

2.5. Bactericidal activity of neutrophils

WT, ΔahpC1 or ΔahpC1 complemented with ahpC1 gene (1 × 10⁷ CFU/ml) were first onpsonized in PBS-glucose containing inactivated autologous human serum (10%) for 20 min at 37 ºC. Human neutrophils (1 × 10⁶ cells/mL) were incubated with each strain (MOI = 1:10) in presence or absence of uric acid (200 and 400 μM), 4-amino benzoic acid hydrazide (50 μM, ABAH, a myeloperoxidase inhibitor), or apocynin (1 mM, APO, a NADPH oxidase inhibitor). After 1 h, Triton X-100 (%0) was added for neutrophil lysis. Cell lysate was serial diluted 10⁻⁵-fold in PBS-glucose, and 10 μL were spread onto MH agar plates for 18–20 h at 30 ºC for CFU counting [28,46].

2.6. Expression and purification of AhpC

The ahpC gene was PCR-amplified from the 07B05 cosmids used in the Xylella fastidiosa Genome Project. The PCR product was cloned into the Ndel and BamHI restrictions sites of pET15b vector. Subsequently, E. coli BL21(DE3) strain was transformed by heat shock method to allow expression of pET15b-ahpC and cultured overnight at 37 ºC in 50 mL of LB-ampicillin, transferred to 1 L of fresh LB-ampicillin and incubated until reaching OD₆₀₀=0.06. Isopropyl-beta-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h of incubation, cells were harvested by centrifugation. The pellet was washed and suspended in 20 mL lysis buffer (Tris-HCl 50 mM, NaCl 100 mM, lysosyme 1 mg/mL, glycerol 10%, 1 tablet EDTA-free protease inhibitor cocktail, pH 7.4). After incubation on ice for 10 min, cells were submitted to sonication at 30% amplitude for 5 min on ice, 20 s on and 40 s off. The suspension was centrifuged at 31,500g for 30 min at 4 ºC and the supernatant was loaded onto a nickel affinity column (HiTrap™, GE Healthcare). The concentrations of protein purification were optimized using the manual gradient of imidazole as described by the manufacturer. SDS-PAGE was used to check the purification quality, and all fractions containing bands with MW ~23 kDa were combined, concentrated through Amicon Ultra 10 kDa ultrafiltration (Millipore Corporate, Germany). Protein concentration was measured spectrophotometrically (ε₂₈₀nm = 27,055 M⁻¹ cm⁻¹) [47].

Basic Local Alignment Search Tool (BLAST) was used to compare the sequence of AHP1 and AHP2 from PA14 (A0A072ZH89 and A0A071KYB2, respectively) with AHPc from Xylella fastidiosa (Q87DE0). AHP1 from PA14 and AHPc from X. fastidiosa share 61% identity, 78% homology, with both peroxidatic Cys47 and resolving disulfide bond Cys 131, respectively [48].

2.7. Pre-reduction of AHPc and quantification of protein thiois

Immediately before the experiments, AHPc was reduced with DTT (in a 5-fold molar excess of thiois) for 2 h at 37 ºC under an argon atmosphere. The remaining DTT was removed by filtration through an Amicon Ultra-15 10K filter (Millipore) against 50 mM phosphate buffer pH 7.4 containing DTDA (100 μM). For all experiments related to AHPc, phosphate buffer was previously treated with bovine catalase (10 μg/mL) before filtration through an Amicon Ultra-15 10K filter (Millipore). Protein thiois were determined by incubating 5 μL samples with 95 μL 0.25 mM 4,4′-dithiopyrididine, 1% SDS for 10 min in the dark and read at 324 nm (ε₃₂₄nm = 21,400 M⁻¹ cm⁻¹).
2.8. Stopped-flow experiment

AhpC oxidation to sulfenic acid was followed by monitoring intrinsic fluorescence decay \[48\]–\[51\]. Pre-reduced AhpC (0.5 μM or 1 μM with \(1.5 \text{ μmol SH/μmol protein}\) was mixed with increasing concentrations of \(\text{H}_2\text{O}_2\) or urate hydroperoxide in a stopped-flow instrument (Applied Photophysics SX20MV, Leatherhead, United Kingdom), excitation \(380\text{nm}\) emission above \(330\text{nm}\). The reactions were performed at 25°C in 50 mM sodium phosphate buffer pH 7.4 containing diethylenetriaminepenta-acetic acid (100 μM, DTPA). Buffer solutions were pre-treated with 10 μg/mL catalase to remove any trace of \(\text{H}_2\text{O}_2\) \[52\]. After that, catalase was removed through filtration using an Amicon Ultra-15 10K filter (Millipore). A slight excess of \(\text{H}_2\text{O}_2\) was used that approximates pseudo-first order conditions. A proper 10-fold excess of \(\text{H}_2\text{O}_2\) is not possible to monitor because the reaction occurs before the dead time of the equipment (~2 ms). A much larger excess of urate hydroperoxide was used to allow pseudo-first order conditions. Observed rate constants (k_{obs}) for fluorescence decrease were determined by fitting data to single exponential equations. The fittings were set from 2 ms. The values of k_{obs} obtained from the decreasing fluorescence were plotted against \(\text{H}_2\text{O}_2\) or urate hydroperoxide concentrations and the corresponding second order rate constants were determined from the slope of these linear fittings.

2.9. Animals

Male C57BL/6 mice (8 weeks old, 25 ± 1 g) were obtained from Anilab (Paulinia, São Paulo, Brazil). Mice were maintained under 12/12 h light/dark cycle with free access to water and commercial standard feed (Pragsoluções, São Paulo) under pathogen-free conditions and controlled temperature (23 ± 3 °C). The procedures were approved by the Ethics Committee for experiments with animals from the Universidade Estadual do Sudoeste da Bahia (#171/2018).

2.10. Intranasal infection, mice survival and tissue collection

WT, ΔahpC1 and ΔahpC1 complemented with ahpC1 were grown as described above. After overnight incubation, bacteria were diluted into fresh LB to OD_{625nm}=0.6, centrifuged at 4500g for 8 min, and the pellet was suspended at 6.7 \times 10^7 CFU/mL in sterile saline. Mice (n = 8/group) were first anesthetized with ketamine (50 mg/kg) and xylazine (16 mg/kg) before nasal instillation of 30 μL bacterial suspension (2 \times 10^9, each strain) or sterile saline \[53\]. Mice were observed twice daily for survival up to 15 days. To avoid excessive suffering, mice were systematically euthanized by cervical dislocation when they were found in a moribund state with at least four of the following distress criteria: inability to maintain upright, associated or not with labored breathing and cyanosis, anorexia and weight loss (>20%), hunching, prostration, impaired motility, ruffled haircoat and dehydration \[54\]. Mice were intranasally inoculated with WT, ΔahpC1, ΔahpC1attaB:ahpC1 or sterile saline, as described above. Twenty-four hours later, they were anesthetized by i.p. administration of a mix of ketamine (50 mg/kg) and xylazine (16 mg/kg) for bronchoalveolar lavage harvesting, followed by cervical dislocation \[55\] for tissue collection as described below.

2.11. Bronchoalveolar lavage

Immediately after anesthesia, mice underwent tracheostomy for tracheal cannulation with infusion catheter (G22) and the lungs were washed three times with 0.5 mL PBS glucose for BAL collection. Cells were diluted in Turk’s solution (NewProv) and total leukocyte count was performed in a Neubauer chamber. Then, 50 μL of BAL was centrifuged at 4,500g for 7 min onto a glass lamina and stained with Rapid Panoptic Kit (NewProv). A total of 100 cells per lamina were assessed by double-blind counting, following standard morphological criteria \[56\]. The remaining BAL was centrifuged at 1500g for 10 min at 4 °C, and the supernatant was kept at −80 °C until nitrite determination.

2.12. Tissue harvesting

The euthanized animals underwent mid-thoracotomy for lung harvesting after BAL harvesting. The right lung was first divided longitudinally in two: one half was used for CFU counting; the other half was snap-frozen in liquid nitrogen and stored at −80 °C for biochemical analysis. One half of the left lung was immersed in Methacarn fixative solution (60% methanol, 30% chloroform and 10% acetic acid) for 24 h, followed by ethanol (70%) washing for histological analyses and the other half was snap-frozen in liquid nitrogen and stored at −80 °C for cytokines levels assessment.

2.13. Survival curve in hyperuricemic mice

Mice were intraperitoneally injected with potassium oxonate (300 mg/kg) to induce hyperuricemia \[57\]. Potassium oxonate was freshly dissolved in 0.5% sodium carboxymethylcellulose and daily injected for 6 days. After 22 h from the first potassium oxonate administration, mice were randomly divided into three groups that were nasally instilled with sterile saline (sham, n = 6), WT (n = 7) or ΔahpC1 (n = 7) PA14 as described above. Mice were observed twice daily for survival up to 5 days after bacteria instillation and excessive suffering was avoided as described above and described elsewhere \[54\]. Concomitantly, a separate group of mice (n = 6) received only 0.5% sodium carboxymethylcellulose for 6 days as vehicle control. On the 7th day following potassium oxonate or vehicle treatment, the sterile saline instilled mice, the only survival ones, were anesthetized with xylazine/ketamine as above and decapitated for blood harvesting to assess serum cytokines and uric acid. Blood samples were centrifuged at 1,500g for 10 min at room temperature and then stored at −80 °C. Serum uric acid was assessed by a mono-reagent kit (Bioclin, Brazil) following the manufacturer’s specification.

2.14. Cytokine levels

IL-1β and TNF-α from serum or lung homogenate were determined using the enzyme-linked immunosorbent assay (ELISA) kits (DY401-05 and DY410-05, respectively) according to the manufacturer’s instructions (R&D System), except by incubation of the samples with the pre-coated microplates for 16 h at 4–8 °C. Lung homogenate was diluted two-fold in diluent reagent before running the ELISA. The cytokine concentrations were expressed as pg/mL or pg/mg of protein for serum or lung homogenate, respectively.

2.15. Determination of nitrite in BAL

Nitrite was measured by the Griess method \[58\]. BAL supernatant (50 μL) was incubated with 50 μL naphthylethnediamine solution (0.1%) and 50 μL sulfanilamide 209 solution (1%) for 10 min at 25°C. Sample absorbance was determined at 540 nm and interpolated against a standard curve of nitrite (1.65–100 μM).

2.16. Tissue CFU counting

Immediately after euthanasia, organs (100 mg/mL) were homogenized in cold PBS buffer using a TissueRuptor system (Qiagen, USA) \[59\]. Ten microliters homogenate were spread onto MH agar plates and incubated for 18–20 h at 30 °C \[17\]. The bacterial colonies were counted and the CFU was expressed by g of wet tissue.

2.17. Histological analyses

Fixed and dehydrated lungs were soaked in paraffin, cut longitudinally in 4 μm-thick microtome sections (Leica RM2125RTS, CN) and
stained with hematoxylin and eosin (H&E). Neutrophils were counted in 30 random fields using a digital camera (Kontron Electronic KS-300, Eching, GE) coupled to a light microscope (BX51, Olympus, JP) under 400× magnification. Neutrophil numbers per field were quantified using Image J 1.44P software (National Institutes of Health, US).

2.18. Peroxidase activity

Lung tissue (100 mg/mL) was homogenized in potassium phosphate buffer (50 mM), pH 6.0 and centrifuged at 4°C, 10,000g for 10 min. Cetyltrimethylammonium bromide (CTAB, 0.5%) was added to the supernatant to prevent MPO adhesion to tube walls. The homogenate (10 μL) was mixed with 110 μL working solution containing 3.3%, 5,5′-tert-ramethylenbenzidine (TMB, 2.9 mM dissolved in 14% dimethylysulfoxide and 86% 150 mM sodium phosphate buffer, pH 5.4). Reaction was initiated by adding 80 μL H₂O₂ (0.75 mM). TMB oxidation was monitored every 30 s at 450 nm (Thermoplate, Tp-reader, US) for 5 min at 25°C (ε₅₀₀nm = 5.9 × 10⁴ M⁻¹ cm⁻¹). The MPO inhibitor, 4-Aminobenzoic acid hydrazide (ABAH, 50 μM) was added to a lung sample from WT-infected mice to evaluate MPO contribution to the peroxidase activity. Results were expressed as specific activity (U/mg protein), where one unit of peroxidase activity was defined as the amount of enzyme that produced 1 μmol of oxidized TMB per min at 25°C [60].

2.19. Quantification of thiobarbituric acid reactive products

Lipid peroxidation was estimated by thiobarbituric acid reactive substances (TBARS) assay [61]. Right lung tissue (100 mg/mL) was homogenized in 50 mM Tris-HCl buffer, pH 8.0, containing butylated hydroxytoluene (BHT, 0.02%) to prevent spurious oxidation. The homogenate was centrifuged at 1,600g for 10 min at 4°C. The supernatant was then diluted (1:2) in 100 μL TrisHCl and incubated with 100 μL trichloroacetic acid (10%) and 800 μL thiobarbituric acid (0.53%, dissolved in 20% acetic acid) for 1 h at 95°C. After cooling on ice for 10 min, samples were again centrifuged at 1,600g for 10 min at 4°C, and absorbance was recorded at 535 nm. TBARs levels were estimated by interpolating sample absorbance on a standard curve of malondialdehyde (Cayman Chemical, US) (0–50 μM), normalized by protein concentration and expressed as μmol of MDA/g of protein.

2.20. Determination of total protein concentration

Total protein concentration in lung homogenates was determined by Bradford colorimetric assay at 595 nm [62] employing serum bovine albumin (Sigma-Aldrich, US) (0–1.4 mg/mL) as standard.

2.21. Statistical analyses

All analyses were performed in GraphPad Prism (v. 5.0). Data are expressed as mean ± standard error of the mean. Statistical significance was calculated with one-way ANOVA and Newman-Keuls as post-test, when appropriate. Kaplan-Meier survival curves were plotted and the significance was calculated using the log-rank test. Differences were considered significant when p < 0.05. Data reproducibility is demonstrated by the number of independent experiments in each figure legend.

3. Results

Initially, we tested the sensitivity of different PA14 mutants to HOCl and among all the strains analyzed ΔahpC1 was the most sensitive (Table 1). A dose-response curve for HOCl inhibiting wild-type and ΔahpC1 survival is demonstrated in Fig. 1A. As depicted in Fig. 1B-C, ΔahpC1 was also more sensitive to H₂O₂ (IC₅₀ 91.2 ± 0.3 μM) than wild-type (IC₅₀ 496.5 ± 6.4 μM). By reinserting the ahpC1 gene to ΔahpC1, the bacterial resistance was restored to wild type level, the IC₅₀ for H₂O₂ was 660.3 ± 2.9 μM for this strain (Fig. 1D).

We then compared the sensitivity of wild-type and ΔahpC1 strains to neutrophils. ΔahpC1 was slightly but significantly more sensitive to neutrophil killing than wild-type. The inhibition of NADPH oxidase by apocynin or myeloperoxidase by ABAH significantly prevented the killing activity of neutrophils (Fig. 2A). The complementation of the ΔahpC1 mutant with ahpC1 again reverted the resistance to the wild-type level (Fig. 2B).

When wild-type and ΔahpC1 mutant strains were instilled via intranasal into mice, an enormous difference in the virulence between both strains was observed. Whereas wild-type PA14 killed 100% mice infected up to 3 days, no death occurred in ΔahpC1 PA14 infected mice up to 15 days. The reinsertion of ahpC1 into the ΔahpC1 PA14 (ΔahpC1 attBahpC1) completely restored the virulence, as this strain also killed 100% mice up to 3 days of infection (Fig. 3A). In accordance with a lower virulence of ΔahpC1 PA14, a much lower number of bacteria (CFU) was found in lung and spleen 24 h after infection with ΔahpC1 compared to wild-type PA14 (Fig. 3B).

As a consequence, a much lower number of total leukocytes and neutrophils was found in bronchoalveolar lavage (BAL) of ΔahpC1 infected mice compared to wild-type (Fig. 4). Total neutrophil was also lower in the lung parenchyma of ΔahpC1 than wild-type infected mice, as confirmed by quantitative hematoxylin-eosin histological counting and peroxidase activity (Fig. 5A and B). The peroxidase activity was likely due to MPO because ABAH completely abrogated the TMB inhibition of HOCl on bacterial growth. Table 1

| Strain   | IC₅₀ HOCl (μM) |
|----------|----------------|
| WT       | 19.1 ± 0.2     |
| ΔahpC1   | 3.2 ± 0.3      |
| ΔahpC2   | 10.0 ± 0.1     |
| ΔahpΔhr  | 13.7 ± 0.4     |
| ΔahpΔaf  | 10.4 ± 0.4     |
| ΔahpΔgex | 4.7 ± 0.2      |

Data are expressed as mean ± SEM of two independent experiments performed in triplicate.

| Strain                     | IC₅₀ HOCl (μM) |
|----------------------------|----------------|
| WT                        | 19.1 ± 0.2     |
| ΔahpC1                    | 3.2 ± 0.3      |
| ΔahpC2                    | 10.0 ± 0.1     |
| ΔahpΔhr                   | 13.7 ± 0.4     |
| ΔahpΔaf                   | 10.4 ± 0.4     |
| ΔahpΔgex                  | 4.7 ± 0.2      |

Previously, we had demonstrated that PA14 was more resistant to the killing by neutrophils in presence of uric acid. We found that the oxidation of uric acid by the neutrophil heme-peroxidase, myeloperoxidase, decreased HOCl formation and, consequently, the microbicidal activity of these cells [28]. Because the oxidation of uric acid by myeloperoxidase generates the oxidants urate free radical and urate hydroperoxide, we asked whether AhpC1 could neutralize urate hydroperoxide and contribute to bacteria resistance against this oxidant. Here, we reproduced those previous findings as physiological concentrations of uric acid (200 and 400 μM) significantly protected PA14 from killing by neutrophils (Fig. 7, white bars). Interestingly, uric acid had the opposite effect to ΔahpC1 mutant since it increased even more the killing capability of neutrophils against this strain (Fig. 7, grey bars). This result suggests that AhpC1 is indeed important for protecting bacteria from alternative oxidants, such as the organic peroxide, urate hydroperoxide. Because urate hydroperoxide is a weaker oxidant than HOCl, it is not as efficient as the latter to kill PA14. However, in the absence of AhpC1, which likely neutralizes urate hydroperoxide, it becomes an efficient microbicidal.
To look further in this direction, we incubated purified urate hydroperoxide with wild-type, \( \Delta \text{ahpC} \) and \( \Delta \text{ahpC} \) attB::ahpC1. Urate hydroperoxide was unable to kill wild-type and the complemented \( \Delta \text{ahpC} \) strain counting at low concentrations (Fig. 7B and D), but it reduced \( \Delta \text{ahpC} \) strain counting at low concentrations (Fig. 7C).

To prove that AhpC1 can indeed neutralize urate hydroperoxide, we calculated the rate constant for the reduction of urate hydroperoxide by the recombinant AhpC from \( X. \text{fastidiosa} \). AhpC1 from \( P. \text{aeruginosa} \) and AhpC from \( X. \text{fastidiosa} \) share 61% identity, 78% homology, with both peroxidatic Cys47 and resolving Cys165 conserved among the two species (Supplem Fig. 1). By mixing AhpC with crescent concentrations of urate hydroperoxide or \( \text{H}_2\text{O}_2 \) a fast drop in the intrinsic fluorescence was observed, which is consistent with the oxidation of the enzyme \[ 48 \] \[ 50 \] (Fig. 8A and C). The plot of \( k_{\text{obs}} \) versus urate hydroperoxide (Fig. 8B) or \( \text{H}_2\text{O}_2 \) (Fig. 8D) concentrations gave a second-order rate constant of \( 2.3 \pm 0.09 \times 10^6 \) and \( 1.5 \pm 0.07 \times 10^8 \text{M}^{-1}\text{s}^{-1} \), respectively. These values are much in agreement with those calculated by the reaction of urate hydroperoxide with human 2-Cys Prx2 \[ 49 \] and by \( \text{H}_2\text{O}_2 \) with human 2-Cys Prx2 and AhpC from \( S. \text{typhimurium} \) \[ 50 , 63 - 65 \]. AhpC also seems to react faster with HOCl, however, we could not determine the rate constant by fluorescence since HOCl also oxidizes the tryptophan residues altering protein fluorescence (data not shown).

We next evaluate the effect of uric acid upon wild-type and \( \Delta \text{ahpC} \) \( P. \text{aeruginosa} \) infection in vivo using a pharmacological model of mouse hyperuricemia. In this model, mice were daily treated with potassium oxonate, an inhibitor of uricase, suspended in carboxymethylcellulose for slow release. A single injection of oxonate is enough to maintain hyperuricemia for at least 24 h \[ 57 \]. Indeed, the concentration of uric acid
was nearly 3-fold higher in mice treated with oxonate (5.25 ± 0.12 mg/dL) versus those treated with vehicle (1.73 ± 0.01 mg/dL) on the 7th day following oxonate injection (Fig. 9B). Hyperuricemia highly enhanced infection-induced mortality. All mice died 24 h after intranasal instillation of wild-type PA14 (Control), WT or ΔahpC1 strains (2 × 10⁶ CFU/mL) and euthanized after 24 h. Tissues were harvested, macerated in PBS-glucose and the supernatant was diluted and spread onto MH agar plates. CFU was counted after growth for 18–20 h at 30°C. Each bar represents mean ± SEM of 8 mice in the infected group and 3 mice in the control group. Statistical analyses were performed by one-way ANOVA analysis of variance followed by Newman-Keuls. *p < 0.05 indicates a significant difference from control (non-infected) and #p < 0.05 indicates a significant difference to ΔahpC1 group.

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hyperuricemic mice instilled with sterile saline. Since all infected mice died within three days, we decided to evaluate how this increase in plasma uric acid was affecting cytokine production and release in this initial period of hyperuricemia. The pro-inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) were significantly higher in hyperuricemic than normouricemic mice on the 7th day following oxonate injection (Fig. 9C). These results corroborate the above and previous findings \[28\] by showing that uric acid promotes PA14 survival and infection severity likely by disrupting HOCl production, increasing inflammatory status and tissue damage.

\(\Delta ahpC1\) PA14 enhanced virulence in hyperuricemic mice reveals that, despite the fact this strain is more sensitive to the product of uric acid oxidation, urate hydroperoxide, the overall decrease in HOCl and increase in inflammatory status by uric acid are dominant to sustain bacterial virulence in vivo.

4. Discussion

The 2-Cys peroxiredoxin AhpC has proven to be an important peroxide detoxifying enzyme \[50\]. It was described to confer \textit{Francisella tularensis} resistance against superoxide, organic peroxides, H\(_2\)O\(_2\) and nitric oxide donors \[66\]. In contrast, another study revealed that deletion of AphC in \textit{Francisella tularensis} did not render a higher susceptibility to H\(_2\)O\(_2\), only to parquat (a superoxide generating compound) and to the peroxynitrite generator, SIN-1 \[67\]. Such difference in H\(_2\)O\(_2\) susceptibility was related to a compensatory effect by catalase that occurs in some \textit{Francisella tularensis} subspecies. Interestingly, the most resistant \textit{Francisella tularensis} SCHU S4 strain was the most sensitive to deletion of AhpC \[66,67\]. This compensatory effect was also detected in \textit{Helicobacter cinaedi} and \textit{Staphylococcus aureus}, since \(\Delta ahpC\) strain had greater catalase activity \[26,27\]. An increase in catalase (kat) expression occurred by a relief in PerR repression in AhpC mutants \[26\]. We did not detect this compensatory mechanism in \(\Delta ahpC1\) PA14 since it was much

Fig. 5. Total neutrophils (A) peroxidase activity (B) and cytokines (C) in lung tissues after 24 h of infection. Representative photomicrography of lung stained with H&E from an animal in the control group (D), inoculated with the WT (E) or \(\Delta ahpC1\) (F) at 400 × magnification. Arrows are pointing to neutrophils. C57BL/6 mice were intranasally infected with \(2 \times 10^6\) CFU/mL PA14. Data are expressed as mean ± SEM of 8 animals for the infected groups and 3 animals for the control group. Statistical analyses were performed by one-way ANOVA analysis of variance followed by Newman-Keuls. \(*p < 0.05\) indicates a significant difference from control (non-infected) and WT+ABAH groups; \(#p < 0.05\) indicates a significant difference from \(\Delta ahpC1\) group.

Fig. 6. TBARS (thiobarbituric acid-reactive substances) in lung tissue (A) and nitrite in BAL (B) after 24 h of infection. C57BL/6 mice were intranasally infected with \(2 \times 10^6\) CFU/mL wild-type (WT) or \(\Delta ahpC1\) strains. TBARs concentration in the lung was obtained by plotting the absorbance in a malondialdehyde (MDA) standard curve and expressed as \(\mu\)mol of MDA equivalents/g protein. Data are expressed as mean ± SEM of 8 animals for the infected groups and 3 animals for the control group. Statistical analyses were performed by one-way ANOVA analysis of variance followed by Newman-Keuls. \(*p < 0.05\) indicates a significant difference from control (non-infected) group \(#p < 0.05\) indicates a significant difference from \(\Delta ahpC1\) group.
Fig. 7. (A) Bacterial survival after incubation with neutrophils in presence or absence of uric acid. Opsonized wild-type or ΔahpC1 (1 × 10⁷ CFU/mL) were incubated with neutrophil (1 × 10⁶ cells/mL) for 1 h in the presence or absence of uric acid. Neutrophils were lysed, samples were diluted 10,000-fold in PBS-glucose. Ten microliters were spread onto MH agar plates and grown for 18–20 h at 30 °C before CFU counting. PA14 survival to urate hydroperoxide in wild-type (B), ΔahpC1 (C) and ΔahpC1 attB:ahpC1 (D). Data were plotted as means ± SEM (n = 4). Statistical analyses were performed by one-way ANOVA analysis of variance followed by Newman-Keuls post-test. *p < 0.05 indicates a significant difference from the group without uric acid in (A) or urate hydroperoxide (B–D). MM: minimal medium; V: urate hydroperoxide vehicle (evaporated mobile phase from HPLC, see methods).

Fig. 8. Kinetics for the reduction of urate hydroperoxide (A, B) and hydrogen peroxide (C, D) by AhpC. (A) Pre-reduced AhpC (1 μM) was mixed with 60 μM urate hydroperoxide (C) Pre-reduced AhpC (0.5 μM) was mixed with 1 μM H₂O₂ in 50 mM sodium phosphate buffer (pH 7.4, 25 °C) and monitored over time (λex = 280 nm, λem > 340 nm) in a stopped-flow device. Data were fitted as a single exponential to obtain the rate constant (kobs) for each peroxide concentration. kobs were plotted against varying urate hydroperoxide (B) or H₂O₂ (D) concentrations to calculate the second order rate constant from the slope of the linear fitting. V: voltage.
more sensitive to H$_2$O$_2$ than the wild-type and the complemented AhpC1. Therefore, despite the antioxidant arsenal present in Pseudomonas aeruginosa, no redundancy for AhpC1 was evidenced.

The compensatory mechanism described in those previous studies was detected using very high concentrations of H$_2$O$_2$ (0.25–1 M) in the halo assay. Noteworthy, catalase is likely to be more much relevant in such high H$_2$O$_2$ concentrations, whereas peroxiredoxins, as AhpC, reduce H$_2$O$_2$ at lower, baseline, concentrations [68]. Indeed, AhpC was the primary detoxifier of endogenous H$_2$O$_2$ generated by aerobic metabolism, whilst KatE played a major role in scavenging exogenous and supraphysiologic levels of H$_2$O$_2$ in Brucella abortus [69].

There is a consensus, however, in the protection afforded by AhpC to organic peroxides [26,27,66]. These previous studies revealed a higher sensitivity of AhpC strains against tert-butyl and cumene hydroperoxides. In the present study we demonstrated, for the first time, the higher sensitivity of an AhpC mutant against a physiological organic peroxide, urate hydroperoxide, which is produced at significant amounts in the inflammatory oxidative burst [30].

We had previously demonstrated that uric acid protected P. aeruginosa against the killing activity of neutrophil-like cells. Uric acid is a substrate for myeloperoxidase and dislocates the production of HOCl [28]. Additionally, uric acid can directly scavenger HOCl [70] [1] [72]. The decrease in HOCl was the main cause for the enhanced P. aeruginosa survival [28]. If on the one hand uric acid decreased HOCl, on the other hand it increased the oxidative status during the inflammatory oxidative burst. Therefore, the protection afforded by uric acid against bacterial killing was not due to a global antioxidant activity. Urate free radical is the first product from the oxidation of uric acid by myeloperoxidase and it can directly dismutate and hydrate to a more stable product, the hydroxyisourate. In presence of superoxide, as in the inflammatory oxidative burst, the combination of urate free radical with superoxide generates the more stable, but strong oxidant, urate hydroperoxide. Our previous findings suggested that urate hydroperoxide was not as efficient as HOCl to kill P. aeruginosa [28]. Here we proved that P. aeruginosa is much more sensitive to HOCl (IC$_{50}$ 19.1 ± 0.2 µM) than to urate hydroperoxide (no killing up to 100 µM) and that this resistance is partially attributed to AhpC1. Noteworthy, deletion of AhpC1 increased the sensitivity to all isolated oxidants: HOCl, urate hydroperoxide and H$_2$O$_2$. AhpC rapidly reduces urate hydroperoxide and H$_2$O$_2$. The rate constants obtained in this study were much similar to that found for the reduction of urate hydroperoxide and H$_2$O$_2$ by the human 2-Cys peroxiredoxin Prx2 and the Salmonella typhimurium AhpC [49,50,64,65]. We could not calculate the rate constant for the reaction of AhpC with HOCl since the latter also oxidizes tryptophan, interfering in the intrinsic protein fluorescence. However, the assessment of AhpC dimer formation under incubation with HOCl suggested that this reaction is also very fast (data not shown).

The key role of AhpC1 in urate hydroperoxide detoxification was confirmed by the distinct effect when neutrophils incubated with uric acid were challenged with wild-type or ΔahpC1 strains. Whereas uric acid protected wild-type PA14 from neutrophil killing, it increased the killing activity against ΔahpC1 PA14. Altogether, these data corroborate the relevance of AhpC1 as a protection mechanism to inflammatory organic peroxides and as a non-redundant factor for P. aeruginosa subversion from the innate immune system.

Deletion of AhpC1 highly attenuated P. aeruginosa virulence to mice. All survived the 15 days of observation when intranasally infected with ΔahpC1, whereas 100% died before the third day of infection when instilled with wild-type or the complemented ΔahpC1 attBΔahpC1. Similarly, all mice infected with ΔahpC1 Francisella tularensis survived the 21 days of observation but those infected with wild-type strain died within 8 days of infection [66]. Another study also revealed a prolonged time to death in mice infected with ΔahpC1 Francisella tularensis [73].

Likewise, a much lower number of ΔahpC1 P. aeruginosa colonized lung and spleen compared to wild-type. As a consequence, a lower number of inflammatory cells was found in bronchoalveolar lavage and lung with lower release of the inflammatory cytokines IL-1β and TNF-α and oxidative stress. Therefore, the absence of AhpC1 in P. aeruginosa attenuated the infection capability, shortening bacteria clearance from lung and decreasing tissue inflammation, which increased host survival.

In agreement, deletion of AhpC impaired Francisella tularensis replication in bone marrow-derived macrophages and in mice organs [67]. The Helicobacter cinaedi ahpC mutant was more susceptible to killing by macrophages than the wild-type strain and the cecal colonizing ability of the ahpC mutant was significantly reduced in mice [27]. Mycobacterium tuberculosis mutant for catalase, which also had less AhpC, had a diminished bacterial growth, lower induction of pro-inflammatory cytokines and significantly reduced pathology scores in infected mice [74]. In contrast, an attenuation in virulence of a double katA ahpC mutant was not verified in Staphylococcus aureus, but bacterial environmental persistence and nasal colonization were strictly dependent on the presence of these enzymes [26].

Brucella abortus ahpC katE double mutant had an extremely attenuated virulence and this effect was not attenuated in mice lacking NADPH oxidase or inducible nitric oxide synthase activities, suggesting that host baseline endogenous H$_2$O$_2$ production represents a relevant stress to this strain [69]. However, deletion of NADPH oxidase was equally lethal to wild-type and ΔahpC1 Francisella tularensis strains, contrasting the

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**Fig. 9.** (A) Hyperuricemic C57BL/6 mice survival after intranasal instillation of sterile saline (non-infected), WT or ΔahpC1 (2 × 10$^6$ CFU/mL). Mice were observed twice a day for survival or any other distress up to 5 days after bacterial instillation. On the 7th day following oxonate treatment, non-infected mice were euthanized for serum uric acid (B) and cytokines (C) measurements. Statistical analyses were performed by one-way ANOVA analysis of variance followed by Newman-Keuls. *p < 0.05 indicates a significant difference from vehicle.
decreased virulence of *AhpC* to wild-type mice [66]. In our hands, inhibition of neutrophils NADPH oxidase or myeloperoxidase significantly increased wild-type and *AhpC* PA14 survival in a similar fashion revealing that neutrophil oxidative burst is crucial to PA14 killing despite the presence of AhpC1.

**ΔahpC1** was also less virulent than wild-type PA14 to hyperuricemic mice, contrasting the effect observed in isolated neutrophils, where incubation with uric acid protected wild-type but increased **ΔahpC1** death. Although it seems contrasting, the establishment of hyperuricemia in mice, contrasting the effect observed in isolated neutrophils, where in inhibition of neutrophils NADPH oxidase or myeloperoxidase significantly decreased wild-type and *AhpC* PA14 survival in a similar fashion revealing that neutrophil oxidative burst is crucial to PA14 killing despite the presence of AhpC1.

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