Unraveling the biological role of LsfA, a 1-Cys Prx involved in the *P. aeruginosa* virulence

“Desvendando o papel biológico de LsfA, uma 1-Cys Prx envolvida na virulência de *Pseudomonas aeruginosa*”

Supervisor: Dr. Luis Eduardo Soares Netto

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Thesis submitted to the Institute of Biosciences of the University of São Paulo, as a partial requirement to obtain the doctoral degree in Biological Sciences.

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“Empty your mind. Be formless. Shapeless. Like water. You put water into a cup, it becomes the cup. You put water into a bottle, it becomes the bottle. You put it in a teapot, it becomes the teapot. Water can flow, or it can crash. Be water, my friend.”

Bruce Lee

“Do or do not. There is no try.”

Master Yoda
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1. General Introduction

1.1. Pseudomonas aeruginosa

1.1.1. General Aspects

*Pseudomonas aeruginosa* is a gamma-proteobacteria, that acts as an opportunistic pathogen in immunocompromised patients. The origin of the word *Pseudomonas* is from the Greek word Pseudo = false, and monas = only unit; whilst *aeruginosa* is from the Latin word aerūgō = rusty copper. *P. aeruginosa* is a bacterium similar to a rod, with 1-5 µm length and 0,5-1 µm height, that presents a green-blue color. It grows as a facultative aerobic bacterium and is able to use more than a hundred different molecules as carbon sources to obtain energy. The ideal temperature of growing is around 37°C, but it can survive between 4-42 °C; in the soil, consuming aromatic hydrocarbons or in water reservoir, as in sewer. The versatility of *P. aeruginosa* is remarkable as this bacterium can occupy several niches, colonizing several different organisms as: plants, ameba, nematodes, and vertebrates (DIGGLE; WHITELEY, 2020; LYCZAK; CANNON; PIER, 2000; WILLIAMS; DEHNBOSTEL; BLACKWELL, 2010).

This bacterium can cause a wide range of pathological processes, being involved in wounds, pulmonary and eye infections; strongly associated with cystic fibrosis patients, which develop pulmonary infection. *P. aeruginosa* infections are often related to the decline of pulmonary functions and patients’ mortality. Biofilm formation and drug-resistant strains represent serious threats that make the corresponding infections almost impossible to eradicate (CIRZ *et al.*, 2006; COURTNEY *et al.*, 2007; WINNIE; COWAN, 1991).

*P. aeruginosa* is among the main causes of nosocomial infections in Brazil and in the world (ANVISA, 2016; GALES *et al.*, 2001; SADER *et al.*, 2015). The beginning of the infection occurs by the colonization of the impaired respiratory epithelium; as the ones suffering with cystic fibrosis or immunocompromised (STREETER; KATOULI, 2016). The contamination occurs mainly due to contaminated hospital material, where this bacterium is capable of adhering, mainly because of its ability to form biofilm (DONLAN, 2001).
Between 2012 and 2013, *P. aeruginosa* was the most abundant gram-negative bacteria found in patients hospitalized in intensive care units in USA (SADER *et al.*, 2015); with 32600 estimated cases in hospitalized patients, causing 2700 deaths in 2017, according to the Center for disease control and prevention (https://www.cdc.gov/drugresistance/biggest-threats.html#pse). In Latin America, this bacterium was the most prevalent among bacterial pathogens on pneumonia patients, the fifth on cases of blood infections and the third most abundant in wound infections, accordingly to SENTRY (Resistance vigilance program) (GALES *et al.*, 2012). In Brazil, *P. aeruginosa* was the fifth etiologic agent found in blood related with central venous catheter at adult, pediatric and new-born intensive care units between 2012-2016; additionally, up to 43% of the isolates presented carbapenem resistance (ANVISA, 2016). *P. aeruginosa* isolates which present antibiotic resistance are another aggravating in infections by this pathogen (MAGIORAKOS *et al.*, 2012). An international observation showed that, in intensive-care units, *P. aeruginosa* represents 16.2% of patient infections and was the cause of 23% of all ICU-acquired infections, mainly related to respiratory infections (VINCENT *et al.*, 2020). Taken all these facts together, the World Health Organization (WHO) classified *P. aeruginosa* in the list of critical priorities for research and Discovery of new drugs (HARBARTH *et al.*, 2017).

1.1.2. Oxidative Stress and Bacterial Antioxidant systems

The initial oxidative stress concept was formulated in 1985 as a disbalance favoring the prooxidant in contraposition to the antioxidant side of a balance, which can provoke cell damage. More recently, this concept evolved in two different situations. Eustress is the oxidative stress generated under normal physiological conditions, while distress is the supraphysiological challenge imposed by high levels of oxidants, generating a pathological condition (reviewed by SIES, 2018).

Different oxidants can be generated during both eu/distress, the most famous ones are the reactive oxygen species (ROS), which includes hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (‘OH) and superoxide anion radical (O$_2$•–). Despite their capacity to damage biological molecules, such as lipids, proteins, and DNA (CROSS *et al.*, 1987), their involvement with the transduction of signals in the cells has been revealed as another
relevant role played by these molecules. Indeed, dysregulation of ROS formation and/or decomposition may impair redox signaling, generating a disease (FINKEL, 2011).

ROS molecules can be generated by different sources. In a eukaryotic cell under physiological conditions, one of the main sources is mitochondria. Initially, superoxide is formed by monoelectronic reduction of molecular oxygen, mainly at complex I and III, but also in complex II, which can then be converted to \( \text{H}_2\text{O}_2 \) by mitochondrial SOD (NAPOLITANO; FASCIOLI; VENDITTI, 2021).

Other important sources of oxidants are the NADPH-dependent oxidases that take part of seven-members family (NOX 1-5 and Duox 1-2), which are involved in the oxidative burst in phagocytic cells and also in other physiological processes (DI MEO et al., 2016; FINKEL, 2011). Superoxide formed by the action of NOX cannot penetrate the bacterial membranes under physiological pH, due to its negative charge. However, superoxide can be dismutated to \( \text{H}_2\text{O}_2 \), which can cross membranes, as this peroxide displays a permeability coefficient similar to water. If the exogenous levels of \( \text{H}_2\text{O}_2 \) exceed 0.2 µM, the influx ratio of \( \text{H}_2\text{O}_2 \) becomes higher than the endogenous amount formed; which happens inside the phagosome during infections, where the levels of \( \text{H}_2\text{O}_2 \) can be elevated by one order of magnitude (HOPKINS, 2017; MISHRA; IMLAY, 2012; SEAVER; IMLAY, 2001).

Nitric oxide (NO\(^{•}\)), a reactive nitrogen species (RNS), can be generated by the activity of nitric oxide synthase (NOS), from the metabolism of the amino acid L-arginine. Three NOS isoforms were identified in mammals: the neuronal (nNOS), the endothelial (eNOS) and the inducible (iNOS), the last one is present in phagocytes (ALDERTON; COOPER; KNOWLES, 2001). While nNOS and eNOS are calcium-dependent, iNOS is not (NATHAN; XIE, 1994; XUE et al., 2018). NO\(^{•}\) produced by nNOS is important for cellular communication between neurons, while NO\(^{•}\) produced by eNOS is involved in the relaxation of endothelial cells. In these cases, NO\(^{•}\) is generated at low levels. In contrast, NO\(^{•}\) is generated in high amounts by iNOS in macrophages, which is involved in killing pathogens. Therefore, depending on its levels, NO\(^{•}\) presents distinct properties (NATHAN; XIE, 1994).

Reaction between NO\(^{•}\) and superoxide anion can form peroxynitrite, another powerful oxidant capable to potentialize the killing capacities of phagocytes (RADI, 2018). Peroxynitrite has a short half-life in physiological pH, but it is membrane permeable,
making this molecule capable of interfering in the surrounding cells. Noteworthy, peroxynitrite can oxidize/nitrate many biomolecules, as proteins, low molecular weight thiols, DNA, unsaturated fatty acids and enzymatic cofactors (CALCERRADA; PELUFFO; RADI, 2011). This molecule is also capable to interfere in many inflammatory, cardiovascular and neurodegenerative conditions (RADI, 2018). It is important to note that different peroxiredoxins are able to reduce peroxynitrite very efficiently (BRYK; GRIFFIN; NATHAN, 2000; MANTA et al., 2009; SANDER et al., 2002; TRUJILLO; FERRER-SUETA; RADI, 2008).

Pathogenic microorganisms evolved strategies to overcome the oxidative challenge imposed by these molecules. Indeed, bacterial defenses were recently analyzed, based on the KEGG database, (https://www.genome.jp/kegg/), comprising 26 different types of proteins, across ~24k bacterial genomes (JOHNSON; HUG, 2019).

**Thioredoxin system:** Thioredoxin is one of the most prevalent thiol disulfide oxidoreductases (JOHNSON; HUG, 2019), displaying essential cellular functions such as the reduction of ribonucleotides into deoxyribonucleotides and modulation of transcription factors’ activity (HIROTA et al., 1999; LUTHMAN; HOLMGREN, 1982; MOORE; REICHARD; THELANDER, 1964). Upon reduction of target disulfides, thioredoxin is oxidized, being re-reduced by thioredoxin reductase, a flavoenzyme that uses electrons from NADPH. Thioredoxin reductases are divided as high molecular weight enzymes and are present in mammals; while low molecular weight thioredoxin reductases are present in organisms such as prokaryotes, fungi and plants (DE OLIVEIRA et al., 2021; WILLIAMS et al., 2000).

**Glutaredoxins:** Other oxidoreductases capable of catalyzing thiol-disulfide exchange reactions, being also involved with the formation of deoxyribonucleotides for DNA synthesis, maintaining the levels of reduced sulfur, signal transduction and oxidative stress defense (reviewed by FERNANDES; HOLMGREN, 2004). In contrast to thioredoxins, glutaredoxins display high affinity for glutathione and their activities are also coupled with glutathione reductase and NADPH. Glutaredoxins are also widespread, enzymes of glutaredoxin 2 subgroup are present only in three bacterial phyla, predicted only in 3.7% of bacterial genomes. The other three glutaredoxins groups described in KEGG are glutaredoxin 1, 3 and the monothiolic ones; all of them more prevalent than Glutaredoxin 2 group (JOHNSON; HUG, 2019). These enzymes can display both a
monothiolic or a dithiolic mechanism, which are distinct by the differences in
deglutathionylation steps (MASHAMAITE; ROHWER; PILLAY, 2015).

**Superoxide scavenging enzymes:** Superoxide dismutase (SOD) are enzymes that
catalyze superoxide dismutation, leading to H₂O₂ production. These enzymes can be
classified according to the nature of its metal cofactor, SOD 1 (Cu/Zn), SOD 2 (Fe/Mn)
and SOD N (Ni). Among all bacterial genomes, SOD 2 is the most abundant one (72.9%),
while SOD 1 and SOD N were only present in 29.2% and 7.9% of genomes respectively
(JOHNSON; HUG, 2019). Additionally, several SODs were already related with both
antioxidant defense and virulence in different bacteria (IIYAMA et al., 2007; KANAFANI;
MARTIN, 1985; KANG; KIM; LEE, 2007; PIDDINGTON et al., 2001; SEYLER;
OLSON; MAIER, 2001).

**Hydroperoxide scavenging enzymes:** Catalase are enzymes capable of reducing
H₂O₂ to water and oxygen; and peroxynitrite to nitrite (GEBICKA; DIDIK, 2009). The
KEGG divide catalases in three groups: catalase, Mn catalase and catalase-peroxidase,
whose prevalence are 41.4%, 13.1% and 33.4% among all bacterial genomes, respectively
(JOHNSON; HUG, 2019; NICHOLLS; FITA; LOEWEN, 2000). Noteworthy, some
catalases are related with virulence in bacteria, and display compensatory effects with other
peroxidases (COSGROVE et al., 2007; LEE et al., 2005; MANDELL, 1975; WOOD et
al., 2003; XU; PAN, 2000).

Glutathione peroxidases are seleno-Cys or sulfur-Cys based proteins that reduce
peroxides in a glutathione (GSH), thioredoxin or even PDI (Protein disulfide isomerase)
dependent manner (CONRAD; FRIEDMANN ANGELI, 2018; TRUJILLO et al., 2022).
In mammals, these proteins are mainly seleno-Cys peroxides, reacting very efficiently
towards several oxidants, such as H₂O₂, peroxynitrite, cholesterol and phosphatidyl choline
hydroperoxides (reviewed by TRUJILLO et al., 2022). In bacteria, these proteins are
sulfur-Cys based, and their genes were found in 54.5% of the genomes analyzed
(JOHNSON; HUG, 2019).

Ohrs (organic hydroperoxide resistance proteins) are enzymes that reduce organic
hydroperoxides highly efficiently in comparison with H₂O₂. Ohrs are also highly efficient
in reducing peroxynitrite and their catalytic power is also based on a reactive cysteine
residue (so called peroxidatic Cys or Cp), which takes part of a catalytic triad together with
fully conserved arginine and glutamate residues (ALEGRIA et al., 2017; DOMINGOS et
Another fully conserved residue among Ohrs is a cysteine residue (resolving Cys or Cₐ) involved in the condensation reaction with a sulfenic acid (Cys-SOH) in Cₚ, resulting in an intra-molecular disulfide bond formation. Afterwards, Ohr is reduced by a lipoylated protein (CUSSIOL et al., 2010). The expression of Ohr genes is regulated in several bacteria by OhrR, a redox, Cys based transcriptional repressor (reviewed by MEIRELES et al., 2022).

Peroxiredoxins (Prx) are widespread, very abundant and highly efficient enzymes, therefore, representing a major group of antioxidant proteins responsible to scavenge different hydroperoxides. These enzymes react very efficiently with a wide range of peroxides with extremely high rate constants (10⁶-10⁸ M⁻¹.s⁻¹), being present in all the three domains of life (RHEE, 2016). These enzymes are also Cys based peroxidases, and the reactive cysteine (also named Cₚ) takes part of a conserved PxxxxT/SxxC motif. Another conserved feature among all Prxs is the presence of a catalytic triad composed of Cₚ, a Thr/Ser and an Arg (HALL et al., 2011). For some Prxs, a second cysteine residue (so-called resolving cysteine or Cₐ) forms a disulfide bond with Cₚ. Additionally, the reduction step varies among Prxs and can involve glutathione (GSH), thioredoxin or ascorbate (FISHER et al., 1999; MONTEIRO et al., 2007; PEDRAJAS et al., 2010, 2016a).

Prxs display different functions in different organisms, such as: defending pathogens from the oxidative burst and its involvement with microorganism virulence (DE OLIVEIRA et al., 2021); sensing and transducing signals (RHEE; WOO; KANG, 2018) and even as PAMPS (pathogen-associated molecular pattern) and DAMPS (host-derived damage- associated molecular patterns) (RHEE, 2016). One sub-family of peroxiredoxins (AhpC/Prx1) is found in 67.2% of all bacterial phyla, but almost exclusively in aerobic bacteria (JOHNSON; HUG, 2019).

1.2. Peroxiredoxins

1.2.1. Overview and the classification

Prxs is part of a large and widespread family of Cys-based peroxidases, involved in functions like regulation of cell proliferation, differentiation, and apoptosis. Prxs can achieve high concentrations in mammalian cells, up to 1% of all soluble protein content; while in Escherichia coli, these peroxidases are among the top ten most abundant proteins (HANSCHMANN et al., 2013).
Beyond Prxs abundance and reactivity (NETTO; ANTUNES, 2016), mammalian Prxs can reduce different types of hydroperoxides, including the fatty acids products derived from lipoxygenases and cyclooxygenases activities (CORDRAY et al., 2007); which are involved in multiple inflammatory pathways (reviewed by KNOOPS et al., 2016). Notably, some cytosolic Prxs are released from necrotic brain cells to the extracellular space, inducing the expression of inflammatory cytokines in macrophages, thereby promoting neural cell death. In contrast, intracellular Prxs are neuroprotective (SHICHITA et al., 2012). Therefore, the extracellular pool of Prxs represents danger signals in the ischemic brain activating Toll-like receptors (SHICHITA et al., 2012).

In general, the catalytic cycle of Prxs consists in the two-electron oxidation of C_P, which is stabilized as a thiolate (C_P-S^-); resulting in sulfenic acid formation and the reduction of the hydroperoxide substrate and releasing a water molecule, nitrite, or the corresponding alcohol, depending on the peroxide used as substrate. For their turnover, thioredoxins (Trxs) (RHEE; CHAE; KIM, 2005) glutathione (GSH) (FISHER, 2011; PEDRAJAS et al., 2016b) or ascorbate (MONTEIRO et al., 2007) among other species can be utilized as reducing agents. Alternatively, C_P-SOH can react with a second peroxide molecule, being hyperoxidized to sulfenic (C_P-SO_H) or sulfonic (C_P-SO_3H) acid. These hyperoxidized species can only be reduced in 2-Cys Prxs (AhpC/Prx1 subfamily) in an ATP dependent manner (HYUN et al., 2005). The high reactivity of the Prxs is related with the fact that these enzymes stabilize the transition state of the nucleophilic substitution reaction between C_P and the peroxide (HALL et al., 2010).

Peroxiredoxins can be classified based on their catalytic mechanism as 1-Cys Prx and 2-Cys Prxs. The 1-Cys Prxs presents only one Cys residue (C_P) throughout the catalytic cycle. Thereby, after the oxidation, C_P in the sulfenic acid form is directly reduced by its reductant (MONTEIRO et al., 2007; PEDRAJAS et al., 2016b)(Fig. 1A). In the other hand, the 2-Cys Prxs presents a second Cys residue (C_R) participating in catalysis (PERKINS et al., 2015). After the oxidation, the C_P-SOH condenses with C_R, generating a disulfide bond that can be intra-subunit (for the so-called atypical 2-Cys Prxs) or inter-subunit (for the so-called typical 2-Cys Prxs) (PERKINS et al., 2015) (Fig. 1B).
Fig. 1: Peroxiredoxins catalytic mechanism, A) 1-Cys Prxs. B) 2-Cys Prxs, divided into typical and atypical. Highlighting the sulfur atom (S) from the Cys residues part of the reaction (C_P and C_R).

In the reduced state of 2-Cys Prxs, the C_P and C_R are more than 10Å apart. Therefore, a structural rearrangement is required for disulfide formation. The last turn of the α helix containing C_P, unfolds, generating the so-called locally unfolded (LU) state. In contrast, the conformation of 2-Cys Prxs in the reduced and highly reactive state is called fully folded (FF). This FF-LU structural switch is proposed to occur in all Prxs, however is largely studied only in 2-Cys Prxs (KARPLUS, 2015).

Beyond this division based on their mechanisms, Prxs are frequently classified according to their primary sequences and structures (POOLE; NELSON, 2016). In this way, six subfamilies are considered: Prx1/AhpC (or only Prx1), Prx5, Prx6, Tpx, BCP/PrxQ (or only PrxQ) e AhpE (POOLE; NELSON, 2016). Here, we describe studies with the PaLsfA protein, which belongs to the Prx6 subfamily, and presents the 1-Cys Prx mechanism.

1.2.2. Prx6 subfamily

Enzymes from the Prx6 subfamily present mostly the 1-Cys Prx mechanism (POOLE; NELSON, 2016), and are one of the less studied Prx groups. This fact is reflected by the low number (around ten) of structures available on the Protein Data Bank (PDB: https://www.rcsb.org/) (HALL et al., 2011). Moreover, the identities of Prx6 biological
reductants are mostly unknown. Our research group showed that 1-Cys Prxs can be reduced by ascorbate, representing a breakthrough in the thiol specific activity paradigm of these peroxidases (MONTEIRO et al., 2007). However, the relevance of this reaction in biological systems is still to be demonstrated.

The best characterized Prx6 enzyme is the mammalian isoform, which the reductive pathway is mostly attributed to glutathione (GSH) and requires a heterodimerization with \( \pi \)-glutathione transferase (ZHOU et al., 2016). Although, it is possible that in some tissues where Prx6, but not \( \pi \)-glutathione transferase, is present, ascorbate might be a relevant reductant.

In the case of *Saccharomyces cerevisiae* 1-Cys Prx, which is named ScPrx1, several reductants were proposed: (i) Thioredoxin (Trx) or GSH (PEDRAJAS et al., 2016b), (ii) glutaredoxin (Grx) with GSH (PEDRAJAS et al., 2010) and ascorbate (MONTEIRO et al., 2007). Noteworthy, ScPrx1 is located both into the matrix and the intermembrane space of yeast mitochondria (GOMES et al., 2017), where distinct reductants might act.

Regarding the oxidative pathway, the human Prx6 (or Prdx6) is well characterized, being capable to reduce very efficiently (~10\(^7\) M\(^{-1}\)s\(^{-1}\)), not only H\(_2\)O\(_2\), but also low molecular weight hydroperoxides, such as tert-butyl hydroperoxide (t-BOOH) and cumene hydroperoxide and peroxynitrite. The Prdx6 has a unique capacity among the Prxs to reduce phospholipids hydroperoxides, making the Prdx6 one of the main enzymes responsible for the reduction of oxidized phospholipids in lungs and other organs (reviewed by FISHER, 2017). Additionally, the reduction of some lipid hydroperoxides by Prdx6 were already described, such as: 9-HpODE ((\(\pm\))9-hydroperoxy-10E,12Z-octadecadienoic acid), 13-HpODE ((9Z,11E)-13-hydroperoxyoctadeca-9,11-dienoic acid) and 15-HpETE ((5Z,8Z,11Z,13E)-15-hydroperoxyicosa-5,8,11,13-tetraenoic acid). In addition, there is a correlation between the peroxidatic activity of Prdx6 and the FAHFAs (fatty acid ester of hydroxy fatty acid) levels in adipose tissue (PALUCHOVA et al., 2022).

A characteristic that makes Prdx6 unique among Prxs is its capacity to hydrolyze glycerophospholipids at the sn-2 position, exhibiting a phospholipase A\(_2\) (PLA\(_2\)) activity. This activity is calcium independent, with a clear preference for phosphatidylcholine, which is more active under acidic pH and regulated by phosphorylation. It is dependent on the S\(^{32}\)-D\(^{140}\)-H\(^{26}\) triad, which is conserved in several members of Prx6 subgroup, but not in 2-Cys Prxs. Noteworthy, both peroxidatic and PLA\(_2\) active sites are distinct (FISHER, 2017; WU et al., 2009). Additionally, another exclusive activity of mammalian Prdx6 is the capacity to acylate lysophosphatidylcholine with a free fatty acid, also called lysoPC-
acyl CoA transferase activity (LPCAT). This activity is governed by the $^{26}\text{HxxxxD}^{31}$ motif that presents a preference for choline. It is important to note that LPCAT activity acts continuously with the PLA$_2$ activity, without the release of the intermediate substrate (FISHER, 2017).

All of these three activities acting together are responsible for preventing: (i) oxidative damage by repairing peroxidized lipids (MANEVICH et al., 2002) and (ii) changes in lipid metabolism in the lungs and, consequently, affecting its surfactant composition. Prdx6 capacity to scavenge PLOOH in mouse lungs is crucial for protection against oxidative stress (LIU et al., 2010). Besides the direct reduction of the damaged phospholipid, the PLA$_2$ together with the LPCAT activity could hydrolyze the phospholipid and reacylate using a non-oxidized fatty acid. Null mutants’ mice for Prdx6 or chemically treated with the PLA$_2$ activity inhibitor (MJ33), exhibited a diminished turnover of the lung surfactant phospholipids, whilst the opposite was observed when overexpressing Prdx6 (FISHER et al., 2005, 2006). Prdx6 can regulate NOX2, which is closely dependent on the PLA$_2$ activity and Prdx6 phosphorylation (CHATTERJEE et al., 2011). Together, those activities can completely regenerate the oxidized phospholipids into the membrane (Fig. 2).
Fig. 2: Scheme of the HsPrdx6 activity in the membrane repair. A) represents a phospholipid membrane in resting state. B) Oxidation of one of the lipidic chain, enabling the action of HsPrdx6, which can C) Directly reduce the phospholipid hydroperoxide by the peroxidatic activity, or D) remove the oxidized chain, by the PLA2 activity and then reinsert the reduced lipidic chain by the LPCAT activity, going back to the original state of the phospholipid membrane (Adapted from FISHER, 2017).

1.2.3. The Prx6 from *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* possess several antioxidant proteins: SOD, heme-peroxidases, and thiol peroxidases, including several Prxs. PaLsfA is the only enzyme that belongs to the Prx6 subgroup, displaying a molecular mass of 24 kDa and only one cysteine residue, C\text{45}, which is the C\text{P}. Besides, PaLsfA also possesses the motif, which in mammalian Prx6 enzymes is involved with their phospholipase activity independent of calcium (aiPLA\textsubscript{2}) and their LPCAT activity (KAIHAMI *et al.*, 2014; KIM; LEE; KIM, 2016).

The expression of PaLsfA was analyzed in genome wide studies, being induced by sulfate (TRALAU *et al.*, 2007) and iron starvation (HEIM *et al.*, 2003); sodium hypochlorite (SMALL *et al.*, 2007) or paraquat challenge (HARE *et al.*, 2011a). In the other hand, PaLsfA expression was diminished when exposed to tributyltin, an immune system inhibitor and endocrine disruptor in humans (DUBEY; TOKASHIKI; SUZUKI, 2006). PaLsfA was also found during planctonic growth of this bacterium (PARK *et al.*, 2014). Additionally, a ChIP-chip analysis identified the promoter region of *lsfA* interacting with the H\textsubscript{2}O\textsubscript{2}-responsive transactivator, OxyR; which is capable to promote the expression of several antioxidant proteins, such as: katA, katB, ahpB and ahpCF (WEI *et al.*, 2012).

The only study specifically focused in PaLsfA revealed an important role of this protein in *P. aeruginosa* virulence, protecting the bacteria against the oxidative burst generated by macrophages (KAIHAMI *et al.*, 2014). The phagocytosis of bacteria lacking PaLsfA (\Delta lsfA) by J774 macrophages (representing the M1 subtype that is more pro-inflammatory) was similar than the wild-type bacterial strain. However, \Delta lsfA strain had their survival impaired within macrophage, which was related to the protection afforded by PaLsfA against the oxidative burst imposed by NOX2. In the wild-type strain, TNF-\alpha and IFN-\gamma production were inhibited by PaLsfA, via the MAPK and NF-kB pathways. In addition, the absence of PaLsfA also affected the recruitment of macrophages and neutrophils, and neutrophils activation in mice’s lungs. Finally, the survival rate of the mice infected by the mutant bacteria to PaLsfA were higher when compared with the mice infected by the wild-
type strain. All these data strongly related the peroxidatic activity of PaLsfA with the virulence of *P. aeruginosa* (KAIHAMI *et al.*, 2014). Although it was clearly shown that PaLsfA interferes with the regulation of inflammatory pathways, the molecules and mechanisms underlying this process remain to be elucidated.

1.3. Inflammatory response to an invading pathogen

1.3.1. Inflammatory response

Inflammatory process represents a protective response by the host immune system that generates some cardinal signs, such as: rubor, calor, tumor and dolor (SERHAN, 2017). Inflammation has been known since the ancient civilizations and occurs in response to a harmful stimulus, including pathogen invasion or damaged cells (CHEN *et al.*, 2018). It is important to note that the inflammatory response involves two distinct phases: acute inflammation and resolution. A natural course of this process is composed by a first, acute inflammation followed by a resolutive step that will lead the organism back to the basal homeostasis, mediated by a concerted temporal production of lipid mediators (SERHAN *et al.*, 2015), among other molecules (Fig. 3). The common inflammatory response involves inflammatory inducers, which will start the cascade. Then, sensor cells detect these inducers and produces the inflammatory mediators, which will affect the target tissues. For bacterial pathogens, after their detection by the host, through Toll-like receptors (TLRs), macrophages produce inflammatory cytokines and chemokines (e.g., TNFα, IL-1, IL-6, CCL2 and CXCL8) and other mediators, such as the prostaglandins (MEDZHITOV, 2010).
The observed immune response can vary according to the bacterial strain (clinical or lab isolates), mouse model, dose of the inoculum, and the time post-infection that is analyzed. However, a lethal challenge to mice occurs in conditions where bacteria generate a hyperinflammatory response, generating a septic shock (LIN; KAZMIERCZAK, 2017).

To invade host lungs, pathogens need to bypass the airway mucus, which possesses a myriad of strategies to combat bacterial invasion, such as: antimicrobial peptides, opsonization, antimicrobial proteins and the alveolar macrophages (LIN; KAZMIERCZAK, 2017). Macrophages represent the first line of the immune response. These phagocytic cells respond to several signals and kill pathogens by the release of several oxidants and by signaling to the recruitment of other phagocytes, such as neutrophils (GWINN; VALLYATHAN, 2006).

Besides its microbicidal activities, oxidants (especially H$_2$O$_2$) can also act as second messengers in the NF-κβ and MAPK dependent pathways, triggering a pro-inflammatory response (GWINN; VALLYATHAN, 2006; MORGAN; LIU, 2011; SON et al., 2011). In this way, multiple redox processes are involved in the regulation of NF-κβ (Fig. 4). For instance, NF-κβ regulates the expression of genes encoding antioxidant enzymes (SODs and thioredoxins); and, NADPH Oxidase (NOX2), Cyclooxygenase-2 (COX-2), 12-lipoxygenase (LOX-12) and LOX-5 (MORGAN; LIU, 2011).

Fig. 3: Time course of the inflammatory process, highlighting the different stages of the inflammation and the lipid mediators presence in each stage (SERHAN et al., 2015).
In addition, lipid molecules promote signaling in both acute inflammation and resolution phases. For instance, prostaglandins and leukotrienes, which are derived from arachidonic acid oxidation through the activities of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), promote the initiation of the inflammatory process. In contrast, specialized pro-resolving mediators (SPM) stop acute inflammation and thereby promote the resolution step (SERHAN, 2017).

Furthermore, Prxs can interfere in signaling pathways related to both inflammatory and resolution processes. For instance, lipopolysaccharide (LPS) increases the expression of human Prx1 that contributes to alter the levels of pro-inflammatory mediators, resulting in NF-κβ activation (LIU et al., 2014). Meanwhile, the gene expression of Prx6 (human orthologue of PaLsfA) is regulated by cyclooxygenases and prostaglandin E2 in primary macrophages (BAST et al., 2010). Curiously, Prdx6 is capable of activating NOX2 and detoxifying lipid hydroperoxides, by its phospholipase and peroxidase activities (reviewed by AREVALO; VÁZQUEZ-MEDINA, 2018; ELKO et al., 2019).

Prxs are also present in pathogenic bacteria, but less is known about their involvement in acute inflammation and resolution. Previously, the involvement of PaLsfA was described in P. aeruginosa virulence (KAIHAMI et al., 2014). Because of its peroxidase activity, PaLsfA decreases the macrophage oxidative state and protects P. aeruginosa from NAPDH oxidase-generated oxidants (KAIHAMI et al., 2014). Additionally, PaLsfA inhibits TNF-α production, influences the recruitment of
macrophages and neutrophils and their activation; thus, playing an important role in virulence in acute pneumonia model (KAIHAMI et al., 2014).

1.3.2. Resolutive response

As mentioned above, the inflammatory process is composed of two phases: acute inflammation and resolution (Fig. 3). Problems related to the occurrence of the resolutive phase generate a state of chronic inflammation, often associated with diseases such as asthma, cardiovascular diseases, diabetes, and rheumatoid arthritis (LEVY; SERHAN, 2014). Lipid mediators (LM) are involved in regulation of the resolutive phase, such as those derived from arachidonic acid, Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and n-3 docosapentaenoic acid (n-3 DPA). Their synthesis is precisely and temporally regulated, starting with the increased levels of leukotrienes and prostaglandins during the acute inflammation, followed by a switch to the production of specialized pro-resolving mediators (SPMs), including lipoxins, resolvins and protectins, and maresins, in a spatio-temporal sequence during the resolutive phase (SERHAN et al., 2015). Specialized pro-resolving mediators are a class, derived from ω-6 and ω-3 essential polyunsaturated fatty acids (PUFA) as arachidonic acid (AA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and eicosapentaenoic acid (EPA) capable to sophisticatedly regulate the resolution of inflammation by the formation of four main classes of lipid mediators: lipoxins, resolvins, maresins, and protectins (LEUTI; MACCARRONE; CHIURCHIÙ, 2019).

Lipoxins, generated from arachidonic acid, have both anti-inflammatory and pro-resolving properties, downregulating neutrophil transmigration and increasing the uptake and removal of apoptotic neutrophils by macrophages (LEVY; SERHAN, 2014). Resolvins, derived from EPA (E-series) and DHA (D-Series) with resolutive properties such the capacity to reduce the polymorphonuclear leukocyte (PMN) infiltration and pro-inflammatory cytokine/chemokine production (ARITA et al., 2006), and are capable to enhances bacterial killing/clearance (CODAGNONE et al., 2018; SPITE et al., 2009). Protectins are derived from DHA and are capable of decreasing leukocyte infiltration in murine peritonitis and reduce neutrophil trans endothelial migration, and enhance human macrophage efferocytosis (LEVY; SERHAN, 2014). Finally, maresins are formed from DHA, capable to restore tissue homeostasis after inflammation, counter regulating the
proinflammatory cytokines such as IL-1β, IL-6, and TNF-α limiting the recruitment of PMNs and neutrophils, and stimulating phagocytosis and efferocytosis (TANG et al., 2018).

All those SPMs have initial steps in which a hydroperoxide is formed into the PUFA by lipoxygenases (15-LOX, 12-LOX and 5-LOX) or cyclooxygenases (COX-2), as exemplified for the DHA pathway (Fig. 5). In the case of the cysteinyl-SPMs, the conjugation with glutathione is required, which is catalyzed by glutathione transferase or leukotriene C4 synthase, with the generations of: resolvin-CTR (RCTRs), maresin conjugates in tissue regeneration (MCTRs) and protectin-CTR (PCTRs). All these SPMs at pico/nanomolar concentration are capable to protect organs by the stimulation of tissue regeneration (JORDAN; WERZ, 2022; SERHAN; CHIANG, 2023; SERHAN; CHIANG; DALLI, 2018).

Fig. 5: Scheme of the formation of SPMs derived from DHA, which involves a formation of a hydroperoxide in the first step by 15-LOX, 12-LOX or COX-2 (JORDAN; WERZ, 2022).

Noteworthy, these SPMs are capable of influencing host protection during bacterial infection, decreasing the requirements for antibiotics (reviewed by SERHAN; LEVY,
Of note, lipoxin A₄ can reduce the virulence of *P. aeruginosa* through the inhibition of quorum sensing and increase the phagocytic ability of the neutrophils (WU et al., 2016). Also, lipoxin A₄ and resolvin (Rv)D2 reduced biofilm formation and virulence gene expression (THORNTON et al., 2021).

Therefore, we hypothesize that PaLsfA can regulate both NF-κβ and MAPK pathways, probably by interfering with both the H₂O₂ and fatty acid hydroperoxides levels. Thus, as PaLsfA can control NF-κβ (KAIHAMI et al., 2014), which in turn regulates the expression of LOX-5 and COX2 genes, possibly the levels of these oxygenases might be affected by PaLsfA, and consequently their products.

In this way, we aimed to deeply characterize PaLsfA on its biological influence in *P. aeruginosa*. First, we investigate its relationship with the only reductant agent described so far (by us), ascorbate. Then characterize the roles of PaLsfA in the oxidative defense of *P. aeruginosa* using different techniques. Finally, using macrophages, we intend to verify how the bacterial protein – PaLsfA – could interfere with the inflammatory/resolutive pathways, looking at the lipid mediators.
2. General Methodology

2.1. Table of Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
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<td>PaLsfA&lt;sup&gt;His37Lys&lt;/sup&gt; Foward</td>
<td>Gtgaagtgcgggcttgagagaagcagcgc</td>
</tr>
<tr>
<td>PaLsfA&lt;sup&gt;His37Lys&lt;/sup&gt; Reverse</td>
<td>Gcgtgcgtctcaagcggccgacttcac</td>
</tr>
<tr>
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<tr>
<td>PaLsfA&lt;sup&gt;His37Phe&lt;/sup&gt; Reverse</td>
<td>gcgtgcgtctcctcgggccgacttcac</td>
</tr>
<tr>
<td>PaLsfA&lt;sup&gt;Thr120Ser&lt;/sup&gt; Foward</td>
<td>Cgccaacgacagctgactgctgctgctggttgc</td>
</tr>
<tr>
<td>PaLsfA&lt;sup&gt;Thr120Ser&lt;/sup&gt; Reverse</td>
<td>gaacagccagacgctgctgctgctgctggttgc</td>
</tr>
</tbody>
</table>

2.2. PaLsfA mutants’ generation and production

Using the pET15b-PaLsfA<sup>WT</sup> plasmid as template, the following mutants were generated: PaLsfA<sup>His37Lys</sup>, PaLsfA<sup>His37Phe</sup>, PaLsfA<sup>Thr120Ser</sup>. Primers were constructed using the Agilent web tool (https://www.agilent.com/store/primerDesignProgram.jsp). All the reactions were carried using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) following the manufacturer instructions. The procedure used for expression and purification of PaLsfA mutants was the same as for the PaLsfA<sup>WT</sup>, previously established by me during my masters (ALEIXO-SILVA, 2018).

2.3. Competition assay between DCPIP and PaLsfA for ascorbate.

Recombinant PaLsfA was first reduced overnight using an 20x excess of DTT (1,4-Dithiothreitol), that was after removed using two HiTrap™ Desalting coupled and the reduced protein was collected following the UV spectra in the FPLC. To obtain the oxidized form (sulfenic acid), PaLsfA was treated using a ratio of 1:1 of H<sub>2</sub>O<sub>2</sub>. Oxidation state of PaLsfA was confirmed using DTDPy (aldrithiol, Cat. 143057 - Sigma-Aldrich). Both DCPIP (ε<sub>600nm</sub> = 20500 M<sup>-1</sup> cm<sup>-1</sup>) and ascorbate (ε<sub>265nm</sub> = 14500 M<sup>-1</sup> cm<sup>-1</sup>) solutions were prepared fresh and quantified spectrophotometrically in the assay buffer (50 mM
potassium phosphate pH 7.2, 50 mM NaCl). Reaction mixtures contained 4 μM ascorbate (injected to start the kinetics), DCPIP (45 μM) and variable protein concentrations (nine replicates per concentration) in the same potassium phosphate buffer described above. In some points of the dataset, we attempted to use the highest possible protein concentration, so the difference between the initial and final absorbance would be higher. Reactions were monitored at 600 nm (oxidized DCPIP) in a Synergy H1 (BioTek® Instruments, Inc.) plate reader with automated injection for 2 minutes at 25 ºC.

2.4. Disk diffusion assay.

This assay was followed according a pre-established protocol (KAIHAMI et al., 2014). An overnight culture of PA14 WT or ΔlsfA (both harboring the empty PJN105) were diluted to O.D.₆₀₀ₙₐₜ=0.1 and grown until O.D.₆₀₀ₙₐₜ=1. In a petri dish containing solid LB, 3 mL of 0.7% soft LB agar + 200 μL bacterial culture was added, in order to form a thin layer of cells. After solidification, sterile paper disks (~6mm diameter), three per plate, were saturated with 2.5% H₂O₂ or 1% t-BOOH and placed on the plate. Plates were incubated for 16 hours/37ºC. and photographed. Inhibition halos were measured using imageJ software, in cm. Experiments performed in triplicates and the results were expressed as a percentage of the WT halo.

2.5. CFU (colony-forming unit) assay.

This experiment was performed as described before by our group (ALEGRIA et al., 2017). Bacterial strains -WT or ΔlsfA (both harboring the empty PJN105) - were grown in LB broth + 50μg/mL gentamycin 37ºC to O.D.₆₀₀ₙₐₜ=1, washed twice with PBS and diluted to O.D.₆₀₀ₙₐₜ=0.1. 200 μL aliquots of cell suspensions were treated with 5mM ATZ for 10 minutes at room temperature, to inhibit catalase. Then, 3 mM SIN-1 or 2.5 mM paraquat were added and incubated for 30 min at 37ºC. Treated cells were serial diluted in 10 mM MgSO₄ and plated on solid LB broth (without antibiotic). Colonies-forming units (CFU) were counted after 16 hours/37ºC. The experiments were expressed as a percentage in relation to the untreated cells. Experiments performed at least in triplicates.
2.6. Ascorbate/Dehydroascorbate in vivo quantification.

PA14 WT was grown overnight in LB media, washed and diluted to O.D. = 1 in 50 mL of PBS containing 10 mM of ascorbate and incubate at 37 C for seven hours, which represented time zero. After 1, 3 or 7 hours, cells were collected by centrifugation and ascorbate/dehydroascorbate content was analysed in the supernatant. In order to quantify the intracellular content, the pellet was washed twice with PBS and resuspended into 2mL of PBS. Cells were disrupted by 1-minute cycles of sonication (15s ON/45s OFF) in ice. Then, the methanol method to quantify both dehydroascorbate and ascorbate were employed as described (BADRAKHAN et al., 2004).

2.7. Hyper7 in P. aeruginosa.

Freshly transformed cells of PA14 WT and ΔlsfA strains, containing both plasmid pUCP18-Hyper7 and pUCP18-Empty, were grown overnight in LB media containing 300 ug/mL of carbenicillin. In the morning, cells were pelleted and diluted to O.D.\textsubscript{600nm}=7 in PBS. Using a Synergy H1 (BioTek® Instruments, Inc.) plate reader with automated injection, 180 µL of the cells were added in duplicates into a 96 wells black plate, with transparent bottom. A 2-minute baseline was measured before the addition of H\textsubscript{2}O\textsubscript{2}, which was automatically added in different concentrations. After addition, the fluorescence was measured for about 20 minutes using excitation at 408 nm and 488 nm and emission at 520 nm (KRITSILIGKOU; SHEN; DICK, 2021), reading the plate from the bottom. Results were expressed as the 488 nm/408 nm ratio of the fluorescence excitations. For the measures of ascorbate reduction, after the pulse of 4mM of H\textsubscript{2}O\textsubscript{2} for 1-minute, different concentrations of ascorbate was added.


Using the human kinase Erk2 (GI: 119554) to phosphorylate the recombinant Prdx6 proteins (150 ng/µL), we performed the assay in a 50 mM Tris-Cl pH 7.5, 20 µM EGTA buffer, containing 10 mM MgCl2 and 2 mM ATP in the presence or absence, of active Erk2 kinase (10 ng/µL). Reactions were carried out for 90 minutes at 30 °C with shaking. For Erk2 dilution, we followed the manufacturer instruction, using the buffer 50 mM Tris-
Cl pH 7.5, 0.1 mM EGTA, with or without 0.1 mM Na$_3$VO$_4$, 0.1% 2-mercaptoethanol and 1 mg/mL bovine serum albumin (BSA).

2.9. Reduction, Alkylation and Tryptic Digestion for MS Samples.

Phosphorylated proteins were mixed with 100 mM ammonium bicarbonate buffer pH 8 plus 8 M urea (1:1) to denature them, reduced with 10 µL of 10 mM dithiothreitol (DTT) for 1 h at 30ºC. Afterwards, samples were alkylated in an amber tube using the Eppendorf ThermoMixer (Eppendorf, Hamburg, Germany) by adding 10 µL of 500 mM iodoacetamide for 30 min at 25ºC (final volume 140 µL). Samples were then diluted with 540 µL of 100 mM ammonium bicarbonate buffer pH 8 and digested by adding 3 µL of Trypsin Gold (Promega, Madison, USA) 40 ng/µL (protein/enzyme ratio of 50:1) for 16 h at 37ºC. Resulting peptides were neutralized by adding 0.1% trifluoroacetic acid and completely dried in speed vac. Finally, samples were concentrated using ZipTip resin (Merck, Darmstadt, Germany) according to the manufacturer’s protocol, dried again and stored at -20ºC until use.

2.10. Evaluation of Thr Phosphorylation by MS

To check the Phosphorylation of Prdx6 proteins, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a nanoACQUITY UPLC system (Waters Corporation, Milford, USA) coupled to a TripleTOF 6600 mass spectrometer (AB SCIEX, Framingham, USA). Analysis was conducted under trap and eluted mode using a nanoACQUITY UPLC-Symmetry (Waters Corporation, Milford, USA) containing a C18 trap column (20mm × 180µm; 5µm) and a separation column (75 µm × 150mm; 3.5 µm). Trapping was done at 10µL/min with 2% of solvent B. Peptides were separated with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.4 µL/min using the following gradient: 2–35% B from 0 to 60 min; 35–85% B from 60 to 61 min; isocratic elution with 85% B from 61 to 65 min; 85–2% B from 65 to 66 min; isocratic elution with 2% B from 66 to 85 min. Nano-electrospray ion source was operated at 2.2 kV (ion spray voltage floating, ISVF), curtain gas 20, interface heater (IHT) 120, ion source gas 1 (GS1) 3, ion source gas 2 (GS2) zero, declustering potential (DP) 80V. Time-of-flight mass spectrometry (TOF-MS) and mass spectrometry analysis (MS/MS) data
were acquired using information-dependent acquisition (IDA) mode. For IDA parameters, a 250ms survey scan in the m/z (mass-to-charge ratio) range of 300–2000 was followed by 25 MS/MS ions in the m/z range of 100–2000 acquired with an accumulation time of 100ms (total cycle time 2.8 s). Switch criteria included intensity greater than 150 counts and charge state 2–5. Former target ions were excluded for 4 s. Software used for acquisition and data processing were Analyst® TF 1.7 (AB SCIEX, Framingham, USA) and PeakView® 2.2 (AB SCIEX, Framingham, USA), respectively. For the analysis of protein modification, MASCOT 2.4 software (Matrix Science Ltd., London, United Kingdom, Redoxoma-FAPESP user license 10.10.1.46/Mascot) was used with mass tolerance of 10ppm for MS experiments and 0.05 Da for MS/MS experiments.

2.11. Hydroperoxides Biosynthesis and Isolation.

Commercial 15-LOX (Soybean, type V, sigma (L6632)) was used to produce hydroperoxides derived from fatty acids as described before (DALLI; COLAS; SERHAN, 2013). Briefly, 30 U of 15-LOX was incubated with 0.5 ug/µl of arachidonic acid (AA)/100 ng of n-3 docosapentaenoic acid (n-3 DPA) / 75 ng of docosahexaenoic acid (DHA)/75 ng of eicosapentaenoic acid (EPA) and submitted to sonication/vortexing cycles (30 seconds each) every four minutes. The reaction was carried for 20 min/ice in a 5 mL of borate buffer, to produce: 15-HpETE, 17-HpDPA, 17-HpDHA, and 15-HpEPE respectively. Then, reaction was stopped by adding one volume of methanol. Samples were submitted to a liquid-liquid extraction by adding 2 volumes of diethyl ether, transferring the top layer, and fully evaporating; at the end, the samples were resuspended in 50 uL of methanol. Finally, the samples were purified using a C18 column in an HPCL system and quantified spectrophotometrically at 234 nm. After the biosynthesis, all the hydroperoxides were confirmed by LC-MS-MS analysis, using NaBH4 to reduce the hydroperoxide and generate the corresponding reduced product, that is more stable to be identified.


To obtain the reduced protein, LsfA was first incubated with 20x excess of DTT (dithiothreitol) overnight in the fridge and then the DTT was removed. Incubation of reduced LsfA (0.2 µM) and different concentrations (indicated in the text) of the
hydroperoxides was performed for 1 minute at room temperature, in the protein buffer (Hepes 20 mM, NaCl 150 mM). The reaction was stopped by adding two volumes of methanol+internal standard (d85HETE). The reduced product for each hydroperoxide was measured by mass spectrometry, comparing samples in presence of LsfA or without this enzyme. The obtained pg/mL concentration was converted to velocity (Mol/second) to obtain the individual k for each hydroperoxide (Hp) concentration, using the following equation:

\[ k = \frac{v}{[LsfA]M + [Hp]M} \]

Finally, to determine the k of LsfA with each hydroperoxide, the individual k were plotted against the hydroperoxide concentration, the slope of the resulting equation is the final k.

2.13. Macrophages culture.

Differentiated macrophages were obtained from peripheral blood mononuclear cells (PBMCs) as described (DALLI; SERHAN, 2012). Briefly, isolated human monocytes were used to obtain M1 macrophages by incubating with GM-CSF (20 ng/ml) for 6 days in RMPI 1640 (supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and penicillin/streptomycin), followed by LPS (100 ng/ml) plus INF-γ (20 ng/ml) treatment for 48 h. To M2 20 ng/ml M-CSF in RPMI 1640 is used for 6 days followed by polarization with 20 ng/ml IL-4 for 48 h.


PA14 WT and ΔlsfA strains were grown overnight in LB media containing gentamicin. In the morning, these cultures were harvested by centrifugation and resuspended into PBS and the optical density was measured. One million differentiated macrophages M1 or M2 were added to the wells of an 12/6 well plate in the RPMI 1640 media without serum. For all the experiments we used a multiplicity of infection (MOI) of 1:50 cells:bacteria. The co-incubation was carried out for 45 minutes or 24 hours and stopped by the addition of methanol or perchloric acid, depending on the subsequent experiment.
2.15. **Lipid mediator profiling.**

At the end of each co-incubation, was added two volumes of methanol + deuterium-labelled internal standards (IS), and the macrophages were scraped from the wells and stored at -80°C until analysis. Extraction procedure and LC–MS–MS were performed as previously described (COLAS; GOMEZ; DALLI, 2020). Resulting peaks were identified and integrated using the SCIEX OS software; Partial Least-Squares Discriminant Analysis (PLS-DA) was performed in the MetaboAnalyst web server (https://www.metaboanalyst.ca/), whilst the pathway analysis figure was generated using the Cytoscape software.
7. General Discussion and Conclusion

Until now, the only description about PaLsfA was describing its involvement with the virulence of *P. aeruginosa* (KAIHAMI et al., 2014) and in proteomic/transcriptomic studies revealing the changes in its levels in response to sulfate or some oxidants (HARE et al., 2011b; QUADRONI et al., 1999; TRALAU et al., 2007). In this way, the work presented in chapter 3 strongly contributed to the understanding of how this antioxidant protein works in *P. aeruginosa*. The kinetic aspect of PaLsfA was the initial step to understand the capacity of this protein in the antioxidant defense. It is well known that the reactivity of the Prxs with different oxidants is in the range $10^6$ – $10^8$ M$^{-1}$s$^{-1}$ (RHEE, 2016), so despite the very efficient reaction between PaLsfA and H$_2$O$_2$, t-BOOH, peroxynitrite (chapter 3), 15 HpETE and 17 HpDPA (chapter 6) this results was not a surprise. Several Prxs were related with the antioxidant defense of microorganisms and with its virulence (DE OLIVEIRA et al., 2021). As an example of its versatility, *P. aeruginosa* possess an outstanding number of antioxidant proteins and systems to support its activity. To note, this bacterium has 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, periplasmic GPx2 and GPx3, osmotically inducible protein C – OsmC, organic hydroperoxide resistance protein – Ohr, bacterioferritin comigratory protein – Bcp, 1-Cys-peroxiredoxin PaLsfA and the 2-Cys-peroxiredoxins alkyl hydroperoxide reductases AhpA, AhpB and AhpC) (https://www.pseudomonas.com). Among them, some have been already characterized and related with its virulence, as the KatA in acute infection in Drosophila (APIDIANAKIS; RAHME, 2009) and peritonitis in mice (LEE et al., 2005). Beyond PaLsfA, another Prx was already related with the virulence of *P. aeruginosa* - AhpC1 – capable to protect the bacterium against the inflammatory oxidative burst caused by urate hydroperoxide (ROCHA et al., 2021). Noteworthy, PaLsfA also contributed to the resistance of *P. aeruginosa* against HOCl (ROCHA et al., 2021).

Members of the Prx6 subgroup typically possess a dimeric or decamer/dodecamer organization, which is shared with the Prx1 subgroup (POOLE; NELSON, 2016). In general, archaeal Prxs tends to present a higher oligomerization state, while the others tend to form only dimers, at least for the Prx6 subgroup. As shown in chapter 3, PaLsfA is a Prx6 member that forms only dimer in all the oxidation states analyzed.
As our group first described the reaction of ascorbate with 1-Cys Prxs (MONTEIRO et al., 2007), the next step was to kinetically characterize this reaction. Using different 1-Cys Prxs, and a 1-Cys like (a Tsa2, from S. cerevisiae, with a mutation in the resolution cysteine), we showed a very consistent second order rate constant of 10^3 M^{-1}.s^{-1} for all of them, as described in chapter 4. We further characterize this reaction with PaLsfA using the solved crystallographic structures and a molecular docking approach. This strategy revealed some amino acids which frequently interact with the ascorbate molecule. The same approach used in chapter 4 was used to characterize this reaction with the mutants for these amino acids. Surprisingly, the resulting second order constant rate was higher in those mutants of the His37 and Thr120. Revealing ascorbate as the only reductant of PaLsfA until now, however the biological implications of this molecule remained unclear.

Another putative activity of several Prx6 is the phospholipase A_2 calcium independent activity, which is well described for the mammalian homologue (CHEN et al., 2000), but never described for any other member of this subgroup before. Here, we characterized, in chapter 5, the phospholipase activity of PaLsfA 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), the main component of the pulmonary surfactant (VELDHUIZEN et al., 1998), as 3.38 ± 0.1 at acidic pH and 0.04 ± 0.002 at neutral pH; additionally to others non-mammalian Prdx 6. Despite the phosphorylation of the Triticum aestivum, Arabidopsis thaliana and Aspergillus fumigatus Prdx6, we could not identify PaLsfA phosphorylation by the human Erk2 kinase, even with the high conservation of the Thr residue in the primary and tertiary structure of these proteins. It is reasonable to propose that this Thr in PaLsfA might be phosphorylated by some endogenous kinase from P. aeruginosa, but not the human Erk2 kinase. For instance, the ppkA protein from P. aeruginosa – a Ser/Thr protein kinase – was already related to environmental adaptation and virulence, variations of biofilm formation, pyocyanin production, tolerance to stress, cell invasion and plant virulence; curiously the response to oxidative stress is also affected by the lack of this protein (PAN et al., 2017). This protein shares ~30% identity with human Erk2.

Biological implications of the phospholipase activity from PaLsfA remains unclear in P. aeruginosa; however, under the tested conditions, it is not related with the role of PaLsfA in the virulence of P. aeruginosa (KAIHAMI et al., 2014). P. aeruginosa, as an extremely versatile pathogen, has several phospholipases, among them is ExoU, which is associated with the type III secretion system. Upon its release, ExoU is capable to disrupt the integrity
of eukaryotic cell membranes and lead to their rapid lysis; and consequently being related with bacterial virulence (VASIL, 2006). Curiously, the cytosolic phospholipase A2 from epithelial cells is activated during the P. aeruginosa infection, resulting in a release of arachidonic acid (KIRSCHNEK; GULBINS, 2006).

In addition to the capacity of protection of P. aeruginosa against different stresses, this bacterium is also capable to sabotage the host signaling pathways to disrupt and interfere into the inflammatory response, specially within the lipid mediators. An epoxide hydrolase, cystic fibrosis transmembrane conductance regulator inhibitory factor (Cif), is capable to disrupt 15-epi LXA4 transcellular biosynthesis and function by the hydrolyzation of 14,15-EET, suppressing the transepithelial neutrophil migration (FLITTER et al., 2017). P. aeruginosa can also produce LoxA – a 15-LOX -, which can catalyze the peroxidation of several free fatty acids (including arachidonic acid) with positional specificity. This enzyme cannot react with 5- or 15-HETEs, and is required biofilm growth in association with the host airway epithelium (DESCHAMPS et al., 2016; VANCE et al., 2004). Further, the activity of LoxA decrease the recruitment of immune cells in the airspace, modulate the cell response of macrophages and neutrophils, and can ultimately induce the generation of LXA4 by neutrophils (MORELLO et al., 2019). ExoU, a bacterial secreted phospholipase, is capable to promote a release of arachidonic acid and the production of prostaglandins PGE2 and PGI2, in addition to the increase in the mono/polymorphonuclear cells recruitment (SALIBA et al., 2005).

Despite the clear capacity of P. aeruginosa interfering into the host inflammatory response, it is unknown to us the involvement of a bacterial antioxidant protein in this process. Here, in chapter 6, we first described the involvement of a peroxiredoxin in the production/signaling of lipid mediators related to the inflammatory and resolutive response. The strong influence of PaLsfA can be noted in the increased release of free PUFAs in the initial steps of co-incubation, for both macrophage subtypes and the lower levels of hydroxides in ΔlsfA strain, revealing the antioxidant capacity of PaLsfA interfering into the signalization of the macrophages. Results from the disbalance caused by PaLsfA are noted after these cells were subjected to a lipid mediator profiling, clearly showing differences in several analytes present in all the pathways analysed. Curiously, maresin 1 – MaR1 – in all cases, was downregulated in the ΔlsfA co-incubation. MaR1 can limit polymorphonuclear neutrophil (PMN) infiltration and enhance human macrophage uptake of apoptotic PMNs, as stimulate efferocytosis; acting as a potent anti-inflammatory
and pro-resolving molecule (SERHAN et al., 2012). It is known that *P. aeruginosa* can survive in a hyperinflammatory state, as the one faced during cystic fibrosis infections. (COHEN; PRINCE, 2012).

Another possible step affected by PaLsfA is through its antioxidant activity and the consequent reduction in macrophages oxidative state (KAIHAMI *et al.*, 2014). Our data revealed a strong impact into the mediators which requires a glutathione transferase activity to be formed, including cysteiny1 leukotrienes, PCTR-s and MCTR-s. The biosynthesis of these compound could be impaired by the alterations in the redox state of the macrophages and consequently in the glutathione pool. Finally, the overall changes in the pathways analysed could suggest a broad interference of PaLsfA by the change in the spatio-temporal production of the initial signalization molecules, disturbing the whole inflammatory/resolutive process.

In conclusion, our findings revealed PaLsfA as a very efficient antioxidant protein, capable to react with a wide range of hydroperoxides, and protect *P. aeruginosa* from superoxide and H$_2$O$_2$. We newly described two crystallographic structures of this protein, which enabled us to deeper characterize the reduction of PaLsfA by ascorbate, its only reductant until now. The description of a PLA$_2$ activity for PaLsfA includes this protein in a group of peroxiredoxins with this activity which included only the mammal isoforms before. Finally, the influence of PaLsfA in the inflammatory lipid mediator biosynthesis opens a new perspective about host-pathogen interaction affected by bacterial antioxidant proteins.
8. References


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9. Abstract

9.1. Abstract

*Pseudomonas aeruginosa* is a ubiquitous, gamma-proteobacteria, which is the main cause of nosocomial infection among all pathogens related to pneumonia in the Intensive Care Unit. We are interested in the redox aspects involved in host-pathogen interactions. PaLsfA belongs to the peroxiredoxin (Prx) family and to the subgroup that contains only one catalytic cysteine (so-called 1-Cys Prx). Prxs are enzymes capable of removing peroxides (including peroxynitrite) at very high rates. As PaLsfA is related to the *P. aeruginosa* virulence we intended in the present thesis, further advance the characterization of this protein, regarding its biological roles in *P. aeruginosa*. We first evaluated the rate constant between ascorbate and PaLsfA, the only reductant of this protein until now. Also, the importance of some amino acids related to the reaction between them, which, surprisingly, for some mutants, increased the reactivity around ten times. By different microbiological approaches, we revealed the importance of PaLsfA for the bacterial defense against H₂O₂ and paraquat (a superoxide generator), but not against SIN-1 (peroxynitrite generator). Using the genetically encoded probe – Hyper7 – we revealed the capacity of PaLsfA protecting *P. aeruginosa* against H₂O₂ and the capacity of ascorbate to act as an intracellular reductant of PaLsfA in this bacterium. Additionally, we first described a phospholipase activity for a bacterial Prx, however the biological implication of this activity remains unclear. Finally, the capacity of PaLsfA influencing the inflammatory process in macrophages is a first description of a bacterial Prx influencing the host inflammatory response, correlating to its involvement with *P. aeruginosa* virulence. In this way, our findings can enlighten the understanding about how this protein affects the virulence of *P. aeruginosa* and make possible future insights into inhibitory mechanisms.
9.2. Resumo

*Pseudomonas aeruginosa* é uma gamma-proteobactéria ubiquita, principal causa de infecção nosocomial entre todos os patógenos relacionados à pneumonia na Unidade de Terapia Intensiva (UTI). Estamos interessados nos aspectos redox envolvidos nas interações hospedeiro-patógeno. A PaLsfA pertence à família da peroxirredoxinas (Prx) e ao subgrupo que contém apenas um cisteína catalítica (chamada 1-Cys Prx). As Prxs são enzimas capazes de remover peróxidos (incluindo peroxinitrito) em altas velocidades. Como a PaLsfA está relacionada à virulência de *P. aeruginosa*, pretendemos na presente tese avançar ainda mais na caracterização dessa proteína, abordando seus papéis biológicos em *P. aeruginosa*. Primeiro, avaliamos a constante de velocidade entre a ascorbato e PaLsfA, o único redutor desta proteína descrito até o momento. Além da importância de alguns aminoácidos relacionados à reação entre eles, que, surpreendentemente, para alguns mutantes, aumentou a reatividade em torno de dez vezes. Por diferentes abordagens microbiológicas, revelamos a importância de PaLsfA para a defesa bacteriana contra H$_2$O$_2$ e paraquat (um gerador de superóxido), mas não contra SIN-1 (gerador de peroxinitrito). Usando a sonda geneticamente codificada - Hyper7 - revelamos a capacidade de PaLsfA de proteger *P. aeruginosa* contra o H$_2$O$_2$ e a capacidade da ascobato de atuar como um redutor intracelular de PaLsfA nesta bactéria. Além disso, descrevemos pela primeira vez uma atividade fosfolipase para uma Prx bacteriana, no entanto a implicação biológica desta atividade ainda necessita ser investigada. Finalmente, a capacidade de PaLsfA em influenciar o processo inflamatório em macrófagos é uma primeira descrição de uma Prx bacteriana influenciando a resposta inflamatória do hospedeiro, correlacionando-se com sua participação na virulência de *P. aeruginosa*. Dessa forma, nossos achados podem esclarecer a compreensão de como essa proteína afeta a virulência da *P. aeruginosa* e possibilitar futuro desenvolvimento sobre mecanismos inibitórios.