Final Report

Project: Identification of a reactive chlorine species-specific transcriptional regulator and its regulon in a human pathogen *Pseudomonas aeruginosa* (MRG5980047)

By

Lect. Adisak Romsang, Ph.D.

and team

April, 2018

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(Comments in this report were from the researchers only, the funders have not recommended)

Abstract

Project Code : **MRG5980047**

Project Title : **Identification of a reactive chlorine species-specific transcriptional regulator and its regulon in a human pathogen *Pseudomonas aeruginosa***

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Project Period : **May, 2016 – May, 2018**

Abstract : *Pseudomonas aeruginosa* is an important opportunistic pathogen that causes severe acute and chronic infection in hospitalized patient and immunocompromised host and is also one of the critical global priority list of antibiotic-resistant bacteria to development of new antibiotics. The treatment difficulties are associated a long-term survival of the pathogens in the hospital environments. One of the major factors for successful infection is the bacterial defense mechanisms against toxic substances from host immune system including reactive oxygen species (ROS) and reactive chlorine species (RCS). Many compounds containing RCS such as sodium hypochlorite (NaOCl) is generally used as bleaching agents in household and disinfectants even in hospitals. The mechanisms to manage ROS in bacteria are well-known but those to deal with RCS are less studied in bacteria and unknown in this pathogenic bacterium. In this project, the bacterial mechanisms to fight against host-generated RCS, both directly and indirectly, will be explored. Several genes encoding ferredoxin reductases (*fprA* and *fprB*), and tRNA thiolating enzyme (*ttcA*) were shown to be involved in the RCS response and role to detoxify the RCS toxicity in the bacterial cell. All of them was independently regulated by their own specific transcriptional regulator, which can sense the redox homeostasis due to the oxidation by cellular RCS. This emphasizes the broad spectrum of cytotoxicity mechanisms of RCS in the bacteria. Moreover, the first RCS-sensing transcriptional regulator in *P. aeruginosa* RcsR was proposed and we showed that RcsR regulated an *rcsA* expression under RCS exposure to increase the detoxification activity against RCS agents including a bleach NaOCl. Finally, these RSC-responsive genes had a role in bacterial pathogenicity in both *C. elegans* and *D. melanogaster* host model systems. This study was to characterize many novel genes in *P. aeruginosa* to expand the virulence network, a cause of nosocomial infections, and could be a part of the reasons in its ability for hospitalization. The highly RCS-sensitive regulators will be used to develop potential biomarkers detecting RCS, which is an insight research leading to the novel strategies for the drug-targeting development or biosensor for RCS toxicity in the environmental contamination.

Keywords : reactive chlorine species, stress response, *fprA*, *ttcA*, *rcsA, Pseudomonas aeruginosa*

บทคัดย่อ

รหัสโครงการ : MRG5980047

ชื่อโครงการ : การค้นหาและศึกษายีนใหม่ที่ถอดรหัสโปรตีนควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการตอบสนองต่ออนุมูลคลอรีนในเชื้อแบคทีเรียก่อโรคในคน สูโดโมนาส แอรูจิโนซา

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บทคัดย่อ : เชื้อสูโดโมนาส แอรูจิโนซา เป็นเชื้อแบคทีเรียฉวยโอกาสที่ก่อโรคในคนและพบได้มากที่สุดชนิดหนึ่ง โดยเฉพาะสามารถทำให้เกิดอาการรุงแรงหรือก่อโรคเรื้อรังได้ในผู้ป่วยที่รักษาตัวที่โรงพยาบาลหรือผู้ที่มีภูมิคุ้มกันบกพร่อง เชื้อชนิดนี้ถูกจัดให้การหาแนวทางในการรักษาหรือคิดค้นตัวยาฆ่าเชื้อชนิดนี้ในระดับการวิกฤต เนื่องจากการรักษาที่ยากและเรื้อรังในโรงพยาบาล ปัจจัยที่ทำให้แบคทีเรียชนิดนี้ก่อโรคได้สำเร็จ คือ กลไกการป้องตัวเองจากสารฆ่าเชื้อที่หลั่งมาจากคนหรือโฮสต์เจ้าบ้าน เช่น สารอนุมูลอิสระออกซิเจนและสารอนุมูลอิสระคลอรีน นอกจากนี้ เรายังนิยมใช้สารอนุมูลอิสระคลอรีนในรูปของน้ำยาฆ่าเชื้อในโรงพยาบาลและน้ำยาซักผ้าขาวตามบ้านเรือนอีกด้วย ทำให้เชื้อสามารถปรับตัวพัฒนาให้เกิดกลไกการต้านสารเหล่านี้ ในโครงการวิจัยนี้ได้วิจัยค้นหาและศึกษายีนใหม่ที่ถอดรหัสโปรตีนควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการตอบสนองต่ออนุมูลคลอรีนในเชื้อแบคทีเรียก่อโรคในคน สูโดโมนาส แอรูจิโนซานี้ พบว่า มีหลายยีนที่เกี่ยวข้องและบางส่วนที่ค้นพบเป็นยีนใหม่ที่ยังไม่เคยมีรายงานมาก่อน กลไกการต้านสารอนุมูลอิสระเหล่านี้มีความซับซ้อนมากมาย มียีนหลากหลายที่ช่วยเสริมฤทธิ์ในการป้องกันและรักษาตัวเองในสภาวะเครียดจากอนุมูลอิสระเหล่านี้ เช่น ยีน *fprA* และ *fprB* เป็นเอนไซม์ที่รักษาสมดุลรีดอกซ์ในเซลล์c]tเป็นส่วนหนึ่งในการสร้างอนุพันธ์เหล็กผสมซัลเฟอร์ ซึ่งใช้เป็นโคแฟกเตอร์ของหลายเอนไซม์ที่จำเป็นต่อการดำรงชีวิตของแบคทีเรีย c]tยีน *ttcA* ยีนที่เติมหมู่ซัลเฟอร์ให้กับ tRNA เพื่อการคัดเลือกในกระบวนการแปลรหัสพันธุกรรมเป็นกรออะมิโน ยีนเหล่านี้ถูกกระตุ้นด้วยสภาวะเครียดที่หลากหลายชนิด ไม่มีความจำเพาะ แต่ขึ้นกับการควบคุมผ่านโปรตีนความคุมการแสดงออกที่ต่างกัน แต่ทุกยีนที่กล่าวมาสามารถถูกกระต้นได้ด้วยสารอนุมูลอิสระคลอรีน ดังนั้นสารนี้จึงมีผลต่อแบคทีเรียในหลายด้าน กลไกในการดื้อสารฆ่าเชื้อยิ่งเกิดได้ง่ายขึ้นเช่นกัน นอกจากนี้ คณะวิจัยยังพบยีนที่มีความจำเพาะต่อสารอนุมูลอิสระคลอรีนเท่านั้นด้วยเช่นกัน คือ ยีน *rcsA* ยีนนี้สำคัญต่อการต้านสารอนุมูลอิสระคลอรีนในรูปน้ำยาฆ่าเชื้อ และเมื่อลบยีนนี้แล้วแบคทีเรียก่อโรคได้น้อยลงด้วย ยีนนี้ถูกควบคุมด้วยโปรตีนความคุมการแสดงออกที่จำเพาะต่อสารอนุมูลอิสระคลอรีนเท่านั้น ผลการวิจัยจากโครงการทำให้สามารถประกอบภาพกลไกที่ซับซ้อนของแบคทเรียชนิดนี้ที่ก่อโรคในคนได้สำเร็จในโรงพยาบาล อันนำไปสู่แนวทางการป้องกัน การตรวจวินิจฉัย และการรักษาโรคติดเชื้อแบคทีเรียสูโดโมนาส แอรูจิโนซา อีกทั้งยังสามารถเป็นแนวทางในการต่อยอดประยุกต์ใช้โปรตีนเพื่อตรวจหาสารอนุมูลอิสระคลอรีนตกค้างในสิ่งแวดล้อมได้อีกด้วย

คำสำคัญ : สารอนุมูลอิสระคลอรีน, การตอบสนองต่อสภาวะเครียด, *fprA*, *ttcA*, *rcsA*, สูโดโมนาส แอรูจิโนซา

Final Report : Identification of a reactive chlorine species-specific transcriptional regulator and its regulon in a human pathogen *Pseudomonas aeruginosa* (MRG5980047)

1. Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen that causes severe acute and chronic infection in hospitalized patient and immunocompromised host and is also one of the critical global priority list of antibiotic-resistant bacteria to development of new antibiotics. The treatment difficulties are associated a long-term survival of the pathogens in the hospital environments. One of the major factors for successful infection is the bacterial defense mechanisms against toxic substances from host immune system including reactive oxygen species (ROS) and reactive chlorine species (RCS). Many compounds containing RCS such as sodium hypochlorite (NaOCl) is generally used as bleaching agents in household and disinfectants even in hospitals. The mechanisms to manage ROS in bacteria are well-known but those to deal with RCS are less studied in bacteria and unknown in this pathogenic bacterium. In this project, the bacterial mechanisms to fight against host-generated RCS, both directly and indirectly, will be explored. Several genes encoding ferredoxin reductases (*fprA* and *fprB*) and tRNA thiolating enzyme (*ttcA*) were shown to be involved in the RCS response and role to detoxify the RCS toxicity in the bacterial cell. All of them was independently regulated by their own specific transcriptional regulator, which can sense the redox homeostasis due to the oxidation by cellular RCS. This emphasizes the broad spectrum of cytotoxicity mechanisms of RCS in the bacteria. Moreover, the first RCS-sensing transcriptional regulator in *P. aeruginosa* RcsR was proposed and we showed that RcsR regulated an *rcsA* expression under RCS exposure to increase the detoxification activity against RCS agents including a bleach NaOCl. Finally, these RSC-responsive genes had a role in bacterial pathogenicity in both *C. elegans* and *D. melanogaster* host model systems. This study was to characterize many novel genes in *P. aeruginosa* to expand the virulence network, a cause of nosocomial infections, and could be a part of the reasons in its ability for hospitalization. The highly RCS-sensitive regulators will be used to develop potential biomarkers detecting RCS, which is an insight research leading to the novel strategies for the drug-targeting development or biosensor for RCS toxicity in the environmental contamination.

2. Executive summary

a. Introduction to Research

Infectious diseases caused by bacteria are still the critical health problems around the world. Although antibiotics are clarified to treat and cure several diseases, bacterial infections still remain the leading causes of death worldwide. The emerging drug resistance of the pathogens made the diseases severe and dearly to treat and thereby increasing mortality rate. Understanding of the bacterial virulence mechanisms and a development of effective antimicrobial drugs would be a successful strategy to fight against infectious diseases. In this proposal, we select one of the major human pathogenic bacterium, *Pseudomonas aeruginosa*, as a principle bacteria.

P. aeruginosa is one of the most important human pathogens causing nosocomial infections of various biological systems in our body such as respiratory infections, genitourinary infections, burned-would infections, eye and skin infections, bacteremia, and particularly in cystic fibrosis patients. According to the Centers for Disease Control and Prevention (CDC) in USA, *P. aeruginosa* is among the top three causes of nosocomial infections. The increased incidence of multidrug resistance isolates of *P. aeruginosa* made its infections are life-threatening and challenging to treat. The treatment difficulties are associated a long-term survival of the pathogens in the hospital environments, thereby enhancing opportunities for transmission of the causative agents between patients through human reservoirs or insentient materials and medical equipment. One of the major factors for successful infection is the bacterial defense mechanisms against toxic substances from host immune system including reactive oxygen species (ROS) and reactive chlorine species (RCS). The mechanisms to manage ROS in bacteria are well-known but those to deal with RCS are still poor. In this proposal, the bacterial mechanisms to fight against host-generated RCS will be explored through a gene encoding an *Escherichia coli* RclR-homologue transcriptional regulator that specifically senses to RCS. Although they share the protein sequence similarity, their putative functional motifs and their putative targeted genes seem to be different. This study will identify and characterize the first RCS-sensing transcriptional regulator in *P. aeruginosa* and the study of this novel regulator gene and its targeted genes in *P. aeruginosa* will expand the virulence network in *P. aeruginosa* and could be a part of the reasons in its ability for hospitalization. This insight research will also lead to the novel strategies for the drug-targeting development against the infectious diseases from this human-pathogenic bacterium.

b. Literature review

A discovery of the first antibiotic, penicillin, by Elexander Fleming in 1928 had an impact on the treatment of infectious diseases. About ten years later, antibiotic resistance microorganisms, however, have conntinued to emerge, and the emergence of multidrug resistant (MDR) bacteria is recently one of paramount health issues worldwide (Ventola, 2015). It is recently beleived that we are in the end of antibiotic era. Many human-pathogenic bacteria that are globally pan-antibiotic resistant are represented for a substantial clinical and econmical burden on the health care system (Ventola, 2015; Phumart, 2012). In the USA, CDC reported the antibiotic resistance threats in 2013 and classified those bacteria as presenting urgent, serious and concerning threats (CDC OoID. Apr, 2013, http://www.cdc.gov/drugresistance/threat-report-2013). The biggest threat is from a global pandemic of resistant Gram-positive *Straphylococcus aureus* and *Enterococcus* species. The most serious Gram-negative antibiotic resistance bacteria are Enterobacteriaceae (mostly *Klebsiella pneumoniae*), *Pseudomonas aeruginosa,* and *Acinetobacter* species. In Thailand, a total of 87,751 hospitalizations developed nosocomial infections due to the top five bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobactor baumannii*, *Pseudomonas aeruginosa* and methicillin- resistant *Staphylococcus aureus*) that are resistant to antibiotics in 2010. The infection by these MDR bacteria resulted in additional of 3.24 million days of hospitalization and 38,481 deaths, that were accounted for 2,538 - 6,084 Million Baht for the treatment in 2010 (Phumart, 2012). Thus, MDR bacterial infection is a priority and urgent health problem in

Thailand that needs comprehensive and systematic approaches at national and local levels to resolve the problem.

In the past decades, only small amount of novel antibiotic was discovered and approved to treat beacuse of several reasons such as more difficulty to discover new antibiotics, high investment, relatively short periods for using antibiotic, rapidly new emerging MDR bacteria, limited funding, and others. Antibiotic development agaisnt Gram-positive bacteria are still interesting of many pharmaceutical companies due to the possibly big market but that against Gram-negative bacteria are not of focus because of smaller market and nature of complicated/rapidly adapted antibiotic resistance mechanisms. Therefore, the study of acquire additive mechanisms in these bacteria against host immune system and resisted antibiotic is such an important research area.

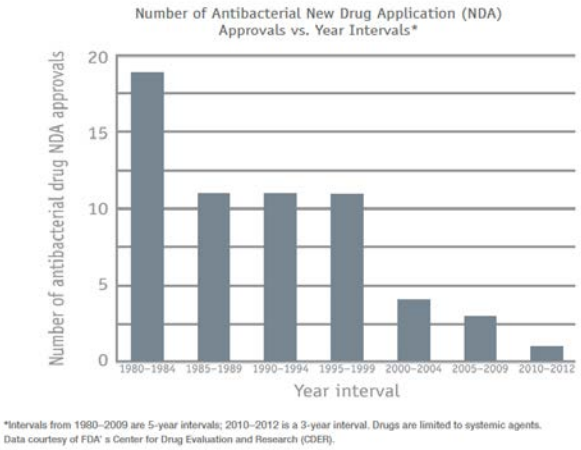


Figure 1: Chart showing the decreased number of new antibiotics developed and approved in the past three decades, leaving the fewer options to treat the resistant bacteria (CDC report, 2013)

The contaminated bacteria cause product yield loss, illness and diseases, in which many countries including Thailand waste a tremendous amount of money to cure bacterial infections as described in above. Development of the novel strategies to enhance an effectiveness of antibacterial agents will be useful. The critical traits contributing to a successful infection of bacteria are the defense mechanisms against toxic level of reactive oxygen species (ROS) produced from host immune system, biofilm formation, and antibiotic resistance. Host defense cells including macrophages and neutrophils establish various enzymes that generate large amount of oxygen-derived free radicals (ROS) and nitrogen-derived free radicals (RNS) in response to bacterial invasion. Additionally, some bacteria can generate hydrogen peroxide to kill or inhibit the growth of other microorganisms that act as competitors. Several antimicrobial agents that used in the clinical treatments block key enzymes and altered metabolic functions leading to production of ROS that damages biological macromolecules. The unchecked productions or accumulation of ROS can damage cell components, proteins,

nucleic acids and cell membranes lead to cell damage, mutations, or lethality. Oxidative stress has been shown as a mechanism related to cell death mediated by antibiotics. Production of hydroxyl radical via the Fenton reaction was proposed as a common mechanism of cellular death of *Escherichai coli* induced by bactericidal antibiotics regardless of drug-target interaction (Kohanski *et al*, 2007). It has also been shown in *P. aeruginosa*, *A. baumannii*, and *Haemophilus influenza* studies that involve in the generation of ROS contributed to antibiotic mediated cell death (Yeom *et al*, 2010; Sampson *et al*, 2012; Choi *et al*, 2015).

During an active infection, bacteria are continuously exposed to ROS and RNS produced from respiratory burst of human immune cells as mentioned above. Reactive oxygen and nitrogen species damage various cellular macromolecules and thus activate several protective mechanisms including antioxidant defense systems and multidrug efflux systems, promoting survival of pathogenic bacteria. To combat or withstand against oxidative stress, the ability to eliminate ROS is the key to the bacterial survival in the environment and the hosts. The bacteria have evolved in a wide variety of stepwise mechanisms to defense oxidative stress. The bacterial management of oxidative stress can be divided into two phases (Romsang *et al*, 2013).

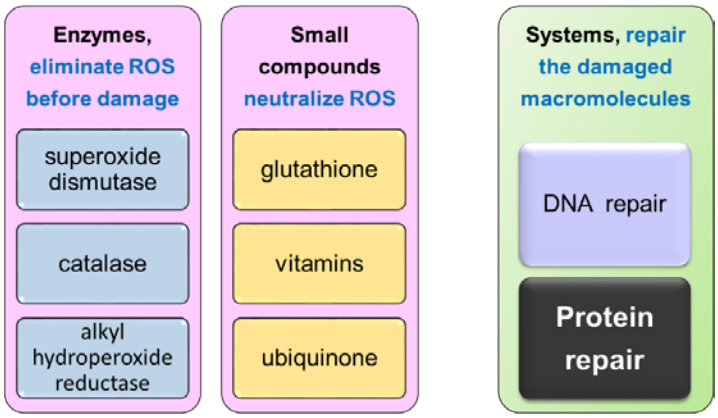


Figure 2: Two phases of ROS managed mechanisms in bacteria, protection and repair

First of all, the elimination of ROS prior to damage occurrence is done by the bacterial antioxidants. These systems include antioxidants and enzymes, such as catalase, superoxide dismutase, heme-oxygenase, and alkyl-hydroxyperoxidases, which have evolved to limit the levels of ROS. Catalase or hydroperoxidase is mostly a heme-associating enzyme, which converts hydrogen peroxide (H_2O_2) to water and oxygen or oxidizes a reductant in the cell with the requirement of H_2O_2. Detoxification of H_2O_2 may decrease formation of hydroxyl radical in the Fenton reaction via transition metal-generating reaction. Alkyl hydroperoxide reductase (AhpCF) plays an important role in defense mechanism against peroxide exposure mediated from the cellular metabolism and the environment. This enzyme is composed of two components, namely AhpC (peroxiredoxin) and AhpF (flavoprotein disulfide reductase). And other scavenging enzymes prevent the accumulation of ROS are produced. For example, peroxiredoxins (Prxs) are omnipresent family of these antioxidants. The prosthetic cofactors are not required for these enzymes and they catalyze the reduction of hydrogen peroxide, organic

hydroperoxide and peroxynitrite to water and alcohol. The antioxidants, small molecules such as glutathione, vitamins, or ubiquitinone sequester or neutralize ROS. The non-enzymatic machineries involves low-molecular-weight antioxidants (LMWA) including NAD(P)H and glutathione. Both of vitamin C and vitamin E can block free radicals by donating H-atom to damage molecules, creating radicals themselves and low reactive, protecting cellular molecules.

Secondly, after the cells have been damaged by ROS, the bacterial repair systems for damaged molecules such as damaged DNA and denatured proteins. There are some well-known DNA repair mechanisms, including repeal of unusual methylation and disobedient methyl transferases, base excision repair and damage of genome, nucleotide excision repair system, and mismatch repair recognized and repairs improperly inserted bases. During the oxidative stress, protein oxidation is one of the most important processes for bacterial survival. Many amino acids within the protein can be modified resulting in oxidatively modified proteins that can lead to the secondary unfolding of proteins and modification by non-protein oxidation products. This oxidative damage on proteins also leads to site-specific amino acid fragmentation, aggregation, modifications and cross-linking, changed in electrical charge and facing properties, and altered susceptibility to proteolysis. If high concentrations of oxidized proteins inside the cell might have adverse effects, the worst outcome can be cell death. Indeed, the cell needs some mechanisms to degrade these toxic oxidized proteins such as protein repair or protein degradation. These processes are more importance because several diseases, such as arteriosclerosis, Parkinson's disease, Alzheimer's disease, and the cell-death pathway, are involved in the accumulation of oxidized proteins. For example, the most effective protein repair system (Methionine sulfoxide reductase, Msr) was characterized in *P. aeruginosa* (Romsang *et al*, 2013). Gene disruption in *msrA* and *msrB* contributed to increased susceptibility against oxidants and attenuated the bacterial virulence. Another study in parallel, genes involved in iron-sulfur cluster (Fe-S) biogenesis and its regulation were found to role in oxidative stress response in *P. aeruginosa* (Romsang *et al*, 2014). ROS targeted Fe-S cluster, which is a key cofactor of the 5 % of the total proteins in bacteria. Damaged Fe-S cluster cannot ligated into the prosthetic group of protein and lead to malfunction of the protein.

Several antioxidant enzymes were characterized to be responsible for degrading ROS in *P. aeruginosa* such as catalases (Kat), superoxide dismutases (SOD), alkyl hydroperoxide reductase (Ahp), organic hydroperoxide resistance (Ohr), and thiol peroxidase (Tpx) (Atichartpongkul *et al*, 2010; Somprasong *et al*, 2012). It is known that these responses depend on select transcriptional regulators, likes OxyR, SoxR, OhrR, and OspR, which are able to specifically sense particular oxidants. These regulators control the expression of genes that contribute directly to ROS detoxification or to the repair of ROS-mediated damage. The ability of redox-sensitive regulators to distinguish among different oxidants is a key factor in redox signaling (Winterbourn and Hampton, 2008).

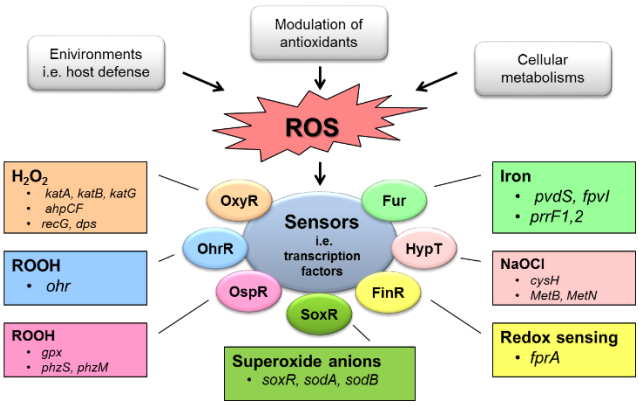


Figure 3: Oxidative stress sensor and response genes in bacteria

Reactive chlorine species (RCS), including hypochlorous acid (HOCl) and chloramines, are powerful antimicrobial oxidants capable of chlorinating and oxidizing a wide range of biomolecules (Deborde and Gunten, 2008; Gray *et al*, 2013; Winterbourn and Kettle, 2013). HOCl is a weak acid that is chemically formed when chlorine dissolved in water or biologically mediated by the reduction of the $OH^{\cdot}$ formation from O_2^{-}. It is the strongest oxidant and can be generated by the reaction between H_2O_2 and phagocyte-generated peroxidases. It is also a major oxidant produced by activated neutrophils. HOCl are powerful oxidants known to have many cytotoxic consequences on bacterial and animal cells (Parker *et al*, 2013). It is an oxidizer, used as bleach, deodorant, and disinfectant. It reacts with a wide variety of biological molecules such as nucleotides, lipids, and proteins and affects in a depletion of DNA replication, an inhibition of adenine nucleotides, an inactivation of glucose oxidase, and unfolding and aggregation of proteins (Parker *et al*, 2013).

The bacterial response against RCS was firstly described in term of the RCS-affected biological pathways and some transcriptional regulators that may represented as RCS sensors were listed (Gray *et al*, 2013). Sulfur-containing molecules including methionine, cysteine, and glutathione (GSH) rapidly react with HOCl. HOCl-generated oxidation of cysteine thiols leads to unstable sulfenyl chloride (R-SCl) intermediates that can react with water to form oxidized cysteine sulfenic acids (R-SOH). These highly reactive intermediates can either be reduced by thioredoxin or be further oxidized to irreversible sulfinic ($R\text{-}SO_2H$) and sulfonic ($R\text{-}SO_3H$) acids, which typically result in protein degradation. Reaction of HOCl with methionine is very fast and predominantly generates methionine sulfoxide and further oxidizes to form irreversible methionine sulfone that is toxic to the cell. Whereas methionine sulfoxide can be *in vivo* repaired by methionine sulfoxide reductases (Ezraty *et al*, 2005). The second most reactive targets of HOCl in proteins are amines. In this reaction, amines are chlorinated to form chloramines (—NHCl), which are considered RCS due to their chlorination and oxidizing properties. But chloramines are less reactive than HOCl and more specific for oxidation of cysteine and methionine. They instead rapidly decompose to their respective aldehydes. N-chlorotaurine, associated with the innate immune system (Nagl *et al*, 2000), are quite stable than others. Chloramines can also react with iron or copper ions to generate nitrogen radicals. HOCl and chloramines also react with nucleotides and lipids (Gray *et al*, 2013). Amines of nucleotide bases in DNA and RNA can be the major targets of chloramines leading to the formation of nitrogen

radicals and stable chlorinated nucleotides. High amounts of HOCl can result in DNA strand breakage. Chlorination of lipids, such as double bonds in unsaturated fatty acids, can lead to the formation of chlorohydrins, which are thought to contribute to HOCl-mediated cellular damage in eukaryotic cells (Carr *et al*, 1997). Radicals mediated by reactions of HOCl and amines/peroxides can result in lipid peroxidation (Niki 2009).

RCS also presented in microbial environments especially during its infection or survivals in host cells. Animal Cells role in the innate immune system produce high levels of oxidative stress-generated agents, including HOCl, to get rid of invading pathogens (Gray *et al*, 2013). The toxic level production of oxidants is mediated by the activation of pathogens' NADPH oxidases placed at the phagosome membrane. High concentrations of heme-containing myeloperoxidase (MPO) are then released into the phagosome, where it generates HOCl by catalyzing chloride in the reaction with H_2O_2 (Khor *et al*, 2004). Neutrophils are the major sources of HOCl production with about 5% of MPO in the innate immune system (Hurst 2012; Khor *et al*, 2004). Other oxidants play only minor roles in bacterial killing by phagocytes due to the high concentrations of the ROS and the prolonged incubation times are required to kill the bacteria (Gray *et al*, 2013). The toxicity of HOCl, which effectively eliminates invading pathogens, can also cause tremendous damage to human tissues. Incorrect cellular trafficking and processing of MPO lead to the release of HOCl into extracellular compartments, where it is involved in the progress of various human diseases, including atherosclerosis, chronic inflammation, and certain cancers (Klebanoff 2005).

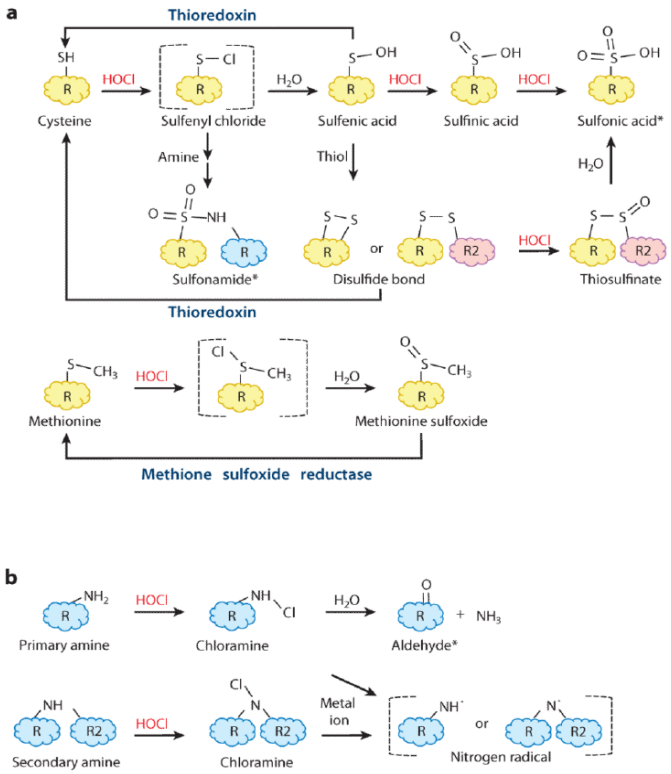


Figure 4: Reactions of hypochlorous acid (HOCl) with biomolecules (Gray *et al*, 2013)

Reaction of HOCl with (a) sulfur-containing compounds or (b) amines

Brackets indicate unstable reactive intermediates

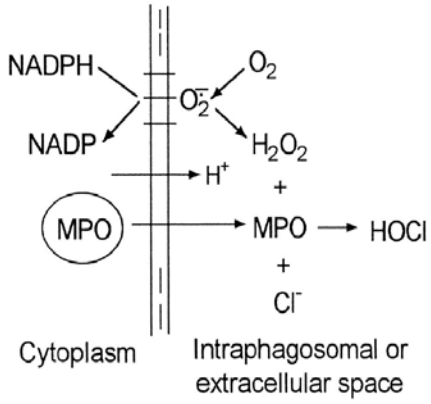


Figure 5: The MPO-H_2O_2-chloride antimicrobial system (Klebanoff 2005)

RCS, like other disinfectants, kill microbes by damaging multiple cellular components. However, the mechanisms that RCS kill bacteria remains incompletely defined and probably varies depending on bacterial species, RCS type, and exposure conditions. This is of interest not only for understanding interactions between bacteria and various RCS-generating eukaryotes, but also potentially for developing strategies to protect human cells from the MPO-generated RCS damage that contributes to inflammatory disease (Gray *et al*, 2013). Thus, an identification of players and mechanisms in bacteria for RCS sensing and response is a key step for understanding the interactions between bacteria and their eukaryotic hosts, with obvious implications for the study of human health and disease.

Recent studies have identified several bacterial transcription factors that respond to RCS treatment including the *E. coli* HypT (Drazic *et al*, 2013) and NemR (Gray *et al*, 2013) and the *Bacillus subtilis* OhrR (Fuangthong *et al*, 2012) and HypR (Palm *et al*, 2012). Unfortunately, HypT is only one proposed to respond to HOCl but the others respond to a variety of other stress signals such as electrophiles and organic hydroperoxides. ROS and electrophiles have a more limited set of cellular targets, whereas RCS are able to damage most of cell components.

Table 2: Names and characteristics of redox sensor in bacteria that sense RCS

Redox sensor	Organism	Signal	Redox-sensing mechanism	Regulon genes	Regulon functions	References
NemR (TetR-type)	*Escherichia coli*	Quinones, Glyoxal, Methylglyoxal N-ethylmaleimide Iodoacetamide HOCl	C106* conserved C21-C116 intersubunit disulfide	*nemR nemA gloA*	TetR-type repressor FMN-dep. reductase for aldehydes, quinones and NEM glyoxalase-I	Umezawa et al, 2008 Gray et al, 2013 Lee et al, 2013 Ozyamak et al, 2013
RcIR (AraC-ytpe)	*Escherichia coli*	HOCl N-chlorotaurine	C21-C89 Intramolecular disulfide	*rclA rclB rclC*	flavoprotein disulfide reductase, periplasmic protein quinone-binding membrane protein	Parker et al, 2013
HypT (LysR-type)	*Escherichia coli*	HOCl	Met123-SO Met206-SO Met230-SO C4-C4 intersubunit disulfide (*in vitro*) C4: HypT dodecamer formation C150: HypT stability	*metB/K/N cysH/K/N, cysPUW, sbp, sufA entC, entH, fecABCDE, fecR, fepCD, ryhB, tonB, yncE*	sulfur, Cys and Met biosynthesis and metabolism Fur-regulon genes involved in iron homeostasis	Gebendorfer et al, 2012 Drazic et al, 2013a Drazic et al, 2013b

In this proposal, we focus on the identification of RclR (formerly known as YkgD), a highly RCS-specific transcriptional activator in *E. coli*. RclR, novel HOCl-specific redox regulator, has been characterized in *E. coli* that are specifically sense to chlorine species (HOCl) but do not sense to ROS, electrophiles or other thiol-reactive compounds (Parker *et al*, 2013). RclR is widely conserved among Gram-negative bacteria and was annotated as redox-sensing transcriptional activator of the AraC family, which uses a thiol-based oxidation mechanism for redox-sensing of HOCl (Parker *et al*, 2013). The redox-sensing mechanism of RclR involves both conserved cysteine residues, Cys21 and Cys89. Both Cys-21 and Cys-89 residues are required for redox-sensing of the HOCl-response *in vivo*, while only Cys21 is essential for redox-sensing *in vitro*. Oxidation of RclR by HOCl leads to specific activation of transcription of the *rclABC* operon that is important for survival of HOCl and N-chlorotaurine. Mutants in each single gene of the *rclABC* operon are sensitive to HOCl suggesting that this operon is an important HOCl protection determinant (Parker et al, 2013). However, the functions of the RclABC proteins for HOCl protection are still unknown that resemble a flavoprotein disulfide reductase, periplasmic protein and possible quinone-binding membrane protein.

A B

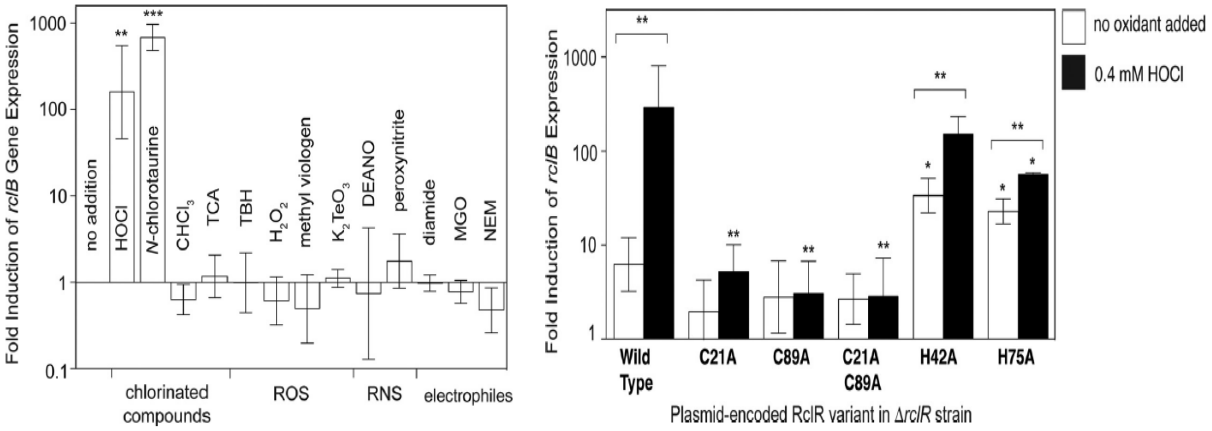


Figure 6: *In vivo* experimental results of *E. coli* RclR study (A) RclR is specifically activated by reactive chlorine species (B) Role of conserved cysteine residues in activation of RclR (Parker *et al*, 2013)

In this proposal, we select one of the major human pathogenic bacterium, *Pseudomonas aeruginosa*, as a principle bacteria. *P. aeruginosa* is one of the top three causes of opportunistic human infections, particularly in cystic fibrosis, cancer and acquired immune-deficiency syndrome (AIDS) patients with compromised host defence mechanisms according to the data from CDC report in 2013.

Figure 7: CDC report page related to MDR *Pseudomonas aeruginosa* in USA

It is widespread around the world and has no specific epidemic distribution. Moreover, *P. aeruginosa*-infected outbreaks were recently reported in all age range. It has been claimed as a cause in outbreaks of many syndromes during the past year such as a contact-lens infection in Thailand, 2011 and a hot-foot syndrome in Germany, 2012. *P. aeruginosa* are often existing even in the hospital. Therefore, hospitalized people are also in high risk to get infections. In Thailand, national antimicrobial resistance surveillance center (NARST) reported antibiotrend of drug-resistant *P. aeruginosa* infection in 2000-2013 (NARST report: http://narst.dmsc.moph.go.th/index.html). The emerging of an imipenem-resistant *P. aeruginosa* was increased since 2008 and more than 50% increasing rate of the emerging in 2013 as shown in figure below. Moreover, among pathogenic bacteria, *P. aeruginosa* was the 2nd highest cause of death after infection and the 4th highest cause of death, due to the antibiotic resistance, in Thailand in 2010 (Phumart, 2012).

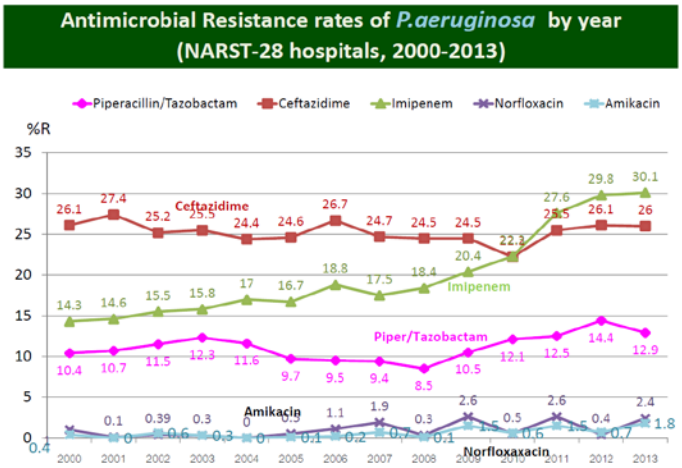


Figure 8: Antibiotrend of drug-resistant *P. aeruginosa* infection in 2000-2013 (NARST report)

The bacteria can spread through food, medical tools and any solution used in the hospitals. Because this bacterium has developed many protective mechanisms or adapted to live in harsh conditions for example weak antiseptics, high temperature, high salts, and even antibiotics. It is often observed growing in tapped water due to its very simple nutritional requirements. Therefore, it can contaminate in any place, live in any environment, and infect anyone in a certain time. *Pseudomonas* infections are more complicated and life threatening.

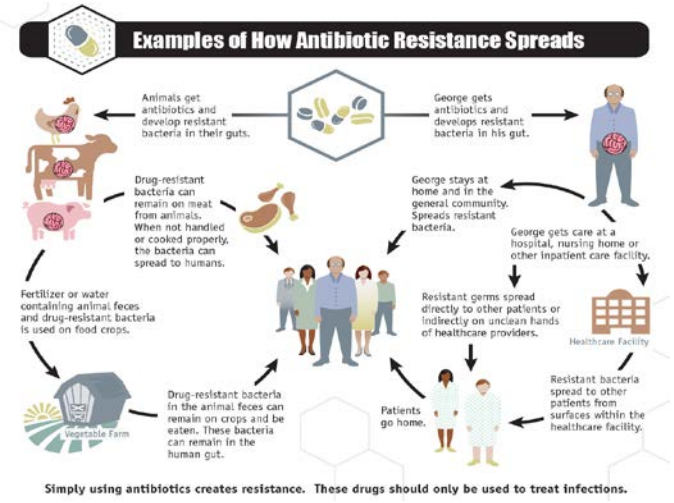


Figure 9: CDC report page related to the examples of how antibiotic resistance spreads

Because of the protective of an outer coat of this bacterium, it can be resistant to many antibiotic drugs. It easily attaches to the bacterial cells. *P. aeruginosa* required very less nutrition for growing at a fast rate. These traits increase the risk of the infection in people with weak immune system. Its incubation period is 24 to 48 hours. *P. aeruginosa* can infect any part of the body such as respiratory system, injured bones, skin, gastric

system and urinary tract. The identification of the bacteria is needed for diagnosis during the infection. Large number of this bacterium is presented in hospitals and anyone even patients can carry this bacterium. Some patients could be the carriers of this bacterium without infected. The fluids of the infected area are taken for laboratory assays to isolate. The factors for identifying this bacterium are generally a fruity odor, replicate at 42°C, non-ferment lactose, and fluorescence under UV exposure.

Since their strong antibiotic resistance, a combination of antibiotics is applied in treatment. The patient would take these antibiotics orally or intravenously. Surgery is needed in some cases, in which tissues are damaged such as eye, ear, heart and bone. In severe case, i.e. wound infections in the legs, amputation may be required. Infections can be completely cured in most cases by medication and surgery. However, for people with immune deficiency, like cancer and AIDS, the prevention of bacterial infection is only one solution. Due to its infection, mortality rate is almost 50% depended on the type of the infection. Infection to the heart and the lung could be high risk of fatal.

Overall data above, the mechanisms to manage ROS in *P. aeruginosa* are well-known in term of protection and repair systems, as shown in our previously studies (such as Somprasong *et al*, 2012; Romsang *et al*, 2013; Romsang *et al*, 2014; Romsang *et al*, 2015), but those to deal with RCS are not know. In this proposal, the *P. aeruginosa* mechanisms to fight against host-generated RCS will be explored through a gene encoding an *Escherichia coli* RclR-homologue transcriptional regulator that specifically senses to RCS. Although they share the protein sequence similarity, their putative functional motifs and their putative targeted genes seem to be different (our preliminary data by using bioinformatics approach). The RCS-involved mechanisms as a neglect puzzle in the virulence circuitry of *P. aeruginosa* will be investigated. This study will expand the virulence network in *P. aeruginosa* and could be a part of the reasons in its ability for additional hospitalization. This insight research will also lead to the novel strategies for the drug-targeting development and a decrease in cost of clinical treatment against the infectious diseases from this human pathogen.

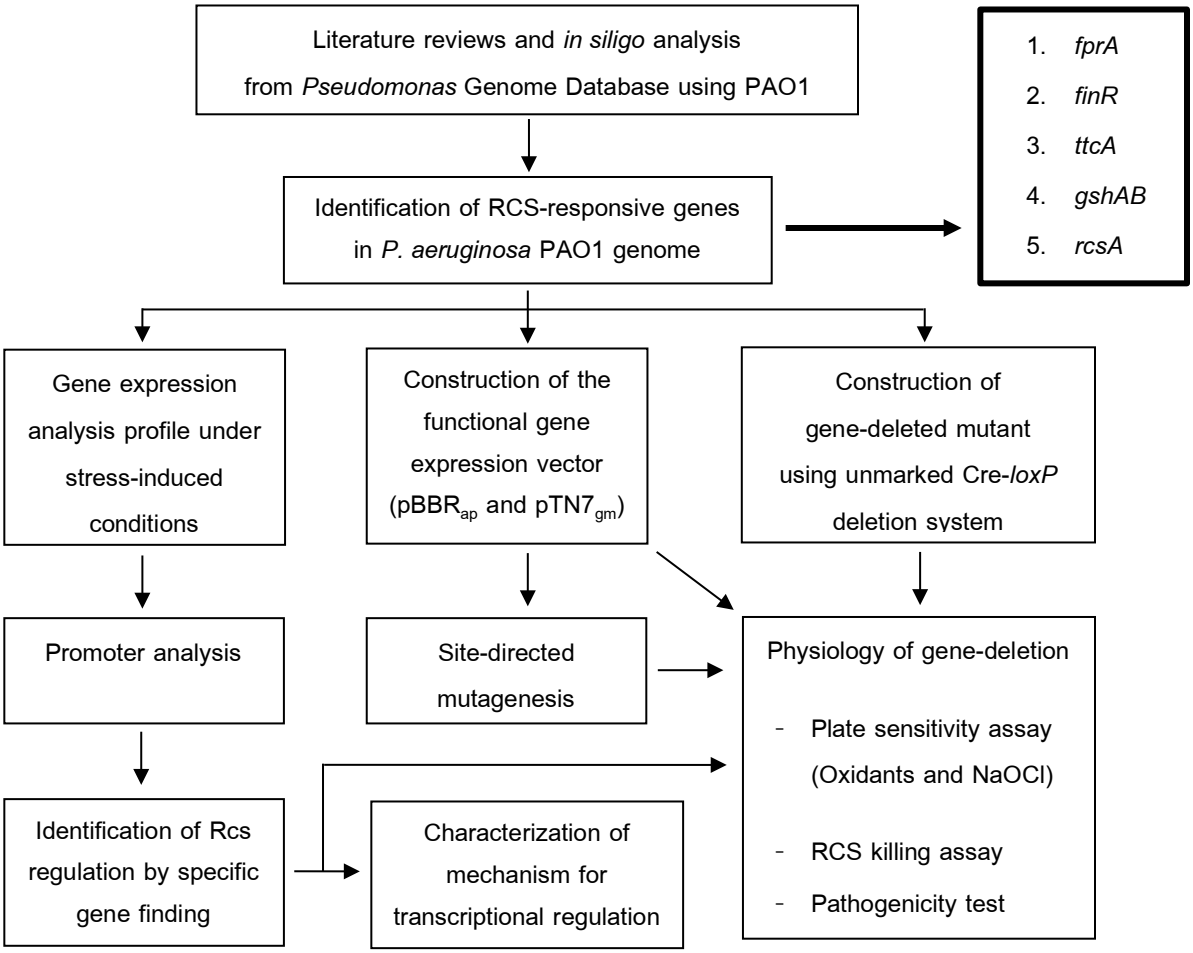
c. Objective

The main objective is to identify a novel gene encoding a reactive chlorine species-specific transcriptional regulator in a human pathogen *P. aeruginosa* PAO1 and to characterize its regulon and mechanisms for sensing and responding to RCS-generated agents and the role in bacterial pathogenicity. In this study, the objective will be divided into 8 sub-objectives as follow:

1. To identify RclR-homologous genes in *P. aeruginosa* PAO1 genome and to construct the gene-deleted mutant
2. To analyze physiological roles of these genes against several stress-generated agents including reactive oxygen species (ROS) and reactive chlorine species (RCS)
3. To construct the functional gene expression vector for complementation assay
4. To determine the key amino acids responsible for the phenotype of the mutants

5. To investigate a gene expression profile under stress inducing conditions
6. To identify the RclR-targeted genes by using specific gene approach
7. To characterize the regulatory elements for transcriptional control
8. To investigate the role of these genes in bacterial pathogenicity

d. Research methodology



1. Bacterial strains, Plasmid and Primers

Bacterial strains and plasmids for this study are showed in Table 1 and 2, respectively.

Table 1. List of bacterial strains

Bacterial strains	Source
Escherichia coli. DH5α	Laboratory stock
Escherichia coli. BW20767	Laboratory stock

Pseudomonas aeruginosa (PAO1)	Laboratory stock

Table 2. List of plasmids

Plasmids	Genotypes	Source
pUC18	Ap^R	Laboratory stock
pCM351	Gm^R, Tet^R	Marx CJ
pCM157	Tet^R	Marx CJ
pBBR1MSC-4	Ap^R	Kovach ME
pUC18-mini-Tn7T-Gm-LAC	Ap^R, Gm^R	Hebert P. Schweizer
pTnS2	Ap^R	Hebert P. Schweizer

2. Strain construction

P. aeruginosa mutant strain will be constructed by homologous recombination techniques using Cre-*loxP* deletion system (Quenee, Lamotte et al. 2005). Briefly, the pUC deleted gene -Gm^R-*loxP* will be transformed into *P. aeruginosa* PAO1 by electroporation. The gene::Gm^R mutant clones will be selected on LB agar containing appropriated antibiotics. The pCM157 containing Cre-recombinase will be used to leave the Gm^R cassette out by transforming into gene::Gm^R strain. Finally, the unmarked gene mutant must be obtained.

The complementary strains will be constructed to prove that the phenotype seen in the mutants is directly contributed by the deleted genes. The full length gene are obtained from PCR reaction using specific primers and separately integrate into pBBR1-MSC4 (Kovach, Elzer et al. 1995) at the multiple cloning sites of vector by using T4 DNA ligase. This recombinant plasmids will be used to study an overexpression of genes in the medium-copy manner by transforming into the P. aeruginosa strains and pBBR1-MSC4 will be used as the empty vector control. To verify the correct DNA sequence of insertion, the DNA sequencing will be done.

In some experiments, the high level of gene expression will lead to the defect in cell physiology. A single-copy chromosomal insertion will be applied by using pUC18mini-Tn7T-Gm vector (Choi and Schweizer 2006). Briefly, full-length of the genes of interest will be cut from pBBR-gene and cloned into a pUC18mini-Tn7T-Gm delivery vector. The recombinant pUC18mini-Tn7T-Gm delivery vectors and a helper plasmid, pTnS2, encoding the Tn7 site-specific transposition pathway will be then co-electroporated into the *P. aeruginosa* strains. The complementation of the mutant with single-copy of gene is generated through transposition of mini-Tn7T-Gm containing gene of interest into bacterial chromosome *atta* specific site and orientation (Choi and Schweizer 2006). The Gm^r transformants will be then selected by growing transformation culture on LB agar plate containing the appropriate concentration of gentamycin. Double cross homologous recombinant mutants will be isolated by antibiotic-selective patching with Gm^r and Cb^s phenotypes. The PCR analysis using a specific primer for mini-Tn7 element (PTn7R) and a bacterium-specific glmS (P_{glmS}) primer will be used for indicating the transposition event in resulting strains (Choi and Schweizer 2006).

3. Site-directed mutagenesis

Site-directed mutagenesis was carried out using PCR-based method to introduce specific base-pair changes at specific locations within the interested gene or DNA fragment. The strategy of this method is explained in the below figure. The first round PCR was to generate two intermediate PCR products AB and CD. The AB fragment was generated by using mutagenic primer b and vector-specific primer a. The CD fragment was generated by mutagenic primer c and vector-specific primer d. The two intermediate PCR products AB and CD were used as template for the second round PCR. The AB and CD fragments shared a part of complementary sequences overlapping the mutated sites that could pair to form duplex DNA. Amplification of product AD in the second round PCR with vector primers, a and d, was amplified the AD fragment. The AD sequence was checked and the fragment was cloned into appropriate vectors, an expression vector. The details and the site specific mutagenesis for each construction are described in results.

4. Bacterial susceptibility assays

Analysis of the killing effects of various reagents on *P. aeruginosa* strains will be performed using plate sensitivity assay (Romsang, Atichartpongkul et al. 2013). The adjusted exponential phase cells will be diluted as 10-fold serial dilution and spotted onto LB agar plate containing either oxidants or RCS-mediated reagents. The plates were incubated at 37°C for 18 hours and observed the difference in growth between these strains. The resistance level against an oxidant was expressed as the percent survivals, defined as the percentage of the CFU on plates containing oxidant divided by the CFU on plates without oxidant.

Analysis of the killing effects of various toxic reagents on *P. aeruginosa* strains will be performed using bacterial killing test (Romsang, Atichartpongkul et al. 2013). The exponential cultures will be adjusted with fresh LB medium to OD_{600} of 0.1 and treated with either oxidants or NaOCl at the final concentration that produce cytotoxicity of in wild type. After 30 min treatment, the treated cultures and untreated cultures will be twice washed and 10-fold serially diluted. The 10 μl of each dilution will be spotted onto LB agar plates. The survival colonies will be scored after incubation at 37°C for 18 h. The percentage survival will be calculated as the number of colony forming unit (CFU) recovered after treatment divided by the number of CFU before treatment and multiplied by 100. The results will be shown in the means and SD from triple biologically independent experiments.

5. *P. aeruginosa* pathogenicity assay

The virulence or the ability to kill the host of P. aeruginosa strains was determined using a *Caenorhabditis elegans* host model system (Tan and Ausubel 2000). The fast killing is known to be mediated by diffusible toxins released from *P. aeruginosa*, which was not need the live bacteria to kill the worms, whereas the slow killing required bacterial growth in the worm gut to exhibit virulence (Mahajan-Miklos et al., 1999, Tan et al., 1999, Cezairliyan et al., 2013). Both slow and fast killing experiments were performed as previously described (Tan and Ausubel 2000, Atichartpongkul, Fuangthong et al. 2010). Briefly, proper staging of L4 nematodes, approximately 30 animals per plate, were used in all experiments. In the fast killing assay, nematodes die in hours when *P. aeruginosa* is grown on a nutrient-rich agar, PGS, whereas, in the slow killing assay, nematodes die in days when *P. aeruginosa* is grown on a nutrient-less agar, NGM agar. Worms was scored as live or dead

based on whether or not touching-reflected movement saw in a dissecting microscope after 3, 6, 9, 12 and 15 h for fast killing and 1, 2, 3, 4, and 5 days for slow killing. The experiments were carried out in blind fashion and analyzed from three biologically independent replicates. Data are the means and standard deviations from three biologically independent experiments.

The virulence of *P. aeruginosa* was also investigated using the *Drosophila melanogater* feeding assay performed according to Haller et.al, Methods Mol Biol 2014 with modifications. Each of the exponential phase cultures of each *P. aeruginosa* strain will be adjusted to an OD_{600} of 0.5 before 800 µL of the homogenized bacterial cells will be overlaid to completely cover the surface of the preservative-free corn flour *Drosophila* medium at the bottom of a glass fly culture vial. Approximately one-week-old adult flies will be feeding starved for 3 h prior to the feeding assay. To anesthetize flies with ethyl acetate using an etherizer, the flies will be exposed to ethyl acetate for 45 s to 1 min and monitor how long they remain asleep. During asleep, twenty flies each will be added to every vial, and each strain of *P. aeruginosa* will be tested at least for three replications. With the right timing of exposure, the flies remain anesthetized up to 20 min. All of the tested flies will be then incubated at 25°C before the number of the viable flies will be monitored every 3-hour phase times compared between different infectious bacterial strains. The experiments will be performed in double-blind fashion and analyzed from nine experiments of three different batches of flies. Data are the means and standard deviations from three biologically independent experiments and shows in a bar chart presentation.

6. RNA isolation, cDNA synthesis, and PCR

Total RNA extraction was done by the hot phenol method and followed by DNase treatment according to the manufacturer's recommendations. First strand cDNA synthesis was performed to in vitro synthesize complementary DNA (cDNA) from mRNA template using RevertAidTM Reverse Transcriptase according to the manufacturer's instructions. The cDNA was stored at -20°C until use as a template for either end-pointed or real time PCR. Endpoint RT-PCR was performed to preliminarily determine the expression level of interested genes. The PCR products were visualized in 1.8% agarose gel electrophoresis. An adjusted 10 ng cDNA was used as a template in a KAPA SYBR FAST qPCR (Kit containing Master Mix 2X, ABI Prism) reaction in an Applied Biosystems StepOnePlus™ real-time PCR system. All reactions were set up according to the manufacturer's instructions. In this test, comparative CT method is used to determine the relative target quantity in the samples using an endogenous control, 16S rRNA, with compared samples and in the reference sample. Measurements are normalized using the endogenous control.

7. 5' RACE-PCR

Rapid Amplification of cDNA Ends (RACE) is a novel procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA. 5' RACE or anchored PCR (amplification with single-sided specificity) is a technique that facilitates the isolation and characterization of 5' ends from low-copy messages. In this study, 5'RACE kit (Roche Life Science, USA) will be applied by using the method according to the manufacture's recommendation with our designed gene-specific primers. Following amplification, 5' RACE products can be cloned into an appropriate vector for subsequent characterization procedures, which may include sequencing,

restriction mapping, preparation of probes to detect the genomic elements associated with the cDNA of interest, or in vitro RNA synthesis.

3. Results

In this project, the bacterial mechanisms to fight against host-generated RCS will be explored through a microarray data analysis to identify the genes induced by RCS in several pathogenic bacteria such as *Escherichia coli* and *Mycobacterium tuberculosis*. Although they share the protein sequence similarity, their putative functional motifs and their putative targeted genes seem to be different. Applying bioinformatics analysis and literature reviews related to the genetic network involved in the RCS response in bacteria, several candidate genes were found to be induced by NaOCl. These genes were encoded the protein played a role in oxidative stress response such as ferredoxin NADP+ reductase (FprA), AhpD-liked protein, NfuA, aspartate ammonia lyase (AspA), and AtfA-homolog factor. The preliminary experiment of these genes' function were determined. This study was to identify and characterize the RCS-mediated response genes in *P. aeruginosa* and the study of novel regulator genes and its targeted genes in *P. aeruginosa* will expand the virulence network in *P. aeruginosa* and could be a part of the reasons in its ability for hospitalization. This insight research will also lead to the novel strategies for the drug-targeting development against the infectious diseases from this human-pathogenic bacterium.

From bioinformatics analysis and literature reviews related to the genetic network involved in the RCS response in bacteria, several candidate genes were found to be induced by NaOCl. These genes were encoded the protein played a role in oxidative stress response such as ferredoxin NADP+ reductase (FprA), AhpD-liked protein, NfuA, aspartate ammonia lyase (AspA), and AtfA-homolog factor. The preliminary experiment of these genes' function were determined.

<u>Part 1</u>: The FinR-regulated essential gene *fprA*, encoding ferredoxin NADP$^+$ reductase: Roles in superoxide-mediated stress protection and virulence of *Pseudomonas aeruginosa*

First, *P. aeruginosa* has two genes encoding ferredoxin NADP(+) reductases, denoted *fprA* and *fprB*. We show here that *P. aeruginosa fprA* is an essential gene. However, the Δ*fprA* mutant could only be successfully constructed in PAO1 strains containing an extra copy of *fprA* on a mini-Tn7 vector integrated into the chromosome or carrying it on a temperature-sensitive plasmid. The strain containing an extra copy of the ferredoxin gene (*fdx1*) could suppress the essentiality of FprA. Other ferredoxin genes could not suppress the requirement for FprA, suggesting that Fdx1 mediates the essentiality of FprA. The expression of *fprA* was highly induced in response to treatments with a superoxide generator, paraquat, or RCS-mediated agents, sodium hypochlorite (NaOCl).

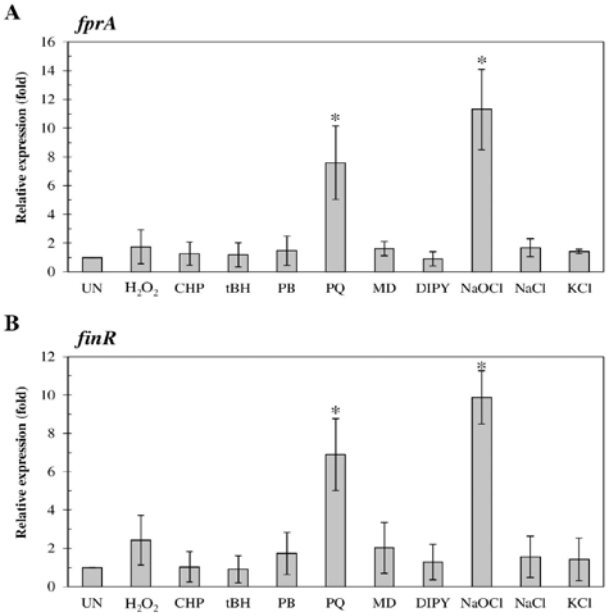


Fig 2. Expression analysis *finR* and *fprA* in response to various stresses. The expression levels of *finR* (A) and *fprA* (B) were determined using real-time RT-PCR. Exponential-phase cultures of *P. aeruginosa* PAO1 were subjected to various stress conditions, including 1 mM H_2O_2, superoxide anion-generating agents (0.5 mM plumbagin [PB], 0.5 mM menadione [MD] and 0.5 paraquat [PQ]), organic hydroperoxides (1 mM cumene hydroperoxide [CHP] and 1 mM *t*-butyl hydroperoxide [tBH]), 1 mM 2,2'-dipyridyl (DIPY), high salts (0.5 M NaCl and 0.5 M KCl), or 0.04% NaOCl for 15 minutes prior to RNA preparation for real-time RT-PCR analysis. Relative expression was analyzed using the 16S rRNA gene as the normalizing gene and was expressed as the fold expression relative to the level of uninduced (UN) PAO1. Data shown are means ± SD of three independent experiments.

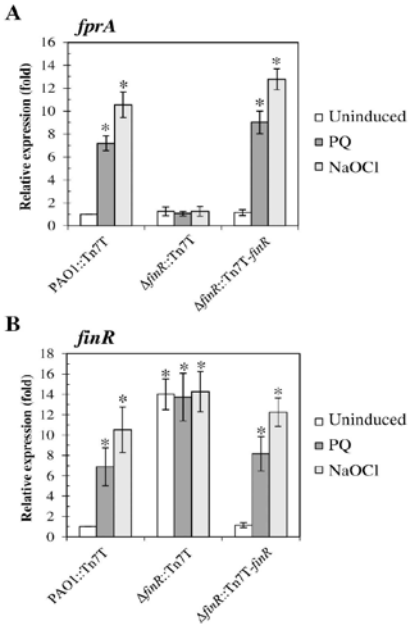


Fig 3. Expression analysis of *fprA* and *finR* in *P. aeruginosa* strains. Expression levels of *fprA* (A) and *finR* (B) in PAO1 wild type (PAO1::Tn7T), Δ*finR* mutant (Δ*finR*::Tn7T) and the complemented mutant (Δ*finR*::Tn7T-*finR*) grown under uninduced, 0.5 mM paraquat (PQ), or 0.04% NaOCl (NaOCl) induced conditions. Relative expression was analyzed using the 16S rRNA gene as the normalizing gene and is expressed as fold expression relative to the level of uninduced PAO1. Data shown are means ± SD of three independent experiments. The asterisks indicate statistically significant differences ($p < 0.01$) compared with the uninduced condition.

The induction of *fprA* by these treatments depended on FinR, a LysR-family transcription regulator. In vivo and in vitro analysis suggested that oxidized FinR acted as a transcriptional activator of *fprA* expression by binding to its regulatory box, located 20 bases upstream of the *fprA* -35 promoter motif. This location of the FinR box also placed it between the -35 and -10 motifs of the *finR* promoter, where the reduced regulator functions as a repressor. Under uninduced conditions, binding of FinR repressed its own transcription but had no effect on *fprA* expression. Exposure to paraquat or NaOCl converted FinR to a transcriptional activator, leading to the expression of both *fprA* and *finR*. The Δ*finR* mutant showed an increased paraquat sensitivity phenotype and attenuated virulence in the *Drosophila melanogaster* host model. These phenotypes could be complemented by high expression of *fprA*, indicating that the observed phenotypes of the Δ*finR* mutant arose from the inability to up-regulate *fprA* expression.

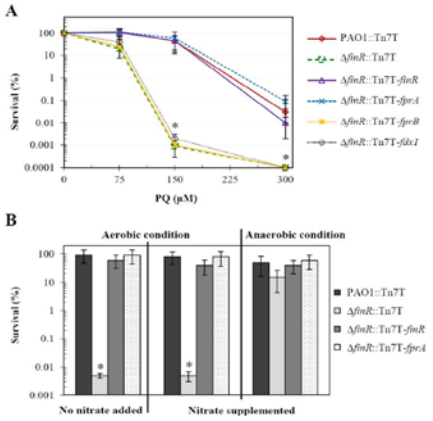


Fig 5. Determination of paraquat resistance levels in *P. aeruginosa* strains. (A) Paraquat resistance levels in PAO1 containing the mnin-Tn7 vector control (PAO1::Tn7T, red) and Δ*finR* mutants containing Tn7T (dotted green), Tn7T-*finR* (purple), Tn7T-*fprA* (dotted blue), Tn7T-*fprB* (yellow), or Tn7T-*fdx1* (dotted black) were determined using plate sensitivity assays. (B) Paraquat (150 μM) resistance levels of *P. aeruginosa* strains were determined using LB with and without 1% (w/v) KNO_3 supplementation and incubated under aerobic and anaerobic atmospheres. The survival is expressed as a percentage of the CFU on LB plates containing paraquat over the CFU on plates without paraquat. Data shown are means ± SD from three independent experiments.

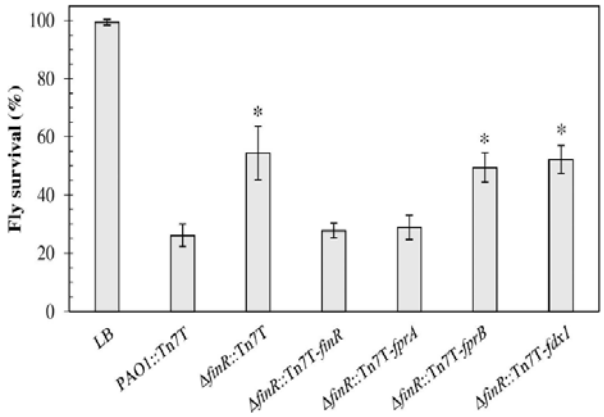


Fig 6. Virulence of *P. aeruginosa* strains. The virulence of PAO1 containing the Tn7T vector control (PAO1::Tn7T) and Δ*finR* mutants containing Tn7T, Tn7T-*finR*, Tn7T-*fprA*, Tn7T-*fprB*, or Tn7T-*fdx1* were determined using the *Drosophila melanogaster* feeding method. The percent fly survival was scored after 18 hours of incubation. Data presented are means ± SD of three independent experiments. The asterisk indicates statistically significant difference ($p < 0.01$) compared with PAO1::Tn7T. LB represents feeding the flies with LB medium.

<u>Part 2</u>: *Pseudomonas aeruginosa ttcA* encoding tRNA-thiolating protein requires an iron-sulfur cluster to participate in hydrogen peroxide-mediated stress protection and pathogenicity

During the translation process, transfer RNA (tRNA) carries amino acids to ribosomes for protein synthesis. Each codon of mRNA is recognized by a specific tRNA, and enzyme-catalysed modifications to tRNA regulate translation. TtcA is a unique tRNA-thiolating enzyme that requires an iron-sulfur [4Fe-4S] cluster to catalyse thiolation of tRNA. In this study, the physiological functions of a putative *ttcA* in *Pseudomonas aeruginosa*, an opportunistic human pathogen that causes serious problems in hospitals, were characterized. A *P. aeruginosa ttcA*-deleted mutant was constructed, and mutant cells were rendered hypersensitive to oxidative stress, such as hydrogen peroxide (H_2O_2) treatment. The Δ*ttcA* mutant exhibited 50-fold lower resistance to H_2O_2 and an 8-fold reduction in the percent survival against NaOCl compared to PAO1. The sensitive phenotype of the Δ*ttcA* mutant against both H_2O_2 and NaOCl was complemented by the expression of a single copy of *ttcA* in Tn7 site, indicating that TtcA plays an important role in the H_2O_2-mediated and NaOCl-derived stress response. In PAO1, the cellular detoxification of H_2O_2 primarily depended on catalase activity levels; however, other mechanisms, such as thiol-peroxidase activity (Tpx) and supporting systems, including haem biosynthesis, were also required to achieve fully responsive functionality against H_2O_2 in *P. aeruginosa*. Catalase activity was lower in the *ttcA* mutant, suggesting that this gene plays a role in protecting against oxidative stress. NaOCl is a bleaching agent that acts as a strong oxidizing agent and can disturb several enzymatic mechanisms, both redox and non-redox, including reactions in tRNA modification processes. Moreover, NaOCl has been shown to generate intracellular ROS, which may increase H_2O_2 levels and lead to mutant susceptibility.

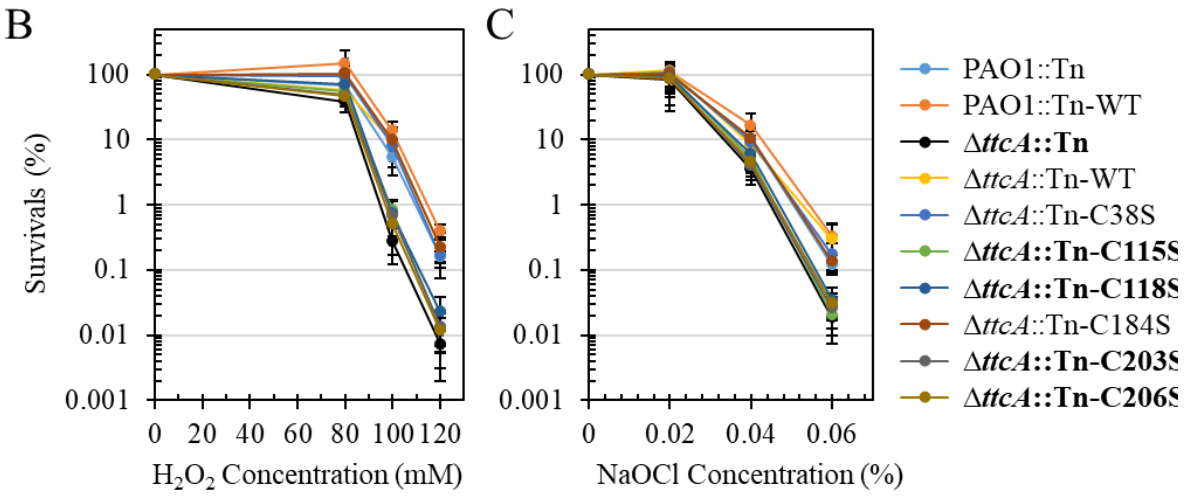


Figure 3. Determination of oxidant resistance levels in *P. aeruginosa* strains. The H_2O_2 and NaOCl resistance levels (at the indicated killing concentrations), respectively, of *P. aeruginosa* PAO1 and Δ*ttcA* mutants with the Tn7 insertion containing either Tn, Tn-TtcA (WT), or site-directed mutagenic cysteines (C38S, C115S, C118S, C184S, C203S, and C206S), determined using a bacterial killing assay.

Moreover, the *ttcA* mutant demonstrated attenuated virulence in a *Drosophila melanogaster* host model (Figure 4). Site-directed mutagenesis analysis revealed that the conserved cysteine motifs involved in [4Fe-4S] cluster ligation were required for TtcA function. Furthermore, *ttcA* expression increased upon H_2O_2 exposure, implying that enzyme levels are induced under stress conditions. Overall, the data suggest that *P. aeruginosa ttcA* plays a critical role in protecting against oxidative stress via catalase activity and is required for successful bacterial infection of the host.

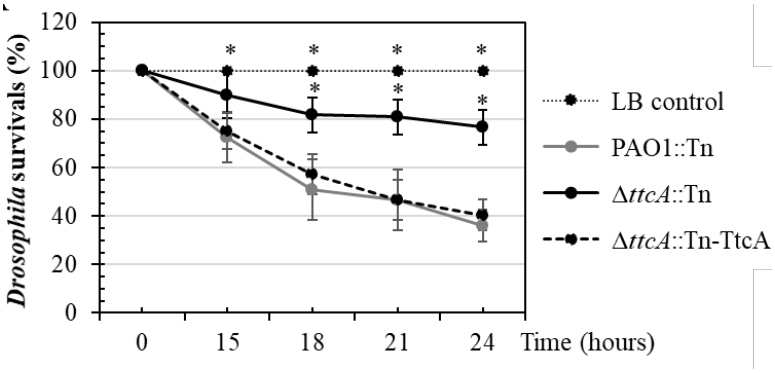


Figure 4. Virulence of PAO1 and Δ*ttcA* mutants containing the Tn7 insertion in either Tn or Tn-TtcA was determined using the *Drosophila melanogaster* feeding method. The percent fly survival was scored at indicated time points of infection after co-incubation.

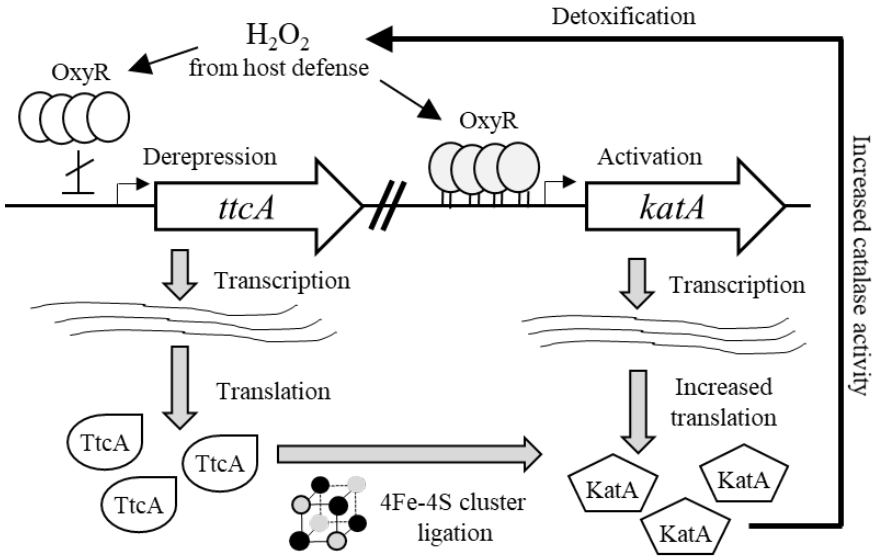


Figure 5. Proposed model of OxyR-regulated *katA* and *ttcA* expression under oxidative stress conditions.

P. aeruginosa OxyR upregulates *katA* and *ttcA* expression to increase catalase activities in response to H_2O_2generated by host defence mechanisms. The iron-sulfur cluster is required for fully functional TtcA activity to have a role in the oxidative stress response via KatA activity and facilitates bacterial survival during infection.

In this study, the physiological role of tRNA modification through Fe-S cluster-ligated TtcA in the pathogenic bacterium *P. aeruginosa* is presented. Under either H_2O_2- or NaOCl-mediated stress, direct transcriptional regulation through *kat* gene expression may not be sufficient to control cellular catalase activity, and translational control through tRNA modification is required. Herein, we proposed an additional model (Fig. 5) involving OxyR regulation to control catalase activity via both direct transcription and indirect translation of TtcA under oxidative stress conditions. During bacterial infection, *P. aeruginosa* OxyR upregulates *katA* and *ttcA* expression to increase catalase activity in response to H_2O_2 generated via host defence mechanisms. Together with iron-sulfur cluster ligation, TtcA has been shown to play an important role in the oxidative stress response and to facilitate bacterial survival during infection of the host, which emphasizes the critical role of the intracellular function of iron-sulfur cluster biogenesis and tRNA modification via IscR and OxyR regulation to mitigate oxidative stress and promote bacterial pathogenicity.

<u>Part 3</u>: *Pseudomonas aeruginosa rcsA1*: AhpD-homologous gene under direct RCS-responsive mechanism

P. aeruginosa rcsR1 and *rcsA1* were characterized in term of an ability required for bacterial survivals under stress conditions. Both of these genes were separately deleted in PAO1 resulting *rcsR1* and *rcsA1* mutants and a chromosomal Tn7-insertion of functional *rcsR1* and *rcsA1* were performed in PAO1 and the mutant strains resulting overexpressed and complemented strains, respectively. The plate sensitivity assay against NaOCl and ROS-mediated agents showed that the *rcsA1* mutant was dramatically susceptible to NaOCl, but not others,

compared to the wild type indicated that *rcsA1* plays an important role in protecting against NaOCl. However, overexpressed and complemented strains have similar level of tested stress sensitivity compared to the level in the wild type suggesting that the NaOCl protection was resulted from the expression of *rcsA1*.

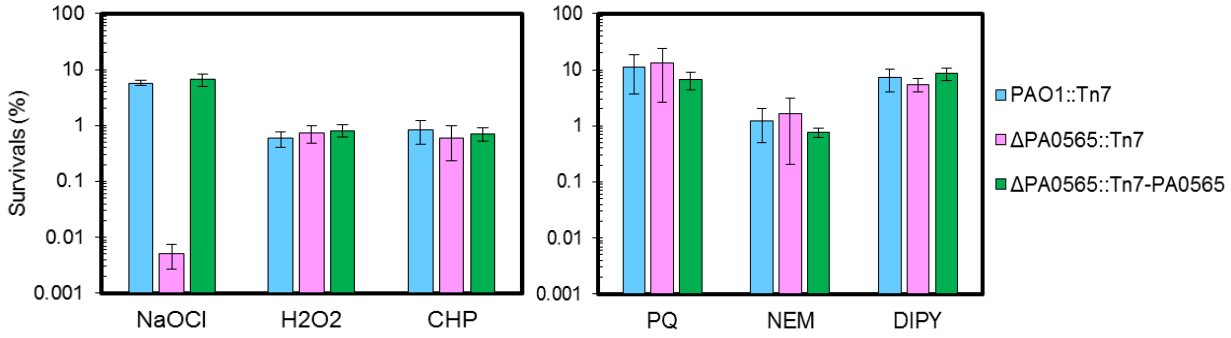


Figure 8. Determination of the resistance levels against oxidative and metal stresses in *P. aeruginosa* strains.

The resistance levels against substances of PAO1::Tn7T, Δ*rcsA*::Tn7T and the Δ*rcsA*::RcsA mutant strains were determined using plate sensitivity assays in plates containing oxidants (A) i.e. 250 µM paraquat, 0.5 mM H_2O_2, 1.6 mM CuOOH, 0.05% NaOCl, 0.35 mM NEM and 1.2 mM 2, 2'-dipyridyl. Survival (%) is defined as the percentage of colony-forming units (CFU) on plates containing oxidant over the number of CFU on plates without oxidant.

Moreover, to identify key amino acids of *rcsA1*, the site-directed mutagenesis was applied by changing amino acid residues in an expression cassette. The results showed that an expression of gene encoding *rcsA1* with either C60S, C63S, or H67A cannot restore the NaOCl susceptibility in the *rcsA1* mutant indicating that C60, C63, H67 are important residues in protecting against NaOCl, which may be targeted at a minor groove of protein (below figure). Finally, gene expression analysis exhibited an increased expression of *rcsA1* under NaOCl-treated condition supporting its physiological function. Overall, *rcsA1* is one of NaOCl-mediated resistance in *P. aeruginosa* and it could be another potential target for developing an effective drug against *P. aeruginosa* infectious diseases.

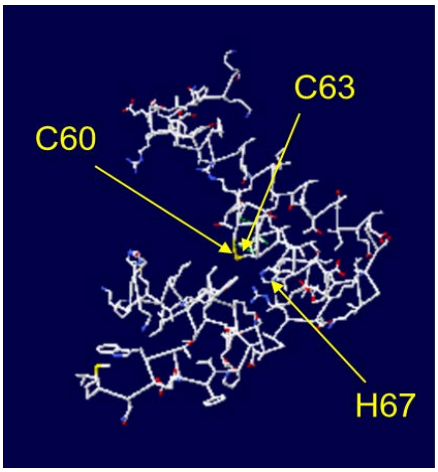


Figure 10. Amino acid analysis of RcsA in *P. aeruginosa* PAO1.

In the other hands, the *rcsR1* mutant exhibited slightly susceptibility against NaOCl compared to the *P. aeruginosa* wild type in the plate sensitivity and the oxidant viability assays suggesting the minor responsive mechanism. Furthermore, the gene expression profile under stress conditions showed that this gene was induced by oxidants suggesting a requirement of this *rcsR1* expression during stresses.

4. Conclusion and Discussion

Pseudomonas aeruginosa is an important opportunistic pathogen that causes severe acute and chronic infection in hospitalized patient and immunocompromised host and is also one of the critical global priority list of antibiotic-resistant bacteria to development of new antibiotics. The treatment difficulties are associated a long-term survival of the pathogens in the hospital environments. One of the major factors for successful infection is the bacterial defense mechanisms against toxic substances from host immune system including reactive oxygen species (ROS) and reactive chlorine species (RCS). Many compounds containing RCS such as sodium hypochlorite (NaOCl) is generally used as bleaching agents in household and disinfectants even in hospitals. The mechanisms to manage ROS in bacteria are well-known but those to deal with RCS are less studied in bacteria and unknown in this pathogenic bacterium.

In this project, the bacterial mechanisms to fight against host-generated RCS, both directly and indirectly, will be explored. Several genes encoding ferredoxin reductases (*fprA* and *fprB*), tRNA thiolating enzyme (*ttcA*) and an iron-sulfur cluster maturation protein (*nfuA*) were shown to be involved in the RCS response and role to detoxify the RCS toxicity in the bacterial cell. The *P. aeruginosa* genes encoding a putative RCS-induced transcriptional regulator and their gene targets were characterized by using recombinant-DNA techniques and physiological susceptibility assays against stress conditions. The result showed that FinR regulated the essential gene *fprA*, which has been shown to involve in detoxification of the oxidative stress and pathogenicity. The *P. aeruginosa ttcA*-deleted mutant was rendered hypersensitive to oxidative stress, such as hydrogen peroxide (H_2O_2) treatment, and also extended to an RCS agent, NaOCl. Catalase activity was lower in

the *ttcA* mutant, suggesting that this gene plays a role in protecting against oxidative stress. Moreover, the *ttcA* mutant demonstrated attenuated virulence in a *Drosophila melanogaster* host model. Site-directed mutagenesis analysis revealed that the conserved cysteine motifs involved in [4Fe-4S] cluster ligation were required for TtcA function. Furthermore, *ttcA* expression increased upon H_2O_2 and NaOCl exposure, implying that enzyme levels are induced under stress conditions and in OxyR-regulated mechanism. Next, deletion of *nfuA* reduced bacterial ability to cope with oxidative stress, iron deprivation conditions and attenuated virulence in the *Caenorhabditis elegans* infection model. Site-directed mutagenesis analysis revealed that the conserved CXXC motif of the Nfu-type scaffold protein domain at the N-terminus was required for NfuA functions in conferring stresses resistance phenotype. Furthermore, anaerobic growth of the *nfuA* mutant in the presence of nitrate was drastically retarded. This phenotype was associated with a reduction in [Fe-S] cluster containing nitrate reductase enzyme activity. However, NfuA was not required for maturation of [Fe-S] containing proteins such as aconitase, succinate dehydrogenase, SoxR and IscR. Taken together, our results indicate the NfuA functions in [Fe-S] clusters delivery to selected target proteins that linking to many physiological processes such as anaerobic growth, bacterial virulence and stresses response in *P. aeruginosa*. Overall, all of these genes was independently regulated by their own specific transcriptional regulator, which can sense the redox homeostasis due to the oxidation by cellular RCS. This emphasizes the broad spectrum of cytotoxicity mechanisms of RCS in the bacteria.

Moreover, the first RCS-sensing transcriptional regulator in *P. aeruginosa* RcsR was proposed and we showed that RcsR regulated an *rcsA* expression under RCS exposure to increase the detoxification activity against RCS agents including a bleach NaOCl. In the study of their molecular mechanism, the thiol group of cysteines in reduced protein was oxidized by RCS and altered to be greater potential for DNA-binding affinity and gene activation in response against RCS stress. Finally, these RSC-responsive genes had a role in bacterial pathogenicity in either *C. elegans* or *D. melanogaster* host model system.

This study was to characterize many novel genes in *P. aeruginosa* to expand the virulence network, a cause of nosocomial infections, and could be a part of the reasons in its ability for hospitalization. The highly RCS-sensitive regulators will be used to develop potential biomarkers detecting RCS, which is an insight research leading to the novel strategies for the drug-targeting development or biosensor for RCS toxicity in the environmental contamination.

5. Output (Acknowledge the Thailand Research Fund)

5.1. International Journal Publication

1. Boonma S, **Romsang A**, Duang-nkern J, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. The FinR-regulated essential gene *fprA*, encoding ferredoxin NADP+ reductase: Roles in superoxide-mediated stress protection and virulence of *Pseudomonas aeruginosa*. PLoS One 2017; 12 (2): e0172071. IF 3.534 / Multidisciplinary Sciences Q1

2. **Romsang A**, Khemsom K, Duang-nkern J, Wongsaroj L, Saninjuk K, Fuangthong M, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa ttcA* encoding tRNA-thiolating protein required an iron-sulfur cluster to role in hydrogen peroxide-mediated stress protection and pathogenicity. (Submitted to Scientific Report, March 2018)
3. Wongsaroj L, Saninjuk K, **Romsang A**, Duang-nkern J, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. 2018. (Submitted to PloS One, April 2018)

5.2. Research Utilization and Application

5.2.1. Invited speaker in medical research and academic institutes

a. **Romsang A**. Hospital environment-induced stress response and antibiotic resistance in *Pseudomonas aeruginosa*. Presented in Micro seminar 2017, Microbiology department, Mahidol-Oxford Tropical Medicine Research Unit; March 8, 2017, the 60th Anniversary Chalermprakiat Building Similan 1-2 room, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

b. **Romsang A**. Multidrug resistance in our life. Presented in Junior Science Club 1/2559, Faculty of Science, Mahidol University; September 14, 2016, K102 Faculty of Science, Mahidol University, Bangkok, Thailand.

5.2.2. Link to academic and research sectors (researchers and students)

a. ได้รับความร่วมมือและข้อเสนอแนะจากอาจารย์ที่ปรึกษา นักศึกษาบัณฑิตศึกษา อาจารย์ และหน่วยวิจัยโรคอุบัติใหม่และอุบัติซ้ำแบคทีเรียในคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และนักวิจัยจากห้างปฏิบัติการเทคโนโลยีชีวภาพ สถาบันวิจัยจุฬาภรณ์ดังรายชื่อปรากฏในผลงานข้างต้น

b. ดำเนินงานวิจัยตามแผนการวิจัยตามเป้าหมายที่ได้ตั้งไว้ทั้งหมด โดยมีนักศึกษาระดับปริญญาตรีเป็นผู้ช่วยในงานวิจัย คือ 1. นส. จารุภา นาคะดำรงวุฒิ ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และได้นำเสนอผลงานวิจัยในงานมหกรรม Science Exhibition 2016 และ 2. นส. ณันนิภา ภู่ภูริพันธุ์ ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และได้นำเสนอผลงานวิจัยในงานมหกรรม Science Exhibition 2017 และ Phuphuripan N, Khemsom K, Duang-nkern J, Mongkolsuk S, **Romsang A**. Protein purification and characterization of transcriptional regulators in response to reactive chlorine species in *Pseudomonas aeruginosa*. Oral presented in the 12th Science and Technology Conference for Youths; June 3-4, 2017; Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

5.3. Others e.g. national journal publication, proceeding, international conference, book chapter, patent

International conferences

1. **Romsang A**, Duang-nkern J, Wongsaroj L, Trinachartvanit W, Dubbs JM, Vattanaviboon P, Mongkolsuk S. Characterization of Reactive Chlorine Species-mediated stress response through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*. Poster presented in the Gordon Research Conference 2016: Microbial Stress Response; July 17-22, 2016, Mount Holyoke College, South Hadley, MA, USA.
2. **Romsang A**, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. AhpD-like protein protects against reactive chlorine species-mediated toxicity in pathogenic bacteria. PST-selected oral presented in the 11th International Symposium of the Protein Society of Thailand (PST); Aug 3-5, 2016, CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand. ***With an outstanding abstract award***
3. **Romsang A**, Duang-nkern J, Saninjuk K, Nakhadamrongwut J, Vattanaviboon P, Mongkolsuk S. Multiple stress responses against a bleaching agent in human pathogen *Pseudomonas aeruginosa*. Platform presented in The 8th Princess Chulabhorn International Science Congress; Nov 13-17, 2016, Shangri-La Hotel, Bangkok, Thailand.
4. Duang-nkern J, **Romsang A**, Vattanaviboon P, Mongkolsuk S. Roles of *Pseudomonas aeruginosa nfuA* in stress conditions, bacterial virulence and regulation. Poster presented in The 8th Princess Chulabhorn International Science Congress; Nov 13-17, 2016, Shangri-La Hotel, Bangkok, Thailand.
5. Saninjuk K, Lorlitiwong W, ***Romsang A***, Mongkolsuk S. LysR-type transcriptional regulator involves in antibiotics resistance and pathogenesis of *Pseudomonas aeruginosa*. Poster presented in The 8th Princess Chulabhorn International Science Congress; Nov 13-17, 2016, Shangri-La Hotel, Bangkok, Thailand.
6. Wongsaroj L, Saninjuk K, **Romsang A**, Trinachartvanit W, Mongkolsuk S. Role of *aspA* a gene encoding aspartate ammonia lyase in Pseudomonas aeruginosa. Poster presented in The 8th Princess Chulabhorn International Science Congress; Nov 13-17, 2016, Shangri-La Hotel, Bangkok, Thailand.
7. Khemsom K, **Romsang A**, Duang-nkern J, Mongkolsuk S. Characterization of a gene encoding AtfA-homolog factor against oxidative stress in *Pseudomonas aeruginosa*. Poster presented in The 8th Princess Chulabhorn International Science Congress; Nov 13-17, 2016, Shangri-La Hotel, Bangkok, Thailand.
8. Saninjuk K, **Romsang A**, Duang-Nkern J, Mongkolsuk S. Molecular characterization of Iron-Sulfur Cluster Regulator IscR-binding motifs in pathogenic bacterium *Pseudomonas aeruginosa*. Poster presented in the American Society for Microbiology (ASM) Microbe 2017; June 1-5, 2017; Ernest N. Morial Convention Center, New Orleans, LA, USA.
9. **Romsang A**, Phuphuripan N, Duang-nkern J, Nontaleerak B, Khemsom K, Mongkolsuk S. Activation of Reactive Chlorine Species-mediated stress response protein through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*. Poster presented in the 5th Conference of the Asia Pacific Protein Association (APPA) and the 12th International Symposium of the Protein Society of Thailand (PST2017); July 11-14, 2017; The Tide resort, Chonburi, Thailand. ***With an outstanding poster award***

10. **Romsang A**, Auwattanamongkol T, Duang-nkern J, Nakhadamrongwut J, Mongkolsuk S. Cross resistance mechanisms between antibiotic, antiseptic, and disinfectant in human pathogen *Pseudomonas aeruginosa*. Oral presented in the 13th Asian Congress on Biotechnology 2017 (ACB2017); July 23-27, 2017; Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.
11. Nontaleerak B, Duang-nkern J, Vattanaviboon P, Mongkolsuk S, **Romsang A**. Functional and expression analysis of a gene encoding peroxiredoxin-liked protein in *Pseudomonas aeruginosa*. Oral presented in the 29th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB2017); November 23-24, 2017, Swissotel Le Concorde, Bangkok, Thailand.
12. **Romsang A**, Duang-Nkern, J, Nontaleerak, B, Wongsaroj, L, Trinachartvanit, W, Vattanaviboon, P, Mongkolsuk, S. Reactive Chlorine Species-Mediated Stress Response through Transcriptional Regulators in a Human Pathogen *Pseudomonas aeruginosa* Involves in Nosocomial Infections. Poster presented in the TRF-OHEC Annual Congress 2018 (TOAC 2018); Jan 10-12, 2018, The Regent Cha-Am Beach Resort, Rayong, Thailand. ***With an outstanding poster award***

6. Appendix (ordered by (5) output) are showed in the next pages.

7. References

- Personal and laboratory website: http://ajrscbt.wix.com/k610-scmu
- Attached Researcher CV
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PLOS ONE

RESEARCH ARTICLE

The FinR-regulated essential gene *fprA*, encoding ferredoxin NADP$^+$ reductase: Roles in superoxide-mediated stress protection and virulence of *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa has two genes encoding ferredoxin NADP(+) reductases, denoted *fprA* and *fprB*. We show here that *P. aeruginosa fprA* is an essential gene. However, the Δ*fprA* mutant could only be successfully constructed in PAO1 strains containing an extra copy of *fprA* on a mini-Tn7 vector integrated into the chromosome or carrying it on a temperature-sensitive plasmid. The strain containing an extra copy of the ferredoxin gene (*fdx1*) could suppress the essentiality of FprA. Other ferredoxin genes could not suppress the requirement for FprA, suggesting that Fdx1 mediates the essentiality of FprA. The expression of *fprA* was highly induced in response to treatments with a superoxide generator, paraquat, or sodium hypochlorite (NaOCl). The induction of *fprA* by these treatments depended on FinR, a LysR-family transcription regulator. In vivo and in vitro analysis suggested that oxidized FinR acted as a transcriptional activator of *fprA* expression by binding to its regulatory box, located 20 bases upstream of the *fprA* -35 promoter motif. This location of the FinR box also placed it between the -35 and -10 motifs of the *finR* promoter, where the reduced regulator functions as a repressor. Under uninduced conditions, binding of FinR repressed its own transcription but had no effect on *fprA* expression. Exposure to paraquat or NaOCl converted FinR to a transcriptional activator, leading to the expression of both *fprA* and *finR*. The Δ*finR* mutant showed an increased paraquat sensitivity phenotype and attenuated virulence in the *Drosophila melanogaster* host model. These phenotypes could be complemented by high expression of *fprA*, indicating that the observed phenotypes of the Δ*finR* mutant arose from the inability to up-regulate *fprA* expression. In addition, increased expression of *fprB* was unable to rescue essentiality of *fprA* or the superoxide-sensitive phenotype of the Δ*finR* mutant, suggesting distinct mechanisms of the FprA and FprB enzymes.

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Competing interests: The authors have declared that no competing interests exist.

Introduction

Pseudomonas aeruginosa is one of the most common opportunistic bacterial pathogens that cause deadly infections in patients with impaired immune systems or in critical condition. Nosocomial infections caused by *P. aeruginosa* are increasing worldwide [1, 2]. The ability of a pathogen to overwhelmingly invade the host is often associated with its ability to rapidly adapt and evade or overcome host immune systems. During pathogen-host interactions, several transcriptional regulators are differentially expressed to fine-tune gene expression networks required for adaptive responses to host-generated stresses [3]. Reactive oxygen species (ROS) are key components of host innate immune responses generated within the phagolysosomes of phagocytes to attack invading microbes. Additionally, normal aerobic metabolism produces ROS as by-products [4]. Consequently, bacteria have evolved mechanisms to protect themselves from oxidative stress. An array of either antioxidant enzymes, such as catalases, superoxide dismutases, and thiol peroxidases or antioxidant molecules, such as glutathione and vitamins are involved in removal of ROS. In addition, there are extensive repaired and rebuilding systems for oxidatively damaged molecules, such as iron-sulfur cluster biosynthesis (Isc), DNA repair (the Mut systems) and proteins repair (methionine sulfoxide reductases, Msr). These systems are necessary for bacterial survivals under stressful conditions [5, 6]. Various transcriptional regulators are involved in coordinating the complex processes of sensing and responding to stresses. The LysR-type transcriptional regulators (LTTRs) represents the most abundant type of transcriptional regulator with an N-terminal DNA-binding helix-turn-helix motif and a C-terminal co-inducer-binding domain as conserved structures. LTTRs exhibit a negative autoregulation and regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility [7–15]. A major regulator in hydrogen peroxide (H_2O_2) defense is OxyR, a LysR-type transcriptional regulator, while the transcription factor SoxR triggers a global stress response against superoxide anions as well as redox cycling drugs [16–19]. Many proteobacterial genomes contain another LysR-type oxidative stress sensing transcriptional regulator, FinR, which is located next to *fprA* (ferredoxin $NADP^+$ reductase, Fpr), an enzyme catalyzing the reversible electron-transferring reaction between NADPH and one-electron carriers such as ferredoxin or flavodoxin. The enzymes are important in maintain NADP(+)/NADPH ratio. Fpr also catalyzes the irreversible electron transfer in diaphorase reaction which drives the oxidation of NADPH in a wide variety of adventitious electron acceptors [20]. In bactria, Fpr has been shown to control the ratio of NADP(+)/NADPH. Fpr participates in many cellular processes, including iron acquisition, iron-sulfur cluster biogenesis and oxidative stress defense [21, 22]. FinR is required for the induction of *fprA* expression upon exposure to superoxide anion stress generated by paraquat [21, 23–25]. However, *Escherichia coli fpr* is a member of the SoxRS regulon, and inactivation of *fpr* increases sensitivity to paraquat [26, 27].

Pseudomonas putida KT2440 contains at least two genes encoding Fpr, namely *fprA* and *fprB* [23, 28]. The *fprA* mutant confers high sensitivity to oxidative and osmotic stresses, while the *fprB* mutant is susceptible only to high osmotic conditions [23, 28]. Like *P. putida*, *P. aeruginosa* PAO1 possesses both *fprA* and *fprB*. Two types of Fprs have their preferred electron transport and redox partners. FprA achieves higher catalytic efficiency when flavodoxin is its redox partner. FprB is important in defenses against multiple stresses including metal, oxidative, and osmotic stresses and is required for the full function of iron-sulfur cluster ([Fe-S])-containing enzymes via its redox partner, Fdx2, involving in the ISC [Fe-S] biogenesis system [29]. For example, in an iron storage complex, the [Fe-S] cluster of bacterioferritin-associated ferredoxin (Bdf) transfers electrons to the heme in bacterioferritin (BfrB) and promotes the release of Fe^{2+} from BfrB by mediating electrons from FprA to BfrB [30]. Moreover, roles for

FprA in sulfate assimilation and siderophore biosynthesis in pseudomonads have been characterized [31]. The expression of *fprB* could be induced by exposure to oxidative stress in an [Fe-S] biogenesis regulator IscR-dependent manner [29]. The physiological function of *P. aeruginosa* FprA remains mysterious due to unsuccessful construction of the *fprA* mutant [31, 32]. This observation raised the possibility that the activity of FprA is essential for bacterial viability. In this communication, *P. aeruginosa fprA* was shown to be essential and was determined to be regulated by FinR.

Results and discussion

fprA is an essential gene in *P. aeruginosa*

P. aeruginosa PAO1 has two annotated *fpr* genes, *fprA* (PA3397) and *fprB* [33]. An open reading frame located next to *fprA* in the opposite orientation was annotated as a putative LysR-type transcriptional regulator, FinR (PA3398). *P. aeruginosa* FinR shares 80.8% and 80.5% amino acid sequence identity with FinR from *P. putida* and *Azotobacter vinelandii*, respectively (S1 Fig). Several attempts to construct the *fprA* mutant in pseudomonads have been met with mixed results. No mutants were obtained in *P. aeruginosa*, but a mutant was constructed in *P. putida* [31, 32]. These observations suggest the essentiality of *fprA* in PAO1. We made several unsuccessful attempts to construct either insertion inactivation or deletion *fprA* mutants. Hence, the notion of the essentiality of *fprA* was tested. A new PAO1 parental strain was constructed that had an extra copy of *fprA* cloned into a mini-Tn7 vector [34], and the recombinant Tn7T-*fprA* transposed into the PAO1 chromosome *att*Tn7 site, giving PAO1::Tn7T-*fprA*. The antibiotic marker of the mini-Tn7 vector was removed by the Flp-FRT system [35]. *fprA* gene deletion by allelic exchange was made by electroporating pUCΔ*fprA*::Gm^r (Table 1) into PAO1::Tn7T-*fprA* and selecting for gentamicin resistance (Gm^r). Several Gm^r and carbenicillin susceptible (Cb^s) colonies were screened by PCR and found to have deleted the functional copy of the chromosomal *fprA* gene. The Δ*fprA* mutant (Δ*fprA*::Tn7T-*fprA*) was successfully constructed. In the control strain, which contains only the mini-Tn7 vector (PAO1::Tn7T), no Gm^r transformants or *fprA* mutants were recovered (Table 1). The results support the notion that *fprA* is an essential gene in PAO1.

An independent approach was conducted to assure the essentiality of *fprA* in *P. aeruginosa* using a plasmid vector with a temperature-sensitive origin of replication that has been recently developed in *P. aeruginosa* [36]. We firstly constructed a temperature-sensitive replication

Table 1. Efficiency of *fprA* deletion in *P. aeruginosa* strains carrying an extra copy of various genes.

***P. aeruginosa* strains**	**Efficiency of *fprA* deletion[a]**
PAO1::Tn7T	ND
PAO1::Tn7T-*finR*	ND
PAO1::Tn7T-*fprA*	1.6×10^2
PAO1::Tn7T-*fprB*	ND
PAO1::Tn7T-*fdx1*	2.1×10^1
PAO1::Tn7T-*fdx2*	ND
PAO1::Tn7T-*rnfB*	ND
PAO1::Tn7T-*fdxA*	ND

[a] Indicated strains of PAO1 were transformed with 1 μg pΔ*fprA*::Gm^r plasmid using electroporation. The transformants with *fprA* deletion were selected by the Gm^r and Cb^s phenotypes. The efficiency of *fprA* deletion is defined as the number of Δ*fprA* mutant obtained per 1 μg pUCΔ*fprA*::Gm^r plasmid. The data shown are means from triple independent experiments. ND, not detectable.

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plasmid by cloning the temperature-sensitive replicon mSF^{ts1} from pSS255 [36] into the broad-host-range vector pBBR1MCS-4 [37], yielding pTS. The plasmid can be maintained at 30˚C but not at the non-permissive temperature of 45˚C. The full-length *fprA* was cloned into the plasmid pTS, generating pTS-*fprA*. Transformants harboring pTS-*fprA* were grown and maintained at 30˚C. Growing bacterial cultures at the non-permissive temperature of 45˚C resulted in the loss of pTS-*fprA*. pUCΔ*fprA*::Gm^r was introduced into PAO1 harboring pTS-*fprA*, and Gm^r transformants were selected and screened for double crossing over and marker exchange events, giving Δ*fprA*::Gm^r/pTS-*fprA*. The Δ*fprA*::Gm^r/pTS-*fprA* mutant strain had normal growth at 30˚C. This mutant strain could not grow on either an agar plate or in LB broth medium when the incubation temperature was shifted to the non-permissive temperature of 45˚C for pTS-*fprA*, indicating the essentiality of *fprA* (Fig 1). The results confirmed that *fprA* is an essential gene that is required for the growth of PAO1. Although *P. aeruginosa* FprA shares the greatest amino acid sequence identity with FrpA from *P. putida* and *A. vinelandii* (S1 Fig), there is no evidence suggesting that it is essential in these two bacteria [31, 38].

Since PAO1 has both *fprA* and *fprB*, we tested the potential cross-functional complementation between *fprB* and *fprA*. Similar approaches that were successfully used to construct the Δ*fprA* mutant were applied to test cross-complementation between *fprA* and *fprB*. A PAO1::Tn7T-*fprB* strain carrying an extra copy of *fpr*B was used for Δ*fprA* mutant construction using pUCΔ*fprA*::Gm^r. After several attempts, no *fprA* mutant was obtained. This indicated the essential function of *fprA* for bacterial growth and showed that expression of *fprB* could not complement *fprA*. This suggests that FprA and FprB have different biochemical and physiological functions. Fpr have essential functions in maintenance of the NAD(P)/NAD(P)H ratio via their reactions with ferredoxins and flavodoxins. In the *fprA* mutant, alterations in the ratio of reduced/ oxidized ferredoxins could contribute to the mutant lethality under tested conditions.

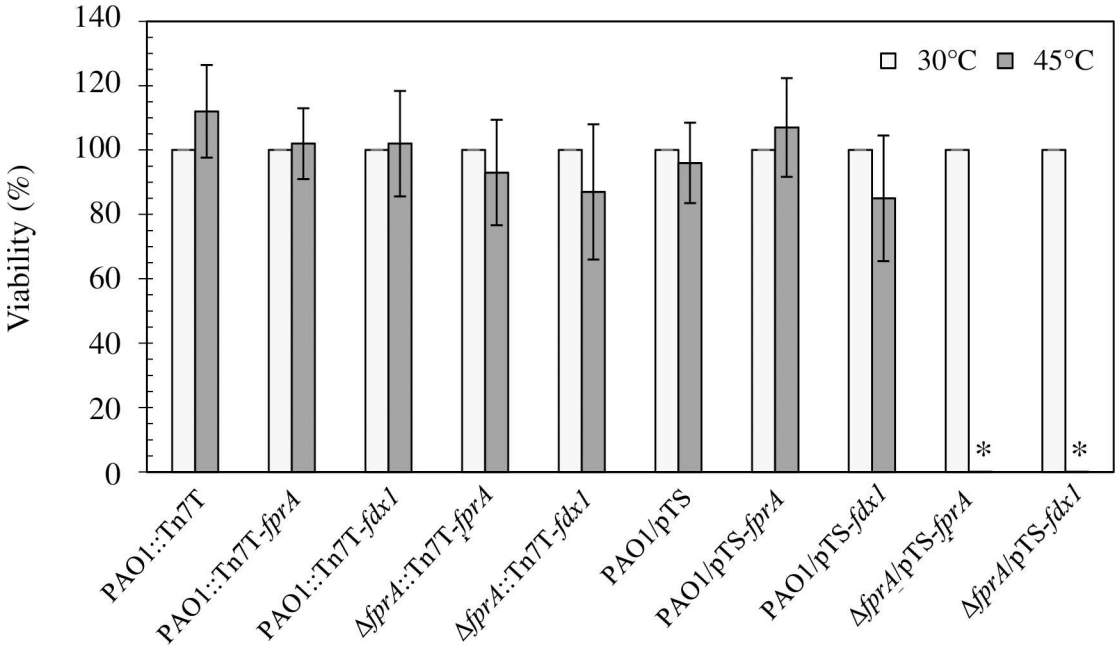

Fig 1. *fprA* is an essential gene in *P. aeruginosa*. The viability of exponential-phase cultures of *P. aeruginosa* PAO1 and Δ*fprA* mutant strains harboring an extra copy of *fprA* or *fdx1* was determined using viable cell count on LB agar plates incubated at either 30˚C or 45˚C. The viability is expressed as a percentage of the CFU of the tested strain over the CFU of the PAO1::Tn7T or PAO1/pTS control.

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The requirement for *fprA* could be complemented by *fdx1* expression

Fpr catalyzes reversible electron transfer between NADPH and electron carriers such as ferredoxins (Fdx), thereby maintaining a balance between NADPH and reduced Fdx pools. Since Fpr is important in maintaining reduced Fdx, we determined whether the expression of *fdx* genes could suppress the essentiality of *fprA*. PAO1 has several genes encoding Fdx of different families, e.g., *fdx1* (PA0362), encoding two[4Fe-4S]-containing bacterial ferredoxin; *fdx2* (PA3809) (a member of the *isc* operon that is involved in iron-sulfur cluster biogenesis), encoding a [2Fe-2S]-containing ferredoxin; *rnfB* (PA3490), encoding a ferredoxin-like protein; and *fdxA* (PA3621), encoding a [4Fe-4S] cluster-containing ferredoxin [33],[6, 39–42]. We tested whether the essentiality of the *fprA* gene was due to its Fdx1 redox partners. Using a similar strategy as used for the construction of the *fprA* mutant, *P. aeruginosa* PAO1 strains were constructed with an extra copy of *fdx1* (PAO1::Tn7-*fdx1*), *fdx2* (PAO1::Tn7-*fdx2*), *rnfB* (PAO1::Tn7-*rnfB*) or *fdxA* (PAO1::Tn7-*fdxA*) and used to test whether Δ*fprA* mutants could be constructed with a suicide plasmid pUCΔ*fprA*::Gm^r. The *fprA* mutant construction was accomplished only in the parental strains PAO1::Tn7-*fdx1* and PAO1::Tn7T-*fprA* (Table 1). In other parental strains tested, no *fprA* mutant could be recovered. The functional complementation of *fprA* by *fdx1* was confirmed by the fact that the Δ*fprA* mutant harboring pTS-*fdx1* could grow at 30˚C and at the non-permissive 45˚C (Fig 1). This finding indicated that expression of *fdx1* can suppress the essential function of *fprA* and permit the growth of the Δ*fprA* mutants. It is likely that Fdx1 functions as a redox partner of FprA. We speculate that deletion of *fprA* severely affects the redox status of Fdx1 by shifting the ratio between reduced and oxidized forms. Increased expression of *fdx1*, either from Tn7T-*fdx1* or pTS-*fdx1* in the mutant was sufficient to compensate for the loss of FprA function by restoring the ratio of reduced/oxidized ferredoxins to a viable levels for *P. aeruginosa*. Fdx1 has been shown to be essential for the viability of PAO1 [40]. The physiological role of Fdx1 in *P. aeruginosa* remains unclear.

fprA and *finR* expression increased in response to exposure to paraquat and NaOCl

The expression patterns of *fprA* under stressful growth conditions were evaluated using real-time RT-PCR. The expression profiles of PAO1 *fprA* challenged with superoxide anion-generating agents (plumbagin, menadione, and paraquat), H_2O_2, organic hydroperoxides (cumene hydroperoxide, and *t*-butyl hydroperoxide), the iron-chelating agent 2,2'-dipyridyl, high salt (NaCl and KCl), and a bleaching agent (NaOCl) were determined. The results in Fig 2A illustrate that *fprA* expression was considerably induced by exposure to paraquat (7.6 ± 2.6-fold) and NaOCl (11.3 ± 2.8-fold), but not by the other oxidants, 2,2'-dipyridyl, or high salt conditions. The expression profiles of *finR* in response to stresses were also determined by real-time RT-PCR. The expression of *finR* could be induced only by exposure to paraquat (6.9 ± 1.9-fold) or NaOCl (9.9 ±1.4-fold) treatments (Fig 2B). Other oxidants and stresses did not significantly (2-fold or less) induce *finR* expression. This induction pattern is similar to the stress response pattern of *fprA*. A previous report indicated that paraquat induction of *fprA* in *Pseudomonas* spp. is significantly affected by the addition of various sources of sulfur [31]. Nonetheless, how intracellular sulfur affects the induction of gene expression by superoxide generator is being investigated.

FinR regulates the expression of *fprA* and itself

To assess whether FinR mediates induction of *fprA* expression upon exposure to oxidative stress, *fprA* expression levels were determined in the Δ*finR* mutant (Δ*finR*::Tn7T) and the

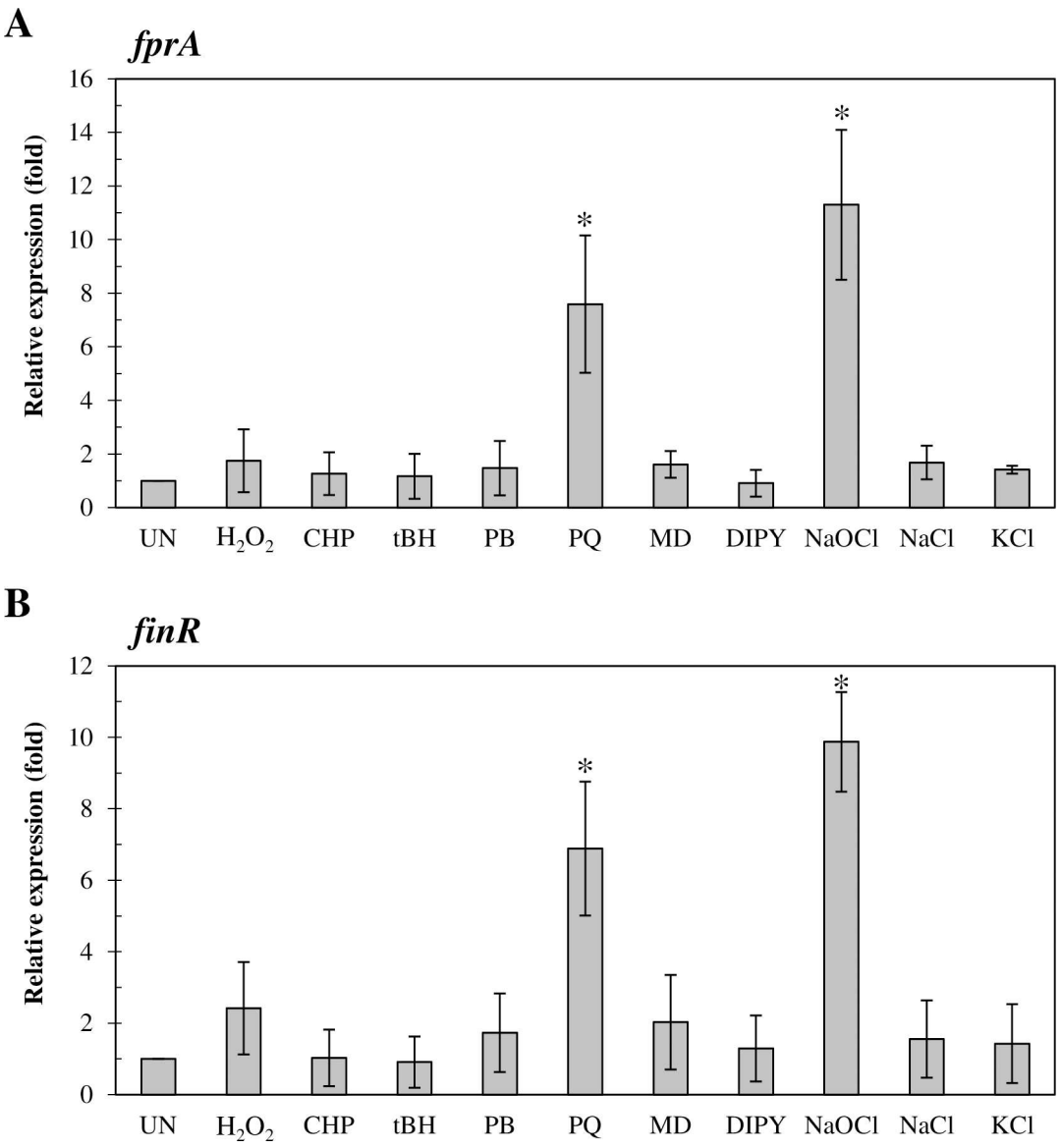

Fig 2. Expression analysis *finR* and *fprA* in response to various stresses. The expression levels of *finR* (A) and *fprA* (B) were determined using real-time RT-PCR. Exponential-phase cultures of *P. aeruginosa* PAO1 were subjected to various stress conditions, including 1 mM H_2O_2, superoxide anion-generating agents (0.5 mM plumbagin [PB], 0.5 mM menadione [MD] and 0.5 paraquat [PQ]), organic hydroperoxides (1 mM cumene hydroperoxide [CHP] and 1 mM *t*-butyl hydroperoxide [tBH]), 1 mM 2,2'-dipyridyl (DIPY), high salts (0.5 M NaCl and 0.5 M KCl), or 0.04% NaOCl for 15 minutes prior to RNA preparation for real-time RT-PCR analysis. Relative expression was analyzed using the 16S rRNA gene as the normalizing gene and was expressed as the fold expression relative to the level of uninduced (UN) PAO1. Data shown are means ± SD of three independent experiments.

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complemented mutant (Δ*finR*::Tn7T-*finR*) using real-time RT-PCR. The results showed that paraquat- and NaOCl-induced expression of *fprA* was abolished in the Δ*finR* mutant and that this could be restored in the complemented mutant strain (Fig 3A). The levels of *fprA* expression in the Δ*finR* mutant in all tested conditions were comparable to those of the uninduced wild-type PAO1 (Fig 3A). Moreover, the *fprA* level in a complemented strain (Δ*finR*::Tn7T-*finR*) was comparable to wild type and a Δ*finR* mutant strain. Thus, oxidized FinR likely

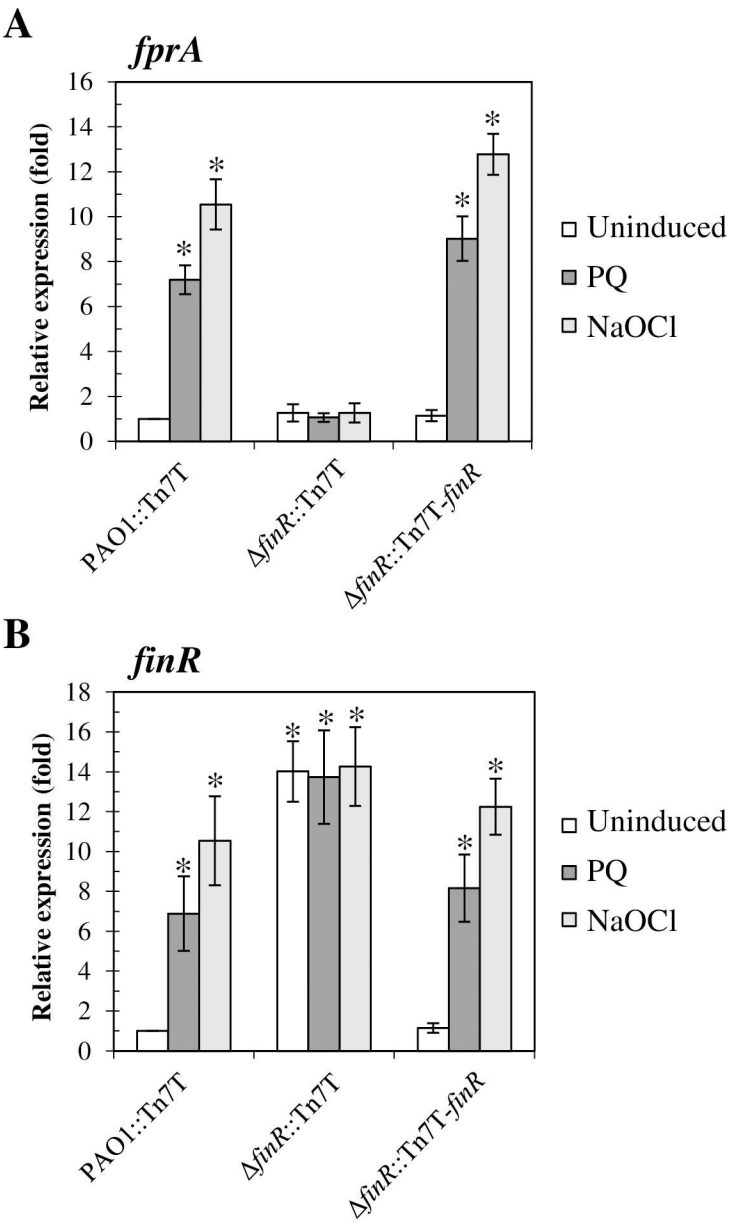

Fig 3. Expression analysis of *fprA* and *finR* in *P. aeruginosa* strains. Expression levels of *fprA* (A) and *finR* (B) in PAO1 wild type (PAO1::Tn7T), Δ*finR* mutant (Δ*finR*::Tn7T) and the complemented mutant (Δ*finR*::Tn7T-*finR*) grown under uninduced, 0.5 mM paraquat (PQ), or 0.04% NaOCl (NaOCl) induced conditions. Relative expression was analyzed using the 16S rRNA gene as the normalizing gene and is expressed as fold expression relative to the level of uninduced PAO1. Data shown are means ± SD of three independent experiments. The asterisks indicate statistically significant differences ($p < 0.01$) compared with the uninduced condition.

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functions as a transcriptional activator on the *fprA* promoter in the presence of the inducers paraquat and NaOCl. However, reduced FinR neither represses nor activates *fprA* expression.

The expression levels of *finR* in response to oxidants were also evaluated in the Δ*finR* mutant and the complemented mutant using real-time RT-PCR with primers located immediately downstream of the transcriptional start site (+1) of *finR* and next to the deletion site (BT3334 and EBI62). The expression levels of *finR* in the Δ*finR* mutant were constitutively

high (~14-fold over wild-type PAO1 levels) in both uninduced and oxidant-induced samples (Fig 3B). The constitutively high expression levels in the *finR* mutant strongly suggest that reduced FinR functions as a transcriptional repressor of its own promoter. Paraquat- and NaOCl-induced *finR* expression could be restored in the complemented Δ*finR* mutant strain (Δ*finR*::Tn7T-*finR*) (Fig 3B). This suggests that reduced FinR functions as a repressor of *finR* expression while oxidized FinR either activates expression or derepresses *finR* expression. The results indicate that *finR* is negatively auto-regulated, which is similar to other transcriptional regulators in the LysR-family [25, 43–45].

FinR binds directly to *finR-fprA* promoter region

fprA is located next to a divergently transcribed gene, *finR*, with a 273-bp intergenic region. To characterize the *fprA* and *finR* promoters, the putative +1 sites were determined using 5' RACE. The +1 site of *fprA* was mapped to an A residue located 54 bp upstream of its translational start codon ATG (Fig 4A). Two sequences (TTTTGC and TAAAAT, separated by 18 bp) that resemble the *E. coli* δ^{70}–35 and -10 promoter motifs were identified. Using a similar technique, the +1 site of *finR* was mapped to a G residue situated 125 bp upstream of the *finR* start codon (ATG) and 97 bp upstream of the putative *fprA* +1 (Fig 4A). The -35 and -10 promoter motifs were identified as TGCTTA and GATAAC and were separated by 18 bp. The *fprA* and *finR* promoter motifs did not overlap with each other (Fig 4A).

The ability of purified FinR to bind to the *fprA-finR* promoter was investigated using electrophoretic mobility shift assays (EMSA). A 6His-tagged FinR protein was purified using an *E. coli* system (25). A [P^{32}]-labeled DNA probe (398 bp) spanning the *fprA-finR* promoters was used in the binding experiments. Purified FinR could bind to the *fprA-finR* promoter sequence at nanomolar concentrations (Fig 4B). The binding specificity of FinR was illustrated by the ability of excess unlabeled *fprA-finR* promoter fragment (CP) but not excess of unrelated DNA (pUC18 plasmid, UP) to compete with the binding of FinR to labeled promoter fragments. Addition of an excess amount of unrelated protein (2.5 μg bovine serum albumin [BSA]) did not affect binding of purified FinR to the *fprA-finR* promoter (Fig 4B). Thus, FinR functions as a transcriptional regulator of *fprA* and *finR* itself through a direct binding to the *fprA-finR* promoter region.

To our knowledge, no consensus sequence for FinR binding box on target gene promoters has been identified. FinR is a member of LysR family of transcription regulators, which often use palindromic DNA sequences as a binding box that the regulator in LysR family binds to modulate expression of the target gene [46]. We identified DNA sequences with two overlaps and almost perfect dyadic symmetry, 5′TATCCATATTCTGGATAAGCATTATCCAGA3′, consisting of the first palindrome 5′TATCCATATTCTGGATA3′ and the second palindrome 5′TCTGGATAAGCATTATCCAGA3′ located between positions -22 and -51 of the *finR* promoter and -46 and -83 of the *fprA* promoter (Fig 4A). The involvement of these two dyadic symmetries in the binding of FinR was evaluated. Site-directed mutagenesis was performed to mutate the putative binding site for FinR from 5′TATCCATATTCTGGATAAGCATTATCCAG A3′ to 5′GCGAACTATTCTGGATAAGCATTATCCA3′ (referred to as MU1) and to 5′TATCC ATATTCTGGATAAGCATGCGAACGA3′ (referred to as MU2) using pP_{fprA} as a DNA template. The mutations essentially changed the first palindrome sequence in MU1 and the second palindrome sequence in MU2. [P^{32}]-labeled *fprA-finR* promoter containing MU1 or MU2 sequences was used in the EMSA experiments. The results in Fig 4C showed that purified FinR bound to the promoter containing MU2 in a similar manner as the native promoter. However, purified FinR at concentration of 200 nM was unable to bind the mutagenized MU1 promoter (Fig 4C). This suggests that the sequence TATCCA of the first palindromic sequence

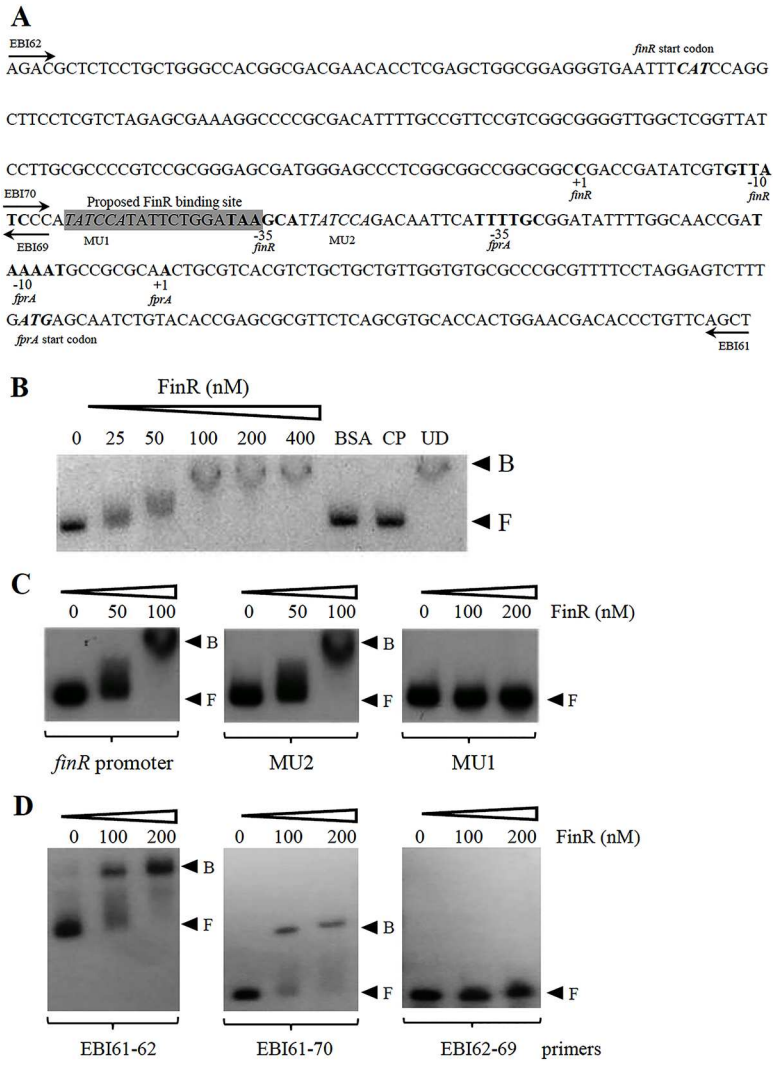

Fig 4. Characterization and binding of purified FinR to the *finR-fprA* promoter. (A) Nucleotide sequence showing the *finR-fprA* promoter structure. +1 indicates the transcriptional start site, and the bold sequences are the putative -35 and -10 promoter motifs. ***CAT*** and ***ATG*** are the translational start codons of FinR and FprA, respectively. The box shaded gray represents the proposed FinR binding site. (B), (C), and (D) Electrophoretic mobility shift assay using purified FinR. A ^{32}P –labeled DNA fragment (B), mutagenized MU1 and MU2 fragments (C), or the promoter fragments (EBI61-62), with (EBI 61–70) and without (EBI 62–69) proposed FinR binding site (D) spanning the *finR-fprA* promoter was incubated with increasing amounts of FinR. BSA represents an unrelated protein (2.5 μg BSA). CP and UD signify the cold probe (100 ng unlabeled promoter fragment) and unrelated DNA (1 μg of pUC18 plasmid), respectively, that were added to the binding reaction mixture containing 100 nM FinR. F and B indicate free and bound probes, respectively.

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5′TATCCATATTCTGGATA3′ is important for in vitro binding of *P. aeruginosa* FinR. To confirm the putative binding site of FinR, the EMSA experiments were performed using the promoter fragment with and without proposed FinR binding site. The results in Fig 4D showed that the purified FinR could bind to the promoter fragment with the proposed FinR-binding site. No FinR binding could be detected when the DNA fragment without the binding site was used (Fig 4D). This supports the site-directed mutagenesis results that in vitro FinR binds specifically to the palindromic sequence 5′TATCCATATTCTGGATA3′.

Δ*finR* mutant shows an increased paraquat sensitivity phenotype that could be suppressed by increasing *fprA* expression

Next, the physiological function of *finR* was assessed using the Δ*finR* mutant. Since FinR is involved in sensing various oxidant resistance levels, the Δ*finR* mutant resistance to oxidants was determined using a plate sensitivity assay. The Δ*finR* mutant exhibited similar levels of resistance to various oxidants, including H_2O_2, cumene hydroperoxide, and NaOCl, as the wild-type PAO1 (data not shown). Nonetheless, Fig 5A shows that the Δ*finR* mutant (Δ*finR*::Tn7T) was much more sensitive (10^4-fold) to paraquat (150 μM) than its parental strain PAO1 (PAO1::Tn7T), and this hypersensitive phenotype of the mutant was fully restored by the expression of a single copy of functional *finR* in a mini-Tn7 vector (Δ*finR*::Tn7T-*finR*). These results indicate a crucial role of *finR* for survival under paraquat stress and are consistent with the previously reported resistance of a *finR* mutant of *P. putida* [25].

Paraquat is a redox cycling drug that has been recognized as a superoxide anion-generating agent in the presence of oxygen by disrupting normal electron flow in aerobic respiration [17]. The drug itself can undergo intracellular transformations and is toxic to cells [19]. The question was raised as to whether hypersensitivity of the *finR* mutant to paraquat was due to reduced ability to detoxify superoxide anions generated from the drug or direct toxicity of the drug. An approach previously described in *E. coli* was used to test the likely mechanism responsible for paraquat sensitivity in the Δ*finR* mutant; this approach used anaerobic cultivation to distinguish between the direct toxicity of the drug and the generation of superoxide anions, which requires oxygen [19]. *P. aeruginosa* did not grow under anaerobic conditions unless nitrate was added to the culture medium [47]. Plate sensitivity assays were performed to re-examine the paraquat sensitivity levels using LB medium supplemented with potassium nitrate (KNO_3, 1% w/v) under anaerobic conditions. All *P. aeruginosa* grew anaerobically (data not shown). The results of the paraquat sensitivity assay under aerobic and anaerobic conditions are shown in Fig 5B. The Δ*finR* mutant (Δ*finR*::Tn7T) was much more sensitive (10^4-fold) to paraquat (150 μM) under aerobic growth than the parental PAO1 (PAO1::Tn7T), whereas no significant change was observed when the plates were incubated under anaerobic conditions. Thus, the killing effects of paraquat are oxygen-dependent and likely occur by generating superoxide anions. Hence, the observed increased sensitivity to paraquat in the Δ*finR* mutant most likely is a result of superoxide killing. PAO1 produces two superoxide dismutase (Sod) isozymes, namely SodA (manganese-containing Sod) and SodB (iron-containing Sod); mutations of either *sodA* or *sodB* enhance sensitivity to superoxide anions generated from paraquat [48]. We tested whether the paraquat-sensitive phenotype of the *finR* mutant was due to lower level of Sod activity; total Sod activity was measured in the *finR* mutant cultivated aerobically. The results showed non-significant differences in the levels of total Sod activity in the *finR* mutant relative to wild-type PAO1 (data not shown). Therefore, alterations in levels of paraquat resistance of the *finR* mutant are independent of total Sod enzyme activity.

We have shown that *fdx1* could suppress the Δ*fprA* essentiality phenotype. Hence, we tested whether expression of *fprB* or the ferredoxin-encoding genes *fdx1*, *fdx2*, *fdxA* and *rnfB* could complement the paraquat hypersensitivity phenotype of the Δ*finR* mutant, and the results showed that expression of these genes could not complement the *finR* mutant phenotype (data not shown). Here, we have established that FinR positively regulates *fprA* expression, and therefore, we speculate that the paraquat hypersensitive phenotype of the Δ*finR* mutant could arise from loss of the ability to activate *fprA* expression upon exposure to paraquat. Expression of *fprA* under the control of the *lac* promoter in a mini-Tn7 vector was transposed into the Δ*finR* mutant, generating Δ*finR*::Tn7T-*fprA*. The paraquat resistance levels of this strain were evaluated. The results in Fig 5A illustrate that increased expression of *fprA* completely restored

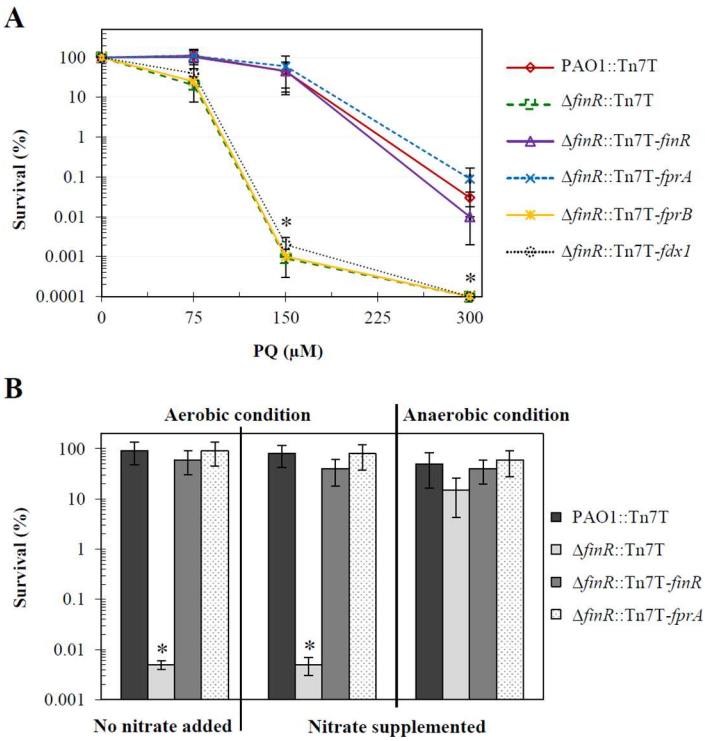

Fig 5. Determination of paraquat resistance levels in *P. aeruginosa* strains. (A) Paraquat resistance levels in PAO1 containing the mnin-Tn7 vector control (PAO1::Tn7T, red) and Δ*finR* mutants containing Tn7T (dotted green), Tn7T-*finR* (purple), Tn7T-*fprA* (dotted blue), Tn7T-*fprB* (yellow), or Tn7T-*fdx1* (dotted black) were determined using plate sensitivity assays. (B) Paraquat (150 μM) resistance levels of *P. aeruginosa* strains were determined using LB with and without 1% (w/v) KNO_3 supplementation and incubated under aerobic and anaerobic atmospheres. The survival is expressed as a percentage of the CFU on LB plates containing paraquat over the CFU on plates without paraquat. Data shown are means ± SD from three independent experiments.

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the paraquat sensitivity of the Δ*finR* mutant to the levels that were attained by the Δ*finR*::Tn7T-*finR* and a wild-type control (PAO1::Tn7T). Since basal levels of *fprA* expression in the Δ*finR* mutant and the parental strain are similar (Fig 3A), the results suggest that the paraquat-hypersensitive phenotype of the Δ*finR* mutant could be due to the inability of the mutant to up-regulate the expression of *fprA* in response to stressful conditions. This suggests that the levels of FprA are critically important, especially under certain stress conditions (i.e., paraquat and NaOCl).

Δ*finR* mutant shows attenuated virulence in a Drosophila host model

FinR positively regulates the expression of an essential gene, *fprA*, in response to oxidative stress; therefore, the contribution of *finR* to the bacterial pathogenicity of *P. aeruginosa* was evaluated using the fruit fly (*Drosophila melanogaster*) as a pathogen-host model as previously described [5, 49]. As shown in Fig 6, feeding the flies with PAO1 cultures resulted in 26.1 ± 3.9% fly survival compared with 99.4 ± 1.0% fly survival when LB medium was fed to the flies as a negative control. Feeding the flies with Δ*finR* mutant cultures resulted in a 2-fold increase in fly survival (54.4 ± 9.2%) compared with feeding with PAO1. Thus, deletion of *finR* attenuated the virulence of *P. aeruginosa* PAO1 in the tested model ($p < 0.01$). The attenuated virulence phenotype of the Δ*finR* mutant could be restored in the complemented mutant strain (Δ*finR*::Tn7T-*finR*), which expressed a functional copy of *finR* (27.8 ± 2.6% fly survival). Additionally, expressing

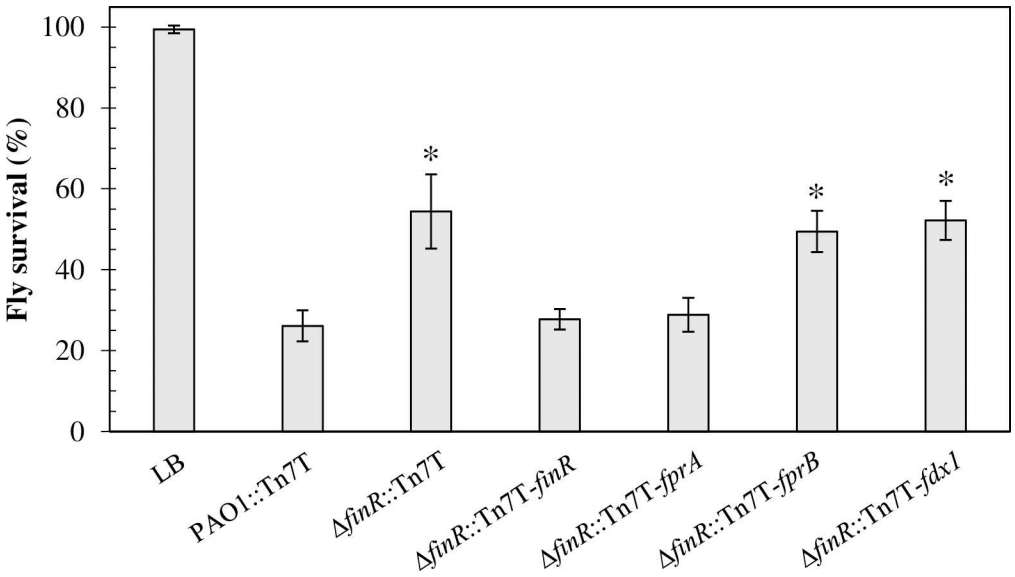

Fig 6. Virulence of *P. aeruginosa* strains. The virulence of PAO1 containing the Tn7T vector control (PAO1::Tn7T) and Δ*finR* mutants containing Tn7T, Tn7T-*finR*, Tn7T-*fprA*, Tn7T-*fprB*, or Tn7T-*fdx1* were determined using the *Drosophila melanogaster* feeding method. The percent fly survival was scored after 18 hours of incubation. Data presented are means ± SD of three independent experiments. The asterisk indicates statistically significant difference ($p < 0.01$) compared with PAO1::Tn7T. LB represents feeding the flies with LB medium.

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fprA could complement the attenuated virulence phenotype of the *finR* mutant (Δ*finR*::Tn7T-*fprA*), as shown by 28.9 ± 4.2% fly survival, while expressing *fprB* (Δ*finR*::Tn7T-*fprB*) could not (49.4 ±5.1% fly survival) (Fig 6). The phenotype of attenuated virulence was consistent with that of paraquat sensitivity levels, in which expression of *fprA* restored the Δ*finR* mutant phenotype (Fig 5A). The facts that increased expression of *fprA* could restore the Δ*finR* mutant phenotype and that basal expression of *fprA* (Fig 4A) in the Δ*finR* mutant was comparable to that of the PAO1 wild type suggested that loss of adaptive expression of *fprA*, which is modulated by FinR, is responsible for the virulence attenuation as well as the paraquat hypersensitivity of the Δ*finR* mutant. We also present here that the paraquat-sensitive phenotype of the Δ*finR* mutant involved superoxide anion-mediated toxicity (Fig 5B). In several plant and animal pathogenic bacteria, defects in superoxide anion detoxification systems, such as knockout of superoxide dismutase genes, render the mutant strains attenuated for virulence in the model hosts [50–52]. Superoxide anions are one of the key components of innate immunity generated by host cells to eradicate invading microbes. In human hosts, superoxide anions are produced within the phagolysosomes of phagocytic cells to kill the engulfed pathogens [53]. Thus, defects in protection against superoxide toxicity of the bacteria would reduce the ability to survive within the host. Hence, the attenuated phenotype could result from the reduced ability of the Δ*finR* mutant to cope with exposure to superoxides during host interactions.

Alternatively, in pseudomonads, FprA plays a role in sulfur metabolism and cysteine biosynthesis, which are important components of [Fe-S] cluster biogenesis [31, 54]. [Fe-S] clusters, which are key cofactors of proteins that are implicated in diverse cellular processes, including respiration and central metabolism, are prone to oxidative damage when cells are exposed to reactive oxygen species (ROS) such as superoxide anions and H_2O_2 [54, 55]. Therefore, impaired [Fe-S] cluster biogenesis during exposure to oxidative stress due to lack of FinR-mediated increased expression of *fprA* would lead to lowered ability of the bacteria to

survive oxidative stress generated by the host's innate immune system. Similar mechanisms could also account for the observed paraquat hypersensitive phenotype. Mutants defective in [Fe-S] cluster biogenesis or repair, for example, deletion of the IscR coding gene, which regulates [Fe-S] cluster biogenesis, show attenuated virulence in host models, including such mutants of *P. aeruginosa* [56–59].

Since *fprA* is essential in PAO1, a direct analysis of the mutant phenotypes is difficult. Analysis of the Δ*finR* mutant provides insight into the importance of *fprA*. Δ*finR* mutant phenotypes (paraquat sensitivity and attenuated virulence) most likely occur as a result of the inability of *fprA* to be up-regulated during stressful conditions. This suggests that the level of FprA is crucial in *P. aeruginosa*. An *fdx1* encoding putative reaction partner of FprA is also an essential gene in PAO1 [40]. Thus, the link between FprA and Fdx1 is important to PAO1 physiology.

Materials and methods

Bacterial strains, plasmids and growth conditions

Both *E. coli* and *P. aeruginosa* (PAO1, ATCC15692) strains were aerobically cultivated in Luria-Bertani (LB) broth (BD Difco, USA) at 37˚C with shaking at 180 rpm unless otherwise stated. To produce synchronous growth, an overnight culture was inoculated into fresh LB medium to give an optical density at 600 nm (OD_{600}) of 0.1. Exponential phase cells (OD_{600} of about 0.6, after 3 h of growth) were used in all experiments. All plasmids used in this study are listed in Table 2.

Molecular techniques

General molecular techniques including DNA and RNA preparations, DNA cloning, PCR amplification, Southern analyses and bacterial transformation were performed according to standard protocols [60]. The oligonucleotide primers used in this study are listed in Table 3.

Construction of *P. aeruginosa* Δ*finR* mutant

The *finR* deletion mutant was constructed using homologous recombination with an unmarked Cre-*loxP* antibiotic marker system as previously described [61]. The primer pairs, EBI73-EBI74 and EBI53-EBI54, were designed to amplify a *finR* fragment containing the C-terminus and N-terminus, respectively, of the *finR* coding region, plus additional flanking regions from the PAO1 genomic DNA. The 1030-bp PCR fragment of the C-terminus was digested with ClaI and SacI and cloned into pUC18Gm^r (pUC18 containing *loxP*-flanked Gm^r, which was constructed by inserting SacI-EcoRI fragments containing *loxP*-flanked Gm^r from pCM351 [61] into pUC18 cut with the same enzymes) at the ClaI and SacI sites, yielding pUC*finR*C::Gm^r. The 926-bp PCR fragment of the N-terminus was digested with EcoRI and NcoI and cloned into pUC*finR*C::Gm^r at the EcoRI and NcoI sites, yielding pUCΔ*finR*::Gm^r. The constructed plasmid resulted in the deletion of 526 bp of the coding region of *finR*. pUCΔ-*finR*::Gm^r was transferred into PAO1, and the putative Δ*finR* mutants that arose from a double crossover event were selected for the Gm^r and Cb^s phenotypes. An unmarked Δ*finR* mutant was created using the Cre-*loxP* system to excise the Gm^r gene as previously described [61], and deletion of *finR* was confirmed by Southern blot analysis.

Construction of the *P. aeruginosa* Δ*fprA* mutant

The *fprA* deletion mutant was constructed using homologous recombination with an unmarked Cre-*loxP* antibiotic marker system using the same protocol as the construction of the Δ*finR* mutant but using primer pairs, EBI75-EBI76 and EBI57-EBI58, which were designed to amplify the *fprA* fragment containing the *fprA* coding region plus additional flanking regions. The

Table 2. List of plasmids used in this study.

Plasmid	Relevant characteristic(s)	Source or Reference
pBBR1MCS-4	Broad-host-range expression vector, Ap^r	[37]
pSS255	Expression vector with a temperature sensitive replicon (mSF^{ts1}), Ap^r	[36]
pTS	pBBR1MCS-4 carrying mSF^{ts1}, Ap^r	This study
pTS-*fprA*	pTS carrying *fprA*	This study
pTS-*fprB*	pTS carrying *fprB*	This study
pTS-*fdx1*	pTS carrying *fdx1*	This study
pTS-*fdx2*	pTS carrying *fdx2*	This study
pTS-*rnfB*	pTS carrying *rnfB*	This study
pTS-*fdxA*	pTS carrying *fdxA*	This study
pUCΔ*finR*::Gm^r	pUC18 containing Gm^r inserted into deleted *finR*, Gm^r	This study
pUCΔ*fprA*::Gm^r	pUC18 containing Gm^r inserted into deleted *fprA*, Gm^r	This study
pCM351	vector containing the *loxP*-Gm^r-*loxP* region, Gm^r	[61]
pCM157	vector containing the Cre-encoding gene, Tet^r	[61]
pUC18-mini-Tn7T::Gm-LAC	mini-Tn7 vector with P_{tac} expression cassette, Gm^r	[34]
pTNS2	Helper plasmid for Tn7 insertion, Ap^r	[34]
pTn-*finR*	pUC18-mini-TN7T::Gm-LAC containing *finR*	This study
pTn-*fprA*	pUC18-mini-TN7T::Gm-LAC containing *fprA*	This study
pTn-*fprB*	pUC18-mini-TN7T::Gm-LAC containing *fprB*	This study
pTn-*fdx1*	pUC18-mini-TN7T::Gm-LAC containing *fdx1*	This study
pTn-*fdx2*	pUC18-mini-TN7T::Gm-LAC containing *fdx2*	This study
pTn-*rnfB*	pUC18-mini-TN7T::Gm-LAC containing *rnfB*	This study
pTn-*fdxA*	pUC18-mini-TN7T::Gm-LAC containing *fdxA*	This study
pP_{fprA}	pUC18 carrying *fprA* promoter	This study
pP_{fprA}MU1	pUC18 carrying mutagenized *fprA* promoter MU1	This study
pP_{fprA}MU2	pUC18 carrying mutagenized *fprA* promoter MU2	This study
pQE30Xa	Vector for expressing N-terminal 6His tagged protein in *E. coli*, Ap^r, Cm^r	Qiagen (Germany)
pQE30Xa-*finR*	pQE30XA carrying full-length *finR*	This study

Gm^r, gentamicin resistance; Ap^r, ampicillin resistance; Tet^r, tetracycline resistance; Cm^r chloramphenicol resistance.

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restriction enzyme sites and plasmids were same as those used in the construction of the Δ*finR* mutant. The obtained plasmid, pUCΔ*fprA*::Gm^r, was used to transform PAO1 wild type and strains containing either an expression plasmid or a temperature-sensitive expression plasmid. PAO1 strains containing an extra copy of various genes (*fdx*, *fdxA*, *fdx2*, *rnfB*, *finR*, *fprA*, *fprB*) was used to test the essentiality of the *fprA* gene and were constructed by transposition of a mini-Tn7 vector containing a target gene into the PAO1 chromosome and the subsequent removal of the Gm^r antibiotic resistance marker gene of mini-Tn7 using the Flp-FRT recombinase as previously described [35].

Construction of plasmid and mini-Tn7 harboring gene coding regions and promoters

To construct pTS, a temperature-sensitive replicon cassette-containing plasmid, a broad-host-range plasmid pBBR1MCS-4 [37] was inserted with a BamHI fragment containing the

Table 3. List of primers used in this study.

Name	Sequence 5'→3'	Purpose
BT2781	GCCCGCACAAGCGGTGGAG	Forward primer for 16S rRNA
BT2782	ACGTCATCCCCACCTTCCT	Reverse primer for 16S rRNA
BT3332	ACGTGCACAACACCGCCC	Forward primer for full-length *finR*
BT3333	CAGGCGGATGTTCAGCGG	Reverse primer for full-length *finR*
BT3334	TAGACGAGGAAGCCTGGATG	Forward primer for *finR* fragment
BT3335	TGTCCCTGGCCAACTGAG	Reverse primer for *finR* fragment
BT3336	GGAGTTCTTCAGCATCAAGG	Forward primer for full-length *fprA*
BT3337	GAAGTACTCGTGTTCCGGCA	Reverse primer for full-length *fprA*
BT3456	GTCTGCTGCTGTTGGTGTG	Forward primer for *fprA* expression
BT3457	GGCAGGGGCTTTCTTCG	Reverse primer for *fprA* expression
BT4443	GTGGCTGTCCGTCGCGGTTG	Forward primer for full-length *fdx1*
BT4444	CAGGCGCCGGCGGGGATCAG	Reverse primer for full-length *fdx1*
BT4479	CCTTGATGCTGAAGAACTCC	Sp2 primer for *fprA*
BT4780	GCAAAATGAATTGTCGTTCGCATGCTTAT	Forward primer for mutated *finR* promoter MU2
BT4781	CTGGATAAGCATGCGAACGACAATTC	Reverse primer for mutated *finR* promoter MU2
BT4782	CTTATCCAGAATAGTTCGCTGGGATAA	Forward primer for mutated *finR* promoter MU1
BT4783	CGTGTTATCCCAGCGAACTATTCTGG	Reverse primer for mutated *finR* promoter MU1
BT3499	GTGCTTTGCGGGACACTAGG	Forward primer for full-length *fprB*
BT3500	GCTATCCGCCGCTACTGC	Reverse primer for full-length *fprB*
BT5019	CCTGGGCGGTGTTGTGCA	Sp1 primer for *finR*
BT5201	GAGGAGAGAACTAGAAAATG	Forward primer for full-length *fdxA*
BT5309	CTTGGCGTATCAGCGCTC	Reverse primer for full-length *fdxA*
EBI01	CATGGGCTTCAGCGGGTTGG	Forward primer for full-length *rnfB*
EBI02	GTGCAGGGCGCTCATGCC	Reverse primer for full-length *rnfB*
EBI53	GGGAATTCGAAGTACTCGTGTTCCGGCA	Forward primer for upstream fragment of *finR*
EBI54	GGCCATGGGAACAGCTTGCAGTCGAACTG	Reverse primer for upstream fragment of *finR*
EBI57	CCGAATTCTCCAGCTCGTAGTGGGCGAC	Forward primer for upstream fragment of *fprA*
EBI58	GGCCATGGTAGTTCGGGCTGGCAATGCTG	Reverse primer for upstream fragment of *fprA*
EBI61	AGCTGAACAGGGTGTCGT	Forward primer for *finR* promoter
EBI62	AGACGCTCTCCTGCTGGG	Reverse primer for *finR* promoter
EBI69	GGGATAACACGATATCGGTCGG	Forward primer for *finR* promoter
EBI70	CGATATCGTGTTATCCCATATCC	Reverse primer for *finR* promoter
EBI73	CCATCGATCGATCAAGCGTGCCGTGGAG	Forward primer for downstream fragment of *finR*
EBI74	CCGGAGCTCTGCTGCTGGGGATCGTCCTG	Reverse primer for downstream fragment of *finR*
EBI75	CCATCGATGGCAAGCTGTTCGAGGACATC	Forward primer for downstream fragment of *fprA*
EBI76	CCGGAGCTCCCTCAGCCAGGGTCACCTGAGC	Reverse primer for downstream fragment of *fprA*
EBI269	GAACTGTCGAGGAATAAGCGAAGATGCC	Forward primer for full-length *fdx2*
EBI270	ATTGCACGCTCCTCTACTAC	Reverse primer for full-length *fdx2*
EBI292	GCGCCTGCAGTCAGGGAATCAGCGGCA	Reverse primer for FinR protein expression
EBI322	ATGAAATTCACCCTCCGC	Forward primer for FinR protein expression

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temperature-sensitive (TS) regulon isolated from vector pTS225 [36] at the BamHI site. pTS-*fprA* was constructed by amplifying the full-length *fprA* from the PAO1 genomic DNA with primers BT3456-BT3457. The 866-bp PCR products were cloned into the pTS cut with SmaI. A similar protocol was used to construct pTS-*fdx1*, pTS-*fdx2*, pTS-*fdxA*, pTS-*rnfB* and pTS-*fprB* for *trans* expression of *fdx1* (PA0362), *fdx2* (PA3809), *fdxA* (PA3621), *rnfB* (PA3490) and *fprB*, respectively. The specific primer pairs for PCR amplification of full-length *fdx1*, *fdx2*,

fdxA, *rnfB*, and *fprB* genes were BT4443-BT4444, EBI269-EBI270, BT5201-BT5309, EBI01-EBI02 and BT3499-BT3500, respectively.

Single-copy complementation was performed using a mini-Tn7 system [34]. The full-length PCR fragments of various genes were PCR amplified with specific primer pairs as described above (and BT3334-BT3335 for *finR*) and cloned into pUC18-mini-Tn7T-Gm-LAC [34], generating *pTn-fprA*, pTn-*fdx1*, pTn-*fdx2*, pTn-*fdxA*, pTn-*rnfB*, pTn-*fprB* and pTn-*finR*. The mini-Tn based recombinant plasmid were then transposed into either PAO1 or mutant strains, generating the complemented strains Δ*finR*::Tn7T-*finR* and Δ*finR*::Tn7T-*fprA*. Confirmation of transposition was carried out as previously described [34].

To construct the plasmids containing the *fprA* promoter region, a putative *fprA* promoter fragment was amplified from the PAO1 genomic DNA with primers EBI61 and EBI62. The 398-bp PCR product was ligated into EcoRV-digested pUC18 and was named pP_{fprA}. PCR-based site-directed mutagenesis at the putative FinR-binding site was performed as previously described [6] using primers BT4782-BT4783 and BT4780-BT4781, and these vectors are referred to as pP_{fprA}-MU1 and pP_{fprA}-MU2, respectively.

5' rapid amplification of cDNA ends (RACE)

5' RACE was performed using a 5'/3' RACE kit (Roche, Germany) as previously described [62]. Essentially, DNase I-treated total RNA was reverse transcribed using specific primers BT3311 and BT3337 as SP1 primers for *finR* and *fprA*, respectively. The first-strand DNA (cDNA) was purified, and poly(A) was added to the 5'-terminus of the cDNA using terminal transferase. Next, poly(A)-tailed cDNA was PCR-amplified using the specific SP2 primer BT4438 for *finR* and BT4479 for *fprA* and an anchored oligo(dT) primer. The purified PCR product was cloned into the pGemT vector, and the +1 site was identified from the DNA sequences.

Real-time RT-PCR

Reverse transcription was performed as described for end-point RT-PCR [63]. Real-time RT-PCR was conducted using 10 ng of cDNA as template, a specific primer pair and a KAPA SYBR® FAST qPCR kit (Kapa Biosystems, USA). The reaction was run on an Applied Biosystems StepOnePlus thermal cycler under the following conditions: denaturation at 95˚C for 20 s, annealing at 60˚C for 30 s, and extension at 60˚C for 30 s, for 40 cycles. The specific primer pairs used for *finR* and *fprA* were BT3334-EBI69 and BT3336-BT3337, respectively. The primer pair for the 16S rRNA gene was BT2781-BT2782, which was used as the normalizing gene. Relative expression analysis was calculated using StepOne software version 2.1 and is presented as expression fold-change relative to the level of PAO1 wild type grown under uninduced conditions. Experiments were repeated independently three times, and the data shown are the means with standard deviations (SD).

Expression and purification of *P. aeruginosa* FinR

The 6His-tagged FinR from *P. aeruginosa* was purified using the pQE-30Xa expression system (Qiagen, Germany). The full-length *finR* gene was amplified from PAO1 genomic DNA with primers EBI322 and EBI292. A 937-bp PCR product was digested with PstI before ligation into pQE-30Xa digested with StuI (blunt ended) and PstI to generate pQE30Xa-*finR* for high-level expression of *finR* containing an N-terminal 6His-tag. An *E. coli* DH5α strain harboring pQE30Xa-*finR* was grown in LB medium containing 100 μg/ml ampicillin at 37˚C to an OD_{600} of 1.0 before being induced with 100 μM IPTG for 60 min. Purification of 6His-tagged FinR was carried out using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column as previously

described [63]. The purity of the FinR protein was more than 90%, as judged by a major band corresponding to the 36.9-kDa protein observed on SDS-PAGE.

Gel mobility shift assay

Gel mobility shift assays were performed using a labeled probe containing either native or mutagenized *fprA*-promoters amplified from pP_{fprA}, pP_{fprA}-MU1, or pP_{fprA}-MU2 as a template and using ^{32}P-labeled BT4691 and BT4692 primers. The promoter fragments (EBI61and EBI62) with and without proposed FinR binding site were amplified from genomic PAO1 using primers, EBI61-EBI70 and EBI62-EBI69, respectively. Binding reactions were conducted using 3 fmol of labeled probe in 25 μl of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.02 mg ml^{-1} bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), 10% (v/v) glycerol, and 200 ng of poly(dI-dC). Various amounts of purified FinR were added, and the reaction mixture was incubated at 25˚C for 20 min. Protein-DNA complexes were separated by electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 4˚C and were visualized by exposure to Hyperfilm (GE Healthcare).

Plate sensitivity assay

A plate sensitivity assay was performed to determine the oxidant resistance level as previously described [63]. Briefly, exponential phase cells were adjusted to OD_{600} of 0.1 before making 10-fold serial dilutions. 10 μl of each dilution was then spotted onto LB agar plate containing appropriate concentrations of testing reagents. The plates were incubated overnight at 37˚C before the colony forming units (CFU) were scored. Percent survival was defined as the CFU on plates containing oxidant divided by the CFU on plates without oxidant and multiply by 100.

Drosophila virulence tests

The virulence of *P. aeruginosa* was investigated using the *Drosophila melanogaster* feeding assay as previously described [5]. Briefly, exponential phase cultures of each *P. aeruginosa* strains were adjusted to an OD_{600} of 0.5 before 800 μL of the bacterial cells were overlaid to completely cover the surface of the preservative-free corn flour *Drosophila* medium at the bottom of a glass fly culture vial. Approximately one-week-old adult flies were starved for 3 h prior to the feeding assay. Twenty flies were added to each vial, and each strain of *P. aeruginosa* was tested for at least three replications. Then, all of the tested flies were incubated at 25˚C for 18 h before the number of the viable flies was observed. The experiments were performed in a double-blind fashion and were analyzed from nine experiments using three different batches of flies.

Statistical analysis

The significance of differences between strains, cultured conditions, or changes of expression level was statistically determined using Student's t-test. $P < 0.05$ is considered significant difference and indicated as an asterisk.

Supporting information

S1 Fig. Multiple amino acid sequence alignment of *P. aeruginosa* FprA and FinR. The deduced amino acid sequence of *P. aeruginosa* (A) FprA ferredoxin NADP(+) reductase A and (B) FinR transcriptional regulator was aligned with those in *Pseudomonas putida* and

Azotobacter vinelandii by using CLASTAL Omega alignment. The asterisk, colon, and period symbols indicate identical residues, conserved substitutions, and semi-conserved substitutions, respectively.
(PDF)

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Project administration: AR PV SM.

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Pseudomonas aeruginosa ttcA encoding tRNA-thiolating protein requires an iron-sulfur cluster to participate in hydrogen peroxide-mediated stress protection and pathogenicity

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ABSTRACT

During the translation process, transfer RNA (tRNA) carries amino acids to ribosomes for protein synthesis. Each codon of mRNA is recognized by a specific tRNA, and enzyme-catalysed modifications to tRNA regulate translation. TtcA is a unique tRNA-thiolating enzyme that requires an iron-sulfur [4Fe-4S] cluster to catalyse thiolation of tRNA. In this study, the physiological functions of a putative *ttcA* in *Pseudomonas aeruginosa*, an opportunistic human pathogen that causes serious problems in hospitals, were characterized. A *P. aeruginosa ttcA*-deleted mutant was constructed, and mutant cells were rendered hypersensitive to oxidative stress, such as hydrogen peroxide (H_2O_2) treatment. Catalase activity was lower in the *ttcA* mutant, suggesting that this gene plays a role in protecting against oxidative stress. Moreover, the *ttcA* mutant demonstrated attenuated virulence in a *Drosophila melanogaster* host model. Site-directed mutagenesis analysis revealed that the conserved cysteine motifs involved in [4Fe-4S] cluster ligation were required for TtcA function. Furthermore, *ttcA* expression increased upon H_2O_2 exposure, implying that enzyme levels are induced under stress conditions. Overall, the data suggest that *P. aeruginosa ttcA* plays a critical role in protecting against oxidative stress via catalase activity and is required for successful bacterial infection of the host.

Introduction

The ability of pathogenic bacteria to successfully invade a host is largely associated with their ability to rapidly adapt to and overcome host immune systems. Reactive oxygen species (ROS) are reactive molecules and free radicals derived from the incomplete reduction of oxygen. ROS are produced as by-products of electron transport during aerobic respiration by phagolysosomes in phagocytic cells, which facilitate attacks on invading microbes[1,2]. ROS also play roles in cellular signalling pathways, including apoptosis, necrosis, gene expression, and the activation of cell signalling cascades[3]. An imbalance between the production and removal of ROS (excess ROS) is referred to as oxidative stress, which causes damage to nucleic acids, lipid peroxidation, protein oxidation, enzyme inhibition, and cofactor inactivation[4]. Accordingly, pathogens have evolved mechanisms to protect themselves against host-generated stresses by scavenging excess ROS with cellular enzymes, such as superoxide dismutase (Sod) and catalase (Kat), and rebuilding and repairing damaged biomolecules, including proteins and cofactors, via the methionine sulfoxide reductase (Msr) and iron-sulfur cluster (Fe-S) biogenesis (Isc) systems, respectively[5-7]. To attain the highest efficiency and execute successful infection, the complex processes underlying bacterial sensing and responses to stress are controlled by specific mechanisms carried out by various transcriptional regulators[8-10]. For example, OxyR, a LysR-type transcriptional regulator, is a global stress response protein involved in hydrogen peroxide (H_2O_2) defence via the activation of genes encoding Kat[11], while SoxR, a [2Fe-2S] cluster-containing transcription factor, triggers a major response to superoxide anions by activating genes encoding Sod[12]. The mechanisms required for adaptive responses to such stresses primarily involve transcriptional controls; however, some bacteria also exhibit adaptive mechanisms for post-transcriptional regulation.

Translational controls in prokaryotes usually involve modifications to tRNA, which is a key molecule for protein synthesis with multiple points of stress-induced regulation[13]. tRNA modifications are catalysed by an enzyme with the potential to influence specific anticodon-codon interactions and regulate translation[14]. A previous study described specific transcripts with particular codon biases encoding stress response proteins that are translationally regulated by dynamic changes in tRNA wobble base modifications[15]. Numerous enzymes have been identified in modification pathways for bacterial tRNAs, such as GidA/MnmE (involved in bacterial

59 virulence in several pathogenic bacteria)[16,17] and TrmJ (functions in the oxidative stress response in
60 *Pseudomonas aeruginosa*)[18]. *Escherichia coli* TtcA, a 2-thiocytidine tRNA biosynthesis protein, catalyses the
61 post-transcriptional thiolation of cytosine 32 as s^2C_{32} in some tRNAs[19]. TtcA contains a redox-active and
62 oxygen-sensitive [4Fe-4S] cluster that is chelated by cysteine residues and is absolutely essential for activity[19].
63 The modified nucleoside s^2C_{32} has thus far been found in tRNAs from organisms belonging to the Archaeal and
64 Bacterial domains[19]. The TtcA protein family is characterized by the presence of both a PP-loop and a Cys-X-X-
65 Cys motif in the central region of the protein but can be divided into two distinct groups based on the presence
66 and location of additional Cys-X-X-Cys motifs in terminal regions of the protein sequence[20,21]. Mutant analysis
67 in *E. coli* showed that both cysteine residues in this central conserved Cys-X-X-Cys motif are required for the
68 formation of s^2C_{32}[19]. The biochemical mechanism of TtcA that catalyses the thiolation of cytosine 32 has been
69 well studied; however, the physiological function of this enzyme has never been reported.

70 *Pseudomonas aeruginosa* is one of the most common opportunistic human pathogens and causes lethal
71 infections in patients with impaired immune systems or in critical condition. Hospital-acquired infections caused
72 by *P. aeruginosa* are increasing with global epidemiology. Expanding our knowledge of the regulatory virulence
73 network in this bacterium will facilitate the identification of potential drug targets. In this study, *P. aeruginosa*
74 *ttcA* encoding TtcA, which contains conserved Cys-X-X-Cys motifs to bind the [4Fe-4S] cluster, was
75 functionally characterized in response to oxidative stress and was found to play a role in the pathogenicity of this
76 bacterium.

77

78 **Results and Discussion**

79 **Identification of *ttcA* in *P. aeruginosa*.**

80 The *P. aeruginosa* PAO1 genome contains the 825-bp open reading frame (ORF) PA1192, annotated as a
81 conserved hypothetical gene encoding a protein with high homology to *E. coli* TtcA, a tRNA 2-thiocytidine
82 biosynthesis protein[22]. *P. aeruginosa* PA1192 has a theoretical molecular mass of 31.3 kDa, and its deduced
83 amino acid sequence shares 67.2% and 66.8% sequence identity with TtcA from *Escherichia coli*[19] and
84 *Salmonella enterica* serovar Typhimurium[21], respectively (Fig. 1A). No paralogous gene of PA1192 in the PAO1

genome was found. The TtcA signature motif (LSGGKDS) in the PP-loop family as well as the iron-sulfur cluster binding domains Cys-X-X-Cys (C115-S-L-C118) and Cys-X-X-Cys (C203-N-L-C206) are conserved in *P. aeruginosa* PA1192 (Fig. 1A). In this study, *P. aeruginosa* PA1192 was annotated as a putative *ttcA* and further noted as *ttcA*.

P. aeruginosa ttcA is located 47 bp upstream of PA1193, a hypothetical protein (Fig. 1B). Analysis of the transcriptional organization of these genes by Northern blotting and RT-PCR using primers located in the *ttcA* and PA1193 genes indicated that they are transcribed separately (data not shown). *ttcA* is arranged 102 bp apart from PA1191, a hypothetical protein partially containing a putative DnaJ-homologous sequence, in the opposite strand (Fig. 1B).

Purified TtcA binds an oxidant-sensitive iron-sulfur cluster.

To detect iron-sulfur cluster-TtcA ligation, *P. aeruginosa* TtcA expression in *Escherichia coli* and TtcA protein purification were performed as described in the Methods. The purified TtcA was then subjected to UV-visible spectroscopy scanning analysis to determine the presence of iron-sulfur clusters. The results in Fig. 2 show significant absorption at 415 nm and 450 nm in the UV-visible spectrum of the purified TtcA, suggesting the presence of a [4Fe-4S] cluster ligated with the protein, similar to the results of previous studies investigating the characteristics of iron-sulfur cluster proteins[23,24]. This finding was supported by an *in silico* analysis of the iron-sulfur cluster binding domains in the *P. aeruginosa* TtcA sequence, which contained two separate Cys-X-X-Cys motifs indicative of [4Fe-4S] cluster ligation.

Oxidative damage occurs when ROS oxidize an exposed Fe^{2+} atom in the [4Fe-4S] cluster through a metal-based oxidation mechanism, resulting in the ejection of an iron atom from the cluster and subsequent reduction of the cluster to the inactive $[3Fe\text{-}4S]^{+}$ oxidation state[25,26]. To determine the effects of H_2O_2 on [4Fe-4S] cluster integrity, purified TtcA was incubated with various concentrations of H_2O_2 prior to performing UV-visible spectroscopy. The results showed decreases in TtcA absorbance at 415 nm and at 450 nm that were H_2O_2 concentration-dependent (Fig. 2), suggesting that ligation of the [4Fe-4S] cluster to TtcA provided targets for H_2O_2-mediated oxidation (5–50 mM), resulting in the destabilization of iron-sulfur clusters bound to the protein. Treatment of the protein with a high concentration (0.5 M) of H_2O_2 led to the total loss of [4Fe-4S] clusters

bound to TtcA, as shown in Fig. 2. Together with the previously described results, we found that *P. aeruginosa* TtcA contains the ROS-sensitive [4Fe-4S] cluster as its cofactor, similar to TtcA in *E. coli*, which contributes to the thiolation of cytosine 32 in tRNA[19]; however, the importance of this cofactor for extended physiological function, particularly under oxidative stress conditions, still needs to be further investigated.

The Δ*ttcA* mutant shows increased susceptibility to H_2O_2 and sodium hypochlorite.

To evaluate the physiological function of the *ttcA* in *P. aeruginosa* PAO1 against oxidative stress, a gene deletion mutant (Δ*ttcA*) was constructed in PAO1, as described in the Methods. Resistance levels against various oxidants, including H_2O_2, an sodium hypochlorite (NaOCl), organic hydroperoxides (cumene hydroperoxide [CHP] and t-butyl hydroperoxide [tBH]), superoxide generators (paraquat [PQ] and menadione [MD]), a thiol-depleting agent (N-ethylmaleimide [NEM]) and an intracellular iron chelating agent 2,2'-dipyridyl (DIPY), were determined using a plate sensitivity assay and were compared to that of wild-type PAO1. There were no significant differences in the resistance levels of the Δ*ttcA* mutant and wild-type PAO1 against organic hydroperoxides, superoxide generators, the thiol-depleting agent and the iron chelator (Fig. 3A). However, the Δ*ttcA* mutant exhibited 50-fold lower resistance to H_2O_2 and an 8-fold reduction in the percent survival against NaOCl compared to PAO1 (Fig. 3A). The sensitive phenotype of the Δ*ttcA* mutant against both H_2O_2 and NaOCl was complemented by the expression of a single copy of *ttcA* in Tn7 site (Fig. 3A), indicating that TtcA plays an important role in the H_2O_2-mediated and NaOCl-derived stress response. In PAO1, the cellular detoxification of H_2O_2 primarily depended on catalase activity levels; however, other mechanisms, such as thiol-peroxidase activity (Tpx) and supporting systems, including haem biosynthesis, were also required to achieve fully responsive functionality against H_2O_2 in *P. aeruginosa*. NaOCl is a bleaching agent that acts as a strong oxidizing agent and can disturb several enzymatic mechanisms, both redox and non-redox, including reactions in tRNA modification processes[27,28]. Moreover, NaOCl has been shown to generate intracellular ROS, which may increase H_2O_2 levels and lead to mutant susceptibility.

Furthermore, PAO1 containing an extra copy of functional *ttcA* did not elevate the levels of resistance against these tested oxidants, suggesting that other components in the tRNA modification process are required or another detoxification system compensates for oxidant sensitivities. In addition to TtcA in *P. aeruginosa* PAO1,

TrmJ, another tRNA-modifying enzyme, has also been shown to function in the oxidative stress response of *P. aeruginosa* PA14[18].

[4Fe-4S] cluster-ligated cysteines are required for the physiological function of TtcA.

To assess the important role of [4Fe-4S] clusters in TtcA-mediated protection against stress conditions, the site-directed mutagenesis of *ttcA* and a complementation assay were performed. Amino acids were changed from cysteine (C) to serine (S) at different positions in the TtcA, including a cysteine next to the PP-loop motif C38; cysteines for Fe-S cluster ligation at C115, C118, C203, and C206; and another conserved cysteine, C184, using pUC18-mini-Tn7T-Gm-*ttcA*; then, mutated genes were transformed and integrated into the chromosome of the Δ*ttcA* mutant. A plate sensitivity assay using lethal concentrations of H_2O_2 and NaOCl was performed to compare the susceptibility of bacterial growth between the transformed Δ*ttcA* mutants. The results in Fig. 3B show that increased susceptibility to H_2O_2 in the Δ*ttcA* mutant was completely restored to wild-type PAO1 levels in Δ*ttcA* mutants containing either the native *ttcA* cassette (WT), the site-directed *ttcA* cassette with C38S, or C184S. However, H_2O_2 susceptibility in the Δ*ttcA* mutant containing the site-directed *ttcA* cassette with either C115S, C118S, C203S or C206S demonstrated similar levels as the Δ*ttcA* mutant (Fig. 3B), indicating no phenotypic restoration among these site-directed mutant strains. Therefore, the four cysteines acting as a putative Fe-S cluster ligand (C115, C118, C203, and C206) were required for fully functional TtcA to play role in the H_2O_2-mediated stress response. Moreover, a similar pattern was obtained with the NaOCl complementation assay, as shown in Fig. 3C, indicating that the site-directed *ttcA* cassette containing the cysteines for Fe-S cluster coordination (either C115S, C118S, C203S or C206S) was unable to restore NaOCl susceptibility of the Δ*ttcA* mutant to wild-type PAO1 levels, resulting in a sensitivity level similar to the Δ*ttcA* mutant. This suggested that these four cysteines for Fe-S cluster ligation were also required for the TtcA functionality in the NaOCl-mediated stress response. Similar observations regarding the importance of this conserved Cys-X-X-Cys motif in the TtcA protein have been reported for the thiolation of the cytidine in position 32 of tRNA in *S.* Typhimurium[21] and in *E. coli*[19].

The Δ*ttcA* mutant exhibits decreased total catalase activity via KatA function.

In several pathogenic bacteria, the cellular detoxification of H_2O_2mainly depends on catalase activity levels. The two major catalases KatA and KatB are responsible for cellular H_2O_2 detoxification in *P. aeruginosa* PAO1[11,29]. To investigate the involvement of TtcA in the H_2O_2-mediated stress response through catalase activity, a total intracellular catalase activity assay was performed in wild-type PAO1 and the Δ*ttcA* mutants. The results showed that total catalase activity in the Δ*ttcA* mutant was 39% and 41% lower than that in wild-type PAO1 under the exponential and stationary phases, respectively, while the Δ*ttcA* mutant harbouring a functional *ttcA* cassette at the Tn7 site showed catalase activity levels similar to that of wild type (Fig. 4A). This result suggested that TtcA is required for full catalase activity in *P. aeruginosa* PAO1 under both the exponential and stationary phases.

To determine whether TtcA has roles in KatA or KatB activity, a catalase gel activity assay was performed. The results in Fig. 4B show that KatA activity in the Δ*ttcA* mutant was decreased compared to that in wild-type PAO1, and activity was restored by the expression of the functional *ttcA* cassette at the Tn7 site, as shown in the complemented strain. However, KatB activity levels were similar for all tested strains. These data support the hypothesis that TtcA might have a direct role in KatA activity. To confirm the contribution of TtcA to KatA activity, a double Δ*katA*Δ*ttcA* mutant was constructed and used to determine H_2O_2 susceptibility levels in a plate sensitivity assay. The results in Fig. 4C show that the Δ*katA* mutant was more than 10^2-fold more sensitive to H_2O_2, while the Δ*ttcA* mutant was approximately 50-fold less sensitive to H_2O_2 compared to the sensitivity of wild-type PAO1. Additionally, the double Δ*katA*Δ*ttcA* mutant exhibited H_2O_2 susceptibility levels similar to the Δ*katA* mutant under a range of H_2O_2 concentrations with differing lethality (Fig. 4C), suggesting that TtcA contributes to KatA activity against H_2O_2 toxicity.

Deletion of *ttcA* causes a change in the expression of genes involved in the oxidative stress response.

To test whether the deletion of *ttcA* contributed to decreased KatA activity either at the transcriptional level or at the post-transcriptional level, expression analysis of *katA* in the Δ*ttcA* mutant compared to wild-type PAO1 was performed using real time RT-PCR analysis. The results in Fig. 5A show that *katA* expression in the Δ*ttcA* mutant was approximately three-fold higher than that in PAO1 under conditions lacking an oxidant, and

increased *katA* expression in the Δ*ttcA* mutant was fully restored to wild-type levels by the extra copy of functional *ttcA* inserted at the Tn7 site. This suggests that decreased KatA activity in the Δ*ttcA* mutant does not result from altered *katA* expression at the transcriptional level; however, it may arise from post-transcriptional control, as it has previously been shown that TtcA has roles in translational control, and decreased KatA activity was observed in this study. To extend our gene expression analysis, the expression profile of genes involved in the oxidative stress response, such as *katB*, *oxyR*, and *tpx*, was determined by performing real time RT-PCR analysis. The results in Fig. 5A show a partial increase in *katB*, *oxyR* and *tpx* expression in the Δ*ttcA* mutant compared to the expression in wild-type PAO1. Furthermore, the expression of *katB* and *tpx* was slightly increased in the Δ*ttcA* mutant under H_2O_2 exposure, but there was no significant difference among these strains under NaOCl treatment (see Supplementary Fig. 1 online). All changes in gene expression in the Δ*ttcA* mutant were restored to wild-type levels by a chromosomal insertion of the extra copy of *ttcA*. This suggested that the Δ*ttcA* mutant caused a defect in H_2O_2 detoxification via KatA-mediated mechanisms, leading to a global change in gene expression, including *katB*, *oxyR* and *tpx* expression, in response to H_2O_2-mediated oxidative stress. This result supports the previous observation in Fig. 4B that KatB activity was slightly increased in the Δ*ttcA* mutant compared to the activity in wild-type PAO1.

Furthermore, to observe the translational efficiency of the *katA* transcript, Western blot analysis was performed using an ectopic 6His-tagged *katA* expression vector on the Δ*katA* mutant background to compare the native *ttcA* (Δ*katA*) and *ttcA* deletion (Δ*katA*Δ*ttcA*) strains. The results shown in Fig. 5B indicate that the relative amounts of 6His-KatA in the *ttcA* deletion mutant (Δ*katA*Δ*ttcA*/p*katA*-6His, 36%) were dramatically lower than those in the native *ttcA* mutant (Δ*katA*/p*katA*-6His, 100%) and were partially restored by the extra copy of *ttcA* under Tn7-mediated expression (Δ*katA*Δ*ttcA*/p*katA*-6His, 67%). These results suggested a defect in the translational efficiency of 6His-tagged *katA* expression in the absence of functional *ttcA* and indicated that *ttcA* plays roles in the oxidative stress response at the post-transcriptional level via KatA activity, and the disruption of functional *ttcA* alters the global expression profile of genes involved in oxidative stress management, including induction of *katB* and expression of *tpx*.

The Δ*ttcA* mutant shows attenuated virulence in a *Drosophila* host model.

The full function of KatA is required for bacterial virulence in several model host systems, as shown in previous studies[30], and TtcA has been shown to respond to oxidative stress via KatA activity; therefore, the contribution of *ttcA* to the bacterial pathogenicity of *P. aeruginosa* was evaluated using *Drosophila melanogaster* as a pathogen-host model. As shown in Fig. 6A, feeding the flies with cultured PAO1 resulted in 50.8 ± 12.5% (after incubation for 12 hours) and 36.0 ± 6.7% (after incubation for 24 hours) fly survival compared with 100 ± 0% (at both time points) fly survival when LB medium was fed to the flies as a negative control. Feeding the flies with Δ*ttcA* mutants resulted in 1.6-fold and 2.1-fold increases in fly survival (81.7 ± 7.4% and 76.7 ± 7.2% after incubation for 12 and 24 hours, respectively) compared with feeding with PAO1 (Fig. 6A). Thus, *ttcA* deletion attenuated the virulence of *P. aeruginosa* PAO1 in the tested model ($p < 0.01$). The attenuated virulence phenotype of the Δ*ttcA* mutant was restored in a Δ*ttcA* mutant expressing a functional copy of *ttcA* (57.2 ± 8.2% and 40.5 ± 6.3% fly survival after incubation for 12 and 24 hours, respectively). The attenuated virulence phenotype was consistent with H_2O_2 sensitivity levels in the Δ*ttcA* mutant (Fig. 3A). In several plant and animal pathogenic bacteria, defects in peroxide detoxification or repair systems, such as knockout of catalase, methionine sulfoxide reductase or iron-sulfur cluster regulator genes, render the mutant strains attenuated for virulence in model hosts[7,30-32]. Hydrogen peroxide is one of the key components of innate immunity generated by host cells to eradicate invading microbes. In human hosts, H_2O_2 is produced within the phagolysosomes of phagocytic cells to kill engulfed pathogens[2]. Thus, defective protection against H_2O_2 toxicity in bacteria would reduce survival within the host. Hence, the attenuated phenotype may result from the reduced ability of the Δ*ttcA* mutant to mitigate exposure to H_2O_2 during host interactions.

Moreover, the iron-sulfur cluster is required to ligate with TtcA to function in tRNA modification and in the response to oxidative stress via catalase activity, as shown in our previous results. To investigate the requirement for iron-sulfur cluster-TtcA ligation in bacterial virulence, complementation with the site-directed *ttcA* mutants was evaluated in a *Drosophila* feeding assay. The results shown in Fig. 6B are similarly to those in Fig. 6A, indicating that feeding the flies with cultured PAO1 either with or without Tn7-mediated insertion of a *ttcA* expression cassette and incubation for 18 hours resulted in approximately 50% fly survival; however, feeding

with Δ*ttcA* mutants resulted in an approximately two-fold increase in fly survival. Feeding with the Δ*ttcA*::Tn-TtcA mutant resulted in a fly survival level similar to that of the PAO1 strain (Fig. 6B). Substitution of iron-sulfur cluster-ligating cysteines, either C115, C118, C203, or C206, with serine in the functional *ttcA* expression cassette and insertion into the Δ*ttcA* mutant chromosome did not restore fly survival levels, while replacing one of the other conserved cysteines (either C38 or C184) in TtcA caused the phenotypic restoration of fly survival to wild-type PAO1 levels (Fig. 6B). This indicated that these iron-sulfur cluster-coordinated cysteines were required for the complete functionality of TtcA in bacterial pathogenicity. Several *P. aeruginosa* genes involved in iron-sulfur cluster biogenesis, including IscR, have been shown to play a role in bacterial virulence, which may correlate with TtcA function in the H_2O_2-mediated oxidative stress response through catalase activity.

ttcA expression is increased in response to H_2O_2 and NaOCl exposure.

Adaptive gene expression is a key component of bacterial defence against environmental stresses. The expression of many genes involved in oxidative stress protection and repair processes is frequently induced by exposure to oxidants[7,33-35]. The expression patterns of *ttcA* in PAO1 cultivated under inducing concentrations of various oxidants were determined using real time RT-PCR. The results illustrated that exposure of PAO1 to organic hydroperoxides, superoxide anion-generating agents, a thiol-chelating agent and an iron-chelating agent did not induce *ttcA* expression (Fig. 7A). By contrast, H_2O_2 and NaOCl treatment of PAO1 highly induced *ttcA* expression by 13.4 ± 1.5-fold and 2.9 ± 1.1-fold, respectively (Fig. 7A). The induction of *ttcA* expression by H_2O_2 and NaOCl treatment correlated with physiological analysis indicating that TtcA contributes to protection against H_2O_2 and NaOCl.

To extend the range of the oxidant-induced gene expression profile, several concentrations of oxidants were applied to bacterial cultures and analysed by real time RT-PCR. The results in Fig. 7B showed that PAO1 cultures were induced with H_2O_2 at concentrations ranging from 0.2 mM to 1 mM and in a dose-dependent manner, which was similar to the gene expression profile obtained for genes in the OxyR regulon, including *katA*, *katB*, *ahpB* and *ahpCF*[11,29]. This hinted at the possibility that *ttcA* expression is regulated by OxyR, the global transcriptional regulator responding to H_2O_2. Moreover, extending the concentration range for NaOCl treatment from 0.001% to 0.02% showed that *ttcA* expression was not significantly altered under 0.005% NaOCl

exposure compared to that in untreated PAO1 (Fig. 7C). This suggested that, unlike H_2O_2 induction, the low concentrations of NaOCl that induced *ttcA* expression may arise from NaOCl reactions generating oxidative stress and probably did not arise via direct NaOCl reactions with the regulator. The NaOCl induction mechanism is under further investigation.

OxyR modulates the expression of *ttcA* to control catalase activity under stress exposure.

To assess whether OxyR regulated the induction of *ttcA* expression upon exposure to oxidative stress, *ttcA* expression levels were examined in an Δ*oxyR* mutant (Δ*oxyR*/pBBR) and a complemented Δ*oxyR*/pBBR-OxyR strain using real time RT-PCR. *oxyR* mutant strains were constructed in PAO1 as described in the Methods. The results showed that under uninduced conditions, the expression of *ttcA* in the Δ*oxyR* mutant was approximately 15-fold higher than *ttcA* levels in PAO1, with $p < 0.05$ (Fig. 8A). H_2O_2 and NaOCl treatments did further not enhance the expression of *ttcA* in the Δ*oxyR* mutant. The expression of *oxyR* from the pBBR1MCS-4 vector in the mutant led to the repression of *ttcA* expression to levels similar to those observed in PAO1 (Fig. 8A). Furthermore, the oxidant-induced expression of *ttcA* expression in the complemented strain was restored to wild-type levels (Fig. 8A). These data strongly suggest that OxyR is a transcriptional repressor of *ttcA* expression. Thus, reduced OxyR likely functions as a transcriptional repressor of *ttcA* expression in the absence of the inducers H_2O_2 and NaOCl. However, due to the presence of oxidants, oxidized OxyR either activates or derepresses *ttcA* expression, leading to upregulated *ttcA* expression, increased catalase activity, and increased resistance to H_2O_2 and NaOCl. OxyR controls a core regulon of oxidative stress defensive genes and other genes involved in the regulation of iron homeostasis, quorum-sensing, protein synthesis and tRNA modification[36,37]. Our results indicate that OxyR is involved in oxidative stress defence through diverse paths of control against H_2O_2 as well as NaOCl.

ttcA promoter analysis was performed and physically mapped *in silico*, and the results are presented in Fig. 8B. *ttcA* is located next to PA1191 with a 102-bp intergenic region. To characterize the *ttcA* promoter, putative +1 sites were investigated using 5' RACE. The +1 site of *ttcA* was mapped to a C residue located 28 bp upstream of its translational ATG start codon (Fig. 8B). Two sequences (GGGCTG and GCGTAAAAT, separated by 18 bp) that resembled the *E. coli* σ^{70} -35 and -10 promoter motifs were identified. Given the limited intergenic space

and a putative promoter sequence analysis, the *ttcA* and PA1191 promoter motifs might overlap with each other. The canonical OxyR promoter recognition sequence was previously proposed to be ATAG-N7-CTAT-N7-ATAG-N7-CTAT[11]. We mapped the *P. aeruginosa ttcA* promoter region and found an upstream sequence (TCGGcgtcgctTTGTgcgtaaaATAGccagcttTTCT) that matched 56% (9 of 16 bases) of the OxyR promoter recognition sequence; therefore, we considered this sequence a putative OxyR binding domain of *ttcA* (Fig. 8B). This putative binding domain overlapped the -10 promoter region, implying an OxyR derepression mechanism for *ttcA* expression in response to oxidative stress. OxyR is a member of the LysR family of transcription regulators, which often use extended palindromic DNA sequences as binding boxes to modulate target gene expression, and diverse consensus sequences for OxyR binding boxes in target gene promoters have been proposed[29]. Direct binding of OxyR and the *ttcA* promoter must be further investigated.

In this study, the physiological role of tRNA modification through Fe-S cluster-ligated TtcA in the pathogenic bacterium *P. aeruginosa* is presented. Under either H_2O_2- or NaOCl-mediated stress, direct transcriptional regulation through *kat* gene expression may not be sufficient to control cellular catalase activity, and translational control through tRNA modification is required. Herein, we proposed an additional model (Fig. 8C) involving OxyR regulation to control catalase activity via both direct transcription and indirect translation of TtcA under oxidative stress conditions. During bacterial infection, *P. aeruginosa* OxyR upregulates *katA*[38] and *ttcA* expression to increase catalase activity in response to H_2O_2 generated via host defence mechanisms. Together with iron-sulfur cluster ligation, TtcA has been shown to play an important role in the oxidative stress response and to facilitate bacterial survival during infection of the host, which emphasizes the critical role of the intracellular function of iron-sulfur cluster biogenesis and tRNA modification via IscR and OxyR regulation to mitigate oxidative stress and promote bacterial pathogenicity.

Methods

Bacterial strains, plasmids and growth conditions.

Both *E. coli* and *P. aeruginosa* (PAO1, ATCC15692) strains were aerobically cultivated in Lysogeny broth (LB from BD Difco, USA) at 37 ºC unless otherwise stated. Exponential phase cells (OD_{600} of about 0.5) were used in all experiments. All plasmids used in this study are listed in Supplementary Table 1 online.

Molecular techniques.

General molecular techniques were performed according to standard protocols[39]. Transformation of plasmids into *P. aeruginosa* strains was carried out using electroporation as previously described[40]. The oligonucleotide primers used in this study are listed in Supplementary Table 2 online.

Construction of *P. aeruginosa* Δ*ttcA* mutants.

The *ttcA* deletion mutant was constructed using homologous recombination with an unmarked Cre-*loxP* system as previously described[41]. A 1271-bp right-flank (RF) PCR product containing the C-terminal of the *ttcA* coding region and a 1093-bp left-flank (LF) PCR product containing the N-terminal was separately amplified from PAO1 genomic DNA using primers EBI1009 and EBI1010 and primers EBI1007 and EBI1008, respectively. The RF fragment was digested with PstI and the 1010-bp RF product fragment was isolated and cloned into pUC18::Gm^{r6} digested with HindIII/blunted and PstI yielding pUC*ttcA*R::Gm^{r}. The LF fragment was digested with NcoI and the 931-bp was isolated and cloned into pUC*ttcA*R::Gm^{r} digested with MunI/blunted and NcoI yielding pUCΔ*ttcA*::Gm^{r}. The constructed plasmid resulted in the deletion of 721 bp of the *ttcA* coding region. pUCΔ*ttcA*::Gm^{r} was transferred into PAO1, and the putative Δ*ttcA*::Gm^{r} mutants were selected for the Gm^{r} and Cb^{s} phenotypes. An unmarked Δ*ttcA* mutant was created using the Cre-*loxP* system to excise the Gm^{r} gene as previously described[41]. To construct the double Δ*katA*Δ*ttcA* mutant, the pUCΔ*ttcA*::Gm^{r} was transferred into Δ*katA* mutant[35] and followed by similar selection and unmarking methods.

Construction of plasmid and mini-Tn7 harbouring *ttcA*-coding regions.

A pBBR-TtcA for ectopic expression of *ttcA* was constructed by amplifying the full-length *ttcA* from the PAO1 genomic DNA with primers BT4673 and BT4674. The 868-bp PCR products were cloned into the medium-copy-number expression vector pBBR1MCS-4[42] cut with SmaI, yielding pBBR-TtcA. Single-copy complementation was performed using a mini-Tn7 system[40]. The full-length *ttcA* were cut from pBBR-TtcA and

cloned into pUC18-mini-Tn7T-Gm-LAC[40] prior to transposing into either PAO1 or mutant strains, generating overexpressed (PAO1::Tn-*ttcA*) or complemented (Δ*ttcA*::Tn-*ttcA*) strains.

Construction of Δ*oxyR* mutant and plasmid harbouring *oxyR*-coding regions.

The *oxyR* deletion mutant was constructed as similar as the *ttcA* deletion mutant construction excepting with primers, BT5910 and BT5911, and a 625-bp deletion site in the *oxyR*-coding region was in between restriction enzymes, XhoI/blunted and SacII. A pBBR-OxyR for ectopic expression of *oxyR* was constructed as similar as pBBR-TtcA construction excepting with primers, EBI1047 and EBI1048.

Site-directed mutagenesis of TtcA.

Site-directed mutagenesis was performed to convert cysteine residues (C38, C115, C118, C184, C203, or C206) to serine residues through PCR-based mutagenesis as previously described[6]. To construct pTn-*ttcA*C38S for the expression of TtcA-C38S, two pairs of primers EBI1011 – TN7S and EBI1012 – BT5250, were used in two-step PCR using pUC18-mini-Tn7T-Gm-*ttcA* as a template. The PCR product was digested with EcoRI and SacI prior to cloning into pUC18-mini-Tn7T-Gm-LAC, generating pTn-*ttcA*C38S. pTn-*ttcA*C115S, pTn-*ttcA*C118S, pTn-*ttcA*C184S, pTn-*ttcA*C203S and pTn-*ttcA*C206S were constructed using the same protocol with different sets of mutagenic primers: EBI1013 and EBI1014 for C115S, EBI1015 and EBI1016 for C118S, EBI1017 and EBI1018 for C184S, EBI1019 and EBI1020 for C203S, and EBI1021 and EBI1022 for C206S. The presence of each mutation was verified by DNA sequencing.

Expression and purification of *P. aeruginosa* TtcA.

6His-tagged TtcA from *P. aeruginosa* was purified using the pQE-30Xa expression system (Qiagen, Germany) as previously described[33]. The full-length *ttcA* gene was amplified from PAO1 genomic DNA with the primers EBI1035 and EBI1036. An 835-bp PCR product was digested with HindIII before ligation into pQE-30Xa digested with StuI/blunted and HindIII to generate pQE30Xa-*ttcA* for the high-level expression of *ttcA* containing an N-terminal 6His-tag. An *E. coli* M15 strain harbouring pQE30Xa-*ttcA* was grown to an OD_{600} of 1.0 before being induced with 100 μM IPTG for 60 min. Purification of 6His-tagged TtcA was carried out using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column as previously described[33]. The purity of the TtcA protein was more than 95%, as judged by a major band corresponding to the 32.3-kDa protein observed on SDS-PAGE.

Plate sensitivity assay.

A plate sensitivity assay was performed to determine the oxidant resistance level as previously described[7]. Briefly, exponential phase cells were adjusted to OD_{600} of 0.1 before making 10-fold serial dilutions. 10 µl of each dilution was then spotted onto LB agar plate containing appropriate concentrations of testing reagents. The plates were incubated overnight at 37 ºC before the colony forming units (CFU) were scored. Percent survival was defined as the percentage of the CFU on plates containing oxidant divided by the CFU on plates without oxidant.

Hydrogen peroxide and NaOCl susceptibility test.

A susceptibility assay was performed to determine the hydrogen peroxide resistance level as previously described[34]. In short, exponential-phase cultures were normalized to an OD_{600} of 0.1 before treating with lethal concentration of either H_2O_2 or NaOCl for 30 min at 37 °C. After treatment, cells were washed twice with fresh LB broth. Cells that survived the treatment were scored using a viable cell count. The resistance levels against H_2O_2 were expressed as the % survival, defined as the percentage of the CFU with treatment divided by the CFU without treatment.

Catalase activity assays.

Total catalase activity in *P. aeruginosa* cells was measured by spectrophotometrically monitoring the decomposition of hydrogen peroxide[6]. Briefly, the reaction was performed by mixing bacterial lysate with 30 mM H_2O_2 in 50 mM phosphate buffer pH 7.0. The absorbance changes at A240 were recorded at time intervals and calculated as the specific activity of catalase (U mg^{-1} protein). One unit of catalase was defined as the amount of enzyme required to hydrolyse 1 µmol of H_2O_2 per min at 25 °C, pH 7.0, and the molar extinction e_{240} was equal to 0.041 cm^2 μmol^{-1}.

The gel activity of Kat was intensely measured from native PAGE of *P. aeruginosa* cell extracts, which were stained for Kat activity as previously described[43]. Thirty milligrams (unheated) of protein were loaded, and protein concentrations were estimated using Bradford assay (Bio-Rad, USA). The stained gel was renatured, and catalase activity was visualized following a previously described method[44] with some modifications by washing twice before soaking with horseradish peroxidase (Sigma, USA) and then removing this enzyme. The gel was

immediately soaked in 5 mM H_2O_2 and stained with 3,3'diaminobenzidine. Catalase activity was visualized as colourless bands against a brownish background.

Western blot analysis.

Western blot analysis was performed as previously described[45]. In brief, crude protein was extracted and isolated before mixing with 6X protein loading dye and boiling for 10 minutes. The stained protein was run under 12.5% SDS-PAGE and transferred to a Hybond PVDF membrane (GE Healthcare) in a semi-dry transfer cell (Bio-Rad). The transferred membrane was blocked and hybridized with anti-6His-peroxidase primary antibody (Roche, Switzerland) and developed with One Step™ Ultra TMB-Blotting Solution (Thermo Scientific) according to the manufacturer's recommendation.

***Drosophila* virulence test.**

The virulence of *P. aeruginosa* was investigated using the *Drosophila melanogaster* feeding assay as previously described[7]. Shortly, 800 µL of exponential phase *P. aeruginosa* cultures were overlaid to completely cover the surface of the preservative-free corn flour *Drosophila* medium in a glass fly culture vial. One-week-old adult flies were starved for 3 hours prior to the feeding assay. Twenty flies were added to each vial and incubated at 25 °C before the number of the viable flies was observed at different time points. The experiments were performed in a double-blind fashion and were analyzed from nine experiments using three different batches of flies.

Real time RT-PCR.

RNA extraction and reverse transcription was performed as previously mentioned[6,34]. Real time RT-PCR was conducted using a SYBR® FAST qPCR kit (KAPA Biosystems, USA). The reaction was run on an Applied Biosystems StepOnePlus thermal cycler under the recommended fast protocol condition. The specific primer pairs used for *ttcA*, *katA*, *katB*, *oxyR*, and *tpx* were BT4675-BT4676, BT797-BT798, BT799-BT800, EBI163-EBI164 and BT3186-BT3787, respectively[34]. The primer pair for the 16S rRNA gene was BT2781-BT2782, which was used as the normalizing gene. Relative expression analysis was calculated using StepOne software and is presented as expression fold-change relative to the level of uninduced conditions. Data shown are the means with standard deviations (SD) from three biologically independent experiments.

5' rapid amplification of cDNA ends (RACE).

5' RACE was performed using a 5'/3' RACE kit (Roche, Switzerland) as previously described[33]. Essentially, DNase I-treated total RNA was reverse transcribed using specific primers EBI341 as SP1 primers. The first-strand DNA (cDNA) was purified, and poly(A) was added to the 5'-terminus of the cDNA using terminal transferase. Next, poly(A)-tailed cDNA was PCR-amplified using the specific SP2 primer BT4991 and an anchored oligo(dT) primer. The purified PCR product was cloned into the pGEM-T Easy vector, and the +1 site was identified from the DNA sequences.

Statistics.

Group data are presented as means ± standard deviation (SD). The Student t-test was used to determine differences between means using the function of Excel (Microsoft, Washington) and the SPSS (version 17.0; SPSS Inc.) statistical package. Unless otherwise is stated, p values of < 0.05 were considered significant.

Ethics statement.

All *P. aeruginosa* and *D. melanogaster* were raised, maintained and all experiments were conducted following procedures, MUSC2016-002 and MUSC60-039-389, approved by the Committee of Biosafety, Faculty of Science, Mahidol University (MUSC) and the MUSC-Institutional Animal Care and Use Committee (IACUC), respectively.

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Author Contributions

Conceived and designed the experiments by AR. Performed the experiments by AR, JD, KK, LW and KS. Analyzed the data by AR, MF and PV. Contributed reagents/materials/analysis tools by AR and SM. Wrote the paper by AR and SM. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

562 **Figure legends**

563 **Figure 1. Multiple alignment of *P. aeruginosa* TtcA and gene organization around *ttcA*.**

564 (A) Alignment of TtcA from *P. aeruginosa* with TtcA sequences from *Escherichia coli* and *Salmonella enterica*
565 serovar Typhimurium. The alignments were performed using the CLUSTALW algorithm. Underlined and bold
566 letters indicate the amino acids responsible for the PP-loop motif and conserved cysteines in TtcA, respectively.
567 The asterisk, colon, and period symbols indicate identical residues, conserved substitutions, and semi-conserved
568 substitutions, respectively. The numbers on top of the alignments indicate the positions of the amino acids. (B)
569 Gene organization of *ttcA* in the *P. aeruginosa* PAO1 genome.

570 **Figure 2. Characterization of iron-sulfur cluster-ligating TtcA in *P. aeruginosa*.**

571 UV-visible absorption spectra of 10 mM purified TtcA protein treated with the indicated concentrations of H_2O_2
572 in 50 mM phosphate buffer were used in the experiments. BSA (10 mM) was used as the non-[Fe-S] protein
573 control.

574 **Figure 3. Determination of oxidant resistance levels in *P. aeruginosa* strains.**

575 (A) Oxidant resistance levels in PAO1 and Δ*ttcA* mutants containing the Tn7 insertion in either Tn or Tn-TtcA
576 were determined using plate sensitivity assays. Resistance levels against H_2O_2 (0.5 mM), NaOCl (0.05%),
577 cumene hydroperoxide (CHP, 1.8 M), tert-butyl hydroperoxide (tBH, 1.2 mM), paraquat (PQ, 200 μM),
578 menadione (MD, 4 mM), N-ethylmaleimide (NEM, 0.35 mM) and 2,2'dipyridyl (DIPY, 1.2 mM) were
579 determined using a plate sensitivity assay. (B) and (C) show the H_2O_2 and NaOCl resistance levels (at the
580 indicated killing concentrations), respectively, of *P. aeruginosa* PAO1 and Δ*ttcA* mutants with the Tn7 insertion
581 containing either Tn, Tn-TtcA (WT), or site-directed mutagenic cysteines (C38S, C115S, C118S, C184S,
582 C203S, and C206S), determined using a bacterial killing assay. All data shown are the mean and standard
583 deviation (SD) of the percent survival after incubation for 18 hours from three independent experiments. The
584 asterisk indicates statistical significance (paired t-test, $p < 0.05$) compared with PAO1::Tn treated under the

same condition. The normal and bold strains indicate statistically significant group differences in resistance levels ($p < 0.05$).

Figure 4. Catalase activity in *P. aeruginosa* strains.

(A) Total intracellular catalase activity was determined in both exponential and stationary cultures of *P. aeruginosa* strains. The data shown are the mean and SD of catalase specific activities in each strain from three independent experiments. The asterisk indicates statistical significance (paired t-test, $p < 0.05$) compared with PAO1::Tn at the same growth phase. (B) KatA and KatB catalase gel activities were investigated among *P. aeruginosa* strains. The band intensity of each tested strain was calculated as the relative intensity (fold change) compared to that of PAO1::Tn, with an asterisk indicating statistical significance (paired t-test, $p < 0.05$). The full-length gel was shown in the Supplementary Fig 2 online. (C) H_2O_2 resistance levels in PAO1, Δ*katA*, Δ*ttcA* and the Δ*katA*Δ*ttcA* double mutant were determined using plate sensitivity assays and are shown as the mean and SD of the percent survival from three independent experiments.

Figure 5. Transcriptional and translational analysis of oxidative stress responsive genes.

(A) Expression levels of *katA*, *katB*, *oxyR*, and *tpx* in wild-type PAO1 and Δ*ttcA* mutants containing either Tn or Tn-TtcA insertions were determined using real time RT-PCR. Bacterial cultures were grown to the exponential cell phase prior to RNA extraction. Relative expression was analysed using the 16S rRNA gene as the normalizing gene and expressed as the fold expression relative to wild-type PAO1 levels. The data shown are the mean and SD from three biologically independent experiments. The asterisks indicate statistically significant differences ($p < 0.01$) compared with PAO1 levels. (B) Western blot analysis of 6His-KatA levels in *P. aeruginosa* strains was determined using a mouse anti-6His antibody. Crude proteins were prepared from an equal amount of *P. aeruginosa* culture, and electrophoresis was carried out using 12.5% SDS–PAGE with protein markers. The full-length blot was shown in the Supplementary Fig 3 online.

Figure 6. Virulence of *P. aeruginosa* strains.

(A) Virulence of PAO1 and Δ*ttcA* mutants containing the Tn7 insertion in either Tn or Tn-TtcA was determined using the *Drosophila melanogaster* feeding method. The percent fly survival was scored at indicated time points of infection after co-incubation. (B) The virulence of PAO1 and Δ*ttcA* mutants containing the Tn7 insertion in either Tn (control), Tn-TtcA (WT), or site-directed mutagenic cysteines in Tn-TtcA (C38S, C115S, C118S, C184S, C203S, and C206S) was determined in the *D. melanogaster* feeding assay, and the percent fly survival was scored after co-incubation for 18 hours. The data presented are the mean of three independent experiments, and the error bars in all graphs represent the SD of the mean. Differences in all graphs were statistically evaluated and found to be significant ($p < 0.05$).

Figure 7. Expression analysis of *ttcA* in *P. aeruginosa* strains.

(A) Expression levels of *ttcA* under oxidant exposure were determined using real time RT-PCR. Cultures of *P. aeruginosa* PAO1 were subjected to various stress conditions, including 0.5 mM H_2O_2, 0.02% NaOCl, 0.5 mM cumene hydroperoxide (CHP), 0.5 mM *t*-butyl hydroperoxide (tBH), 0.5 mM plumbagin (PB), 0.5 mM paraquat (PQ), 0.5 mM menadione (MD), 0.1 mM N-ethylmaleimide (NEM) or 1 mM 2,2'-dipyridyl (DIPY) for 15 minutes prior to RNA preparation for real time RT-PCR analysis. Expression levels of *ttcA* in PAO1 under the indicated concentrations of H_2O_2 (B) and NaOCl (C) were determined as in previous experiments. Relative expression and data interpretation were performed as described in previous experiments. The asterisks indicate statistically significant differences ($p < 0.01$) compared with uninduced conditions.

Figure 8. OxyR-dependent *ttcA* expression and promoter analysis.

(A) Expression levels of *ttcA* in wild-type PAO1 (PAO1/pBBR), the Δ*oxyR* mutant (Δ*oxyR*/pBBR) and the complemented mutant (Δ*oxyR*/pOxyR) grown under uninduced, 0.5 mM H_2O_2, or 0.02% NaOCl induced conditions were investigated using real time RT-PCR and analysed as described in previous experiments. (B) Nucleotide sequence showing the *ttcA* promoter structure. The putative −10 and −35 promoter elements are indicated as underlined text, and the +1 transcription start site (obtained from the 5' rapid amplification of cDNA ends (RACE) results) and the ATG translation start site are bolded. The box shaded grey represents the putative OxyR binding site from computational analysis. (C) Proposed model of OxyR-regulated *katA* and *ttcA*

expression under oxidative stress conditions. *P. aeruginosa* OxyR upregulates *katA* and *ttcA* expression to increase catalase activities in response to H_2O_2generated by host defence mechanisms. The iron-sulfur cluster is required for fully functional TtcA activity to have a role in the oxidative stress response via KatA activity and facilitates bacterial survival during infection.

A

```
                                                    38
PAE   -------MGTLSVNQNKLQKRLRRLAGEAITDFNMIEDGDKVMVCLSGGKDSYTMLDILL
ECO   MQENQQITKKEQYNLNKLQKRLRRNVGEAIADFNMIEEGDRIMVCLSGGKDSYTMLEILR
STM   MQEIQKNTKKEQYNLNKLQKRLRRNVGEAIADFNMIEEGDRIMVCLSGGKDSYTMLEILR
               . . * ********* .****:******:**::**************:**

PAE   YLQKVAPIRFEIVAVNMDQKQPGFPEHVLPEYLKSIGVEYHIVEKDTYSVVKEKIPEGKT
ECO   NLQQSAPINFSLVAVNLDQKQPGFPEHVLPEYLEKLGVEYKIVEENTYGIVKEKIPEGKT
STM   NLQQSAPINFSLVAVNLDQKQPGFPEHILPAYLEQLGVEYKIVEENTYGIVKEKIPEGKT
       **: ***.*.:****:**********:** **:.:****:***::**.:**********

      115 118
PAE   TCSLCSRLRRGTLYTFADEIGATKMALGHHRDDILETFFLNMFYGGTLKAMPPKLLADDG
ECO   TCSLCSRLRRGILYRTATELGATKIALGHHRDDILQTLFLNMFYGGKMKGMPPKLMSDDG
STM   TCSLCSRLRRGILYRTATELGATKIALGHHRDDILQTLFLNMFYGGKMKGMPPKLMSDDG
      ********** ** * *:****:**********:*:********.:*.*****::***

               184               203 206
PAE   RNVVIRPLAYCSEKDIEAYSQLKEFPIIPCNLCGSQENLQRQVVKEMLLEWERKSPGRTE
ECO   KHIVIRPLAYCREKDIQRFADAKAFPIIPCNLCGSQPNLQRQVIADMLRDWDKRYPGRIE
STM   KHIVIRPLAYCREKDIIRFAEAKAFPIIPCNLCGSQPNLQRQVIADMLRDWDKRYPGRIE
      :::******** **** ::: * ************ ******: :** :*::: *** *

PAE   IMFRALQNVVPSQLADRNLFDFANLRIDENATPRFLDVMNL------------------
ECO   TMFSAMQNVVPSHLCDTNLFDFKGITHGSEVVNGGDLAFDREEIPLQPACWQPEEDENQL
STM   TMFSAMQNVVPSHLCDTNLFDFKGITHGSEVVDGGDLAFDREEIPLQPAGWQPEEDDTAL
       ** *:******:*.* ***** .: ..:.. .::

PAE   ----------- (100%)
ECO   DELRLNVVEVK (67.2%)
STM   EALRLDVIEVK (66.8%)
```

B

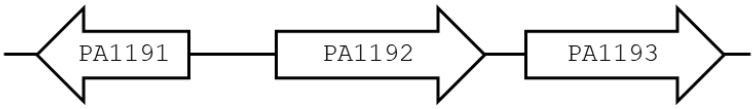
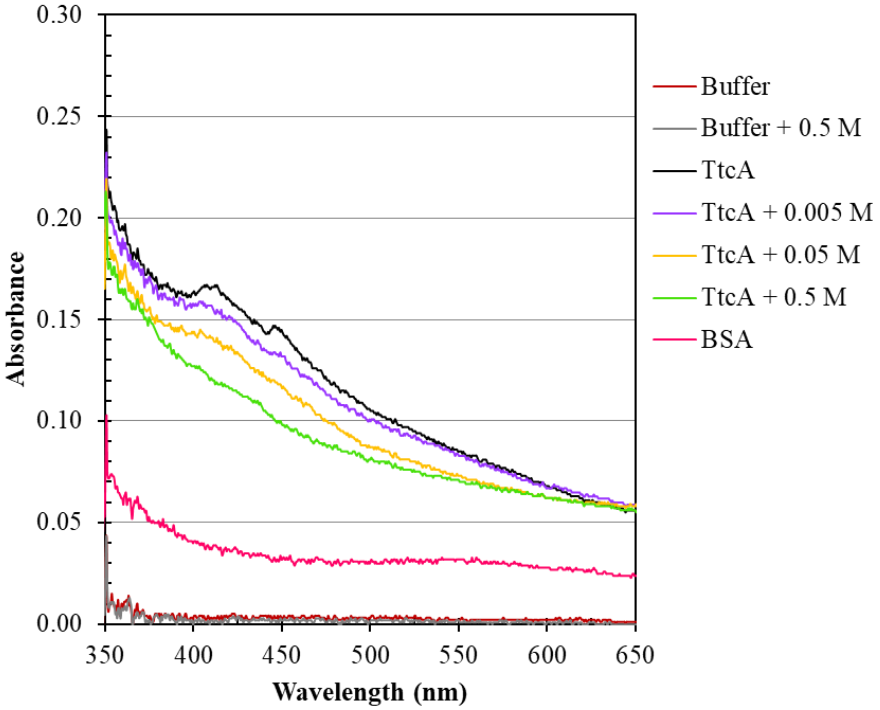


Fig. 1

637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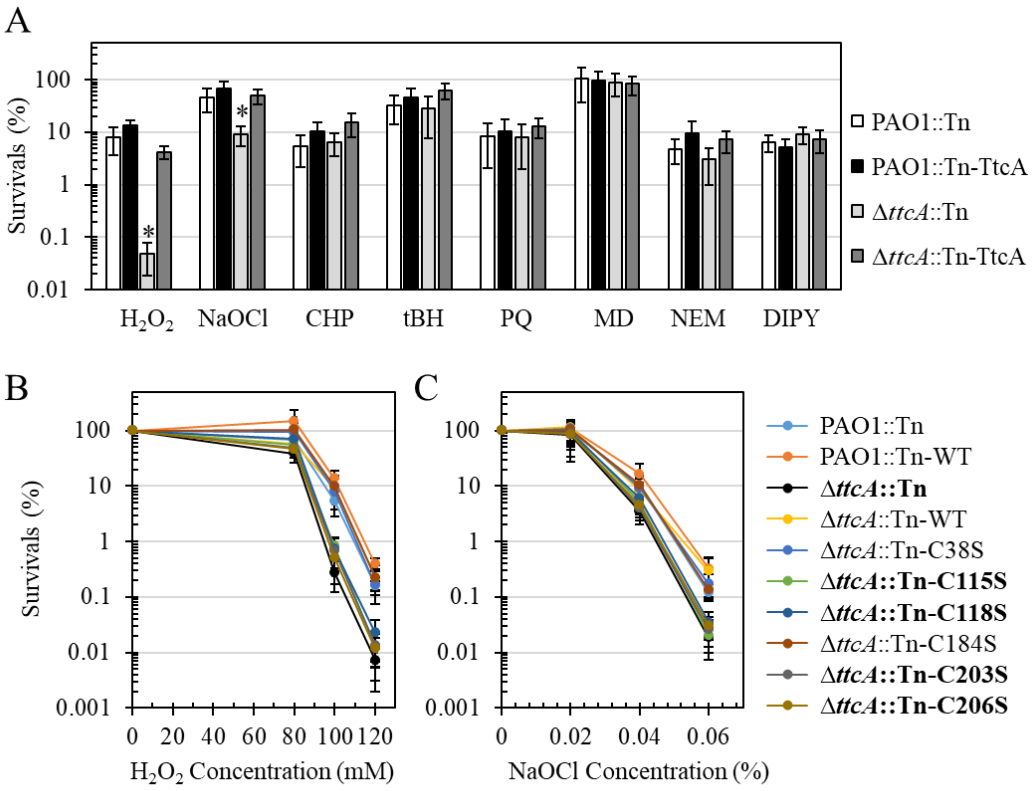
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
350
400
450
500
550
600
650
Wavelength (nm)
Buffer
Buffer + 0.5 M
TtcA
TtcA + 0.005 M
TtcA + 0.05 M
TtcA + 0.5 M
BSA

Fig. 2

638


A
Survivals (%)
100
10
1
0.1
0.01
*
*
H2O2
NaOCl
CHP
tBH
PQ
MD
NEM
DIPY
PAO1::Tn
PAO1::Tn-TtcA
ΔttcA::Tn
ΔttcA::Tn-TtcA
B
Survivals (%)
100
10
1
0.1
0.01
0.001
0 20 40 60 80 100 120
H2O2 Concentration (mM)
C
100
10
1
0.1
0.01
0.001
0 0.02 0.04 0.06
NaOCl Concentration (%)
PAO1::Tn
PAO1::Tn-WT
ΔttcA::Tn
ΔttcA::Tn-WT
ΔttcA::Tn-C38S
ΔttcA::Tn-C115S
ΔttcA::Tn-C118S
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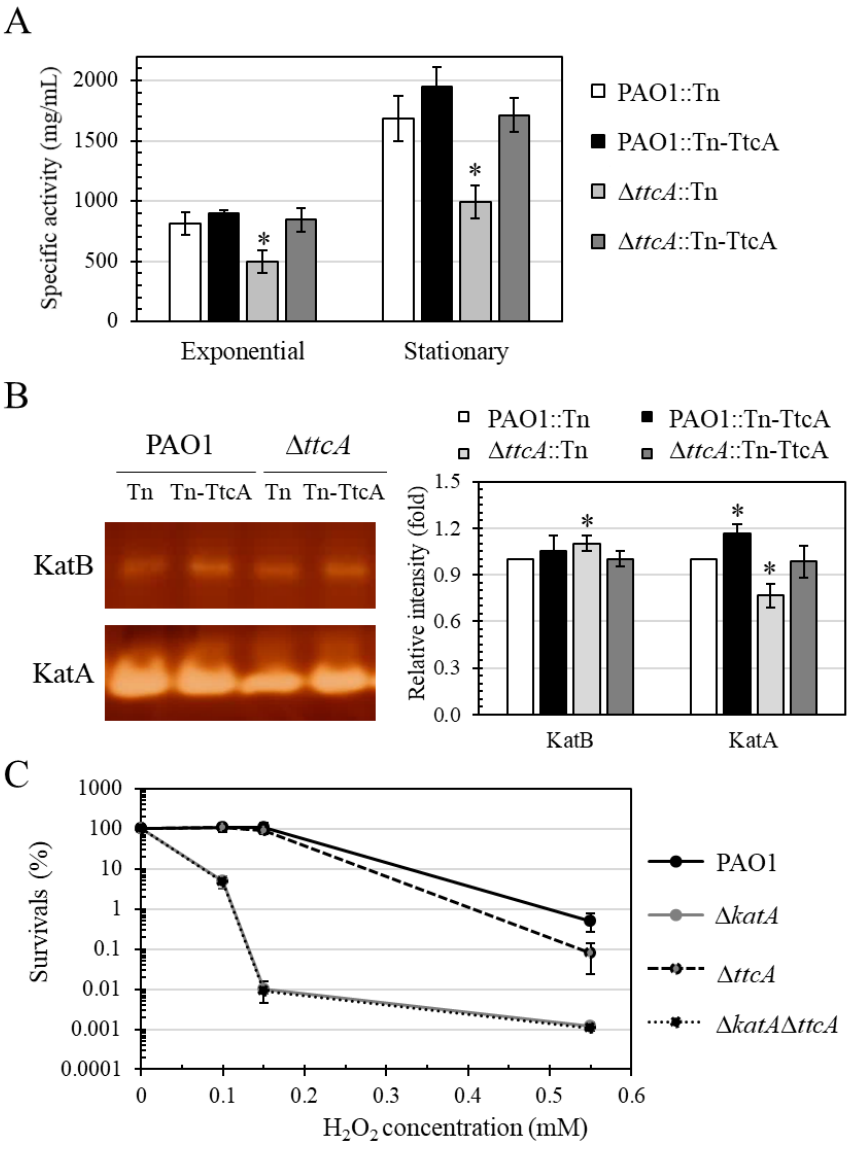
ΔttcA::Tn-C206S

Fig. 3

639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Specific activity (mg/mL)
2000
1500
1000
500
0
Exponential
Stationary
PAO1::Tn
PAO1::Tn-TtcA
ΔttcA::Tn
ΔttcA::Tn-TtcA
B
PAO1
ΔttcA
Tn
Tn-TtcA
Tn
Tn-TtcA
KatB
KatA
Relative intensity (fold)
1.5
1.2
0.9
0.6
0.3
0.0
KatB
KatA
C
Survivals (%)
1000
100
10
1
0.1
0.01
0.001
0.0001
0
0.1
0.2
0.3
0.4
0.5
0.6
H2O2 concentration (mM)
PAO1
ΔkatA
ΔttcA
ΔkatAΔttcA

Fig. 4

640

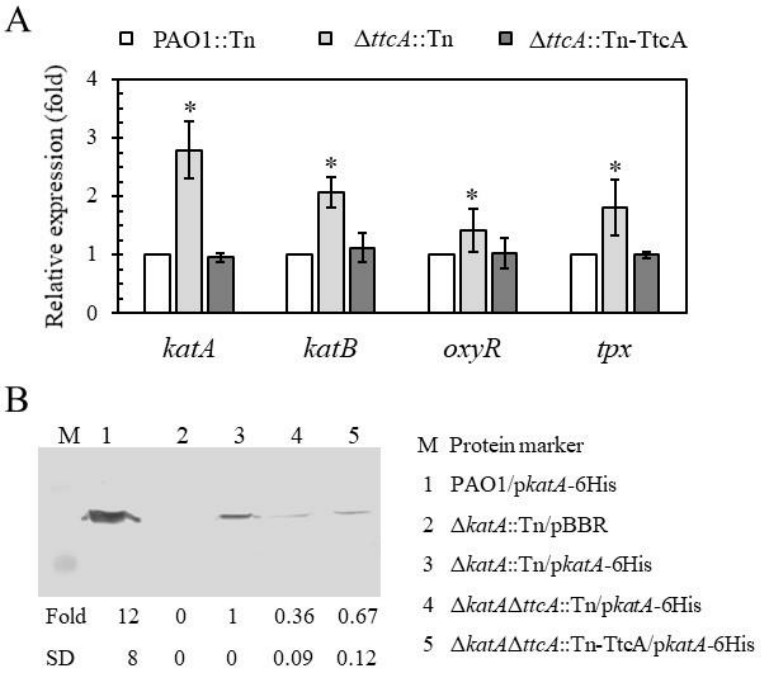
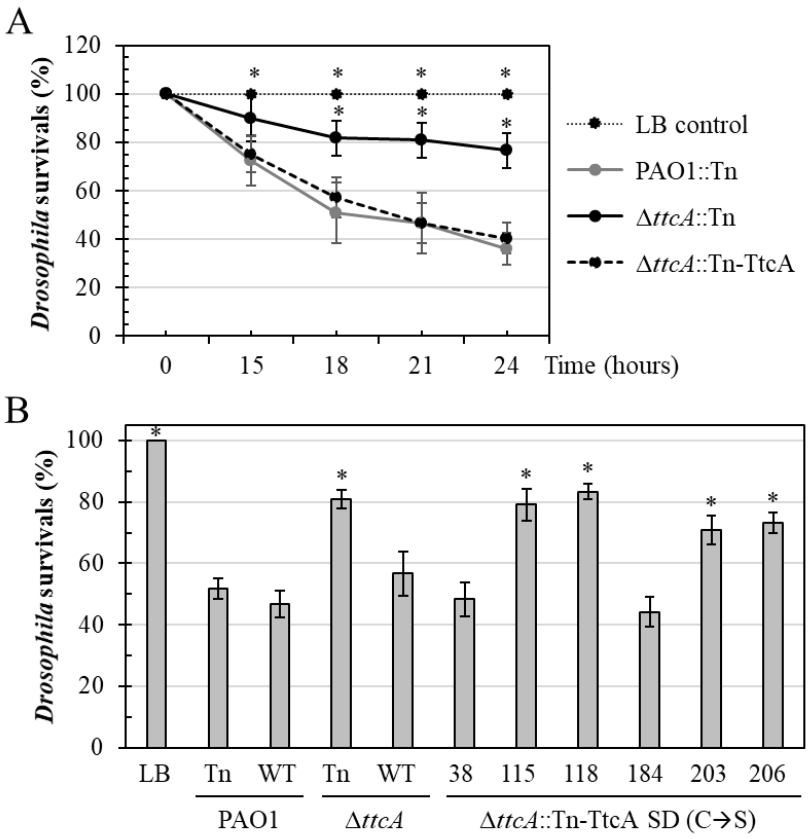

A
PAO1::Tn
ΔttcA::Tn
ΔttcA::Tn-TtcA
Relative expression (fold)
0
1
2
3
4
*
katA
katB
oxyR
tpx
B
M 1 2 3 4 5
Fold 12 0 1 0.36 0.67
SD 8 0 0 0.09 0.12
M Protein marker
1 PAO1/pkatA-6His
2 ΔkatA::Tn/pBBR
3 ΔkatA::Tn/pkatA-6His
4 ΔkatAΔttcA::Tn/pkatA-6His
5 ΔkatAΔttcA::Tn-TtcA/pkatA-6His

Fig. 5

641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Drosophila survivals (%)
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100
80
60
40
20
0
0
15
18
21
24
Time (hours)
LB control
PAO1::Tn
ΔttcA::Tn
ΔttcA::Tn-TtcA
B
Drosophila survivals (%)
100
80
60
40
20
0
LB
Tn
WT
Tn
WT
38
115
118
184
203
206
PAO1
ΔttcA
ΔttcA::Tn-TtcA SD (C→S)

Fig. 6

642

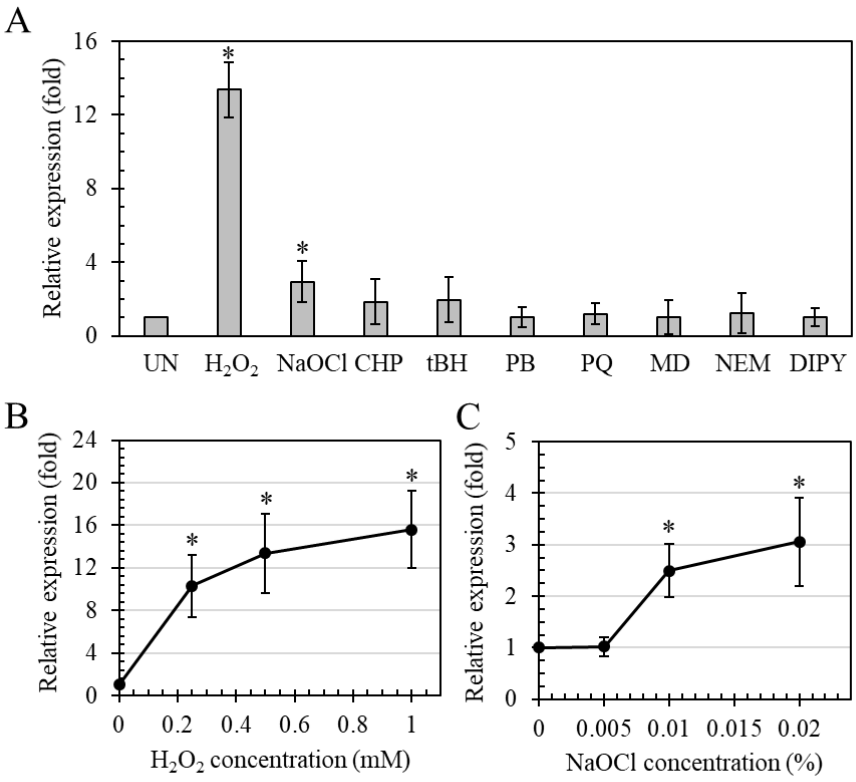
A
Relative expression (fold)
16
12
8
4
0
*
*
UN
H2O2
NaOCl
CHP
tBH
PB
PQ
MD
NEM
DIPY
B
Relative expression (fold)
24
20
16
12
8
4
0
*
*
*
0
0.2
0.4
0.6
0.8
1
H2O2 concentration (mM)
C
Relative expression (fold)
5
4
3
2
1
0
*
*
0
0.005
0.01
0.015
0.02
NaOCl concentration (%)

Fig. 7

643

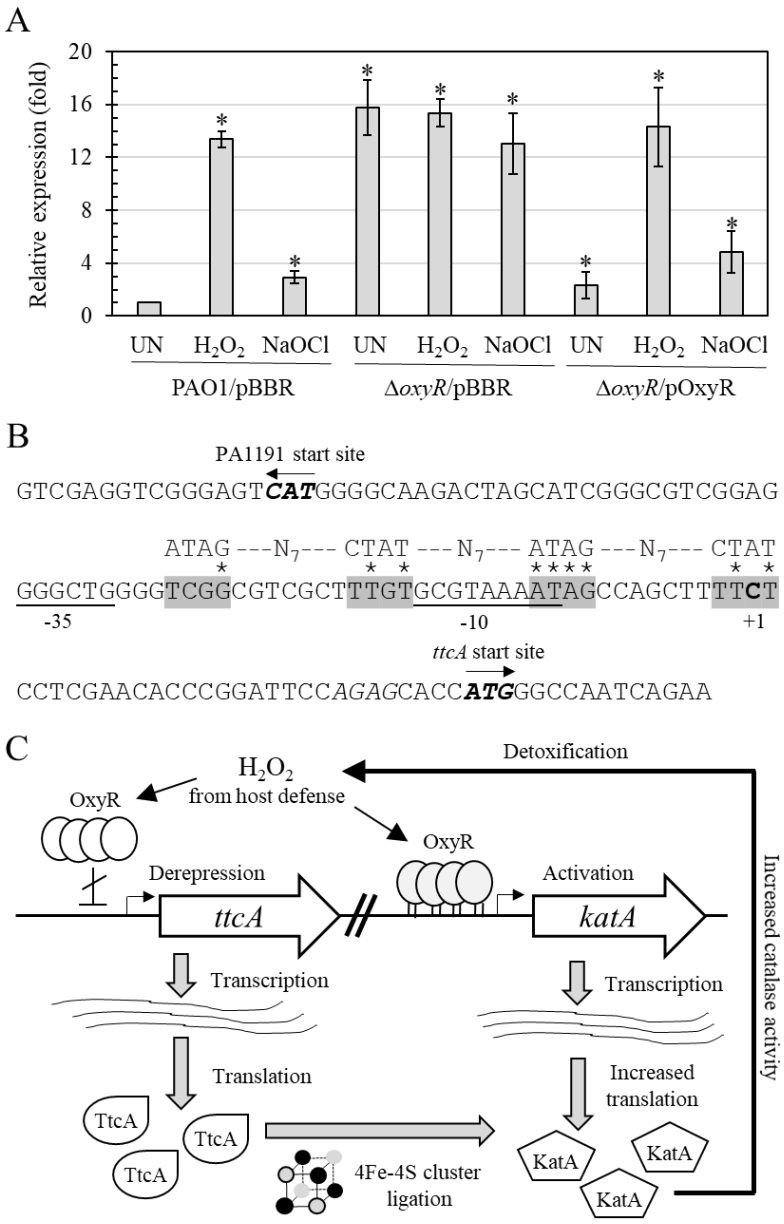
A
Relative expression (fold)
20
16
12
8
4
0
UN
H2O2
NaOCl
UN
H2O2
NaOCl
UN
H2O2
NaOCl
PAO1/pBBR
ΔoxyR/pBBR
ΔoxyR/pOxyR
B
PA1191 start site
GTCGAGGTCGGGAGTCATGGGGCAAGACTAGCATCGGGCGTCGGAG
ATAG ---N7--- CTAT ---N7--- ATAG ---N7--- CTAT
GGGCTGGGGTCGGCGTCGCTTTGTGCGTAAAATAGCCAGCTTTTCT
-35
-10
+1
ttcA start site
CCTCGAACACCCGGATTCCAGAGCACCATGGGCCAATCAGAA
C
H2O2
from host defense
Detoxification
OxyR
OxyR
Derepression
Activation
ttcA
katA
Transcription
Transcription
Translation
Increased
translation
TtcA
TtcA
TtcA
4Fe-4S cluster
ligation
KatA
KatA
KatA
Increased catalase activity

Fig. 8

644

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Pseudomonas aeruginosa glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation

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Abstract:	Pseudomonas aeruginosa PAO1 contains gshA and gshB genes, which encode enzymes involved in glutathione biosynthesis. Challenging P. aeruginosa with hydrogen peroxide (H2O2), cumene hydroperoxide (CHP) and t-butyl hydroperoxide (tBH) increased the expression of gshA and gshB. The physiological roles of these genes in P. aeruginosa oxidative stress, bacterial virulence and biofilm formation were examined using P. aeruginosa ΔgshA, ΔgshB, and double ΔgshAΔgshB mutant strains. These mutants exhibited significantly increased susceptibility to methyl viologen, thiol-depleting agent, and methylglyoxal compared to PAO1. Expression of functional gshA, gshB or exogenous supplementation with GSH complemented these phenotypes, which indicates that the observed mutant phenotypes arose from their inability to produce GSH. Virulence assays using a Drosophila melanogaster model revealed that the ΔgshA, ΔgshB and double ΔgshAΔgshB mutants exhibited attenuated virulence phenotypes. An analysis of virulence factors, including pyocyanin, pyoverdine, and cell motility (swimming and twitching), showed that these levels were reduced in these gsh mutants compared to PAO1. In contrast, biofilm formation increased in mutants. These data indicate that the GSH product and the genes responsible for GSH synthesis play multiple crucial roles in oxidative stress protection, bacterial virulence and biofilm formation in P. aeruginosa.
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If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
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Additional data availability information:	

Editor
PLOS ONE

5-04-18

Dear Editor

Enclosed with this letter is a manuscript entitled "*Pseudomonas aeruginosa* glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation" submitted as a "*research article*" to the PLOS ONE. The manuscript described the physiological role of *gshA* and *gshB*, genes that are responsible for GSH biosynthesis in *P. aeruginosa*. We found that the inactivation of *gshA* and *gshB* genes increased the susceptibility to oxidative stress-generating agents and attenuated virulence due to defects in pigment production, siderophore, and motility. GSH biosynthesis controlled biofilm formation. The data demonstrated that GSH was not essential but played centrally important roles in various physiological processes that were important to survival in the diverse environmental conditions that *P. aeruginosa* encountered.

We also attached the related manuscript entitled "Disruption of glutathione biosynthesis pathway alters antibiotic susceptibility levels of *Pseudomonas aeruginosa*" that has been submitted for consideration to published in the Journal of Antimicrobial Chemotherapy for your information.

If you need more information on any topics please let me know.

Looking forward to hearing from you.

Yours sincerely,

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Pseudomonas aeruginosa glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation

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Abstract

Pseudomonas aeruginosa PAO1 contains *gshA* and *gshB* genes, which encode enzymes involved in glutathione biosynthesis. Challenging *P. aeruginosa* with hydrogen peroxide (H_2O_2), cumene hydroperoxide (CHP) and *t*-butyl hydroperoxide (tBH) increased the expression of *gshA* and *gshB*. The physiological roles of these genes in *P. aeruginosa* oxidative stress, bacterial virulence and biofilm formation were examined using *P. aeruginosa* Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutant strains. These mutants exhibited significantly increased susceptibility to methyl viologen, thiol-depleting agent, and methylglyoxal compared to PAO1. Expression of functional *gshA, gshB* or exogenous supplementation with GSH complemented these phenotypes, which indicates that the observed mutant phenotypes arose from their inability to produce GSH. Virulence assays using a *Drosophila melanogaster* model revealed that the Δ*gshA*, Δ*gshB* and double Δ*gshA*Δ*gshB* mutants exhibited attenuated virulence phenotypes. An analysis of virulence factors, including pyocyanin, pyoverdine, and cell motility (swimming and twitching), showed that these levels were reduced in these *gsh* mutants compared to PAO1. In contrast, biofilm formation increased in mutants. These data indicate that the GSH product and the genes responsible for GSH synthesis play multiple crucial roles in oxidative stress protection, bacterial virulence and biofilm formation in *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that causes nosocomial infections in hospitalized patients with AIDS, cancer, and cystic fibrosis (CF). During infection, *P. aeruginosa* is first eliminated by innate immune cells, such as phagocytic cells, in which NADPH oxidase-dependent reactive oxygen species (ROS) are generated as bactericidal substances [1]. ROS are also generated as a by-product of mitochondrial electron transport [2]. Oxidative stress occurs when cells are exposed to ROS, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and peroxide (ROOH), which causes oxidative damage to the cell via interactions with cellular components, including lipids, DNA and proteins [3]. These reactions lead to lipid peroxidation, DNA mutation, DNA-protein crosslinking, protein oxidation and fragmentation. *P. aeruginosa* has evolved mechanisms to protect itself from oxidative stress to survive during these conditions. Several antioxidant enzymes degrade ROS toxicity, such as catalases, superoxide dismutases, alkyl hydroperoxide reductases, and thiol peroxidases [4,5,6]. Antioxidant molecules, such as vitamins and glutathione (GSH), also play roles in ROS removal. Biomolecular repair enzymes, such as methionine sulfoxide reductases (MSR), are required during high oxidative damage conditions [7].

The tripeptide GSH is a thiol molecule that is found in most Gram-negative bacteria and all eukaryotic cells [8]. GSH is an important compound in cells because it is involved in the maintenance of cellular homeostasis, regulation of sulfur transport, conjugation of metabolites, xenobiotic detoxification, antibiotic resistance, enzymatic regulation and the expression of stress response genes [9]. GSH is the most abundant antioxidant molecule in cells, and it protects against oxidative stress via direct and indirect interactions with ROS [10]. GSH donates its electrons directly to $O_2^{\cdot-}$, $^{\cdot}OH$, peroxy radical ($ROO^{\cdot}$) and peroxynitrite ($ONOO^-$), which leads to

glutathione disulfide (GSSG), and catalase or glutathione peroxidase decompose H_2O_2 using GSH [3]. GSH reacts with free radicals, and it is oxidized to form GSSG [8]. Glutathione reductase reduces GSSG back to GSH for recycling during the redox process in cells [8].

A two-step process catalyzed by γ-glutamyl-cysteine synthetase and glutathione synthetase is required to synthesize GSH. γ-glutamyl-cysteine synthetase is encoded by the *gshA* gene, and it catalyzes the bonding formation between glutamate and cysteine to form γ-L-glutamylcysteine [8]. Glutathione synthetase is encoded by the *gshB* gene, and it catalyzes the formation of the addition glycine and cysteine in γ-L-glutamylcysteine to form GSH [8]. *Escherichia coli* that lack the GSH biosynthesis gene (*gshA* or *gshB*) are sensitive to diamide [11]. The absence of *gshA* in *Salmonella* sp. increased susceptibility to H_2O_2 and nitrosative stress [12]. *Salmonella* without *gshA* exhibited attenuated virulence in a murine model [12].

The aim of this work was to investigate the roles of glutathione biosynthesis genes *gshA* (PA5203) and *gshB* (PA0407) in the oxidative stress protection and bacterial virulence of *P. aeruginosa*.

Results and discussion

Expression profiles of *gshA* and *gshB* in response to stress

The *P. aeruginosa* PAO1 genome contains *gshA* (PA5203), which encodes the glutamate-cysteine ligase, and *gshB* (PA0407), which encodes γ-glutamyl-cysteine synthetase and glutathione synthetase [51]. The gene expression patterns of *gshA* and *gshB* under stress conditions were investigated using real-time RT-PCR. PAO1 cultures were challenged with 1 mM H_2O_2, a superoxide generator (0.5 mM plumbagin [PB], 0.5 mM menadione [MD], 0.5 mM paraquat [PQ]), organic hydroperoxides (1 mM cumene hydroperoxide [CHP], and 1 mM *t*-butyl

hydroperoxide [tBH]) and a thiol-depleting agent (0.5 mM N-ethylmaleimide [NEM]). Fig 1 shows that peroxides, including H_2O_2 (2.6 ± 0.3-fold), CHP (6.3 ± 0.2-fold), and tBH (2.7 ± 0.2-fold), considerably increased *gshA* expression compared to non-induced levels. However, other oxidants, including superoxide generators and NEM, did not significantly induce *gshA* expression (Fig 1). Exposure to H_2O_2 (2.1 ± 0.2-fold), CHP (3.3 ± 0.4-fold), and tBH (3.7 ± 0.3-fold) in *gshB* expression, but PQ, MD, PB, and NEM treatments only marginally induced expression (approximately 50%) compared to the non-induced condition. There were some similarities between the patterns of *gshA* and *gshB* expression. Notably, treatment of PAO1 with MD and PB induced a small (approximately 40%) reduction in *gshA* expression compared to PAO1. NEM treatment produced an over 4-fold reduction in *gshA* expression (Fig 1). These treatments unexpectedly induced a small increase in *gshB* expression (2-fold) (Fig 1). The contrasting patterns of *gshA* and *gshB* responses to these oxidants suggest a complex response involving GSH and its intermediates. The oxidant expression profiles of *gshA* and *gshB* shared some similarities, but these patterns did not fit any know oxidant sensing/responding transcription regulators (IscR, Fur, or OxyR) [13,14]. These novel patterns suggest that single or multiple unknown regulators differentially modulated these two genes. These hypotheses are being investigated. The oxidant expression profiles of these genes suggest that these genes play a role in protecting cells from oxidants that highly induce their expression [15,16].

Fig 1. The expression of *gshA* and *gshB* in response to stress. The expression levels of *gshA* and *gshB* were determined using real-time RT-PCR. Exponential-phase cells of *P. aeruginosa* PAO1 were subjected to various stress conditions, including 1 mM H_2O_2, 1 mM cumene hydroperoxide (CHP), 1 mM *t*-butyl hydroperoxide (tBH), 0.5 mM paraquat (PQ), 0.5

mM menadione (MD), and 0.5 mM plumbagin (PB)], and 0.5 mM N-ethylmaleimide (NEM) for 15 minutes prior to RNA preparation for real-time RT-PCR analysis. Relative expression was normalized to the 16S rRNA gene, and results are expressed as the fold-expression relative to the level of uninduced condition (UN). Data shown are means ± SD of three independent experiments.

gsh mutants exhibit increased susceptibilities to paraquat (PQ) and *N*-ethylmaleimide (NEM)

A plate sensitivity assay was performed to compare plate growth efficiency in the presence of oxidants between the PAO1 and *gsh* mutants and investigate the physiological roles of GshA and GshB in oxidative stress protection. Fig 2A shows the results. All *gsh* mutants, including Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants [17], were 10^3-fold more sensitive to PQ (0.25 mM) treatment compared to wild-type PAO1. The PQ-sensitive phenotype of Δ*gshA* and Δ*gshB* was complemented in the mutant strains transposed with a mini-Tn7 vector containing the full-length gene and showed levels similar to PAO1, which suggests that the PQ susceptibility in these mutants was the result of a lack of functional GshA or GshB. GSH (2 mM) was supplemented in the medium to confirm whether GSH, which is a product of GshA and GshB, was involved in protection against PQ toxicity in *P. aeruginosa*. Bacterial survival was determined after 0.25 mM paraquat treatment. The survival rates of Δ*gshA*, Δ*gshB*, double Δ*gshA*Δ*gshB* mutants and PAO1 grown in 2 mM GSH supplemented medium after paraquat treatment increased significantly (10^3) in all *gsh* mutants compared to mutants grown in LB without 2 mM GSH (Fig 2A). There were no significant differences in survival rates after paraquat treatment in PAO1, Δ*gshA*::*gshA*, and *gshB*::*gshB* complemented strains grown in LB

with 2 mM GSH compared with strains grown in LB alone (Fig 2A). This result suggests that the PQ susceptibility in these *gsh* mutants resulted from defects in glutathione biosynthesis and the exogenous GSH restored the PQ-sensitive phenotype. This result suggests that GSH plays a role in the protection against PQ toxicity in PAO1.

Fig 2. Determination of PQ and NEM resistance levels in *gsh* mutants and PAO1. (A) Plate sensitivity assay was performed with and without 2 mM GSH supplementation in LB plates containing 0.25 mM PQ and 0.3 mM NEM. (B) Plate sensitivity assay against 0.25 mM PQ using LB plates plus 1% $NaNO_3$ and incubated under aerobic and anaerobic conditions. Data presented are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.05$) relative to PAO1.

PQ is a superoxide generator that undergoes an intracellular redox cycling reaction via the acceptance of an electron from NADPH and transfers that electron to oxygen to produce a superoxide anion [18]. However, PQ itself exerts its toxicity in an oxygen-independent manner via intracellular transformations [19]. The plate sensitivity assay was performed under aerobic and anaerobic conditions, as described in the Materials and Methods, to determine whether PQ toxicity in *gsh* mutants was produced from superoxide anion generation or direct toxicity. The 10^3-fold increase in PQ sensitivity of Δ*gshA*, Δ*gshB*, and Δ*gshA*Δ*gshB* mutants was abolished under the anaerobic condition compared to the aerobic condition (Fig 2B). These *gsh* mutants exhibited a similar PQ susceptibility under the anaerobic condition as PAO1. Therefore, the increased PQ susceptibility of the Δ*gsh* mutant required oxygen and most likely resulted from superoxide anion-mediated toxicity. These results support the hypothesis that GSH acts as an

antioxidant agent to scavenge this superoxide radical and defects in GSH biosynthesis contribute to oxidative stress that leads to cell death.

The Δ*gshA* and double Δ*gshA*Δ*gshB* mutants were 10^2-fold more susceptible to NEM (0.3 mM) than the wild-type bacteria (Fig 2A). However, the Δ*gshB* mutant exhibited similar susceptibility levels to NEM as the wild type (Fig. 2A), which suggests that the lack of GshB activity did not affect thiol depletion. Complementation of the *gshA* mutant (Δ*gshA*::*gshA*) strain produced similar susceptibility levels as PAO1 (Fig 2A).

NEM is a thiol-depleting compound that reacts with the sulfhydryl group of cysteine residues in several proteins. NEM causes cellular thiol depletion and contributed to the NEM hypersensitive phenotype of *gshA* mutant, which suggests that GSH biosynthesis is important to NEM resistance. GSH protects proteins from NEM-induced modification to maintain the function of these proteins under NEM exposure [20]. GSH also reacts chemically with NEM to lower toxic concentrations [20]. The deletion of *gshA* resulted in the lack of GSH and its intermediates, and cells with deleted *gshB* gene still produced γ-glutamylcysteine, which is an intermediate of GSH biosynthesis that exhibits antioxidant properties [11]. GSH detoxifies NEM toxicity via direct conjugation to produce an *N*-ethylsuccinimido-*S*-glutathione (ESG) adduct [20], which activates potassium efflux systems and decreases cytoplasmic pH to protect cells from electrophile toxicity [21]. The ESG adduct is degraded to a non-toxic metabolite, *N*-ethylmaleamic acid, during NEM detoxification prior to release from the cell [20]. NEM also activates *P. aeruginosa* glutathione-gated potassium efflux (GGKE), which leads to K^+ and Ca^{2+} efflux and H^+ influx, and alters biofilms to result in detachment [22]. GSH and its intermediates may provide general thiol-buffering effects to protect bacteria against the thiol-depleting agent NEM.

gsh mutants are sensitive to methylglyoxal

Reactive electrophilic species (RES) are highly reactive molecules that contain α, β-unsaturated carbonyl or electrophilic groups [23]. RES cause stress to the cell via reactions with nucleophilic macromolecules, including proteins and DNA, and produce irreversible damage and mutation [23]. Methylglyoxal is an RES-generating molecule that is highly toxic to cells. The broth microdilution assay was performed using *gsh* mutants to investigate the role of GSH biosynthesis in the protection from methylglyoxal toxicity. The susceptibility level was expressed as the MIC values of each bacterial strain. The Δ*gshA* and Δ*gshA*Δ*gshB* mutants exhibited a 4-fold reduction in MIC level (0.01%) against methylglyoxal, and the Δ*gshB* exhibited a 2-fold reduction in MIC (0.02%) compared to PAO1 (0.04%) (Table 1). The introduction of *gshA* or *gshB* completely restored the increased susceptibility to methylglyoxal of both mutants to the PAO1 level (0.04%). The reduction in MIC against methylglyoxal in *gsh* mutants suggests that cellular GSH is important in the protection of *P. aeruginosa* against methylglyoxal-mediated RES, which was observed in other bacteria [24,25]. Exogenous GSH was supplemented into the culture medium, and the phenotypes were re-examined to determine whether GSH was required for methylglyoxal resistance in this bacterium. Supplementation of 2 mM GSH increased methylglyoxal resistance in *gsh* mutants to levels similar to the PAO1 level (MIC, 0.04%). These results suggest that the methylglyoxal susceptibility of *gsh* mutants resulted from the malfunction of GSH biosynthesis, which decreased GSH levels in the cell.

Table 1. MIC of methylglyoxal for *P. aeruginosa* PAO1 and *gsh* mutants.

Strains	MIC of Methylglyoxal (%)	
	No GSH	**2 mM GSH**
PAO1	0.04	0.04
Δ*gshA*	0.01	0.04
Δ*gshA*:: *gshA*	0.04	0.04
Δ*gshB*	0.02	0.04
Δ*gshB*::*gshB*	0.04	0.04
Δ*gshA*Δ*gshB*	0.01	0.04

The data shown are the mode of at least three independent experiments .

Methylglyoxal uses different mechanisms to exert its antimicrobial activity, including inhibition of protein, DNA, and RNA synthesis [26,27]. Bacterial methylglyoxal detoxification is carried out mainly by glyoxalase I and II enzymes [23]. In *E. coli*, Glyoxalase I requires GSH as a cofactor in the converting of methylglyoxal to the intermediate *S*-lactoylglutathione, which activates the potassium efflux pump and NEM-GSH adduct (ESG) [28]. The acidic cytoplasm contributes to cell survival against the methylglyoxal toxicity. Notably, *P. aeruginosa* expresses two glyoxalase I enzymes, which belong to different metal activation classes [29]. Glyoxalase II further converts *S*-D-lactoylglutathione to glycolic and lactic acids [23]. Therefore, cellular GSH plays a direct role in the full activity of glyoxalase I in the detoxification of methylglyoxal in this bacterium.

Δ*gsh* mutants attenuate virulence in a *Drosophila* host model

GSH is responsible for ROS and RES protections in *P. aeruginosa*, and these factors contribute to bacterial pathogenicity. The virulence of the *P. aeruginosa gsh* mutant strains was tested using a fruit fly *Drosophila melanogaster* feeding assay, as described in the Materials and Methods. Feeding flies with *P. aeruginosa* wild-type PAO1 produced 50 ± 6.3% fly survival compared to 100% LB feeding as a negative control after a 20-h incubation (Fig 3). The percentage of fly survival increased significantly to 80 ± 4.2%, 80 ± 3.9%, and 81 ± 4.5% when the Δ*gshA*, Δ*gshB*, and Δ*gshA*Δ*gshB* mutants were fed to *D. melanogaster*, respectively ($P < 0.05$). These results indicate that deletion of *gshA* or *gshB* attenuates the virulence of *P. aeruginosa* PAO1 in the tested model. Functional *gshA* or *gshB* restored the attenuated virulence in these mutants because similar levels of percent fly survival as the wild-type flies were observed (58 ± 3.0% and 62 ± 5.8% fly survival, respectively). These results suggest that GSH biosynthesis plays important roles in the pathogenicity of *P. aeruginosa* in the fruit fly *Drosophila* host model.

Fig 3. Virulence testing for *gsh* mutants and PAO1 using fruit fly *Drosophila melanogaster* feeding assay. Surviving flies were counted after 20 h of incubation, and results are expressed as the percent survival. Data presented are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.05$) compared with PAO1.

Fly immunity is a multilayered system that includes at least 7 defense mechanisms to protect flies from invading pathogens [30]. One of these mechanisms that regulates bacteria in

the fly gut is antimicrobial peptides (AMPs), and ROS, particularly superoxide anions produced from midgut epithelial cells, is a first-line defense mechanisms [31]. In the infected fly gut, ROS was produced from the NADPH oxidase dDuox protein of epithelial cells, which is triggered by invading bacteria [32]. *P. aeruginosa* PAO1 protected itself from the oxidative stress generated by the host cells via the use of GSH as an antioxidant agent, which lead to growth in the fly gut and host death from bacterial infection. GSH is responsible for virulence attenuation and the superoxide hypersensitivity of *gsh* mutants. Loss of *gshA* or *gshB* in *P. aeruginosa* attenuated the virulence ability to cause fly death, likely because of a reduced ability to survive within the host. Therefore, *gsh* mutants were killed more rapidly by host-produced ROS.

Virulence factors play an important role in bacterial infection, colonization, and invasion within the host cell [33]. Different virulence factors are required in two forms of bacterial stages during infection: the planktonic form is involved in acute infection, and biofilm is involved in chronic infection [34]. Planktonic bacteria produce several virulence factors to infect the host, including phenazine pyocyanin, which generates ROS and promotes inflammation, motility factors that facilitate bacterial movement through host cells, siderophores that trap extracellular iron, and toxins that damage host cells [34]. Biofilm formation is associated with persistent infection and antibiotic resistance within host cells [35].

Glutathione plays important roles in pyocyanin production

P. aeruginosa pyocyanin is a terminal signaling factor in a quorum sensing network and a virulence factor from oxidative stress pathways, which is involved in the pathophysiological effects in cystic fibrosis patients [36]. The amount of pyocyanin in *gsh* mutant strains was measured and compared to PAO1. The results in Fig 4A show that the wild-type PAO1 culture medium contained 6.32 ± 0.3 μg/ml pyocyanin, and the Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB*

mutant culture mediums contained significantly lower pyocyanin (3.01 ± 0.0, 3.84 ± 0.3, and 3.63 ± 0.4 μg/ml, respectively). The amount of pyocyanin in the culture medium of the Δ*gshA*::*gshA* complemented strains was similar to PAO1, and the Δ*gshB*::*gshB* strain exhibited increased pyocyanin levels up to 17.72 ± 0.05 μg/ml (3-fold higher than PAO1). The effect of constitutive expression of *gshB* from the Tn7 expression vector promoter on pyocyanin production was unexpected. This result may be due to deregulation of *gshB* expression, which leads to a much higher level of pyocyanin via unknown mechanisms. The significantly decreased pyocyanin levels in these *gsh* mutants suggest that bacterial GSH biosynthesis is required for pyocyanin production.

Fig 4. Pyocyanin production in *P. aeruginosa* wild-type PAO1 and *gsh* mutants. (A) PAO1, Δ*gshA*, Δ*gshA*::*gshA*, Δ*gshB*, Δ*gshB*::*gshB* and double Δ*gshA*Δ*gshB* mutant strains were cultured for 24 h, and the supernatant was collected for pyocyanin measurement. (B) Real-time RT-PCR analysis of *phzA1* and *phzA2* expression. Total RNA was isolated from PAO1, Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* strains. Data shown are the fold change in expression relative to wild-type PAO1 level. (C) Northern blot analysis of mRNA samples probed with radioactively labeled *phzA2*. Total RNA (20 μg) prepared from the cultures of PAO1 and the *gsh* mutants were loaded into each lane. The number below each band represents the fold change in band intensity relative to the level of the wild-type determined using densitometric analysis. The asterisk indicates a statistically significant difference ($P < 0.05$) compared with PAO1.

Production of pyocyanin in *P. aeruginosa* involves two homologous systems encoded by the *phzA1B1C1D1E1F1G1* (*phzA1*) and *phzA2B2C2D2E2F2G2* (*phzA2*) gene clusters [37]. The

expression of *phzA1* and *phzA2* in *gsh* mutants was determined using real-time RT-PCR. The level of *phzA1* exhibited a small decrease (less than 50%) compared to PAO1 levels in *gsh* mutants (Fig 4B). The expression of *phzA2* was 2-fold lower in the Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants compared with the PAO1 (Fig 4B). These results suggest that GSH biosynthesis is required for the full expression of *phzA1* and *phzA2* operons via unknown mechanisms, and the expression levels of these operons contribute to overall pyocyanin production in *P. aeruginosa* [38]. Northern blot analysis was performed to confirm the expression of *phzA* in the *gsh* mutants compared to PAO1. The results demonstrate that *phzA2* genes were transcribed mostly as polycistronic transcripts, and *phzA*2 expression was reduced approximately 2-fold in the Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants relative to PAO1 level (Fig 4C). These results are consistent with the results of the real-time RT-PCR analysis. A similar observation was reported for the *gshB* mutant in *P. aeruginosa* [38].

GSH interferes with the ability of pyocyanin to interact with extracellular DNA (eDNA) via a direct reaction with pyocyanin [39]. The reaction of eDNA and pyocyanin is important in biofilm formation. Therefore, the balance of pyocyanin-eDNA-GSH is altered in the absence of GSH, which could lead to the observed decrease in pyocyanin production (Fig 4A).

gsh mutants produce lower pyoverdine levels

Pyoverdine is a green fluorescent siderophore that is also involved in *P. aeruginosa* pathogenicity. It is secreted from *P. aeruginosa* under iron-limiting conditions for the chelation of ferric ions in the environment into cells [40]. The amount of pyoverdine in *P. aeruginosa* strains was quantified using fluorescent spectrometry to investigate whether GSH affected pyoverdine production. The intensity of fluorescence was normalized to cell density (OD_{600}), and the results are shown as percent relative fluorescence intensity. The amounts of pyoverdine in

305 Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants were significantly lower (21.9 ± 2.4%, 19.1 ±
306 1.8%, and 15.8 ± 1.1%, respectively) than PAO1 (100%) (Fig 5). The expression of functional
307 *gshA* and *gshB* restored pyoverdine secretion in the GSH mutants to the PAO1 level (Fig 5).
308 These data suggest that GSH biosynthesis is involved in pyoverdine production in *P. aeruginosa*.
309
310 **Fig 5. Pyoverdine production in *P. aeruginosa.*** PAO1, Δ*gshA*, Δ*gshA*::*gshA*, Δ*gshB*,
311 Δ*gshB*::*gshB* and double Δ*gshA*Δ*gshB* mutants were incubated in Pseudomonas F medium
312 overnight at 37°C. Pyoverdine was fluorometrically measured by recording the emission at 477
313 nm and excitation at 400 nm in a luminescence spectrometer. The asterisk indicates a statistically
314 significant difference ($P < 0.05$) compared to PAO1.
315
316 Siderophore pyoverdine is secreted by the type VI secretion system (T6SS) under iron-
317 limiting conditions in *P. aeruginosa* to scavenge Fe^{3+} in the extracellular environment [48].
318 Ferripyoverdine (pyoverdine-Fe^{3+} complex) binds the FpvA outer membrane receptor and
319 imports iron into the cell. FpvA interacts with FpvR antisigma factor in the periplasm and
320 transmits the signal to the cytoplasmic domain of FpvR. Two sigma factors, σ^{PvdS} and σ^{FpvI}, are
321 activated and bind to RNA polymerase to initiate the transcription of pyoverdine synthesis genes
322 and *fpvA*, respectively [48].
323 Another siderophore in *P. aeruginosa* is pyochelin. Ferripyochelin is transported into the
324 cell via the FptA outer membrane receptor [49]. Ferripyochelin iron reductase located in the
325 periplasm and cytoplasm catalyzes the reduction of Fe^{3+} and releases Fe^{2+} from the pyochelin
326 [49]. GSH and NADH are electron donators for ferripyochelin iron reductase [50]. Therefore, the

re-adjustment of iron uptake processes, such as reduced pyoverdine synthesis, is required in the absence of thiols.

Δ*gsh* mutants exhibit impaired swimming and twitching motility

Flagella and type IV pili play important roles in bacterial virulence during acute and chronic *P. aeruginosa* infections. Flagella are also required for biofilm formation and contribute to persistent colonization, and type IV pili mediate adherence to the epithelial cell surface and contribute to biofilm formation [41]. *P. aeruginosa* uses a single flagellum for swimming in a liquid environment, and twitching is flagella-independent. Twitching is powered by an extension and retraction of pili. Table 2 shows that the swimming motility of wild-type PAO1 was 47.0 ± 1.7 mm after 48 h of incubation. Defects in swimming motility in the Δ*gshA* (33.0 ± 2.0 mm), Δ*gshB* (38.5 ± 2.1 mm), and Δ*gshA*Δ*gshB* mutants (35.0 ± 2.0 mm) were observed compared to PAO1. The Δ*gshA*::*gshA* and the Δ*gshB*::*gshB* complemented strains exhibited restored motility (43.3 ± 1.2 and 45.3 ± 5.7 mm, respectively) to a similar level as PAO1 (Table 2). Table 3 also shows that twitching motility was reduced significantly in the Δ*gshA*, Δ*gshB*, and Δ*gshA*Δ*gshB* mutants (32.3 ± 3.1, 33.7 ± 3.2, and 32.3 ± 2.5 mm, respectively) compared to PAO1 (43.3 ± 1.5 mm). Swimming and twitching motility were defective in the Δ*gsh* mutants, but the colony sizes of these mutants on the LB agar were similar to PAO1 (data not shown).

Table 2. Swimming and twitching motility in *P. aeruginosa* after 48 h incubation.

Strains	Motility Zone (mm ± SD)	
	Swimming	**Twiching**
PAO1	47.0 ± 1.7	43.3 ± 1.5
Δ*gshA*	33.0 ± 2.0 *	32.3 ± 3.1 *
Δ*gshA*::*gshA*	43.3 ± 1.2	43.0 ± 2.0
Δ*gshB*	38.5 ± 2.1 *	33.7 ± 3.2 *
Δ*gshB*::*gshB*	45.3 ± 5.7	41.3 ± 1.5
Δ*gshA*Δ*gshB*	35.0 ± 2.0 *	32.3 ± 2.5 *

The data shown are the means ± SD of motility zone (mm) at 48 h incubation of three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.01$) relative to PAO1.

GSH is transported from the bacterial cytoplasm to the periplasm via the CydDC transporter, which is an ATP-binding cassette-type transporter [42]. GSH exportation maintains the redox environment and protects cells from external toxicity or electrophilic compounds in *S. typhimurium* and *E. coli* [43]. Periplasmic GSH participates in disulfide bond formation and protein folding via the disulfide bond protein (Dsb) pathway [44]. These correct folding proteins are important for the proper assembly of flagella motors and pili. *E. coli cydD* mutants exhibited defective cell motility due to disrupted flagella assembly. The *gshA* mutant also exhibited defective flagella function. These results indicate that the transportation of GSH to the periplasm plays an important role in cell motility [42].

Deletion of genes encoding glutathione biosynthesis increases biofilm formation

Biofilm formation is one virulence factor in *P. aeruginosa*. Biofilm contributes to resistance to various stresses, including antimicrobial stress, and it is an important component of chronic infections [45]. Biofilm formation was determined in PAO1 and *gsh* mutants in the present study. Biofilm levels in the Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants (1.9 ± 0.2-fold, 2.3 ± 0.1-fold, and 1.9 ± 0.1-fold, respectively) were significantly higher than PAO1 (Fig 6). The Δ*gshA*::*gshA* and Δ*gshB*::*gshB* complemented strains exhibited restored biofilm formation to the PAO1 level (Fig 6). These results demonstrated that a defect in GSH biosynthesis via deletion of *gshA* or *gshB* increased biofilm formation, which suggests a role for GSH in the repression of biofilm formation. GSH (2 mM) was added to the bacterial culture during the assay to determine whether GSH inhibited biofilm production in *P. aeruginosa*. Fig 6 shows that the addition of GSH decreased biofilm formation in Δ*gshA*, Δ*gshB*, and double Δ*gshA*Δ*gshB* mutants to a level similar to PAO1. GSH inhibition of biofilm formation in *P. aeruginosa* PAO1 is consistent with previous observations that GSH disrupted mature and immature biofilms of the clinical *P. aeruginosa* strain [46]. Pyocyanin pigment activates eDNA release from *P. aeruginosa* via H_2O_2-mediated cell lysis, which leads to the binding of pyocyanin to eDNA and facilitation of biofilm formation [39]. GSH non-enzymatically interacts with pyocyanin to form a pyocyanin-GSH complex that inhibits pyocyanin-mediated cell lysis, the release of eDNA, binding to eDNA, and biofilm formation [39]. A small increase in *gshA* expression levels (1.1-fold) in non-clonal cystic fibrosis isolates *P. aeruginosa* was observed when the isolates switched from planktonic to biofilm growth, which suggests that biofilm cells require GSH to control production [47].

Fig 6. Biofilm formation assay in *P. aeruginosa.* PAO1, Δ*gshA*, Δ*gshA*::*gshA*, Δ*gshB*, Δ*gshB*::*gshB* and double Δ*gshA*Δ*gshB* mutants were cultured in LB medium with or without 2 mM GSH supplementation for 24 h. The biofilm layer of PAO1 and indicated strains was stained with 1% crystal violet solution. The biofilm biomass (OD_{600}) of *gsh* mutants and complemented strains compared with PAO1 were quantified. The asterisk indicates a statistically significant difference ($P < 0.05$) compared to PAO1

GSH biosynthesis was required for the activation of virulence factors in planktonic cells, including pyocyanin pigment, siderophore, and motility to promote virulence, in *P. aeruginosa* for infection and survival within the host cell (Figs 4 and 5, and Table 3). GSH acted as an anti-biofilm in chronic infection and adjusted the metabolic protection and stress response mechanisms.

Conclusion

gshA and *gshB* are responsible for GSH biosynthesis in *P. aeruginosa*. These findings demonstrated that the inactivation of *gshA* and *gshB* genes increased the susceptibility to ROS- and RES-mediated agents and attenuated virulence due to defects in pigment production, siderophore, and motility (Fig 7). GSH biosynthesis controlled biofilm formation. The data demonstrated that GSH was not essential but played centrally important roles in various physiological processes that were important to survival in the diverse environmental conditions that *P. aeruginosa* encountered.

Fig 7. Overview function of GSH biosynthesis in *P. aeruginosa.* GshA and GshB catalyzed GSH biosynthesis. Bacteria at the early stage of infection encounter various stresses, including ROS and RES, which are generated by the host cell. GSH biosynthesis plays a primary protective role in the detoxification of these oxidative stresses. *P. aeruginosa* required GSH for activation of pigment, siderophore, and motility, which promote bacterial virulence in planktonic cells. GSH also disrupts biofilm formation to control the amount of biofilm.

Materials and methods

Bacterial strains

All bacterial strains used in this study are listed in Table 3. *P. aeruginosa* strains were grown in lysogeny broth (LB) with shaking at 180 rpm at 37°C. The overnight culture was inoculated in fresh LB medium and incubated with shaking. Cells in the exponential phase (optical density at 600 nm (OD_{600}) of 0.5 after 3 h of growth) were used in all experiments.

Table 3. Bacterial strains and plasmids used in this study.

Strains	**Relevant characteristics**	**Source or Reference**
P. aeruginosa		
PAO1	Wild type carrying mini-Tn7T	[17]
Δ*gshA*	*gshA* mutant, derivative of PAO1 in which a part of *gshA* was deleted and carrying mini-Tn7T	[17]
Δ*gshA*::*gshA*	*gshA* mutant carrying mini-Tn7Tcontaining *gshA*	[17]

Δ*gshB*	*gshB* mutant, derivative of PAO1 in which a part of *gshB* was deleted and carrying mini-Tn7T	[17]
Δ*gshB*::*gshB*	*gshB* mutant carrying mini-Tn7Tcontaining *gshB*	[17]
Δ*gshA*Δ*gshB*	Double *gshA* and *gshB* mutant, derivative of *gshA* mutant in which a part of *gshB* was deleted and carrying mini-Tn7T	[17]
E. coli		
DH5α	ϕ80d *lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, hsdR17(r_k^-, m_k^+), *supE*44, *relA*1, *deoR*, Δ(*lacYMAargF*)U169	Stratagene Inc. (USA)

Molecular techniques

General molecular techniques, including DNA and RNA preparations, DNA cloning, PCR amplification, Southern blot analysis and bacterial transformation, were performed according to standard protocols [48].

Expression analysis of *gshA, gshB, phzA1* and *phzA2* using endpoint and real-time RT-PCR

Total RNA samples were extracted from exponential phase PAO1 culture with and without oxidant treatment for 15 min at 37ºC. Total RNA was treated with DNase I (Thermo Scientific, USA) prior to performing cDNA synthesis using 5 µg DNase I-treated RNA, RevertAid Reverse Transcriptase and random hexamer primers (Thermo Scientific, USA). Endpoint reverse transcription (RT)-PCR was performed using 10 ng cDNA to determine gene expression levels using primers EBI11 (5'-CGCTACGGCAAGACCATG-3') and EBI12 (5'-GCGCTCCAACTGGCTCGG-3') for *gshA*, BT5458 (5'-CGCATGCGCCCGCTGAAGG-3') and BT5459 (5'-GCGCGCCAGGCAGTAGGG-3') for *gshB*, EBI315 (5'-

CGGTCAGCGGTACAGGGAA-3') and EBI316 (5'-GCGAGAGTACCAACGGTTGAAA-3') for *phzA1*, and EBI316 and EBI317 (5'-CGTCGCACTCGACCCAGAA-3') for *phzA2*. The 16S rRNA gene was amplified using primers BT2781 (5′-GCCCGCACAAGCGGTGGAG-3′), and BT2782 (5′-ACGTCATCCCCACCTTCCT-3′) was used as an internal control to normalize cDNA samples. RT-PCR products were visualized using 1.8% agarose gel electrophoresis.

Northern blot analysis

Total RNA isolation, agarose-formaldehyde gel electrophoresis, blotting, and hybridization were performed as previously described [48]. Purified total RNA (20 μg) was loaded into the gel. A 305-bp fragment of the *phzA2* coding region was used as the DNA probe and amplified using primers EBI316 (5′-GCGAGAGTACCAACGGTTGAAA-3′) and EBI317 (5′-CGTCGCACTCGACCCAGAA-3′). Radioactively labeled probes were prepared using random-primed labeling with [α-^{32}P] dCTP.

Determination of the oxidant resistance level

To determine the oxidant resistance level, a plate sensitivity assay was performed as previously described [7]. Briefly, exponential phase cells were adjusted to an OD_{600} of 0.1 prior to a 10-fold serial dilution in LB medium. Each dilution (10 μl) was spotted onto an LB plate containing 0.25 mM paraquat (PQ) and 0.3 mM *N*-ethylmaleimide (NEM). The plates were incubated overnight at 37°C prior to quantification of colony forming units (CFUs). Percent survival was defined as the CFUs on the plates containing oxidant divided by the CFUs on plates without oxidant and multiplied by 100.

Plate sensitivity assays for anaerobic conditions were performed using LB medium supplemented with sodium nitrate ($NaNO_3$, 1% w/v). The culture plates were incubated in an

anaerobic jar containing an anaerobic gas pack (AnaeroGen, Oxoid, UK) and incubated at 37°C for 48 h.

MIC determination

The minimum inhibitory concentrations (MICs) of antibiotics were determined using a broth microdilution assay. *P. aeruginosa* PAO1 and *gsh* mutant strains were grown in LB medium at 37°C under aerobic conditions until reaching the exponential phase. The LB broth supplemented with different concentrations of agents with and without 2 mM reduced GSH (Sigma Aldrich, USA) was incubated with bacteria at 37°C for 18 h. The lowest concentration of antibiotic that inhibited bacterial growth after 18 h of incubation was determined as the MIC value.

Drosophila melanogaster virulence assay

The virulence of *P. aeruginosa* and mutants were evaluated using the *Drosophila melanogaster* model as previously described [7]. Essentially, the exponential phase cultures of *P. aeruginosa* strains were adjusted to an OD_{600} of 0.5 in 800 µl of LB broth prior to the overlaying of cell suspensions onto the surface of preservative-free corn flour *Drosophila* medium (350 ml water, 32 g corn flour, 9 g yeast, 20 g sugar, and 8 g agar) at the bottom of glass fly culture vials. The 12-day-old adult flies were starved for 3 h prior to placement in each vial covered with bacterial cells (20 flies per vial). The vials were incubated at 25ºC for 20 h, and the number of viable flies was counted.

Biofilm formation assay

Biofilm formation assays were performed as previously described [49]. Overnight cultures of *P. aeruginosa* strains were diluted 1:100 in fresh LB medium, and 150 µl of medium was transferred into 96-well plates. Plates were incubated at 37°C for 24 h without shaking, and

480 the cell suspension was removed and rinsed with 200 µl of phosphate buffered saline (PBS). The
481 biofilm layer was stained with a 0.1% crystal violet solution and incubated at room temperature
482 for 15 min. The plate was rinsed with water and dried. Ethanol (200 µl) was added to solubilize
483 the crystal violet dye. The absorbance was measured at OD_{600} using a spectrophotometer to
484 quantify biofilm formation.

485 ## Bacterial motility assay

486 Swimming motility was tested in M8 minimal medium supplemented with 1 mM $MgSO_4$,
487 0.5% Casamino acids, and 0.2% glucose and solidified with 0.3% agar for several hours [50].
488 Overnight bacterial cultures were spotted on agar and incubated at 37ºC for 48 h. Twitching
489 motility was tested using stab inoculation with a toothpick through a 1% agar LB layer to the
490 bottom of the Petri dish and incubated 37ºC for 48 h.

491 ## Pyocyanin pigment production

492 *P. aeruginosa* strains were grown in glycerol alanine minimal medium (GA medium)
493 containing 1% glycerol, 67.3 mM L-alanine, 8 mM $MgSO_4$, 0.44 mM K_2HPO_4, 0.065 mM
494 $FeSO_4$ at 37ºC for 24 h, and pyocyanin production was quantified based on the absorbance of
495 pyocyanin at 520 nm (OD_{520}) in an acidic solution as describe previously [51]. Briefly, the
496 supernatant from bacterial culture was collected by centrifugation at 6,000 rpm for 10 min. The
497 pyocyanin in the supernatant was extracted by mixing 3 ml of chloroform into 5 ml of
498 supernatant. The lower chloroform layer containing pyocyanin was collected, and 1 ml of 0.2 M
499 HCl was added to extract pyocyanin into the aqueous phase. The pyocyanin was quantified as
500 OD_{520}. The pyocyanin concentration is expressed as micrograms of pyocyanin per milliliter of
501 culture supernatant and determined by multiplication of the OD_{520} by 17.072 [51].
502

Pyoverdine measurement

P. aeruginosa PAO1 and *gsh* mutant strains were incubated at 37°C in *Pseudomonas* F medium (BD Difco, USA) overnight. Pyoverdine in the supernatant was fluorometrically measured via recording of the emission at 477 nm and excitation at 400 nm in a luminescence spectrometer and normalized to the OD_{600} of the corresponding cultures.

Statistical Analysis

The difference between two samples was determined using a paired *t*-test, and $P < 0.05$ was considered statistically significant.

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648 genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability
649 of the two anthranilate synthases and evolutionary implications. J Bacteriol 172: 884-900.

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Figure 1

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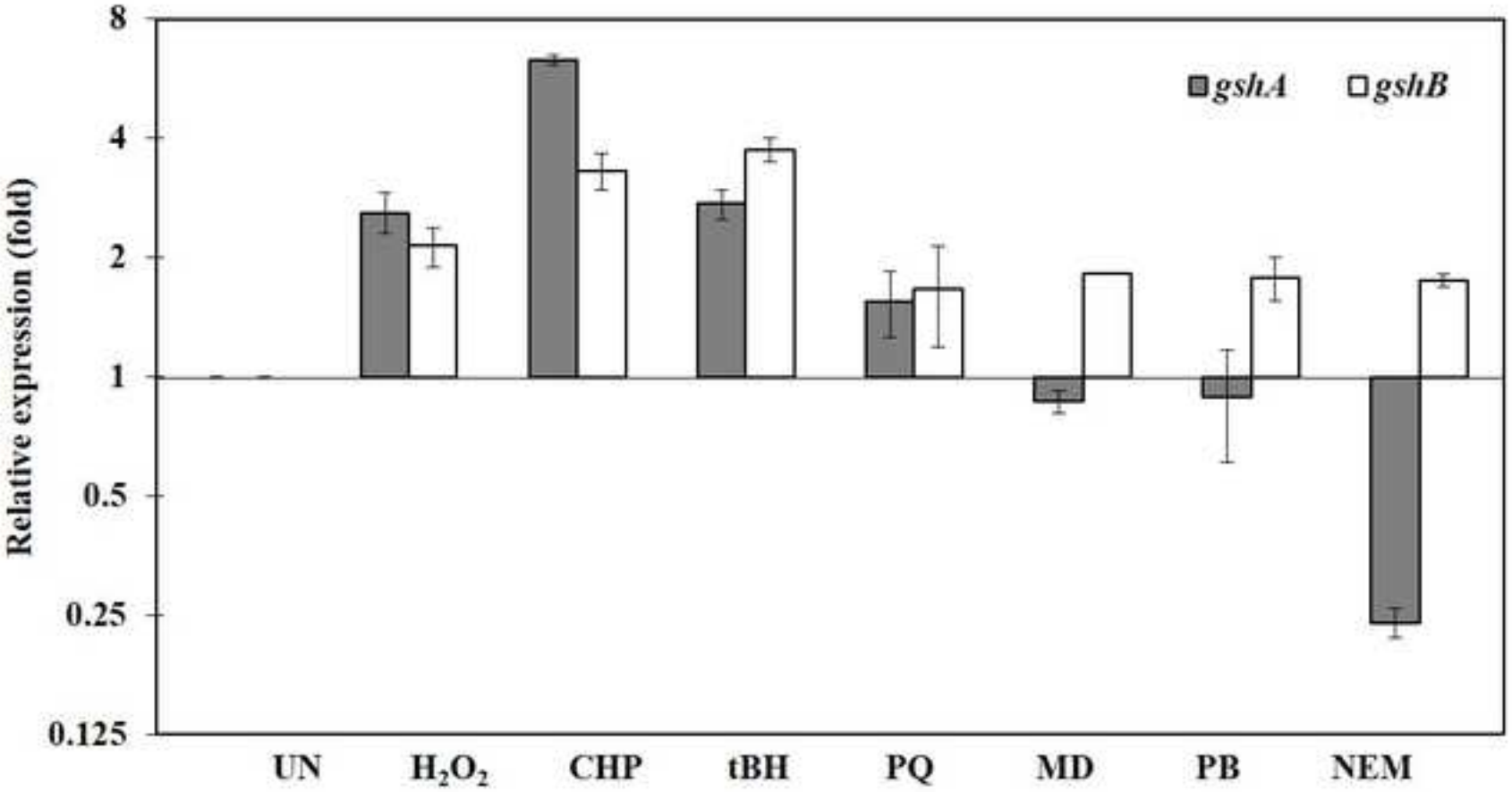

Figure 2

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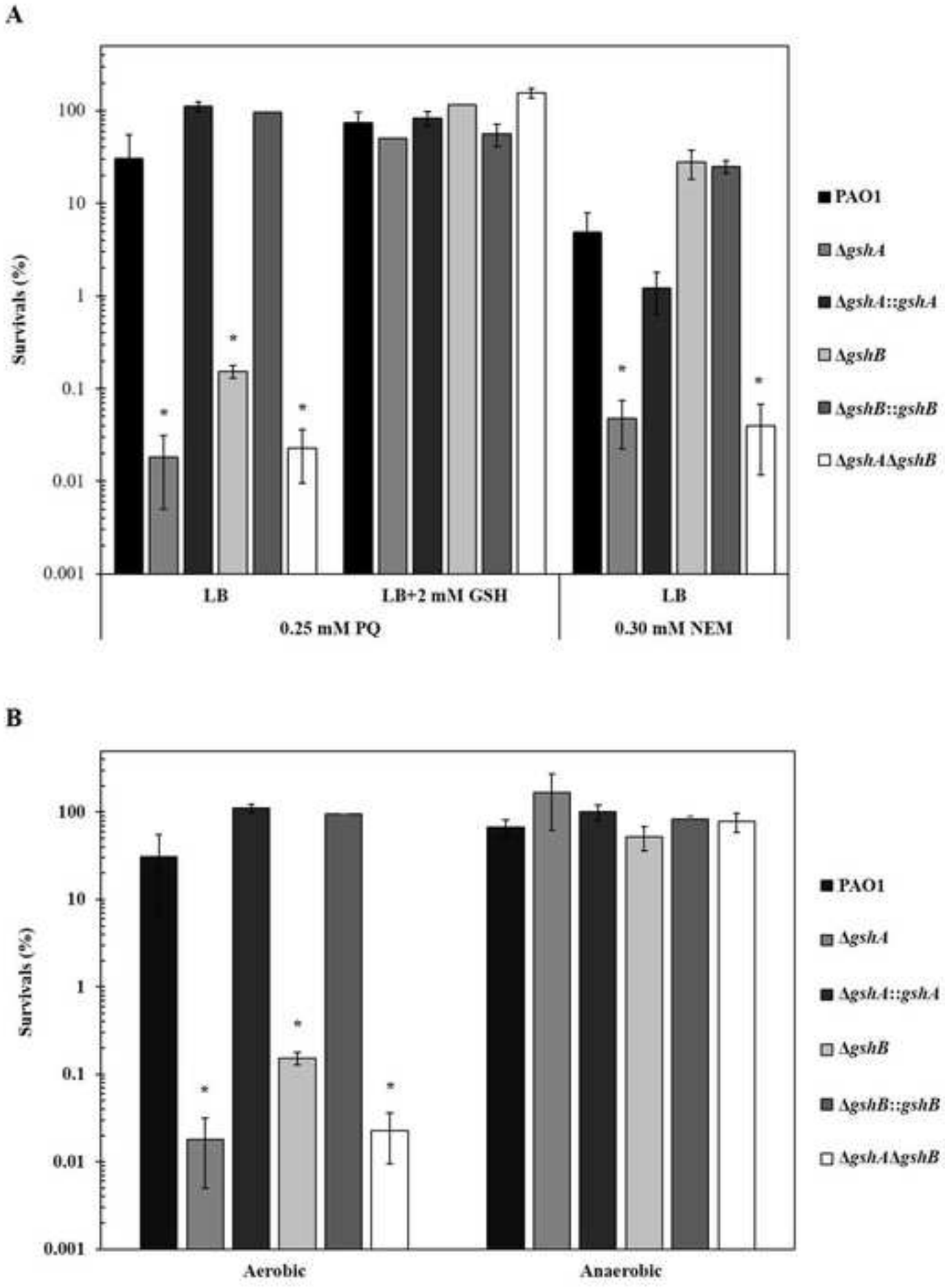

Figure 3

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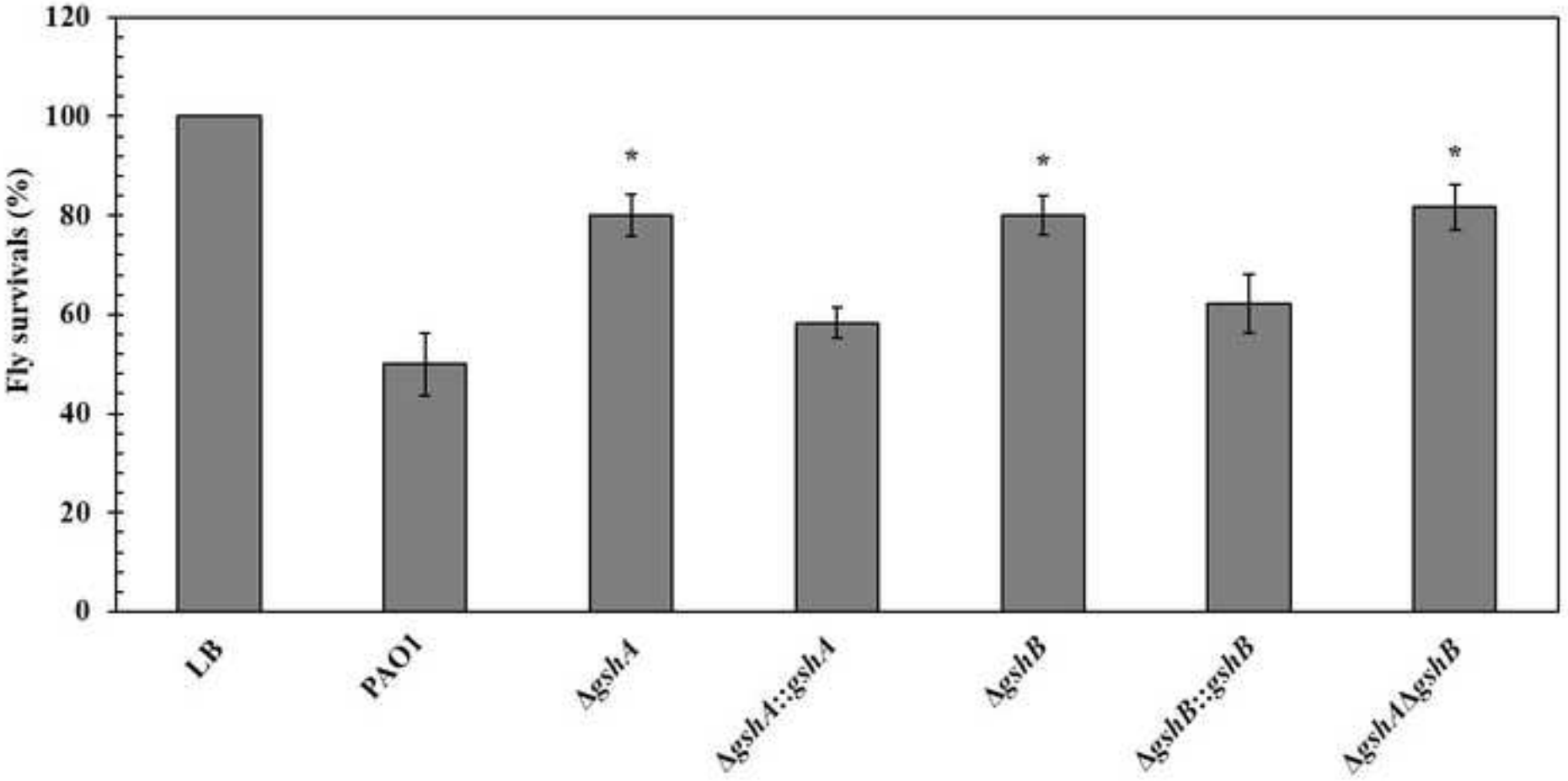

Figure 4

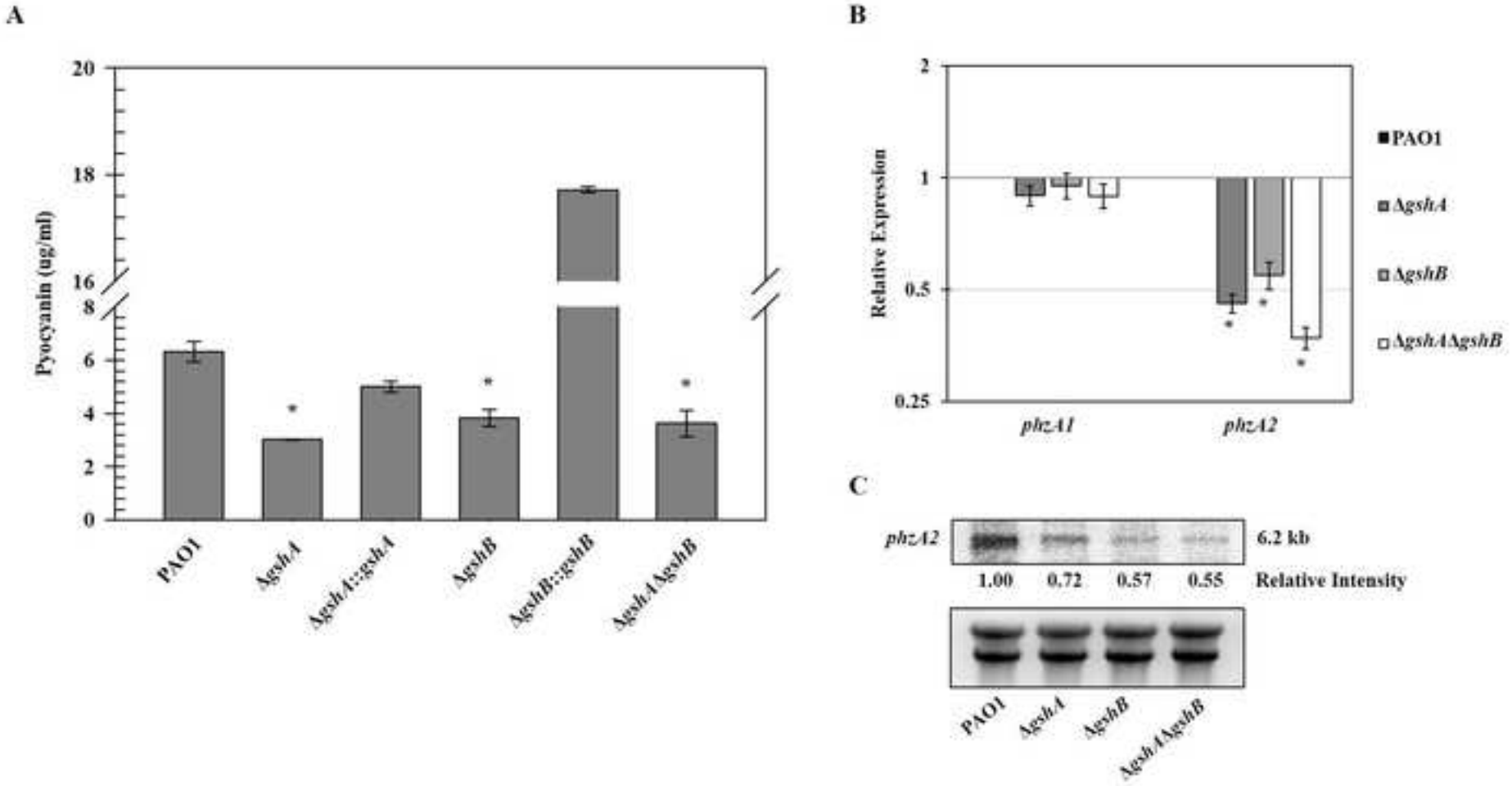

Figure 5

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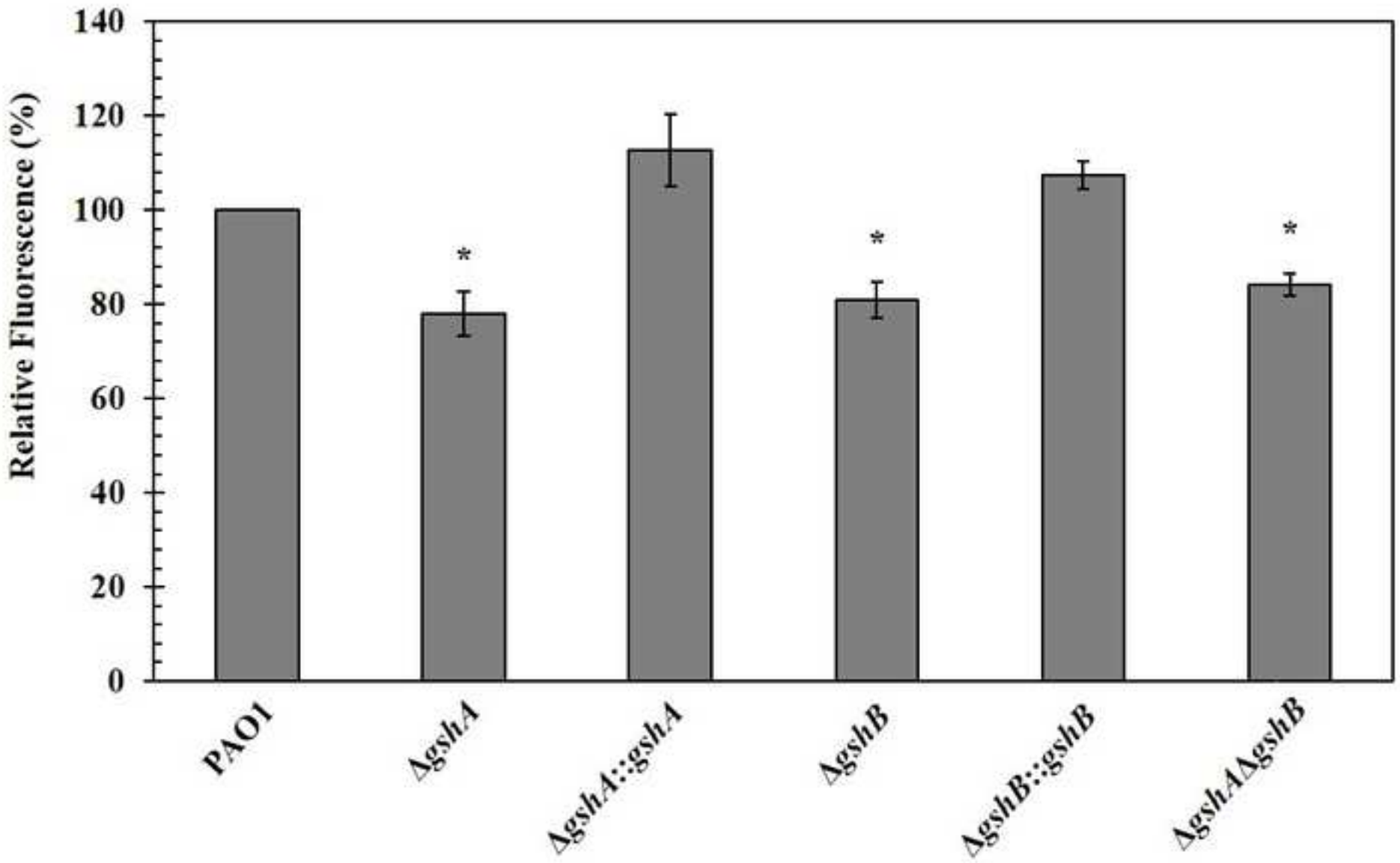

Figure 6

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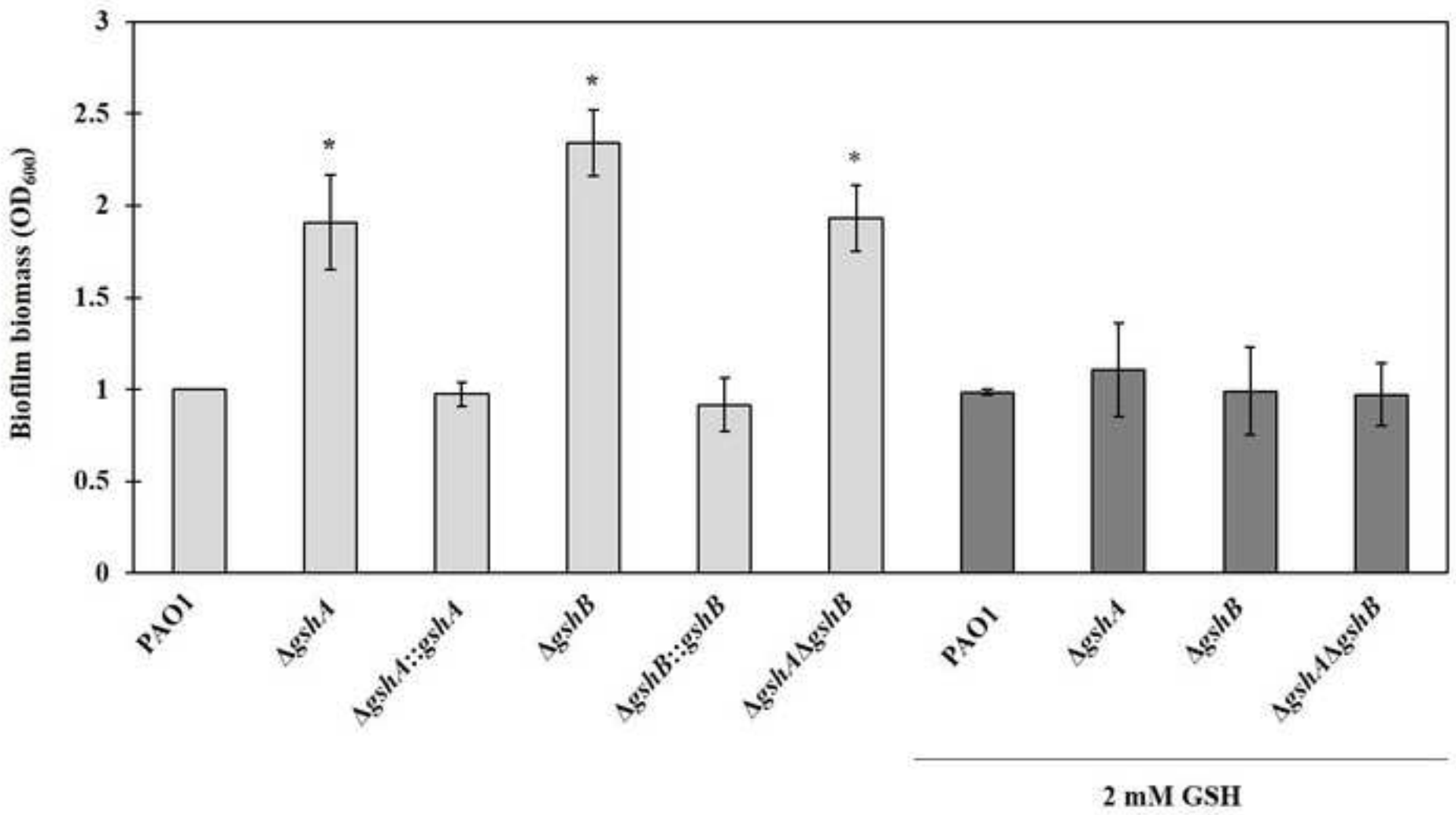

Figure 7

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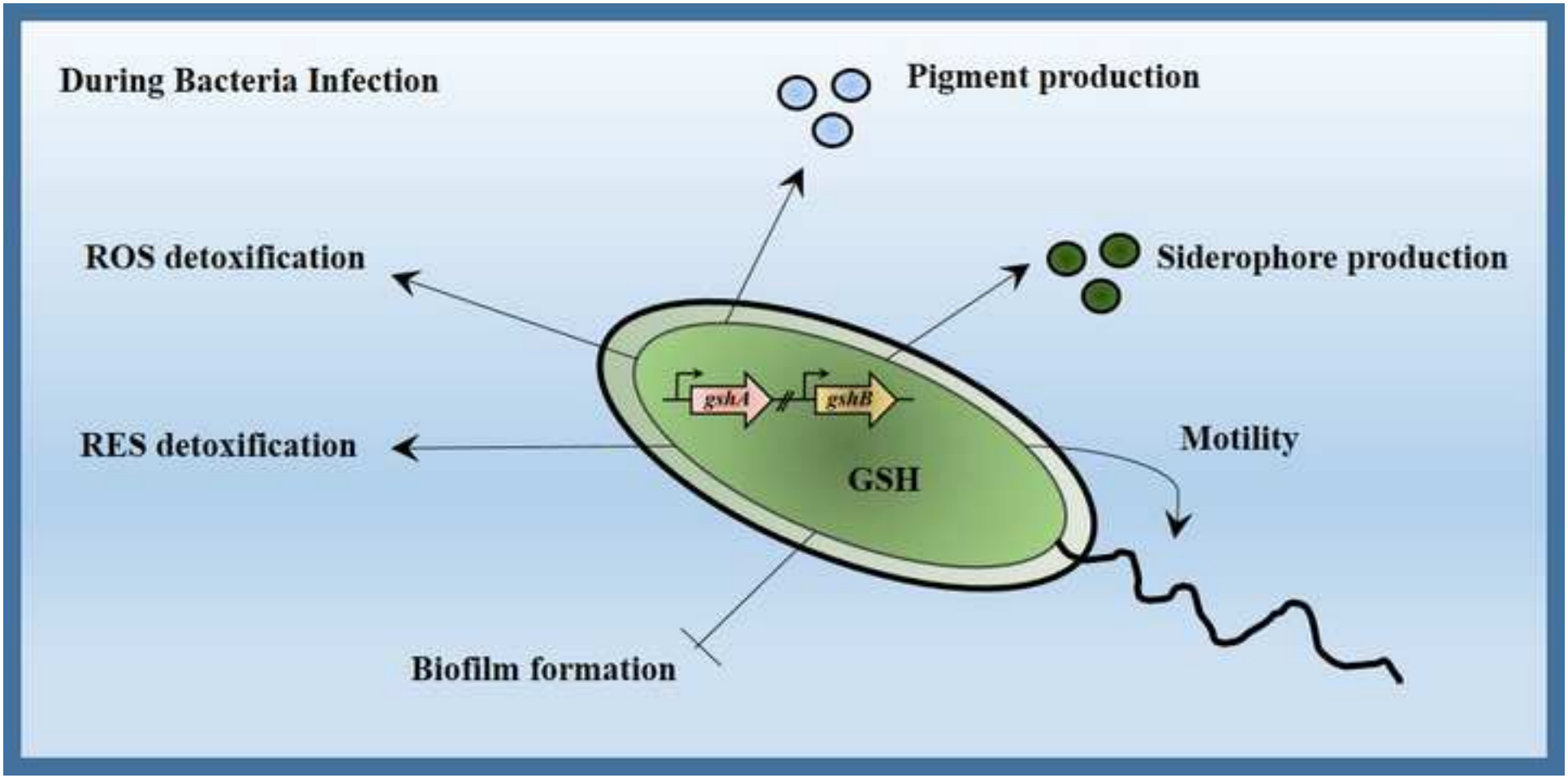


Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400 Thailand
☎: 662 203 6333 Fax: 662 354 9169

6th February 2017

To: Dr Adisak Romsang

Re: Speaker for Micro Research Seminar (Microbiology department, Mahidol-Oxford Tropical Medicine Research Unit)

Dear Dr. Adisak,

We are pleased to invite you to the "Micro seminar 2017" which will be held at the 60th Anniversary Chalermprakiat Building Similan 1-2 room on 8th March 12.30 - 13.30 at the Mahidol-Oxford Tropical Medicine Research Unit.

It is an honour to invite you to participate in this Seminar as the main Speaker. We believe that your work and your contribution to this field will be of great benefit to our colleagues.

We look forward to hearing from you. Kindly reply to Ms. Suparat Giengkam at suparat@tropmedres.ac

Yours faithfully,

Professor Nicholas Day
Director
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine
Mahidol University

นักศึกษาทุนฯ นักศึกษาวิทยาศาสตร์ และผู้สนใจทั่วไปเข้าร่วมกิจกรรม Junior Science Club ครั้งที่ 1/2559 ในหัวข้อ "Multidrug resistance in our life" โดยวิทยากรพิเศษ ดร. อดิศักดิ์ ร่มแสง ภาควิชาเทคโนโลยีชีวภาพ ในวันพุธที่ 14 กันยายน 2559 เวลา 13.30 – 16.30 น. ณ ห้อง K-102 อาคารเฉลิมพระเกียรติ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล พญาไท

Activation of Reactive Chlorine Species-mediated stress response protein through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*

Adisak Romsang[1,2,*], Nannipa Phuphuripan[1], Jintana Duang-nkern[3], Benya Nontaleerak[1], Khwannarin Khemsom[1], Skorn Mongkolsuk[1,2,3,4]

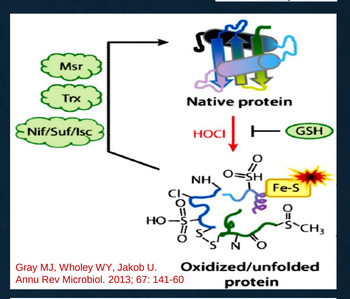
Mahidol University
Faculty of Science
Department of Biotechnology

[1]Department of Biotechnology; [2]Center for Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok 10400, Thailand;
[3]Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, Thailand; [4]Center of Excellence on Environmental Health and Toxicology (EHT), Ministry Of Education, Thailand
***Correspondence e-mail**: adisak.rom@mahidol.ac.th

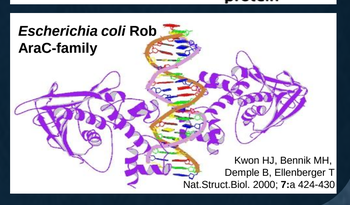
Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen that causes severe infections in patient with a long-term hospitalized environment. One of the major factors for successful infection is the bacterial defense mechanisms against Reactive Oxygen Species and **Reactive Chlorine Species (RCS)** generated from host immune system and disinfectant used in hospitals.

RcsR is a RCS-specific transcriptional regulator that regulates the nearby genes involved in the RCS stress response. *P. aeruginosa* genome contains several genes participated during RCS exposure such as Ohr, Msr and Rcs, which are our interests.



Gray MJ, Wholey WY, Jakob U. Annu Rev Microbiol. 2013; 67: 141-60



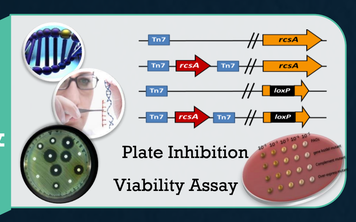
Kwon HJ, Bennik MH, Demple B, Ellenberger T Nat.Struct.Biol. 2000; 7:a 424-430

Objectives

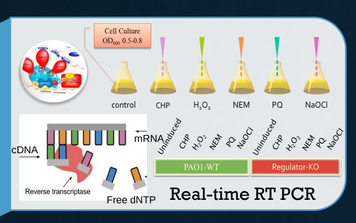
- To study the physiological function of *P. aeruginosa* genes encoding RCS-involved transcriptional regulator and its target
- To characterize the gene expression and gene regulation under RCS stress
- To purify the RcsR proteins and to characterize their properties
- To identify the DNA-binding site of RcsR and its key amino acids for gene activation

Methodology

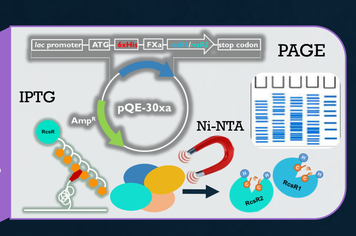
Strain construction & Physiological role analysis



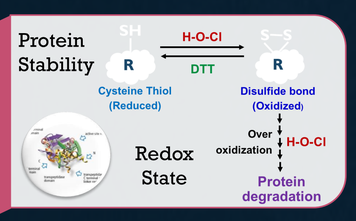
Gene expression & regulation



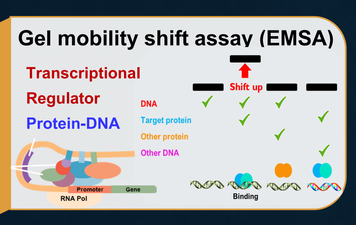
Protein expression & purification



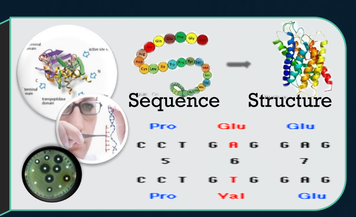
Protein characterization



DNA-binding site of RcsR



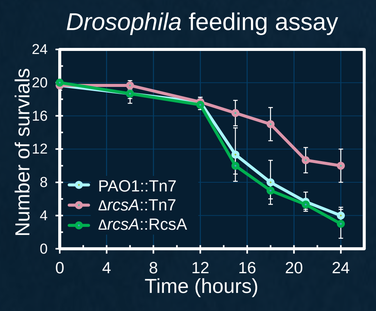
Site-directed mutagenesis

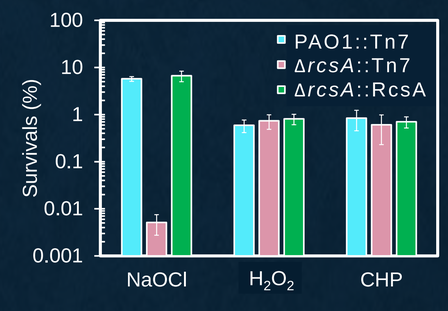


Results and Discussion

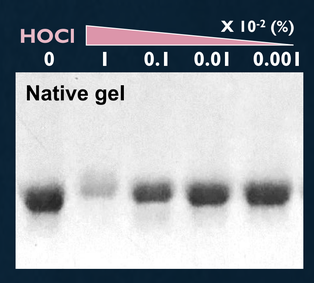
Physiological study

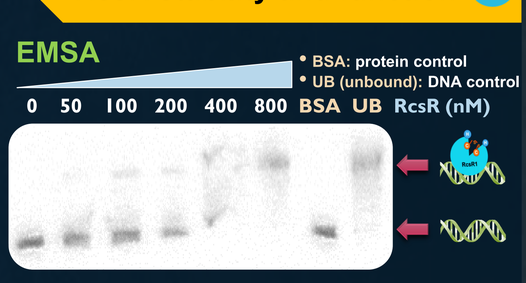
Drosophila feeding assay



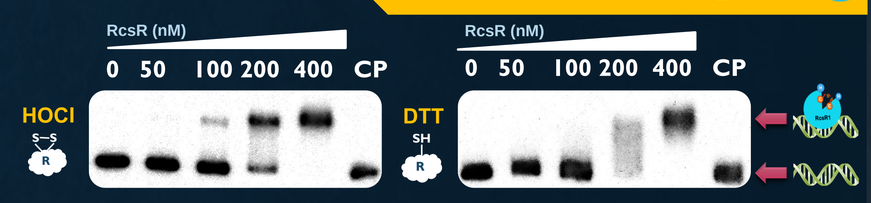


RcsR stability and function



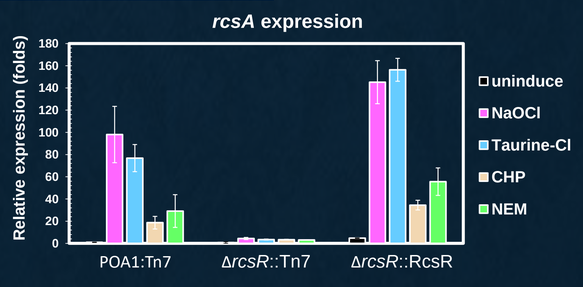


Oxidized RcsR-binding DNA

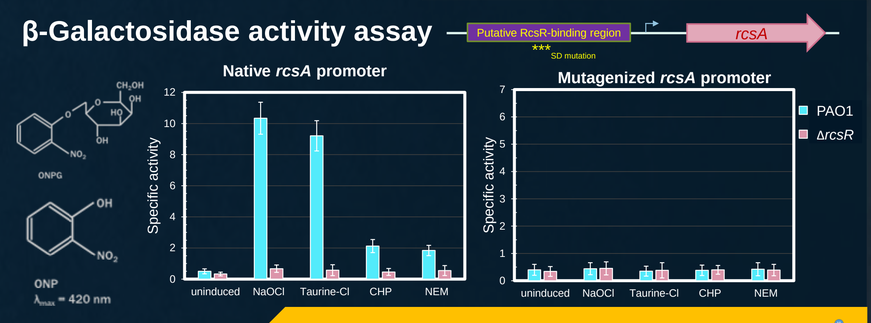


Gene expression and regulation

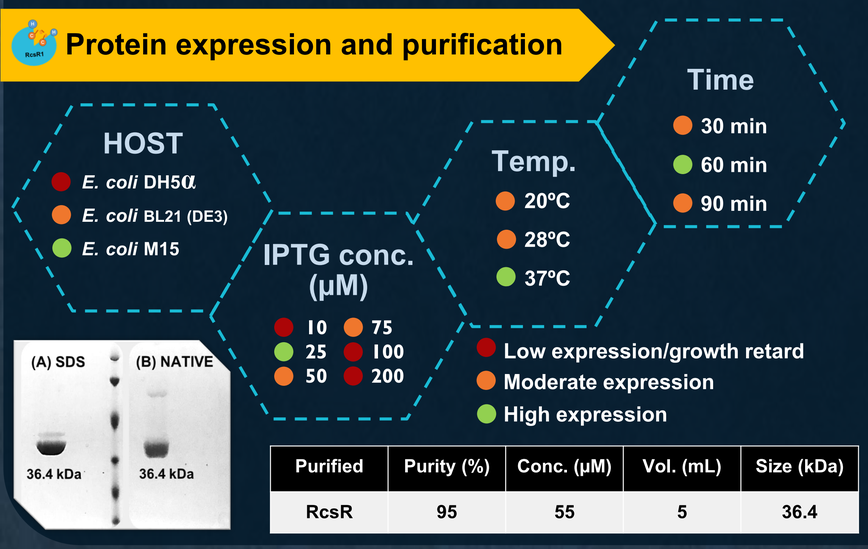
Gene	*rcsA*	*rcsR*
Untreated	1.00	1.00
NaOCl	98.03	5.64
Taurine-Cl	78.33	4.58
H_2O_2	2.01	1.81
tBH	21.01	3.27
CHP	23.67	4.09
PB	1.08	1.06
PQ	1.86	2.16
MD	1.01	0.98
NEM	32.09	3.78



RcsR-binding DNA motif

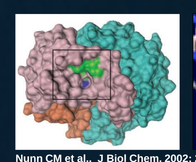


Protein expression and purification



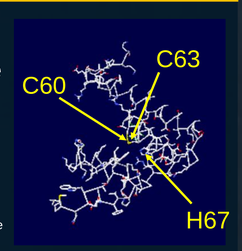
Purified	Purity (%)	Conc. (μM)	Vol. (mL)	Size (kDa)
RcsR	95	55	5	36.4

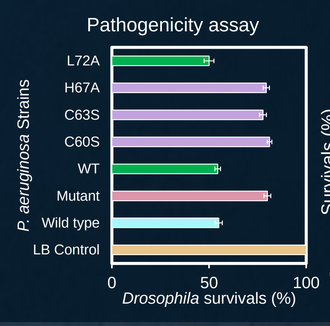
Key amino acids in RCS response

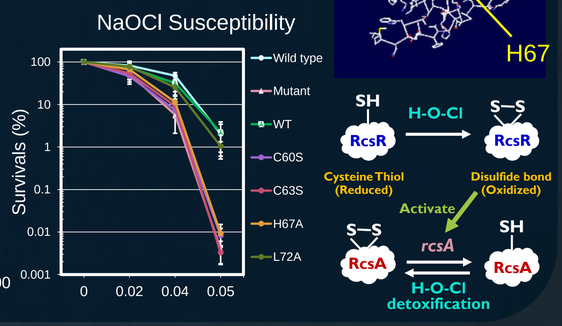


Nunn CM et al., J Biol Chem. 2002; 277(22): 20033-40.

Narrow groove
Cysteine & Histidine for interacting with RCS molecule

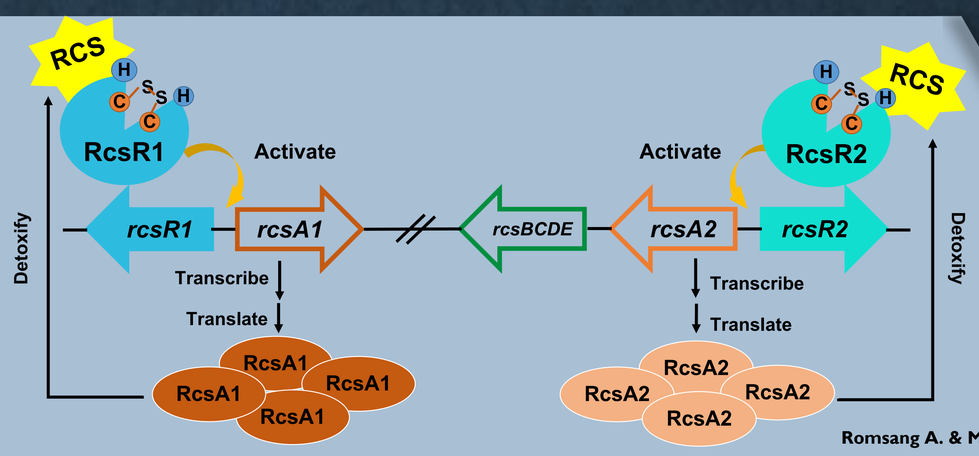






Conclusion

Overall results indicated the molecular mechanism of proteins, RcsR, the RCS-sensing transcriptional regulators in order to activate their targeted genes in *Pseudomonas aeruginosa* genome. This could be a part of the reasons for *P. aeruginosa* persistence and successful infection in hospitals worldwide.



Romsang A. & Mongkolsuk S. (2017)

K610 Laboratory, Department of Biotechnology and Center for Emerging Bacterial Infections
Faculty of Science, Mahidol University
http://ajrscbt.wix.com/k610-scmu

This research was supported by grants from Faculty of Science, Mahidol University and the joint funding of OHEC (MU) and Thailand Research Fund (MRG5980047).

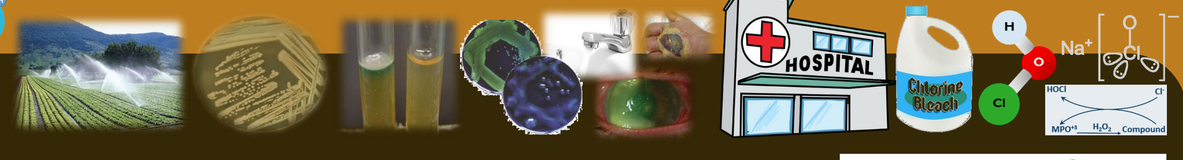
Reactive Chlorine Species-Mediated Stress Response through Transcriptional Regulators in a Human Pathogen *Pseudomonas aeruginosa* Involves in Nosocomial Infections

Adisak Romsang[1,2,*], Nannipa Phuphuripan[1], Jintana Duang-nkern[3], Benya Nontaleerak[1], Lampet Wongsaroj[4], Wachareeporn Trinachartvanit[5], Paiboon Vattanaviboon[2,3], Skorn Mongkolsuk[1,2,3,4]

[1]Department of Biotechnology; [2]Center for Emerging Bacterial Infections, [4]Molecular Medicine Graduate Program; [5]Department of Biology; Faculty of Science, Mahidol University, Bangkok 10400, Thailand; [3]Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210
***Correspondence e-mail**: adisak.rom@mahidol.ac.th

TRF-OHEC Annual Congress 2018
Jan 10-12, 2018 (4-6 PM) @ Grand Sema Hall
The Regent Cha-Am Beach Resort, Phetchaburi

Introduction



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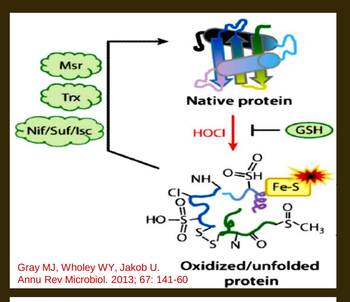

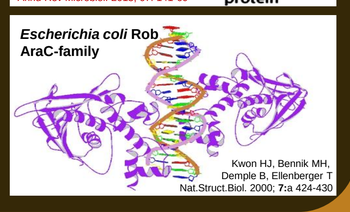

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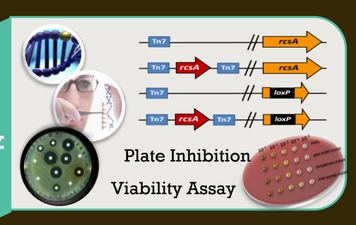
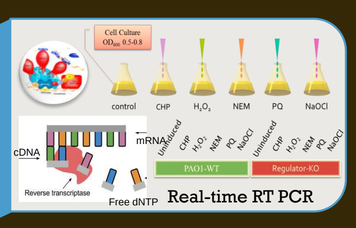
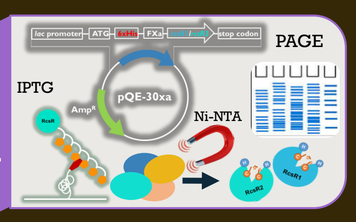


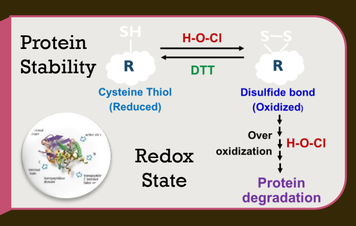
Gene expression & regulation



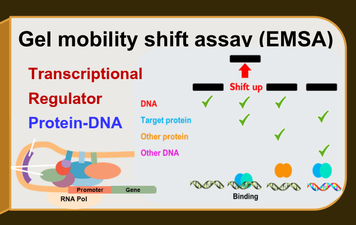
Protein expression & purification



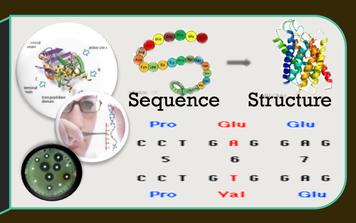
Protein characterization



DNA-binding site of RcsR

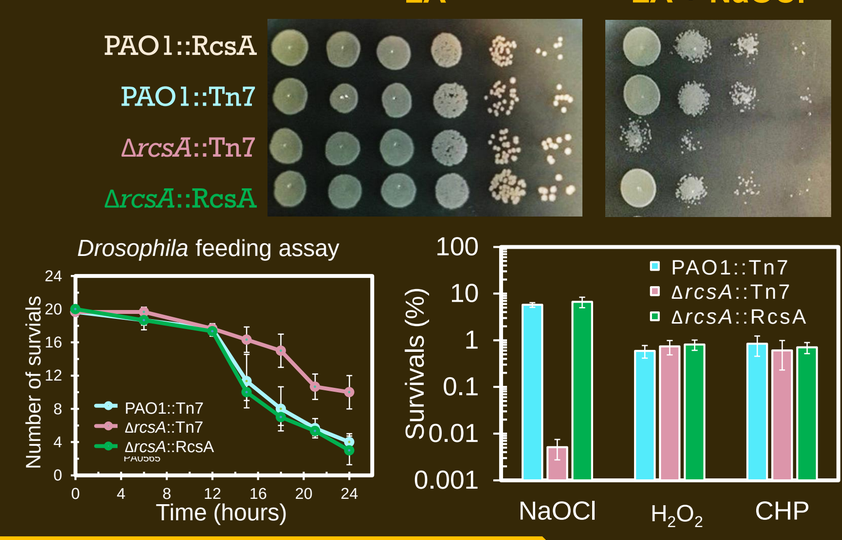


Site-directed mutagenesis

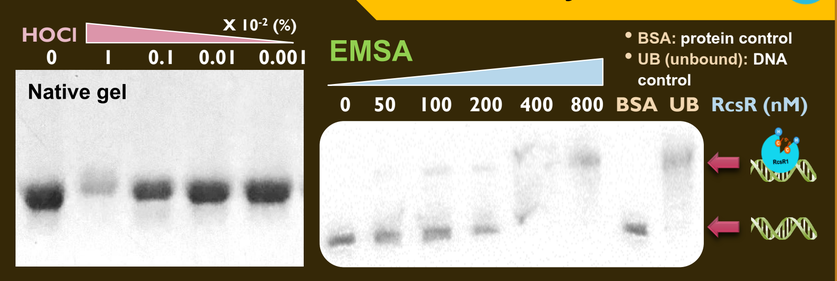


Results and Discussion

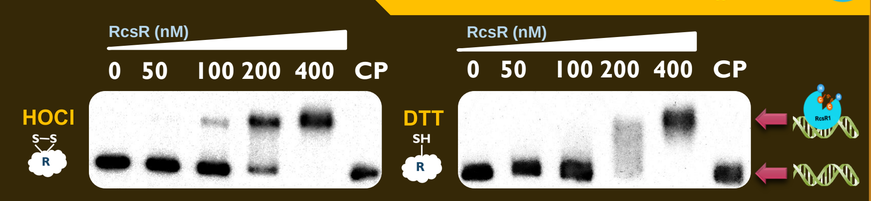
Physiological study



RcsR stability and function



Oxidized RcsR-binding DNA



Gene expression and regulation

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Untreated	1.00	1.00
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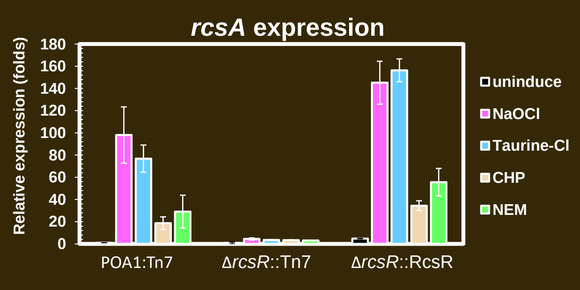
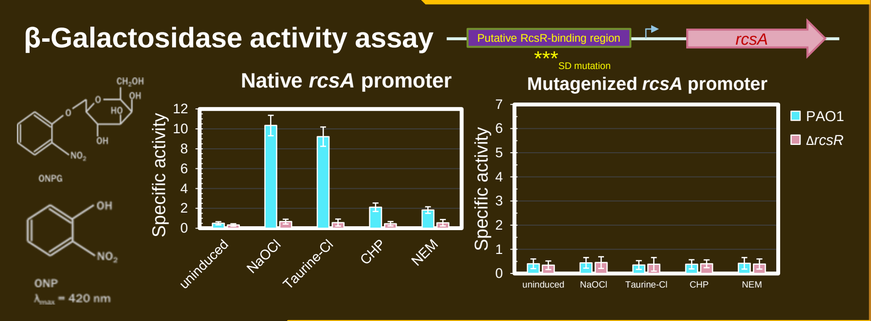
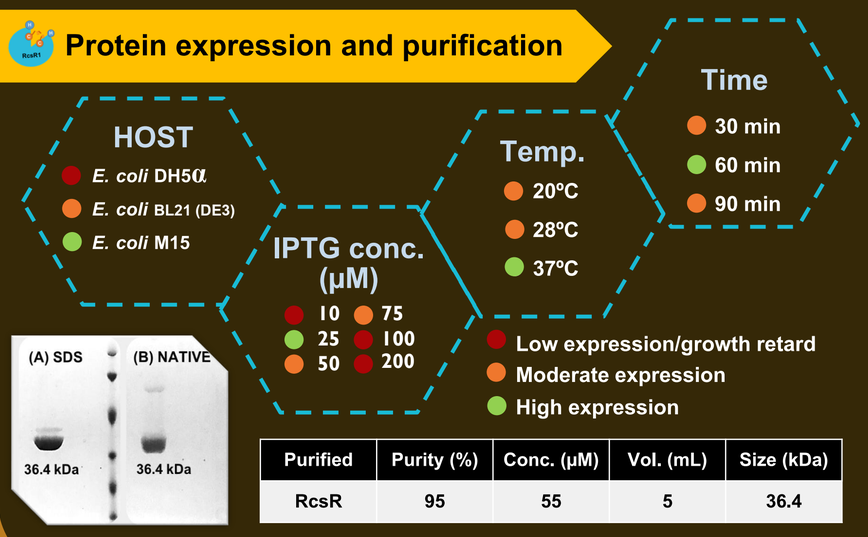
Taurine-Cl	78.33	4.58
H_2O_2	2.01	1.81
tBH	21.01	3.27
CHP	23.67	4.09
PB	1.08	1.06
PQ	1.86	2.16
MD	1.01	0.98
NEM	32.09	3.78



RcsR-binding DNA motif

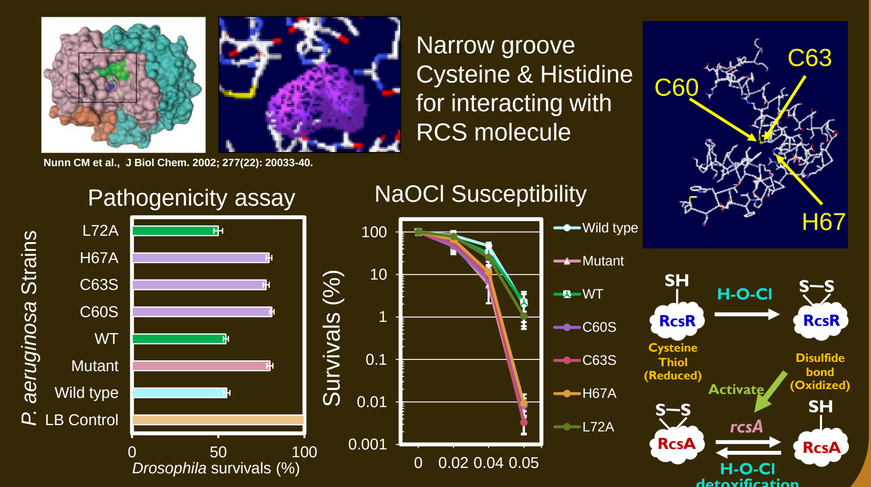


Protein expression and purification



Purified	Purity (%)	Conc. (μM)	Vol. (mL)	Size (kDa)
RcsR	95	55	5	36.4

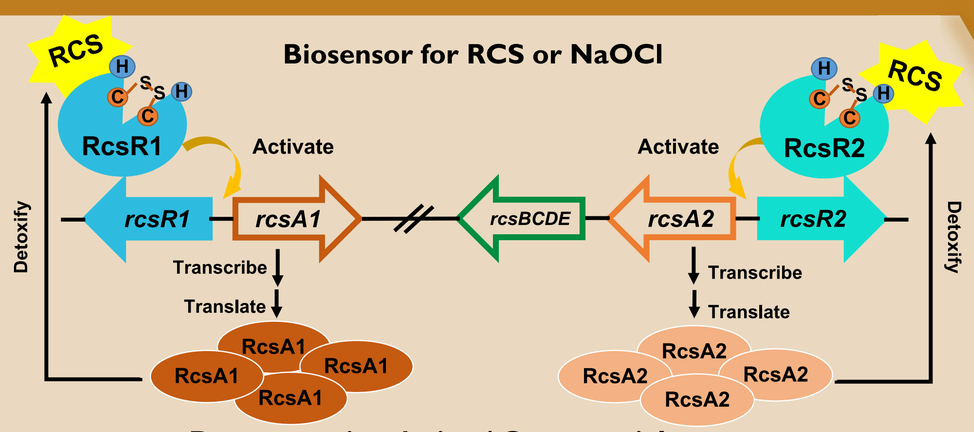
Key amino acids in RCS response



Conclusion

Overall results indicated the molecular mechanism of proteins, RcsR, the RCS-sensing transcriptional regulators in order to activate their targeted genes in *Pseudomonas aeruginosa* genome. This could be a part of the reasons for *P. aeruginosa* persistence and successful infection in hospitals worldwide.

Published output: Boonma S, Romsang A, Duang-nkern J, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. PLoS One 2017; 12(2): e0172071.



K610 Laboratory, Department of Biotechnology and Center for Emerging Bacterial Infections
Faculty of Science, Mahidol University
http://ajrscbt.wix.com/k610-scmu

Romsang A. and Mongkolsuk S. (2018)

Acknowledgement

This research was supported by a grant from the joint funding of OHEC (MU) and Thailand Research Fund (MRG5980047).

PA-33

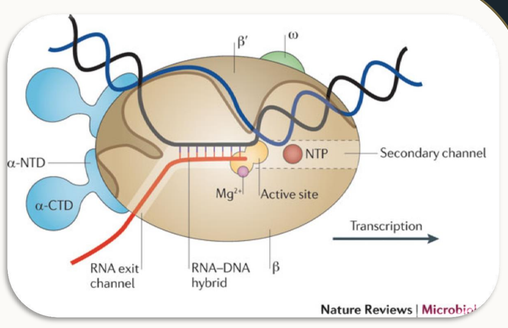
Characterization of a gene encoding AtfA-homolog factor against oxidative stress in *Pseudomonas aeruginosa*

Khwannarin Khemsom[1], Adisak Romsang[1,2], Jintana duang-nkern[3], Skorn Mongkolsuk[1,2,3]

[1]Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand, [2]Center for Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok 10400, Thailand, [3]Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, Thailand

Introduction

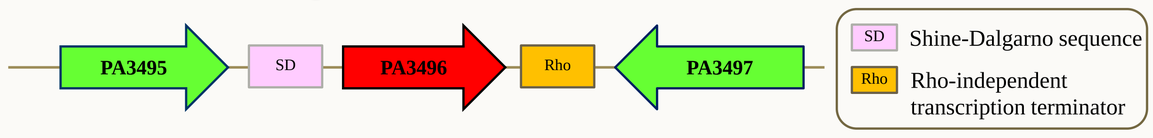
A small **acidic transcription factor A** (AtfA) is an RNA polymerase interacting protein that is widely distributed in γ-Proteobacteria. It has been shown to have an important role in multiple cellular processes including cell viability, motility, biofilm formation and antibiotic susceptibility [1].



The physiological function of this conserved protein has never been investigated in ***Pseudomonas aeruginosa***, a pathogenic bacterium classified in the γ-Proteobacteria.

P. aeruginosa is a ubiquitous bacterium that can be found in nature as well as in hospital and on medical equipment because it can tolerate antiseptic chemicals and need very few nutrients for surviving.

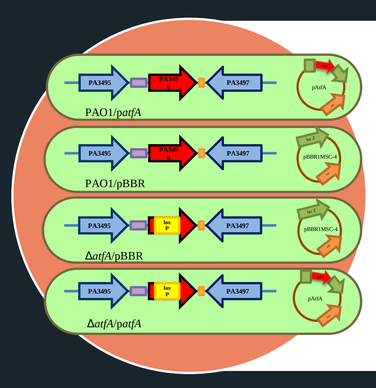
P. aeruginosa AtfA contains 59 amino acids with 180 nucleotides. It has 60% similarity to AtfA in *Acinetobactor* spp. and consists of an acidic properties (pI = 4.25). From *Pseudomonas* genome database, the *atfA* in *P. aeruginosa* PAO1 has its own ribosome binding site (Shine-Dalgarno sequence) and Rho-independent transcription terminator indicating its mono-cistronic transcript. In this study, the roles of *P. aeruginosa atfA* in response to stresses was investigated.



Objectives

- To construct the Δ*atfA* mutant by gene deletion technique
- To study physiology of Δ*atfA* mutant under oxidative stress and antibiotic exposure
- To investigate an *atfA* expression profile during cell growth

Methodology

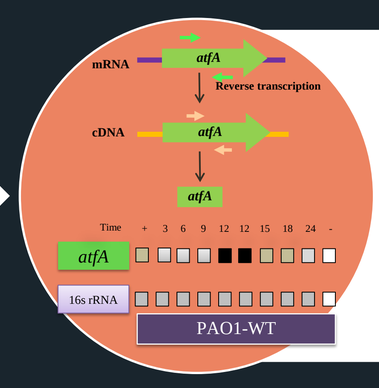


Strains construction

- Overexpressed strain
- Wild-type PAO1
- *atfA*-deleted mutant
- Complemented strain

Physiological study

- Plate sensitivity assay
- Disk diffusion assay
- Biofilm formation test



Gene expression

- RT-PCR

Results & Discussion

Part I: Bacterial strains construction

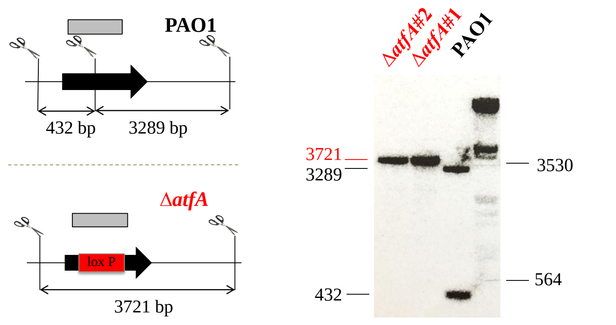


Fig 1. Southern analysis of Δ*atfA* by MscI digestion using *atfA* PCR product as DNA probe and Lambda DNA marker

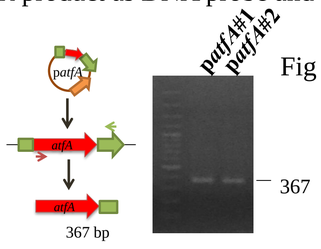


Fig 2. Confirmation of *atfA*-containing plasmid by PCR, agarose gel electrophoresis and DNA sequencing (data not shown)

Part II: Physiological study of Δ*atfA* mutant in *P. aeruginosa*

Plate sensitivity assay

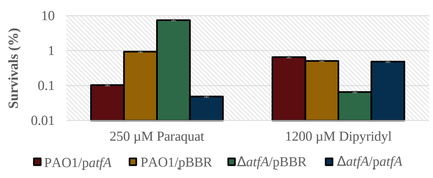


Fig 3. Susceptibility profile under stress of *P. aeruginosa atfA*-mutant strains

Biofilm formation

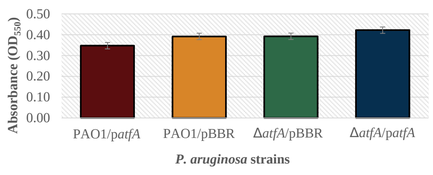


Fig 4. The ability of *P. aeruginosa atfA*-mutant strains to form biofilms

Antibiotic disk diffusion assay

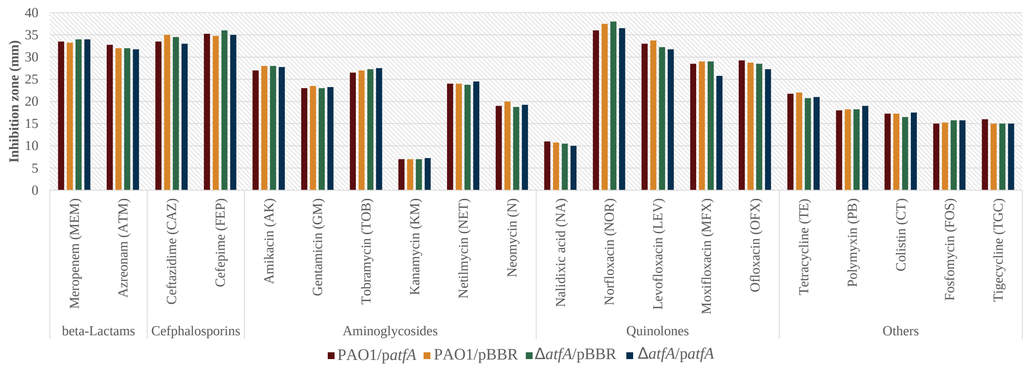
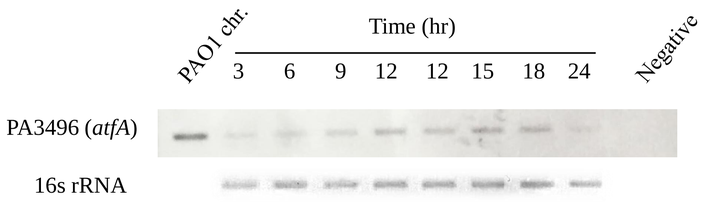


Fig 5. Determination of antibiotic susceptibility in *P. aeruginosa atfA*-mutant strains

Alteration of an *atfA* expression as shown in gene deletion and overexpression affected on cell susceptibility under stresses. In contrast, *atfA* is not required for biofilm formation and antibiotic susceptibility indicating that *atfA* in *P. aeruginosa* plays different roles in stress response compared to that in *Acinetobactor* spp.

Part III: RNA analysis



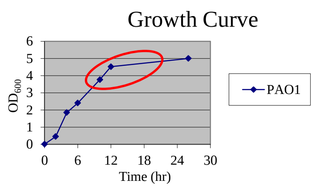
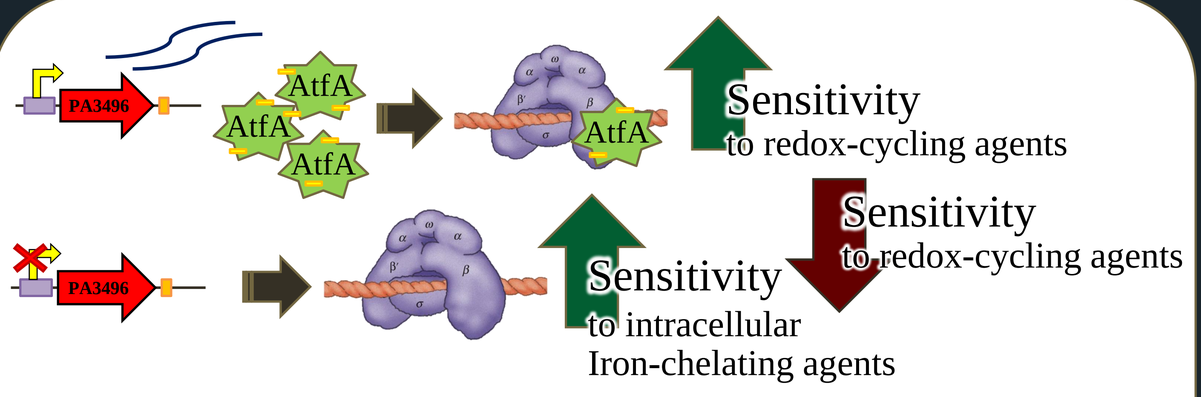


Fig 6. Endpoint RT-PCR analysis of *atfA* expression during bacterial growth and 16s rRNA as control

The expression profile of *atfA* during cell growth suggesting a highly *atfA* expression in late exponential and stationary phases.

The results indicated that, *atfA* is not required for cell growth and proliferation. It may be necessary for detoxification.

Conclusion



This finding exhibited a novel biological function of the transcription factor AtfA in response to oxidative stress in *Pseudomonas aeruginosa*.

Acknowledgements

This research was supported by grants from Faculty of Science, Mahidol University, Chulabhorn Research Institute, Scholarship for Young Scientists 2015, SCMU (to K.K.), and the joint funding of OHEC and TRF, MRG5980047 (to A.R.).

Reference

1. Withers R, Doherty GP, Jordan M, Yang X, Dixon NE, Lewis PJ. AtfA, a new factor in global regulation of transcription in *Acinetobacter* spp. *Molecular Microbiology* 2014; 93(6): 1130–1143.

Role of *aspA* a gene encoding aspartate ammonia lyase in *Pseudomonas aeruginosa*

PA-35

***Lampet Wongsaroj*[1], Kritsakorn Saninjuk[2], Adisak Romsang[2,3], Wachareeporn Trinachartvanit[4], Skorn Mongkolsuk[2,3,5]**

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[2]*Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand*
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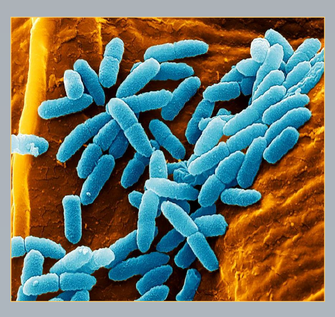
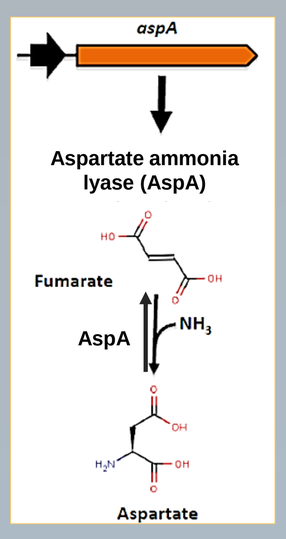
Abstract

Pseudomonas aeruginosa (PAO1) is an opportunistic human pathogen. The aspartate ammonia lyase (*aspA*) is an enzyme involving amino acid metabolism and acid tolerance in *P. aeruginosa*. In this study, Δ*aspA* deletion mutant was constructed. Plate sensitivity assay was performed to observe phenotype of the mutant under oxidative stress conditions. The Δ*aspA* mutant was sensitive to oxidants compared to the PAO1 wild type. The pH changes in the presence of aspartate in different strains were tested by using phenol red test to confirm that AspA deaminates aspartate to produce ammonia thereby increasing the pH. The ammonia production in Δ*aspA* mutant was decreased compared to that in the wild-type. Moreover, the pathogenicity testing using fruit fly was performed. Overall data suggest that Δ*aspA*, a gene encoding aspartate ammonia lyase, plays a role in oxidative and acid stress response in *P. aeruginosa*.

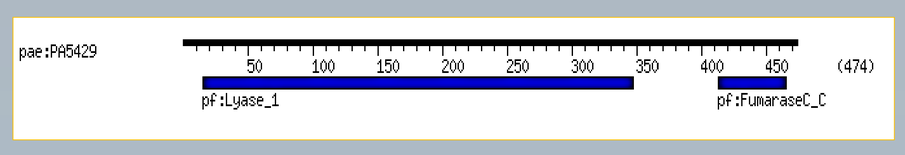
Introduction



Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen, which causes serious problems in bloodstream, urinary tract, surgical wound and pneumonia in hospitalized patients. During the infection, this pathogen is exposed to oxidative and acid stress conditions that are generated by host defense system. The ability to encounter host defense of *P. aeruginosa* is a key process for survival.

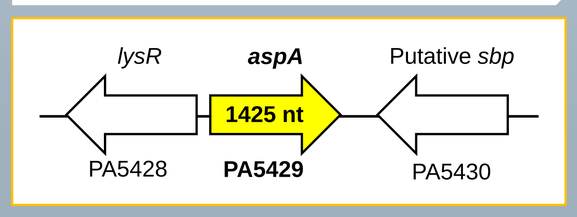


Aspartate ammonia lyase (AspA; EC 4.3.1.1) is an enzyme that catalyzes the deamination of aspartate to form fumarate and ammonia. The AspA is an enzyme involving amino acid metabolism and acid tolerance (Hu et al., 2010).



Motifs: Pfam:Lyase_1 Lyase
Pfam:FumaraseC_C Fumarase C C-terminus

Methods



Mutant construction

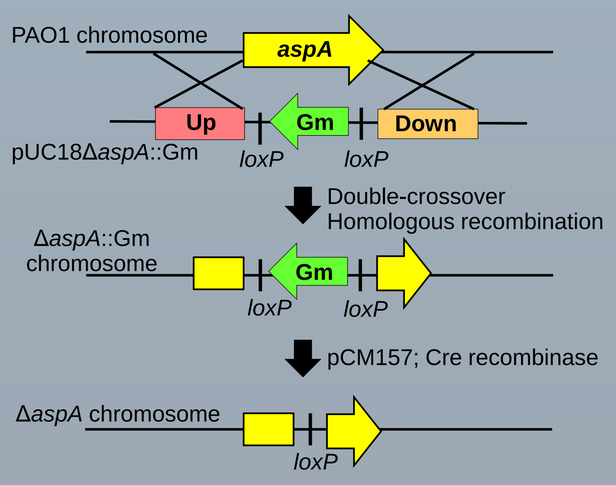


Plate sensitivity assay

Cells were serially diluted and spotted on LB agar containing various oxidants.

Complementation

Full-length *aspA* gene was amplified and cloned into pBBR1MSC-4

⬇ Electroporation

Transformed into Δ*aspA* mutant, generating Δ*aspA*/pBBR-A*spA*

Phenol red test

10^8 bacteria were resuspended in phenol red buffer and incubated at 37ºC. Pictures were taken after 18 h.

Pathogenicity Test

Flies D. Melanogaster were infected with *P. aeruginosa* strains. After18 h post-infection, flies were measured mortality.

Results and Discussion

The Δ*aspA* mutant was more sensitive to oxidants and antibiotics when compared to that in the PAO1 wild type. The complemented strains showed the phenotypic restoration compared to the mutant.

Figure 1 Plate sensitivity assay of *aspA* mutant and its complementation with pBBR-*AspA* against hydrogen peroxide (H_2O_2), paraquat (PQ), neomycin (Neo) and kanamycin (Km).

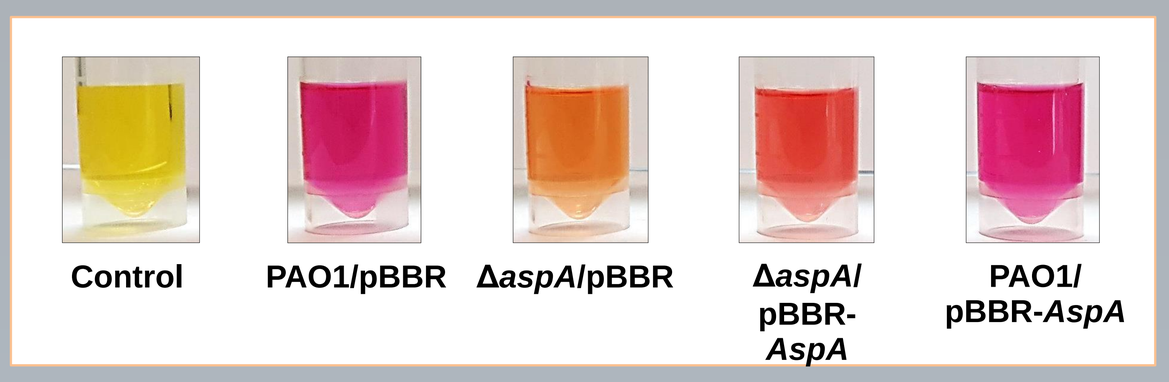


Figure 2 Test of pH changes by phenol red assay in the presence of aspartate

Phenol red is a pH indicator, which turns from yellow to red when pH goes from acidic to alkaline. The buffer color was changed from yellow to pink in wild type, indicating AspA deaminates aspartate to produce ammonia and thus increased the pH. Partial pH change was detected in Δ*aspA*.

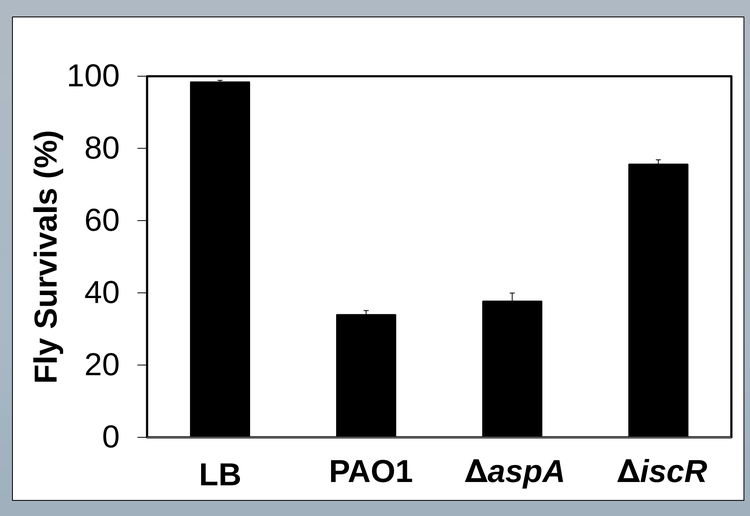


Figure 3 Virulence of the mutant was measured based on the mortality of *Drosophila* after 18 h infection. PAO1 and the iscR mutant were used as controls.

The Δ*aspA* decreased percentage of fruit fly survival as similar to that in the PAO1. This suggested that the *aspA* may not be require for bacterial virulence in *P. aeruginosa*.

Conclusion

A gene encoding aspartate ammonia lyase, *aspA*, plays a role in oxidative and acid stress response, and antibiotic resistance in *P. aeruginosa*.

Acknowledgements

This research was supported by grants from Faculty of Science, Mahidol University, Chulabhorn Research Institute, Royal Golden Jubilee Ph.D. (PHD/0132/2557), Thailand Research Fund (to L.W.), and the joint funding of OHEC and TRF (MRG5980047), Thailand (to A.R.).

Characterization of Reactive Chlorine Species-mediated stress response through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*

Adisak Romsang[a], Jintana Duang-Nkern[b], Lampet Wongsaroj[c], Wachareeporn Trinachartvanit[d], James M. Dubbs[b], Paiboon Vattanaviboon[b,e], Skorn Mongkolsuk[a,b,c,e]

[a]Department of Biotechnology, Faculty of Science, Mahidol University, Ratchathewi, Bangkok 10400, Thailand; [b]Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok 10210, Thailand
[c]Molecular Medicine Graduate Program, Faculty of Science, Mahidol University, Bangkok, Thailand; [d]Department of Biology, Faculty of Science, Mahidol University, Ratchathewi, Bangkok 10400, Thailand
[e]Center of Excellence on Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok, Thailand E-mail: adisak.rom@mahidol.ac.th and skorn.mon@mahidol.ac.th

Microbial Stress Response
Gordon Research Conference
July 17-22, 2016 Mount Holyoke College, MA

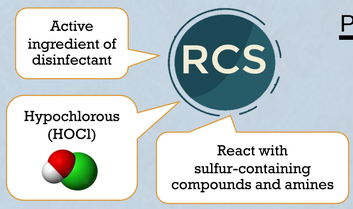
Abstract

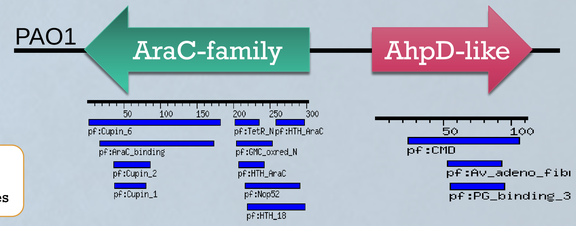
Pseudomonas aeruginosa is an important opportunistic pathogen that causes severe infections in hospitalized patient. The treatment difficulties are associated a long-term survival of the pathogens in the hospital environments. One of the major factors for successful infection is the bacterial defense mechanisms against **reactive oxygen species (ROS)** and **reactive chlorine species (RCS)** generated from host immune system.

In this study, *P. aeruginosa* gene encoding an AraC-family transcriptional regulator, PA0564, was characterized. Gene expression and physiological function analysis were shown that PA0564 bind to the promoter of a gene PA0565 encoding an AhpD-liked protein and activated the PA0565 expression in specifically response to RCS-mediated stress. The binding site of PA0564 on the PA0565 promoter region was identified by using β-galactosidase activity assay and site-directed mutagenesis. Moreover, deletion of PA0565 conferred the hypersensitivity against NaOCl, but not against any ROS-generated agent, in the PA0565 mutant compared to that in the wild-type PAO1. Finally, the virulence assay using *Drosophila melanogaster* model host system were performed to determine the RCS-mediated effect on the bacterial pathogenicity. It could be another specific RCS-sensing transcriptional regulator in *P. aeruginosa* and will expand the virulence network in *P. aeruginosa* that is a part of the reasons in its ability for hospitalization.

Introduction

Pseudomonas aeruginosa has become increasingly recognized as an opportunistic human pathogen in hospitals, especially in immunocompromized individuals. An ability of *P. aeruginosa* to resist antibiotics and reactive chlorine species, such as sodium hypochlorite (NaOCl), has permitted this organism to persist in hospitals.





Objective

To characterize the roles of *P. aeruginosa* genes encoding an AraC-family regulator and its targets in RCS-mediated stress response and pathogenicity

Methodology

Gene expression
- Real time RT-PCR
- Stress induction
- RCS and ROS

Gene regulation
- Real time RT-PCR
- Promoter-driven enzyme assay

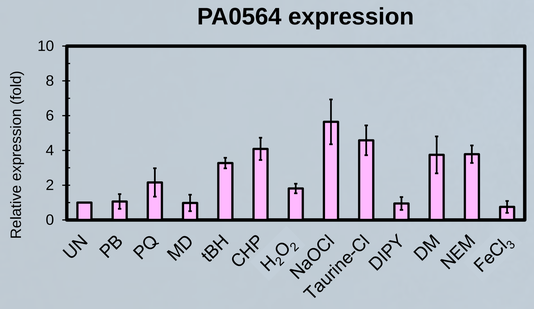
Strain construction
- Gene deletion
- Expression vector
- Site-directed mutagenesis

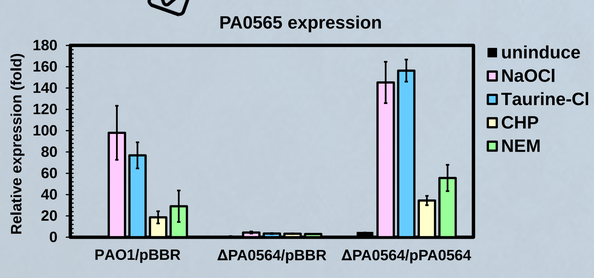
Physiological function against stresses
- RCS stress: NaOCl
- ROS stress

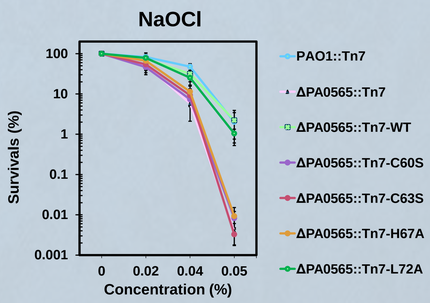
Pathogenicity test
- *Drosophila* model host system

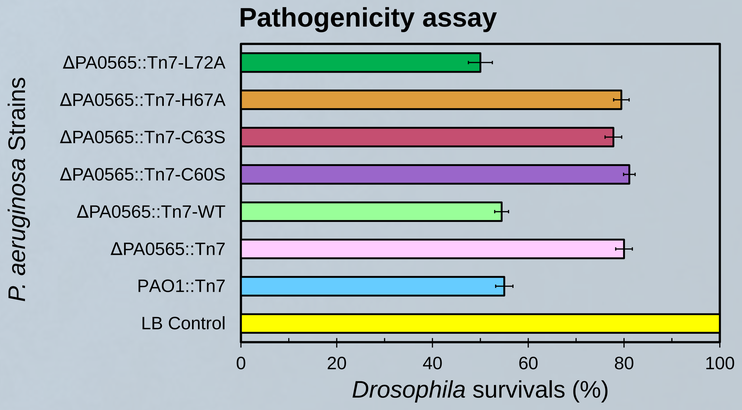
Antibiotic resistance
- Antibiotics
- Disc diffusion

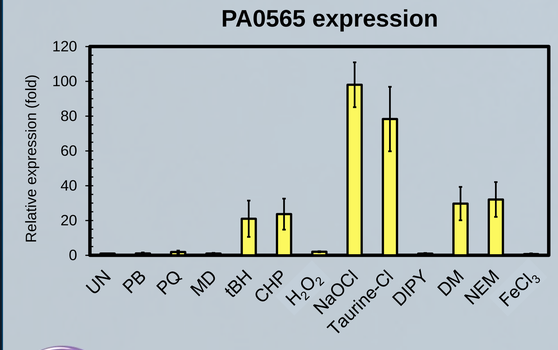
Results & Discussion





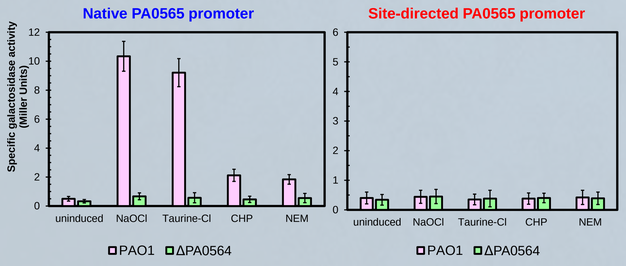


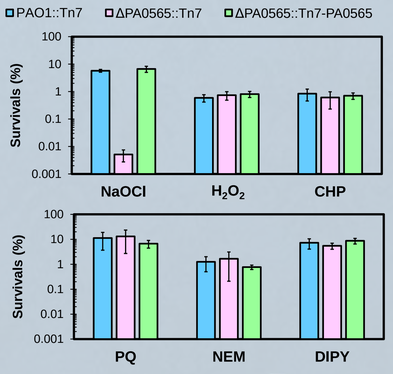




…PA0564
CGGTCGAGTGGGTC**CAT**GGGATGAGCCGATCAGGTCGAAAGTAGAGACGCATGGGCAGGCGGCGGTCCAGAC

AraC-binding site Shine-Dalgarno PA0565…
TGGTCGAGACTGGTAACCCGA*CGCGCCACTCCCGGCGCG*ACAGCAAC*GGAGA*ATATCACC**ATG**CTGAACAACTG
*CG**AAAA**ACTCCC**AAAA**CG*





Antibiotics	Conc (mg/L)	Inhibition zone (mm)		
		PAO1	ΔPA0564	ΔPA0565
Carbenicillin	100	20.0	20.0	20.0
Aztreonam	30	22.0	22.0	22.5
Imipenem	10	24.5	24.0	24.5
Ceftriaxone	30	16.0	16.0	16.0
Ceftazidime	30	21.5	22.0	22.0
Polymyxin B	300	15.0	14.5	15.0
Colistin	25	14.0	14.0	14.5

Antibiotics	Conc (mg/L)	Inhibition zone (mm)		
		PAO1	ΔPA0564	ΔPA0565
Gentamicin	10	12.0	12.0	12.5
Kanamycin	30	13.5	13.5	13.5
Norfloxacin	10	27.5	27.0	27.5
Ciprofloxacin	1	31.5	31.5	32.0
Levofloxacin	5	25.0	25.0	25.0
Tetracycline	30	15.0	15.0	15.0
Fosfomyxin	50	12.5	12.5	13.0

Summary

Overall data showed that a NaOCl-induced PA0565 expression via an AraC-family transcriptional regulator (PA0564) control contributes to ability of *Pseudomonas aeruginosa* for surviving in RCS-generated stress. Cysteine at position 60 and 63, and histidine at position 67 of PA0565 were important for fully function in protecting bacteria when expose to RCS-mediated agent. Moreover, PA0565 was required for pathogenicity during *Drosophila* model host infection. This is one of potential mechanisms that allow this bacterium to persist in the hospitals.

Acknowledgment

This research was supported by Faculty of Science and Faculty of Graduate Studies, Mahidol University. AR was supported by the Thailand Research Fund (MRG5980047) with SM as his mentor. The authors thank Dr. Mayuree Fuangthong, Dr. Rojana Sukchawalit, and members in K610 lab at SCMU and 805C lab at CRI for their valuable advices.

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ROLES OF PSEUDOMONAS AERUGINOSA NFUA IN STRESS CONDITIONS, BACTERIAL VIRULENCE AND REGULATION

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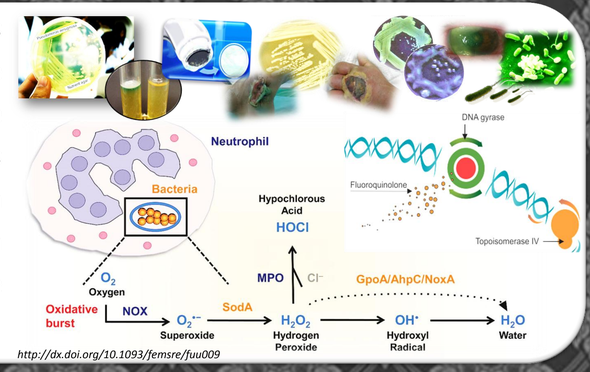
PC8 ENVIRONMENTAL HEALTH: INTER-LINKAGES AMONG THE ENVIRONMENT, CHEMICALS AND INFECTIOUS AGENTS
November 13 - 17, 2016, Shangri-La Hotel, Bangkok, Thailand
Organized by the Chulabhorn Research Institute

Introduction

Iron sulfur [Fe-S] cluster is a cofactor of many proteins that participate in redox reactions. In prokaryote, there are at least three distinct [Fe-S] clusters assembly systems, including Isc (iron sulfur cluster), Suf (sulfur formation) and Nif (nitrogen fixation). These three systems share a common feature in the scaffold proteins that mobilize sulfur atoms from a cysteine desulfurase and iron atoms from an iron donor to synthesize a cluster. Fluoroquinolone antibiotics are bacterial DNA gyrase inhibitors with efficacy against *Pseudomonas aeruginosa*. The mechanism of bacterial cell death from fluoroquinolones is partly due to the increased generation of hydroxyl radicals ($OH^{\cdot}$). During infection, superoxide anions, which are a part of host defense response, can oxidize [Fe-S] cluster-containing proteins resulting in a release of free irons, which lead to the production of $OH^{\cdot}$ via the Fenton reaction. *P. aeruginosa* is an opportunistic human pathogen and wildly distributed in soil and water. It is resist to many antibiotics. We here investigated the role of *nfuA* in the susceptibility of *P. aeruginosa* to oxidative stress and fluoroquinolone antibiotics and identified the important domain in NfuA function.



http://dx.doi.org/10.1093/femsre/fuu009

Methodology

Strains Construction
- Clean deletion
- Transposon
- Site directed mutagenesis

Antibiotic Test
- Disc diffusion assay
- Minimum Inhibitory Concentrations

Physiological Function
- Plate sensitivity assay
- Growth observation

Pathogenicity Assay
- *Caenorhabditis elegans* model host system

Gene Expression and Regulation
- Real time RT-PCR
- EMSA

Results and Discussion

Identification of *nfuA* in *P. aeruginosa* genome

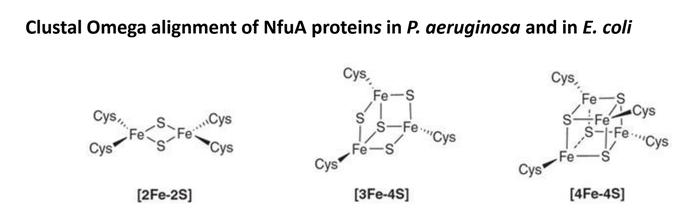
```
                                          Cys43  Cys47
E.coli          --MIRISDAAQAHFAKLLANQ-EEGTQIRVFVINPGTPNAECGVSYCPPDAVEATDTALK
P.aeruginosa    MSAITITEAAQAYLAELLEKQSTPGIGIRIFITQPGTQYAETCIAYCKPGEEKVEDTAIA
                   * *:.****:.:*:** :*   *  **:*: :***  **  ::** *.  :. ***:

E.coli          FDLLTAYVDELSAPYLEDAEIDFVTDQLGSQLTLKAPNAKMRKVADDAPLMERVEYMLQS
P.aeruginosa    LKDFTAWIDAVSEPFLEDAVVDYATDRMGGQLTIKAPNAKVPMVNEDSPITERINYYLQT
                :. :**:.* :* *:**** :*:.**::*.***:******:  * :*:*: **:.* **:

                                  Cys152  Cys155
E.coli          QINPQLAGHGGRVSLMEITEDGYAILQFGGGCNGCSMVDVTLKEGIEKQLLNEFPELKGV
P.aeruginosa    EINPGLASHGGQVSLVDVVEDNIAVLRFGGGCQGCGMVDMTLKDGVEKTLIERIPELKGV
                :*** **.***:***:::.**. *:*:*:*****:**.***:***:*:** *:.:******
                                  CXXC motif
E.coli          RDLTEHQRGEHSYY
P.aeruginosa    RDVTDHSNKENAYY
                **:*:*.. *::**
```

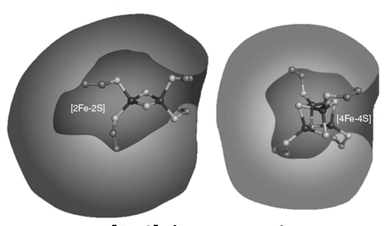
Clustal Omega alignment of NfuA proteins in *P. aeruginosa* and in *E. coli*

The results from Clustal Omega alignment of *P. aeruginosa* NfuA showed 52% identity to NfuA from *E. coil* and revealed the two functional cysteine residues (CXXC motif) that coordinate with [Fe-S] cluster in the assembly mechanism are conserved at Cys43-Cys47 and Cys152-Cys155.



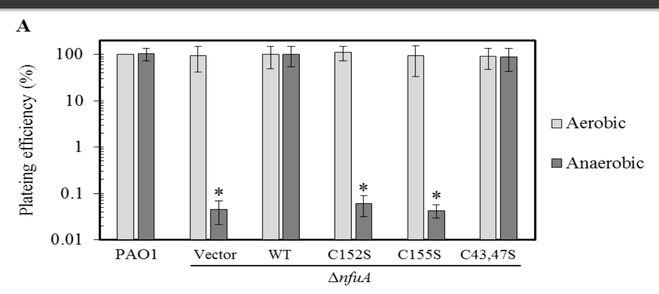
Structure of canonical [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters

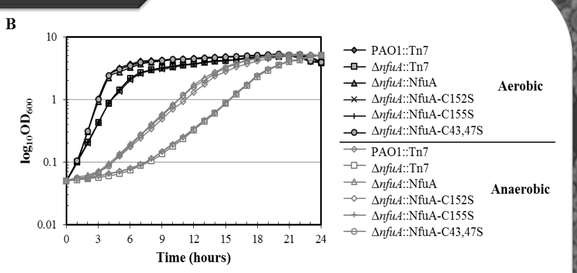
http://www.nature.com/nchembio/journal/v2/n4/fig_tab/nchembio0406-171_F1.html



[Fe-S] clusters protein

P. aeruginosa growth under aerobic and anaerobic conditions



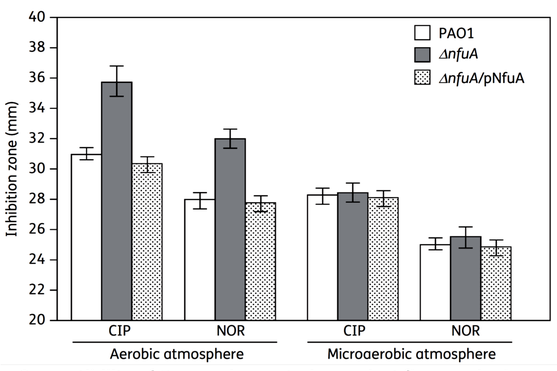


P. aeruginosa strains were spread onto plates containing 1% KNO_3 and incubated under aerobic and anaerobic conditions. The plating efficiency of Δ*nfuA* was reduced more than 10^3-fold relative to that of PAO1 wild type. This indicates that *nfuA* was required for *P. aeruginosa* growth especially under anaerobic condition. The U-type domain of NfuA plays an important role for the phenotypes.

The growth of *P. aeruginosa* strains in LB broth supplemented with 1% KNO_3 under aerobic and anaerobic conditions was determined.

The Δ*nfuA* mutant showed a growth retardation compared to PAO1 indicated the contribution of *nfuA* to normal aerobic growth of *P. aeruginosa*.

Antibiotics susceptibility test



Susceptibility of *P. aeruginosa* strains against fluoroquinolones

Strains	CIP (mg/L)	NOR (mg/L)
PAO1/Tn7	0.2	2
Δ*nfuA*/Tn7	0.05	0.5
Δ*nfuA*/NfuA	0.2	2
Δ*nfuA*/NfuA C152S	0.05	0.5
Δ*nfuA*/NfuA C155S	0.05	0.5
Δ*nfuA*/NfuA C43,47S	0.2	2

MICs of *P. aeruginosa* strains to fluoroquinolones

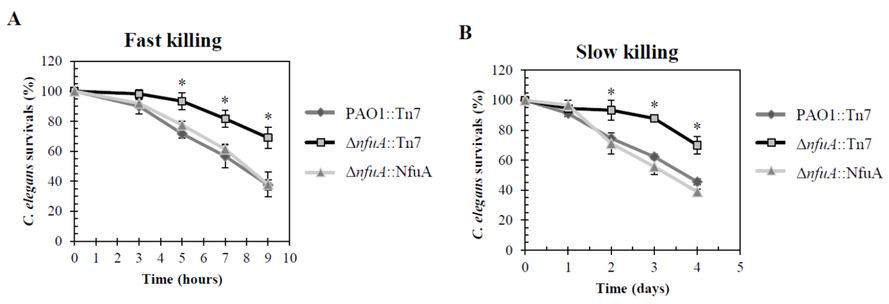
The Kirby-Bauer disk diffusion assay was performed. The results illustrated that the deletion of *nfuA* was more susceptible to fluoroquinolone antibiotics, including ciprofloxacin and norfloxacin than the PAO1.

We further investigated whether the increased susceptibility to fluoroquinolone antibiotics in the *ΔnfuA* involved antibiotic-induced ROS, by testing antibiotic susceptibility levels under microaerobic conditions. The fluoroquinolone susceptible phenotype of the Δ*nfuA* mutant was abolished when cells were grown under microaerobic condition.

It indicated that the *nfuA* is necessary for the protection of *P. aeruginosa* from fluoroquinolone antibiotic treatment in aerobic condition and oxidative stress participates in the increased fluoroquinolone susceptibility.

Pathogenicity test

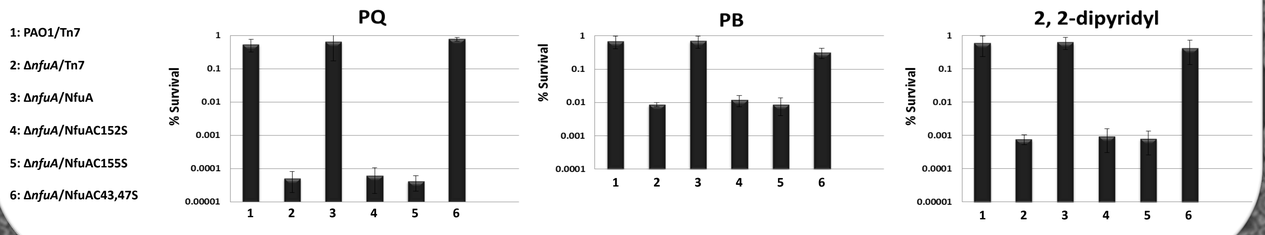
Fast (A) and slow (B) killing assays in *Caenorhabditis elegans* model host system were performed to determine the virulence of *P. aeruginosa* strains. Live worms was scored in hours for fast killing and in days for slow killing experiments. The Δ*nfuA* mutant was attenuated in virulence by showing a roughly two-fold reduction in its ability to kill *C. elegans* compared to PAO1 wild type. Thus, the *nfuA* contributes to a full virulence of *P. aeruginosa*.



Physiological function againts oxidative stresses

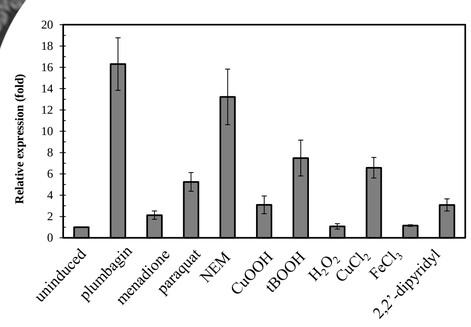
The resistance levels against substances of *P. aeruginosa* strains were determined using plate sensitivity assays in plates containing oxidants 250 µM paraquat (PQ), 2mM plumbagin (PB) and 1.2 mM 2, 2'-dipyridyl.

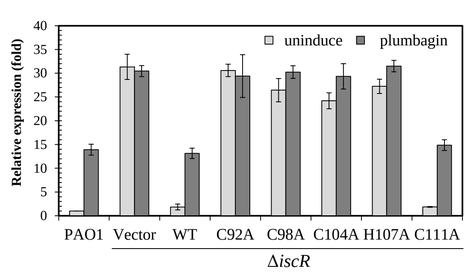
The Δ*nfuA* mutant reduced bacterial ability to cope with either oxidative stress or iron deprivation conditions. Site-directed mutagenesis analysis revealed that the conserved CXXC motif of the U-type domain, but not the A-type domain, was required for NfuA function.



Determination of the resistance levels against oxidative stress (PQ and PB) and iron deprivation in *P. aeruginosa* strains

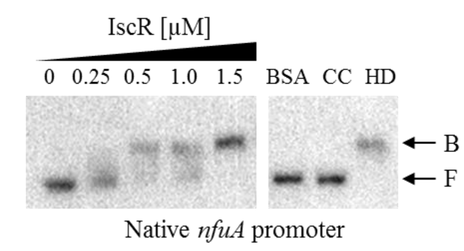
Gene expression and regulation





Real Time RT-PCR showing the *nfuA* expression during normal growth and in response to various oxidants. The expression of *nfuA* was induced in response to superoxide generators, thiol-depleting agent and iron depletion condition. The stress inducible pattern of *nfuA* expression is comparable to that of the *iscR* regulon, where the expression is mediated by IscR, a transcription regulator containing [Fe-S] cluster.

The expression of wild-type (WT) IscR in the Δ*iscR* mutant could repress the *nfuA* transcript to the level comparable to that of PAO1 and pretreatment with plumbagin induced high expression of *nfuA*. However, the expression of IscR variant (C94A, C98A, C104A or H107A) failed to repress the *nfuA* expression in contrasted to IscR-C111A. The results support an idea that halo-IscR contributes to a repression of *nfuA* gene expression.

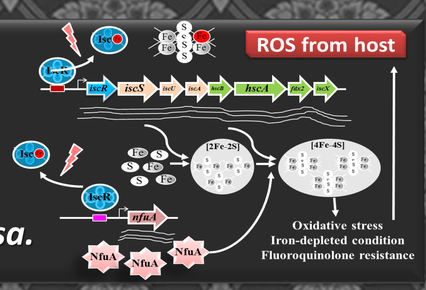


Electrophoretic mobility shift assay (EMSA) was performed using ^{32}P-labeled native *nfuA* promoter fragments and increasing concentrations of purified IscR. CC and HD represent an addition of unlabeled *nfuA* promoter and heterologous DNA. F and B indicate free and bound probes, respectively. The result showed that purified IscR was able to bind the *nfuA* promoter fragment.

This results supported the role of IscR as a transcriptional repressor for *nfuA* expression.

Summary

The *nfuA* involves in [Fe-S] cluster biogenesis and protects against fluoroquinolone antibiotics and oxidative stress as well as virulence in *P. aeruginosa*.



This research was supported by grants from Chulabhorn Research Institute, Mahidol University, and Center for Emerging Bacterial Infection MUSC.
A.R. was supported by the joint funding of OHEC and TRF (MRG5980047).

LYSR-TYPE TRANSCRIPTIONAL REGULATOR INVOLVES IN ANTIBIOTICS RESISTANCE AND PATHOGENESIS OF *PSEUDOMONAS AERUGINOSA*

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PA-34

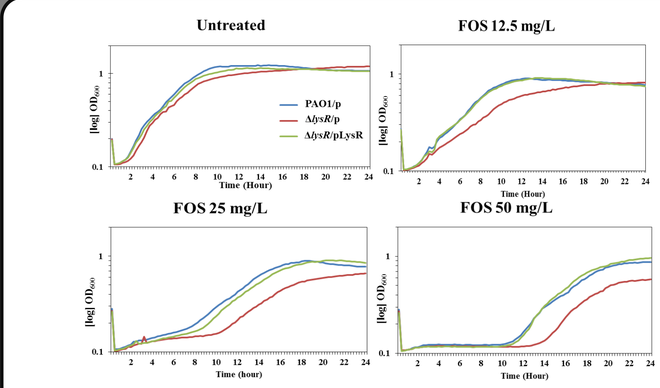
- The LysR-type transcriptional regulator family (LTTR) is highly conserved and being the largest family in prokaryotic DNA-binding protein. LTTRs control many biological pathways, including metabolism, quorum sensing, motility, oxidative stress responses, and virulence in pathogenic bacteria.
- *P. aeruginosa* (PA) genome contains many LTTR-encoded genes including *lysR*, which is annotated as a LysR-type transcriptional regulator.
- In this study, the *lysR*-deficient mutant was constructed from wild-type PAO1 strain. The mutant was more susceptibility to a broad-spectrum antibiotic than the PAO1 because LysR regulated the expression of gene encoding an antibiotic transporter that is homologous to glycerol-triphosphate transporter (GlpT). In addition, the mutant has an defect in pigment production, motility, and biofilm formation. The attenuated pathogenicity in the *lysR* mutant is observed.

LysR-type transcriptional regulator (LTTR) and *P. aeruginosa*

LTTR family is a group of transcriptional regulators and is highly conserved and ubiquitous among bacteria. LTTR is global transcriptional regulator, acting as either activators or repressor of single or operonic genes.

Pseudomonas aeruginosa is one of opportunistic pathogens of human, which causes of hospital-acquired infections with high mortality rate because of its inherited resistance to array of antibiotics and biocides, making it difficult to treat infections with this bacterium.

Antibiotics susceptibility of Δ*lysR* mutant



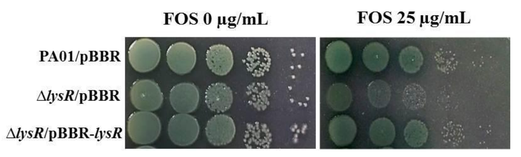


Figure 1. Growth of the Δ*lysR* mutant against various concentration of fosfomycin.

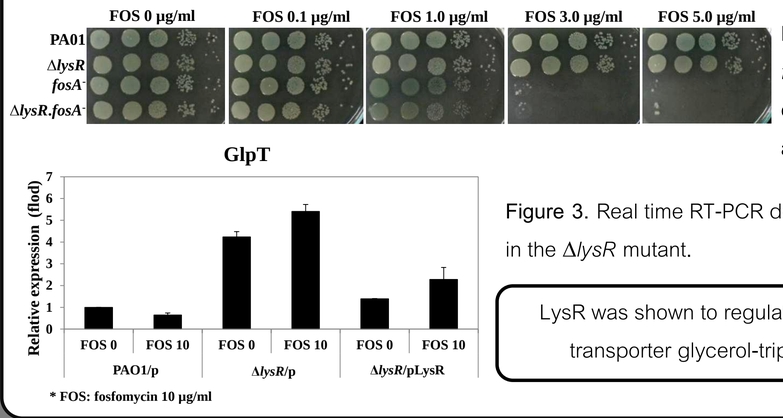
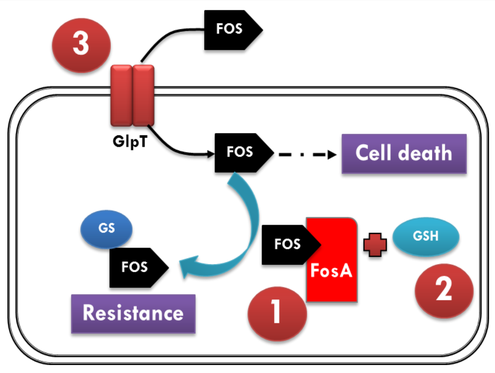


Figure 2. The resistance level of Δ*lysR* and *fosA*⁻ mutants against antibiotic were determined using antibiotic plate sensitivity assay.

Figure 3. Real time RT-PCR determining an expression level of *glpT* in the Δ*lysR* mutant.

LysR was shown to regulate a gene encoding an antibiotic transporter glycerol-triphosphate transporter (GlpT).

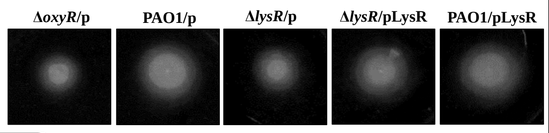
Fosfomycin resistance mechanisms in *P. aeruginosa*



(1) *fosA* overexpression induces resistance by enzymatic addition of glutathione (GSH), the fosfomycin (FOS) inactive (2)
(3) GlpT transports FOS into bacterial cell cytoplasm, causing cell death

Bacterial pathogenicity determination assays

Figure 4. Determination of swimming motility of *P. aeruginosa* strains. An Δ*oxyR* mutant was used as a positive control.



The swimming motility was 26% reduced in Δ*lysR* mutant compared to that in the wild type. This phenotype was restored as shown in the complemented strain. This suggested that the *lysR* may be involved in the regulation of swimming motility.

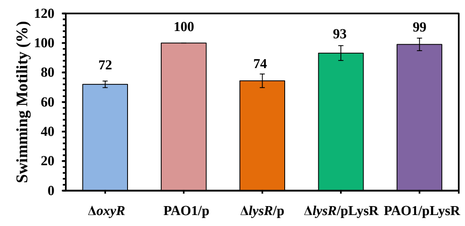


Figure 5. Determination of biofilm formation of *P. aeruginosa* strains. An Δ*oxyR* mutant was used as a positive control.

The biofilm formation was 28% reduced in Δ*lysR* mutant compared to that in the wild type. The fully complemented phenotype was observed in the complemented strain. This suggested that the *lysR* may involved in the regulation of biofilm formation.

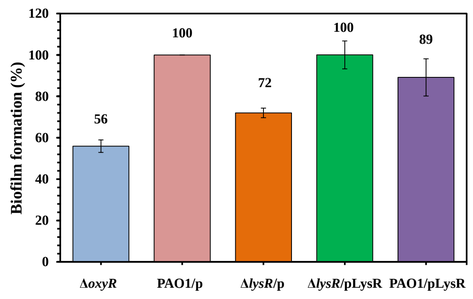
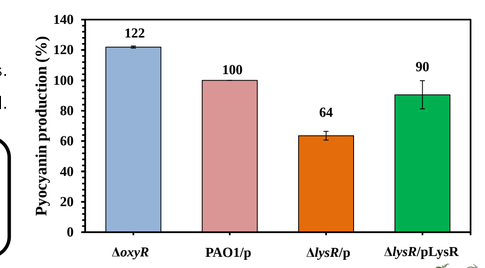
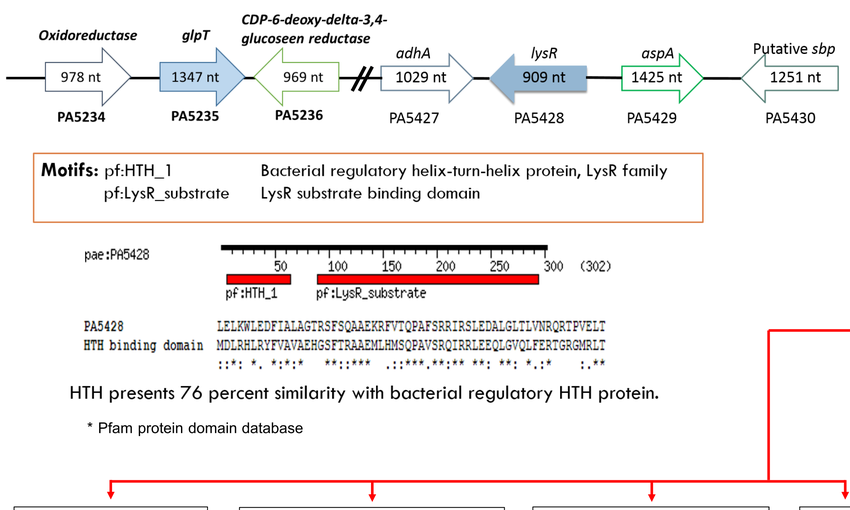


Figure 6. Determination of pyocyanin production of *P. aeruginosa* strains. An Δ*oxyR* mutant was used as a positive control.

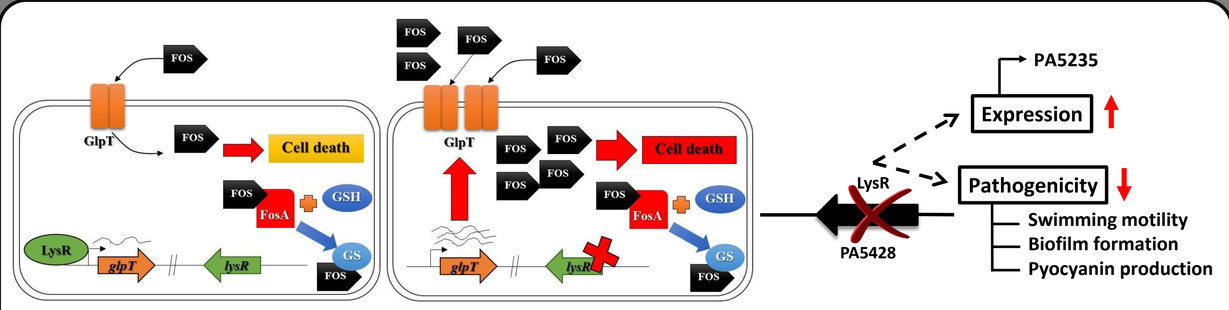
The pyocyanin production in the Δ*lysR* mutant was 37% reduced compared to that in the wild type. The partially complemented was observed in the complemented strains. This suggested that the *lysR* may involved in the regulation of pyocyanin production.



P. aeruginosa LTTR, PA5428 (*lysR*)



Summary



- The *lysR* is found to be important for resistance to broad-spectrum antibiotic by regulates gene encoding an antibiotic transporter, GlpT.
- The *lysR* may be one of a key virulence factor in regulation of swimming motility, biofilm formation and pyocyanin production during infections.
- Overall, the data indicate an importance of this gene encoding LTTR for the antibiotics resistance and the pathogenicity.

Acknowledgments

This research was supported by grants from Faculty of Science, Mahidol University, Chulabhorn Research Institute, Royal Golden Jubilee Ph.D. (PHD/0047/2557), Thailand Research Fund (to K.S.), and the joint funding of OHEC and TRF (MRG5980047), Thailand (to A.R.).

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AhpD-like protein protects against reactive chlorine species-mediated toxicity in pathogenic bacteria

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[5] Center of Excellence on Environmental Health and Toxicology (EHT), Ministry Of Education, Thailand

Grants from the Faculty of Science, Mahidol University and the Thailand Research Fund (MRG5980047), Thailand

AhpD-like Protein Protects against Reactive Chlorine Species-Mediated Toxicity In Pathogenic Bacteria

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Pseudomonas aeruginosa has become increasingly recognized as an opportunistic human pathogen in hospitals, especially in immunocompromized individuals. An ability of *P. aeruginosa* to resist antibiotics and reactive chlorine species, such as sodium hypochlorite (NaOCl), has permitted this organism to persist in hospitals. In this study, *P. aeruginosa rcsA*, a gene encoding AhpD-like protein, was characterized in term of an ability required for bacterial survivals under stress conditions. The gene encoding RscA was deleted and overexpressed by using recombinant DNA techniques. The plate sensitivity assay against NaOCl and ROS-mediated agents showed that the *rcsA* mutant was dramatically susceptible to NaOCl, but not others, compared to the wild type indicated that *rcsA* plays an important role in protecting against NaOCl. Moreover, to identify key amino acids of RcsA, the site-directed mutagenesis was applied by changing amino acid residues in an expression cassette. The results showed that a complementation by *rcsA* with either C60S, C63S, or H67A cannot restore the NaOCl susceptibility in the *rcsA* mutant indicating that C60, C63, H67 are important residues of RcsA in protecting against NaOCl. Finally, gene expression analysis exhibited an increased expression of *rcsA* under NaOCl-treated condition supporting its physiological function. Furthermore, protein purification and protein activity assay in NaOCl-detoxifying mechanism are under investigating. Overall, *rcsA* is one of NaOCl-mediated resistance in *P. aeruginosa* and one of causes that allow this organism to persist in hospitals.

Supported by grants from the Faculty of Science, Mahidol University and the Thailand Research Fund (MRG5980047), Thailand

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Dr. Adisak Romsang
Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand

June 14, 2017

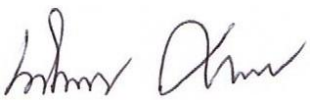
Dear Dr. Adisak Romsang,

On behalf of the Vice President of Thai Society for Biotechnology (TSB) and the chair person of The 13rd Asian Congress on Biotechnology (ACB 2017) "Bioinnovation and Bioeconomy", I would like to inform you that your abstract is accepted to give the **oral presentation** entitled "Cross Resistance Mechanisms between Antibiotic, Antiseptic, and Disinfectant in Human Pathogen *Pseudomonas aeruginosa*" at our congress. To complete the registration, please pay the registration fee with an early bird registration rate, THB 6,900 (Non-TSB member) or THB 5,900 (TSB member) or THB 3,900 (Student). Detail of AFOB membership can be found at www.afob.org.

Enclosed please find our tentative program. More detail of ACB 2017 can be found at www.acb2017thailand.org. Should you have any question, please feel free to contact Miss Duangporn Lakasong, the secretary of the congress at acb2017thailand@hotmail.com.

We are looking forward to meeting you at the Congress in July.

Sincerely yours,



(Associate Professor Dr. Penjit Srinophakun)

Vice President of Thai Society of Biotechnology

Chair person of ACB 2017

Email: fengpjs@ku.ac.th

Molecular Characterization of Iron-Sulfur Cluster Regulator IscR-Binding Motifs in Pathogenic Bacterium *Pseudomonas aeruginosa*

Kritsakorn Saninjuk[1], Adisak Romsang[1,2], Jintana Duang-Nkern[3], Skorn Mongkolsuk[1,2,3]*

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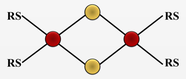
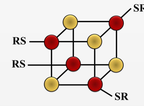
[3]Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, Thailand *Email: skorn@cri.or.th



[2Fe-2S]



[4Fe-4S]

ABSTRACT

Pseudomonas aeruginosa is a Gram-negative bacterium with its ability to cause human infection especially in immunocompromised patients. Iron-sulfur cluster Fe-S is a key cofactor of several proteins required for various cellular activities, including respiration, metabolism, nitrogen fixation, RNA modification, and gene regulation. In *P. aeruginosa*, IscR is globally dimeric transcriptional regulator in Fe-S cluster biogenesis, modulates a cellular iron homeostasis, and responds to environmental stresses including oxidative stress from the host immune system. The *P. aeruginosa* PAO1 contains an *isc* gene cluster consisting of *iscRSUA-hscBA-fdx2-iscX*. Unlike the multiple Fe-S biogenesis systems in enteric bacteria including *Escherichia coli*, *P. aeruginosa* has one stress-induced ISC system. In this study, site-directed mutagenesis of was conducted and the results showed that four conserved amino acid residues i.e. C92, C98, C104 and H107 involved in Fe-S ligation and regulatory mechanism of IscR. The Fe-S cluster-containing protein in purified IscR with either C92A, C98A, C104A or H107A was decreased compared to that in wild-type IscR by UV-visible scanning. The results from in vitro binding assay illustrated that the ligation of Fe-S cluster was required for repression mechanism of IscR and holo-IscR bound to two IscR binding sites located on the *isc* operon's promoter. The sequence-specific binding of IscR to DNA was performed using DNase protection assay and showed that the IscR protected region spanning the sequence region of -67 to -18 covering the RNA polymerase-binding region (-35). The sequence upstream of *P. aeruginosa iscR* contains two IscR-binding motifs, -67 to -42 (Site A) and -43 to -18 (Site B). The nucleotide substitution of first AT rich region of each sites with CCC indicated that both Site A- and Site B-binding sites were important for Isc-binding on the isc promoter. Overall data presented the molecular characterization of IscR-binding motifs in this pathogenic bacterium.

INTRODUCTION

Figure 1: *Pseudomonas aeruginosa*

P. aeruginosa commonly found in soil, water, plants, animals and also humans. *P. aeruginosa* causes healthcare-associated infections (HAI) with high mortality rate because of its inherited resistance to array of antibiotics and biocides, making it difficult to treat infections with this bacterium.

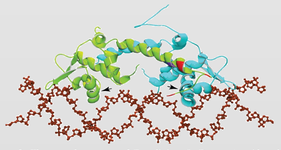


Figure 2: The crystal structure of *E. coli* Apo–IscR, carrying Ala substitutions at Cys92, Cys98, Cys104, and His107, bound to DNA (Romsang A., et al, 2016)

Iron-Sulfur (Fe-S) Cluster contains iron and inorganic sulfide. The Fe-S clusters are cofactors of enzymes or proteins involved in several cellular functions such as electron transfer, enzymatic catalysis, gene regulation, and central metabolism. Fe-S clusters are found in all tree kingdom of life, including archaea, bacteria and eukaryotes. The most common forms of Fe-S cluster are the **cubic [4Fe-4S]** and the **rhombic [2Fe-2S]**.

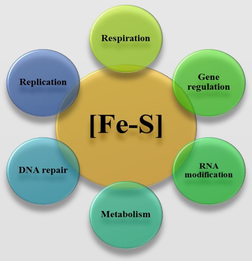


Figure 3: Roles of Fe-S cluster

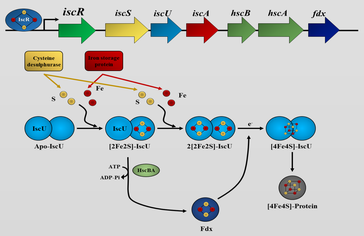


Figure 4: *Escherichia coli*, Fe-S cluster biogenesis (Béatrice Py & Frédéric Barras, 2010, Nature Reviews)

In *Escherichia coli*, the ***isc* operon** encompasses genes encoding a regulator (IscR), a cysteine desulfurase (IscS), a scaffold (IscU), an A-type protein (IscA), DnaJ-like co-chaperone (HscB), a DnaK-like chaperone (HscA) and a ferredoxin (Fdx). The Isc system is the housekeeping biogenesis pathway, in which its inactivation leads to reduction of [Fe-S]-enzyme activity under normal growth condition.

IscR is a [2Fe-2S] global transcriptional regulator, which is encoded by the first gene in the operon of Fe-S cluster assembly pathway (***iscRSUA-hscBA-fdx***). IscR exists in two major forms: **apo-IscR** (**clusterless**) and **holo-IscR ([2Fe-2S] ligated IscR)**. Mutagenic studies have shown that IscR requires three cysteine residues (**Cys-92, Cys-98, and Cys-104**) and one histidine residue (**His-107**) for Fe-S cluster ligation.

P. aeruginosa IscR

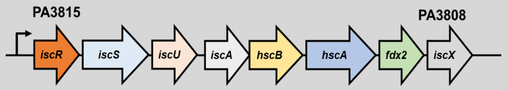


Figure 5: Gene organization of an *isc* operon in *P. aeruginosa* PAO1

- *P. aeruginosa* PAO1 revealed the homologues of the *isc* gene cluster *iscRSUA-hscBA-fdx2-iscX* (**PA3815-PA3808**) that was involved in Fe-S cluster biogenesis (**Fig. 5**), stress response (Romsang A., et al, 2014, PLOS ONE), and virulence (Romsang A., et al, 2016).

```
                                                92      98          104  107
P. aeruginosa                   PGGGYQLSRHMSGIHVAQVIDAVNESVDATRCQGQGDCHS----GDTCLTHHLWCDLSLQ
E. coli                         PGGGYLLGKDASSIAVGEVISAVDESVDATRCQGKGGCQG----GDKCLTHALWRDLSDR
Acidithiobacillus ferooxidants  PGGGYRLAMPDSEIPLTRIVEAVNESISTTQCGGDPHLCCKGD-GQQCLTHDLWEELGNR
Burkholderia mallei             PGGGYNLARRAQDVTVADIIIAVDEPLDATQCGGKGACEGSKQPDGHCMTHELWSTLNQK
                                ***** *.   . : :  :: **:*.:.:*:* *.         .  *:** **  *. :
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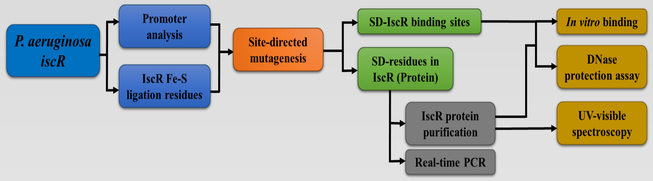
Figure 6: The CLUSTALW sequence alignment of IscR homologues

- **PA3815** is identified as ***iscR***. IscR contains the essential residues for Fe-S cluster ligation, **three cysteines (C92, C98, and C104)** and a **histidine (H107)** (**Fig. 6**).

HYPOTHESIS

***P. aeruginosa* IscR regulates its expression by recognizing Type-I binding sites in the presence of Fe-S cluster**

EXPERMENTAL OUTLINE



RESULTS

Fe-S cluster Ligation Residues

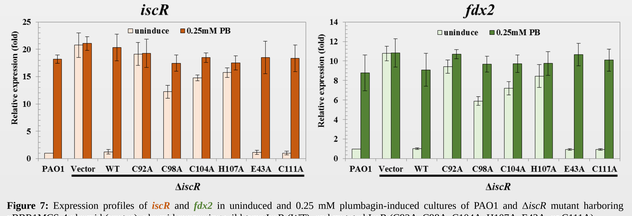


Figure 7: Expression profiles of *iscR* and *fdx2* in uninduced and 0.25 mM plumbagin-induced cultures of PAO1 and Δ*iscR* mutant harboring pBBR1MCS-4 plasmid (vector), plasmid expressing wild-type IscR (WT), and mutated IscR (C92A, C98A, C104A, H107A, E43A, or C111A)

- Under uninduced conditions, the expression of ***iscR*** and ***fdx2*** in the Δ*iscR* mutant was 20- and 10-fold higher than the levels in PAO1, respectively. Plumbagin treatments did not further enhance the expression of these genes in the Δ*iscR* mutant.
- The Δ*iscR* mutant harboring pBBR-*iscR* repressed expression of ***iscR* (17-fold)** and ***fdx2* (10-fold)** compared with the *iscR* mutant harboring empty vector.
- The expression of mutated IscR-C92A, C98A, C104A and H107A did not repress the expression of ***iscR*** and ***fdx2***.
- The inability of mutant IscR-C92A, C98A, C104A and H107A to repress ***iscR*** and ***fdx2*** expression suggests that these mutations prevent the ligation of Fe-S cluster to IscR, which is sufficient to prevent the repression of the *isc* operon, (Romsang A., et al, 2014, PLOS ONE).

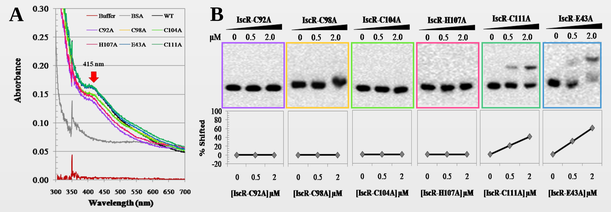


Figure 8: UV-visible spectra of IscR variants (C92A, C98A, C104A, H107A, E43A, and C111A) **(A)**. *In vitro* binding assay **(B)**.

- To address the importance of Fe-S cluster ligation, purified 6xHis-tagged IscR wild-type and IscR variants with amino acid substitutions (**C92A**, **C98A**, **C104A**, and **H107A**) were subjected to spectrum analysis using UV-visible spectroscopy.
- IscR variant proteins (**C92A**, **C98A**, **C104A** and **H107A**) contained a reduced amount of Fe-S clusters relative to wild-type IscR (**Fig. 8A,** Romsang A., et al, 2014, PLOS ONE).
- *In vitro* binding assay was performed to determine binding ability of IscR-variants protein on *iscR* promoter. All of the IscR varaints were unable to bind the *iscR* promoter (**Fig 8B**).
- **Fe-S cluster ligation residues are important for binding of IscR to Fe-S cluster and regulatory mechanism of [Fe-S]-IscR to its promoter.**

Promoter Analysis of *iscR*

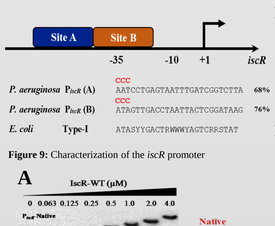


Figure 9: Characterization of the *iscR* promoter

- Sequence upstream of *P. aeruginosa iscR* contains 2 putative Type-I IscR-binding motifs denoted **Site A** between positions −43 and −67, and **Site B** spanning positions −18 to −42 relative to the *iscR* transcription start (**Fig. 9**).
- These sequence share high identity **(68% and 76%,** respectively) with the consensus sequence of ***E. coli* Type-I IscR binding motif**.

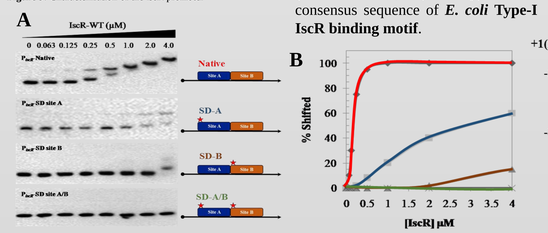


Figure 10: IscR binding with *iscR* promoter mutations

- Result from *In vitro* binding assay showed that Purified IscR protein bound to *iscR* promoter (**Fig. 10A**).
- The **AAT** (of **Site A**) and **ATA** (of **Site B**) were substituted with **CCC** using PCR-based site-directed mutagenesis
- Mutation of **Site A** reduced binding ability of IscR and P*iscR* SD-A since 50% binding complex was observed at 2.0 μM of IscR (**Fig. 10B**)
- Mutation of **Site B** drastically reduced binding ability of IscR and P*iscR* SD-B as 50% binding complex was observed at more than 4.0 μM of IscR (**Fig. 10B**)

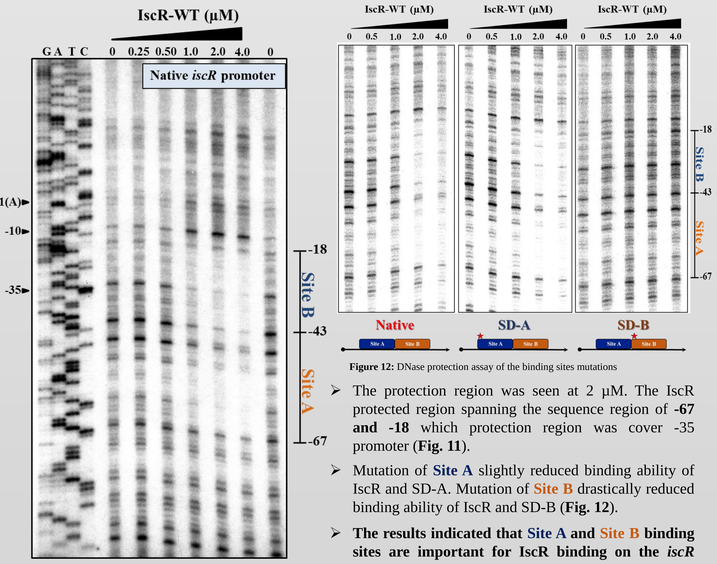


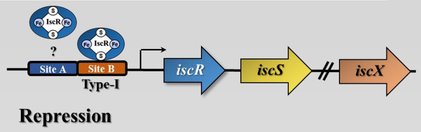
Figure 11: DNase protection assay of IscR-WT and *iscR* promoter

Figure 12: DNase protection assay of the binding sites mutations

- The protection region was seen at 2 μM. The IscR protected region spanning the sequence region of **-67 and -18** which protection region was cover -35 promoter (**Fig. 11**).
- Mutation of **Site A** slightly reduced binding ability of IscR and SD-A. Mutation of **Site B** drastically reduced binding ability of IscR and SD-B (**Fig. 12**).
- **The results indicated that Site A and Site B binding sites are important for IscR binding on the *iscR* promoter.**

CONCLUSION

- ***P. aeruginosa* IscR contains conserve Fe-S cluster-ligation residues, C92, C98, C104 and H107, which are crucial for binding of IscR to Fe-S cluster and regulatory mechanism of [Fe-S]-IscR to its promoter.**
- **The Site B exhibits higher affinity for IscR binding *in vitro,* while mutations in Site A slightly affects on repression.**



ACKNOWLEDGEMENTS

- **Supported by Chulabhorn Research Institute, Faculty of Science, Mahidol University and Royal Golden Jubilee (PHD/0047/2557), Thailand Research Fund**
- **Mathee' Lab, Florida International University**

Curriculum vitae

Adisak Romsang, Ph.D.

Lecturer, K610 Laboratory, 6th floor, Charlermprakiet Building,
Department of Biotechnology, Faculty of Science, Mahidol University,
Bangkok 10400, Thailand Tel. +66 2201 5962 E-mail: adisak.rom@mahidol.ac.th
Websites: http://ajrscbt.wix.com/k610-scmu, http://www.sc.mahidol.ac.th/academics/staff/AC_a/Adisak_R.htm

Personal details

Sex: Male **Age:** 32 years
Date of Birth: January 3rd, 1986
Place of Birth: Bangkok, Thailand
Military status: Exempted reservist (finished Military Service Training of Territorial Defense Course)

Marital Status: Married with two children
Citizenship: Thai
Blood Group: O
Religion: Buddhism (ordained in 2006)

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Education background

- 2008 – 2013 Ph.D. in Biotechnology (2013), Mahidol University, Bangkok, Thailand (GPAX: 3.94)
  Scholarship: Royal Golden Jubilee (PHD/0294/2550), Thailand Research Fund
  Dissertation: Protein repair systems during oxidative stress in *Pseudomonas aeruginosa*
- 2004 - 2007 B.Sc. in Biotechnology (2008), Mahidol University, Bangkok, Thailand
  GPAX: 3.43 (Second Class Honor)
- 2001 - 2003 High-school, Yothin Burana School (English Program), Bangkok, Thailand
  GPAX: 3.82

Research experiences

- Jun, 2015 – present Lecturer & researcher, Department of Biotechnology & Center for Emerging Bacterial Infections Room K610, 6th floor, Charlermprakiet Building, Faculty of Science, Mahidol University, Bangkok 10400, Thailand (Research in medical biotechnology based on bacterial genetics)
- Aug, 2013 – May, 2015 Researcher (Post-Ph.D.) in Laboratory of Biotechnology, 8th Floor, Biomedical Science Building, Chulabhorn Research Institute, Kamphaeng Phet 6, Laksi, Bangkok 10210, Thailand
  CRI Project: "Identification of novel genes that involve in bacterial pathogenicity"
- Mar, 2008 – Jul, 2013 Ph.D. Research: Genetic approach against oxidative stress in *Pseudomonas aeruginosa*
  - Center for Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
  - Laboratory of Biotechnology, Chulabhorn Research Institute, Laksi, Bangkok 10210, Thailand
- Nov 20 – Dec 2, 2012 Visiting scientist in Schweizer's Laboratory at Department of Microbiology, Colorado State University, Fort Collins, USA
  (Working on fosmid library construction in pathogenic bacterium, *Burkhoderia pseudomallei*)
- May 16 – Dec 12, 2012 Visiting scientist in Helmann's Laboratory at Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY, USA (Working on *Bacillus subtilis* molecular genetics and biochemistry: "Bacillithiol and metal stress response")
- Oct, 2007 – Feb, 2008 Senior Project at School of Biological Science, Flinders University, Adelaide, South Australia
  (Working on fungal genetics and recombination: "Knockout of the recombination hotspot cog^+ and its effect on crossing-over and gene-conversion at *his-3* in *Neurospora crassa*")
- April 2 – May 30, 2007 Summer Training at Bioassay Laboratory, BIOTEC CRU, BIOTEC, NSTDA, Thailand
  (Working on animal cell culture, optimization growth and differentiation conditions, screening anti-diabetic compounds from fungal crude extracts)

Academic committee and activities

- Jan, 2017 – present — Executive board of the Thai Society for Biotechnology, BIOTEC, NSTDA, Thailand
- June, 2015 – present — Lecturer, Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, THA
  Undergraduate: SCBT203 (Bact), SCBT305 (Industrial BT), SCBT308 (Myco), SCBT343, 493 (Topics in BT and Seminar in BT), SCBT401 (Bioinformatics), SCBT485-6 (Special Project)
  Invited lecturer for undergraduate at Mahidol University International College (MUIC - Biotech)
  Graduate: SCBT502 (Recomb DNA Tech), SCBT605 (Mini-thesis), SCBT594-6 (Adv Topics in BT)
  Invited lecturer for graduate: SCMI605 (Microbial Genetics)
- July, 2016 – present — Committee, Undergraduate Program in Science, Faculty of Science, Mahidol University, Thailand
  Secretory of both Undergraduate Program Committee and Undergraduate Program Development Committee, Department of Biotechnology, Faculty of Science, Mahidol University, Thailand
- 2016 – present — MU LabPass Committee, Mahidol University; Biosafety Committee, Faculty of Science, Mahidol University; Chemical Safety and Waste Management Committee, Faculty of Science, Mahidol University; Committee for Occupational Safety, Health, and Environment Management, Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
- Aug, 2015 – present — Committee for 1st year and for scholar students in Faculty of Science, Mahidol University, Thailand
- July, 23-27, 2017 — Scientific committee, The 13th Asian Congress on Biotechnology (ACB2017), Khon Kaen, Thailand
- Mar 25-27, 2017 — Mentor in the MU-English Camp 2017 at Singapore for English talent of scholar students
- September 7, 2016 — Invited speaker "Plan Unpredictable Future" in Orientation and Advisor Meeting for scholar students, L01 Hall, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, Thailand
- April – Aug, 2016 — Research Mentor for Biotechnology Youth 2106, World Biotech Tour 2016, Bangkok, Thailand
- June 10-11, 2016 — Academic committee (Biology), the 11th Conference on Science and Technology for Youths: 2016, Bangkok International Trade and Exhibition Centre, Bangkok, Thailand
- Aug, 2013 – present — Research assistance and technical supports for graduate students in Prof. Skorn Mongkolsuk's Laboratory (both Mahidol University and Chulabhorn Graduated Institute)
- February 1-2, 2012 — Conference assistance, the 23rd Annual Meeting of the Thai Society for Biotechnology (TSB2011), "Systems Biotechnology", the Imperial Queen's Park Hotel, Bangkok, Thailand
- July – August, 2010 — Teaching Training under Graduated Program in Biotechnology (SCBT413 Bioprocess Engineering Lab – Filter Press and semi-industrial-scaled centrifuge applications)
- March 25-29, 2008 — Participate in The 6th International CSSI Workshop, Chulabhorn Research Institute, Bangkok, THA

Honors and awards

- Jan 10-12, 2018 — Outstanding research poster award in the 17th TRF-OHEC Annual Congress 2018 (TOAC 2018), The Regent Cha-Am Beach Resort, Rayong, Thailand.
- July 11-14, 2017 — Outstanding poster award (2nd prize) in the 5th Asia Pacific Protein Association Conference and the 12th International Symposium of the Protein Society of Thailand, The Tide Resort, Bangsaen, THA
- August 3, 2016 — Outstanding abstract award in the 11th International Symposium of the Protein Society of Thailand (PST), CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand
- November 18, 2015 — The 2015 Taguchi Prize for outstanding thesis from Thai Society for Biotechnology, Thailand
- August 9, 2015 — Awards for Graduates with Distinctions, Academic Year 2013 (DEAN'S LIST): a plaque and a certificate of honor from the Faculty of Graduate Studies, Mahidol University, Thailand
- July 16, 2015 — Outstanding abstract award in the 10th International Symposium of the Protein Society of Thailand (PST), CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand
- February 7, 2014 — Best Oral Presentation Award (Biological Science) in the 2nd ASEAN Plus Three Graduate Research Congress (AGRC), S31 Sukhumvit Hotel, Bangkok, Thailand (O-BS002)
- April 3, 2011 — Outstanding Oral Presentation Award in RGJ-Ph.D. Congress XII, Thailand Research Fund, Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand (S2-09)
- April 20, 2010 — A certificate for Teaching Training under Graduated Program in Biotechnology
- 2009 - 2010 — Two certificates for the excellent committee in graduated student association
- March 13, 2008 — A certificate of excellence in practical work for senior project of science program; Oral presentation in the 9th Science Project Exhibition, Faculty of Science, Mahidol University, Bangkok, Thailand
- February 19, 2004 — A plaque of *KonDeeSriYoThin* for the Excellent Student of 2003, Yothin Burana School, BKK, THA

Publications

Journal articles (8 articles; >100 citations; *h*-index 5; *i*10-index 4; IF, Impact factor; Q, Quartile)

1. Boonma S, **Romsang A**, Duang-nkern J, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. The FinR-regulated essential gene *fprA*, encoding ferredoxin NADP$^+$ reductase: Roles in superoxide-mediated stress protection and virulence of *Pseudomonas aeruginosa*. PLoS One **2017** Jan; 12 (2): e0172071. IF 3.534 / Multidisciplinary Sciences Q1
2. **Romsang A**, Duang-nkern J, Wirathorn W, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* IscR-regulated ferredoxin NADP(+) reductase gene (*fprB*) functions in iron-sulfur cluster biogenesis and multiple stress response. PloS One **2015** Jul; 10 (7): e0134374. IF 3.534 / Multidisciplinary Sciences Q1 / 2 citations

3. **Romsang A**, Leesukon P, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Mutation on gene encoded monothiol-Glutaredoxin (GrxD) in *Pseudomonas aeruginosa* increases susceptibility to polymyxins. Int J Antimicrob Ag **2015** Mar; 45 (3): 314-8. IF 4.259 / Microbiology Q1 / 5 citations
4. Fuangthong M, Jittawuttipoka T, Wisitkamol R, **Romsang A**, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. IscR plays a role in oxidative stress resistance and pathogenicity of a plant pathogen, *Xanthomonas campestris*. Microbiol Res **2015** Jan; 170 (C): 139-146. IF 1.939 / Microbiology Q3 / 7 citations
5. Ma Z, Chandrangsu P, Helmann TC, **Romsang A**, Gaballa A, Helmann JD. Bacillithiol is a major buffer of the labile zinc pool in *Bacillus subtilis*. Mol Microbiol **2014** Nov; 94 (4): 756-70. IF 5.026 / Microbiology Q1 / 42 citations
6. **Romsang A**, Duang-nkern J, Leesukon P, Saninjuk K, Vattanaviboon P, Mongkolsuk S. The iron-sulfur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance against oxidants in *Pseudomonas aeruginosa*. PLoS One **2014** Jan; 9 (1): e86763. IF 3.534 / Multidisciplinary Sciences Q1 / 12 citations
7. **Romsang A**, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. Gene expression and physiological role of *Pseudomonas aeruginosa* methionine sulfoxide reductases during oxidative stress. J Bacteriol **2013** Aug; 195 (15): 3299-308. IF 3.177 / Microbiology Q1 / 17 citations
8. Somprasong N, Jittawuttipoka T, Duang-nkern J, **Romsang A**, Chaiyen P, Schweizer H, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* thiol-peroxidase protects against hydrogen peroxide toxicity and displays atypical patterns of gene regulation. J Bacteriol **2012** Aug; 194 (15): 3904-11. IF 3.825 / Microbiology Q1 / 16 citations

Manuscripts in preparation (4)

1. **Romsang A**, Khemsom K, Duang-nkern J, Wongsaroj L, Saninjuk K, Fuangthong M, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa ttcA* encoding tRNA-thiolating protein required an iron-sulfur cluster to role in hydrogen peroxide-mediated stress protection and pathogenicity. (Submitted to Scientific Report, March 2018)
2. Wongsaroj L, Saninjuk K, **Romsang A**, Duang-nkern J, Trinachartvanit W, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. 2018. (Submitted to PloS One, April 2018)
3. **Romsang A**, Duang-nkern J, Saninjuk K, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa nfuA*: gene regulation and its role in sustaining growth under stress, anaerobic, and virulence conditions. (preparing for PloS One)
4. Wongsaroj L, Saninjuk K, **Romsang A**, Vattanaviboon P, Mongkolsuk S. Disruption of glutathione biosynthesis pathway alters antibiotic susceptibility levels of *Pseudomonas aeruginosa*. 2018. (preparing for IJAA)

Chapter in Book (1)

1. **Romsang A**, Dubbs JM, Mongkolsuk S. The iron-sulfur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in *Pseudomonas aeruginosa*. In: "Stress and Environmental Control of Gene Expression in Bacteria." (Frans J. de Bruijn ed.) John Wiley & Sons, USA. **2016**; 2: 1090-1102. 2 citations

Presentations

International conferences (26)

[1]**Romsang A,** Duang-Nkern, J, Nontaleerak, B, Wongsaroj, L, Trinachartvanit, W, Vattanaviboon, P, Mongkolsuk, S. Reactive Chlorine Species-Mediated Stress Response through Transcriptional Regulators in a Human Pathogen *Pseudomonas aeruginosa* Involves in Nosocomial Infections. Poster presented in the TRF-OHEC Annual Congress 2018 (TOAC 2018); Jan 10-12, 2018, The Regent Cha-Am Beach Resort, Rayong, Thailand.

Nontaleerak B, Duang-nkern J, Vattanaviboon P, Mongkolsuk S, **Romsang A**. Functional and expression analysis of a gene encoding peroxiredoxin-liked protein in *Pseudomonas aeruginosa*. Oral presented in the 29th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB2017); November 23-24, **2017**, Swissotel Le Concorde, Bangkok, Thailand.

Romsang A, Auwattanamongkol T, Duang-nkern J, Nakhadamrongwut J, Mongkolsuk S. Cross resistance mechanisms between antibiotic, antiseptic, and disinfectant in human pathogen *Pseudomonas aeruginosa*. Oral presented in the 13th Asian Congress on Biotechnology 2017 (ACB2017); July 23-27, **2017**; Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.

[1]**Romsang A**, Phuphuripan N, Duang-nkern J, Nontaleerak B, Khemsom K, Mongkolsuk S. Activation of Reactive Chlorine Species-mediated stress response protein through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*. Poster presented in the 5th Conference of the Asia Pacific Protein Association (APPA) and the 12th International Symposium of the Protein Society of Thailand (PST2017); July 11-14, **2017**; The Tide resort, Chonburi, Thailand.

Saninjuk K, **Romsang A**, Duang-Nkern J, Mongkolsuk S. Molecular characterization of Iron-Sulfur Cluster Regulator IscR-binding motifs in pathogenic bacterium *Pseudomonas aeruginosa*. Poster presented in the American Society for Microbiology (ASM) Microbe 2017; June 1-5, **2017**; Ernest N. Morial Convention Center, New Orleans, LA, USA.

Romsang A, Duang-nkern J, Saninjuk K, Nakhadamrongwut J, Vattanaviboon P, Mongkolsuk S. Multiple stress responses against a bleaching agent in human pathogen *Pseudomonas aeruginosa*. Platform presented in the 8th Princess Chulabhorn International Science Congress; Nov 13-17, **2016**, Shangri-La Hotel, Bangkok, Thailand.

Duang-nkern J, **Romsang A**, Vattanaviboon P, Mongkolsuk S. Roles of *Pseudomonas aeruginosa nfuA* in stress conditions, bacterial virulence and regulation. Poster presented in the 8th Princess Chulabhorn International Science Congress; Nov 13-17, **2016**, Shangri-La Hotel, Bangkok, Thailand.

Saninjuk K, Lorlitiwong W, **Romsang A**, Mongkolsuk S. LysR-type transcriptional regulator involves in antibiotics resistance and pathogenesis of *Pseudomonas aeruginosa*. Poster presented in the 8th Princess Chulabhorn International Science Congress; Nov 13-17, **2016**, Shangri-La Hotel, Bangkok, Thailand.

Wongsaroj L, Saninjuk K, **Romsang A**, Trinachartvanit W, Mongkolsuk S. Role of *aspA* a gene encoding aspartate ammonia lyase in *Pseudomonas aeruginosa*. Poster presented in the 8th Princess Chulabhorn International Science Congress; Nov 13-17, **2016**, Shangri-La Hotel, Bangkok, Thailand.

Khemsom K, **Romsang A**, Duang-nkern J, Mongkolsuk S. Characterization of a gene encoding AtfA-homolog factor against oxidative stress in *Pseudomonas aeruginosa*. Poster presented in the 8th Princess Chulabhorn International Science Congress; Nov 13-17, **2016**, Shangri-La Hotel, Bangkok, Thailand.

Romsang A, Klowoothtipat S, Duang-nkern J, Wongsaroj L, Trinachartvanit W, Fuangthong M, Mongkolsuk S. Role of a tRNA-modification gene in oxidative stress response in a pathogenic bacterium *Pseudomonas aeruginosa*. Oral presented in Biotechnology International Congress (BIC) 2016; Sep 20-23, **2016**, BITEC Bang Na, Bangkok, Thailand.

[2]**Romsang A**, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. AhpD-like protein protects against reactive chlorine species-mediated toxicity in pathogenic bacteria. PST-selected oral presented in the 11th International Symposium of the Protein Society of Thailand (PST2016); Aug 3-5, **2016**, CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

Romsang A, Duang-nkern J, Wongsaroj L, Trinachartvanit W, Dubbs JM, Vattanaviboon P, Mongkolsuk S. Characterization of Reactive Chlorine Species-mediated stress response through AraC-family transcriptional regulator in *Pseudomonas aeruginosa*. Poster presented in the Gordon Research Conference 2016: Microbial Stress Response; July 17-22, **2016**, Mount Holyoke College, South Hadley, MA, USA.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Protein repair: a neglected puzzle of virulence circuitry in pathogenic bacteria. Oral presented in the 27th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB2015); November 17-20, **2015**, Mandarin Hotel, Bangkok, Thailand.

Romsang A. Protein repair systems during oxidative stress in *Pseudomonas aeruginosa*. Poster presented in the 27th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB2015); November 17-20, **2015**, Mandarin Hotel, Bangkok, Thailand. *(The 2015 Taguchi Prize for Outstanding Doctoral Degree Thesis)*

[2]**Romsang A**, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Redox enzymes in *Pseudomonas aeruginosa*: Novel mechanisms for drug-targeting design against infectious diseases. PST-selected oral presented in the 10th International Symposium of the Protein Society of Thailand (PST); July 15-17, **2015**, CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Insight into strategies for drug development design combatting infectious diseases caused by pathogenic bacteria. Oral presented in the 26th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB2014); November 26-29, **2014**, Mae Fah Lunag University, Chiang Rai, Thailand.

Duang-nkern J, **Romsang A,** Fuangthong M, Vattanaviboon P, Mongkolsuk S. IscR: a global regulator for iron-sulfur cluster biogenesis involves in oxidative stress response and essentials for virulence of pathogenic bacteria. Poster presented in the 26th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2014); November 26-29, **2014**, Mae Fah Lunag University, Chiang Rai, Thailand.

[3]Saninjuk K, Wirathorn W, **Romsang A,** Mongkolsuk S. LysR-type transcriptional regulator (LTTR) involves in oxidative stress response and importance for pathogenicity of human pathogen *Pseudomonas aeruginosa*. Poster presented in the 26th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2014); November 26-29, **2014**, Mae Fah Lunag University, Chiang Rai, Thailand.

Boonma S, **Romsang A,** Mongkolsuk S. Physiological analysis of gene encoding FinR transcriptional regulator and its regulon under oxidative stress in *Pseudomonas aeruginosa*. Poster presented in the 26th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2014); November 26-29, **2014**, Mae Fah Lunag University, Chiang Rai, Thailand.

Romsang A, Duang-nkern J, Boonma S, Saninjuk K, Vattanaviboon P, Mongkolsuk S. Iron-sulfur cluster: a key prosthetic group modulates regulatory mechanisms of transcriptional regulator IscR in *Pseudomonas aeruginosa*. Poster presented in the Joint 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand; August 6-8, **2014**, Miracle Grand Convention Hotel, Bangkok, Thailand.

[4]**Romsang A**, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Redox Enzymes in *Pseudomonas aeruginosa*: Novel strategies for drug-targeting design against infectious diseases. Oral presented in the 2nd ASEAN Plus Three Graduate Research Congress (AGRC); February 5-7, **2014**, S31 Sukhumvit Hotel, Bangkok, Thailand.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Iron-sulfur cluster homeostasis: a global regulatory network is essential for stress responses and virulence in *Pseudomonas aeruginosa***.** Oral presented in the 25th Annual Meeting of the Thai Society for Biotechnology & International Conference (TSB 2013); October 16-19, **2013**, The Emerald Hotel, BKK, THA.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. The *Pseudomonas aeruginosa* iron-sulfur cluster regulator, IscR, controls genes in response to oxidative stress and antibiotic resistance. Poster presented in the *Pseudomonas* conference 2013: Genetic manipulation of Pseudomonads: applications in biotechnology and medicine; September 7-11, **2013**, University of Lausanne, Lausanne, Switzerland.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Physiological analysis of ferredoxin NADP(+) reductase subclass II roles in cellular stresses and iron–sulfur cluster biogenesis in *Pseudomonas aeruginosa.* Poster presented in the Gordon Research Conference 2012: Microbial Stress Response; July 15-20, **2012**, Mount Holyoke College, South Hadley, MA, USA.

[5]**Romsang A**, Rattanaprapanporn S, Trinachartvanit W, Fuangthong M, Mongkolsuk S. Methionine sulfoxide reductases (Msr) response against oxidative stress and pathogenicity in *Pseudomonas aeruginosa.* Oral presented in RGJ-Ph.D. Congress XII, Thailand Research Fund; April 1-3, **2011**, Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand.

[1]Outstanding poster award; [2]Outstanding abstract; [3]Best poster award; [4]Best oral presentation; [5]Outstanding oral presentation

National conferences (11)

Phuphuripan N, Khemsom K, Duang-nkern J, Mongkolsuk S, **Romsang A**. Protein purification and characterization of transcriptional regulators in response to reactive chlorine species in *Pseudomonas aeruginosa*. Oral presented in the 12th Science and Technology Conference for Youths; June 3-4, **2017**; Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, THA.

Auwattanamongkol T, Khemsom K, Mongkolsuk S, **Romsang A**. Construction of *Pseudomonas aeruginosa* genomic library in order to identify antibiotic and antiseptic resistance mechanisms. Oral presented in the 12th Science and Technology Conference for Youths; June 3-4, **2017**; Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

Nasathit B, Khemsom K, Ngamwongsatit B, **Romsang A**. Effect of natural extracts on bacterial growth and biofilm formation in *Salmonella* spp. to replace an antibiootucs treatment. Oral presented in the 12th Science and Technology Conference for Youths; June 3-4, **2017**; Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

Pinyosiritorn P, Khemsom K, Oonsuk S, Thepsingha W, **Romsang A**. Characterization of bacterial strains in bacteriology laboratory classes and risk assessment analysis in biosafety. Poster presented in the 12th Science and Technology Conference for Youths; June 3-4, **2017**; Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Inactivation of genes involved in iron-sulfur cluster biogenesis alters an antibiotic resistance in *Pseudomonas aeruginosa*. Poster presented in Next Generation Sequencing for Genetic and Genomic Studies Conference (NGS2014); July 29-30, **2014**, Windsor Suites Hotel, Bangkok, Thailand.

Romsang A, Saninjuk K, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* MsrA and MsrB play a differential role against superoxide-generated stress response and virulence. Poster presented in Mahidol University Research Expo 2013; January 27-28, **2014**, Srisavarindhira Building, Siriraj Hospital, Bangkok, Thailand.

Boonyakanog A, **Romsang A**, Duang-nkern J, Mongkolsuk S. Physiological analysis of *Pseudomonas aeruginosa* ferric uptake regulator, Fur, through its intermediates against oxidative stress and antibiotic resistance. Poster presented in Mahidol University Research Expo 2013; January 27-28, **2014**, Srisavarindhira Building, Siriraj Hospital, Bangkok, Thailand.

Saninjuk K, **Romsang A**, Mongkolsuk S. Physiological role of biotin sulfoxide reductase against oxidative stress in *Pseudomonas aeruginosa***.** Poster presented in the 39th Congress on Science and Technology of Thailand (STT39); October 21-23, **2013**, Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, Thailand.

Romsang A, Duang-nkern J, Vattanaviboon P, Mongkolsuk S. Protein repair: the neglected puzzle of virulence network in a successful human pathogen *Pseudomonas aeruginosa*. Poster presented in Center of Excellence on Environmental Health and Toxicology (EHT/PERDO) Conference; August 24-25, **2013**, CRI Convention Center, Chulabhorn Research Institute, THA.

Boonyakanog A, **Romsang A**, Duang-nkern J, Mongkolsuk S. Metal toxicity in environment altered the mutation on *fur* in *Pseudomonas aeruginosa* that potent to increase in antibiotics resistance. Poster presented in EHT/PERDO Conference; August 24-25, **2013**, CRI Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

Romsang A, Vattanaviboon P, Mongkolsuk S. Protein repair systems during oxidative damages in *Pseudomonas aeruginosa.* Oral presented in the 37th Congress on Science and Technology of Thailand (STT37); October 10-12, **2011**, Centara Grand Hotel & Bangkok Convention Center at Central World, Bangkok, Thailand.

Invited speech (5)

Romsang A. Hospital environment-induced stress response and antibiotic resistance in *Pseudomonas aeruginosa*. Presented in Micro seminar 2017, Microbiology department, Mahidol-Oxford Tropical Medicine Research Unit; March 8, **2017**, the 60th Anniversary Chalermprakiat Building Similan 1-2 room, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Romsang A. Multidrug resistance in our life. Presented in Junior Science Club 1/2559, Faculty of Science, Mahidol University; September 14, **2016**, K102 Faculty of Science, Mahidol University, Bangkok, Thailand.

Romsang A. Bacteria or Human: Who's gonna be the winner in an infectious battle? Presented in Seminar in Biotechnology, Faculty of Science, Mahidol University; August 20, **2015**, Faculty of Science, Mahidol University, Bangkok, Thailand.

Romsang A. Iron-sulfur cluster homeostasis: a global regulatory network is essential for stress responses and virulence in pathogenic bacteria. Presented in Chulabhorn Research Institute November Seminar; November 13, **2014**, Chulabhorn Research Institute, Bangkok, Thailand.

Romsang A, Mongkolsuk S, Helmann JD. Genes regulated by the iron-sulfur cluster containing regulator, IscR, during oxidative stress in *Pseudomonas aeruginosa*. Presented in Graduate Research Seminar in Microbiology (BioMI 7980); November 6, **2012**, Riley Robb Hall, Cornell University, NY, USA.

Memberships

2015 – Present	Protein Society of Thailand (PST), Thailand; L58-0128
2014 – Present	Thai Society for Biotechnology (TSB), Thailand; สช-960
2014 – Present	Mahidol University Alumni Association, Thailand; 1-004432
2014 – Present	Mahidol University Science Alumni Association (MSCA), Thailand
2012 – Present	Cornell Thai Association, Ithaca, NY

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Grant/Funding

2016 - 2018	MRG, Thailand Research Fund (TRF)
2015 - 2017	Talent Management, Mahidol University
2007 - 2017	Grant for Center of EBI, Mahidol University
2008 - 2013	Royal Golden Jubilee Ph.D., TRF

Special skills

Language	Fluent English (Good reading, writing, speaking, and listening)
Computer	Good knowledge of PC hardware and software: MS-Offices, databases, communication and searching
Delivery Licenses	5 years-driving license (Private car) NO. 48009876 since 2009, expiry date: January 3rd, 2023
Science and Research	Apply molecular genetics and biological tools to study in bacteria, fungi, plant tissues and animal cells Able to use most of laboratory equipment, chemical analyzers, and semi-industrial scale machines
Certified/Trained	อบรมในโครงการพัฒนารายวิชา/บทเรียนออนไลน์แบบ SPOC หรือ MOOC ภายใต้ระบบ Mux รุ่นที่ 1/2560 ใบรับคำขอรับใบอนุญาตใช้สัตว์ เลขที่ U1-05931-2559 (December 16, 2016) ESPReL for K610 Laboratory เลขทะเบียน: 2-0130-0050-2 สาขาเทคโนโลยีชีวภาพการแพทย์ (2015 - present) Conference for Biosafety Officer Workshop (June 27 – July 1, 2016: SD-Avenue Hotel, Bangkok, Thailand) LSI's course in "Laboratory Safety for Global Competitiveness" (Sep 11, 2015: Thailand Lab 2015) โครงการเสริมสร้างศักยภาพอาจารย์ ม. มหิดลขั้นพื้นฐานด้านการจัดการเรียนการสอน (June 8-12, 2015) Good Clinical Research Practice: ICH-GCP Training with 2-year certification (April 28, 2015: CCC) Mass Spectrometer Research Seminar: "AB SCIEX Academic Seminar" (Nov 17, 2014: CRI) Merck Millipore solutions for perfection in analytical HPLC sample preparation (March 11, 2014: CRI) Characterization of recombinant protein and glycoprotein using LC/MS (March 4-6, 2014: CGI)

Interests

- **Personal preference:** photography, theatre, high-technology gadgets, travel, cook, hike and swim
- **Research:**
  - Novel genes involved in microbial pathogenicity and antibiotic resistance
  - Novel transcriptional regulators in microbes responsible for environmental changes
  - Transcriptional networks and Next Generation Sequencing in microorganisms
  - Protein expression, isolation, analysis and engineering for biotechnological applications
- **Teaching:** Bacteriology, Microbiology, Genetics, Bioinformatics, Microbial Gene Regulation, recombinant DNA technology

References

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  Head, Laboratory of Biotechnology, Chulabhorn Research Institute, Laksi, Bangkok 10210, Thailand Tel. +66 2553 8557 E-mail: skorn@cri.or.th
- Prof. John D. Helmann — Head, Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101, USA Tel. +60 7255 1517 E-mail: jdh9@cornell.edu