

RESEARCH ARTICLE

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

Lampet Wongsaroj¹, Kritsakorn Saninjuk², Adisak Romsang^{1,2,3}, Jintana Duang-nkern⁴, Wachareeporn Trinachartvanit⁵, Paiboon Vattanaviboon^{4,6}, Skorn Mongkolsuk^{1,2,3,4*}

1 Molecular Medicine Graduate Program, Faculty of Science, Mahidol University, Bangkok, Thailand, **2** Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand, **3** Center for Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok, Thailand, **4** Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok, Thailand, **5** Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand, **6** Program in Applied Biological Sciences: Environmental Health, Chulabhorn Graduate Institute, Bangkok, Thailand

* skorn@cri.or.th



OPEN ACCESS

Citation: Wongsaroj L, Saninjuk K, Romsang A, Duang-nkern J, Trinachartvanit W, Vattanaviboon P, et al. (2018) *Pseudomonas aeruginosa* glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. PLoS ONE 13(10): e0205815. <https://doi.org/10.1371/journal.pone.0205815>

Editor: Roy Martin Roop, II, East Carolina University Brody School of Medicine, UNITED STATES

Received: April 5, 2018

Accepted: October 2, 2018

Published: October 16, 2018

Copyright: © 2018 Wongsaroj et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from the Chulabhorn Research Institute (<http://www.cri.or.th/en/index.php>) and Mahidol University (<https://www.mahidol.ac.th>). L.W. and K.S. were supported by the Royal Golden Jubilee Ph.D. Scholarship (PHD/0132/2557 and PHD/0047/2557) from

Abstract

Pseudomonas aeruginosa PAO1 contains *gshA* and *gshB* genes, which encode enzymes involved in glutathione (GSH) biosynthesis. Challenging *P. aeruginosa* with hydrogen peroxide, cumene hydroperoxide, and *t*-butyl hydroperoxide 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* $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ 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 $\Delta gshA$, $\Delta gshB$ and double $\Delta gshA\Delta gshB$ 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.*

Thailand Research Fund (<http://rgj.trf.or.th/eng/rgje11.asp>). A.R. was supported by the grants from the Center for Emerging Bacterial Infections (EBI) and the Central Instrument Facility (CIF grant) of Faculty of Science, the Mahidol University (<http://science.mahidol.ac.th>) and the joint funding of the Office of the Higher Education Commission and the Thailand Research Fund (MRG5980047), Thailand (<https://www.trf.or.th>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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 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 organic hydroperoxide (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 cross-linking, 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 [4–6]. Antioxidant molecules, such as vitamins and glutathione (GSH), also play roles in ROS removal. Biomolecular repair enzymes, such as methionine sulfide 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 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 -glutamylcysteine synthetase and glutathione synthetase is required to synthesize GSH. γ -Glutamylcysteine 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 -glutamylcysteine synthetase and glutathione synthetase. 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 , superoxide generators (0.5 mM plumbagin [PB], 0.5 mM menadione [MD], and 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 uninduced 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) increased

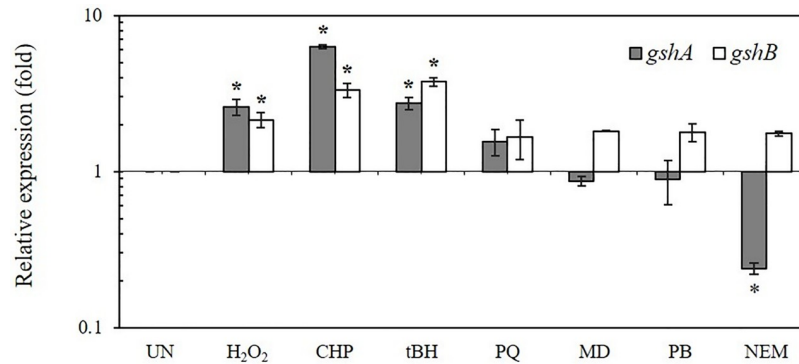


Fig 1. The expression of *gshA* and *gshB* in response to stress. The expression levels of *gshA* and *gshB* were determined using real-time RT-PCR. Exponential-phase cells of *P. aeruginosa* PAO1 were subjected to various stress conditions, including 1 mM H₂O₂, 1 mM CHP, 1 mM tBH, 0.5 mM PQ, 0.5 mM MD, 0.5 mM PB, and 0.5 mM NEM for 15 min prior to RNA preparation for real-time RT-PCR analysis. Relative expression (RE) was normalized to the 16S rRNA gene, and results are expressed as the fold-expression relative to the level of uninduced condition (log₁₀ RE = 1). Data shown are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$ by one-way ANOVA with Bonferroni correction).

<https://doi.org/10.1371/journal.pone.0205815.g001>

gshB expression, but PQ, MD, PB, and NEM treatments only marginally induced expression (approximately 50%) compared to the uninduced 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 known 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].

***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. All *gsh* mutants, including $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ mutants, were 10³-fold more sensitive to PQ (0.25 mM) treatment compared to wild-type PAO1 (Fig 2A). The PQ-sensitive phenotype of $\Delta gshA$ and $\Delta gshB$ 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. Exogenous 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 PQ treatment. The survival rates of $\Delta gshA$, $\Delta gshB$, double $\Delta gshA\Delta gshB$ mutants, and PAO1 grown in 2 mM GSH supplemented medium after paraquat treatment increased significantly (10³-fold) 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

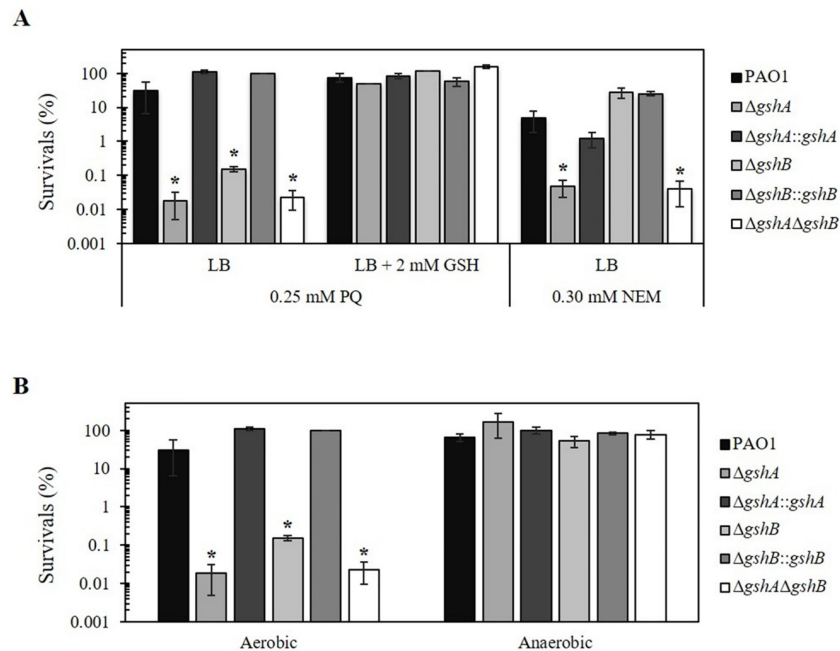


Fig 2. Determination of PQ and NEM resistance levels in *gsh* mutants and PAO1. (A) Plate sensitivity assay was performed with and without 2 mM GSH supplementation in LB plates containing 0.25 mM PQ and 0.30 mM NEM. (B) Plate sensitivity assay against 0.25 mM PQ using LB plates plus 1% NaNO₃ and incubated under aerobic and anaerobic conditions. Data presented are means \pm SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.01$ by one-way ANOVA with Bonferroni correction) relative to PAO1.

<https://doi.org/10.1371/journal.pone.0205815.g002>

treatment in PAO1, $\Delta gshA::gshA$, 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. A similar observation has shown that *P. aeruginosa gshA* mutant is more sensitive to PQ [17].

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 10³-fold increase in PQ sensitivity of $\Delta gshA$, $\Delta gshB$, and $\Delta gshA\Delta gshB$ 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 Δgsh mutant might require 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 [17].

The $\Delta gshA$ and double $\Delta gshA\Delta gshB$ mutants were 10²-fold more susceptible to NEM (0.30 mM) than the wild type (Fig 2A). However, the $\Delta gshB$ 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 ($\Delta gshA::gshA$) 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 $\Delta gshA$ and $\Delta gshA\Delta gshB$ mutants exhibited a 4-fold reduction in MIC level (0.01%) against methylglyoxal, and the $\Delta gshB$ 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
$\Delta gshA$	0.01	0.04
$\Delta gshA::gshA$	0.04	0.04
$\Delta gshB$	0.02	0.04
$\Delta gshB::gshB$	0.04	0.04
$\Delta gshA\Delta gshB$	0.01	0.04

The data shown are the MICs of three independent experiments with SD equal to 0.

<https://doi.org/10.1371/journal.pone.0205815.t001>

RES are produced as by-product of metabolisms in several bacteria, animals, and human. Methylglyoxal is generated by the fragmentation of triose phosphates (a glycolysis intermediate). Then, dihydroxyacetone phosphate (DHAP, a glycolysis intermediate) could be converted to methylglyoxal by methylglyoxal synthase [23, 26]. Methylglyoxal uses different mechanisms to exert its antimicrobial activity, including inhibition of protein, DNA, and RNA synthesis [27, 28]. 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) [26]. 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.

Δgsh 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. The Kaplan-Meier survival curves showed that median lifespans of flies infected with $\Delta gshA$, $\Delta gshB$, and $\Delta gshA\Delta gshB$ mutants were significantly increased to 12, 12, and 16 h, respectively, compared to 8 h in PAO1 (Log-Rank $p < 0.01$) (Fig 3). Loss of *gshA* and *gshB* genes in *P. aeruginosa* extended lifespan of the infected *Drosophila*. The $\Delta gshA$, $\Delta gshB$, and $\Delta gshA\Delta gshB$ mutants had reduced virulence compared to the PAO1, as indicated by a greater survival of infected flies. The wild-type PAO1 infected flies showed $10 \pm 2.8\%$ fly survival compared to 100% LB feeding as a negative control after a 16 h incubation. The $\Delta gshA$, $\Delta gshB$, and

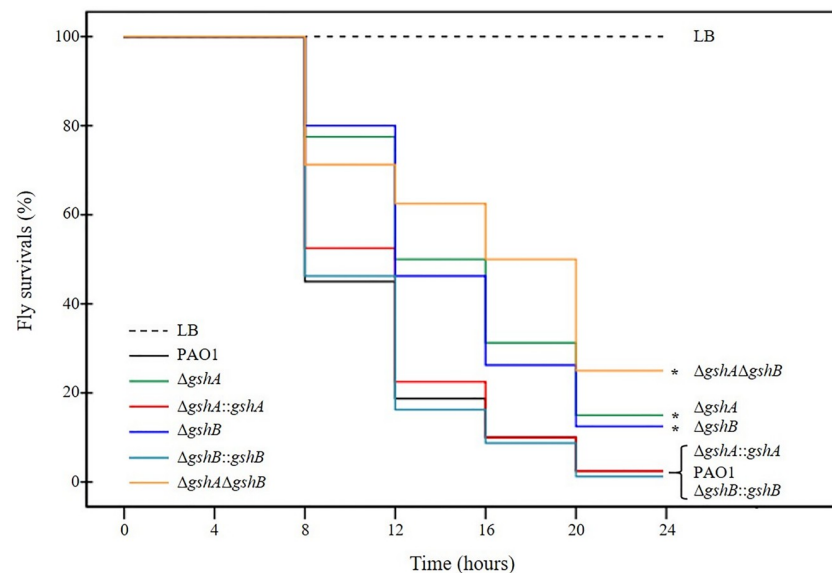


Fig 3. Kaplan-Meier survival curve of fruit fly *Drosophila melanogaster* infected with PAO1 and mutants. Each group of 20 *D. melanogaster* was infected with PAO1, *gsh* mutants, and complemented strains. Experiments were independently repeated for six times and the pooled data were used to build the survival curves (n = 120). Negative control groups were treated with fresh LB medium. The asterisk indicates a statistically significant difference. Statistical significance was measured by using Log-Rank test $p < 0.01$ as compared to the wild type.

<https://doi.org/10.1371/journal.pone.0205815.g003>

$\Delta gshA\Delta gshB$ mutants showed $31 \pm 3\%$, $26 \pm 2.8\%$, and $50 \pm 5.8\%$ fly survival at 16 h, respectively, compare to that of the PAO1. The percentage of fly survival increased significantly at 8–16 h time intervals when the $\Delta gshA$, $\Delta gshB$, and $\Delta gshA\Delta gshB$ mutants were fed to *D. melanogaster*, respectively ($p < 0.05$ by one-way ANOVA with Bonferroni correction) relative to PAO1. 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 at the same time ($10 \pm 1.6\%$ and $8.5 \pm 3\%$ 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.

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 (DUOX) 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 $6.32 \pm 0.3 \mu\text{g ml}^{-1}$ pyocyanin, and the $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ mutant culture medium contained significantly lower pyocyanin (3.01 ± 0.0 , 3.84 ± 0.3 , and $3.63 \pm 0.4 \mu\text{g ml}^{-1}$, respectively). The amount of pyocyanin in the culture medium of the $\Delta gshA::gshA$ complemented strains was similar to PAO1, and the $\Delta gshB::gshB$ strain exhibited increased pyocyanin levels up to $17.72 \pm 0.05 \mu\text{g ml}^{-1}$ (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. *P. aeruginosa gshA* mutants have been shown to produce reduced pyocyanin [17].

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

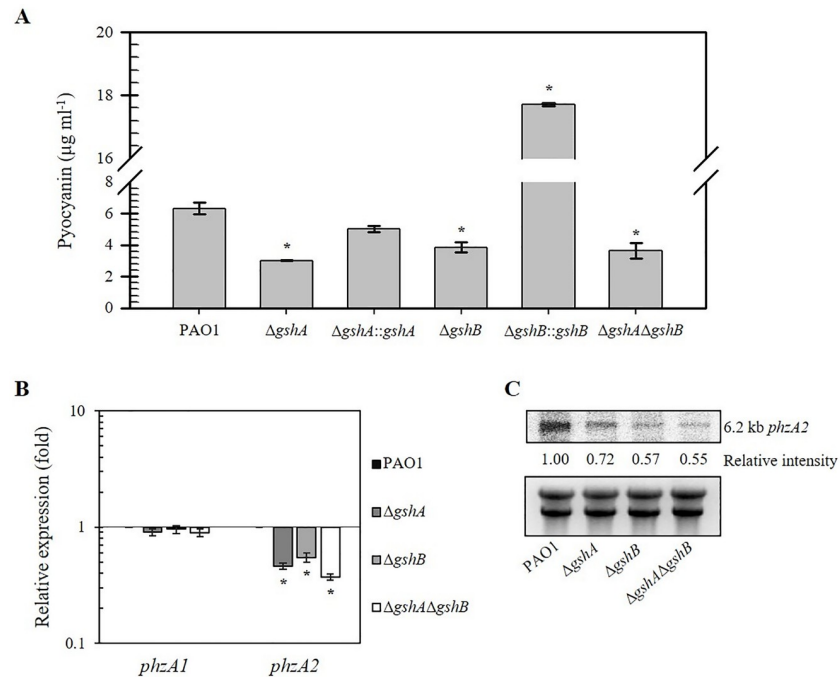


Fig 4. Pyocyanin production in *P. aeruginosa* wild-type PAO1 and *gsh* mutants. (A) PAO1, ΔgshA, ΔgshA::gshA, ΔgshB, ΔgshB::gshB and double ΔgshAΔgshB mutant strains were cultured for 24 h, and the supernatant was collected for pyocyanin measurement. (B) Real-time RT-PCR analysis of *phzA1* and *phzA2* expression. Total RNA was isolated from PAO1, ΔgshA, ΔgshB, and double ΔgshAΔgshB 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 ($p < 0.01$ by one-way ANOVA with Bonferroni correction) compared with PAO1.

<https://doi.org/10.1371/journal.pone.0205815.g004>

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 ΔgshA, ΔgshB, and double ΔgshAΔgshB 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 *phzA2* in the *gsh* mutants compared to PAO1. The results demonstrate that *phzA2* genes were transcribed mostly as polycistronic transcripts, and *phzA2* expression was reduced approximately 2-fold in the ΔgshA, ΔgshB, and double ΔgshAΔgshB 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 was 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

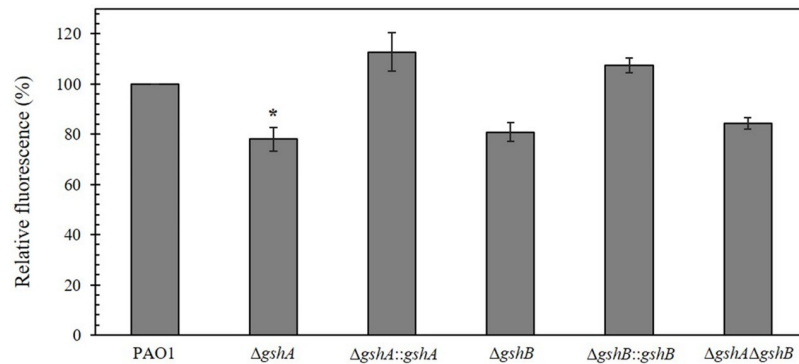


Fig 5. Pyoverdine production in *P. aeruginosa*. PAO1, $\Delta gshA$, $\Delta gshA::gshA$, $\Delta gshB$, $\Delta gshB::gshB$ and double $\Delta gshA\Delta gshB$ mutants were incubated in *Pseudomonas* F medium overnight at 37 °C. Pyoverdine was fluorometrically measured by recording the emission at 477 nm and excitation at 400 nm in a luminescence spectrometer. The asterisk indicates a statistically significant difference ($p < 0.05$ by one-way ANOVA with Bonferroni correction) compared to PAO1.

<https://doi.org/10.1371/journal.pone.0205815.g005>

ions in the environment into cells [40]. It has been shown that pyoverdine is essential for *P. aeruginosa* virulence [41]. Addition of GSH to clinically isolated *P. aeruginosa* strain has been shown to increase pyoverdine concentration in both the pyoverdine and ferripyoverdine forms [42]. The amount of pyoverdine in PAO1 and *gsh* mutants were quantified using fluorescent spectrometry. The intensity of fluorescence was normalized to cell density (OD_{600nm}), and the results are shown as percent relative fluorescence intensity. The percent relative fluorescence intensity of pyoverdine in $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ mutants were $78.0 \pm 2.4\%$, $80.9 \pm 1.8\%$, $84.3 \pm 1.1\%$, respectively, compared with 100% for PAO1 (Fig 5). Hence, the amount of pyoverdine produced by *gsh* mutants were 15 to 20% lower than PAO1. The expression of functional *gshA* and *gshB* restored pyoverdine secretion in the GSH mutants to the PAO1 level (Fig 5). These data suggest that GSH biosynthesis is involved in pyoverdine production in *P. aeruginosa*.

Siderophore pyoverdine is secreted by the type VI secretion system (T6SS) under iron-limiting conditions in *P. aeruginosa* to scavenge Fe^{3+} in the extracellular environment [43]. Ferripyoverdine (pyoverdine- Fe^{3+} complex) binds the FpvA outer membrane receptor and imports iron into the cell. FpvA interacts with FpvR antisigma factor in the periplasm and transmits the signal to the cytoplasmic domain of FpvR. Two sigma factors, σ^{PvdS} and σ^{FpvI} , are activated and bind to RNA polymerase to initiate the transcription of pyoverdine synthesis genes and *fpvA*, respectively [40].

Another siderophore in *P. aeruginosa* is pyochelin. Ferripyochelin is transported into the cell via the FptA outer membrane receptor [44]. Ferripyochelin iron reductase located in the periplasm and cytoplasm catalyzes the reduction of Fe^{3+} and releases Fe^{2+} from the pyochelin [44]. GSH and NADH are electron donors for ferripyochelin iron reductase [44]. Therefore, the re-adjustment of iron uptake processes, such as reduced pyoverdine synthesis, is required in the absence of thiols.

Δgsh mutants exhibit impaired swimming and twitching motility

Motility is an essential factor contributing to the bacteria virulence [45]. 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 [46]. *P. aeruginosa* uses a single flagellum for swimming in a liquid environment,

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

Strains	Motility Zone (mm ± SD)	
	Swimming	Twitching
PAO1	47.0 ± 1.7	43.3 ± 1.5
$\Delta gshA$	33.0 ± 2.0 *	32.3 ± 3.1 *
$\Delta gshA::gshA$	43.3 ± 1.2	43.0 ± 2.0
$\Delta gshB$	38.5 ± 2.1 *	33.7 ± 3.2 *
$\Delta gshB::gshB$	45.3 ± 5.7	41.3 ± 1.5
$\Delta gshA\Delta gshB$	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 ($p < 0.01$ by one-way ANOVA with Bonferroni correction) relative to PAO1.

<https://doi.org/10.1371/journal.pone.0205815.t002>

and twitching is flagellum-independent. Twitching is powered by an extension and retraction of pili. Flagellar assembly is required the correct folding proteins in either motor switch or hook for efficient movement. GSH is involved in disulfide bond formation and protein folding [47]. This study, we assessed whether GSH deficiency affect bacterial cell motility. 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 $\Delta gshA$ (33.0 ± 2.0 mm), $\Delta gshB$ (38.5 ± 2.1 mm), and $\Delta gshA\Delta gshB$ mutants (35.0 ± 2.0 mm) were observed compared to PAO1. The $\Delta gshA::gshA$ and the $\Delta gshB::gshB$ 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 2 also shows that twitching motility was reduced significantly in the $\Delta gshA$, $\Delta gshB$, and $\Delta gshA\Delta gshB$ 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 Δgsh mutants, but the colony sizes of these mutants on the LB agar were similar to PAO1 (data not shown).

GSH is transported from the bacterial cytoplasm to the periplasm via the CydDC transporter, which is an ATP-binding cassette-type transporter [48]. GSH exportation maintains the redox environment and protects cells from external toxicity or electrophilic compounds in *S. typhimurium* and *E. coli* [49]. Periplasmic GSH participates in disulfide bond formation and protein folding via the disulfide bond protein (Dsb) pathway [47]. These correct folding proteins are important for the proper assembly of flagellar motors and pili. *E. coli cydD* mutants exhibited defective cell motility due to disrupted flagellar assembly. The *gshA* mutant also exhibited defective flagella function. These results indicate that decreased GSH level could affect cell motility [17].

Deletion of genes encoding glutathione biosynthesis increases biofilm formation

Biofilm formation is one virulence factor in *P. aeruginosa*. It is an indicator for the pathogenicity of *P. aeruginosa* because it contributes to resistance to various stresses, including antimicrobial stress, and it is an important component of chronic infections [50, 51]. GSH addition disrupted mature and immature biofilms of a clinical *P. aeruginosa* strain [42]. Biofilm formation was determined in PAO1 and *gsh* mutants in the present study to assess whether lack of GSH has any effects on biofilm. Biofilm levels in the $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ 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 $\Delta gshA::gshA$ and $\Delta gshB::gshB$ complemented strains exhibited restored biofilm formation to the PAO1 level (Fig 6). These results demonstrated that a defect

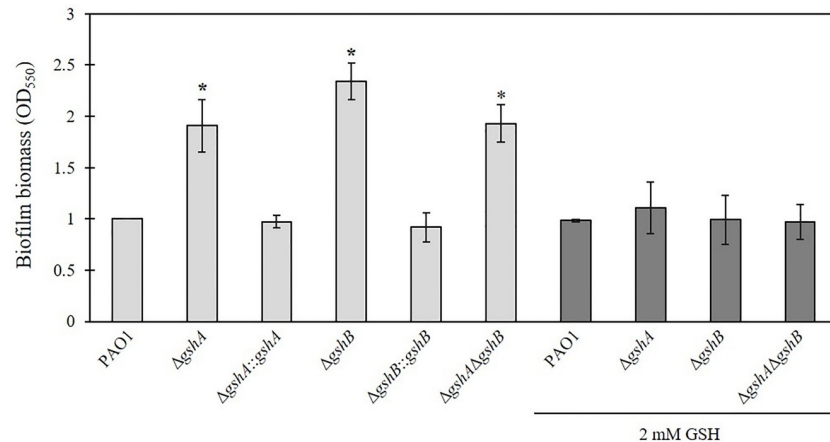


Fig 6. Biofilm formation assay in *P. aeruginosa*. PAO1, $\Delta gshA$, $\Delta gshA::gshA$, $\Delta gshB$, $\Delta gshB::gshB$ and double $\Delta gshA\Delta gshB$ mutants were cultured in LB medium with or without 2 mM GSH supplementation for 24 h. The biofilm layer of PAO1 and indicated strains was stained with 1% crystal violet solution. The biofilm biomass (OD_{550nm}) of *gsh* mutants and complemented strains compared with PAO1 were quantified. The asterisk indicates a statistically significant difference ($p < 0.01$ by one-way ANOVA with Bonferroni correction) compared to PAO1.

<https://doi.org/10.1371/journal.pone.0205815.g006>

in GSH biosynthesis via deletion of *gshA* or *gshB* increased biofilm formation, which suggests a role for GSH in the repression of biofilm formation. Exogenous 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 $\Delta gshA$, $\Delta gshB$, and double $\Delta gshA\Delta gshB$ mutants to a level similar to PAO1. These results are contrasted to a previous observation that the *gshA* mutant produced less biofilm [17]. GSH inhibition of biofilm formation in *P. aeruginosa* PAO1 is consistent with previous observations that GSH disrupted mature and immature biofilms of a clinical *P. aeruginosa* strain [42]. Pyocyanin pigment activates eDNA release from *P. aeruginosa* via H₂O₂-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 [52].

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 2). 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.

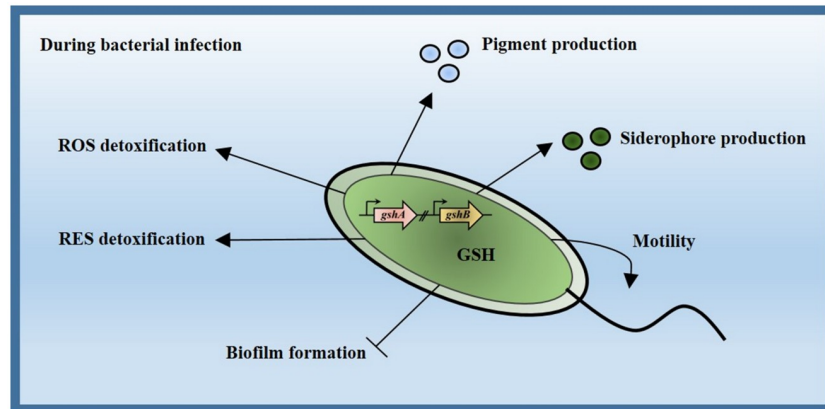


Fig 7. Overview function of GSH biosynthesis in *P. aeruginosa*. GshA and GshB catalyzed GSH biosynthesis. Bacteria at the early stage of infection encounter various stresses, including ROS which are generated by the host cell and RES generated from metabolism. GSH biosynthesis plays a primary protective 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.

<https://doi.org/10.1371/journal.pone.0205815.g007>

Materials and methods

Bacterial strains

All bacterial strains used in this study are listed in [S1 Table](#). *P. aeruginosa* strains were grown in Luria-Bertani (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_{600nm}] of 0.5 after 3 h of growth) were used in all experiments.

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 [53].

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

Total RNA samples were extracted from exponential phase PAO1 culture (OD_{600nm} of approximately 0.5 after 3 h of growth) with and without oxidant treatment for 15 min at 37 °C. 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). Real-time PCR was performed as previously described [54] using 10 ng cDNA to determine gene expression levels using primers EBI11 (5' -CGCTACG GCAAGACCATG-3') and EBI12 (5' -GCGCTCCAACCTGGCTCGG-3') for *gshA*, BT5458 (5' -CGCATGCGCCCCGCTGAAGG-3') and BT5459 (5' -GCGCGCCAGGCAGTAGGG-3') for *gshB*, EBI315 (5' -CGGTCAGCGGTACAGGGAA-3') and EBI316 (5' -GCGAGAGTACCAACG GTTGAAA-3') for *phzA1*, and EBI316 and EBI317 (5' -CGTCGCACTCGACCCAGAA-3') for *phzA2*. The 16S rRNA gene was amplified using primers BT2781 (5' -GCCCGCACAAAGCG GTGGAG-3'), and BT2782 (5' -ACGTCATCCCCACCTTCCT-3') was used as an internal control to normalize cDNA samples. The real-time RT-PCR was performed on Applied Biosystems StepOnePlus. Relative expression analysis was calculated using StepOne software v2.1

and is expressed as fold-expression relative to the level of wild-type PAO1 grown under uninduced condition.

Northern blot analysis

Total RNA isolation, agarose-formaldehyde gel electrophoresis, blotting, and hybridization were performed as previously described [7, 55]. Purified total RNA (20 μ g) 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'-CGTCGCAC TCGACCCAGAA-3'). Radioactively labeled probes were prepared using random-primed labeling with [α -³²P] dCTP.

Determination of the oxidant resistance level

To determine the oxidant resistance level, a plate sensitivity assay was performed as previously described [56]. Briefly, exponential-phase cells (OD_{600nm} of approximately 0.5 after 3 h of growth) were adjusted to an OD_{600nm} of 0.1 prior to a 10-fold serial dilution in LB medium. Each dilution (10 μ l) was spotted onto an LB plate containing 0.25 mM PQ and 0.30 mM 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 (NaNO₃, 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 methylglyoxal were determined as previously described using a broth microdilution assay [57]. *P. aeruginosa* PAO1 and *gsh* mutant strains were grown in LB medium at 37 °C under aerobic conditions until reaching the exponential phase (OD_{600nm} of 0.5). The culture was adjusted to 0.5 McFarland (OD_{600nm} of approximate 0.132) and was diluted 1:20 in fresh medium. The LB broth supplemented with different concentrations of agents with and without 2 mM 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, 58]. Essentially, the late-exponential-phase cultures (OD_{600nm} of 0.8) of *P. aeruginosa* strains were adjusted to an OD_{600nm} of 0.5 in 800 μ l 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 25 °C, and the number of viable flies was counted every four hours after flies were infected with bacteria.

Biofilm formation assay

Biofilm formation assays were performed as previously described [59]. Overnight cultures of *P. aeruginosa* strains were diluted 1:100 in fresh LB medium, and 150 μ l of medium was transferred into 96-well plates. Plates were incubated at 37 °C for 24 h without shaking, and the cell

suspension was removed and rinsed with 200 μ l of phosphate buffered saline (PBS). The biofilm layer was stained with a 0.1% crystal violet solution and incubated at room temperature for 15 min. The plate was rinsed with water and dried. Ethanol (200 μ l) was added to solubilize the crystal violet dye. The absorbance was measured at OD_{550nm} using a spectrophotometer to quantify biofilm formation.

Bacterial motility assay

Swimming motility was tested in M8 minimal medium supplemented with 1 mM MgSO₄, 0.5% Casamino acids, 0.2% glucose, and solidified with 0.3% agar for several hours [60]. Overnight bacterial cultures were spotted on agar and incubated at 37 °C for 48 h. Twitching motility was tested using stab inoculation with a toothpick through a 1% agar LB layer to the bottom of the Petri dish and incubated 37 °C for 48 h.

Pyocyanin pigment production

P. aeruginosa strains were grown in glycerol alanine minimal medium (GA medium) containing 1% glycerol, 67.3 mM L-alanine, 8 mM MgSO₄, 0.44 mM K₂HPO₄, and 0.065 mM FeSO₄ at 37 °C for 24 h, and pyocyanin production was quantified based on the absorbance of pyocyanin at 520 nm (OD_{520nm}) in an acidic solution as describe previously [61]. Briefly, the supernatant from bacterial culture was collected by centrifugation at 6,000 rpm for 10 min. The pyocyanin in the supernatant was extracted by mixing 3 ml of chloroform into 5 ml of supernatant. The lower chloroform layer containing pyocyanin was collected, and 1 ml of 0.2 M HCl was added to extract pyocyanin into the aqueous phase. The pyocyanin was quantified as OD_{520nm}. The pyocyanin concentration is expressed as micrograms of pyocyanin per milliliter of culture supernatant and determined by multiplication of the OD_{520nm} by 17.072 [61].

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_{600nm} of the corresponding cultures.

Statistical analysis

Group data are presented as means \pm standard deviation of at least three independent experiments. The level of statistically significant difference between samples was determined using one-way ANOVA with Bonferroni correction, and *p* value of < 0.05 was considered statistically significant.

Ethics statement

All *P. aeruginosa* and *D. melanogaster* were raised, maintained and all experiments were conducted following procedures, MUSC2017-001 and MUSC60-052-402, approved by the Committee of Biosafety, Faculty of Science, Mahidol University (MUSC) and the MUSC-Institutional Animal Care and Use Committee (IACUC), respectively.

Supporting information

S1 Table. Bacterial strains used in this study.
(DOCX)

Acknowledgments

The authors thank Wilaiwan Wirathorn for technical assistance. Parts of this work are from L. W. dissertation submitted for a Ph.D. degree from Mahidol University.

Author Contributions

Conceptualization: Wachareeporn Trinachartvanit, Skorn Mongkolsuk.

Formal analysis: Lampet Wongsaroj, Kritsakorn Saninjuk, Adisak Romsang, Jintana Duang-nkern.

Funding acquisition: Skorn Mongkolsuk.

Investigation: Lampet Wongsaroj, Kritsakorn Saninjuk, Jintana Duang-nkern.

Methodology: Lampet Wongsaroj, Kritsakorn Saninjuk, Jintana Duang-nkern, Wachareeporn Trinachartvanit.

Project administration: Paiboon Vattanaviboon.

Resources: Skorn Mongkolsuk.

Supervision: Skorn Mongkolsuk.

Writing – review & editing: Lampet Wongsaroj, Kritsakorn Saninjuk, Adisak Romsang, Paiboon Vattanaviboon, Skorn Mongkolsuk.

References

1. Cifani N, Pompili B, Anile M, Patella M, Diso D, Venuta F, et al. Reactive-oxygen-species-mediated *P. aeruginosa* killing is functional in human cystic fibrosis macrophages. PLoS One. 2013; 8(8):e71717. <https://doi.org/10.1371/journal.pone.0071717> PMID: 23977124
2. Albesa I, Becerra MC, Battan PC, Paez PL. Oxidative stress involved in the antibacterial action of different antibiotics. Biochem Biophys Res Commun. 2004; 317(2):605–9. <https://doi.org/10.1016/j.bbrc.2004.03.085> PMID: 15063800
3. Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol. 2002; 30(6):620–50. <https://doi.org/10.1080/01926230290166724> PMID: 12512863
4. Somprasong N, Jittawuttipoka T, Duang-Nkern J, Romsang A, Chaiyen P, Schweizer HP, et al. *Pseudomonas aeruginosa* thiol peroxidase protects against hydrogen peroxide toxicity and displays atypical patterns of gene regulation. J Bacteriol. 2012; 194(15):3904–12. <https://doi.org/10.1128/JB.00347-12> PMID: 22609922
5. Ochsner UA, Hassett DJ, Vasil ML. Genetic and physiological characterization of *ohr*, encoding a protein involved in organic hydroperoxide resistance in *Pseudomonas aeruginosa*. J Bacteriol. 2001; 183(2):773–8. <https://doi.org/10.1128/JB.183.2.773-778.2001> PMID: 11133975
6. An BC, Lee SS, Lee EM, Lee JT, Wi SG, Jung HS, et al. A new antioxidant with dual functions as a peroxidase and chaperone in *Pseudomonas aeruginosa*. Mol Cells. 2010; 29(2):145–51. <https://doi.org/10.1007/s10059-010-0023-1> PMID: 20082221
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; 195(15):3299–308. <https://doi.org/10.1128/JB.00167-13> PMID: 23687271
8. Smirnova GV, Oktyabrsky ON. Glutathione in bacteria. Biochem (Mosc) Suppl Ser A Membr Cell Biol. 2005; 70(11):1199–211. <https://doi.org/10.1007/s10541-005-0248-3> PMID: 16336178
9. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids. 2012; 2012:736837. <https://doi.org/10.1155/2012/736837> PMID: 22500213
10. Jamieson DJ. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast. 1998; 14(16):1511–27. [https://doi.org/10.1002/\(SICI\)1097-0061\(199812\)14:16<1511::AID-YEA356>3.0.CO;2-S](https://doi.org/10.1002/(SICI)1097-0061(199812)14:16<1511::AID-YEA356>3.0.CO;2-S) PMID: 9885153

11. Carmel-Harel O, Storz G. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu Rev Microbiol*. 2000; 54:439–61. <https://doi.org/10.1146/annurev.micro.54.1.439> PMID: 11018134
12. Song M, Husain M, Jones-Carson J, Liu L, Henard CA, Vazquez-Torres A. Low-molecular-weight thiol-dependent antioxidant and antinitrosative defences in *Salmonella pathogenesis*. *Mol Microbiol*. 2013; 87(3):609–22. <https://doi.org/10.1111/mmi.12119> PMID: 23217033
13. Romsang A, Duang-Nkern J, Leesukon P, Saninjuk K, Vattanaviboon P, Mongkolsuk S. The iron-sulphur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in *Pseudomonas aeruginosa*. *PLoS One*. 2014; 9(1):e86763. <https://doi.org/10.1371/journal.pone.0086763> PMID: 24466226
14. Panmanee W, Charoenlap N, Atichartpongkul S, Mahaviahakanont A, Whiteside MD, Winsor G, et al. The OxyR-regulated *phnW* gene encoding 2-aminoethylphosphonate: pyruvate aminotransferase helps protect *Pseudomonas aeruginosa* from *tert*-butyl hydroperoxide. *PLoS One*. 2017; 12(12): e0189066. <https://doi.org/10.1371/journal.pone.0189066> PMID: 29216242
15. Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, et al. Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. *Appl Environ Microbiol*. 2009; 75(19):6110–23. <https://doi.org/10.1128/AEM.00914-09> PMID: 19666735
16. Li K, Hein S, Zou W, Klug G. The glutathione-glutaredoxin system in *Rhodobacter capsulatus*: part of a complex regulatory network controlling defense against oxidative stress. *J Bacteriol*. 2004; 186(20):6800–8. <https://doi.org/10.1128/JB.186.20.6800-6808.2004> PMID: 15466032
17. Van Laar TA, Esani S, Birges TJ, Hazen B, Thomas JM, Rawat M. *Pseudomonas aeruginosa gshA* mutant is defective in biofilm formation, swarming, and pyocyanin production. *mSphere*. 2018; 3(2). <https://doi.org/10.1128/mSphere.00155-18> PMID: 29669887
18. Dietrich LE, Kiley PJ. A shared mechanism of SoxR activation by redox-cycling compounds. *Mol Microbiol*. 2011; 79(5):1119–22. <https://doi.org/10.1111/j.1365-2958.2011.07552.x> PMID: 21338412
19. Gu M, Imlay JA. The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol*. 2011; 79(5):1136–50. <https://doi.org/10.1111/j.1365-2958.2010.07520.x> PMID: 21226770
20. McLaggan D, Rufino H, Jaspars M, Booth IR. Glutathione-dependent conversion of N-ethylmaleimide to the maleamic acid by *Escherichia coli*: an intracellular detoxification process. *Appl Environ Microbiol*. 2000; 66(4):1393–9. <https://doi.org/10.1128/AEM.66.4.1393-1399.2000> PMID: 10742217
21. Ferguson GP, Munro AW, Douglas RM, McLaggan D, Booth IR. Activation of potassium channels during metabolite detoxification in *Escherichia coli*. *Mol Microbiol*. 1993; 9(6):1297–303. <https://doi.org/10.1111/j.1365-2958.1993.tb01259.x> PMID: 7934942
22. Zhang W, McLamore ES, Wu R, Stensberg M, Porterfield DM, Banks MK. Glutathione-gated potassium efflux as a mechanism of active biofilm detachment. *Water Environ Res*. 2014; 86(5):462–9. <https://doi.org/10.2175/106143013X13807328849855> PMID: 24961073
23. Lee C, Park C. Bacterial responses to glyoxal and methylglyoxal: reactive electrophilic species. *Int J Mol Sci*. 2017; 18(1):169. <https://doi.org/10.3390/ijms18010169> PMID: 28106725
24. Ferguson GP, Booth IR. Importance of glutathione for growth and survival of *Escherichia coli* cells: detoxification of methylglyoxal and maintenance of intracellular K⁺. *J Bacteriol*. 1998; 180(16):4314–8. PMID: 9696786
25. Riccillo PM, Muglia CI, de Bruijn FJ, Roe AJ, Booth IR, Aguilar OM. Glutathione is involved in environmental stress responses in *Rhizobium tropici*, including acid tolerance. *J Bacteriol*. 2000; 182(6):1748–53. <https://doi.org/10.1128/JB.182.6.1748-1753.2000> PMID: 10692382
26. MacLean MJ, Ness LS, Ferguson GP, Booth IR. The role of glyoxalase I in the detoxification of methylglyoxal and in the activation of the KefB K⁺ efflux system in *Escherichia coli*. *Mol Microbiol*. 1998; 27(3):563–71. <https://doi.org/10.1046/j.1365-2958.1998.00701.x> PMID: 9489668
27. Midorikawa Y, Hibasami H, Gasaluck P, Yoshimura H, Masuji A, Nakashima K, et al. Evaluation of the antimicrobial activity of methylglyoxal bis (guanyldiazone) analogues, the inhibitors for polyamine biosynthetic pathway. *J Appl Bacteriol*. 1991; 70(4):291–3. <https://doi.org/10.1111/j.1365-2672.1991.tb02938.x> PMID: 2055790
28. Murata-Kamiya N, Kamiya H. Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA. *Nucleic Acids Res*. 2001; 29(16):3433–8. <https://doi.org/10.1093/nar/29.16.3433> PMID: 11504881
29. Sukdeo N, Honek JF. *Pseudomonas aeruginosa* contains multiple glyoxalase I-encoding genes from both metal activation classes. *Biochim Biophys Acta*. 2007; 1774(6):756–63. <https://doi.org/10.1016/j.bbapap.2007.04.005> PMID: 17513180

30. Dionne MS, Schneider DS. Models of infectious diseases in the fruit fly *Drosophila melanogaster*. *Dis Model Mech*. 2008; 1(1):43–9. <https://doi.org/10.1242/dmm.000307> PMID: 19048052
31. Ryu JH, Kim SH, Lee HY, Bai JY, Nam YD, Bae JW, et al. Innate immune homeostasis by the homeobox gene *caudal* and commensal-gut mutualism in *Drosophila*. *Science*. 2008; 319(5864):777–82. <https://doi.org/10.1126/science.1149357> PMID: 18218863
32. Vallet-Gely I, Lemaitre B, Boccard F. Bacterial strategies to overcome insect defences. *Nat Rev Microbiol*. 2008; 6(4):302–13. <https://doi.org/10.1038/nrmicro1870> PMID: 18327270
33. Ben Haj Khalifa A, Moissenet D, Vu Thien H, Khedher M. Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation. *Ann Biol Clin (Paris)*. 2011; 69(4):393–403. <https://doi.org/10.1684/abc.2011.0589> PMID: 21896403
34. Phillips PL, Schultz GS. Molecular mechanisms of biofilm infection: biofilm virulence factors. *Adv Wound Care (New Rochelle)*. 2012; 1(3):109–14. <https://doi.org/10.1089/wound.2011.0301> PMID: 24527289
35. Furukawa S, Kuchma SL, O'Toole GA. Keeping their options open: acute versus persistent infections. *J Bacteriol*. 2006; 188(4):1211–7. <https://doi.org/10.1128/JB.188.4.1211-1217.2006> PMID: 16452401
36. Rada B, Leto TL. Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections. *Trends Microbiol*. 2013; 21(2):73–81. <https://doi.org/10.1016/j.tim.2012.10.004> PMID: 23140890
37. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol*. 2001; 183(21):6454–65. <https://doi.org/10.1128/JB.183.21.6454-6465.2001> PMID: 11591691
38. Zhang YN, Shao XL, Wang SH, Zhang SQ. Effect of glutathione on pyocyanin production in *Pseudomonas aeruginosa*. *Asian J Chem*. 2014; 26(11):3265–9. <https://doi.org/10.14233/ajchem.2014.17507> PMID: 99891524
39. Das T, Ibugo AI, Klare W, Manefield M. Role of pyocyanin and extracellular DNA in facilitating *Pseudomonas aeruginosa* biofilm formation. *Microbial Biofilms-Importance and Applications 2016*.
40. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 2002; 99(10):7072–7. <https://doi.org/10.1073/pnas.092016999> PMID: 11997446
41. Meyer JM, Neely A, Stintzi A, Georges C, Holder IA. Pyoverdine is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun*. 1996; 64(2):518–23. PMID: 8550201
42. Klare W, Das T, Ibugo A, Buckle E, Manefield M, Manos J. Glutathione-disrupted biofilms of clinical *Pseudomonas aeruginosa* strains exhibit an enhanced antibiotic effect and a novel biofilm transcriptome. *Antimicrob Agents Chemother*. 2016; 60(8):4539–51. <https://doi.org/10.1128/AAC.02919-15> PMID: 27161630
43. Chen WJ, Kuo TY, Hsieh FC, Chen PY, Wang CS, Shih YL, et al. Involvement of type VI secretion system in secretion of iron chelator pyoverdine in *Pseudomonas taiwanensis*. *Sci Rep*. 2016; 6:32950. <https://doi.org/10.1038/srep32950> PMID: 27605490
44. Cox CD. Iron reductases from *Pseudomonas aeruginosa*. *J Bacteriol*. 1980; 141(1):199–204. PMID: 6766439
45. Reimmann C, Ginet N, Michel L, Keel C, Michaux P, Krishnapillai V, et al. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology*. 2002; 148(Pt 4):923–32. <https://doi.org/10.1099/00221287-148-4-923> PMID: 11932439
46. Murray TS, Ledizet M, Kazmierczak BI. Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of *Pseudomonas aeruginosa* clinical isolates. *J Med Microbiol*. 2010; 59(Pt 5):511–20. <https://doi.org/10.1099/jmm.0.017715-0> PMID: 20093376
47. Shepherd M. The CydDC ABC transporter of *Escherichia coli*: new roles for a reductant efflux pump. *Biochem Soc Trans*. 2015; 43(5):908–12. <https://doi.org/10.1042/BST20150098> PMID: 26517902
48. Pittman MS, Robinson HC, Poole RK. A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. *J Biol Chem*. 2005; 280(37):32254–61. <https://doi.org/10.1074/jbc.M503075200> PMID: 16040611
49. Owens RA, Hartman PE. Export of glutathione by some widely used *Salmonella typhimurium* and *Escherichia coli* strains. *J Bacteriol*. 1986; 168(1):109–14. <https://doi.org/10.1128/JB.168.1.109-114.1986> PMID: 3531162
50. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol*. 2002; 292(2):107–13. <https://doi.org/10.1078/1438-4221-00196> PMID: 12195733

51. Choy MH, Stapleton F, Willcox MD, Zhu H. Comparison of virulence factors in *Pseudomonas aeruginosa* strains isolated from contact lens- and non-contact lens-related keratitis. *J Med Microbiol*. 2008; 57(Pt 12):1539–46. <https://doi.org/10.1099/jmm.0.2008/003723-0> PMID: 19018027
52. Manos J, Arthur J, Rose B, Tingpej P, Fung C, Curtis M, et al. Transcriptome analyses and biofilm-forming characteristics of a clonal *Pseudomonas aeruginosa* from the cystic fibrosis lung. *J Med Microbiol*. 2008; 57(12):1454–65. <https://doi.org/10.1099/jmm.0.2008/005009-0> PMID: 19018014
53. Sambrook J, Russell DW. *Molecular cloning: a laboratory manual*: Cold spring harbor laboratory press Cold Spring Harbor, New York.2001.
54. 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; 10(7):e0134374. <https://doi.org/10.1371/journal.pone.0134374> PMID: 26230408
55. Romsang A, Duang-Nkern J, Saninjuk K, Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa nfuA*: gene regulation and its physiological roles in sustaining growth under stress and anaerobic conditions and maintaining bacterial virulence. *PLoS One*. 2018; 13(8):e0202151. <https://doi.org/10.1371/journal.pone.0202151> PMID: 30092083
56. Boonma S, Romsang A, Duang-Nkern J, Atichartpongkul S, Trinachartvanit W, Vattanaviboon P, et al. 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. <https://doi.org/10.1371/journal.pone.0172071> PMID: 28187184
57. Romsang A, Leesukon P, Duangnkern J, Vattanaviboon P, Mongkolsuk S. Mutation of the gene encoding monothiol glutaredoxin (GrxD) in *Pseudomonas aeruginosa* increases its susceptibility to polymyxins. *Int J Antimicrob Agents*. 2015; 45(3):314–8. <https://doi.org/10.1016/j.ijantimicag.2014.10.024> PMID: 25593012
58. Romsang A, Duang-Nkern J, Khemsom K, Wongsaroj L, Saninjuk K, Fuangthong M, et al. *Pseudomonas aeruginosa ttcA* encoding tRNA-thiolating protein requires an iron-sulfur cluster to participate in hydrogen peroxide-mediated stress protection and pathogenicity. *Sci Rep*. 2018; 8(1):11882. <https://doi.org/10.1038/s41598-018-30368-y> PMID: 30089777
59. Barraud N, Moscoso JA, Ghigo JM, Filloux A. Methods for studying biofilm dispersal in *Pseudomonas aeruginosa*. *Methods Mol Biol*. 2014; 1149:643–51. https://doi.org/10.1007/978-1-4939-0473-0_49 PMID: 24818939
60. Caiazza NC, Shanks RM, O'Toole GA. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol*. 2005; 187(21):7351–61. <https://doi.org/10.1128/JB.187.21.7351-7361.2005> PMID: 16237018
61. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol*. 1990; 172(2):884–900. <https://doi.org/10.1128/JB.172.2.884-900.1990> PMID: 2153661