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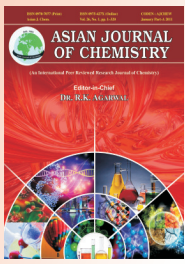

Effect of Glutathione on Pyocyanin Production in *Pseudomonas aeruginosa*

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Pyocyanin secreted by *Pseudomonas aeruginosa* is a redox active virulence factor, generating superoxide and H_2O_2 in host cells. Glutathione (GSH) is the most effective antioxidant for removing these reactive oxygen species in cells. However, many pulmonary diseases such as cystic fibrosis are associated with the lower levels of glutathione in the epithelial lining fluid (ELF) than those in healthy individuals. Oxidative injury inflicted by *P. aeruginosa* is one of the major causes to aggravating cystic fibrosis disease. We hypothesized that glutathione plays an important role during *P. aeruginosa* mediated pathogenesis. To test this hypothesis, a glutathione-null *gshB* mutant was constructed. The result demonstrated that the *gshB* mutant has less pyocyanin than the wild type PAO1 ($p < 0.01$), while *P. aeruginosa* strains treated with glutathione increased pyocyanin levels. This demonstrated glutathione can activate pyocyanin production. These findings may bring new insights into the molecular pathogenesis of *P. aeruginosa* infections and lead to novel therapeutic intervention for inhibiting *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, Glutathione, Pyocyanin, Cystic fibrosis.

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen causing infections in patients suffering from cystic fibrosis, chronic obstructive pulmonary disease, or severe burns and in patients in intensive care units[1-3]. It poses an over whelming threat to these individuals, leading to a high risk of morbidity and mortality. Using a mechanism of gene regulation called quorum sensing (QS), *P. aeruginosa* regulates the production of extracellular virulence factors, including elastase, alkaline protease, hemolysin, rhamnolipids and pyocyanin[4,5].

Pyocyanin is a blue-green redox-active secondary metabolite, secreted by *P. aeruginosa* and it is considered to be an important virulence factor for the organism by oxidative stress[6-9]. Two homologous operons are involved in the pyocyanin production: phzA1B1C1D1G1 (phzA1) and phzA2B2C2D2G2 (phzA2)[10]. Evidence suggests that phzA1 operon is regulated by quorum sensing system[11]. Using alternative model hosts and mice have clearly demonstrated that pyocyanin plays crucial roles in oxidative injury inflicted by *P. aeruginosa* in the hosts[12-15]. When it enters a host, pyocyanin can directly accepts electrons from either NADH or NADPH. Under aerobic conditions pyocyanin passes those electrons to O_2 and generates reactive oxygen species (ROS), superoxide (O_2^-) and hydrogen peroxide (H_2O_2), in posing oxidative stress to the cells.

Glutathione (GSH) is one of the most important intra and extracellular antioxidants, providing protection against oxidative stress[9,16,17]. Glutathione scavenges and inactivates reactive oxygen species (ROS), thereby reducing their cytotoxicity. When cells are exposed to oxidant species, GSH is able to reduce ROS and itself is oxidized into glutathione disulfide (GSSG). Glutathione disulfide is reduced back to GSH through the action of glutathione reductase. This cycling of GSH is an important means of limiting oxidative damage in the cells[16,18]. Growing evidence has demonstrated many pulmonary diseases infected with *P.aeruginosa* are associated with lower levels of GSH in the epithelial lining fluid than those in healthy individuals[19, 20].

Therefore, inhaled GSH therapy has been used in clinical trials to increase the GSH levels in the ELF of cystic fibrosis patients[21,22]. Our previously reported that an effect of GSH and its oxidized form GSSG altered the bacterium's sensitivity to different antibiotics[23]. We hypothesized that there is still a strong association between GSH and the pathogenicity of *P. aeruginosa*. In this paper, to test this hypothesis, we selected glutathione synthetase gene (*gshB*) related with glutathione in *P. aeruginosa's* cell and constructed a glutathione-null *gshB* mutant in the wild type *P. aeruginosa* strain (PAO1). Our analysis of PAO1 and PAO1(Δ*gshB*) showed that PAO1 (Δ*gshB*)

down regulate *phzA1* and results in a lower pyocyanin level. We further demonstrated that adding the GSH to *P. aeruginosa* strains could also increase the production of pyocyanin. These results suggest that GSH has an important role in pyocyanin production. Since pyocyanin is directly involved in successful disease control, understanding the regulatory systems of it contribute to the control of *P. aeruginosa* infection under various field conditions.

EXPERIMENTAL

Bacterial strains, plasmids and growth conditions: Bacterial strains and plasmids used in this study are listed in Table-1. Bacteria strains were grown at 37 ºC on LB agar plates or in LB broth with shaking at 200 rpm. The antibiotics used in this study were as follows: (1) For *E. coli* in all media, kanamycin (Kan) at 50 µg/mL, ampicillin (at 100 µg/mL and tetracycline (TC) at 15 µg/mL; (2) for *P. aeruginosa*: gentamicin (Gen) at 50 µg/mL, trimethoprim (Tmp) at 300 µg/mL and tetracycline (TC) at 150 µg/mL.

The plasmid pMS402 carrying a promoterless *luxCDABE* reporter gene cluster was used to construct promoter-*luxCDABE* reporter fusions[24]. The promoter regions of the gene were PCR amplified using *Pfx* DNA polymerase and primers synthesized according to the PAO1 genome data[25]. The construct plasmid was transformed into PAO1 by electroporation. DNA manipulation, PCR and transformation were performed following general procedures[26].

Monitoring gene expression: The gene expression was measured as counts per sec (cps) of lux light production in a Victor^2 Multilabel Counter (Perkin-Elmer) as before[24]. Assays were carried out in a 96-well black plate with a transparent bottom. Using bacterial culture prior to inoculation, overnight cultures of the strains were cultivated in fresh medium for three additional hours. Using plate wells containing 95 µL medium and 5 µL fresh cultures, 45 µL filter-sterilized mineral oil was added to each well to prevent evaporation during the assay. Both the expression of the promoter-reporter fusions and bacterial growth (OD_{595}) were measured every 0.5 h for 24 h under different conditions in the Victor^2 multilabel counter, the strain carrying pMS402 was used as a control. All the experiments were repeated at least three times and the figures shown are representative of similar profiles.

Construction of *P. aeruginosa* PAO1 (Δ*gshB*) mutant: The *gshB* gene knockout mutant was generated by allelic replacement through site-directed homologous recombination followed by *sacB*-Gm based counter selection as previously described[27]. The *gshB* gene was cloned in the suicide plasmid pEX18Ap carrying a *sacB* gene. The primer pairs for amplified of the *gshB* gene were gshb3 (5'-ACTGGATCCTCA TTGCGGATCGTGGTG-3'), containing a *Bam*HI site and *gahb4* (5'-ATCAAGCTTATCACGTCGCAACCGACC-3'), containing a *Hind*III site. A *sph*I-digested *lacZGm*^{r} resistance cassette derived from pZ1918-lacZ was cloned at the *Sph*I site on the PCR fragment, 944-bp downstream of the *gshB* start codon, the resulting suicide plasmid was named pSB*gshB*. The plasmids pSB*gshB* were transferred to PAO1 by mobilized with pRK2013 and used to taken the place of the original *gshB* gene. Subsequently, strains that have undergone homologous recombination can be selected by plating them on LB plus 5 % sucrose containing 50 µg/mL Gm. The mutant was verified by PCR analysis using primers gshb3, gshb4 and a *lacZ* primer (52-AGATCGCACTCCAGCCAG-32) that binds at the 52-end of *lacZ*. The *gshB* knockout mutant was named PAO1(D*gshB*).

Complementation of PAO1(Δ*gshB*): Complementation experiments were performed with the vector mini-CTX1. The gene *gshB* fragment was generated by standard PCR methods using primers *gshb3* and *gshb4*. The PCR product was digested with *Bam*HI and *Hind*III and inserted into mini-CTX1. The recombinant plasmid was transferred to PAO1(Δ*gshB*) mutant by a biparental meeting using SM10λ*pir* as donor and integrated at the *att*B site in the genome. Briefly, overnight cultures of recombinant plasmid Sm10λ*pir* containing the *gshB* gene fragment and PAO1 (Δ*gshB*) mutant were pelleted and resuspended in PBS, respectively, then mixed and spotted onto the center of an LB agar plate. After incubation overnight at 37 ºC, bacterial growth was resuspended in PBS. Transformants

TABLE-1
BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Strain and plasmids	Genotype or phenotype	Reference
	E. coli	
DH5α	F$^-$ mcrA Δ (*mrr-hsd*RMS-*mcrBC*)80d*lacZ* Δ M15 Δ *lacX*74 *deoR recA1 endA1 araD139* Δ (*ara leu*)7697 *galU galK*λ$^-$ *rpsL nupG*	Invitrogen
SM10	Mobilizing strain, RP4 integrated in the chromosome, Kn^r	38
	P. aeruginosa	
PAO1	Wild type, lab strain	Invitrogen
PAO1(Δ*gshB*)	PA0407 replacement mutant of PAO1, PAO1(Δ*gshB*) Gm^r	This study
GshbC	PAO1 (Δ*gshB*) complemented strain; PAO1 (Δ*gshB*) at tB PA0407	This study
	Plasmids	
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE*; Kn + Tmpr	Lab collection
pEX18Ap	*oriT*$^+$ *sacB*$^+$ gene replacement vector with multiple-cloning site from pUC18, Ap^r	39
pZ1918-lacZGm	Source plasmid of Gm^r cassette	40
pRK2013	Broad-host-range helper vector; Tra$^+$, Kn^r	41
Mini-CTX1	Integration plasmid; Tet^r	39
pSB*gshB*	PA0407 knockout plasmid pEX18Ap with 564-bp downstream region,Gm^r-*lacZ* fragment pZ1918lacZGm and 944 bp upstream of PA0407 cloned between *Hind*III and *BamH*I sites; Ap^{r}Gm^r	This study
pKD-*phzA1*	pMS402 containing *phzA1* promoter region	42

were selected and cultured in LB with 150 µg/mL tetracycline. The result was checked by PCR analysis. The complemented mutant strain was named PAO1(C*gshB*).

Quantification of pyocyanin: The pyocyanin assay is based upon the absorbance of excreted pyocyanin at 520 nm in acidic solution using previously reported methods[28]. 5 mL supernatant of *P. aeruginosa* strain or derivatives culture (22 h) was mixed with 3 mL of chloroform. Then the lower chloroform phase was mixed with 1 mL of 0.2 N HCl, after shaking and centrifugal, the colour layer was removed and measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072.

Effect of glutathione on pyocyanin quantitation: Glutathione was obtained from Sigma. To find out whether GSH affected the pyocyanin quantification, PAO1 and PAO1 (Δ*gshB*) were treated with GSH (3 µg/µL). The control strains were similarly grown, but in the absence of GSH. Pyocyanin was determined by the up method.

Statistical analysis: All data were analyzed using SPSS 10.0 statistical package (SPSS Inc.). Statistical tests were two-tailed, with $p < 0.05$ taken as the threshold for significance.

RESULTS AND DISCUSSION

To investigate whether GSH can affect the pyocyanin, the promoter regions of *phzA1* gene was amplified by PCR and cloned in pMS402 upstream of the promoterless luxCDABE reporter operon. These plasmid was introduced into the wild type PAO1 and PAO1(Δ*gshB*). The results demonstrated the *phzA1* expression level in PAO1 (Δ*gshB*) differs significantly from that in PAO1. The expression of *phzA1* was decreased in PAO1 (Δ*gshB*) compared with PAO1. The significant decrease of *phzA1* expression in the PAO1 (Δ*gshB*) mutant due to the absence of GSH, suggests that *gshB* is required for the full expression of *phzA1*(Fig. 1).

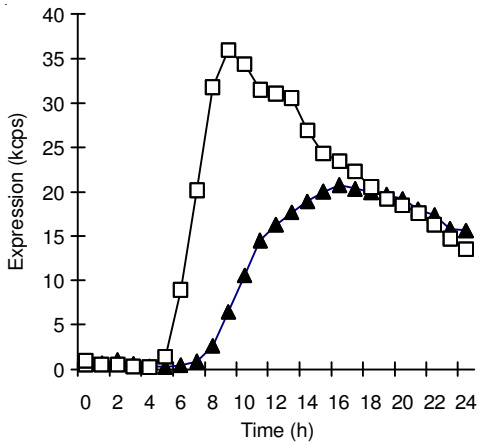
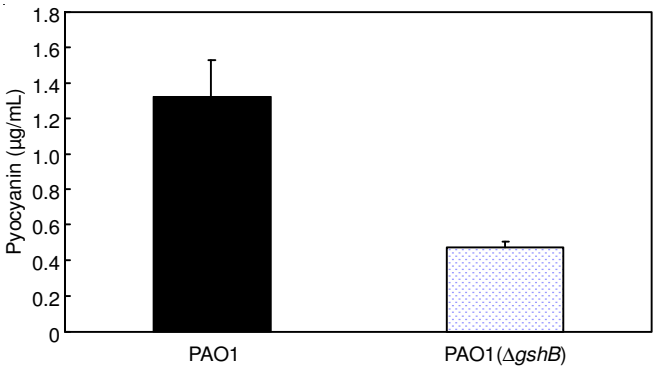


Fig. 1. Expression of the *phzA1* in PAO1 and PAO1(Δ*gshB*) mutant. The data from PAO1 are shown by squares and those from PAO1(Δ*gshB*) by triangles. The assays were independently repeated at least three times and the data shown are representative of comparable results

Decreased pyocyanin production in PAO1 (Δ*gshB*): It was noticed that PAO1(Δ*gshB*) exhibited unusual pigment phenotypes compared to PAO1 when cultivated in LB broth. The cultures of PAO1 were bluish green due to production of pyocyanin, whereas the cultures of PAO1 (Δ*gshB*) were much lighter (Fig. 2A). Quantification of pyocyanin production indicate that the PAO1 (Δ*gshB*) produced markedly less pyocyanin than the wild type strain PAO1 ($p < 0.01$), confirming the requirement of *gshB* for full level of pyocyanin production (Fig. 2B).

(a)



(b)

Fig. 2. Pyocyanin production in PAO1 and PAO1(Δ*gshB*) mutant. (A): The stationary-phase PAO1 and PAO (Δ*gshb*) cultures in LB were immediately centrifuged and placed in a cuvette, vortexing 5 min at room temp. (B): Data are expressed in the means ± STDEVD and were analyzed by ANOVA. $p < 0.01$, compared between PAO1 and PAO1 (Δ*gshB*) mutant. The assays were independently repeated at least three times

Effects of treatment with glutathione on pyocyanin production: To further determine whether adding GSH to *P. aeruginosa* strains had an effect on the pyocyanin production, we compared the pyocyanin production of strains in both the presence and the absence of 3 µg/µL GSH. As expected, we observed an increase in the pyocyanin production after the strain was treated with GSH. PAO1 and PAO1 (Δ*gshB*) treated with GSH produced larger amounts of pyocyanin than the control ($p < 0.01$) (Fig. 3). This result indicates that GSH can increase pyocyanin production.

Complementation of PAO1 (Δ*gshB*) mutant: To confirm the role of the *gshB* in the expression of the *phzA1* and the production of pyocyanin, we introduced the functional *gshB* gene of PAO1 by chromosomal insertion into PAO1 (Δ*gshB*). This result indicated the expression of *phzA1* and pyocyanin production were restored to PAO1 (Fig. 4). This result confirmed that PAO1 (Δ*gshB*) accounts for the observed phenotype.

Cystic fibrosis patients infected by *P. aeruginosa* are characterized by an increase in oxidant production and this excessive production is thought to be one of the main reasons through which the lung epithelium becomes damaged in cystic fibrosis[29,30]. In the cystic fibrosis lung, GSH, as one of the first

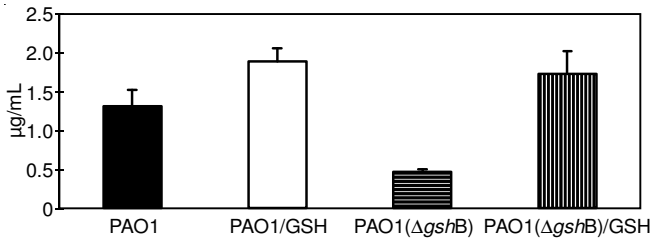


Fig. 3. Effect of GSH on pyocyanin production. PAO1 and PAO1 (Δ*gshB*) mutant were cultured in the presence of GSH (3 μg/μL) or in the absence of GSH for 22 h, following from which pyocyanin productions were determined. Data are expressed in the means ± STDEVD and were analyzed by ANOVA. The assays were independently repeated at least three times. $p < 0.01$, compared between PAO1and PAO1 (Δ*gshB*) mutant. $p < 0.01$, compared between PAO1 (Δ*gshB*) mutant and PAO1 (Δ*gshB*) mutant treated with GSH. $p < 0.05$, compared between PAO1 mutant and PAO1 treated with GSH

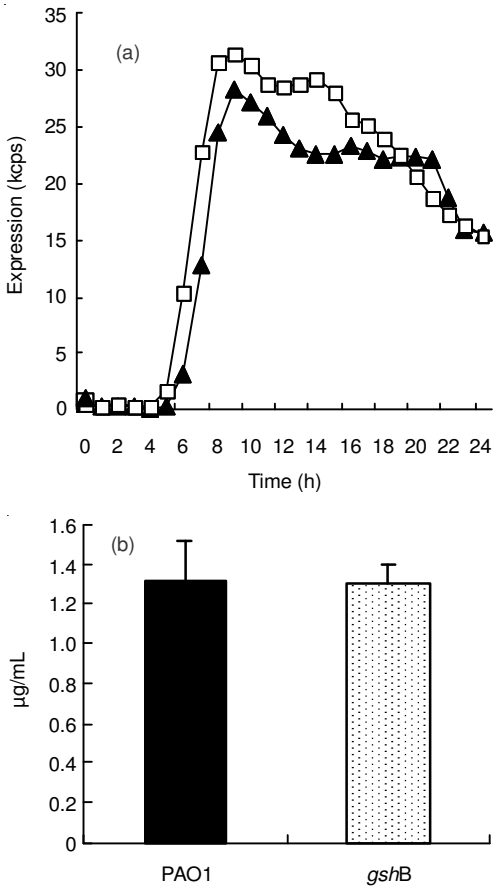


Fig. 4. Results of PAO1 (Δ*gshB*) mutant complement to restore the wild type phenotype. A: The expression of the *gshB*. The data from the wild type are shown by squares and those from the mutant by triangles. B: pyocyanin production

lines of defense against oxidant stress, is deficient in ELF. Evidence has shown PYOCYANIN depletes cellular GSH (up to 50 %) and increases oxidized GSH[31]. In the bronchiolar epithelial cell[32], GSH seems to contribute to the persistence of *P. aeruginosa* in the lungs of patients with cystic fibrosis. Glutathione plays an important role in the protection of the cystic fibrosis lung against oxidant-induced lung injury and might influence the pathogenicity of *P. aeruginosa*. Thus, previous research thought inhaled antioxidant therapy may be a more appropriate method of increasing GSH levels in the ELF of cystic fibrosis patients[22]. A variety of antioxidant therapies have been attempted in cystic fibrosis patients, including supplementation with vitamins C and E or N-acetylcysteine (a GSH precursor), GSH, or intravenous treatment with polyethylene glycol-conjugated superoxide dismutase(SOD) and catalase[21,33-35]. These studies thought inhaled antioxidants could improve lung epithelial functions[21, 22, 36,37]. However, O'Malley *et al.*[31] reported that pyocyanin can directly and rapidly oxidize GSH, leading to the formation of O_2^-, H_2O_2 and other ROS. These could in turn react with GSH to form GSSG in the antioxidant mode of the molecule and contribute to the cellular depletion of epithelial cell GSH over time. O'Malley *et al.*[31] suggests that the therapeutic approach of adiministration of GSH in cystic fibrosis patients was ineffective, or perhaps even deleterious.

To date, the benefits of the therapeutic approach of adiministration of GSH in cystic fibrosis infected with *P. aeruginosa* have been equivocal at best. In contrast, no data are available concerning the GSH effect on the pathogenicity of *P. aeruginosa.* In this study, we compared the characteristics of PAO1(Δ*gshB*) with that of wild type parent PAO1. The PAO1(Δ*gshB*) greatly decreased the expression of *phzA1* gene and reduced pyocyanin production. We further demonstrated that PAO1 and PAO1(Δ*gshB*) treated with GSH could produced higher levels of pyocyanin than the control. Our other experiments showed that in the presence of 3 μg/μL GSH, the sensitivity of *P. aeruginosa* was decreased against kanamycin, erythromycin, cefotaxime sodium, ciprofloxacin, carbenicillin and streptomycin[23]. These study does elucidate that GSH plays an important role in pathogenicity of *P. aeruginosa.*

In summary, in the present study we have shown that GSH can increase the pyocyanin production in *P. aeruginosa* and treatment using GSH in patients infected with *P. aeruginosa* needs to be discussed further. These findings may be of considerable interests to the pathophysiology of *P. aeruginosa.*

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