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Impact of glutathione on the gene expression of *exoY* and *exoS* in *Pseudomonas aeruginosa*

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Abstract: **[Objective]** To study the impact of GSH (glutathione) on the gene expression of *exoY* and *exoS* in *Pseudomonas aeruginosa*. **[Methods]** We treated *P. aeruginosa* with BSO (buthionine sulfoximine) and DEM (diethylmaleate) to deplete GSH, or construct the *P. aeruginosa* mutant containing a *lacZ*Gm disrupted *gshB* (glutathione synthetase) gene by homologous recombination technology. The expression of *exoY* and *exoS* was determined by measuring light production of the *lux*-based reporters on pMS402. **[Results]** The expression of *exoY* and *exoS* decreased in the *gshB* mutant and *P. aeruginosa* treated with BSO and DEM. **[Conclusion]** GSH in the *P. aeruginosa* can increase the expression of the genes *exoY* and *exoS*. Furthermore, this result provided possibilities to elucidate the molecular mechanisms of pathogenesis and immune response triggered by *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; glutathione; *exoS*, *exoY*
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1 INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen that primarily causes infections in immunocompromised individuals, burn victims, and CF (cystic fibrosis) patients[1]. Due to its ubiquitous nature, exposure to *P. aeruginosa* in the hospital setting is also prevalent, making it one of the more common nosocomial infections[2]. The type Ⅲ secretion system, a major virulence determinant, allows *P. aeruginosa* to inject secreted toxins through a syringe-like apparatus directly into the eukaryotic cytoplasm. Four of these determinants are known: ExoY, ExoS, ExoT, and ExoU and all participate, at varying levels, in the cytotoxicity of *P. aeruginosa* leading to the invasion and dissemination of *P. aeruginosa*[3].

Oxidative injury inflicted by *P. aeruginosa* in CF lungs is one of the causes in the disease manifestation[4]. GSH is considered to be one of the body's most important intra- and extracellular antioxidants, providing protection against exposure to high levels of reactive oxygen species[5-6]. When cells are exposed to oxidant species, GSH is converted to its oxidized form, GSSG (glutathione disulfide), by the action of glutathione peroxidase. GSSG is reduced back to GSH through the action of glutathione reductase. This cycling of GSH is an important means of limiting cellular exposure to cytotoxicity from oxidative damage[4]. However, studies have demonstrated that GSH is in high concentrations in normal respiratory ELF (epithelial lining fluid) and is deficient in CF ELF, this consumption and cellular loss of GSH plays an essential role in the onset and progression of CF[7].

With this background, we hypothesized that there is a strong association between GSH and the pathogenicity of *P. aeruginosa*. To address this hypothesis, *P.*

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aeruginosa was treated with either GSH or GSH depletion by BSO[8] and DEM[9], the result demonstrated that GSH affects the expression of *exoY* and *exoS*. We therefore generated a *gshB* mutant in the wild-type PAO1 strain. Our analysis of PAO1 and *gshB* mutant showed an unexpected result: GSH increased the expression of *exoS* and *exoY*.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Chemicals: Glutathione, glutathione disulfide, buthionine sulfoximine, and diethylmaleate were purchased from Sigma-Aldrich.

1.1.2 Bacterial strains, plasmids and growth conditions: All bacterial strains and plasmids used in this study are listed in Table1, *P. aeruginosa* strains and derivatives were grown at 37℃ on LB plates or in LB broth with shaking at 200 r/min. The antibiotics used in this study were: for *E. coli*, kanamycin (kan) at 50 μg/ml, Ampicillin (Amp) at 100 μg/ml; for *P. aeruginosa*, gentamicin (Gm) at 50 μg/ml, Trimethoprim (Tmp) at 300 μg/ml. The plasmid pMS402 carrying a promoterless luxCDABE reporter gene cluster was used to construct promoter-luxCDABE reporter fusions. The promoter regions of the gene were PCR amplified using *Pfx* DNA polymerase (Invitrogen) and primers synthesized according to the PAO1 genome data[10]. The promoter regions of *exoS* and *exoY* were cloned into the *Bam*HI-*Xho*I site upstream of the *lux* genes on pMS402. Using these lux-based reporters, gene expression was measured as counts per second (cps) of light production in a Victor^2 Multilabel Counter (Perkin-Elmer).

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference
E. coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lac*X74 *deoR recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL endA1 nupG*	Invitrogen
P. aeruginosa		
PAO1	Wild type, lab strain	
PAO(Δ*gshB*)	PA0407 replacement mutant of PAO1; PA0407::Gm^r	This study
Plasmids		
pMS402	Expression reporter plasmid carrying the promoterless *lux*CDABE; Km^r Tmp^r	[11]
pEX18Ap	*oriT*$^+$ *sacB*$^+$ gene replacement vector with multiple-cloning site from pUC18; Ap^r	[12]
pZ1918-*lacZ*Gm	source of gentamicin cassette; Gm^r	[13]
pRK2013	Broad-host-range helper vector; Tra$^+$ Km^r	[13]
pKD-*exoY*	pMS402 containing *exoY* promoter region	[11]
pKD-*exoS*	pMS402 containing *exoS* promoter region	[11]

1.2 Monitoring gene expression

Overnight cultures of the reporter strains were cultivated in fresh medium for three additional hours before use as an inoculant. Assays were carried out in a 96-well black plate with a transparent bottom. Five microliters of the fresh cultures were inoculated into the wells containing a total of 95 μl medium. Fifty microliters of filter-sterilized mineral oil were added to each well to prevent evaporation during the assay. Luminescence was measured every 30 min for 24h under different conditions. Bacterial growth was monitored at the same time by measuring the OD_{595} in the Victor^2 multilabel counter. All the experiments were repeated at least three times, and the figures shown are representative of similar profiles.

1.3 Construction of *P. aeruginosa gshB* mutants

The *gshB* knockout mutant was generated by allelic replacement through site-directed homologous recombination followed by *sacB*-Gm based counterselection. First, the *gshB* was amplified by PCR and then cloned into pEX18Ap, second, a *Sph*I DNA fragment containing the *lacZ*Gm from pZ1918-*lacZ*Gm was cloned at the *Sph*I site on the PCR fragment, 944-bp downstream of the *gshB* start codon, yielding a new suicide plasmid pSB*gshB*. The primer pairs for amplification of the *gshB* gene were gshb1 (5′-ACTGGATCCTCATTGCGGATCGTGGTG-3′), containing a *Bam*HI site, and gshb2 (5′-ATCAAGCTTATCACGTCGCAACCGACC-3′), containing a *Hin*d Ⅲ site. The resulting suicide vector

pSB*gshB* was used to replace the *gshB* gene. Briefly, overnight cultures of *E. coli* DH10B containing pSB*gshB* plasmid, *E. coli* DH10B containing pRK2013, and PAO1 were pelleted and resuspended respectively in the PBS, then mixed together and spotted onto the LB agar plate. Following incubation overnight at 37℃, bacterial cells were resuspended in PBS and appropriate dilutions were plated on PIA containing 150 μg/ml Gm. Subsequently, strains that had undergone a double-recombination event at this locus were selected by plating them on LB plus 10% sucrose containing 50 μg/ml Gm. Mutation was confirmed by PCR analysis and DNA sequencing. The *gshB* knockout mutant was named PAO(Δ*gshB*).

1.4 Effect of GSH on *exoS* and *exoY*

pKD-*exoS* and pKD-*exoY* were transformed into PAO1 by electroporation, respectively. The strains were grown in LB broth in the presence of 3 μg/μl or 1.5 μg/μl GSH. Control strains were similarly grown, but in the absence of GSH. This experiment was conducted in a 96-multiwell plate platform.

For GSH depletion, *P. aeruginosa* strains were grown in LB broth with 1.5 mmol/L BSO and 1mmol/L DEM. Control strains were not treated with BSO and DEM.

1.5 Measurement of intracellular total glutathione

The concentrations of total glutathione in *P. aeruginosa* cells were determined using a commercially available kit (Beyotime Institute of Biotechnology, Jiangsu, China). All procedures were completely complied with the manufacture's instructions.

2 RESULTS

2.1 Measurement of glutathione

To verify the effects of BSO and DEM treatment and the glutathione concentration in PAO(Δ*gshB*), total cellular GSH levels were measured in *P. aeruginosa* cells. The mean level of total GSH was 2.4 nmol/mg in PAO1. The combination of 1.5 mmol/L BSO and 1 mmol/L DEM led to a reduction of 80% in total cellular GSH, without affecting cell viability. This result demonstrated that treatment of BSO and DEM is a valid strategy to deplete GSH in *P. aeruginosa* cells. A significant GSH depletion in the PAO (Δ*gshB*) also was observed, indicating that the *gshB* gene was mutated successfully.

2.2 Effect of GSH on *exoS* and *exoY*.

To investigate whether GSH can affect the pathogenicity of *P. aeruginosa*, a group of 32 well-characterized *P. aeruginosa* genes that are related to pathogenicity were tested in LB broth with different concentrations of GSH. The promoter regions of these genes or their accommodating operons were polymerase chain reaction (PCR) amplified, cloned in pMS402 and transferred back into *P. aeruginosa*[11]. The results indicated that *exoY* and *exoS* were affected by GSH, whereas no effect was observed on any of the other genes. ExoS and ExoY are secretion toxins, belong to the type Ⅲ system of *P. aeruginosa*. ExoY is an adenylate

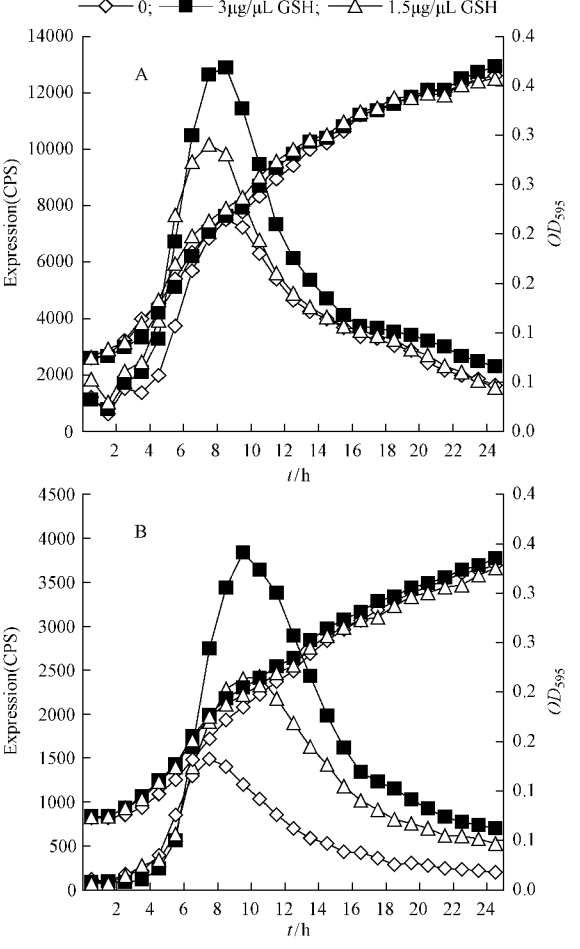

Fig. 1 Activation of *exoS* and *exoY* expression by GSH. The *exoS* and *exoY* expression profile and bacterial growth in the absence of GSH and in the presence of 1.5 ug/ml GSH and 3 ug/ml GSH. The assays were independently repeated at least three times, and the data shown are representative of comparable results.
A: Activation of *exoS* expression by GSH. B: Activation of *exoY* expression by GSH.

cyclase whose activity is associated with profound morphological changes in epithelial cells[14]. ExoS is a 453 amino acid, and have ADP-ribosylating activity toward low-molecular-mass GTP-binding proteins of the Ras family[15]. As shown in the Fig. 1, the expression of *exoY* and *exoS* were greatly enhanced in the presence of 1.5 μg/μl or 3 μg/μl GSH while bacterial growth was not affected. During a 24 h culture course, there is a rise in the expression of *exoY* and *exoS* with increasing GSH concentration.

2.3 The change of the expression of *exoS* and *exoY* in the PAO (Δ*gshB*) mutant

To further identify that GSH indeed affect the expression *exoY* and *exoS*, PAO (Δ*gshB*) was constructed. pKD-*exoS* and pKD-*exoY* were transferred into PAO (Δ*gshB*). The results indicate that the expression of the *exoS* and *exoY* is decreased compared to that of the parent strain PAO1.

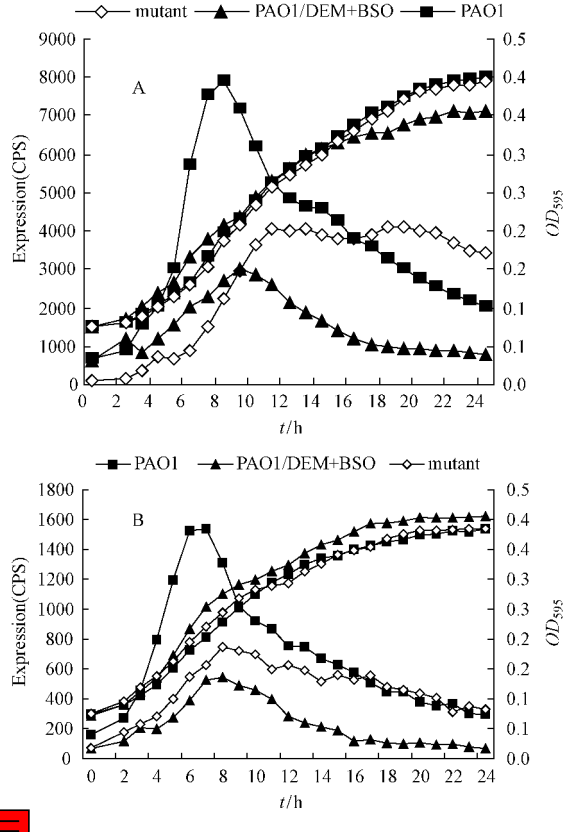

Fig. 2 Expression profiles and corresponding growth curves are shown for *exoS* and *exoY* in the wild type PAO1, PAO (Δ*gshB*) mutant and PAO1 treated with 1.5 mmol/L BSO and 1 mmol/L DEM. A: The expression profiles and corresponding growth curves of *exoS*; B: The expression profiles and corresponding growth curves of *exoY*.

To confirm the above results, we studied the effect of GSH depletion by BSO and DEM, to investigate whether the expression of *exoY* and *exoS* require GSH. We performed a side-by-side comparison among the PAO1、PAO (Δ*gshB*) and PAO1 was treated with BSO and DEM. The analysis revealed that the expression of *exoY* and *exoS* showed a difference between the PAO1 and PAO1 treated with BSO and DEM, however there was no major difference between the PAO (Δ*gshB*) and PAO1 treated with BSO and DEM (Fig. 2). PAO1 treated with BSO and DEM reduced the expression of *exoY* and *exoS* to that of the PAO (Δ*gshB*) level, this result corresponded well to the results derived from the PAO (Δ*gshB*).

3 DISCUSSION

P. aeruginosa secretes a variety of factors provoking a strong inflammatory condition in compromised patients. In most cases, *P. aeruginosa*'s cytotoxicity has been associated with its ability to generate superoxide and H_2O_2. Several studies have linked CF lung disease with increased oxidative stress[16]. This oxidative burden compromises antioxidant defenses, leading to protein oxidation[16] and lipid peroxidation, and is thought to contribute to the destruction of lung tissue in CF. On the other hand, multiple studies have revealed that the GSH levels are decreased in cystic fibrosis patients infected with *P. aeruginosa*[17]. GSH is a potent antioxidant capable of scavenging a variety of oxidant molecules, thereby protecting cells and tissues from damage by oxidant released by inflammatory cells or delivered from other exogenous sources[18]. When placed under increased oxidative stress cells often exhibit a decrease in GSH, as well as a decrease in the ratios of GSH/GSSG[19]. Thus, an increase in GSH levels in lungs of CF patients may be warranted for the prevention and treatment of CF lung disease[20]. 17 CF patients inhaled GSH and showed the GSH levels in the bronchoalveolar lavage fluid was increased three-to fourfold, but no obvious change before or after GSH treatment was found in the number, area, and density of oxidized proteins[21]. Thus the administration of GSH is ineffective in the regulation of

the oxidative stress or perhaps even deleterious in the airways of patients infected with *P. aeruginosa* strains. Pyocyanin directly oxidizes GSH, leading to the generation of ROS (reactive oxygen species) superoxide and hydrogen, so the GSH could enhance the formation of ROS, rather than serving an antioxidant function in the cell[4]. These results are consistent with our oberservations.

In our study, to test the relation between GSH and *P. aeruginosa*, we depleted the GSH of *P. aeruginosa* by two methods, construction of the PAO (Δ*gshB*) mutant or treated cells with DEM and BSO. The results showed that the GSH content existing in the culture of the *P. aeruginosa* is important to the expression of the *exoS* and *exoY*. In the PAO(Δ*gshB*) the expression of *exoS* and *exoY* are decreased. As expected, adding GSH to *P. aeruginosa* causes the increased expression of *exoS* and *exoY*. This data raise the possibility that GSH plays an important role in the regulation of pathogenicity of *P. aeruginosa*, but using GSH to treat patients infected with *P. aeruginosa* may be dangerous. Our studies indicated that, directly or indirectly, the GSH activates expression of *exoY* and *exoS*, so GSH treatment may be a risk for patients infected with *P. aeruginosa*. It has been reported the rhl system represses the expression of exoenzymes, but there is no evidence demonstrating that GSH affects the quorum sensing systems in our studies. And in recent years, it has been found that cellular GSH is not only the "redox tone" of the intracellular environment, but also plays an important regulatory role in inflammatory processes via gene transcription[22]. It suggests there is another mechanism involved in the regulation of GSH. In the present study we demonstrate that addition of GSH to *P. aeruginosa* would provoke more *P. aeruginosa* cytotoxicity.

In conclusion, our data have shown that GSH can increase the expression the *exoS* and *exoY*. Further studies are currently underway to analyze the role of GSH on *exoS* and *exoY* regulation.

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谷胱甘肽对铜绿假单胞菌 *exoS* 和 *exoY* 基因的影响

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摘要:【目的】研究谷胱甘肽对铜绿假单胞菌 *exoS* 和 *exoY* 基因表达的影响。【方法】利用丁硫氨酸亚砜胺和马来酸二乙酯同时耗竭细胞内的谷胱甘肽,并构建包含被 *lacZ*Gm 破坏的谷胱甘肽合成酶基因的突变体。通过分别连有 *exoS* 和 *exoY* 基因启动子的 pMS402 质粒上 Lux 报道子发光值大小检测 *exoS* 和 *exoY* 基因表达变化情况。【结果】*exoS* 和 *exoY* 基因的表达在用化学药品耗竭的细胞中或是在谷胱甘肽合成酶突变体中都降低。【结论】铜绿假单胞菌细胞内的谷胱甘肽可以促进 *exoS* 和 *exoY* 的表达。这将为进一步研究铜绿假单胞菌的感染以及致病性机理提供一定的理论基础。

关键词:铜绿假单胞菌;谷胱甘肽;*exoS*;*exoY*

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