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Original article

Impact of *pauR* transcription factor deletion in the polyamine degradation pathway on antimicrobial stress responses in *Pseudomonas aeruginosa*

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Abstract

Background: The *pauR* transcription factor in *Pseudomonas aeruginosa (P. aeruginosa)* acts as a global regulator of enzymes for polyamine utilization, and polyamine transporters encoded by *spuDEFGH* operon. Although *spuDEFGH* is linked to the type III secretion system, its role under oxidative and antibiotic stress is unclear. As some PauR-regulated genes potentially contribute to multidrug resistance, we deleted *pauR* instead of targeting individual genes. This study explores the impact of *pauR* deletion on stress responses.

Objectives: This study aimed to construct *pauR*-deleted and *pauR*-expressing strains in *P. aeruginosa* and to investigate the role of *pauR* in response to antimicrobial agents,including antibiotics and oxidants.

Methods: *P. aeruginosa pauR* strain was generated using Gibson assembly and the Cre/*loxP* gene-deletion system. Antibiotic and oxidant susceptibility profiles of both wild-type and mutant strains were assessed using disc diffusion and plate sensitivity assays, respectively.

Results: The *pauR*-deleted mutant was constructed and verified through PCR-based and local genomicDNA sequence analyses. The disc diffusion test revealed no significant difference in antibiotic susceptibility levels between the tested strains. However, exposure to the superoxide generator resulted in a significantly decreased susceptibility in the *pauR* mutant compared to the wild type, with a 99% of confidence interval. Furthermore, an ectopic *pauR* expression in the *pauR* mutant increased its susceptibility.

Conclusion: While *pauR* does not appear to play a key role in antibiotic resistance, it may contribute to resistance against the oxidant in *P. aeruginosa*. In further experiments, genes within the *pauR* regulon that contribute to the oxidative stress response mechanism will be identified.

Keywords: Antibiotic, antimicrobial resistance, oxidative stress, *Pseudomonas aeruginosa*.

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Pseudomonas aeruginosa (P. aeruginosa) is a multidrug-resistant human pathogen that commonly infects immunocompromised patients. Identifying alternative killing strategies is essential. The global transcription factor PauR regulates 29 genes distributed across 12 genomic loci, [1] including some *pauABCD*, which are involved in polyamine catabolism via the γ-glutamylation pathway. [2] The PauR-regulated *spuDEFGH* operon encodes ATP-binding cassette (ABC) transporters, which are implicated not only in polyamine transport [3] but also in the induction of the type III secretion system (T3SS), a major virulence factor. [4] Along with *Pseudomonas* genome database, several loci under PauR regulation contained predicted membrane transporters that potentially involve in drug extrusion. These transporters not only transport its physiological substrates such polyamines, but also expel several toxic substances. [5]

Beyond PauR-regulated genes, polyamines mitigate cell-surface damage caused by membrane-targeting antimicrobials, including both oxidants and antibiotics. [6] Although some PauR regulons are well-studied in virulence, their link to antimicrobial stresses remain poorly defined. This study investigates the collective role of *pauR*-regulated genes in antimicrobial resistance by deleting *pauR* and assessing the phenotypes of the resulting mutants under oxidative and antibiotic stress.

Materials and methods

Bacterial strains, plasmids, and growth conditions

All *P. aeruginosa* and *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco, USA) at 37°C with continuous shaking at 180 rpm under aerobic conditions. For *E. coli* cultivation, the medium was supplemented with 100 μg/ml ampicillin (Amp), whereas for *P. aeruginosa* cultivation, the medium was supplemented with 200 μg/ml carbenicillin (Cb), 30 μg/ml tetracycline (Tet), and 30 μg/ml gentamicin (Gm) as required for selective marker of plasmid. All bacterial strains listed in **Table 1**, with or without plasmids described in **Table 2**, were cultured and maintained, all experiments were conducted in the BSL-2 laboratory according to the procedure MUSC2022-029, approved by the Biosafety Committee of the Faculty of Science at Mahidol University, Thailand.

Construction of *P. aeruginosa* pauR deletion mutant

All PCR products were amplified using Phusion high-fidelity DNA polymerase system (Thermo Scientific) and primers list in **Table 3**. The three DNA fragments include a *loxP*-Gm cassette fragment was PCR amplified from pCM351 using primer LGFW and LGRV. The other two, left-flanking (LF) and right-flanking (RF) fragments were PCR amplified from *P.*

Table 1. Bacterial strains used in this study.

Bacterial strains	Genotype or characteristics	Sources
Escherichia coli	λ^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*	
(E. coli) DH5α	(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	Laboratory stock
P. aeruginosa PAO1	Wild type	Laboratory stock
Δ*pauR*	*pauR* mutant, gene deletion in *pauR* coding region	This study

Table 2. Plasmids used in this study.

Plasmids	Genotype or characteristics	Sources
pBBR1MCS-4	Medium-copy-number expression vector, Ap^r	Laboratory stock
pBBR-*pauR*	pBBR1MCS-4 containing full length *pauR*, Ap^r	This study
pUC18	High-copy-number cloning vector, Ap^r	Laboratory stock
pCM351	Plasmid contains *loxP*-flanked Gm^r cassette	Laboratory stock
pUC18-Gm-*loxP-pauR*	pUC18-Gm^r-*loxP* containing deleted *pauR*, Ap^r, Gm^r	This study
pCM157	Cre recombinase expression vector, Tet^r	Laboratory stock

Table 3 Primers used in this study.

Primers	Sequence (5'- 3')	Purposes
FLFW	ATA AGG AGG ACT CCA CCC	Full length forward primer
FLRV	CGG GGT CTT GTG CTT CAG	Full length reverse primer
LFFW	GGATCCCCGGGTACCCTACAGCACCGAGCGTAG	Forward primer for left-flank deletion
LFRV	CGTGTTAACCGGTCGTTTGGCGAGTTCGCGC	Reverse primer for left-flank deletion
LGFW	CGACCGGTTAACACGCGTACGTAGG	Forward primer for *loxP*-Gm cassette
LGRV	GAAAACGGTGCCCTGACGTCTAGATCTGA	Reverse primer for *loxP*-Gm cassette
RFFW	CAGGGCACCGTTTTCGCAATCCCTACGATGTG	Forward primer for right-flank deletion
RFRV	TGATTACGAATTCGAGCTCCTTGTCGCCAACCTTCGG	Reverse primer for right-flank deletion
M13F	GTAAAACGACGGCCAGT	Universal forward primer

aeruginosa PAO1 genomic DNA (primer LFFW and LFRV for LF fragment, and primer RFFW and RFRV for RF fragment), then both were used for flanking a *loxP*-Gm cassette fragment in Gibson assembly process (NEBuilder® HiFi DNA Assembly Master Mix) with *Kpn*I and *Sac*I linearized the pUC18 plasmid vector (**Figure 1A**) yielding pUC18-Gm-*loxP-pauR*. The plasmid pUC18-Gm-*loxP-pauR* had been transformed into PAO1 wildtype using electroporation method resulting in Δ*pauR*::Gm, then the Δ*pauR*::Gm transformants were selected by Gm^r phenotype and PCR verification. The pCM157 was electroporated into Δ*pauR*::Gm contributing to Tet^r Δ*pauR*/pCM157, and subsequently induced loss of pCM157, which finally generate markerless strain Δ*pauR* confirmed by PCR amplification (**Figure 1B)**.

Construction of P. aeruginosa full-length

The full-length *pauR* DNA fragment was Phusion-PCR amplified from the *P. aeruginosa* PAO1 genomic DNA with primers FLFW and FLRV. The PCR product was cloned into expression vector pBBR1MCS-4. PAO1 wildtype and Δ*pauR* were electroporated with empty and full-length *pauR* pBBR1MCS-4, then transformants were screened by Ap^r selectable marker and were PCR checked to verify DNA insert orientation using primers M13F and FLRV, and DNA sequencing was performed to verify the DNA insert sequence.

Disc diffusion test

Disc diffusion assay determine susceptibility in *P. aeruginosa* strains against antibiotics by comparing size of inhibition zone. The culture were grown in Mueller Hinton (MH) broth at 37ºC with continuous shaking 180 rpm. The cultures were grown overnight and then subcultured into the log phase and adjusted cell concentration before the assay. The commercial antibiotic discs were placed on bacteria-containing agar. The plates were overnight incubated, then clear zone was recorded.

Plate sensitivity assay

Plate sensitivity assay was performed to examine susceptibility level against oxidazing agents as previously described. [7] Luria-Bertani agar (LA) plates were contained different sorts of oxidizing agents. The plates were overnight incubated statically at 37°C and the bacterial growth among these strains was compared.

Results

Strain constructions

P. aeruginosa pauR mutant, Δ*pauR*, was constructed by a Cre-*loxP* gene deletion technique as mentioned in the methods, and overall key events of gene deletion are shown in **Figure 1A**. In **Figure 1B**, the three clones of markerless strain Δ*pauR* in lane 1-3, were verified by PCR amplification with primer LFFW and RFRV, which represent the gene deletion of shorter PCR product size compared to longer PCR product sizes of Δ*pauR*::Gm and native *pauR* in lane 4 and 5, respectively. The full-length *pauR* on pBBR plasmid was analyzed by PCR in **Figure 1C** and DNA sequencing (data not shown).

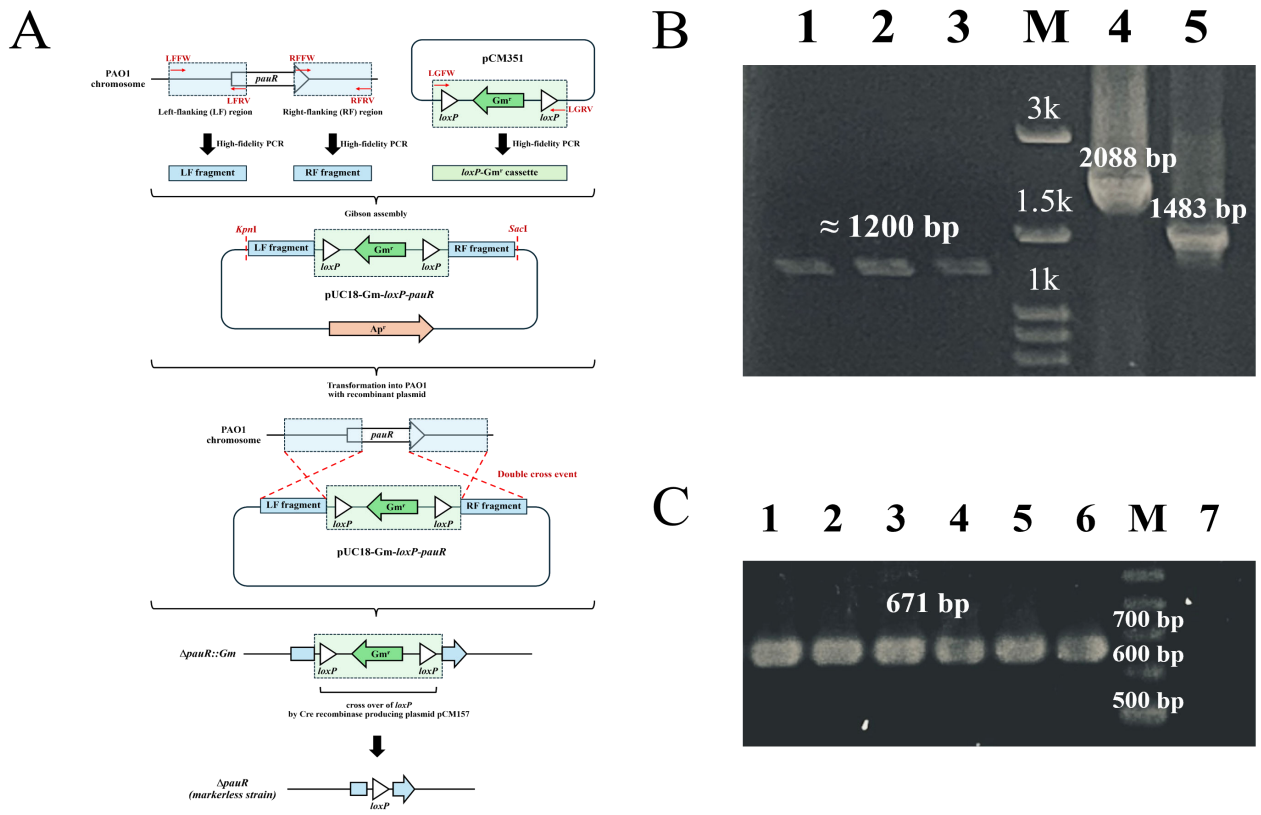

Figure 1. Diagram of gene replacement and deletion of suicide vector pUC18-Gm-*loxP-pauR* on PAO1 chromosome **(A)**, and confirmation of Δ*pauR* deletion **(B)** and pBBR/FL-*pauR* **(C)** by PCR amplification. In **(B)** section, the Δ*pauR* was confirmed by PCR with primers, LFFW and RFRV; lane 1-3 represented approximate 1200-bp PCR products of three Δ*pauR* clones without a Gm^r gene; lane 4 represented a 2088-bp PCR product of Δ*pauR*::Gm^r; lanes 5 represented a 1483-bp PCR product of PAO1 wild type. In **(C)** section, the three clones of PAO1/pBBR-FL-*pauR* (overexpression strain, lane 1-3) and Δ*pauR*/pBBR-FL-*pauR* (complementation strain, lane 4-6) were confirmed by PCR with specific primers yielding 671-bp PCR product of both strains; lane 7, empty pBBR used as negative control. Lane M of both **(B)** and **(C)** is a 100 bp+3K DNA ladder (SMOBIO, Inc., Hsinchu City, Taiwan).

Disc diffusion assay determining antibiotic susceptibility profile

P. aeruginosa constructed strains (PAO1/pBBR-*pauR*, overexpression; PAO1/pBBR, wildtype control; Δ*pauR* /pBBR, deletion; and Δ*pauR* /pBBR-*pauR*, complementation) were tested with six different antibiotics consisting Tobramycin (a representative of aminoglycosides), Ofloxacin (a representative of quinolones), Imipenem and Ceftazidime (representatives of β-lactams, carbapenem and cephalosporin, respectively), Colistin and Tetracycline (representatives of uncatagorized group). The antibiotic abbreviates and concentration (in μg unit) are described in **Figure 2**. Overall, the results show no significant difference in antibiotic susceptibility levels between the tested strains due to their similar levels of inhibition zone towards all tested antibiotics analyzed by Student's *t*-test (two-sample unequal varience, $P < 0.05$). By screening the antibiotic susceptibility profile using the disc diffusion test, *P. aeruginosa pauR* does not appear to play a key role in antibiotic resistance.

Plate sensitivity assay determining oxidative stress response

To investigate the role of *P. aeruginosa pauR* in oxidative stress response, D*pauR* and *pauR*-expressed mutants were used to tested against five oxidants with a growth-inhibiting concentration ranges as indicated in **Figure 3**. By performing the plate sensitivity assay, the results showed no significant difference in the susceptibility levels against peroxides (hydrogen peroxide, H_2O_2; and cumene hydroperoxide, CHP), thiol-chelating agent (N-Ethylmaleimide, NEM) and strong oxidizing agent (sodium hypochlorite, NaOCl) among these tested strains.

In contrary, an exposure to the superoxide generator, paraquat, resulted in a significantly decreased susceptibility level in the Δ*pauR* mutant compared to the wild type, with a confidence interval of 99%.

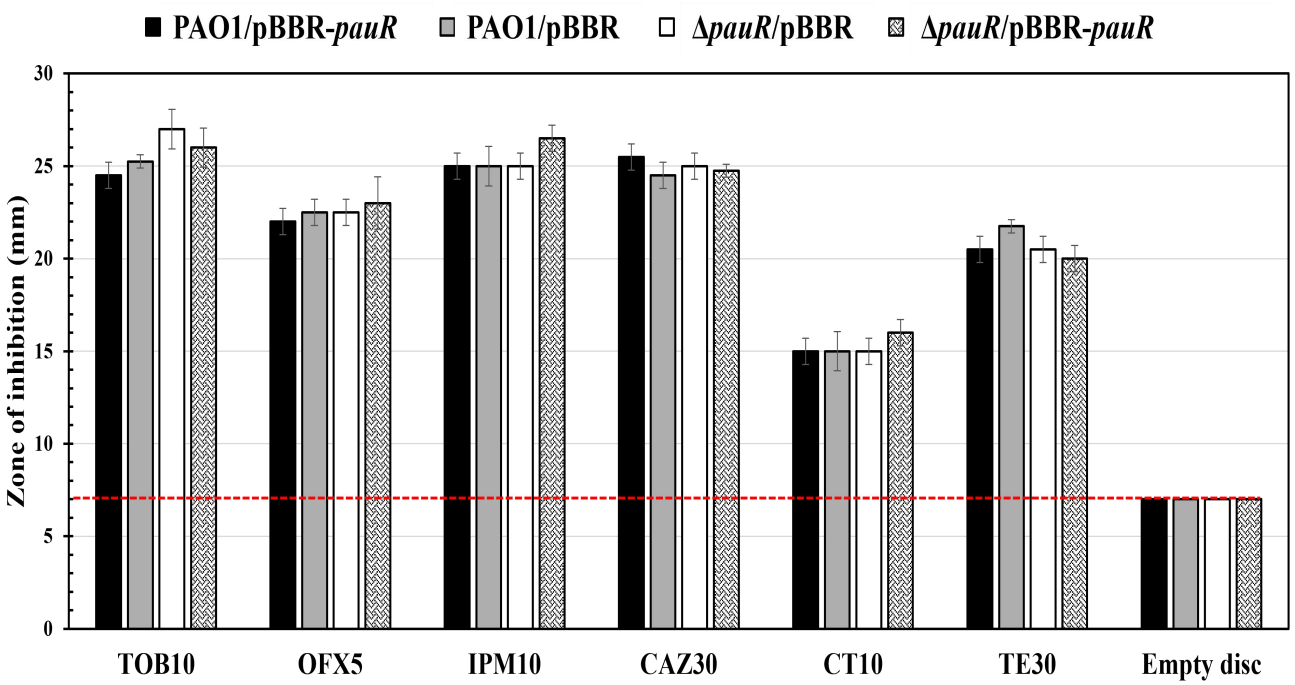

Figure 2. Disc diffusion assay was used to determine antibiotic susceptibility levels in various *P. aeruginosa* PAO1 strains including PAO1/pBBR-*pauR* (Black), PAO1/pBBR (Grey), Δ*pauR*/pBBR (White), and Δ*pauR*/pBBR-*pauR* (Weave) strains. Antibiotics used in this study include Tobramycin (TOB10), Ofloxacin (OFX5), Imipenem (IPM10), Ceftazidime (CAZ30), Colistin (CT10) and Tetracycline (TE30). The diameters of the clear zones were measured after overnight incubation, with the empty disc diameter being 7 mm. Bars indicate the mean ± standard deviation (SD) of two biologically independent experiments.

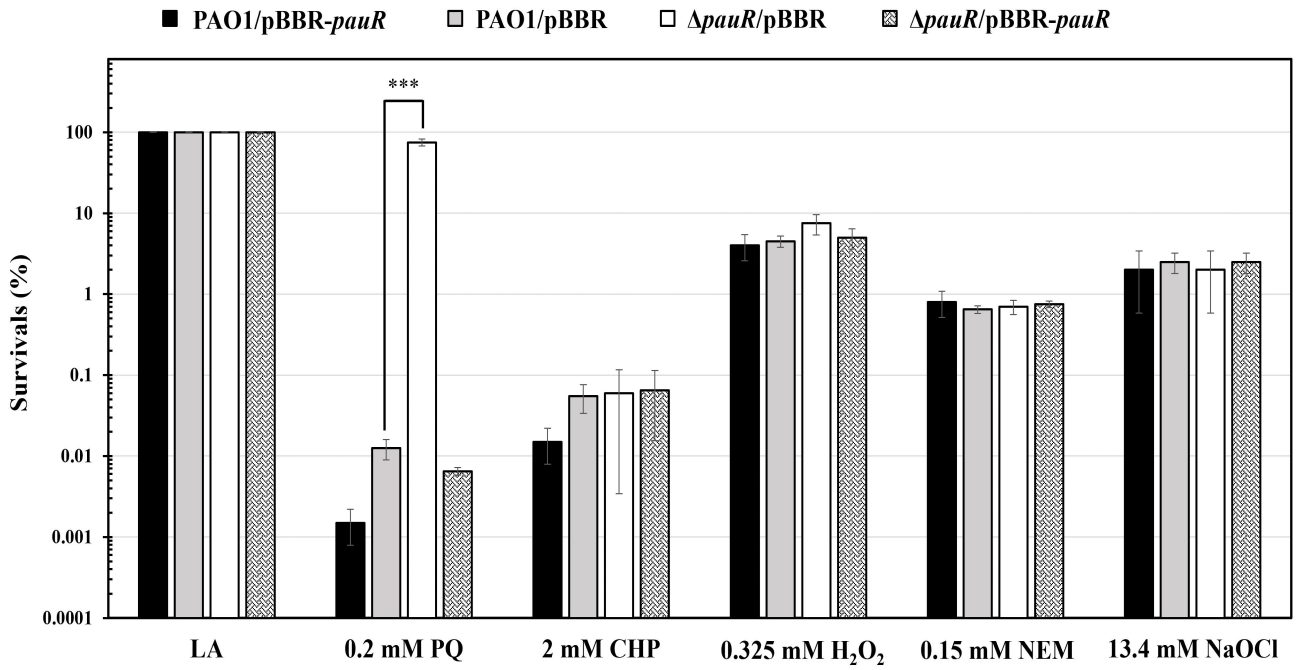

Figure 3. Plate sensitivity assay was used to evaluate oxidative stress response in *P. aeruginosa* strains including PAO1/pBBR-*pauR* (Black), PAO1/pBBR (Grey), Δ*pauR*/pBBR (White), and Δ*pauR*/pBBR-*pauR* (Weave) strains. Oxidants used in this study include paraquat (0.2 mM PQ); cumene hydroperoxide (2 mM CHP), hydrogen peroxide (0.325 mM H_2O_2), N-ethylmaleimide (0.15 mM NEM) and sodium hypochlorite (13.4 mM NaOCl). The percentage survival was calculated by number of CFU on plate with oxidants divided by CFU on plate without oxidant times 100. Data shown are the means ± SD of percent survivals at overnight incubation from two independent experiments. The three asterisk indicates a statistically significant difference (***$P < 0.01$) using *t*-test relative to the PAO1 wild type.

Furthermore, *pauR* expression in the *pauR* mutant increased its susceptibility to paraquat in both complementation and overexpression strains **(Figure 3)**. Indeed, *P. aeruginosa pauR* may contribute to resistance against paraquat-induced oxidative stress. A possibility that PauR may regulate the gene(s) that function(s) in mechanism to maintain the growth from the toxicity of paraquat.

Discussion

A superoxide generator, paraquat, and the polyamine are interconnected through polyamine utilization group of genes. The genes within the *pauR* regulon that contribute to the paraquat resistance mechanism will be further identified. In *Pseudomonas putida*, polyamines alleviate H_2O_2 damage by inducing the production of cytoplasmic siderophores, thereby reducing the availability of free iron and subsequent H_2O_2formation. [8] Dynamic interaction influences stress responses, making polyamine metabolism a key factor in mitigating oxidative stress. [9] Overall data hightlight that targeting polyamine metabolism to enhance oxidative stress or boosting polyamine levels to improve host defense could be alternative ways to fight againt bacterial infections.

The construction of *pauR*-deleted and *pauR*-expressed mutant strains were successfully done in this study and used to determine in the physiological function assays. The results in the disc diffusion test showed that *P. aeruginosa pauR* does not appear to play a key role in antibiotic resistance. Next, oxidant susceptibility results showed that deletion of *pauR* caused a decreased susceptibility level while an overexpression of *pauR* contributed to an increased susceptibility level in *P. aeruginosa*. This suggests that PauR may contribute to resistance against paraquat-induced oxidative stress in *P. aeruginosa*.

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Conflict of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors have any conflicts of interest to disclose.

Data sharing statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request for noncommercial purposes.

References

1. Trouillon J, Ragno M, Simon V, Attrée I, Elsen S. Transcription Inhibitors with XRE DNA-Binding and Cupin Signal-Sensing Domains Drive Metabolic Diversification in *Pseudomonas*. mSystems 2021; 6: e00753-20.
2. Chou HT, Li JY, Peng YC, Lu CD. Molecular characterization of PauR and its role in control of putrescine and cadaverine catabolism through the γ-glutamylation pathway in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2013;195: 3906-13.
3. Lu CD, Itoh Y, Nakada Y, Jiang Y. Functional analysis and regulation of the divergent *spuABCDEFGH-spuI* operons for polyamine uptake and utilization in *Pseudomonas aeruginosa* PAO1. J Bacteriol 2002;184: 3765-73.
4. Lin Q, Wang H, Huang J, Liu Z, Chen Q, Yu G, Xu Z, Cheng P, Liang Z, Zhang LH. Spermidine Is an intercellular signal modulating T3SS expression in pseudomonas aeruginosa. Microbiol Spectr 2022; 10:e0064422.
5. Short FL, Liu Q, Shah B, Clift HE, Naidu V, Li L, et al. The acinetobacter baumannii disinfectant resistance protein, AmvA, is a spermidine and spermine efflux pump. Commun Biol 2021;4:1114.
6. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-Localized Spermidine Protects the *Pseudomonas aeruginosa* Outer Membrane from Antibiotic Treatment and Oxidative Stress. J Bacteriol 2012;194:813-26.
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;195:3299-308.
8. Barrientos-Moreno L, Molina-Henares MA, Pastor-García M, Ramos-González MI, Espinosa-Urgel M. Arginine biosynthesis modulates pyoverdine production and release in Pseudomonas putida as part of the mechanism of adaptation to oxidative stress. J Bacteriol 2019 21;201:e00454-19.
9. Solmi L, Rosli HG, Pombo MA, Stalder S, Rossi FR, Romero FM, et al. Inferring the significance of the polyamine metabolism in the phytopathogenic bacteria *Pseudomonas syringae*: A meta-analysis approach. Front Microbiol 2022;13:893626.