MvaT negatively regulates pyocin S5 expression in *Pseudomonas aeruginosa*

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A R T I C L E   I N F O

A B S T R A C T

Regulatory mechanisms that direct the synthesis and release of pyocin S5, a surface-acting bacteriocin produced by *Pseudomonas aeruginosa*, are relatively unknown. This study aims to identify transcription factors that regulate pyocin S5 expression in *P. aeruginosa* PAO1. We captured the transcription factor MvaT using the promoter region upstream of *S5* gene (S5P). Further, we demonstrated specific binding of MvaT and its paralog MvaU to S5P using a gel-shift assay. Lastly, we showed that MvaT negatively regulates the *S5* gene expression by gene deletion and transcriptomic analysis. Our findings provide valuable insights into the regulation of pyocin S5 production, which paves the way to develop novel therapeutics against *P. aeruginosa* infections.

1. Introduction

Pyocins are antimicrobial bacteriocins produced by many *Pseudomonas aeruginosa* strains that target opportunistic pathogens of the same genus. In *P. aeruginosa*, three types of pyocins (R, F, and S) have been reported.[1] Both R- and F-type pyocins resemble phage tails, whereas S-type pyocins are colicin-like small soluble proteins that comprise multiple domains for receptor binding, translocation and killing. To date, the bactericidal properties of various pyocins[1–9] and the functions of associated immunity proteins[10] have been extensively characterized, thus establishing pyocins as next-generation therapeutics against infections caused by *Pseudomonas*[3,4,7,11] in addition to antimicrobial peptides.[12]

Pyocin production is inducible by treatments that cause DNA damage,[1,13–15] and under the conditions of low oxygen[16] and denitrification.[17] Briefly, DNA-damaging mutagens increase *recA* gene expression and activate RecA protein. The active RecA protein cleaves repressor PrtR, leading to the expression of an activator PrtN. In turn, PrtN binds to a P-box in the promoters of pyocin genes and activates pyocin production.[18] PrtR autoregulates itself to maintain a relatively stable level, limiting pyocin production and contributing to *P. aeruginosa* pathogenesis and resistance against ciprofloxacin19. This P-box-regulatory mechanism has been elucidated for producing pyocins, including S1, S2, AP41, R2, and F2, where P-boxes were found in the DNA upstream regions.[20]

Previously, we reported the bactericidal activity of pyocin S5 (PA0985, encoded by the region nt1066321-1067817) against the *P. aeruginosa* clinical isolates through membrane damage.[4] We reported a predicted immunity protein that confers resistance to pyocin S5 in a sensitive strain of *P. aeruginosa*.[10] In another study, probiotic *E. coli* Nissle (EcN) was engineered to express pyocin S5 and colicin E7. The engineered EcN has been proven to eliminate and prevent *P. aeruginosa* gut infection in animal models.[21] McCaughey et al. have shown that pyocin S5 at a low concentration shows strong efficacy in a murine model of *P. aeruginosa* lung infection.[22] Pyocin S5 also improves survival in a *Galleria mellonella* infection model and a murine model of *P. aeruginosa* sepsis.[23]

As pyocin production can stimulate biofilm formation and affect bacterial population,[24,25] understanding the regulation of pyocin S5 production could potentially enhance the effectiveness of pyocin S5 as therapeutics against *P. aeruginosa*. Noticeably, the upstream region of *S5* and its immunity gene (*S5I*, PA0984) lacks the characteristic P-box regulatory motif which modulates the synthesis, release and activation of other pyocins.[26,27] In perspective, the regulatory mechanisms for *S5* and *S5I* could differ from the typical P-box-regulatory mechanism. To date, few transcription factors that regulate *S5* and *S5I* expression have been identified.

In this study, we aimed to identify transcription factors that regulate pyocin S5 expression. Briefly, we cloned the upstream region of S5 gene (S5P) and used S5P to capture transcription factor from the proteome.

We sequenced the captured transcription factor. Next, we overexpressed the identified transcription factor and its paralog and verified their binding activities. Lastly, we studied their roles in regulating *S5* expression by gene deletion and transcriptomic analysis in *P. aeruginosa*.

2. Materials and methods

2.1. Strains, plasmids, and chemicals

Pseudomonas aeruginosa PAO1 (ATCC 15692) was used for this study. *Escherichia coli* Top10 and BL21(DE3) were used for gene cloning and protein expression, respectively. Plasmids pET30b(+), pGEM-T, and pUCP18 were used for protein overexpression, gene deletion and complementation, respectively. The strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown at 37 °C in LB broth or on LB agar plates, and antibiotics (100 μg/ml ampicillin, 50 μg/ml kanamycin, 200 μg/ml carbenicillin, streptomycin 100 μg/ml) were added when necessary. Chemicals were purchased from Sigma Aldrich (USA) unless otherwise specified.

2.2. Transcription-factor capture and sequencing analysis

To isolate transcription factors that bind to the upstream region of *S5* gene, we prepared DNA fragment and total protein according to [28] with modifications. First, we amplified a *S5* upstream region −404 to −1 (S5P) from the genomic DNA of *P. aeruginosa* PAO1 by PCR using iProof DNA polymerase (Biorad, USA) and 5′-biotinylated primers (Table S1), followed by gel purification using QIAGEN Gel Extraction Kit (Qiagen, Germany). Second, we extracted the total protein from *P. aeruginosa* PAO1 cells at exponential phase after cell lysis by ultra-sonication. The biotin-labeled PCR product (15 μg) was incubated with streptavidin-coupled Dynabeads T1 (Life Technologies, USA), and the unbound DNA was removed by magnetic separation. The S5P-coupled Dynabeads were equilibrated with binding buffer (50 mM Tris-HCl at pH 8.0, 0.5 mM EDTA, 100 mM NaCl, 0.05 mM Triton X-100), and incubated with 0.5 g of total protein by shaking for 3 h. Salmon sperm DNA at 10 mg/ml was added to reduce unspecific binding. After washing thrice with binding buffer, the captured protein was eluted with 1 M NaCl and separated by SDS-PAGE.

After gel electrophoresis, protein bands were excised for MALDI-TOF mass spectrometry analysis.[29] The protein with the highest MASCOT protein search score was selected and PA numbers were retrieved from

the Pseudomonas Genome Database (http://www.pseudomonas.com).

2.3. Protein overexpression and purification

To characterize the *in vitro* binding activity of transcription factors against S5P, we fused a 6 x His-tag to protein candidates and overexpressed them in *E. coli*. *mvaT* and *mvaU* genes were amplified from PAO1 genomic DNA by using iProof DNA polymerase and gel purified. The purified PCR products and plasmid pET30b(+) were digested by restriction enzymes *Nde*I and *Xho*I (New England Biolabs, USA). The digested DNA fragments were purified and ligated by T4 DNA ligase and transformed into *E. coli* Top10 cells. Successful genetic constructs (pE-T, and pE-U) were screened by colony PCR and validated by by DNA sequencing.

Next, recombinant plasmids were transformed into *E. coli* BL21 (DE3) and induced for protein overexpression using 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG). The overexpression of MvaT-His$_6$ and MvaU-His$_6$ were confirmed by SDS-PAGE analysis and purified by fast protein liquid chromatography (FPLC).[4]

2.4. Electrophoretic mobility shift assay (EMSA)

The binding activities of MvaT and MvaU to S5P were investigated by EMSA. The differing amounts of MvaT-His$_6$ or MvaU-His$_6$ protein (0 ng, 20 ng, 40 ng, 60 ng, and 80 ng) were incubated with S5P (50 ng) for 20 min, in the presence of *rpoD* DNA fragment (30 ng) as a negative control. The mixture was then separated by nondenaturing polyacrylamide gel electrophoresis. Gel was stained by SYBR® Green DNA stain (Life Technologies, USA), and DNA bands were visualized by 300 nm UV transillumination. Thereafter, the same gel was stained by SYPRO® Ruby EMSA protein gel stain (Life Technologies, USA), and protein bands were visualized by 300 nm UV transillumination.

2.5. Gene deletion, complementation, and quantitative PCR

To investigate the regulatory role of MvaU in *S5* expression, we deleted *mvaU* gene in *P. aeruginosa* PAO1. First, the upstream (MvaUU) and downstream (MvaUD) regions of *mvaU* gene were amplified and fused by overlap extension PCR. The MvaUU-MvaUD fusion fragment was digested by *Sac*I and *Sph*I, cloned into pGEM-T vector, and sequenced. Second, we amplified a streptomycin-resistant gene (*Sm*) from a standard biological part BBa_K156011 (http://www.parts.igem.org) and cloned *Sm* gene into pUCP18, leading to the recombinant plasmid containing a Plac-*Sm* cassette. After verification by sequencing, the resultant plasmid was digested by using *Sma*I and *Xba*I, and the Plac-*Sm* cassette was cloned into a locus between the upstream and downstream regions of *mvaU* gene previously cloned into pGEM-T vector. The resultant *mvaU*-deletion cassette sequence was verified by sequencing, and the streptomycin resistance was confirmed on LB agar plates with 100 μg/ml of streptomycin.

We amplified the *mvaU*-deletion cassette, and transformed the PCR product into *P. aeruginosa* PAO1 by electroporation.[30] The transformants were screened on streptomycin-containing LB agar plates. To obtain positive *mvaU*-deficient transformants, we performed colony PCR by using the primers specific to the upstream and downstream regions of *mvaU* on the genome. The correct *mvaU* deletion mutant Δ*mvaU* and Δ*mvaT*[31] were selected for gene complementation and expression analysis as described below.

We overexpressed *mvaT* in Δ*mvaT* and *mvaU* in Δ*mvaU* by cloning them into plasmid pUCP18 and transforming the correct recombinant plasmid (pUCP-Tc, pUCP-Uc) into Δ*mvaT* and Δ*mvaU* respectively by electroporation. The transformants were screened on LB agar plates with 200 μg/ml of carbenicillin. Upon complementation, the *S5* gene expression level in the corresponding gene deletion mutants could be restored of that observed in wild type strain PAO1. Transcription levels of *S5* gene in wild type PAO1 with pUCP18, PAO1 Δ*mvaU* with pUCP18

Table 1
Strains and plasmids used in this study.

Name	Description	Resources
Strains		
P. aeruginosa PAO1	Wild type	ATCC
P. aeruginosa Δ*mvaT*	*mvaT*-deletion mutant of PAO1	[40]
P. aeruginosa Δ*mvaU*	*mvaU*-deletion mutant of PAO1	This study
E. coli Top10	Gene cloning host	Lab strain
E. coli BL21(DE3)	Host for protein overexpression	Merck
Plasmids		
pET30b(+)	Expression vector, Kan^R	Merck
pET-T	Recombinant pET30b-derived plasmid for MvaT overexpression, Kan^R	This study
pET-U	Recombinant pET30b-derived plasmid for MvaU overexpression, Kan^R	This study
pGEM-T	Plasmid for deletion cassette construction, Amp^R	Promega
pUCP18	Plasmid for gene complementation, Carb^R	[41]
pUCP-*Sm*	Plasmid containing Sm^R cassette	This study
pUCP-*Uc*	Recombinant pUCP18-derived plasmid for MvaU complementation, Carb^R	This study
pUCP-*Tc*	Recombinant pUCP18-derived plasmid for MvaT complementation, Carb^R	This study

and with pUCP-Uc, and PAO1 *ΔmvaT* with pUCP18 and with pUCP-Tc were quantified by quantitative PCR (qPCR).

Specifically, the total RNA of *P. aeruginosa* cells at exponential phase was extracted using RNeasy Mini Kit (Qiagen, Germany), qualified and quantified by agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific, USA), respectively. Complementary DNA (cDNA) was synthesized from the total RNA by using Biorad iScript Reverse Transcription Supermix, and an equal amount of cDNA (50 ng) was used as a template for qPCR with Biorad SsoFast EvaGreen Supermix and the primers specific to *S5* and *rpoD* genes (Table S1). With *rpoD* as a reference gene, the expression of *S5* was normalized to that in wild type (PAO1) with pUCP18.

2.6. Transcriptomic analysis

To understand the gene profile of *ΔmvaT* compared to wild type (WT), we performed RNA sequencing according to the standard workflow. In total RNA, ribosomal RNA was removed by an rRNA removal kit, and rRNA free residue was cleaned up by ethanol precipitation. Subsequently, sequencing libraries were generated using the rRNA-depleted RNA and performing procedures. The generated libraries were pooled and sequenced on Illumina platform NovaSeq 6000 PE150. RNA sequencing data were analyzed using Geneious Prime® 2022.0.1 (https://www.geneious.com). Briefly, the sequences were imported as paired reads and the adapters, primers, and PhiX reads were trimmed using the BBDuk plugin. The annotated genome of *P. aeruginosa* PAO1 (NCBI Acc. No. NC_002516.2) was imported and used as the reference genome. The trimmed reads were then mapped to the reference genome and expression levels were calculated using default settings. Comparison of expression levels was performed using the Deseq2 plugin in Geneious Prime® 2022.0.1 under the Annotate and Predict function.

3. Results and discussion

3.1. Capture and identification of transcription factors

We captured the transcription factors that potentially regulate *S5* gene expression using the upstream region of *S5* gene (S5P). Fig. 1 shows two positive bands of proteins captured by S5P. By MALDI-TOF analysis, the two proteins were identified as ATP-dependent protease (PA0779, 88.59 kDa) and MvaT (PA4315, 14.17 kDa). To our knowledge, PA0779 (AsrA) is involved in protective responses to nitrogen oxide[32] and heat shock responses to lethal levels of tobramycin but not involved in the regulation of *S5* expression.[33] MvaT is a histone-like nucleoid structuring protein and a global regulator of virulence factors and house-keeping genes.[34] MvaT has also been shown to interact with its paralog MvaU (PA2667) to regulate phase-variable cup gene expression in *P. aeruginosa*.[35] Collectively, we hypothesized that MvaT and MvaU possibly regulate *S5* expression. Hence, we selected MvaT and MvaU to study their roles in regulating *S5* expression in *P. aeruginosa*.

3.2. In vitro binding activity of MvaT and MvaU to S5P

Based on the hypothesis that MvaT and MvaU may be transcription factors that regulate *S5* gene expression, we performed a gel-shift assay to confirm the binding activity of MvaT and MvaU against S5P. MvaT and MvaU were individually overexpressed in *E. coli*. Fig. 2 shows successful overexpression of MvaT and MvaU proteins upon IPTG induction. After FPLC purification, increasing amounts of MvaT and MvaU (0 ng, 20 ng, 40 ng, 60 ng, and 80 ng) were incubated with S5P for the gel-shift assay. Fig. 3A shows that MvaT's binding to S5P significantly retarded the electrophoretic migration of S5P-MvaT complex. An increasing amount of MvaT protein up to 80 ng increased the intensity of the retarded S5P complex and decreased the intensity of the unbound S5P DNA fragment. In contrast, the intensity of *rpoD* DNA fragment (a negative control) remained constant. These trends of DNA intensities
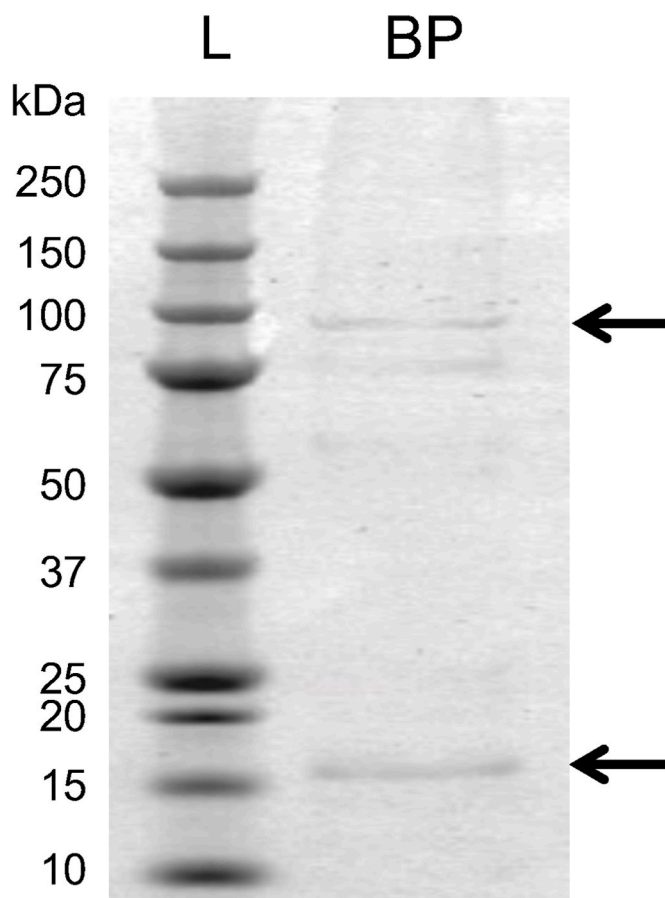

Fig. 1. Capture of *S5* promoter-binding proteins. The upstream DNA fragment of *S5* gene (S5P, 404 bp) was amplified and labeled with biotin. S5P was used to capture protein28. Two proteins (indicated by arrows) were captured. L, protein ladder, BP, binding proteins.

indicate that MvaT specifically bound to S5P in a dose-dependent manner. MvaU also showed specific binding to S5P but showing a lower S5P DNA intensity (Fig. 3B), suggesting a lower binding activity than MvaT. Overall, the results of *in vitro* binding activity assays provided a preliminary evidence on the role of MvaT and MvaU in regulating *S5* expression.

3.3. The regulatory role of MvaT and MvaU in S5 gene expression

Following the *in vitro* binding assays, we studied the regulatory role of MvaT and MvaU in *S5* gene expression in *ΔmvaT* and *ΔmvaU* respectively. First, we analyzed *S5* expression in single mutants *ΔmvaT* and *ΔmvaU*, respectively by qPCR. To complement their deficiency, we overexpressed MvaT in *ΔmvaT* and MvaU in *ΔmvaU*, followed by *S5* expression analysis by qPCR. Fig. 4A shows that the *mvaT* deletion significantly enhanced *S5* gene expression by 2.1-fold compared to the wild-type PAO1 (WT), suggesting that MvaT is a negative regulator of S5 expression, consistent with a previous study showing *S5* upregulation in the cells lacking *mvaT*.[36] The overexpression of *mvaT* under *lac* promoter in *ΔmvaT* (*ΔmvaT* + MvaT) did not restore the *S5* gene expression level to its level in WT. In *ΔmvaU*, there was no change in S5 gene expression compared to WT, likely due to the presence of MvaT which possibly represses *S5* expression in the absence of MvaU. The overexpression of MvaU in *ΔmvaU* (*ΔmvaU* + MvaU) enhanced *S5* expression by 1.6-fold over WT (Fig. 4B). We hypothesize that mvaU alone unlikely represses *S5*.

We performed transcriptomic analysis to further understand MvaT's regulatory role. Most genes (Table S2, Fig. 5A) were upregulated in
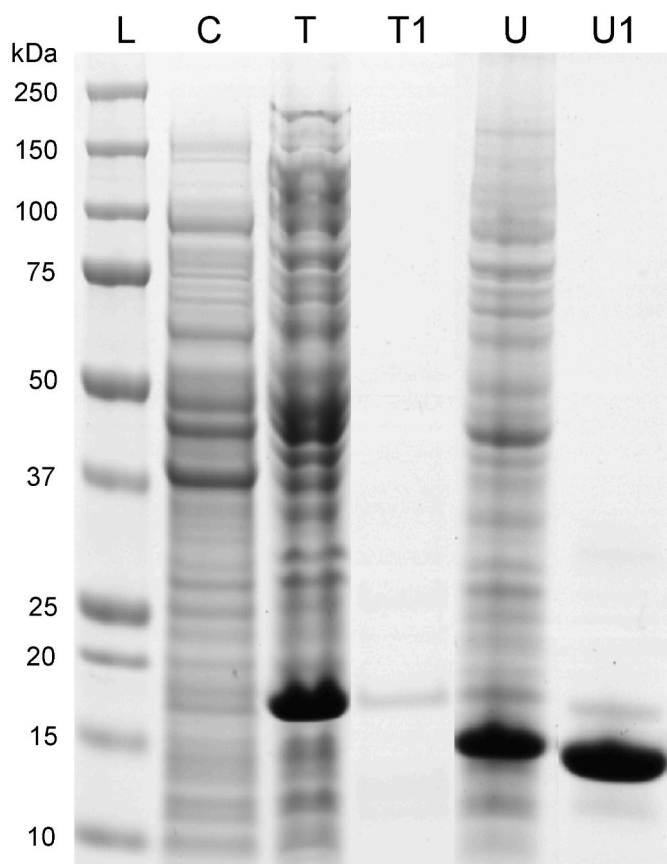
Fig. 2. Overexpression and purification of MvaT (A) and MvaU (B) proteins. Genes were cloned into expression vector pET30b(+) in *E. coli* BL21 (DE3), induced by 1 mM IPTG, and the His$_6$-tagged Mva proteins were purified by using FPLC. L, protein ladder, C, pET30b(+), T, MvaT, T1, purified MvaT, U, MvaU, U1, purified MvaU.

ΔmvaT. *MvaU* was upregulated (FC2.8) in *ΔmvaT*, suggesting a negative regulatory role of MvaT in *mvaU* expression. Target genes of MvaT suggested by the RegulomePA[37]—a database of transcriptional regulatory interactions in PAO1, i.e., m*exEF* and *oprN* in *ΔmvaT* were also upregulated. Other regulated genes are related to fimbrial synthesis,

oxidative stress, drug resistance, quorum sensing (upregulated) and dipeptide metabolism (downregulated).

4. Discussion

Previously, two genome regions (>8 kb) associated with *S5* were found enriched by MvaT and MvaU, respectively.[34,36] Here, we determined a 404-bp *S5* upstream region specifically bound by MvaT or MvaU, which provides a further evidence on Mva-*S5* upstream interactions. In line with the interactions, MvaT is involved in regulating *S5* expression.[31,38] Its regulatory mechanisms differ from the PrtR/N-involved regulatory mechanism,[18,19] consistent with the lack of a P-box regulatory motif in the *S5* upstream sequence.

In this study, we demonstrated the role of MvaT in repressing *S5* expression. We could not capture MvaU using S5P. Given the possibility of dimerization,[35,39] we hypothesize that hetero- and homodimers, i.e., MvaT-MvaU and MvaT-MvaT in the WT and MvaT-MvaT in *ΔmvaU*, which can bind to the *S5* promoter region (Fig. 5B), limit the occupancy by RNA polymerase and ultimately repress *S5* expression in WT and *ΔmvaU*.[36] Theoretically, MvaU-MvaU homodimer might also be formed in WT and contribute to *S5* repression along with MvaT-MvaT and MvaT-MvaU dimers. In *ΔmvaT*, MvaU-MvaU dimer might be formed but unable to repress *S5* like that in WT, allowing *S5* upregulation. In *ΔmvaT* + *mvaT* and *ΔmvaU* + *mvaU*, *S5* was upregulated (Fig. 4), likely due to competency or interaction between homodimers and heterodimer (MvaT-MvaT, MvaU-MvaU and MvaT-MvaU). Here, we hypothesize that MvaT plays a more dominant role in *S5* regulation than MvaU, consistent with the higher MvaT-S5P DNA intensity than MvaU-S5P shown in Fig. 3. Nevertheless, the dimerization of MvaT and MvaU and the MvaT-MvaU-S5P interactions are complex, and the underlying mechanism of *S5* regulation by MvaT and MvaU remains to be further elucidated.

5. Conclusions

In this study, using *in vitro* promoter-binding assays, we captured the transcription factor MvaT that binds to the upstream region of pyocin S5 gene, demonstrated the specific binding of transcription factors MvaT and its paralog MvaU to *S5* upstream region, and confirmed the dominant role of MvaT as a negative regulator of *S5* expression in *P. aeruginosa*. We would put future efforts into elucidating the mechanisms of MvaT-MvaU-S5P interactions and precise regulation of *S5* expression through manipulating transcriptional factors, including
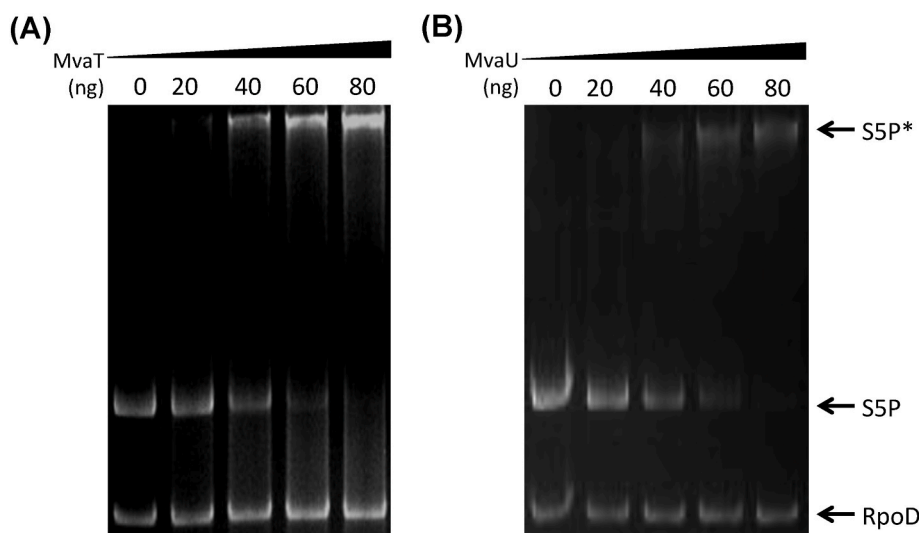

Fig. 3. Electrophoretic mobility shift assay of MvaT (A) and MvaU (B) against S5P. The differing amounts of MvaT or MvaU proteins (0 ng, 20 ng, 40 ng, 60 ng, and 80 ng) were incubated with 50 ng S5P in the presence of *rpoD* DNA fragment (30 ng) as negative control. S5P*, S5P-MvaT or S5P-MvaU complex.
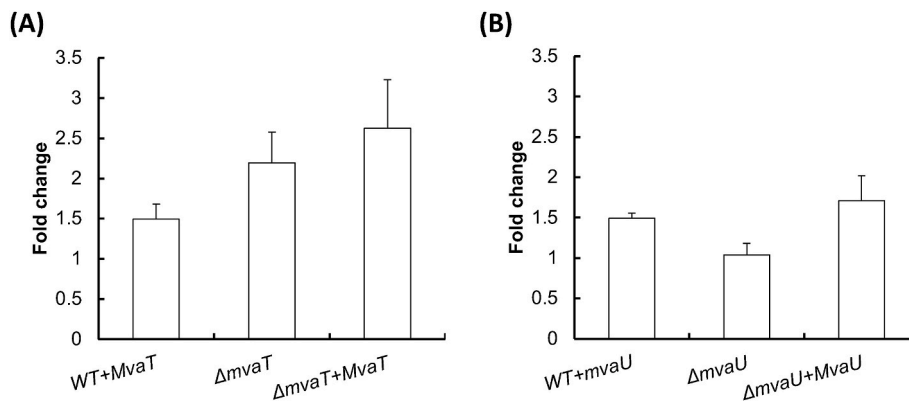
Fig. 4. Expression of *S5* in *P. aeruginosa* wild type, *mvaT* and *mvaU* mutant. The expression levels of *S5* in wild type (WT) cells with pUCP18 were set to 1.0, and the *S5* expression was normalized to *rpoD*. Values are the mean of three biological replicates ± SE.
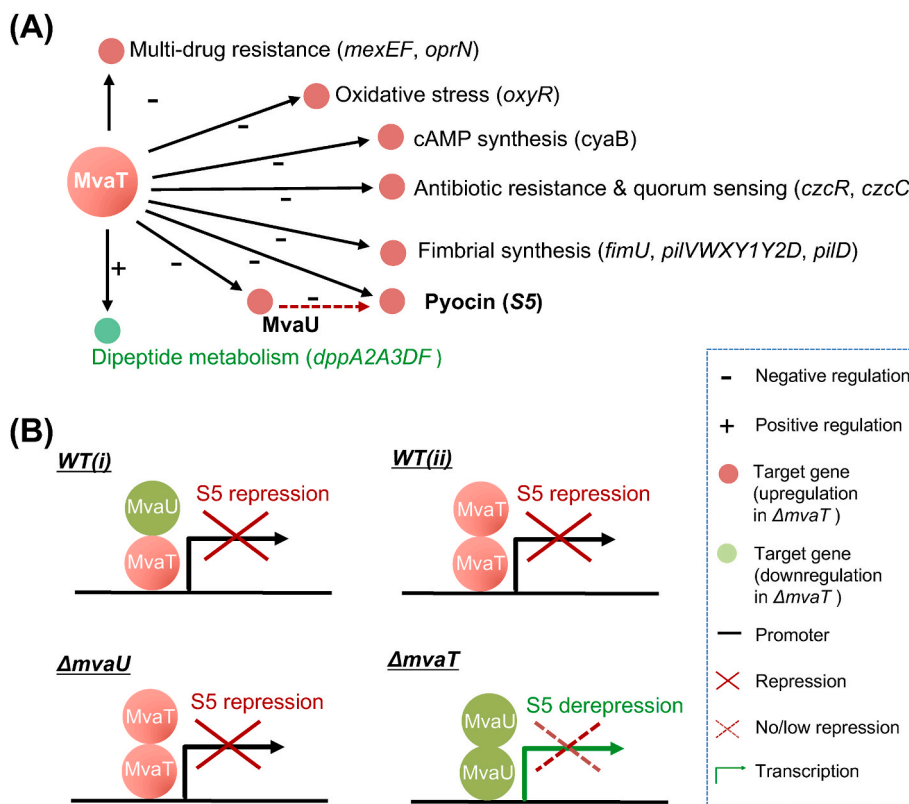

Fig. 5. A summary of differentially regulated genes and associated functions in *ΔmvaT* (A) and proposed mechanism on S5 regulation by MvaT and MvaU dimers (B). Homodimers (MvaU-MvaU and MvaT-MvaT) and heterodimer MvaU-MvaT can bind to the *S5* promoter region.

MvaT and MvaU in various *P. aeruginosa* WT and mutants.

Author contributions

HL conceived the project and designed the experiments; RL and RBDS performed the experiments and analyzed the data; RL and HL wrote the manuscript. HL supervised the project. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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