JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 271-278

Identification of Genes Affecting *phzA1*/*phzA2* Expression in *Pseudomonas aeruginosa*

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(Received: 07 January 2013; accepted: 18 February 2013)

Phenazine compounds produced by *P. aeruginosa* play important roles in pathogenicity as virulence factors as well as signaling molecules. Previously, we have identified genes that altered *phzA1* expression in the presence of subinhibitory concentrations of tetracycline. In this study, we found both *phzA1* and *phzA2* were activated by subinhibitory concentrations of spectinomycin (Spc). Using *luxCDABE* based reporters we screened transposon libraries and identified 12 genes whose mutation changed the activation of *phzA1* and/or *phzA2* by Spc, and therefore potentially involved in phenazine regulation. Among these genes, the interruption of *lasI*, *pqsR* and *pqsE* abolished *phzA1* and *phzA2* expression while the mutation of *vqsM* stopped *phzA2* expression, and therefore Spc lost its effect in all these mutants. The disruption of 3 fimbrial related genes, *pilY, pilX,* and *pilH* independently increased the degrees of Spc activation of *phzA2* whereas the *algC* and *truA* mutations decreased the Spc activation of *phzA2*. *Ppk* and *pcnB* mutants had increased Spc activation of *phzA1* expression. Complementation assays with cloned genes carried on a plasmid in the *truA* mutant restored the Spc activation of *phzA2*, confirming the involvement of this gene in Spc regulation of *phzA2* operon. The *phzA1* expression in the presence of Spc was only partially restored by *pcnB* complementation, suggesting other genes in the *pcnB* operon may play a role. The information obtained in this study provides a basis for further investigations.

Key words: *Pseudomonas aeruginosa*, phenazine, spectinomycin, transposon mutagenesis.

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing serious infections in humans, especially those who are immune-compromised or suffering chronic diseases. As a human pathogen, this bacterium is the leading source of gram-negative nosocomial infections. Phenazine compounds produced by *P. aeruginosa* are biologically active metabolites that play important roles in competitiveness[1], suppressing other bacteria, and in bacterial virulence when the bacterium infects human and animal hosts[2]. Besides, phenazines can also function as active signal molecules and as antibiotics[3,4]. Phenazine compounds are produced by a series of enzymes encoded on two homologous operons *phzA1B1C1D1E1F1G1* (*phz1*) and *phzA2B2C2D2E2F2G2* (*phz2*) in *P. aeruginosa*[5]. The expression of the *phzA1* and *phzA2* operons is regulated by a complex regulatory network. It is positively regulated by *Pseudomonas* quinolone signal (PQS) and *rhl* quorum sensing system[6], and repressed by QscR[7]. In addition, the gene products

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of *phzM*, *phzH*, and *phzS* are required to convert the intermediate phenazine-1-carboxylic acid (PCA) to other final products, including phenazine-1-carboxamide, 1-hydroxphenazine, and pyocyanin. Despite the important roles of phenazine compounds, the regulatory networks of phenazine productions still need to be fully understood.

Antibiotics are widely used to treat infections in the clinics depending on their killing and/or inhibitory activity toward bacterial pathogens. Recent studies have demonstrated that subinhibitory concentrations of antibiotics play important roles in regulation of gene expression including virulence factor genes as signaling molecules[3, 8, 9]. Several studies have reported that subinhibitory concentrations of antibiotics such as ceftazidime and tobramycin, reduces the quorum sensing signals[10] and azithromycin reduces quorum sensing activity and virulence factors expression[11, 12], while some antibiotics at the subinhibitory concentrations have been proved to activate the expression of quorum-sensing controlled virulence factors[13, 14]. However, the mechanisms of virulence gene regulation by the subinhibitory concentrations of antibiotics are complex and remain unclear.

In this study, we observed that both *phzA1* and *phzA2* were activated by subinhibitory concentrations of spectinomycin (Spc). To uncover the mechanism of Spc activation, we constructed transposon mutagenesis libraries using *luxCDABE* based reporters and identified 12 new genes that are potential components of the Spc regulation pathways.

MATERIALS AND METHODS

Bacterial strains, growth conditions and antibiotic reagents

The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and derivatives were routinely grown on LB agar or in LB broth at 37°C. Antibiotics (Amresco, USA) were used at the following concentrations: for *P. aeruginosa*, gentamicin (Gm) at 50 µg/ml in LB or 150 µg/ml in *Pseudomonas* isolation agar (PIA) and carbenicillin (Cb) at 250 µg/ml in LB; for *E. coli*, ampicillin (Ap) at 100 µg/ml and Gm at 15 µg/ml in LB, spectinomycin (Spc) was prepared in deionized water and added as indicated.

Table 1. Bacterial strains and plasmids used in this stud

Strain or plasmid	Relevant characteristics	Source
E. coli		
SM10	Mobilizing strain, RP4 integrated in the chromosome; Kn	[15]
P. aeruginosa		
PAO1	Wild type	This lab
Plasmids		
pBT20 Mini	TnM delivery vector; Ap Gm 25	[16]
PAK1900	Multicopy *E. coli-P. aeruginosa* shuttle vector	[17]
CTX-*phzA1*	pMS402 containing *phzA1*promoter region; Kn^r, Tmp^r	[18]
CTX-*phzA2*	pMS402 containing *phzA2* promoter region; Kn^r, Tmp^r	[18]

Expression monitoring assay

Using *lux*-based reporters, gene expression in liquid cultures was measured as light level (in counts per second) (c.p.s.) in a Victor^3 multilabel plate reader (Perkin-Elmer)[19]. The reporter strains were cultivated in LB broth overnight. The overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and cultivated for two additional hours before being used as inoculants. The cultures were inoculated into parallel wells on a 96-well black plate with a transparent bottom. 5 µl fresh cultures were inoculated into the wells containing a total of 95 µl medium plus other component. 50 µl of filter-sterilized mineral oil (Sigma) was added to prevent evaporation. Promoter activities were measured every 30 min for 24 h and the growth of bacterial was monitored at the same time by measuring the OD_{600} in the Victor^3 multilabel plate reader. Expression on solid medium was measured by

plating the reporter strains in soft top LB agar. Spc were spotted on spaced ûlter discs laid on top of the agar. These plates were incubated at 37°C overnight and imaging was performed using the LAS-3000 imaging system (Fuji Corp.).

Construction of tansposon mutagenesis library

The donor strain *E. coli* SM10-ë *pir* containing pBT20, and the recipient *P. aeruginosa* PAO1 carrying the reporter *phzA1-lux* or *phzA2-lux* on its chromosome were grown separately on solid media overnight and the cells were collected, resuspended in LB and spotted on a fresh LB agar plate at a ratio of 2:1. After incubation for 2 hours, the mixed culture was diluted and spread on PIA containing 150 µg/ml gentamicin and Spc of 100 µg/ml (for *phzA1*) or 25 µg/ml (for *phzA2*). After incubation at 37°C overnight, colonies with obvious altered luminescence were picked into 384-well plates. For long term reserved, 10% glycerol was added into the wells and frozen at -70°C.

Complementation of trasposon mutants Δ*pcnB* and Δ*truA*

The multicopy *E.coli-P. aeruginosa* shuttle vector pAK1900 was used to complement of trasposon mutants Δ*pcnB* and Δ*truA*. The coding sequence of gene *pcnB* and *truA* were PCR amplified, and PCR products were ligated with plasmid pAK1900 to generate constructs pAK-*pcnB* and pAK-*truA*. These two constructs were electroporated into transposon mutants Δ*pcnB* and Δ*truA* respectively. The generated complemented strains were measured for *phzA1/phzA2* expression in 96-well plate.

RESULTS

Regulation of *phzA1/phzA2* by Spectinomycin

It has been shown that the *phzA1* expression could be positively regulated by the subinhibitory concentrations of tetracycline[8]. To test whether *phzA2* could be regulated by antibiotics, we used the *lux*-based reporter to monitor the expression of *phzA2* in *P. aeruginosa* under conditions where different concentrations of antibiotics were present. The reporter had a promoterless *luxCDABE* reporter fused downstream of the *phzA2* promoter and therefore the gene expression levels can be measured as the amount of light production. Filter discs impregnated with antibiotics on solid media were used to monitor gene regulation by antibiotics. The light emission beyond the zone of inhibition monitored by an imaging system represented the expression of *phzA2*. As shown in Fig.1, *phzA2* expression was highly activated in the presence of Spc, and the same result was also observed in *phzA1* expression on Spc spotted plate. Further analysis of *phzA1/phzA2* regulation by Spc was carried out in multiwell plates, and expression was measured as light production (counts per second) in a multilabel plate reader. The *phzA1* expression was activated the most when Spc at 100µg/ml (Fig. 2A), while *phzA2* expression was activated the most

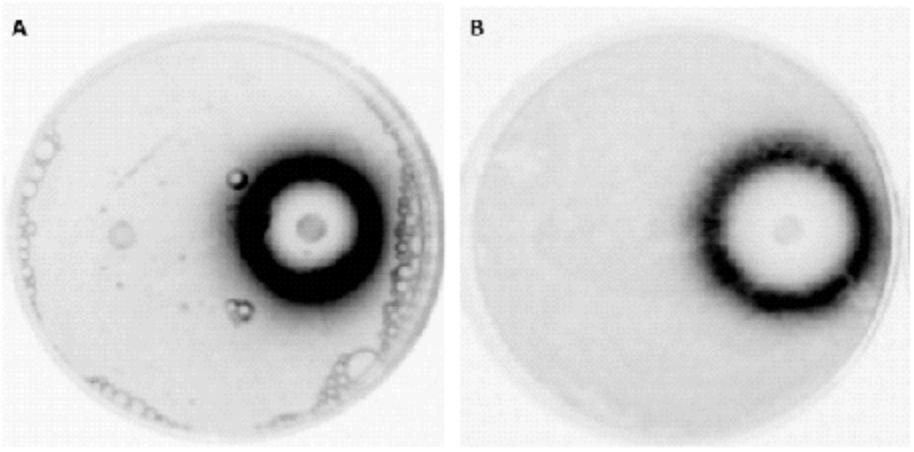

Fig. 1. The activation of *phzA1* (A) and *phzA2* (B) expression by Spc. Spc was spotted on the right filter discs. The arrow showed the discs with solvent control. The experiment was repeated three times and the data shown isrepresentatives of similar results

when Spc at 25μg/ml (Fig. 2B). The regulated expression of *phzA1* and *phzA2* reached 30 folds and 4 folds respectively.

Identification of genes involved in the activation of *phzA1/phzA2* by Spc

To identify genes involved in activating *phzA1* and *phzA2* expression, *phzA1* and *phzA2* were used as the reporters and transposon mutagenesis was carried out. There were 18,000 mutants (for *phzA1*) and 40,000 mutants (for *phzA2*) screened. After rescreening 40 mutants exhibiting *phzA1/phzA2* expression altered were selected for further studies. Arbitrary PCR and subsequent sequencing the PCR products were used to identify the insertion sites of the mutants. Total of 12 genes were eventually identified affecting *phzA1* or *phzA2*

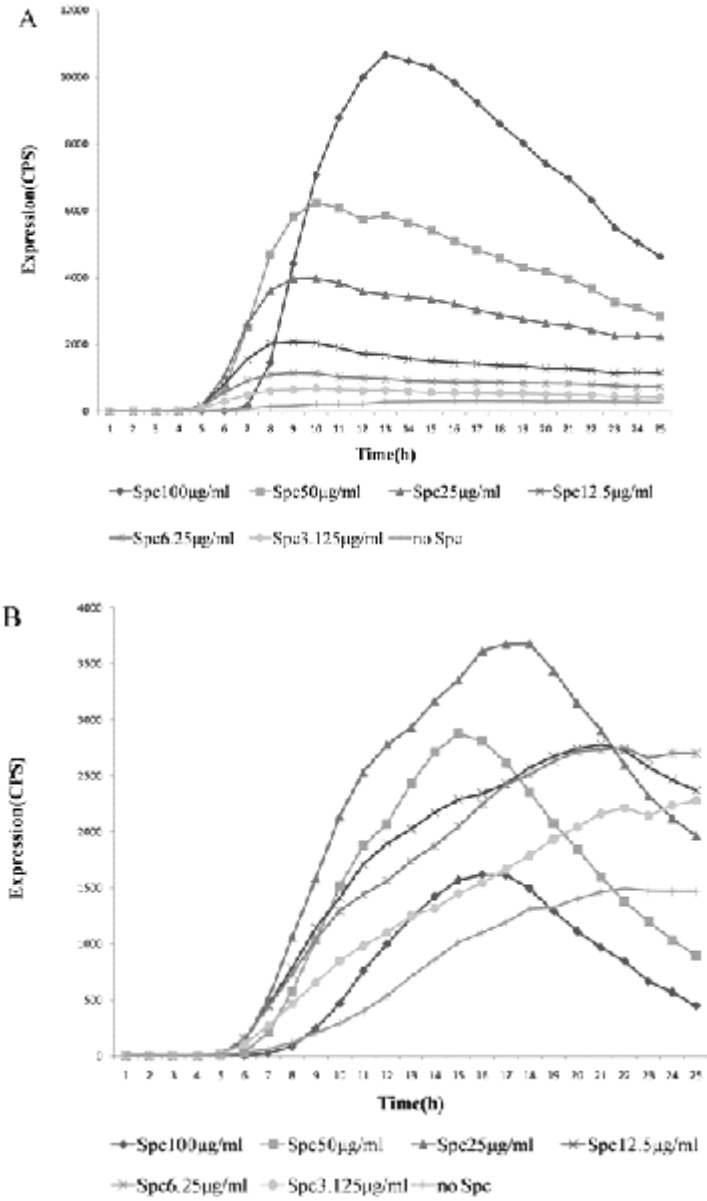

Fig. 2. Regulation of *phzA1*(A) and *phzA2*(B) by subinhibitory Spc at serial 2-fold dilutions of concentrations.(A) The expression of *phzA1* at different concentrations of Spc. (B) The expression of *phzA1* at different concentrations of Spc. The experiments were repeated three times and the data shown are representatives of similar results.

expression in the presence of spec, i.e. exhibiting changes in Spc activation profiles compared to the wild type (Table 1). Because of the effect on Spc activation they are potentially components in the regulation of *phz* by Spc. In the transposon mutants of *ΔlasI*, *ΔpqsR*, *ΔvqsM* and Δ*rhlI*, the expression of *phzA1* and *phzA2* became extremely low, indicating that these genes were required for the expression of *phzA1* and *phzA2*. However, it was difficult to determine if these genes were directly involved in the Spc activation of *phzA1/phzA2*. PA5242 encodes the polyphosphate kinase, and the absence of which increased the *phzA1* expression even more than in the wild type when exposed to Spc. PA4727 encoding a poly (A) polymerase also increased the *phzA1* activation by Spc. So did PA4554, PA0409 and PA4553 which encode the type 4 fimbrial biogenesis proteins. The mutation of PA3114 which encodes the tRNA-pseudouridine sythase I abolished, the activation of *phzA2* by Spc, so did the mutation of *rsaL* encoding QS related transcriptional regulator. PA5322 encodes the phosphomannomutase whose mutation decreased the *phzA2* activation by Spc.

Complementation analysis of *pcnB* and *truA* mutants

Among the identified genes that interacted with *phzA1* and *phzA2*, *pcnB* and *truA* were selected for further studies. To confirm the roles of these genes in *phzA1/phzA2* expression regulation, complementation assays were carried out. The complementation constructs were generated using PCR products of *pcnB*/*truA* coding sequences ligated to pAK1900 plasmid; the new constructs were then transformed into the transposon mutants of Δ*pcnB* and Δ*truA* respectively. After generating the new complementation strains, the expression of *phzA1* and *phzA2* were measured in the presence and absence of Spc. As shown in Fig. 3C, the activation of *phzA2* by Spc was restored to the wild type level when *truA* was complemented, confirming the involvement of *truA* in Spc activation. The *pcnB* complementation only restored partially the Spc effect on *phzA1* expression, indicating other genes in the *pcnB* operon may be involved considering the possible polar effect of the transposon insertion at *pcnB*

Table 2. Mutated genes identified affecting *phzA1*/*phzA2* expression and/or Spc activation

PA number	Characteristics of encoded protein	Operon affected	Change of *phzA1* or *phzA2* activation by Spc
PA5242	Ppk, polyphosphate kinase	*phzA1*	Increased*
PA4727	PcnB, poly(A) polymerase	*phzA1*	Increased
PA0409	PilH, type 4 fimbrial biogenesis protein	*phzA2*	Increased
PA4554	PilY, type 4 fimbrial biogensis protein	*phzA2*	Increased
PA4553	PilX, type 4 fimbrial biogensis protein	*phzA2*	Increased
PA3114	TruA, tRNA-pseudouridine synthase 1	*phzA2*	No activation#
PA5322	AlgC, phosphomannomutase	*phzA2*	Decreased
PA1431	RsaL, regulator protein	*phzA2*	No activation
PA1000	PqsE, quinolone signal response protein	*phzA2*	No expression‡
PA1432	LasI, autoinducer synthesis protein	*phzA1* and *phzA2*	No expression
PA2227	VqsM, Ara C-type transcriptional regulator	*phzA1* and *phzA2*	No expression
PA1003	PqsR, transcriptional regulator	*phzA1* and *phzA2*	No expression

Notes: * Bigger fold change in the mutant (from the expression level without Spc to expression level with Spc) than in the wild type; # Spc activation absent in the mutant; ‡ No expression observed with and without Spc.

DISCUSSION

Phenazine compounds play important roles in pathogenicity as virulence factors of *P. aeruginosa*. In addition, they also act as antibiotics against other bacteria and cell-cell signaling molecules. Previously, we have identified genes that altered *phzA1* expression in the presence of subinhibitory concentrations of tetracycline. This

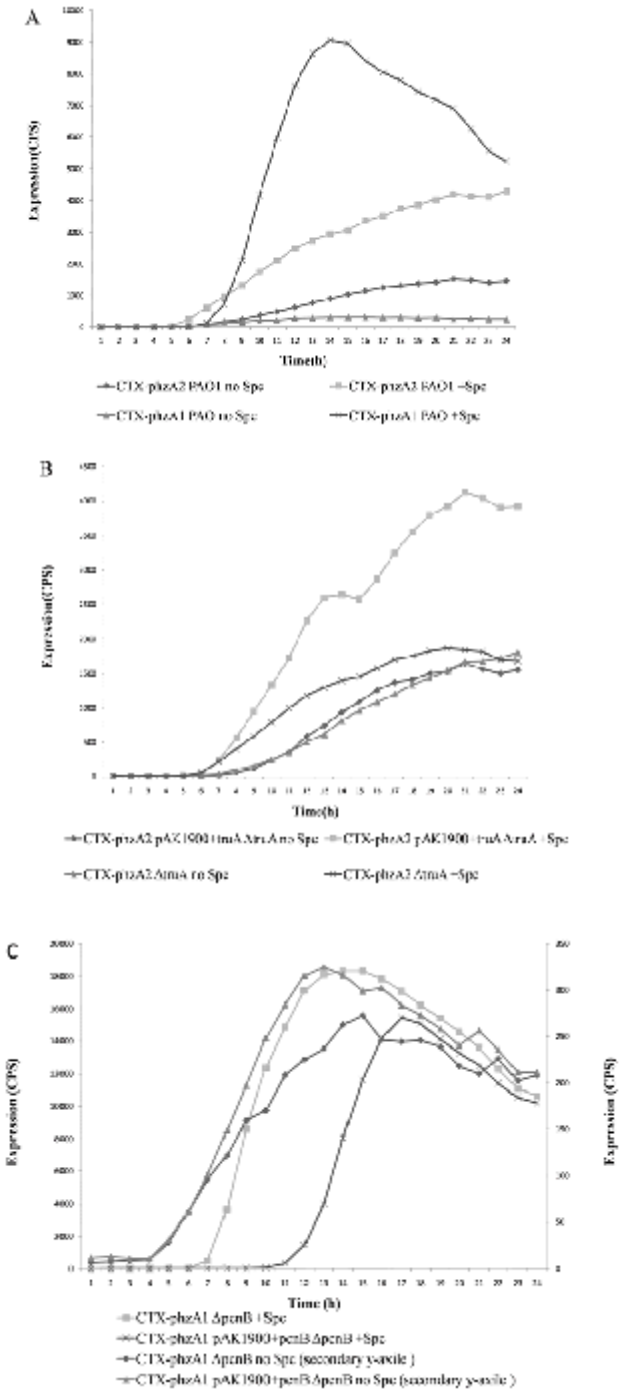

Fig. 3. The altered effect of Spec on the expression of *phzA1/phzA2* in *pcnB* and *truA* mutants and the restoration of Spc regulation by gene complementation. (A) The activation of *phzA1* and *phzA2* expression by Spc in the wild type strain PAO1. (B) The effect of Spc on *phzA2* expression in *truA* mutant and in the complementation strain containing plasmid PAK-*truA* (PAK1900+*truA*). (C) The effect of Spc on *phzA1* expression in *pcnB* mutant and in the complementation strain containing plasmid PAK-*pcnB* (PAK1900+*pcnB*). The experiments were repeated three times and the data shown are representatives of similar results

report presents the evidence that the *phzA1* and *phzA2* operons are activated by subinhibitory concentrations of Spc and 12 genes were identified to involve in Spc activation. Some of the genes affecting *phzA1/phzA2* expression in the presence of subinhibitory Spc are known regulators of the operon. Spc lost its effect in *lasI*, *pqsR*, *pqsE and vqsM* mutants, since the expression of *phzA1/ phzA2* were totally abolished in these mutants[6, 20, 21].

We observed that the *phzA1* expression in *ΔpcnB* was even higher than the expression in wide type PAO1when exposing to Spc, which may indicate that *pcnB* inhibits the activation of *phzA1* expression by Spc. PA4727 (*pcnB*) encodes the poly (A) polymerase that functions in stabling mRNA in *P. aeruginosa*[22]. PcnB belongs to the CbrB regulon in *Pseudomonas fluorescen*[23], which composes with CbrA involved in carbon and nitrogen utilization *P. aeruginosa*[24]. The two component system CbrA-CbrB regulated the swarming, biofilm formation and antibiotic resistance in *P. aeruginosa*[25]. So we propose that pcnB interaction with Spc activation may related with two component system CbrA-CbrB. However, further study should be required to investigate the mechanism of *pcnB* interaction with Spc activation.

PA3014 is tRNA-pseudouridine sythase I encoding gene. It is reported that tRNA-pseudouridine sythase I guarantee the stability of tRNA structure, which further making the correct open reading frame for protein expression[26]. In this study, we found that the disruption of *truA* abolished the spectinomycin activation for *phzA2* expression. Whether the interaction with Spc activation by Spc is related with the function of *truA* for tRNA stabilization should be further investigated. The information obtained should provide a basis for further investigations.

Antibiotics are bioactive compounds displaying inhibitory activity toward other microorganisms at high concentrations. However, antibiotics may be at lower concentrations in ecological environments and likely function as signaling molecules[3, 8, 9]. Phenazine compounds produced by *P. aeruginosa* are biologically active metabolites that play important roles in competitiveness[1], suppressing other bacteria, and in bacterial virulence when the bacterium infects human and animal hosts. Be similar to the activation of phenazine compound synthesis by subinhibitory concentrations of Tet molecules[13], subinhibitory concentrations of Spc also enhanced the expression of phenazine compound synthesis, which confirmed that the antibiotics in a microbial community could act as signal molecules[27], which alert *P. aeruginosa* to the existence or aggression of other bacteria. The increased pyocyanin production would help *P. aeruginosa* to compete with the other microbes.

ACKNOWLEDGEMENTS

This work was supported by PCSIRT (No.IRT1174) and URPG (University of Manitoba), and also by a grant from NFSC China (No.81171620).

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