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PltR expression modulated by the global regulators GacA, RsmA, LasI and RhlI in *Pseudomonas* sp. M18

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Abstract

Pseudomonas sp. M18 can suppress certain soil-borne phytopathogenic fungi by producing two antibiotics, pyoluteorin (Plt) and phenazine-1-carboxylic acid (PCA). *pltR* encodes an LysR-type transcriptional activator required for expression of divergently transcribed Plt biosynthetic genes. Here we provide evidence that Plt biosynthesis is negatively regulated by the quorum-sensing (QS) signal molecule (*N*-acyl homoserine lactone, AHL) synthase gene *lasI* and positively regulated by the orphan LuxR-type regulatory gene *vqsR*. *pltR* expression is modulated by four important global regulators including the two-component response regulatory gene *gacA*, the RNA-binding repressor RsmA, and the QS signal molecule synthase genes *lasI* and *rhlI*. *pltR* translation was almost completely inhibited in the *gacA* mutant M18G when compared with that in the wild-type strain of M18. We also observed significant enhancement of *pltR* translation in the *rsmA* mutant compared with wild-type strain M18. Moreover, mutation in *lasI* or *rhlI* gave rise to a significantly elevated level of *pltR* transcription in strain M18. However, no obvious difference was observed in *pltR* expression between the *vqsR* mutant and the wild-type of strain M18. These results highlight the fact that PltR likely at least partly plays an important mediator role in Gac/Rsm and the Las/Rhl regulatory pathways involved in Plt biosynthesis.

Keywords: Antifungal compound; Pyoluteorin; LysR-type transcriptional regulator; Global gene regulation; Two-component signal transduction; Quorum sensing

1. Introduction

Pyoluteorin (Plt), an aromatic polyketide antibiotic composed of a non-ribosomal peptide synthetase(NRPS)-derived pyrrole moiety and a polyketide synthase(PKS)-derived resorcinol ring, was produced by certain *Pseudomonas* strains, including *Pseudomonas fluorescens* Pf-5 [24], CHA0 [29] and *Pseudomonas* sp. M18 [12,15]. It has been used to control certain plant diseases caused by soil-borne phytopathogenic fungi, especially damping-off caused by *Pythium ultimum* [8]. Pyoluteorin provides a well-established model for understanding the molecular regulation of antibiotic production in *Pseudomonas*. The gene cluster responsible for Plt biosynthesis (*pltLABCDEFG* and *pltM*), regulation (*pltR* and *pltZ*), transport and resistance (*pltHIJKNO*) has been cloned in strains of Pf-5 [5,24] and M18 [14,15]. The *pltR* gene encodes a predicted protein containing 343 amino acid residues with a molecular mass of 37,777 Da [24,34]. A putative promoter/operator region upstream of the *pltR* gene is located within the 653 bp intergenic region between the *pltL* and *pltR* genes [15]. PltR, like other members of the LysR family of prokaryotic transcriptional regulatory proteins, contains a helix-turn-helix DNA binding motif at its N-terminus and a LysR-substrate binding domain at its C-terminus [24,34]. The majority of the LysR-type proteins appear to be transcription activators, and most are known to negatively regulate their own expression [28]. PltR functions as a transcriptional activator of the divergently transcribed Plt biosynthetic operon *pltLABCDEFG* [24,34].

In addition to the above-mentioned pathway-specific regulators, Plt biosynthesis is also under the control of several important global regulators or regulatory systems [9]. The GacS/GacA two-component signal transduction system globally

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controls production of Plt and other extracellular biocontrol or pathogenicity factors in *Pseudomonas* spp. strains [9]. In *Pseudomonas* sp. M18, Plt production is positively regulated and phenazine-1-caboxylic acid (PCA) negatively regulated by the GacS/GacA system [6]. Expression of GacS/GacA-controlled genes is post-transcriptionally repressed by RNA binding proteins RsmA and RsmE, which are titrated by three sRNAs (RsmXYZ) in *P. fluorescens* CHA0. In turn, the RsmXYZ RNAs are transcriptionally activated by the GacS/GacA system [9,18]. In addition, the quorum-sensing (QS) regulators or systems are involved in regulation of antibiotic production in several phenazine-producing *Pseudomonas* strains[32]. Two QS systems, LasIR and RhlIR, positively regulate expression of phenazine biosynthetic genes in *P. aeruginosa* PAO1 [22]. More specifically, however, the Rhl QS system has a significant negative impact on Plt production and on its biosynthetic gene expression in *Pseudomonas* sp. M18 [33]. The orphan LuxR-type QS regulator VqsR is required for production of signaling molecules (AHLs) and phenazine antibiotics in *P. aeruginosa* [16,17]. On the contrary, VqsR has no obvious impact on the production of AHL (C4-, C6-HSL) and phenazine antibiotics (PCA) in *Pseudomonas* sp. M18 (data not shown). From what has been mentioned above, it would be reasonable to believe that both Plt and PCA production are controlled by particular regulatory mechanisms in *Pseudomonas* sp. M18. To further understand the unique and complex regulatory pathways controlling Plt biosynthesis, we thus need to investigate the correlation between global regulators or systems and pathway-specific regulators.

In this paper, we report that Plt biosynthesis is negatively controlled by *lasI* and positively controlled by *vqsR*. We studied the influence of five important global regulators (GacA, RsmA, RhlI, LasI and VqsR) on expression of the transcriptional activator (PltR) of the *plt* operon at the transcriptional and post-transcriptional level in strain M18, and found that the *gacA* genomic mutation almost fully inhibited *pltR* translation, but not transcription. Moreover, RsmA was found to negatively regulate *pltR* expression at the translational level. Significant enhancement of *pltR* expression was also observed in the *lasI* and *rhlI* genomic mutants compared with that in the wild-type strain of M18. However, no obvious difference in *pltR* expression was found between the *vqsR* mutant and the wild-type M18 strain.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Pseudomonas sp. strain M18 was isolated from the rhizosphere of sweet melon in a Shanghai suburb in 1996 [12,15]. Other bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was generally cultured at 37 °C in a Luria–Bertani (LB) broth [26] and *Pseudomonas* sp. strain M18 and its derivatives were grown at 28 °C in either LB broth or King's medium B (KMB) broth [20]. Antibiotic supplements were used at the following concentrations (μg ml^{-1}): for pseudomonads, gentamicin (Gm) 40, kanamycin (Km) 50, spectinomycin (Sp) 100 and tetracycline (Tc) 125; for *E. coli*, Km 50, Gm 15, and Tc 25.

2.2. DNA manipulations and sequence analyses

Standard procedures were used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation and transformation [26]. Restriction endonucleases, Taq and Pfu DNA polymerase, DNA molecular weight markers, DNA purifying kits and other associated products were used as recommended by the manufacturers (MBI Fermentas or TaKaRa).

A database search for similar protein sequences was carried out with the BLASTX of NCBI [35]. Putative promoters were predicted with NNPP (promoter prediction by neural network) [25], and the probable domain homology search was performed with Pfam (protein family database) HMM (hidden Markov model) [1].

2.3. Construction of lasI and vqsR chromosomal mutant strains

A 0.94 kb fragment containing the *lasI* coding region and its flanking sequence was PCR-amplified with *Pfu* polymerase from M18 chromosomal DNA with primers PlasI-A (5′-ATA CTT GGA TCC GTT TCC TGG CTT TCC C-3′) and PlasI-B (5′-ACA GCT AAG CTT GGA TGC CTG ATA GCG A-3′). The underlined letters indicate the added restriction sites for *Bam*HI and *Hin*dIII, respectively. The PCR product was purified and digested with *Bam*HI and *Hin*dIII, and cloned into the suicide plasmid pEX18Tc to create pEX18TcLasI. The 0.89 kb gentamicin resistance cassette from pUCGm was inserted into pEX18TcLasI at the *Pst*I site (at nucleotide 397 of the *lasI* gene) to construct plasmid pEX18TcLSG carrying the *lasI*::Gm^R fragment. The plasmid pEX18TcLsG was mobilized from donor *E. coli* SM10 into M18 by bi-parental mating. Transconjugants were selected on LB plates containing Sp to counterselect SM10 and Gm. After a second crossing-over, Gm^R Tet^S Sac^R recombinants were obtained. The resultant marker-exchanged mutant designated M18LSG was further confirmed by PCR with primers PlasI-A and PlasI-B, restriction enzyme digestion with *Pst*I, and DNA sequencing analysis with the wild-type strain M18 as control. After *Pst*I restriction enzyme digestion, three DNA fragments of 0.89 kb (gentamicin resistance cassette), 0.77 kb and 0.34 kb were seen in PCR products from M18LSG, while only two DNA fragments of 0.77 kb and 0.34 kb were seen in PCR products from the wild-type strain M18 (data not shown).

Plasmid pUCX is a *vqsR* gene-containing derivative of pUC18 obtained from the shotgun library of pLAP2. The 1.3 kb *Eco*RI–*Xba*I fragment containing *vqsR* and its flanking sequence from pUCX was inserted into suicide plasmid pEX18Gm to construct pEXX. The 1.7 kb kanamycin resistance cassette from pDSK519 was inserted into pEXX at the blunt-ended *Bam*HI site to construct plasmid pEXXK