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Temperature-Dependent Expression of *phzM* and Its Regulatory Genes *lasI* and *ptsP* in Rhizosphere Isolate *Pseudomonas* sp. Strain M18[▽]

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***Pseudomonas* sp. strain M18, an effective biological control agent isolated from the melon rhizosphere, has a genetic background similar to that of the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1. However, the predominant phenazine produced by strain M18 is phenazine-1-carboxylic acid (PCA) rather than pyocyanin (PYO); the quantitative ratio of PCA to PYO is 105 to 1 at 28°C in strain M18, while the ratio is 1 to 2 at 37°C in strain PAO1. We first provided evidence that the differential production of the two phenazines in strains M18 and PAO1 is related to the temperature-dependent and strain-specific expression patterns of *phzM*, a gene involved in the conversion of PCA to PYO. Transcriptional levels of *phzM* were measured by quantitative real-time PCR, and the activities of both transcriptional and translational *phzM'-'lacZ* fusions were determined in strains M18 and PAO1, respectively. Using *lasI*::Gm and *ptsP*::Gm inactivation M18 mutants, we further show that expression of the *phzM* gene is positively regulated by the quorum-sensing protein LasI and negatively regulated by the phosphoenolpyruvate phosphotransferase protein PtsP. Surprisingly, the *lasI* and *ptsP* regulatory genes were also expressed in a temperature-dependent and strain-specific manner. The differential production of the phenazines PCA and PYO by strains M18 and PAO1 may be a consequence of selective pressure imposed on *P. aeruginosa* PAO1 and its relative M18 in the two different niches over a long evolutionary process.**

Phenazines, known for over 150 years, are a group of the nitrogen-containing secondary metabolites synthesized mainly by *Pseudomonas* spp. and a few other bacterial strains. Advances within the past 2 decades have provided significant new insights into the genetics, biochemistry, and regulation of phenazine synthesis, as well as the mode of action and functional roles of these compounds in various environments (33, 44). Despite the fact that the phenazine biosynthesis locus is highly conserved among various *Pseudomonas* spp., individual strains differ in the range of phenazine compounds they produce and the relative amounts of the possible phenazines are strongly influenced by growth conditions (7, 30, 34, 61). The crucial roles of phenazine-1-carboxylic acid (PCA) in plant root disease suppression has been well documented in studies with several biological control strains, such as *Pseudomonas fluorescens* 2-79, *P. chlororaphis* 30-84, and *P. chlororaphis* PCL1391, where PCA can be converted into phenazine-1-carboxamide (PCN) (6, 33, 35, 37, 50). Nevertheless, PCA is considered a predominant phenazine (44) in these strains, and its secretion mainly contributes to the biocontrol activity against various fungal phytopathogens such as *Gaeumannomyces graminis* var. *tritici* (41, 49, 50). Chromosomal insertion of genes involved in the PCA biosynthetic pathway enhances the efficacy of damping-off disease control by *P. fluorescens*. The phenazine-deficient strains *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 have reduced survival rates and a diminished ability to compete with the resident microflora (5, 51).

Pyocyanin (PYO), a derivative of PCA, is the other major phenazine compound produced by *P. aeruginosa* and functions as an important virulence factor, although PCA and PCN could also be produced by this species (26, 48). PYO imparts the characteristic blue or blue-green color to *P. aeruginosa* cultures and the fluids in sputum from cystic lung fibrosis patients (11). Recently, PYO was thought to be not required for fungal killing in *P. aeruginosa* (16). It has been postulated that the conversion of PCA to PYO is a two-step process that involves N-methylation by PhzM and hydroxylation by PhzS in pseudomonads. *P. aeruginosa* with *phzM* or *phzS* inactivated by gene-targeted insertion developed a PYO-deficient yellow-reddish phenotype (34).

The strain used in this study, *Pseudomonas* sp. strain M18, is an effective biocontrol agent against various soil-borne phytopathogens and was isolated from the sweet melon rhizosphere (20). *Pseudomonas* sp. strain M18 has a genetic background similar to that of *P. aeruginosa* PAO1; the nucleotide sequences of the 16S rRNA gene and several global regulatory genes such as *gacA*, *rsmA*, *rpoS*, *rpoD*, *qscR*, *vqsR*, *lasI*, and *rhlI* from strain M18 show high identities with those in *P. aeruginosa* PAO1 (4, 14, 15, 22, 54, 59, 62). In addition, there exist two sets of *phz* biosynthesis gene clusters (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*), each encoding enzymes required to convert chorismic acid to PCA and two phenazine modification genes, *phzM* and *phzS*, flanking the *phzA1B1C1D1E1F1G1* cluster in the strain M18 genome, which are also similar to those in *P. aeruginosa* PAO1 (unpublished data). *Pseudomonas* sp. strain M18 possesses *las* and *rhl*, two sets of acyl-homoserine lactone quorum-sensing (QS) systems that are the same as those in *P. aeruginosa* PAO1 as well (4, 59). Interestingly, strain M18 had developed several unusual fea-

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tures which were similar to those of biocontrol strains inhabiting soil and quite different from those of *P. aeruginosa* PAO1. First, the predominant phenazine produced in *Pseudomonas* sp. strain M18 is PCA rather than PYO, as shown in this report. Second, *Pseudomonas* sp. strain M18 is the first strain that was reported to produce two antibiotics, PCA and pyoluteorin (Plt). The Plt synthesis cluster of approximately 30 kb, including its flanking regulatory region, is highly homologous to that of biocontrol strain *P. fluorescens* pf-5. Third, several specific features of the regulatory mechanism of the two antibiotics of PCA and Plt production were described, such as that PCA is negatively regulated and Plt is positively regulated by a global regulator called GacA (14); however, the reverse relationship occurs in *Pseudomonas* sp. strain M18 through another global regulator, RsmA (62). In a more recent study, we demonstrated the negative effect of the regulator QscR on PCA production, but not on Plt production, in *Pseudomonas* sp. strain M18 (54). The differential regulation of PCA and Plt production by potential acyl-homoserine lactone QS signaling molecules secreted by this strain has also been studied (4, 59). Besides, the interrelationship between the *las* and *rhl* QS systems in *Pseudomonas* sp. strain M18 is also different from that in strain PAO1 (unpublished data).

As reported previously, PhzM is believed to be involved in the conversion of PCA into PYO in *P. aeruginosa* PAO1 (34, 40). PhzM is a predicted 36-kDa protein similar to enzymes involved in the methylation of polyketide antibiotics by *Streptomyces* spp. (60). Though the 1.8-Å crystal structure of PhzM from *P. aeruginosa* was determined by single-wavelength anomalous dispersion (40), little is known about the features of *phzM* gene expression and regulation in pseudomonads. All of these interesting phenomena found in strain M18, especially the different accumulation of PCA and PYO produced by strains M18 and PAO1, encouraged us to further investigate *phzM* gene expression and regulation and to clarify the differential production of the phenazines PCA and PYO. In this report, several characteristics of *phzM* gene expression with its regulation in strains M18 and PAO1 are newly described.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and chemicals. *Pseudomonas* sp. strain M18 is a fluorescent pseudomonad strain isolated from the sweet-melon rhizosphere; it is an unusual strain that shares some distinct features with both *P. aeruginosa* and the other biocontrol pseudomonads (14, 20, 23). The bacterial strains and plasmids used in this study are listed in Table 1.

Escherichia coli was routinely grown at 37°C in Luria-Bertani medium (46). The fungus *Fusarium oxysporum* var. *cucumerinum* was grown at 28°C on potato dextrose agar (PDA) plates (49). *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1 were incubated in King's medium B (KMB) for growth (24). For secondary metabolite PCA and PYO production, pigment-producing medium (PPM) was used (28). Antibiotics were added at the following concentrations: gentamicin (Gm) at 40 $\mu g\ ml^{-1}$, kanamycin (Km) at 50 $\mu g\ ml^{-1}$, spectinomycin (Sp) at 100 $\mu g\ ml^{-1}$, and tetracycline (Tc) at 125 $\mu g\ ml^{-1}$ for pseudomonads and Km at 50 $\mu g\ ml^{-1}$, Gm at 15 $\mu g\ ml^{-1}$, Tc at 15 $\mu g\ ml^{-1}$, and ampicillin (Ap) at 100 $\mu g\ ml^{-1}$ for *E. coli*. Restriction endonucleases, DNA-modifying enzymes, *Taq* and *Pfu* DNA polymerases, DNA molecular mass markers, and other associated products were purchased and utilized as recommended by the manufacturers (TaKaRa, Dalian, China, and MBI Fermentas, Shenzhen, China). Small-scale preparations of plasmid DNA were performed with a MiniBEST plasmid purification kit, version 2.0 (Biodev-tech, Beijing, China). Genomic DNA was extracted and purified from *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1 with an EZ-10 spin column genomic DNA isolation kit (Bio Basiv Inc., Shanghai, China). Restriction fragments were purified from agarose gels with a DNA gel extraction kit (Axygen Scientific, Inc., Hangzhou, China).

Sequencing of the *phzM* gene from *Pseudomonas* sp. strain M18 and mapping of its transcriptional start sites in strains M18 and PAO1. Based on the *P. aeruginosa* PAO1 sequence, primers phzM1F and phzM1R were designed as listed in Table 2 (underlined letters are the EcoRI and HindIII restriction sites). The *phzM* gene coding region with its regulatory element from strain M18 was PCR amplified with *Pfu* DNA polymerase as recommended by the manufacturer. The PCR-amplified fragment was recovered and directly sequenced by Invitrogen (Shanghai, China).

RNA was isolated at the early stationary growth phase (optical density at 600 nm [OD_{600}], 1.0) from strain M18 and PAO1 cultures with RNAwiz and a DNA-Free kit (Invitrogen). Rapid amplification of the 5′ cDNA end was performed with the 5′ RACE (rapid amplification of cDNA ends) system (Invitrogen) to identify the transcriptional start site of *phzM* according to the manufacturer's instructions (29). Briefly, based on the *phzM* gene sequence, specific primer phzMsp1 was designed and reverse transcriptase was used for first-strand cDNA synthesis. A homopolymeric tail was then added to the 3′ end of the cDNA with TdT and dCTP/dATP. PCR amplification was accomplished with a nested gene-specific primer, phzMsp2, a novel deoxyinosine-containing anchor primer, and the poly(C) tail cDNA as the template. PCR products were directly sequenced, and transcriptional start sites were identified.

Construction of mutants with inactivated genes and fusion plasmids. (i) Construction of mutants. All of the primers used for plasmid construction are listed in Table 2 with enzyme site sequences underlined. A 1,250-bp PCR fragment carrying the open reading frame of the Km resistance (Km^r)-encoding gene with its flanking regions was amplified with primers kan-F and kan-R from plasmid pET28a. The PCR product digested with SacI was ligated into plasmid pUCGm to generate a pUCKm vector. To inactivate the *phzM* gene, a 1,062-bp PCR fragment carrying the *phzM* open reading frame with flanking regions was amplified by the primer pair phzM2F-phzM1R from *Pseudomonas* sp. strain M18 chromosomal DNA, digested, and ligated into the vector pEX18Tc at the EcoRI and HindIII sites. The resulting plasmid, pEX18Tc-*phzM*, was digested with KpnI and SmaI to delete a 215-bp fragment in the *phzM* gene, blunted, and then ligated with a SmaI-digested Km^r cassette amplified from plasmid pUCKm to generate pEX18Tc-*phzM-kan*. Plasmid pEX18Tc-*phzM-kan* was transferred into *Pseudomonas* sp. strain M18 or *P. aeruginosa* PAO1 to generate the isogenic *phzM* inactivation mutant M18pM or PAO1pM with by allelic exchange and the Flp-*FRT* recombination protocol as described previously (19). The same method was used to construct chromosomal *ptsP*::Gm M18PP with primers ptsP1 and ptsP2. The successful inactivation of *phzM*::Km and *ptsP*::Gm in strains M18 and PAO1 was further verified by PCR. In addition, the *lasI*::Gm mutant M18LSG derived from wild-type strain M18 had been constructed previously (4). Plasmids pME6032*lasI* and pME6000*ptsP* (a 2.4-kb PCR product of the *ptsP* gene generated by primers ptsP1 and ptsP2 with EcoRI-HindIII and inserted into pME6000) were introduced into the *lasI*::Gm inactivation and *ptsP*::Gm mutants of M18, respectively, forming complemented mutants M18LSG and M18PP in *trans*.

(ii) Construction of translational and transcriptional *phzM′-′lacZ* fusion and *ptsP′-′lacZ* fusion plasmids. A 704-bp and a 620-bp PCR product, containing the promoter region with the first nine or eight codons of the *phzM* gene and the *ptsP* gene, respectively, from the M18 genome were generated with primer pairs PM9F-PM9R and ptsPPF-ptsP620fR and inserted into sites upstream of *lacZ* in pME6015, forming translational fusion plasmids pPML and pPPL, respectively. Meanwhile, to construct *phzM′-′lacZ* transcriptional fusion plasmids pPMC and pPMCO, the respective 407-bp or 408-bp PCR product containing the upstream region from the transcriptional start site was generated with strains M18 and PAO1 and primer pairs PM9F-TMPR and PM9F-TMPRO, respectively, and then inserted upstream of *lacZ* into pME6522 (see Fig. 4). The *lasI′-′lacZ* translational fusion plasmid pLIL was constructed in our previously published study (4).

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from the various target strains, and the amounts of target cDNAs obtained by reverse transcription were quantified with the MiniOpticon RT-PCR system (Bio-Rad) with SYBR green I stain. Primer pairs RTphzM1-RTphzM2, RTlasI1-RTlasI2, RTptsP1-RTptsP2, and PSRMRPOD1-PSRMRPOD2 were designed based on the *phzM* (193 bp), *lasI* (169 bp), *ptsP* (156 bp), and *rpoD* (173 bp) sequences, respectively, in *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1. The housekeeping gene *rpoD* was used as a reference. Twenty-five microliters of each reaction mixture was prepared according to standard PCR protocols. No-template controls containing distilled water and no-amplification controls containing total RNA instead of the cDNA as the template were included in each RT-PCR run to check for DNA contamination. The target cDNA (*phzM*, *lasI*, or *ptsP*) and reference cDNA (*rpoD*) were amplified in separate wells. PCRs were run in an MJ Mini personal thermal cycler (Bio-Rad) with the following program: one step

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169 deoR* λ (ϕ80d*lacZ*ΔM15)	46
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	46
Pseudomonas strains		
P. aeruginosa PAO1	Wild type	Dieter Haas
Pseudomonas sp. strain M18	Wild type, PCA, Plt producer, Sp^r	Lab collection
M18pM	PYO defective; M18 *phzM*::Km Sp^r Km^r	This study
PAO1pM	PYO defective; PAO1 *phzM*::Km Sp^r Km^r	This study
M18PP	PYO overproducer; M18 *ptsP*::Gm^r Sp^r Gm^r	This study
PAO1PP	PYO overproducer; PAO1 *ptsP*::Gm^r Sp^r Gm^r	This study
PAOLSG	PAO1 *lasI*::Gm^r Sp^r Gm^r	This study
M18LSG	M18 *lasI*::Gm^r Sp^r Gm^r	4
Plasmids		
pBluescript SK	ColE, cloning and sequencing vector, Amp^r	Lab collection
pUCGm	Source of Gm^r cassette, Gm^r	Dieter Haas
pET28a	Expression vector, Km^r	Novagen
pUCKm	Source of Km^r cassette, Km^r	This study
pEX18Tc	Gene replacement vector with multiple cloning site from pUC18, *oriT*$^+$ *sacB*$^+$ Tc^r	19
pEXTphzM	pEX18Tc with EcoRI-HindIII insert of 1.06 kb, including *phzM* gene and partial flanking sequence, Tc^r	This study
pEXTphzMKm	*phzM*:: Km in pEXT*phzM*, Tc^r Km^r	This study
pEXTptsP	pEX18Tc with EcoRI-HindIII insert of 2.4 kb, including *ptsP* gene and partial flanking sequence, Tc^r	This study
pEXTptsPGm	*ptsP*::Gm in pEXT*ptsP*, Tc^r Gm^r	This study
pME6000	Broad-host-range cloning vector, Tc^r	32
pME6000ptsP	pME6000 with 2.4-kb EcoRI-HindIII insert, including *ptsP* gene with partial flanking sequence, Tc^r	This study
pME6032	pVS1-p15A *E. coli-Pseudomonas* shuttle vector, *lacI'-ptac* expression vector, Tc^r	18
pME6032lasI	pME6032 with 1.2-kb BglI-EcoRI insert, including *lasI* gene with partial flanking sequence, Tc^r	4
pME6015	pVS1-p15A *E. coli-Pseudomonas* shuttle vector for translational *lacZ* fusions and promoter probing, Tc^r	18
pME6522	pVS1-p15A *E. coli-Pseudomonas* shuttle vector for transcriptional *lacZ* fusions and promoter probing, Tc^r	2
pPML	*phzM'-'lacZ* translational fusion, 704-bp EcoRI-PstI PCR-amplified fragment containing 407 nucleotides upstream and 297 nucleotides downstream of transcriptional start site of *phzM* cloned into pME6015, Tc^r	This study
pPMC	*phzM'-'lacZ* transcriptional fusion, 407-bp EcoRI-PstI PCR-amplified fragment from *Pseudomonas* sp. strain M18 cloned into pME6522, Tc^r	This study
pPMCO	*phzM'-'lacZ* transcriptional fusion, 408-bp EcoRI-PstI PCR-amplified fragment from *P. aeruginosa* PAO1 cloned into pME6522, Tc^r	This study
pPMCD	*phzM'-'lacZ* transcriptional fusion, 333-bp EcoRI-PstI PCR-amplified fragment lacking the *lux* box from *Pseudomonas* sp. strain M18 cloned into pME6522, Tc^r	This study
pLIL	*lasI'-'lacZ* translational fusion plasmid, Tc^r	4
pPPL	*ptsP'-'lacZ* translational fusion, 620-bp EcoRI-PstI PCR-amplified fragment containing 24-bp DNA sequence downstream of translational start site of *ptsP* cloned into pME6015, Tc^r	This study

of 10 min at 95°C and 40 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 20 s. The evolution of fluorescence intensity in each reaction mixture was recorded continuously with a MiniOpticon RT-PCR detector (Bio-Rad). The PCR products amplified from cDNA were further confirmed by sequencing. PCR analyses of each target strain in different growth phases were repeated three times. The data analysis method used was described previously (59). The amount of *phzM*, *lasI*, or *ptsP* cDNA, normalized to the level of the reference *rpoD* and calibrated relative to strain M18, was expressed as a $2^{-\Delta\Delta CT}$ value, which represents the change in target gene expression in a given strain relative to that in strain M18. Statistical significance was computed by an unpaired Student *t* test; a *P* value of <0.05 was considered statistically significant.

Assays of β-Gal activity and PCA and PYO production. *phzM'-'lacZ*, *lasI'-'lacZ*, and *ptsP'-'lacZ* translational fusion plasmids pPML, pLIL, and pPPL, respectively, and *phzM'-'lacZ* transcriptional fusion plasmid pPMC were transformed into the various target strains, while only plasmid pPMCO was introduced into strain PAO1. Subsequently, these transformed strains harboring the various fusion plasmids were cultivated at 28°C or 37°C with shaking at 200 rpm in 150-ml conical flasks containing 50 ml PPM broth supplemented with 0.05% Triton X-100. Samples were collected after a certain time of growth, and β-galactosidase (β-Gal) specific activity was assayed according to the method of Miller (38).

PPM was used for PCA and PYO production. PCA was extracted at pH 4 and quantified in chloroform as described by Ge et al. (14). Quantification of PYO production was done at neutral pH in chloroform as described by Essar et al. (12, 45, 58).

Fungal inhibition assay. Fungal inhibition by strains M18 and PAO1 was determined by measuring their ability to inhibit mycelium plug growth of *F. oxysporum* var. *cucumerinum* on a PDA agar plate as follows. Briefly, a 5-mm plug from the leading edge of a culture of *F. oxysporum* var. *cucumerinum* grown for 5 days at 28°C on a PDA plate was placed in the center of a fresh PDA plate.

TABLE 2. Primers used in this study

Primer[a]	Nucleotide sequence	Restriction site[b]
phzMsp1	5′-TGGAATGCCAGGTTGCTC-3′	
phzMsp2	5′-CGCCGTAGAACAGCACCAT-3′	
kan-F	5′-CCGGGAGCTCGAATTCAGCAGAGCGAGGTATGTAGG-3′	SacI
kan-R	5′-CCGGGAGCTCGAATTCCAGGTGGCACTTTTCGGGGA-3′	SacI
phzM1F	5′-GGCCGAATTCTCTCCAGGTATGCCGGAGAA-3′	EcoRI
phzM1R	5′-GGCCAAGCTTGAAAGTTCCGATTCAGGCCC-3′	HindIII
phzM2F	5′-GGCCGAATTCGGTACTTCTCGGGTTACGGA-3′	EcoRI
ptsP1	5′-CCGCGCGAATTCGACAGGTGGTGACCTTCAAG-3′	EcoRI
ptsP2	5′-CCGCGC AAGCTT GAAATCAGGGCTGGACGGTA-3′	HindIII
PM9F	5′-GGCCGAATTCTCTCCAGGTATGCCGGAGAA-3′	EcoRI
PM9R	5′-CCGGCTGCAGCGCAGCAGCAAGATTCGAAT-3′	PstI
DLasbF	5′-GGCCGAATTC GCCAGACAGGGTATGCGGGA-3′	EcoRI
TMPR	5′-CCGGCTGCAG AGCATCAGCTTAGCAATCCC-3′	PstI
TMPRO	5′-CCGGCTGCAG AAGCATCAGCTTAGCAATCC-3′	PstI
ptsPPF	5′-GCGCGAATTCTGTATCCTCCCCGCACGAGT-3′	EcoRI
ptsP620fR	5′-CGGCCTGCAGGATCTTGCGCAGCGTGTTGA-3′	PstI
RTphzM1	5′-GGATCGACAGCGACGAGACG-3′	
RTphzM2	5′-CTCGCCGTAGAACAGCACCAT-3′	
PSRMRPOD1	5′-GAGCGGGAGGAGCGTTTAC-3′	
PSRMRPOD2	5′-CGGGCAAAAAATAAGCAGAGG-3′	
RTlasI1	5′-AACTGCTGGGCGAGATGC-3′	
RTlasI2	5′-CGCCAGCAACCGAAAACC-3′	
RTptsP1	5′-AAGTGAACTCCGCCAAGG-3′	
RTptsP2	5′-TGGAACGCTTGTTGAGGC-3′	

[a] All primers were obtained from Invitrogen (Shanghai, China).
[b] The restriction sites used in the cloning procedure are underlined.

Bacterial cultures grown overnight on a KMB plate were placed 25 mm from the PDA plate center. The coculture plates were incubated at 28°C and scored after 4 or 5 days by measuring the distance in millimeters between the edges of the bacterial colony and the fungal mycelium plug as described by Thomashow and Weller (16, 49). Each experiment was repeated twice with three replicates.

Sequences analysis. The primers used in this study are listed in Table 2. PCR products were recovered and sequenced directly by a commercial service (Invitrogen).

Nucleotide sequence accession numbers. The nucleotide sequences of the *phzM* and *ptsP* genes from the *Pseudomonas* sp. strain M18 chromosome were deposited in GenBank with accession no. FJ535573 and FJ790321, respectively.

RESULTS

The PCA and PYO pigments are produced by strains M18 and PAO1 in a temperature-dependent and strain-specific manner. In our previously published works, we demonstrated that strain M18 isolated from rhizosphere niches showed a genetic background similar to that of strain PAO1 and was a strong biocontrol strain (15, 54, 59). When *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1 were inoculated onto KMB plates as described in Materials and Methods and incubated at 28°C or 37°C, respectively, for 1 to 2 days, the two strains produced distinct pigments, as shown in Fig. 1A and B. Strain M18 produced a yellow-reddish pigment, while strain PAO1 produced a yellow pigment with a little blue color at 28°C. However, when the plates were incubated at 37°C, strain M18 still produced the yellow-reddish pigment, while strain PAO1 produced a blue-green pigment. To assay fungal inhibition by the two strains, the fungus *F. oxysporum* var. *cucumerinum* was cocultured with strains M18 and PAO1 on a fungal growth PDA plate at 28°C. Mycelium growth of the fungus *F. oxysporum* var. *cucumerinum* was totally inhibited by strain M18, but strain PAO1 showed almost no fungal killing activity (Fig. 1C). These results indicated that strains M18 and PAO1

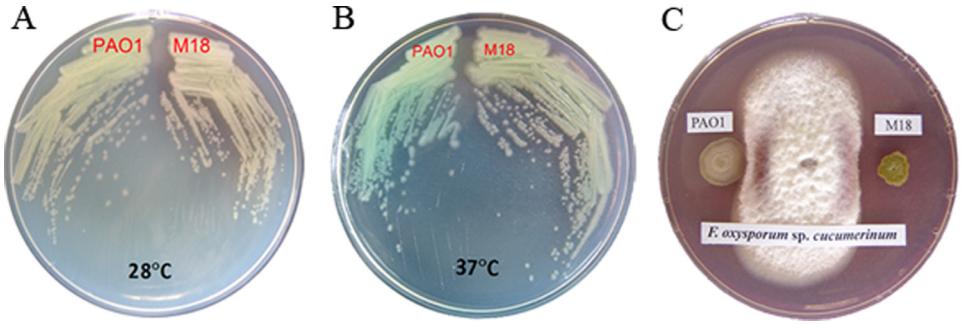

FIG. 1. Pseudomonad growth and plate cocultures of *F. oxysporum* var. *cucumerinum* with *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1. Shown are (A) pigments produced by strains M18 and PAO1 grown for 16 to 24 h at 28°C and (B) pigments produced by strains M18 and PAO1 grown for 16 to 24 h at 37°C on KMB plates. (C) A 5-mm plug of the fungus *F. oxysporum* var. *cucumerinum* was placed in the center of a PDA plate, and cultures of *Pseudomonas* strains M18 and PAO1 were inoculated 25 mm from the center on each side of the plate.

showed different biological control abilities. It is well known that phenazine compounds are the major contributors to the colony color of various pseudomonads (17, 33, 44). PYO produces a characteristic blue-green color, while PCA generates a yellow-reddish color, where the final colony color appearance on the plates suggests that different phenazines are produced by the two strains in a temperature-dependent and strain-specific manner.

The PCA and PYO production of the two strains was further measured at 28°C and 37°C, respectively, and the results are shown in Fig. 2. The growth curves of strains M18 and PAO1 were similar at both 28°C and 37°C (Fig. 2A). However, 28°C was more suitable for cell growth of the two strains, and cell density dropped rapidly at 37°C during the late logarithmic phase. It was evident that the predominant phenazine produced by strain M18 and strain PAO1 was different at the different temperatures, as shown in Fig. 2B and C. The suitable temperature for PCA production was apparently 28°C, where the major phenazine produced by strain M18 was PCA, reaching up to 147 mg/liter or ninefold over that of *P. aeruginosa* PAO1 at that temperature. The quantitative ratio of PCA to PYO was very high, reaching up to 105:1 at 28°C and was 5:1 even at 37°C in strain M18. PYO was largely produced by strain PAO1 at 37°C, and the quantitative ratio of PYO to PCA was 2:1 at this temperature. The PYO production of strain PAO1 reached 24 mg/liter and was 3.5-fold greater than that in strain M18 at 37°C. In addition, the production of PCA by strain M18 was 4-fold lower at 37°C than at 28°C, while PYO production by strain PAO1 at 37°C was 20-fold greater than that at 28°C. There was no PYO detected in *phzM*::Km inactivation mutants M18pM and PAO1pM, but the total PCA produced by the two strains accumulated differently (data not shown).

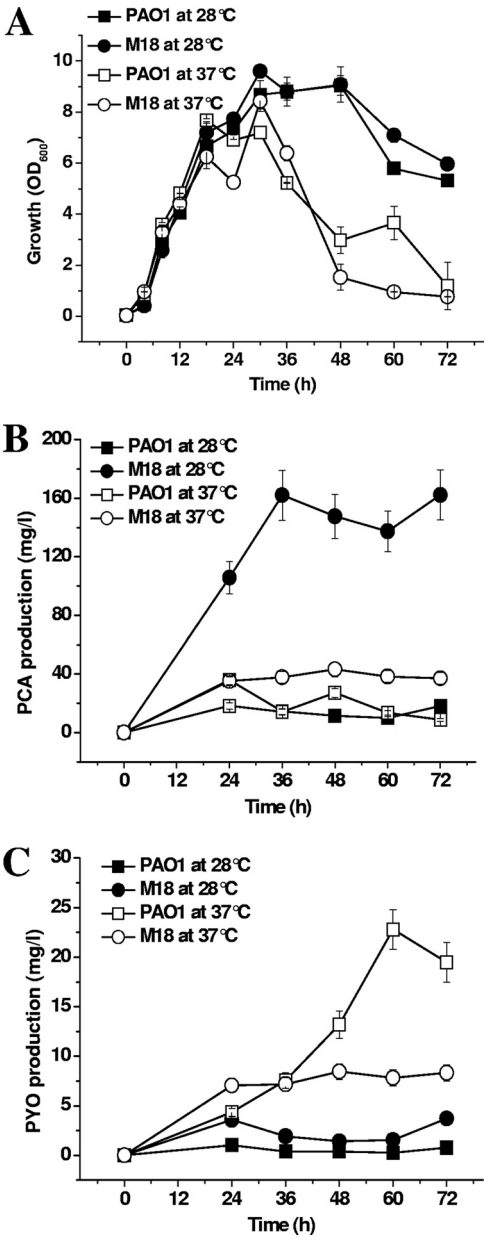

FIG. 2. Dynamic growth curves and different accumulations of PCA and PYO in strains M18 and PAO1. Shown are (A) the dynamic growth curves and (B) PCA and (C) PYO production of *Pseudomonas* sp. strain M18 (circles) and *P. aeruginosa* PAO1 (squares) grown in PPM broth at 28°C (solid symbols) and 37°C (open symbols). Values are the means ± standard deviations of triplicate cultures.

Mapping of the *phzM* gene transcriptional start sites in strains M18 and PAO1. To clarify whether the temperature-dependent and strain-specific *phzM* gene expression in the two strains comes from differences in their gene coding regions, we first compared the nucleotide sequences of the *phzM* gene coding region with its flanking sequences in strains M18 and PAO1. A 1,694-bp fragment of a PCR product including the 1,005-bp gene coding region from the chromosomal DNA of strain M18 was sequenced directly by Invitrogen and then complemented with that from strain PAO1 by using NCBI BLASTX. We found that the full nucleotide sequence of the PCR fragment from strain M18 covering the whole gene structure and its flanking region showed 99% similarity to the *phzM* gene-containing region in *P. aeruginosa* PAO1. The *phzM* genes of both strains were closely located upstream of the *phzA1B1C1D1E1F1G1* operon and transcribed divergently from it (Fig. 3A). The functional roles of the *phzM* gene were confirmed in the two strains by insertional inactivation of the gene. The results indicated that the production of PYO was abolished completely and the quantity of the PCA product increased in both mutant strains M18pM and PAO1pM (data not shown). We then investigated the transcriptional start sites of the *phzM* genes of *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1 by 5′ RACE as described in Materials and Methods. With the poly(C)-tailed cDNA as a template, PCR amplification with the nested gene-specific primer and the novel deoxyinosine-containing anchor primer resulted in a single-band PCR product. This PCR product was analyzed by DNA sequencing, and the result revealed that there was only a single nucleotide difference between the two transcriptional start points in strains M18 and PAO1. The *phzM* transcriptional start site was at 269 bp upstream (C nucleotide) of the translation start site in strain PAO1 but was 270 bp upstream (T nucleotide) of the translation start site in strain M18, as shown in Fig. 3B. A putative *lux* box was found between bp 326 and 307 upstream of the translational start site in both genes

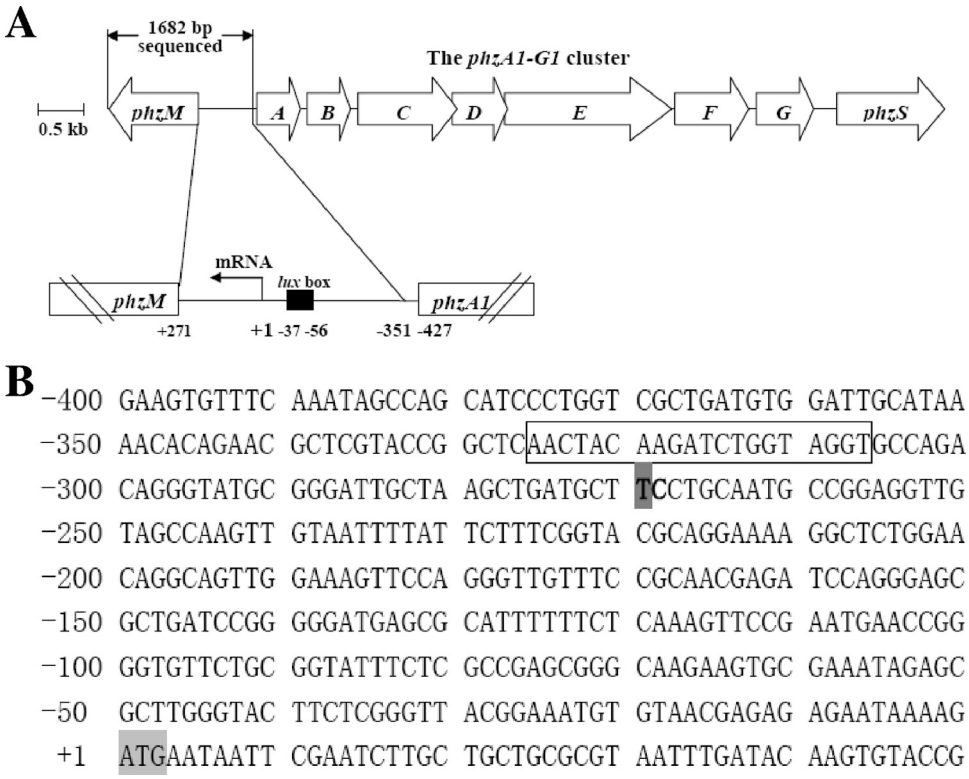

FIG. 3. Location of the *phzM* gene with its transcriptional start site and the nucleotide sequence of the *phzM* promoter region in *Pseudomonas* sp. strain M18. (A) Location of *phzM* with its transcriptional start site. Numbering is relative to the transcriptional start site. A putative *lux*-like box (black box) is located upstream of the transcription start site. The length of the space between *phzM* and *phzA1* is 696 bp. (B) Nucleotide sequence of the *phzM* promoter region. In strain M18, the mRNA start site of *phzM*, mapped by 5′ RACE, is at position −270, which is occupied by the T nucleotide indicated by a dark gray box in bold, while in strain PAO1, it is at position −269, which is occupied by the C nucleotide in bold. The potential *lux*-like box is enclosed in a rectangle. The ATG translational start codon is shaded light gray.

(Fig. 3B) and exhibited substantial homology to other reported *lux* box sequences (29, 39, 45, 55, 59).

The temperature-dependent and strain-specific expression of the *phzM* gene occurred at the transcriptional level. Since the *phzM* product is involved in the conversion of PCA to PYO in *P. aeruginosa* PAO1 (34, 40) and production of PCA and PYO by the two strains was determined to be temperature dependent and strain specific (Fig. 2), we investigated further to see if the *phzM* gene is expressed in a the temperature-dependent manner in the two strains and to determine if the *phzM* gene expressional regulation occurs at the translational, transcriptional, or both levels. Based on the *phzM* transcriptional start sites determined as described above, *phzM′-′lacZ* translational and transcriptional fusions were constructed to identify the *phzM* regulatory expression levels in strains M18 and PAO1. The same *phzM′-′lacZ* translational fusion plasmid, pPML, was introduced into strains M18 and PAO1, and β-Gal activity was measured at both 28°C and 37°C. Compared with the β-Gal activity in strain M18/pPML at 28°C, threefold and fivefold increased activities were detected in strains M18/pPML and PAO1/pPML, respectively, at 37°C, and the β-Gal activity increased sharply in PAO1/pPML when the OD_{600} reached 3.0 (Fig. 4B). The same trends were also found by analysis of the activities of the two transcriptional fusion products in strains M18/pPMC and PAO1/pPMCO; the β-Gal activities in both strains were about three to four times higher at 37°C than that at 28°C (Fig. 4C). The β-Gal activity in PAO1/pPMCO was threefold higher than that in M18/pPMC at the late logarithmic phase. Thus, we can conclude that the temperature-dependent expression of the *phzM* gene in strains M18 and PAO1 occurred mostly at the transcriptional level, since we obtained nearly the same increased expression ratio at both the translational and transcriptional levels at the two temperatures. It was found that the strain-specific expression of the *phzM* gene occurred mainly at the late logarithmic growth phase, when the OD_{600} of the two strains reached 3.0.

The expression of the *phzM* gene was positively regulated by LasI at the transcriptional level and negatively regulated by PtsP at both the transcriptional and translational levels in strain M18. We further investigated the effects of six global regulatory genes, *gacA*, *rsmA*, *rhlI*, *qscR*, *lasI*, and *ptsP*, on *phzM* gene expression. It was found that the regulators GacA and RsmA had no obvious effect on *phzM* gene expression, while LasI and RhlI had a positive effect but QscR and PtsP had a negative effect on *phzM* gene expression (unpublished data). Interestingly, the *lasI* and *ptsP* regulatory genes, which exerted a strong regulatory effect on *phzM*, were also expressed in a temperature-dependent and strain-specific manner. The evidence showing the regulatory effect of *lasI* and *ptsP* on *phzM* expression is presented in the following sections.

The construction of mutant M18PP with *ptsP*::Gm inactivated is described in Materials and Methods. The full nucleo-

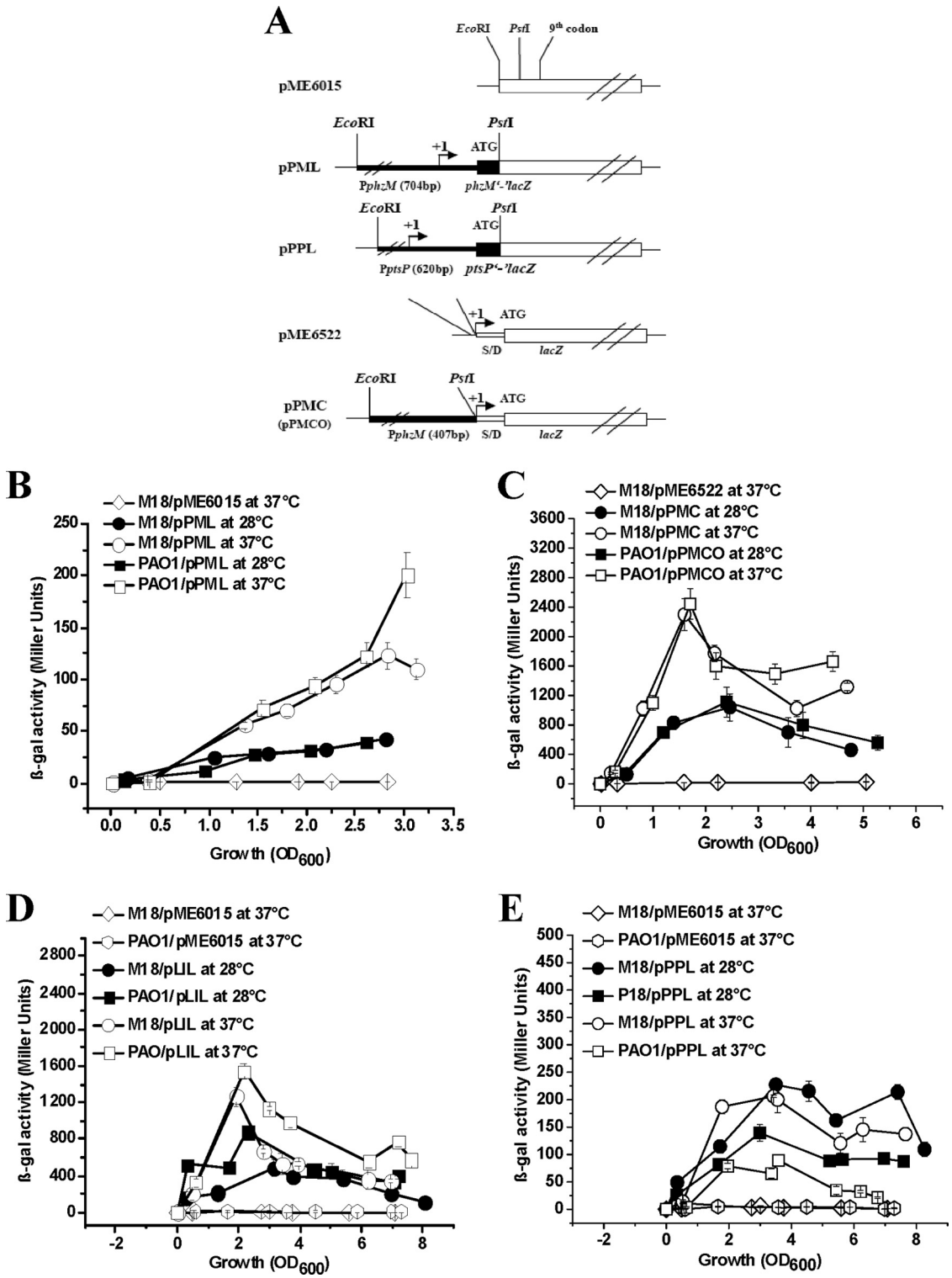

FIG. 4. Temperature-dependent and strain-specific expression of the *phzM* gene and its regulatory genes *lasI* and *ptsP* determined by assaying β-Gal activity in *Pseudomonas* sp. strain M18 and *P. aeruginosa* PAO1. (A) Physical maps of *phzM'-'lacZ* translational fusion plasmid pPML and transcriptional fusion plasmid pPMC in strain M18 and pPMCO and *ptsP'-'lacZ* translational fusion plasmid pPPL in strain PAO1. *lasI'-'lacZ* translational fusion plasmid pLIL was constructed by the same method as in our previous work (4). SD, putative Shine-Dalgarno sequence. *phzM* and *ptsP* sequences are shown by heavy black lines, and *lacZ* sequences are shown by white boxes. P*phzM* and P*ptsP* are the *phzM* and *ptsP* gene promoters. (B) β-Gal activities of M18/pPML (circles) and PAO1/pPML (squares) at 28°C (solid symbols) and 37°C (empty symbols) and strain M18 (diamonds) containing control plasmid pME6015. (C) β-Gal activities of M18/pPMC (circles) and PAO1/pPMCO (squares) at 28°C (solid symbols) and 37°C (empty symbols) and strain M18 (diamonds) containing control plasmid pME6522. (D) β-Gal activities of M18/pLIL (circles) and PAO1/pLIL (squares) at 28°C (solid symbols) and 37°C (empty symbols) and strain M18 (diamonds) and PAO1 (hexagons) containing control

tide sequence of the whole *ptsP* gene coding region with its flanking regions and the encoded PtsP protein sequence of strain M18 show 97% and 100% identities, respectively, with those of *P. aeruginosa* PAO1. The mutant M18LSG with *lasI*::Gm inactivated was constructed in our previously published work (4). The cell growth rate was promoted by *lasI* inactivation and inhibited by *ptsP* inactivation, compared with that of wild-type strain M18 (Fig. 5A).

To determine whether LasI and PtsP regulate *phzM* expression, we tested the translational and transcriptional *phzM'-'lacZ* fusions in wild-type M18, *lasI*::Gm mutant M18LSG, and *ptsP*::Gm mutant M18PP (Fig. 5). In *lasI*::Gm mutant M18LSG, *phzM* gene expression was strongly inhibited and delayed at both the translational and transcriptional levels. β-Gal activity was nearly undetectable at the early logarithmic phase. Only 20 to 30% activity was found at the late logarithmic phase, when the OD_{600} reached 3.0 to 4.0, compared with that in wild-type M18. In addition, the β-Gal activity from putative *lux* box deletion-containing fusion plasmid pPMCD was undetectable in strains M18 and M18LSG (data not shown). These data suggested that *phzM* expression was positively controlled by the *las* system at the transcriptional level mostly via the *lux* box upstream of the transcriptional start site, in agreement with other reports on the regulation of LasI on *vqsR* (29). Inactivation of *ptsP* induced large amounts of PYO production and promoted cell lysis (data not shown); therefore, cell growth was inhibited and the OD_{600} of M18PP could not exceed 5.0 (Fig. 5A). The β-Gal activity from translational *phzM'-'lacZ* fusion plasmid pPML increased by 3-fold in *ptsP*::Gm mutant M18PP when the OD_{600} reached 2.0, compared with that in wild-type M18 (Fig. 5B), while at the transcriptional level in M18PP it was only 1.5-fold greater than that of wild-type M18 (Fig. 5C). This result suggested that PtsP inhibited *phzM* expression mainly at the translational level and not at the transcriptional level. This negative regulation of *phzM* by PtsP disappeared at later growth phases at an OD_{600} of about 4.0, perhaps due to the impaired cell growth ability of strain M18PP. The induction of *phzM* expression corresponded to the increase in PYO production by the *ptsP*::Gm mutant.

To confirm the proposed regulatory roles of LasI and PtsP in *phzM* expression, the *phzM* transcripts were assessed in wild-type M18, the *lasI*::Gm mutant, the *ptsP*::Gm mutant, and the complemented strains in *trans* by qRT-PCR at both the early exponential phase and the late exponential phase, as summarized in Table 3. When cells reached an OD_{600} of 2.0 to 2.4 in the early exponential phase, the normalized *phzM* levels in strains M18LSG and M18PP were 0.28 and 4.26, respectively, relative to that in strain M18. At the late exponential phase, the normalized *phzM* levels in M18LSG and M18PP reached 0.37 and 4.66, respectively. The results indicated that *phzM* expression was reduced in the *lasI* mutant and increased in the *ptsP* mutant. The transcriptional level of *phzM* was recovered in the complemented mutants in both the early exponential phase ($P < 0.05$) and the late exponential phase ($P < 0.05$).

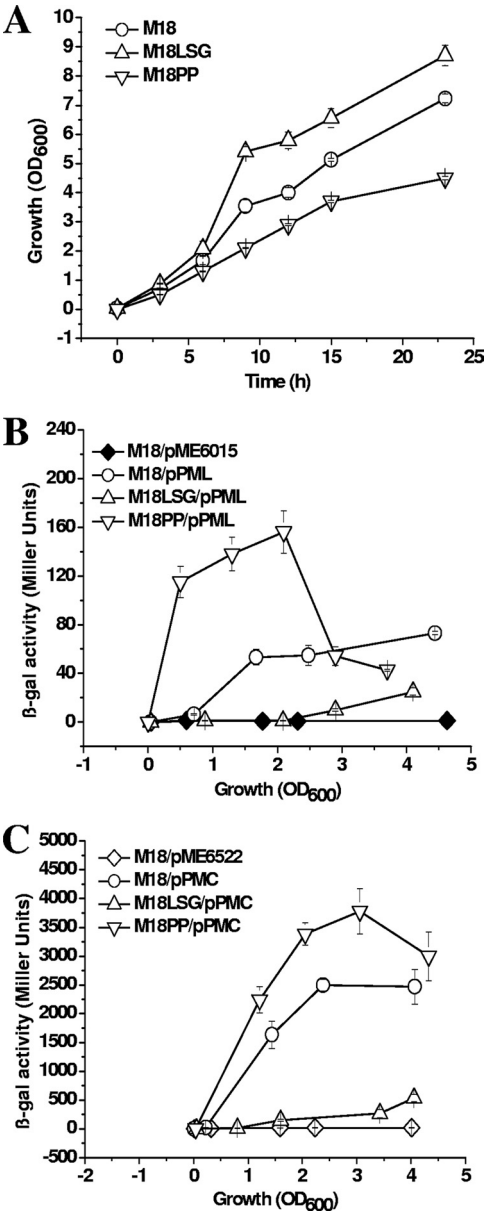

FIG. 5. Effects of LasI and PtsP on *phzM* gene expression in *Pseudomonas* sp. strain M18. Shown are the growth curves of wild-type M18 (circles), corresponding *lasI*::Gm mutant M18LSG (triangles), and *ptsP*::Gm mutant M18PP (inverted triangles) (A) and the β-Gal activities of translational fusion plasmid pPML (B) and transcriptional fusion plasmid pPMC (C) in wild-type strain M18 (circles), *lasI*::Gm mutant M18LSG (triangles), and *ptsP*::Gm mutant M18PP (inverted triangles). pME6015 and pME6522 are the control plasmids transformed into strain M18 (diamonds). Growth was in PPM broth. The values are means ± standard deviations of triplicate cultures.

plasmid pME6015. (E) β-Gal activities of M18/pPPL (circles) and PAO1/pPPL (squares) at 28°C (solid symbols) and 37°C (empty symbols) and M18 (diamonds) and PAO1 (hexagons) containing control plasmid pME6015. Growth was in PPM broth. The values are means ± standard deviations of triplicate cultures.

TABLE 3. Relative *phzM* transcription levels in wild-type strain M18, *lasI*::Gm mutant M18LSG, complemented M18LSG, *ptsP*::Gm mutant M18PP, and complemented M18PP determined by qRT-PCR during early and late exponential phase

Strain	Early log phase				Late log phase			
	$C_{T,\ rpoD}$[a]	$C_{T,\ phzM}$[a]	ΔC_T[b]	M[c]	$C_{T,\ rpoD}$[a]	$C_{T,\ phzM}$[a]	ΔC_T[b]	M[c]
M18	20.05 ± 0.5	23.94 ± 0.1	3.89 ± 0.1	1.00	20.14 ± 0.01	22.09 ± 0.2	1.71 ± 0.05	1.00
M18LSG	19.78 ± 0.09	25.48 ± 0.12	5.70 ± 0.1	0.28	20.28 ± 0.03	23.65 ± 0.05	3.37 ± 0.04	0.37
Complemented M18LSG	20.07 ± 0.02	23.96 ± 0.07	3.89 ± 0.02	1.01	20.10 ± 0.02	21.81 ± 0.03	1.702 ± 0.03	1.19
M18PP	20.06 ± 0.27	21.05 ± 0.89	0.99 ± 0.1	4.26	19.76 ± 0.08	19.20 ± 0.66	-0.56 ± 0.04	4.66
Complemented M18PP	19.60 ± 0.03	21.84 ± 0.26	2.24 ± 0.1	1.79	19.68 ± 0.09	20.67 ± 0.70	0.99 ± 0.07	1.59

[a] Values were measured during early exponential phase at an OD_{600} of 2.0 to 2.4 and during late exponential phase at an OD_{600} of 5.0 to 6.0. All values are means ± standard deviations of three independent experiments.
[b] ΔC_T = mean $C_{T,\ phzM}$ − mean $C_{T,\ rpoD}$.
[c] $M = 2^{-\Delta\Delta CT}{}_{phzM}$ normalized amount of cDNA from the *phzM* gene in different strains relative to that in wild-type *Pseudomonas* sp. strain M18; $\Delta\Delta C_T$ = mean ΔC_T − mean $\Delta C_{T,\ M18}$.

These data, together with the results of analyses by qRT-PCR and *phzM'-'lacZ* fusional expression, clearly confirmed that the expression of *phzM* is positively regulated mainly at the transcriptional level by the QS protein LasI and negatively regulated at both the transcriptional and translational levels by the PtsP protein.

Quantitative analysis of temperature-regulated *phzM*, *lasI*, and *ptsP* transcripts by qRT-PCR in strains M18 and PAO1. According to the previous results of *phzM'-'lacZ* fusional expression shown in Fig. 4, the *phzM* gene was expressed in a temperature-dependent and strain-specific manner. We further investigated the expression of *phzM* with its regulatory genes *lasI* and *ptsP* by measuring the transcripts by qRT-PCR at both 28°C and 37°C in strains M18 and PAO1, with the housekeeping gene *rpoD* as a reference (Table 4). In early and late exponential phase, compared with the transcripts in strain M18, the normalized *phzM* levels in strain PAO1 at 37°C were high, at six- and sevenfold over that at 28°C and increased to over twofold in strain M18 at 37°C or in strain PAO1 at 28°C. The results indicated that temperature-dependent *phzM* expression at the transcriptional level occurs in a strain-specific manner since *phzM* transcriptional activity was much higher in strain PAO1 than in strain M18, especially at 37°C. The lower expression of the *phzM* gene in strain M18, especially at 28°C, corresponded to the higher quantity of PCA produced by *Pseudomonas* sp. strain M18 at 28°C. Therefore, we can conclude that the different amounts of phenazine produced by strains M18 and PAO1 are related to *phzM* gene expression in a temperature-dependent and strain-specific manner.

In addition, the transcriptional expression of *phzM*, as well as its positive regulatory gene *lasI* and negative regulatory gene *ptsP*, was also assayed by qRT-PCR. As expected, the levels of the *lasI* transcripts at 37°C were twofold greater than those at 28°C and were much higher in strain PAO1 than in strain M18 at both temperatures. The results revealed that *lasI* had a similar temperature-dependent and strain-specific expression pattern, with the highest level in strain PAO1 at 37°C and the lowest level in strain M18 at 28°C. However, *ptsP* expression had an adverse temperature-dependent and strain-specific pattern; the highest level of *ptsP* transcripts was observed in strain M18 at 28°C, while the lowest level of *ptsP* transcripts was observed in strain PAO1 at 37°C. The normalized *ptsP* levels decreased to 0.24, 0.86, and 0.12, respectively, in strain M18 at 37°C and strain PAO1 at 28°C and 37°C, relative to that in strain M18 at 28°C, when the OD_{600} reached 2.0 to 2.4.

Temperature-dependent and strain-specific expression of *lasI* and *ptsP* in strains M18 and PAO1 as determined by assaying fusion β-Gal activities. The temperature-regulated transcripts of *lasI* and *ptsP* were first identified in strains M18 and PAO1 by qRT-PCR as shown in Table 4; further, *lasI'-'lacZ* and *ptsP'-'lacZ* translational fusion plasmids pLIL and pPPL were constructed and then transformed into strains M18 and PAO1, respectively. Their β-Gal activities were assayed at 28°C and 37°C, as shown in Fig. 4D and E. The β-Gal activity detected in PAO1/pLIL was 2.5-fold higher than that in M18/pLIL, and the β-Gal activities were 2.5 to 3 times higher at 37°C than at 28°C in both strains M18/pLIL and PAO1/pLIL. The highest β-Gal activity was detected in PAO1/pLIL at 37°C

TABLE 4. Relative transcriptional levels of *phzM* and its regulatory genes *lasI* and *ptsP* in strains M18 and PAO1 determined by qRT-PCR during early and late exponential phase

Strain and temp (°C)	Early log phase							Late log phase						
	$C_{T,\ rpoD}$[a]	$C_{T,\ phzM}$[a]	$C_{T,\ lasI}$[a]	$C_{T,\ ptsP}$[a]	M[b]	L[b]	P[b]	$C_{T,\ rpoD}$[a]	$C_{T,\ phzM}$[a]	$C_{T,\ lasI}$[a]	$C_{T,\ ptsP}$[a]	M[b]	L[b]	P[b]
M18														
28	19.75 ± 0.2	24.76 ± 0.1	18.91 ± 0.4	20.65 ± 0.15	1.00	1.00	1.00	20.06 ± 0.1	23.08 ± 0.2	19.14 ± 0.4	27.10 ± 0.5	1.00	1.00	1.00
37	20.05 ± 0.13	23.94 ± 0.1	18.70 ± 0.1	23.01 ± 0.52	2.17	1.42	0.24	20.14 ± 0.3	22.09 ± 0.05	18.06 ± 0.3	28.52 ± 0.6	2.10	2.23	0.40
PAO1														
28	19.61 ± 0.32	23.44 ± 0.1	18.05 ± 0.2	20.73 ± 0.40	2.27	1.65	0.86	20.17 ± 0.2	21.91 ± 0.13	18.84 ± 0.5	27.27 ± 0.8	2.43	1.33	0.96
37	19.36 ± 0.54	21.77 ± 0.2	16.28 ± 0.2	23.35 ± 0.95	6.06	4.72	0.12	20.03 ± 0.3	20.20 ± 0.15	17.48 ± 0.3	28.94 ± 0.9	7.21	3.10	0.27

[a] Values were measured during early exponential phase at an OD_{600} of 2.0 to 2.4 and during late exponential phase at an OD_{600} of 5.0 to 6.0. All values are means ± standard deviations of three independent experiments.
[b] $M = 2^{-\Delta\Delta CT}{}_{phzM}$, $L = 2^{-\Delta\Delta CT}{}_{lasI}$, and $P = 2^{-\Delta\Delta CT}{}_{ptsP}$ (normalized amounts of cDNA from the *phzM*, *lasI*, and *ptsP* genes in different strains relative to that in wild-type *Pseudomonas* sp. strain M18 at 28°C; $\Delta\Delta C_T$ = mean ΔC_T − mean $\Delta C_{T,\ M18}$ at 28°C).

(Fig. 4D). Meanwhile, the β-Gal activities produced by the *ptsP′-′lacZ* translational fusion in strains M18 and PAO1 were assayed. The results (Fig. 4E) showed that the β-Gal activities were threefold higher at 28°C than at 37°C in both strains M18/pPPL and PAO1/pPPL. At the same temperature, the β-Gal activity was two to three times higher in M18/pPPL than in PAO1/pPPL. The highest β-Gal activity was detected in M18/pPPL at 28°C. These measurements of β-Gal activity (Fig. 4D and E), together with the transcriptional analysis by qRT-PCR (Table 4), show clearly that the expression of *lasI* and *ptsP* is also regulated in a temperature-dependent and strain-specific manner.

DISCUSSION

An unexpected finding of this study was that *phzM* and its regulatory genes *lasI* and *ptsP* were expressed in a temperature-dependent and strain-specific manner in *P. aeruginosa* PAO1 and the related strain *Pseudomonas* sp. strain M18. *P. aeruginosa* is the most-studied phenazine-producing microorganism, and the characteristic blue-green pigment PYO is the most well-described phenazine and is associated with 90 to 95% of *P. aeruginosa* isolates (33, 48). *P. aeruginosa* is a ubiquitous environmental bacterium that is one of the top three causes of opportunistic human infections, while the major PYO produced by *P. aeruginosa* functions as an important virulence factor (26, 27, 43). Several strains of *P. aeruginosa* have also been found to be good biocontrol agents by being well adapted to the rhizosphere (1), where they can display strong activities against fungal phytopathogens (52). However, the antifungal metabolites produced by *P. aeruginosa* have been identified as PCN, PCA, or 5′-methyl-PCA, rather than PYO (5, 16, 20, 25). In our previously published work (20, 21, 59), we reported that *Pseudomonas* sp. strain M18 is another effective biocontrol agent and that the major virulence factor against soil-borne phytopathogens is PCA. *Pseudomonas* sp. strain M18 has a genetic background similar to that of *P. aeruginosa* PAO1, but the different phenotypic characteristics and phenazines produced from strain M18 have not been studied in detail. In the present study, we used *P. aeruginosa* PAO1, a representative strain isolated from a human patient, and *Pseudomonas* sp. strain M18, a strain related to *P. aeruginosa* and isolated from the rhizosphere, as two models from different ecological niches to characterize their phenazine production phenotypes and their biosynthetic regulatory genes.

We first described the differences in phenotype and fungal inhibition between these two strains (Fig. 1). Much stronger activity against fungal growth was detected in strain M18 than in strain PAO1 under suitable fungal growth conditions. It is known that PCA and PYO are the two important varieties of phenazines produced in various pseudomonads, although the relative quantities and functional roles of PCA and PYO produced in pseudomonads were not strictly distinguished and carefully studied in many publications. PCA and PYO were considered to have similar physiological and biochemical functions in cells (13); however, the different functional roles of PCA and PYO have been described in a few publications recently (10, 16, 28, 57). Based on the methods used in this study, it would be quite difficult to compare our PCA and PYO measurements with those in other studies, where PCA was mainly assayed at pH 4.0 by UV spectroscopy of high-performance liquid chromatography at 248 nm, while PYO was assayed at pH 7.0 by measuring OD_{520} (9, 14, 45, 58, 59). We first quantified the relative amounts of PCA and PYO produced by strains M18 and PAO1 and, interestingly, found that the main phenazine accumulated in strain M18 was PCA, especially at 28°C, while it was PYO in strain PAO1, especially at 37°C. PCA is considered to play critical roles in the protection of plants by biocontrol pseudomonad strains in the rhizosphere against infection and phytopathogenic disease (37, 44), while PYO is not essential for fungal killing (16). The PDA plate assay confirmed the different fungal killing abilities of PCA and PYO, and the purified PCA and PYO products need to be tested in fungal inhibition experiments to further validate the results. The differential production of the phenazines PCA and PYO by strains M18 and PAO1 might be a consequence of selective pressures imposed on *P. aeruginosa* PAO1 and its relative strain M18 in different ecological niches over a long evolutionary process.

PCA is believed to be converted to PYO by the sequential actions of the putative *S*-adenosylmethionine-independent *N*-methyltransferase PhzM and the putative flavin-containing monooxygenase PhzS in *P. aeruginosa* PAO1 during in vitro experiments (34, 40). The characteristics of *phzM* expression and regulation were first detailed in this report, although the expression and regulation of the *phzS* gene remain to be characterized. Nucleotide sequence determination indicated that the coding region and the upstream regulatory sequence of *phzM* show 99% similarity between strains M18 and PAO1, with only a nucleotide difference between the two transcriptional start sites of *phzM* in strains M18 and PAO1. The one nucleotide difference between the two transcriptional start sites had no effect on the transcriptional expression of *phzM* in the two strains (data not shown). We found that *phzM* expression is regulated in a temperature-dependent and strain-specific manner by quantitative analysis of *phzM* transcript levels by qRT-PCR and by analyzing the β-Gal activities from *phzM′-′lacZ* translational and transcriptional fusion constructs in these two strains. In contrast to M18, strain PAO1 displayed substantially higher levels of *phzM* expression at 37°C, especially in the late logarithmic phase. These results were consistent with data showing the differential accumulation of different phenazines and suggested that the regulation of *phzM* in *P. aeruginosa* PAO1 and *Pseudomonas* sp. strain M18 evolved to meet the host-driven survival and fitness requirements dictated by different environmental conditions, although further evidence is required to determine the activities of the PhzM protein at 28°C and 37°C. However, the relationship and interaction between the precise sequences in *cis* in the *phzM* gene regulatory region and the concrete factor or factors in *trans* which are involved in the temperature-dependent and strain-specific expression of *phzM* at both the transcriptional and translational levels in the two strains await further investigation. Other temperature-related regulators will be investigated by performing genome-wide transcriptome profiling in our laboratory as described by Chan et al. (3).

Besides the temperature-dependent and strain-specific expression of *phzM*, the results provided in this study suggest that the differential accumulation of PCA in the two strains might result from multiple mechanisms involved in the expression of

the phenazine biosynthetic genes developed during evolution in different niches. We demonstrated that significantly greater amounts of PCA were produced in both wild-type M18 and the *phzM*::Km inactivation mutant M18pM than in both wild-type PAO1 and the *phzM*::Km inactivation mutant PAO1pM, although PYO was completely abolished in both *phzM* inactivation mutant strains M18pM and PAO1pM (data not shown). The amount of PCA produced by both strains M18 and M18pM at 28°C was almost 10-fold higher than that produced by both strains PAO1 and PAO1pM. Meanwhile, PYO was predominant at 37°C only in wild-type strain PAO1. These results suggest that another factor or factors besides *phzM* gene expression may be involved in the differential accumulation of phenazine production by the two strains. Regardless, expression of the *phz* phenazine biosynthetic gene cluster is strongly influenced by environmental conditions, as well as the other global regulators and QS systems (17, 42). Two *phz* biosynthetic clusters were found in both strains M18 and PAO1. Recently, 32 genes were found to be involved in *phzA1 B1C1D1E1F1G1* gene expression, which functions mainly for PYO synthesis in PAO1 (8, 31), but the *phzA2B2C2D2E2F2G2* cluster functions mainly for PCA synthesis in *Pseudomonas* sp. strain M18 (unpublished data). The environmental conditions and other regulators involved in *phz* gene cluster expression and the different amounts of PCA accumulation should also be further investigated.

The functions of the global regulatory genes *gacA*, *rsmA*, *rhlI*, *lasI*, *ptsP*, and *qscR* in *phzM* gene expression were also investigated. Only *lasI* and *rhlI* showed strong positive effects, while *ptsP* and *qscR* showed a negative effect on *phzM* expression in both strains M18 and PAO1 (Table 3 and Fig. 5). Interestingly, we found that the expression of *lasI* and *ptsP* also occurs in a temperature-dependent and strain-specific manner, as shown in Table 4 and Fig. 4D and E. These results indicated that the two strains from different niches had developed their delicate regulatory systems to adapt to their environments. This is the first report of the temperature-dependent and strain-specific expression of *lasI* and *ptsP* and their regulatory effects on *phzM*. From previous reports (4, 41, 56), LasI produces the diffusible signal molecule 3-oxo-C_{12}-HSL, which binds to the transcriptional regulator protein LasR to form a complex, which can then activate the transcription of the *lux* operon by binding to the *lux* box in the promoter region. A conserved dyad symmetry DNA sequence, termed the putative *lux* box, is present upstream of *phzM* (Fig. 3), suggesting that the *las* system directly activates *phzM* by recognizing the putative *lux* box. In previous studies (36, 58), inactivation of *ptsP* resulted in PYO overproduction by *P. aeruginosa* PAO1, leading to a significant decrease in the expression of *qscR* and twofold and threefold increases in the expression of *lasI* and *rhlI*, respectively, and suggested that PtsP inhibited PYO production by activating *qscR* and repressing the *las* and *rhl* system in *P. aeruginosa*. Furthermore, PtsP may regulate *phzM* indirectly by other regulators. As far as we know, the exact regulation mechanism of PtsP has not yet been clarified completely and may involve the utilization of carbon and nitrogen, rather than a promoter-specific regulatory factor (36, 53, 58). In this report, we provide evidence that the conversion of PCA to PYO by PhzM activity, especially at 37°C, is activated by LasI and inhibited by PtsP, although the precise regulatory mechanism remains to be further investigated.

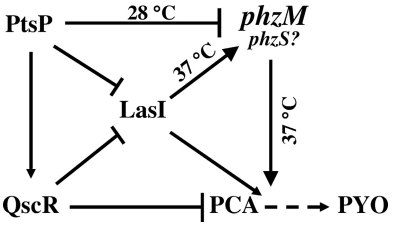

FIG. 6. Network of the regulation on *phzM* expression and phenazine PCA and PYO production by pseudomonads. Arrows indicate positive regulation, lines with flat ends indicate negative regulation, and the broken arrow indicates a putative pathway.

In conclusion, we demonstrated several characteristics of *phzM* gene expression and regulation. It was revealed that *phzM* gene expression occurs in a temperature-dependent and strain-specific manner and the *phzM* gene was positively regulated by the *las* system and negatively regulated by *ptsP*, while both regulators were also expressed in a temperature-dependent manner. The network related to *phzM* gene expression and its regulators involved in the conversion of PCA into PYO are summarized in Fig. 6. The results of this study provide preliminary information for future studies to more fully understand *phzM* expression and global regulatory networks involved in the virulence factors of and antibiotic production by strain *P. aeruginosa* and its relative *Pseudomonas* sp. strain M18 (47). Although strain M18 has a genetic background similar to that of strain PAO1, it has distinct unusual phenotypic and biochemical features to meet the requirements of its ecological fitness and competitiveness in its living niche. We cannot make the final determination of whether strain M18 belongs to a new subspecies of *P. aeruginosa* or a new pseudomonad species at present. The final decision about the classification of strain M18 is dependent on the pending results of whole-genome nucleotide sequencing, which is currently being carried out in our laboratory. Only when that has been completed can we determine the differences between strains M18 and PAO1, as well as other pseudomonad strains, on the whole-genome level and determine the taxonomic position of strain M18 based on phylogenetic analysis.

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