The resistance-nodulation-division efflux pump EmhABC influences the production of 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* 2P24

Tao Tian,¹ Xiao-Gang Wu,¹ Hui-Mei Duan¹ and Li-Qun Zhang^{1,2}

Correspondence
Li-Qun Zhang
zhanglq@cau.edu.cn

¹Department of Plant Pathology, China Agricultural University, Beijing, 100193, PR China

²The Key Laboratory of Plant Pathology, Ministry of Agriculture, Beijing, 100193, PR China

The polyketide metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) plays a major role in the biological control of soil-borne plant diseases by *Pseudomonas fluorescens* 2P24. Two mutants (PM810 and PM820) with increased extracellular accumulation of 2,4-DAPG were isolated using transposon mutagenesis. The disrupted genes in these two mutants shared >80 % identity with the genes of the EmhR–EmhABC resistance-nodulation-division (RND) efflux system of *P. fluorescens* cLP6a. The deletion of *emhA* (PM802), *emhB* (PM803) or *emhC* (PM804) genes in strain 2P24 increased the extracellular accumulation of 2,4-DAPG, whereas the deletion of the *emhR* (PM801) gene decreased the biosynthesis of 2,4-DAPG. The promoter assay confirmed the elevated transcription of *emhABC* in the EmhR disrupted strain (PM801) and an indirect negative regulation of 2,4-DAPG biosynthetic locus transcription by the EmhABC efflux pump. Induction by exogenous 2,4-DAPG led to remarkable differences in transcription of chromosome-borne *phlA::lacZ* fusion in PM901 and PM811 (*emhA*[−]) strains. Additionally, the EmhABC system in strain 2P24 was involved in the resistance to a group of toxic compounds, including ampicillin, chloramphenicol, tetracycline, ethidium bromide and crystal violet. In conclusion, our results suggest that the EmhABC system is an important element that influences the production of antibiotic 2,4-DAPG and enhances resistance to toxic compounds in *P. fluorescens* 2P24.

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INTRODUCTION

Fluorescent pseudomonads are well-known Gram-negative bacteria with prominent adaptability, nutritional versatility and multitudinous production of secondary metabolites (Goldberg *et al.*, 2008). Plant-associated pseudomonads produce many secondary metabolites, such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide, phenazine, pyoluteorin and pyrrolnitrin, that are also able to suppress micro-organisms (Dwivedi & Johri, 2003). Among them, 2,4-DAPG is a well-characterized compound due to its broad spectrum of activity and *in situ* inhibitory action on phytopathogens (Bangera & Thomashow, 1999). It functions as a major ingredient in biocontrol agents, such as *Pseudomonas fluorescens* strains Q2-87, F113,

CHA0 and 2P24, used to protect crops against a number of plant diseases such as wheat take-all caused by *Gaeumannomyces graminis*, sugar beet seedlings damping-off caused by *Pythium ultimum*, tobacco black root rot caused by *Thielaviopsis basicola*, cotton rhizoctoniosis caused by *Rhizoctonia solani* and tomato bacterial wilt caused by *Ralstonia solanacearum* (Bangera & Thomashow, 1999; Fenton *et al.*, 1992; Schnider-Keel *et al.*, 2000; Zhou *et al.*, 2005; Wei & Zhang, 2006).

The genetic locus *phlACBD* is responsible for the biosynthesis of 2,4-DAPG in a number of pseudomonad strains (Bangera & Thomashow, 1999; Fenton *et al.*, 1992; Vincent *et al.*, 1991). Recent reports revealed that besides environmental factors and its autoregulation (Abbas *et al.*, 2002; Duffy & Defago, 1999; Schnider-Keel *et al.*, 2000), the production of 2,4-DAPG is regulated by a series of transcriptional and translational factors, including the repressor PhlF (Delany *et al.*, 2000; Haas *et al.*, 2000), the two-component regulatory system GacS/GacA (Haas & Defago, 2005; Laville *et al.*, 1992), regulatory PrrB (Aarons *et al.*, 2000) and RsmY (Valverde *et al.*, 2003) RNA, global regulator proteins MvaT and MvaV (Baehler *et al.*, 2006) and disulfide interchange protein DsbA (Mavrodi *et al.*, 2006).

Abbreviations: 2,4-DAPG, 2,4-diacetylphloroglucinol; EB, ethidium bromide; KB, King's B; LB, Luria–Bertani broth; RND, resistance nodulation division; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession number for the DNA sequence containing the *emhR–emhABC* genes of *P. fluorescens* 2P24 is FJ807391.

Four supplementary figures and a supplementary table are available with the online version of this paper.

In addition to production and secretion of numerous secondary metabolites, bacteria in their natural environments also encounter various harmful compounds, including antibiotics, dyes, detergents, free fatty acids, aromatic compounds and heavy metal cations (Aendekerk *et al.*, 2002; Hearn *et al.*, 2003; Nikaido, 1996). Bacteria respond to these agents via multiple defence systems, such as active efflux pumps, a permeability barrier, modifying the target structure and producing enzymes capable of inactivating antibiotics (Poole, 2004). A bacterial resistance-nodulation-division (RND) efflux system is typically composed of a membrane fusion protein, an inner-membrane protein and an outer-membrane protein. The RND system contributes to the resistance of the bacteria to a wide array of antibiotics and toxic molecules by exporting compounds to the environment (Poole, 2004). Additionally, it

affects quorum sensing (Chan & Chua, 2005; Pearson *et al.*, 1999; Yang *et al.*, 2006), the stationary-phase-specific σ factor (Yang *et al.*, 2006) and ExsA, the master regulator of the type III secretion system (T3SS) (Linares *et al.*, 2005).

In this paper, we have described the identification of the EmhR–EmhABC efflux pump in *P. fluorescens* 2P24 and provided genetic evidence that the EmhABC transporter influences resistance to toxic compounds and indirectly affects 2,4-DAPG production.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1. Strains and plasmids used in this study

Ap^r, Gm^r, Km^r, Sm^r and Tc^r indicate resistance to ampicillin, gentamicin, kanamycin, streptomycin and tetracycline, respectively.

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	F [−] <i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i> Δ (<i>lacZYA-argF</i>) <i>U169</i> λ [−] (ϕ 80 <i>dlacZ</i> Δ M15)	This lab
<i>P. fluorescens</i>		
2P24	Wild-type, Ap ^r	Wei & Zhang (2005)
PM801	<i>emhR</i> in-frame deletion in strain 2P24, Ap ^r	This study
PM802	<i>emhA</i> in-frame deletion in strain 2P24	This study
PM803	<i>emhB</i> in-frame deletion in strain 2P24	This study
PM804	<i>emhC</i> in-frame deletion in strain 2P24	This study
PM810	<i>emhA</i> ::Tn5 in strain 2P24	This study
PM811	<i>emhA</i> in-frame deletion in mutant PM901	This study
PM820	<i>emhC</i> ::Tn5 in strain 2P24	This study
PM901	Chromosomal <i>phlA</i> :: <i>lacZ</i> reporter fusion in strain 2P24	This study
Plasmids		
pBluescript II SK(+))	Cloning vector, Ap ^r	Stratagene
pHSG299	ColE1 origin, cloning vector, Km ^r	TaKaRa
pRK415	<i>Escherichia</i> – <i>Pseudomonas</i> shuttle vector, Tc ^r	Keen <i>et al.</i> (1988)
pLAF-5- <i>emhABC</i>	Cosmid clone containing entire EmhABC gene, Tc ^r	This study
pBS- <i>emhABC</i>	A derivative plasmid of pBluescript II SK(+) containing a 6.7 kb <i>EcoRI</i> fragment, Ap ^r	This study
pUTKm	Delivery plasmid for Tn5; R6K replicon, Ap ^r Km ^r	Herrero <i>et al.</i> (1990)
pRG970	Cloning vector containing promoterless <i>lacZYA</i> for construction of transcriptional fusions, Sm ^r	Van den Eede <i>et al.</i> (1992)
pRG970Km	A derivative plasmid of pRG970, Km ^r	Yan <i>et al.</i> (2009)
p970K- <i>phlA</i>	pRG970Km containing <i>phlA</i> :: <i>lacZ</i> transcriptional fusion, Km ^r	This study
p970K- <i>emhA</i>	pRG970Km containing <i>emhA</i> :: <i>lacZ</i> transcriptional fusion, Km ^r	This study
p299 Δ <i>emhR</i>	Suicide plasmid containing deleted <i>emhR</i> on pHSG299, Km ^r	This study
p299 Δ <i>emhA</i>	Suicide plasmid containing deleted <i>emhA</i> on pHSG299, Km ^r	This study
p299 Δ <i>emhB</i>	Suicide plasmid containing deleted <i>emhB</i> on pHSG299, Km ^r	This study
p299 Δ <i>emhC</i>	Suicide plasmid containing deleted <i>emhC</i> on pHSG299, Km ^r	This study
p299- <i>phlAFR</i>	Suicide plasmid containing deleted <i>phlA</i> on pHSG299, Km ^r	This study
p299- <i>phlAFRZ</i>	A promoterless <i>lacZ</i> inserted into p299- <i>phlAFR</i> with <i>Bam</i> HI site, Km ^r	This study
p415- <i>emhR</i>	<i>emhR</i> was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study
p415- <i>emhA</i>	<i>emhA</i> was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study
p415- <i>emhB</i>	<i>emhB</i> was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study
p415- <i>emhC</i>	<i>emhC</i> was cloned downstream of the <i>lac</i> promoter in pRK415, Tc ^r	This study

P. fluorescens strains were grown in Luria–Bertani (LB) broth, King's B (KB; King *et al.*, 1954) or ABM (Chilton *et al.*, 1974) medium at 28 °C. *Escherichia coli* DH5 α , which was used for cloning experiments, was grown in LB broth at 37 °C. When required, the growth medium was supplemented with X-Gal (40 $\mu\text{g ml}^{-1}$), ampicillin (50 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), tetracycline (20 $\mu\text{g ml}^{-1}$) or chloramphenicol (20 $\mu\text{g ml}^{-1}$).

DNA manipulations and sequencing. Plasmid DNA extraction and other molecular assays were performed according to standard procedures (Sambrook & Russell, 2001) or as recommended by the manufacturer. Electroporation of bacterial cells with plasmid DNA was performed as described previously (Wei & Zhang, 2005). DNA sequencing was performed by Sun Biotechnology and analysed by using the National Center for Biotechnology Information BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST>).

Transposon mutagenesis and cloning of Tn5 insertion sites. To screen for novel transcriptional regulators of the *phlACBD* operon, the 2P24 strain of *P. fluorescens* was subjected to random Tn5 insertional mutagenesis using plasmid pUT-Km (Herrero *et al.*, 1990). Four out of about 4000 kanamycin-resistant colonies of this 2P24 strain showed increased red pigment production, which is a characteristic phenotype known to be linked with 2,4-DAPG production (Bangera & Thomashow, 1999; Raaijmakers *et al.*, 1997). The genomic DNA fragment flanking the kanamycin resistance gene in transposon Tn5 was cloned into the pBluescript II SK(+) (pBS; Stratagene) vector at the *EcoRI* site and sequenced with primers zhang-O and zhang-I (Supplementary Table S1, available with the online version of this paper) localized at the two ends of the Tn5 transposon.

Cloning of the *emhR*–*emhABC* efflux pump. The cosmid pLAF-5-*emhABC* was selected by PCR using primers PA1538 and PA3070 (Supplementary Table S1), which were designed according to the Tn5 flanking sequence, from a 2P24 strain genomic DNA library (Wei &

Zhang, 2005). A 6.7 kb *EcoRI* fragment from pLAF-5-*emhABC* was subcloned into pBS, giving rise to a new plasmid pBS-*emhABC*. Sequencing analysis revealed intact *emhA*, *emhB* and *emhC* genes and a partial *emhR* gene. The entire *emhR* sequence was obtained by further subcloning and sequencing the cosmid pLAF-5-*emhABC*. When pieced together, a 7787 bp contig comprising five complete ORFs was identified (Fig. 1).

Construction and complementation of *emhR*, *emhA*, *emhB* and *emhC* deletion mutants. The individual deletion mutants of *emhR*, *emhA*, *emhB* and *emhC* genes were constructed using a two-step homologous recombination strategy. The in-frame deletion structures of *emhR*, *emhA*, *emhB* and *emhC* were ligated into the plasmid pHSG299 (TaKaRa) to generate p299 Δ *emhR*, p299 Δ *emhA*, p299 Δ *emhB* and p299 Δ *emhC*, respectively (Supplementary Fig. S1). Allelic exchange using these plasmids with the wild-type strain 2P24 resulted in mutants PM801 (*emhR*[−]), PM802 (*emhA*[−]), PM803 (*emhB*[−]) and PM804 (*emhC*[−]) (Fig. 1), and all deletion mutants were confirmed by PCR amplification (data not shown). The schematic diagrams for the relative location of primers and primer sequences are provided in Supplementary Fig. S1 and Supplementary Table S1, respectively.

Complementary plasmids for the *emhR*, *emhA*, *emhB* and *emhC* deletion mutants were constructed using the shuttle vector pRK415 (Keen *et al.*, 1988) and named p415-*emhR*, p415-*emhA*, p415-*emhB* and p415-*emhC*, respectively (Fig. 1).

Construction of *emhA*::*lacZ* and *phlA*::*lacZ* transcriptional fusion reporters. The upstream and downstream regions of the *phlA* gene were amplified by PCR using primers phl1370/phl3010 and phl3440/phl5830 (Supplementary Table S1), digested and cloned into pHSG299 to produce the plasmid p299-*phlAFR*. A promoterless *lacZ* gene (Yan *et al.*, 2009) was then inserted into p299-*phlAFR* at the *Bam*HI site, resulting in p299-*phlAFRZ*, which was introduced into the *P. fluorescens* 2P24 strain to generate a reporter mutant PM901 (*phlA*::*lacZ*) by a two-step homologous recombination strategy. The

Fig. 1. Organization of the *emhABC* cluster. The single-headed open arrows represent the location and orientation of the genes in the *P. fluorescens* 2P24 chromosome. The locations of the Tn5 insertions in strains PM810 and PM820 are indicated by black arrows. The main restriction enzyme sites are shown. The construction of deletion mutants PM801, PM802, PM803 and PM804 and of complementation plasmids p415-*emhR*, p415-*emhA*, p415-*emhB* and p415-*emhC* are described in Methods. The solid bars correspond to the deleted fragments and the complementation segments are indicated as the solid diamond-ended bars.

mutant PM811 (*emhA*⁻, *phlA*::*lacZ*) was constructed from PM901 using the p299Δ*emhA* plasmid.

To construct plasmid-borne reporters, the promoter regions of *emhA* and *phlA* were amplified by PCR using primers PA1541/ PA2150 and phl2267/phl3010 (Supplementary Table S1) and were cloned ahead of a promoterless *lacZ* in pRG970Km (Yan *et al.*, 2009) derived from pRG970 (Van den Eede *et al.*, 1992). The resulting plasmids p970K-*emhA* and p970K-*phlA* were used for *emhA* and *phlA* promoter analysis, respectively.

MIC determination. MIC analysis was carried out in 96-well microtitre plates by the standard broth microdilution method (Schwalbe *et al.*, 2007). LB broth was inoculated with an overnight culture diluted at 1/1000 and incubated for 16 h at 28 °C with shaking. The culture was adjusted with LB to a 0.5 McFarland nephelometer standard (1×10^8 cells ml⁻¹) and then diluted 100-fold with LB (final inoculum density of 1×10^6 cells ml⁻¹). Bacterial growth was determined 20 h after incubation at 28 °C.

Extraction and detection of 2,4-DAPG. *P. fluorescens* 2P24 and its derivatives were cultivated with shaking in 100 ml flasks containing 30 ml liquid KB medium at 140 r.p.m. at 28 °C; 2,4-DAPG production was quantified by HPLC using the method described by Shanahan *et al.* (1992). The extracellular 2,4-DAPG was extracted from the centrifuged supernatant, and the cell-associated 2,4-DAPG was extracted from broken cells prepared by a freeze-thaw procedure.

β-Galactosidase activity assay. To measure β-galactosidase activity, *P. fluorescens* strains were grown with shaking in 100 ml flasks containing 30 ml liquid medium at 140 r.p.m. at 28 °C. Cultures were sampled at different time points and β-galactosidase-specific activities were quantified by using the Miller method (Miller, 1972).

In the assay for the induction of *phlA*::*lacZ* expression, 2,4-DAPG was added when the growth of strains PM901 (*phlA*::*lacZ*) and PM811 (*emhA*⁻, *phlA*::*lacZ*) reached an OD₆₀₀ of 0.8. The β-galactosidase activity was assayed 1 h after induction.

RESULTS

Identification of the RND efflux pump EmhABC in *P. fluorescens* 2P24

The production of the antibiotic 2,4-DAPG was linked with the appearance of an uncharacterized red pigment in a number of *P. fluorescens* biocontrol agents (Bangera & Thomashow, 1999; Raaijmakers *et al.*, 1997), including strain 2P24 (Supplementary Fig. S2). To identify the putative regulators mediating the production of 2,4-DAPG in strain 2P24, a random mini-Tn5 mutagenesis was performed using the red pigment as the selective marker. Approximately 4000 transposon insertion mutants were generated and four mutants displayed obviously increased expression of the red pigment (Supplementary Fig. S2) in liquid culture compared with the wild-type strain 2P24. In addition to the increased extracellular yield of 2,4-DAPG (Fig. 2), two mutant strains PM810 and PM820 lost their ampicillin resistance (data not shown). Sequence analysis revealed that the transposon-flanking DNA regions in strains PM810 and PM820 are 84 and 83 % identical with the *emhA* and *emhC* genes of *P. fluorescens* cLP6a,

Fig. 2. The analysis of extracellular 2,4-DAPG production by *P. fluorescens* 2P24 and its Tn5 mutants. Extractions were made from at least three independent cultures in 30 ml KB medium; 2,4-DAPG production was determined by HPLC. Error bars indicate SD.

respectively (Hearn *et al.*, 2003), and are very similar to the *emhA*-like and *emhC*-like genes in *P. fluorescens* Pf0-1 (GenBank accession no. CP000094, 87 and 85 % identity) and *P. fluorescens* Pf-5 (GenBank accession no. CP000076, 84 and 83 % identity). In strain cLP6a, *emhA* and *emhC* genes encode the components of the RND efflux pump which actively exports certain antibiotics and polycyclic aromatic hydrocarbons (Hearn *et al.*, 2003).

A cosmid pLAF-5-*emhABC* was selected by PCR from a 2P24 strain genomic DNA library, and a 7787 bp contig comprising five complete ORFs was obtained by subcloning (Fig. 1). The ORF1 encodes a hypothetical protein with no significant similarity to other proteins listed in GenBank. The nucleotide sequences of *emhA*, *emhB* and *emhC* are homologous to those of the membrane fusion protein, the inner-membrane pump and the outer-membrane channel of the efflux system in *P. fluorescens* cLP6a, with 84, 86 and 83 % identity, respectively. *EmhB* was also predicted to contain a 12-transmembrane helical domain structure with two large periplasmic loops between transmembrane segments 1–2 and 7–8, a characteristic structure of the inner-membrane component of the RND family (Tusnady & Simon, 2001; Tseng *et al.*, 1999). Another ORF is 80 % identical to the *emhR* gene of strain cLP6a and encodes a 210 aa protein of the TetR family. In addition, sequence analysis suggested that the *emhA* gene is co-transcribed with *emhB* and *emhC* genes in *P. fluorescens* 2P24.

EmhR acts as a repressor of the EmhABC RND efflux pump

Sequence analysis implied that *EmhR* may serve as a repressor of expression of the *emhABC* genes in strain

2P24. To test this possibility, the expression of *emhABC* in PM801, an *emhR* in-frame deletion mutant, was measured in ABM medium using an *emhA::lacZ* transcriptional fusion reporter (p970K-*emhA*). The transcription of *emhA* was significantly elevated (threefold) from the early exponential phase (14 h) in the *emhR* deletion strain PM801 compared with that in the wild-type 2P24 strain. A much more significant difference (sixfold) was observed when the bacteria grew into late exponential and stationary phases (Fig. 3). The overexpression of the *emhABC* operon in the *EmhR* deletion mutant suggested that *EmhR* acts as a transcriptional repressor of the *emhABC* genes.

Substrate specificities of the *P. fluorescens* 2P24 EmhABC efflux pump

The susceptibilities of the 2P24 strain and its derivatives PM801 (*emhR*[−]), PM802 (*emhA*[−]), PM803 (*emhB*[−]) and PM804 (*emhC*[−]) to an array of antimicrobial agents were assayed as described in Methods (Table 2 summarizes the MICs). The wild-type strain 2P24 is highly resistant to a battery of compounds, including ampicillin, chloramphenicol, erythromycin, rifampicin, SDS and ethidium bromide (EB); moderately resistant to streptomycin and crystal violet; and susceptible to gentamicin, tetracycline, polymyxin B and kanamycin. In the *emhABC*-overexpressed mutant PM801, increased resistance to ampicillin, chloramphenicol, tetracycline and crystal violet was observed. The deletion of the pump component *emhA* or *emhB* resulted in a similar level of increased susceptibility to ampicillin, chloramphenicol, tetracycline, EB and crystal violet. The deletion of *emhC* increased susceptibility to ampicillin, chloramphenicol, tetracycline, EB and crystal violet compared with that achieved by deletion of *emhA*

and *emhB*. The deletion of *emhA* or *emhB* had no effect on the susceptibility to kanamycin, gentamicin, streptomycin and erythromycin, while *emhC* deletion made the 2P24 strain more susceptible to these agents. The susceptibility of PM802, PM803 and PM804 to the tested agents was fully restored to that of the wild-type by the corresponding plasmid-borne wild-type allele complementation. The antibiotic susceptibility of PM801 complementary strain was restored to higher than wild-type levels. Our data suggested that *EmhABC* is an efflux pump responsible for the resistance of the strain to a number of toxic agents.

The presence of a functional EmhABC pump decreases the production of 2,4-DAPG in the 2P24 strain

The extracellular accumulation of the antibiotic 2,4-DAPG in the *EmhABC* pump mutants of the 2P24 strain was assayed by HPLC (Fig. 4a). The extracellular accumulation of 2,4-DAPG increased dramatically (13-fold) in the PM802 (*emhA*[−]), PM803 (*emhB*[−]) and PM804 (*emhC*[−]) mutants, but reduced significantly (twofold) in the PM801 mutant (*emhR*[−]) compared with that in the wild-type strain 2P24. These changes in the mutant strains PM802, PM803 and PM804 could be restored by wild-type allele complementation. However, complementation of the PM801 strain by the introduction of the plasmid p415-*emhR* resulted in the overproduction of 2,4-DAPG, possibly due to the strong inhibition of *emhABC* expression by multi-copy *emhR* genes (Fig. 4a).

To further investigate whether the transcription of the 2,4-DAPG biosynthetic locus *phlACBD* was altered in mutant strains, plasmid p970K-*phlA* harbouring a *phlA::lacZ* transcriptional fusion was introduced into the mutants PM801, PM802, PM803, PM804 and their parental strain 2P24. The expression of *phlA::lacZ* decreased to half the level in the *emhR* mutant PM801, but increased about sevenfold in *emhABC* mutants PM802, PM803 and PM804, compared with their parental strain 2P24 in KB broth (Fig. 4b). These findings suggested that the presence or absence of a functional *EmhABC* pump results in alteration in the biosynthesis of 2,4-DAPG in our strain.

The influence of EmhABC on the transcription of *phlACBD* is 2,4-DAPG-dependent

The expression of the *phlACBD* operon in *P. fluorescens* strains CHA0 and F113 is autoinduced by its biosynthetic product 2,4-DAPG (Abbas *et al.*, 2002; Schnider-Keel *et al.*, 2000). To investigate whether a similar regulation mechanism exists in strain 2P24, a reporter strain PM901 that does not produce 2,4-DAPG was constructed by introducing a chromosomally integrated *phlA::lacZ* fusion. The *phlACBD* operon was constitutively expressed at a low level in PM901 in LB medium. Exogenous 2,4-DAPG did not influence the transcription of *phlACBD* obviously at low concentrations ($\leq 1 \mu\text{M}$), but drastically enhanced the

Fig. 3. Regulation of *EmhR* on the transcription of *emhA::lacZ* fusion in *P. fluorescens* 2P24 (circles) and its *emhR* mutant PM801 (triangles). β -Galactosidase activity (filled symbols) and the growth rate (open symbols) of each strain were measured at various time points after inoculation into 30 ml ABM medium. All experiments were performed in triplicate; error bars indicate SD.

Table 2. Susceptibilities of *P. fluorescens* 2P24 and its constructed mutants to various antimicrobial agents

ND, Not determined due to tetracycline resistance gene encoded on vector pRK415.

Antimicrobial agent	MIC (µg ml ⁻¹)								
	2P24	PM801	PM801/p415emhR	PM802	PM802/p415emhA	PM803	PM803/p415emhB	PM804	PM804/p415emhC
Gentamicin	4	4	4	4	4	4	4	0.25	4
Streptomycin	16	16	16	16	16	16	16	2	16
Kanamycin	1	1	1	1	1	1	1	0.25	1
Ampicillin	256	>512	32	16	256	16	256	2	256
Erythromycin	256	256	256	256	256	256	256	8	256
Polymyxin B	1	1	1	1	1	1	1	1	1
Tetracycline	2	4	ND	1	ND	<0.25	ND	<0.25	ND
Chloramphenicol	128	256	64	32	128	32	128	4	128
Rifampicin	>512	>512	>512	>512	>512	>512	>512	>512	>512
Ethidium bromide	>512	>512	>512	256	>512	256	>512	128	>512
Crystal violet	16	32	16	8	16	8	16	4	16
SDS	>512	>512	>512	>512	>512	>512	>512	>512	>512

phlACBD expression at high concentrations ($\geq 10 \mu\text{M}$) (Fig. 5a), suggesting that the transcription of the *phlACBD* operon was induced by exogenous 2,4-DAPG in strain 2P24. Taking into consideration the autoinduction of the *phlACBD* operon by 2,4-DAPG and the efflux pump function of EmhABC, it was inferred that the negative regulation of EmhABC on the transcription of the *phlACBD* operon was possibly mediated via the efflux of 2,4-DAPG. To verify this assumption, the effect of 2,4-DAPG on the expression of *phlA::lacZ* was analysed in the PM901 strain and its *emhA*-negative derivative PM811. No transcriptional differences were observed between these two strains without the addition of exogenous 2,4-DAPG. However, upon addition of $1 \mu\text{M}$ 2,4-DAPG, there was a significant enhancement of *phlA* transcription in the PM811 strain, but not in the PM901 strain. A much stronger induction of *phlA* expression was observed in the PM811 strain when the concentration of exogenous 2,4-DAPG was $10 \mu\text{M}$ (Fig. 5b).

We also detected the cell-associated 2,4-DAPG production in the 2P24 strain and its EmhABC pump mutants. From both exponential and stationary phase cultures, the intracellular production of 2,4-DAPG was significantly higher in the PM802 (*emhA*⁻), PM803 (*emhB*⁻) and PM804 (*emhC*⁻) mutants, but lower in the PM801 mutant (*emhR*⁻) compared with that in the wild-type strain 2P24 (Fig. 6a, b), indicating that the disruption of the EmhABC pump led to an elevation of intracellular 2,4-DAPG accumulation, which subsequently enhanced the transcriptional feedback of the *phlACBD* operon.

These results collectively indicated that the influence of EmhABC on the transcription of *phlACBD* operon is 2,4-DAPG-dependent, and the EmhABC RND efflux system may play an important role in the maintenance of a normal intracellular concentration of 2,4-DAPG in *P. fluorescens* 2P24.

DISCUSSION

The *emhR-emhABC* locus of *P. fluorescens* 2P24 encodes a TetR repressor (EmhR) and a typical three-component RND efflux system comprising a membrane fusion protein (EmhA), a proton transporter (EmhB) and an outer-membrane protein (EmhC). The RND efflux pump is known for its broad substrate spectrum. EmhB in *P. fluorescens* 2P24 is closely related to that of *P. fluorescens* cLP6a, which actively effluxes polycyclic aromatic hydrocarbons, antibiotics and hydrophobic dyes (Hearn *et al.*, 2003, 2006); the TtgB efflux pump, which transports antibiotics and solvents in *Pseudomonas putida* DOT-T1E (Ramos *et al.*, 1998); the ArpB antibiotic efflux pump in *P. putida* S12 (Kieboom & de Bont, 2001); and the MexB in *Pseudomonas aeruginosa*, which is involved in the efflux of an array of antibiotics and tolerance to the toxic organic solvents hexane and xylene (Li *et al.*, 1995). We therefore speculated that EmhABC might be involved in the efflux of

Fig. 4. The EmhABC pump negatively regulated the production of 2,4-DAPG at a transcriptional level. (a) Analysis of extracellular 2,4-DAPG accumulation by *P. fluorescens* 2P24 and its derivatives. Extractions were made from at least three independent cultures in 30 ml KB medium, and the 2,4-DAPG production level was determined by HPLC. The numbers 1–9 represent strains 2P24-pRK415, PM801-pRK415, PM801-p415emhR, PM802-pRK415, PM802-p415emhA, PM803-pRK415, PM803-p415emhB, PM804-pRK415 and PM804-p415emhC, respectively. (b) Expression of the plasmid transcriptional *phlA::lacZ* fusion in *P. fluorescens* 2P24 (squares) and its derivatives (PM801/p970K-*phlA*, circles; PM802/p970K-*phlA*, triangles; PM803/p970K-*phlA*, diamonds; PM804/p970K-*phlA*, hexagons). β-Galactosidase activity (filled symbols) and the growth rate (open symbols) of each strain were measured at various time points after inoculation into 30 ml KB medium. Error bars indicate SD.

antibiotics and other toxic agents in *P. fluorescens* 2P24. This hypothesis was supported by the fact that the strain with overexpressed EmhABC (*emhR*⁺) displayed elevated resistance and that the susceptibility to some compounds was higher for the EmhABC mutants than the wild-type strain 2P24. However, the *emhC* deletion mutant PM804 was significantly more susceptible to a wider range of agents than the *emhA* and *emhB* knockouts (Table 2). These genetic findings demonstrated that all three components of EmhABC were required for antibiotic efflux and resistance in strain 2P24, but individual components might contribute to the antibiotic resistance

Fig. 5. Induced transcription of the chromosomal *phlA::lacZ* fusion by exogenous 2,4-DAPG in strains PM901 (filled bars) (a) and PM811 (open bars) (compared with PM901) (b). 2,4-DAPG was added when the growth of strains PM901 (*phlA::lacZ*) and PM811 (*emhA*[−], *phlA::lacZ*) reached an OD₆₀₀ of 0.8. The β-galactosidase activity was assayed 1 h after the addition of 2,4-DAPG. All experiments were performed in triplicate; error bars indicate SD.

at different levels. In bacteria, a particular outer-membrane protein may operate in several export systems with overlapping substrate specificity. For example, the outer-membrane protein TolC in *E. coli* associates with both RND counterparts AcrAB and AcrEF, and the outer-membrane protein OprM is shared by MexAB and MexXY RND transporters in *P. aeruginosa* (Poole, 2001). TolC even supports the function of MexCD and MexXY, the efflux transporters in *P. aeruginosa*. Moreover, MexCD possesses its specific outer-membrane channel OprJ and is still functional when OprJ is replaced by OprM (Zgurskaya *et al.*, 2003). The promoter analysis revealed that *emhABC* genes are expressed at a similar level in mutants PM802, PM803 and PM804, indicating that the different antibiotic susceptibility of the *emhC* mutant (PM804) was not due to its distinct influence on the *emhABC* transcription (Supplementary Fig. S3). A speculative explanation is that

Fig. 6. Effect of EmhABC on intracellular accumulation of 2,4-DAPG in *P. fluorescens* 2P24 and its derivatives. Bacterial cells were sampled 12 h (a) and 24 h (b) after inoculation. Cell-associated 2,4-DAPG was extracted from freeze–thaw cell lysates and quantified by HPLC. The numbers 1–5 represent strains 2P24, PM801, PM802, PM803 and PM804, respectively. The results were determined from at least three independent cultures in 30 ml KB medium. Error bars indicate SD.

EmhC has a broader role. Perhaps EmhC in the 2P24 strain operates with some unknown RND efflux pumps that contribute to drug resistance and share overlapping substrate specificity with the EmhABC transporter. However, additional experimentation is required to prove this hypothesis.

The multidrug efflux pump is known not only for its broad substrate spectrum against antibiotics and other toxic substances but also for its regulatory function in gene expression (Poole, 2008). In *E. coli*, the deletion of the AcrAB RND transporter delayed expression of *rpoS*, a gene encoding the stationary-phase-specific σ factor; and the overexpression of *acrAB* enhanced *rpoS* expression (Rahmati *et al.*, 2002). Overproduction of either MexCD-OprJ or MexEF-OprN in the *P. aeruginosa* strain PAO1 V reduced the expression of *exsA*, a master regulator of the T3SS, and therefore, decreased the transcription of the

T3SS regulon (Linares *et al.*, 2005). Nonetheless, the underlying regulatory pathways remain elusive. Our data showed that the RND efflux system EmhABC in *P. fluorescens* 2P24 negatively regulated the yield of 2,4-DAPG through, at least partially, the negative regulation of the transcription of *phlACBD* in a 2,4-DAPG-dependent manner, since EmhABC was unable to mediate the transcription of *phlACBD* when the 2,4-DAPG production was abolished. We also confirmed that 2,4-DAPG positively autoregulated the transcription of the *phlACBD* locus in the 2P24 strain (Fig. 5a), as was the case in the strains CHA0 and F113 (Abbas *et al.*, 2002; Schnider-Keel *et al.*, 2000). Thus, it is plausible that EmhABC indirectly regulates the transcriptional feedback of *phlACBD* by altering the intracellular concentration of 2,4-DAPG. Hence, we propose that EmhABC functions as an efflux pump for 2,4-DAPG. The abolishment of EmhABC might result in a rapid accumulation of 2,4-DAPG in the cells which, in turn, triggers or accelerates the transcription of the *phlACBD* locus. This model is supported by the induction assay in 2,4-DAPG non-producing mutants PM901 and PM811 by exogenous 2,4-DAPG at different concentrations (Fig. 5b), and by the quantification of cell-associated 2,4-DAPG in wild-type 2P24 and its EmhABC mutants (Fig. 6a, b). However, our model did not rule out other potential factors involved in the transport of 2,4-DAPG. For example, the putative permease PhIE has been suggested to function in 2,4-DAPG export (Bangera & Thomashow, 1999). In *P. fluorescens* F113, a time-course experiment was performed to monitor the ratio of cell-associated:free 2,4-DAPG, and it was proposed that PhIE could not be the only factor determining the distribution of 2,4-DAPG between the cells and the medium (Abbas *et al.*, 2004). In strain 2P24, the ratios of cell-associated:extracellular 2,4-DAPG in mid-exponential phase were much higher in pump component deletion strains than that in strains 2P24 and PM801 (Supplementary Fig. S4a), suggesting that EmhABC serves as an important factor in determining the distribution of 2,4-DAPG between the cells and the medium. However, in the stationary phase, the ratios of cell-associated:free 2,4-DAPG in EmhABC mutants were lower than those for strain 2P24 and PM801 (Supplementary Fig. S4b). Since the level of cell-associated 2,4-DAPG in EmhABC mutants was similar in the exponential and stationary phases (Fig. 6a, b), and the 2,4-DAPG ratios in all the tested strains were significantly lower in the stationary phase than those in the exponential phase (Supplementary Fig. S4a, b), we reasoned that the lower 2,4-DAPG ratios in EmhABC mutants in the stationary phase were due to the dramatically increased extracellular accumulation of 2,4-DAPG. This is consistent with our previous assay that the mutation on the EmhABC pump results in an increase in the extracellular accumulation of 2,4-DAPG (Fig. 4a).

Overall, these data suggest that under our experimental conditions, EmhABC is not the only mechanism of 2,4-DAPG transportation, but it is an important factor in

adjusting the intracellular concentration of 2,4-DAPG. Further investigations are needed in *P. fluorescens* 2P24 to elucidate the function of PhIE and other potential regulatory elements in 2,4-DAPG transportation.

In summary, we identified EmhABC as a new regulator of 2,4-DAPG production in *P. fluorescens* 2P24. Genetic analysis indicated the possibility that EmhABC could play an important role in the secretion of 2,4-DAPG. Future work will concentrate on exploring direct evidence that 2,4-DAPG is one of the substrates of the EmhABC efflux pump.

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