PhlG mediates the conversion of DAPG to MAPG in *Pseudomonas fluorescens* 2P24

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The antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), produced by the Gram-negative rod-shaped bacterium *Pseudomonas fluorescens* 2P24, is active against various soil-borne bacterial and fungal pathogens that cause plant diseases. Biosynthesis of 2,4-DAPG is controlled by regulating expression of the *phlACBD* operon at the post-transcriptional level. The *phlG* gene is located between the *phlF* and *phlH* genes, upstream of the *phlACBD* biosynthetic operon. Herein, we cloned the *phlG* gene, generated a *phlG* deletion mutant, and investigated its regulatory role in 2,4-DAPG biosynthesis. The results showed that deletion of *phlG* had no effect on the biosynthesis of 2,4-DAPG, but it affected conversion of 2,4-DAPG to its precursor monoacetylphloroglucinol (MAPG). The global regulatory factor encoded by gacS positively regulated expression of *phlG*, while rsmE negatively regulated its expression. Deleting *phlG* did not alter the ability of the bacterium to colonise plants or promote plant growth. These results suggest that *phlG* collaborates with other factors to regulate production of the antibiotic 2,4-DAPG in *P. fluorescens* 2P24.

The antibiotic 2,4-diacetylphloroglucinol (DAPG) is produced by several *Pseudomonas* sp., including 2P24, CHA0, Pf-5, and YGJ3, and it plays a key role in inhibiting the growth of pathogenic microorganisms surrounding the plant rhizosphere1–4. As a phenolic secondary metabolite, 2,4-DAPG from some bacteria above has shown the capacity to control various plant pathogens. For example, *P. fluorescens* CHA0 protects plants against tobacco black root rot, *P. fluorescens* F113 protects sugar beet against *Pythium* damping-off, and *P. fluorescens* 2P24 protects against tomato bacterial wilt and wheat take-all diseases5–11. To further improve its potential applications, chemically synthesised 2,4-DAPG analogues have been developed and tested against plant diseases, and MP4, one of analogue of 2,4-DAPG, exhibited particularly potent antifungal activity, with inhibition rates of 84% and 63% against *Penicillium digitatum* and *Penicillium italicum*, respectively, and lower toxicity toward human cells compared with a fungicide widely used to treat harvested citrus fruit12. Such discoveries may assist the utilisation of DAPG analogues as novel biological fungicides for controlling plant diseases.

In *P. fluorescens*, the DAPG locus contains the four biosynthetic genes *phlACBD* that together produce 2,4-DAPG. PhlA, PhlC and PhlB are required for transacetylation of the monoacetylphloroglucinol (MAPG) precursor to generate DAPG13,14, and PhlD is critical for the biosynthesis of MAPG. In *P. fluorescens* 2P24, multiple factors in the GacS/GacA two-component system are involved in the biosynthesis of 2,4-DAPG during the late exponential and stationary phases2,15,16. The small RNA-binding proteins RsmA and RsmE, the resistance-nodulation-division efflux pump EmhABC, and the sigma factors RpoD, RpoN and RpoS are also associated with 2,4-DAPG biosynthesis1,17–21. In addition, PsrA is also a regulator of a sigma factor and involves in 2,4-DAPG biosynthesis PsrA negatively regulates *phlA* expression via either direct binding to an operator in the PhlA promoter region, or post-transcriptionally by affecting RpoS and RsmA expression. Inactivation of PsrA leads to a significant increase in 2,4-DAPG biosynthesis.

The *phlG* gene is also present in the DAPG biosynthetic locus of *P. fluorescens* 2P24, located between *phlF* and *phlH*. The PhlG protein is a DAPG hydrolase that may also modulate DAPG production10. The three-dimensional structure of PhlG revealed that the enzyme converts DAPG into MAPG by cleaving the carbon-carbon bond that...
regulators PhlF and PhlH, and PhlG does not affect root colonization or plant growth in P. fluorescens 2P24. PhlG mediates the conversion of DAPG to MAPG. Deletion of PhlG does not affect the growth of 2P24 and PM901. Growth rate of wild-type (WT) P. fluorescens 2P24 and the phlG deletion mutant.

Figure 1. (A) Schematic diagram of the construction of the phlG deletion mutant in Pseudomonas fluorescens 2P24 and PM901. (B) Growth rate of wild-type (WT) P. fluorescens 2P24 and the phlG deletion mutant.

Attaches the acetyl group to the phenolic ring, hence PhlG differs functionally from classical α/β-hydrolases in terms of catalytic mechanism and substrate specificity. Expression of PhlG is controlled by the pathway-specific regulators PhlF and PhlH, and phlH and PhlG impose negative feedback regulation on 2,4-DAPG biosynthesis.

To further investigate the function of PhlG in the synthesis and metabolism of 2,4-DAPG in P. fluorescens 2P24, we performed thin-layer chromatography (TLC) and liquid chromatography (LC) assays to analyse 2,4-DAPG degradation, and generated and characterised PhlG mutants. The results confirm that PhlG converts DAPG to MAPG, and rsmE and gacS negatively or positively regulate phlG gene expression.

Results
Deletion of PhlG does not affect the growth of P. fluorescens 2P24. To investigate the influence of the phlG gene on the growth of P. fluorescens 2P24, the PhlG deletion (2P24-ΔG) and complementary line (2P24-G) were generated and transformed into 2P24 and PM901 as shown in Fig. 1A. The bacteria growth of 2P24, 2P24-ΔG and 2P24-G were evaluated at different time points (Fig. 1B). The results showed that the growth rate of the deletion mutant 2P24-ΔG was comparable with that of the WT and complementary strain 2P24-G, suggesting that deletion of phlG did not affect bacterial cell growth.

PhlG mediates the conversion of DAPG to MAPG. A previous report showed that strain CHA0 degrades the potent antimicrobial agent DAPG to the much less toxic MAPG. To investigate whether PhlG is also involved in this function in P. fluorescens 2P24, we deleted the phlG gene in strain PM901, a derivative of 2P24 in which phlA is deleted, rendering the strain unable to produce DAPG and MAPG. TLC and LC assays were performed to examine the potential regulatory role of PhlG in 2,4-DAPG biosynthesis. The culture of PM901 (the phlA mutant, ΔphlA), PM901-ΔG (the phlA and phlG double mutant, ΔphlAΔphlG) and PM901-G (the phlG complementary strain) were incubated in the presence (100 μM) and absence of DAPG. The results showed that the timing of DAPG reduction and MAPG increase is quite similar. With the PM901 strain, the DAPG concentration declined and the compound was undetectable after 48 h of incubation (Fig. 2A and Table S2). However, the DAPG concentration was decreased from 10 mg to 2 mg in the culture medium of PM901-ΔG strain lacking PhlG after 60 h incubation. When the PhlG is complemented in PM901-G strain, the accumulation of DAPG is similar to those of PM901, whereas the MAPG concentration increased rapidly from 12 h and was maintained at an elevated level at 60 h.

Consistent with Fig. 3A, the results of TLC assays showed that DAPG degradation after 24 h was accompanied by the temporary accumulation of MAPG in the PM901 culture medium (Fig. 2B, C). In PM901-ΔG, the accumulation of DAPG is maintained at higher level after 24 h and decreased at 60 h. By the contrast, the formation of MAPG increased from 24 h and showed high accumulation at 60 h. When PhlG was complemented in PM901-G, the patterns of DAPG and MAPG were similar to those of PM901. This suggested that PhlG is functional in conversion of DAPG to MAPG in P. fluorescens 2P24.

GacS and RsmZ regulate phlG expression in P. fluorescens 2P24. In the biocontrol strain P. fluorescens CHA0, posttranscriptional repression of GacS/GacA-controlled genes relies highly on the RNA-binding protein RsmE and RsmA. To explore whether DAPG biosynthesis is regulated by the GacS/GacA regulatory cascade through affecting the PhlG expression, gacS and rsmE genes in 2P24-LacZ-G were mutated and β-galactosidase activity was measured. We found that expression of phlG was completely inhibited when gacS gene was deleted, whereas phlG expression was markedly increased following deletion of rsmE (Fig. 3 and Table S3). Taken together, these results indicate that the global regulatory factor gacS positively regulates expression of phlG, while rsmE negatively regulates its expression.

PhlG does not affect root colonization or plant growth in P. fluorescens 2P24. In order to investigate whether the colonization ability of the 2P24 strain is influenced by phlG, the seed germination rate was determined after soaking seeds in cultures of strains 2P24, PM901, 2P24-ΔG and PM901-ΔG. The number of bacteria...
present on seeds and roots was measured at 7 d and 10 d post-sowing. As shown in Fig. 4A and Table S4, deletion of \( \text{phlG} \) did not alter the colonisation capacity. The effects of strains 2P24, PM901, 2P24-\( \Delta \)G and PM901-\( \Delta \)G on plant growth were also examined. There was no significant difference in plant height and root length when roots were colonised by 2P24-\( \Delta \)G, PM901-\( \Delta \)G and the respective WT strains (Fig. 4B, C and Table S5). This indicates that PhilG does not affect root colonization or plant growth in \( P. \) fluorescens 2P24.

Figure 2. (A) Requirement of PhilG for the conversion of DAPG to MAPG. Strains PM901, PM901-\( \Delta \)G (PhilG mutant), and PM901-G (PhilG complemented mutant) were grown at 27 °C in media supplemented with 100 \( \mu \)M DAPG, and bacterial cell growth and the concentrations of DAPG and MAPG were measured at the indicated time periods. Values are means and standard deviations from three independent cultures, and all experiments were performed in triplicate. (B) Degradation of 2,4-DAPG is regulated by PhilG. D, 2,4-DAPG at start of experiment; M, MAPG at start of experiment; measurements were made at 12, 24, 36, 48, and 60 h after inoculation. CK: Degradation profile of 2,4-DAPG without any treatment in 60 h. The original pictures of gels are shown in the supplementary information Fig. S3. (C) The signals of Fig. 3B from the original picture (Fig. S3) were quantified by Quantity ONE software.

Figure 3. GacS and RsmZ regulate \( \text{phlG} \) expression in \( P. \) fluorescens 2P24.
Discussion

2,4-DAPG is a key secondary metabolite in the biocontrol bacterium *Pseudomonas fluorescens* that inhibits the growth of pathogenic microorganisms in the plant rhizosphere. Our data showed that PhlG does not affect the antifungal activities of 2P24 against *Rhizoctonia solani* Kühn, which is different with the role of PhlG in CHA05.

The protein encoded by the *phlG* gene catalyses the conversion of DAPG into the much less toxic MAPG by cleaving a carbon-carbon bond linking an acetyl group to the phenolic ring24. In this study, we confirmed the function of the product of the *phlG* gene that is located in the DAPG biosynthetic cluster in *P. fluorescens* 2P24, which is consistent with that *phlG* might also be associated with the DAPG biosynthetic locus in *P. fluorescens* strains Pf-5, Q2-87, F113 and CHA03,8,10,13,14.

Together with a previous report in *P. fluorescens* CHA010, our results of mutation of the *phlG* gene and DAPG degradation assays are underling that PhlG probably encodes a hydrolase that converts DAPG to MAPG. This suggests that PhlG-mediated degradation of DAPG is a conserved feature among DAPG-producing pseudomonads. In addition, we also found that deletion of *phlG* slowed down but did not eliminate degradation of 2,4-DAPG, suggesting other factors involved in the DAPG degradation in this bacterium. MAPG is a direct precursor of DAPG biosynthesis10,14,25. We therefore propose that MAPG is also a degradation product of DAPG generated by PhlG, although the biological significance of this conversion remains controversial. PhlG appears to act on both the DAPG metabolite itself, and the DAPG biosynthetic operon. For example, accumulation of MAPG was increased in the *phlA* deletion mutant. By converting DAPG to the much less toxic MAPG, PhlG may help to prevent accumulation of the toxic metabolite, as demonstrated in strains CHA0 and F11310.

In *P. fluorescens*, the GacS/GacA system positively regulated the transcription of noncoding small RNAs, which further bind with repressor proteins RsmA and RsmE and release them from their target mRNAs22,23. The GacS mutant was used to investigate whether PhlG is regulated by the GacS/GacA system in *P. fluorescens*, and expression of *phlG* gene was markedly decreased when gacS was deleted, suggesting that this global regulatory factor positively regulates *phlG* expression. This further suggests that DAPG biosynthesis is highly dependent on the GacS/GacA regulatory cascade14,16. In *P. fluorescens* CHA0, the RNA-binding protein RsmA is a key regulatory element in the GacS/GacA signal transduction pathway that acts at the posttranscriptional level. The 64 amino acid polypeptide RsmE is a homolog of RsmA in strain CHA0, and RsmA and RsmE function together to cause maximal repression in the GacS/GacA cascade in this organism21. Deletion of the *rsmE* gene significantly up-regulated the expression of *phlG* in *P. fluorescens* 2P24, suggesting the negative regulation of RsmE on PhlG-mediated DAPG degradation. Additionally, PhlG expression is also negatively regulated by PhlF, a known pathway-specific transcriptional repressor of DAPG gene expression21.

Theoretically, mutant 2P24-ΔG should be more inhibitory to the bacterial or fungal pathogens than 2P24, because it produces more DAPG. However, it appears that PhlG-mediated DAPG degradation does not affect
either the root colonisation or plant growth-promoting activity of *P. fluorescens* 2P24. In *P. fluorescens* strains CHA0 and Pf-5, DAPG biosynthesis is negatively affected by pyoluteorin 13,14,26-27, we thus speculate that other unknown factors probably mediate the antifungal activity of *P. fluorescens* 2P24 in the absence of PhlG. This means PhlG might have no direct effect on the synthesis of 2,4-DAPG; instead, phlG regulates the metabolism of 2,4-DAPG by assisting the conversion of DAPG to MAPG, which indicating that the deletion of phlG may have an indirect effect on the total amount of DAPG in the cell.

Materials and Methods

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* and *P. fluorescens* were cultured as described previously in 28.

**Plasmid construction and transformation.** To generate the phlG deletion mutant p299ΔphlG, fragments flanking the phlG gene were amplified with four pair of primers using 2P24 genomic DNA as template. Forward primer Ga (5′-GGCGGTACCTACCGGTACGATG-3′) and reverse primer Gd (5′-CCGAGCTCGTTGGAACACATCTCG-3′) were used to amplify the right flanking sequence of the phlG gene, and the right flanking sequence was amplified with forward primer Gc (5′-GGAGATCTCGGTAGGACCTCTC-3′) and reverse primer Gd (5′-GTCATCTTCTGGAAGACAGATCGG-3′). After digestion with the relevant restriction enzymes, PCR fragments Gab and Gcd were cloned into pHSG299 and pHSG399 (TaKaRa), respectively. After digestion of p399ΔGcd with Hind III and BamHI, the excised fragment was ligated into the corresponding plasmid p299ΔphlG, and the two fragments were ligated at the BamHI I site, resulting in the p299ΔphlG mutant, which resulted in the sequence of 317 bp (247-567) of PhlG gene (GenBank: DQ083928.1) was deleted (Fig. S1). The construct was verified by diagnostic PCR by perimer pair of G1/G2 and Ga/Gd using 2P24 genomic DNA and phlG p299ΔphlG (Fig. S1A, B). We found that 300 bp fragment from both diagnostic PCR was lost in p299ΔG.

To generate the complementation plasmid, the coding region of the phlG gene was amplified by PCR with primer pair G1 (5′-ATGATCCGTCATGCTTGTGACATCGCG-3′) and G2 (5′-ATGTCGACCTCTCGTTGGAACACATCTCG-3′). The amplified fragment was digested and ligated with plasmid pRK415 to generate recombinant plasmid p415-phlG referenced to Wei et al., 2005 and Wu et al., 2012, which was transformed into 2P24 and PM901, resulting in strains 2P24-G and PM901-G.

Plasmid p299-phlG and 3 kb LacZ fragments were digested with BamHI I and ligated to create p299-LacZ-G. The correct plasmid was transferred to the strain 2P24. The homologous recombination mutant 2P24-LacZ-G was screened X-gal-containing LB plates. Using strains 2P24-LacZ-G and 2P24-ΔG as templates, using primer G1: 5′-ATGTCGACCATCTTCTGGAACAGG-3′ and G2: 5′-ATGTCGACCATCTTCTGTTGGAACACATCTCG-3′ were subjected to PCR examination (Fig. S2C).

To obtain gacS gene from *P. fluorescens* 2P24, the chromosomal DNA of *P. fluorescens* 2P24 was digested with *Mob I* and ligated to pLAFLRS with BamHI I and Sac I. Then, it was transfected into *E. coli* DH5a after in *vitro* packaging to construct a genomic library. According to the published sequence of gacS gene, primers S-H: 5′-TGGCGATCAACCACGATGC-3′ and S-G: 5′-TGGCGTTCTCGGCGGGTGAACCT-3′ in the conserved region were designed. PCR amplification was performed using the strain 2P24 genome DNA as a template. The resulting fragment (0.54 kb) was verified by sequencing, and the 2P24 genomic DNA was screened by PCR using this as a marker. The verified PCR containing 3.2 kb fragment and 2.5 kb was shown in Fig. S2D. The resulting positive fragment was sub-cloned and ligated into plasmid pBluescript and verified by sequencing.

The construction of 2P24ΔGacS is referenced to Hailei Wei and Liqun Zhang 29. Based on the obtained gacS gene sequence from 2P24 strain, primer S1: 5′-GATAAGCTTGGCACTC-3′ and S3186: 5′-ATGGATCC TATGAGTGGGCGTTTCAGCC-3′ were designed. PCR amplification was carried out using the strain 2P24 genome DNA as a template. The PCR product was digested with the corresponding enzyme and ligated with pBlueScript to construct the recombinant plasmid pS-BH. pS-BH was digested with *EcoR I* and self-ligated to obtain the recombinant vector pS-BEH. The pS-BEH was digested with *Sal I* and *BamHI* I, and ligated with the corresponding digested pSR47s to construct a suicide vector (pSR47sΔS). The pSR47sΔS was transformed into 2P24 by the method of parental hybridization, and the recombinant bacteria were screened by using kanamycin as a marker. The selected recombinant bacteria were cultured at 28°C for 36 h without antibiotic pressure, and continuously transferred to culture for 4 times. The strains which lost the kanamycin resistance were screened, and the second recombinant strain was produced, thereby obtaining the deletion mutant (2P24ΔGacS).

The *rsmE* gene of *P. fluorescens* 2P24 was found in the genome sketch of *P. fluorescens* 2P24 by homology alignment according to the sequence of other *rsmE* genes of *P. fluorescens* that have been reported. The left flanking fragment (1.015 kb) was amplified by primers RsmE-2285 (5′-ATC TGCAGAAGGGCGCGTGCTG-3′) and RsmE-3330 (5′-ATGGATTTCATTAATGCGGCTTATCATCGC-3′) using 2P24 genome DNA as template. After digestion with *Pst I* and *BamHI* I, the fragment was ligated into the corresponding digested pHSG399 to obtain p399RsmE (L) and confirmed by sequencing. The primers RsmE-3498 and RsmE-4685 were designed for PCR amplification using the 2P24 strain genome as a template. The right flanking fragment (1.108 kb) was amplified with primers RsmE-3498 (5′-ATGATCCTCACCGGGCCACAGCCGCC-3′) and RsmE-4658 (5′-CCGCGTGGTTCGAAATGTTGCA-3′) using 2P24 genome DNA as template. The same digestion strategy was followed as in p399RsmE (L) construction. Then, the plasmid p399RsmE (L) was cultured and digested with *Pst I*. The digested fragment was recovered and then ligated into the corresponding digested pHSG399RsmE(R), and the resulting vector was named pHSG399ΔRsmE.

The pHSG399ΔRsmE was digested with *EcoR I* and Sac I. The fragment was recovered and then ligated into the corresponding digested pBSKm. The resulting vector was named pBSKmΔRsmE. The vectors containing GacS and RsmE mutant were introduced into the mutant 2P24-LacZ-G by electroporation, and the two-step homologous recombination mutants lacking the GacS and RsmE genes were screened by PCR (Fig. S2E). The β-galactosidase enzyme activity of the mutant strain was simultaneously determined.
Measurement of β-galactosidase activity. The procedure for the construction of reporter fusions and measurement of β-galactosidase activity is described in Wu et al. (2012).

Determination of DAPG and MAPG production by TLC and LC assays. Strains were cultured overnight and adjusted to an absorbance of 600 nm (OD600) of 0.8 with LB. Control (CK) cultures were not mock inoculated. Next, 10 mg of 2,4-DAPG dissolved in methanol was added after 10 h and cultures were incubated at 28 °C with shaking at 150 rpm for 12, 24, 36, 48, or 60 h. Cells were centrifuged at 7000 g for 10 min and the supernatant was acidified to pH 2.0 with 1 M HCl. The organic phase was extracted by rotary evaporation with an equal volume of ethyl acetate, and the dry solid was dissolved in 0.15 mL of methanol. Samples were spotted on the bottom of a silicone plate, dried with a sterilised airflow, and separated by TLC using a solvent system comprised of chloroform:acetone (19:1 v:v). A 1–5% ferric chloride ethanol solution spray was applied for visualisation of the developed plate. LC was used to assess 2,4-DAPG and MAPG standards.

Root colonisation test and wheat growth assay. Wheat seeds of uniform size without visible wounds were selected and used to investigate the response to the bacterial root-colonising fluorescent Pseudomonas sp. YGJ3. Wild-type (WT) 2P24 and PM901 strains were included as controls. Strains 2P24, PM901, 2P24-ΔG and PM901-ΔG were separately inoculated into 5 mL of LB medium and incubated at 28 °C with shaking at 150 rpm for 24 h. When the culture concentration reached an OD600 value of 0.056 or 5×107 colony forming units (cfu)/mL, wheat seeds were soaked inside the culture for overnight. The wheat seeds were planted into a pot containing 30 g mixture of meteorite and soil. Three seeds per pot and 3 pots per treatment were prepared. At 7 days and 10 days, soil was gently removed from roots by shaking, and roots were placed in test tubes containing 10 mL of sterile water and agitated at 150 rpm for 10 min. The number of bacteria in the test tube was calculated, and the root length and plant fresh weight were determined. All tests were repeated for three times.

Statistical analysis. GraphPad Prism software version 5.01 (Graphpad Software, Inc.) was used for analysis of variance, followed by multiple comparisons using one way ANOVA, and p < 0.05 was considered statistically significant.

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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