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Systematic Analysis of Two Tandem GGDEF/EAL Domain Genes Regulating Antifungal Activities in *Pseudomonas glycinae* MS82

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Abstract: Cyclic diguanylate (c-di-GMP) affects bacterial physiological and biochemical functions like biofilm, motility, virulence, and bacterial secretion systems. GGDEF/EAL-domain proteins, participating in c-di-GMP synthesis and degradation, are widely present in *Pseudomonas*, with various structures and functions. *Pseudomonas glycinae* MS82 is a rhizosphere bacterium that protects mushroom against the pathogenic fungi. Although 14 genes encoding GGDEF/EAL-domain proteins have been identified in the genome of MS82, c-di-GMP regulation is poorly understood as a facilitator or repressor of physiological phenotypes. Here, *PafQ* and *PafR*, encoding the proteins with the tandem GGDEF/EAL domain, were functionally analyzed and found to regulate antifungal activity. Individual deletion mutants of *PafQ* and *PafR* were constructed in *P. glycinae* MS82 through biparental conjugation and homologous recombination. Subsequently, antifungal activity, biofilm formation, motility, and expression of the genes related to antifungal substance synthesis were examined and contrasted with those of wild-type *P. glycinae* MS82. Most phenotypes of physiological activities were significantly reduced after knocking out *PafQ* or *PafR*. In other members of the genus *Pseudomonas*, homologous genes of *PafQ* and *PafR* possess different functions in c-di-GMP regulation. In *P. glycinae*, the positive regulation of *PafQ* and *PafR* on fungistatic substance synthesis, biofilm formation, and motility is crucial in the biocontrol of mushroom diseases.

Keywords: *Pseudomonas glycinae*; *PafQ* and *PafR*; c-di-GMP; biocontrol; mushroom disease

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1. Introduction

Pseudomonas spp., a type of rhizospheric microorganism, are termed plant growth-promoting rhizobacteria (PGPR) because of their effect of promoting plant growth and controlling plant diseases [1]. *Pseudomonas* spp. can produce a variety of antibiotic substances, such as phenazines [2], pyrrolnitrin (PRN) [3], 2,4-diacetylphloroglucinol (DAPG) [4], pyoluteorin (PLT) [5], hydrocyanic acid (HCN) [6], cyclic lipopeptide (CLP) [7], etc. Recently, increasing attention has been paid to the effective control of diseases of edible fungi by using the antifungal substances produced by *Pseudomonas* spp. These antifungal substances had obvious inhibitory effects on brown blotch disease [8], dry bubble disease [9], and so on. *Pseudomonas glycinae* MS82, formerly known as *P. fluorescens* MS82, isolated from the rhizosphere of a soybean plant, was shown to have good prospects of biocontrol application through in vivo and in vitro tests. A striking feature of *P. glycinae* MS82 is its antifungal activity against the pathogenic fungi *Trichoderma viride* and *Mycogone perniciosa*, which affect edible mushroom, but not against the fungus *Agaricus bisporus*, the most commonly and widely consumed mushroom globally [10,11].

Cyclic diguanylate monophosphate (c-di-GMP) is a ubiquitous in bacteria and serves as an important second messenger that regulates important physiological and biochemical functions of bacteria, such as biofilm [12], motility [13], virulence [14], bacterial secretion systems [15], quorum sensing [16] and other physiological activities and metabolism. The metabolism and intracellular levels of c-di-GMP in bacteria are predominantly regulated by diguanylate cyclase (DGC) and phosphodiesterase (PDE), while DGC and PDE are mainly determined by the highly conserved GGDEF and EAL domains [17]. There is a class of genes in bacteria that possess have both GGDEF and EAL domains. However, while it appears that these two domains with opposite catalytic functions exist in the same gene, in fact either the GGDEF domain has weak or no enzyme activity [18], or only one of the activities is displayed [19].

The cyclic diguanylate signaling pathway is well known for regulating bacterial antimicrobial functions. C-di-GMP in *Pseudomonas fluorescens* could improve the effect of biological control through promoting the formation of biofilm and enhancing its colonization ability in plant rhizosphere [20]. But the higher intracellular levels of c-di-GMP is the inhibitory signal for the antifungal substance HSAF secreted by *Lysobacter enzymogenes* OH11 [21]. Our previous research showed that the tandem GGDEF/EAL domain gene *PafR* is required for the antifungal activity of *P. gylcinae* MS82 against *T. viride* and *M. perniciosa* [10]. This study aimed to further investigate the regulation of the genes containing the tandem GGDEF/EAL domain on bacterial phenotypes including synthesis of antifungal active substances in *P. gylcinae* MS82 by quantitative analyses of antifungal activity and other related functions in the corresponding mutants.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids used in this work are detailed in Table 1. Bacteria strains were cultured at 28 °C (*P. gylcinae*) or 37 °C (*Escherichia coli*) in liquid LB (Luria-Bertani) or on LB-agar plates [22]. The concentrations of ampicillin (Amp) and gentamicin (Gm) were both 50 ng/mL in the different media. *P. glycinae* MS82 and mutants were grown on LB-agar plates for determination of biofilm formation. In the bacterial motility assay, the swimming medium was adjusted to LB medium containing 0.3% (*w*/*v*) agar, the swarming medium was adjusted to LB medium containing 0.7% (*w*/*v*) agar, and twitching motility medium was adjusted to LB medium containing 1% (*w*/*v*) agar.

Table 1. Strains and plasmids used in this work.

Strains and Plasmids	Genotype or Phenotype [1]	Source
Strains		
E. coli		
DH5α	*supE*44 Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	TSINGKE, Beijing, China
S17-1λ	*RP*4-2(Km::Tn7,Tc::Mu-1), *pro*-82, *LAMpir*, *recA*1, *endA*1, *thiE*1, *hsdR*17, *cre*C510	WEIDI, Shanghai, China
P. gylcinae		
MS82	Amp^r [1], Wild type	[23]
MT13385	Amp^r, *PafQ* deletion mutant derived from MS82	This work
MT24120	Amp^r, *PafR* deletion mutant derived from MS82	This work
T. virens		
NJ1	A fungal pathogen collected from *Pleurotus ostreatus* substrate	Lab stock
Plasmids and vectors		
pEX18GM	Gm^r, Suicide vector, *SacB*	FENGHUI, Changsha, China
pEX18-13385	Gm^r, up- and down-stream region of *PafQ* in PEX18GM	This work
pEX18-24120	Gm^r, up- and down-stream region of *PafR* in PEX18GM	This work

[1] Amp^r, Gm^r stand for resistance to ampicillin and gentamicin.

2.2. Construction of PafQ and PafR Deletion Mutants

Deletion mutants were constructed using the homologous recombination and parental combination method as described previously [24]. Briefly, the up- and down-stream regions of *PafQ* and *PafR* were amplified with four pairs of primers (see Table 2), respectively. The plasmid pEX18-13385 (or pEX18-24120) was obtained by digesting both regions with the selected restriction enzyme. Finally, the *PafQ* (or *PafR*) deletion mutant MT13385 (or MT24120) clones could grow on LB media containing ampicillin but not on the media supplemented with both ampicillin and gentamicin.

Table 2. Primers used for deletion of *PafQ* and *PafR*.

Primer Name	Sequence 5′→3′	Restriction Enzyme Site [1]	Length (bp)	Annealing Temperature (°C)
13385-F1	CGGAATTCGAATACCTCAACAGACACTC [1]	*Eco* RI	387	55
13385-R1	GGGGTACCCCTTGGCAGTAGCAATGCGC	*Kpn* I		
13385-F2	GGGGTACCGGAAATCACCGAAACCACCG	*Kpn* I	425	55
13385-R2	GCTCTAGAATGACCTGCGATTAGCGGCT	*Xba* I		
24120-F1	CGGAATTCCAGCCAGACCGCAGGATTAC	*Eco* RI	404	55
24120-R1	GGGGTACCGCTGTCGCTGAGGATTTTTC	*Kpn* I		
24120-F2	GGGGTACCGCGACGAGGTTCAGGGTTAT	*Kpn* I	302	55
24120-R2	GCTCTAGAATCGGGGGCAGAAAAGGGG	*Xba* I		

[1] The restriction enzyme site is underlined in the primer sequence.

2.3. Bioassay of Fungistatic Activity

The fungistatic activities of *P. glycinae* MS82, MT13385, and MT24120 were performed with the inhibition zone method as described previously [25]. In brief, 10 µL bacterial suspension (the concentration is approximately 2×10^8 CFU/mL) was inoculated in the center of a LB-agar plate. The *T. virens* strain NJ1 spore suspension (about 2×10^8/mL spore) was oversprayed onto the plate with a TLC Reagent Sprayer after the bacterial inoculum dried. The inoculated plates were incubated at 28 °C for two days in the dark. Three independent replicates were conducted and the diameter of the antifungal zone of each treatment was measured.

2.4. qRT-PCR Analysis

P. glycinae MS82 produces several substances with antifungal activities [11]. To understand whether deletion of the two genes had any effect on the expression of genes involved in the synthesis of these antimicrobial substances, expressions of some related synthesis genes in different mutants were quantitatively measured by qRT-PCR analysis. Total RNA was extracted from bacteria cells (10^6 cells per sample) with TSP412 reagent (TSINGKE, Beijing, China) and reverse transcription was performed using a Goldenstar RT6 cDNA Synthesis Kit Ver 2 (TSINGKE, Beijing, China) as per the manufacturer's protocols. Relative mRNA quantification was calculated using a standard curve. Target gene expression was normalized to that of the housekeeping gene *gyrB*. Primers used for qRT-PCR are listed in Table 3.

2.5. Analysis of Biofilm Formation

Biofilm formation was analyzed at different time points as described by Huertas-Rosales et al. [26]. The assay was performed on the sterile 96-well flat-bottom plates. Inoculated plates were incubated at 28 °C up to 48 h. Observations were made each 12 h for a total of four times. The experiments were repeated 4 times with three technical replicates each.

Table 3. Primers for qRT-PCR in RNA samples of mutants.

Gene Locus_tag [1]	Gene Function	Primer Name	Primer Sequence 5′→3′
DBV33_00020	DNA gyrase subunit B	gyrB-F	CGGCACCCAGATTCACTT
		gyrB-R	GGAGTTGAGGAAGGACAGTT
DBV33_00835	adenylyl-sulfate kinase	835-F	AGTCGTGGTCTGCAAAGTGT
		835-R	CTGCACCAGACCTCGCAATA
DBV33_09910	non-ribosomal peptide synthetase (NRPS)	9910-F	CGTCAGACTGCTCAACACCT
		9910-R	TTGACCGATCGGCATTGTCA
DBV33_09945	membrane dipeptidase	9945-F	ATCGGGTTCAAGGACAACCC
		9945-R	TCCTTGTCGACTTCGTTCCC
DBV33_09975	ornithine monooxygenase	9975-F	CAACAATGCCACCGGTGAAG
		9975-R	AAGCCCTGCATGTACAGACC
DBV33_11760	NRPS	11760-F	CTGGCAGCGATCCATGTGTA
		11760-R	TGAATGACAACTCGCGACCA
DBV33_11765	NRPS	11765-F	AGCATCTGGACGAACCTGTG
		11765-R	CAGGTCGAGGCGGAAGTATC
DBV33_12065	NRPS	12065-F	GTTGAAGTGTGGCCGTTGTC
		12065-R	CGTTGCTGATCCGGACGATA
DBV33_12075	NRPS	12075-F	GGTATCGGGCCAATCCTGAG
		12075-R	ACCGCTGCCACTCAAATACA
DBV33_17145	(2Fe-2S)-binding protein	17145-F	CAATGCACTGCCTAGAAAGAACC
		17145-R	TGAGCACGGTTTCGCCAATAG

[1] The gene locus_tag stands for the gene number in the GenBank database under accession number CP028826.1.

2.6. Analysis of Motility

Analyses of swimming, swarming, and twitching motility were conducted as described previously [27]. For swimming and swarming assay, 10 µL liquid cultures of *P. glycinae* MS82, MT13385, and MT24120 were spotted in the center of medium plates, then grown statically at 28 °C for 12 h. For twitching assay, three different clones were seeded on the base of the twitching plates and grown statically at 28 °C. 72 h later, the agar was removed and the empty plate was dealt with 0.01% crystal violet for half an hour. Finally, spreading diameters in the three kinds of motility assay were measured. Three replicates were performed for each assay.

3. Results

3.1. Deletion of PafQ and PafR

According to the genome of *P. glycinae* MS82 deposited in the GenBank database under accession number CP028826.1, *PafQ* (locus_tag: DBV33_13385) and *PafR* (locus_tag: DBV33_24120) are two of the 14 tandem GGDEF/EAL domain genes encoding proteins associated with biosynthesis of DGCs and/or PDEs of c-di-GMP. *PafQ* is a 2082-bp gene consisting of a MHYT-GGDEF-EAL domain, while *PafR* is a 3822-bp gene and contains three PAC domains, two PAS motifs, and one GGDEF/EAL domain.

To investigate potential biological functions of *PafQ* and *PafR*, two deletion mutants were constructed from *P. glycinae* MS82. The length of amplified fragments containing up- and downstream regions of *PafQ* or *PafR* were 812 bp and 706 bp from strains MT13385 and MT24120, respectively (Figure 1A-1,B-2). The deletion mutants were confirmed through PCR combined with restriction enzyme digestion, and the fragment sequences were subjected to BLAST from the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 25 November 2021). Genetic stability of the mutant strains were confirmed by continuously culturing.

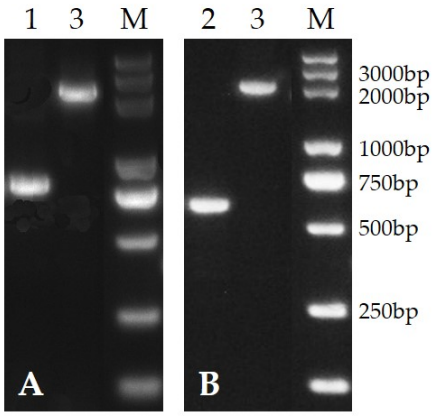

Figure 1. PCR confirmation of *PafQ* and *PafR* deletion mutants. (**A**), *PafQ*; (**B**), *PafR*; M, marker 5000; 1, *P. glycinae* mutant MT13385; 2, *P. glycinae* mutant MT24120; 3, *P. glycinae* MS82 (wild-type strain).

3.2. Bioassay of Fungistatic Activity

Fungistatic activities of *P. glycinae* MS82 and two mutants against the fungal pathogen *T. virens* were significantly different (Figure 2); when *PafQ* and *PafR* were knocked out separately, the antifungal activities of both strains decreased markedly. In addition, the antifungal activity of strain MT13385 (*PafQ* deletion mutant, 15.67 ± 1.15 mm) was significantly higher than that of strain MT24120 (*PafR* deletion mutant, 1.67 ± 0.58 mm).

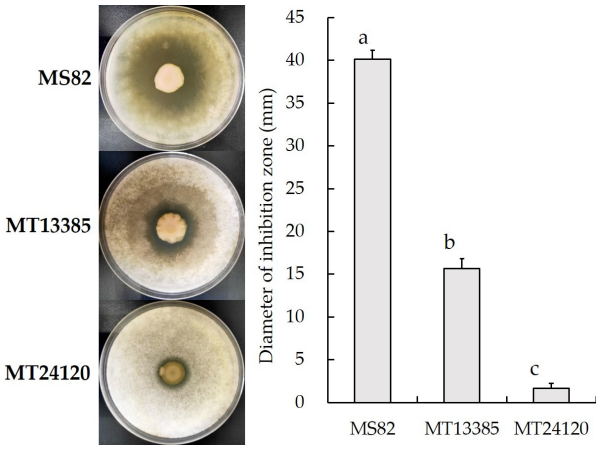

Figure 2. Inhibition of *PafQ* and *PafR* deletion mutants against *T. virens*. Different letters indicate values are significantly different ($p < 0.05$). MS82, *P. glycinae* wild-type strain; MT13385, *PafQ* deletion mutant; MT24120, *PafR* deletion mutant.

3.3. qRT-PCR Analysis

Transcriptomic analysis was conducted using qRT-PCR to evaluate the regulatory function(s) of *PafQ* and *PafR* on nine genes related to the synthesis of antifungal substances (Figure 3). After deletion of the gene *PafQ*, the expression of eight of the examined genes was significantly reduced compared with the expression levels in strain MS82; the exception was the gene 17145, which exhibited increased expression in strain MT13385. However, the expression of all the nine genes in the *PafR*-deletion mutant MT24120 was significantly lower compared with that in strain MS82, and the expression of six genes in strain MT24120 was significantly lower compared with that in the *PafQ*-deletion mutant MT13385. The differences in the expression of these genes related to antifungal substance synthesis in different mutants was correlated with the differences in antibacterial activity.

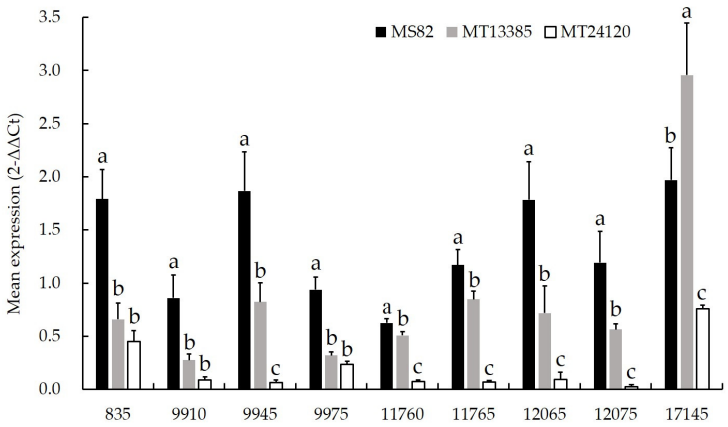

Figure 3. qRT-PCR analysis of nine genes in *PafQ* and *PafR* deletion mutants. The nine genes are shown as identification numbers shown in the genome GenBank accession number CP028826.1. Different letters indicate values are significantly different ($p < 0.05$). MS82, *P. glycinae* the wild-type strain; MT13385, *PafQ* deletion mutant; MT24120, *PafR* deletion mutant.

3.4. Analysis of Biofilm Formation

Effects of deletion of genes *PafQ* and *PafR* on bacterial biofilm formation were analyzed and shown in Figure 4. At 12 h, the amounts of biofilm formed by both mutants were lower compared with that of strain MS82. However, at 24 h, the amount of biofilm formed by MT13385 was significantly higher than those of strains MT24120 and MS82. After 36 h, the biofilm formation amount of strain MS82 was significantly lower compared with that of strain MT13385 and significantly higher than that of strain MT24120. These data suggest deletions of genes *PafQ* and *PafR* have opposing effects on bacterial biofilm formation.

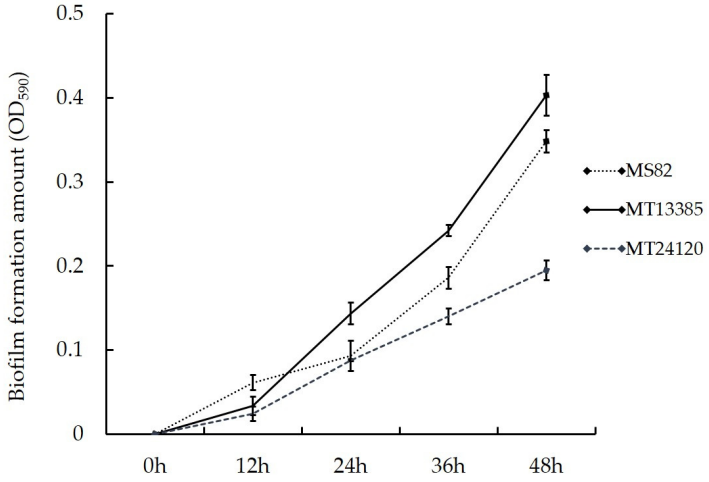

Figure 4. Biofilm formation growth curves of *PafQ* and *PafR* deletion mutants. MS82, *P. glycinae* wild-type strain; MT13385, *PafQ* deletion mutant; MT24120, *PafR* deletion mutant.

3.5. Analysis of Motility

The motility assay showed slightly different results among the three types of motility (Figure 5). The diameters of the two deletion mutants (strains MT13385 and MT24120) for all three motility assays were significantly lower than those of the wild-type strain MS82. In the swimming and swarming motility assays, the diameters between the two mutants was not different significant; however, in the twitching motility assay, the diameter of strain MT24120 was significantly higher than that of strain MT13385. These results indicated that both *PafQ* and *PafR* had positive effects on motility ability, but the extent of the effects was different.

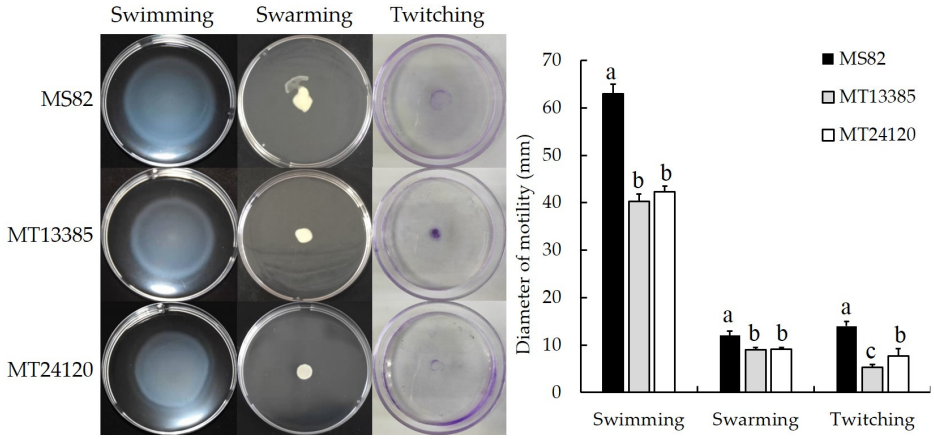

Figure 5. Motility assay of *PafQ* and *PafR* deletion mutants. Different letters indicate values are statistically different ($p < 0.05$). MS82, *P. glycinae* the wild-type strain; MT13385, *PafQ* deletion mutant; MT24120, *PafR* deletion mutant.

4. Discussion

In the past decade, most research on c-di-GMP focused on pathogenic bacteria, especially *Pseudomonas aeruginosa*, an important pathogenic bacterium in humans and animals. The c-di-GMP signaling pathway regulates the physiological functions of motility [28], toxicity [29], exopolysaccharides (EPS) [17], and biofilm formation [30]. Most of these traits are closely related to the pathogenicity or drug resistance of bacteria. Roles of c-di-GMP in pathogenesis has been extensively studied, and the c-di-GMP signaling pathway is known to influence the bacteriostatic ability of bacteria through the regulation of metabolite synthesis, biofilm formation, and motility. However, its function in biocontrol traits of PGPR remains to be further investigated.

One of the most common mechanisms of *Pseudomonas* as biocontrol bacteria is to inhibit the growth of pathogens by secreting secondary metabolites. The synthesis of some metabolites is regulated by c-di-GMP-related genes. For example, the production of 2,4-DAPG is negatively regulated by c-di-GMP through the RsmA and RsmE proteins. Furthermore, three of 23 GGDEF/EAL domain proteins are imvovled in regulation of biological control traits in *P. fluorescens* 2P24 [31]. PigX, a GGDEF/EAL domain protein, has high homology in biocontrol strain *Serratia plymuthica* G3 and pathogenic strain *Serratia* sp. ATCC 39006 and exhibited a positive impact on PRN production in the former [32] but negative impacts on prodigiosin antibiotic biosynthesis in the latter [33]. The results of this study also suggest that the two GGDEF-EAL domain genes in strain MS82 could positively regulate the production of fungistatic substances.

The colonization ability of biocontrol bacteria is another key factor affecting biocontrol function. Biofilm formation and motility are closely related to colonization ability [34,35]. The process of biofilm formation in *Pseudomonas* is regulated by a variety of c-di-GMP-binding receptors, such as PilZ domain proteins (FlgZ, Alg44, and MapZ) [36–38], transcription regulatory factors (FleQ and BrlR) [39,40], degenerated GGDEF/EAL domain proteins (FimX and PelD) [41,42], T2SSE_N (MshEN) domain proteins (GspE) [43], GIL domain proteins (BcsA) [44], etc. These effector proteins regulate motility and EPS synthesis, and then affect biofilm formation [30]. Alg44, an endometrial protein with a PilZ domain, binds c-di-GMP, and the combining of c-di-GMP with Alg44 can activate the enzyme activity of glycosyltransferase Alg8 and then promote alginate synthesis and biofilm formation [45]. FleQ, as a positive regulator, directly binds the promoter regions of flagellar and EPS genes to promote biofilm production and colonization on plants [46]. FimX and PelD, each containing degenerated EAL and GGEDF domains, can combine with c-di-GMP through these degenerated domains, and both proteins positively affected biofilm formation by promoting the assembly of type IV pilus and the synthesis of EPS, respectively [42,47].

Genes *PafQ* and *PafR* also exhibited similar positive regulation on biofilm formation and motility in this study.

GGDEF and EAL domains, often linked in series, are widely found in proteins associated with c-di-GMP metabolism [48]. GGDEF/EAL domain proteins may be monofunctional or double-functional enzymes, and their activities may activate or inhibit each other. For example, there are six GGDEF/EAL domain proteins in *Acetobacter xylinum*, of which three show DGC activity and the other three exhibit PDE activity. They regulate bacterial cellulose synthesis [49]. In *Shewanella baltica*, the GGDEF-EAL domain protein Sbal_3235, which regulates the biofilm formation and spoilage activity, has both DGC and PDE activities [50]. PafQ, containing a tandem GGDEF-EAL domain and a conserved membrane-sensing MHYT domain, is highly homologous with MucR, a membrane-anchored protein that contains a GGDEF/EAL domain and exhibits both DGC and PDE activity [51]. MucR specifically exerts DGC activity to regulate alginate biosynthesis by activating alginate production through the formation of a localized c-di-GMP pool in the vicinity of Alg44 [52]. Binding of the MHYT domain of MucR with nitric oxide (NO) in the environment activates the PDE activity of MucR and inhibits its DGC activity. MucR promotes the degradation of c-di-GMP and affects the production of biofilms [53]. PafR, containing three PAS domains and a tandem GGDEF-EAL domain, is also highly homologous with MucR. The similarity between the gene *PafR* in MS82 and the gene *MorA* in *P. putida* PNL-MK25 was 89.16% with BLAST from the NCBI. MorA, a novel regulator localized in membrane, is conserved among diverse species of the genus *Pseudomonas*, and homologs are present in all genomes of members of the genus *Pseudomonas* sequenced thus far [54]. In *P. putida*, MorA restricts flagellar protein biosynthesis and assembly to the late growth stage of the bacterial cells [55]. In *P. aeruginosa*, MorA promotes the biofilm formation and negatively regulates protease secretion via the type II secretion system (T2SS) [56]. In *P. glycinae* MS82, PafR demonstrated positive regulation on antifungal activity, biofilm formation, and motility. These differences in the phenotypes may result from the different species of *Pseudomonas* used in the different studies [57].

5. Conclusions

This study confirmed the positive regulatory roles of tandem GGDEF/EAL-domain genes *PafQ* and *PafR* in the antifungal activity of biocontrol bacteria *P. glycinae* MS82. Most phenotypes of physiological activities, such as antifungal activity, biofilm formation, motility, were significantly reduced for the *PafQ* or *PafR* deletion mutant. In *P. glycinae*, the positive regulation of *PafQ* and *PafR* on fungistatic substance synthesis, biofilm formation, and motility is crucial in the biocontrol of mushroom diseases. Further investigations are needed to understand the detailed mechanisms by which the cyclic-di-GMP signaling pathway affects metabolites. Although c-di-GMP receptors have been reported extensively, it is difficult to foresee possible intermediary players without solid experimental data. Nevertheless, proteins with predicted c-di-GMP binding canonical motifs can be used to explore their roles as receptors in this signaling pathway.

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