Scientific Research Publishing

Advances in Microbiology, 2017, 7, 217-230
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

The *PafR* Gene Is Required for Antifungal Activity of Strain MS82 against *Mycogone perniciosa*

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How to cite this paper: Ma, L., Wang, X.Q., Deng, P., Baird, S.M., Liu, Y.Z., Qu, S.X. and Lu, S.-E. (2017) The *PafR* Gene Is Required for Antifungal Activity of Strain MS82 against *Mycogone perniciosa*. *Advances in Microbiology*, **7**, 217-230.
https://doi.org/10.4236/aim.2017.74018

Received: March 2, 2017
Accepted: April 11, 2017
Published: April 14, 2017

Abstract

Bacterial strain MS82, isolated from the rhizosphere of a soybean plant, belongs to the species *Pseudomonas fluorescens*. The most important feature of strain MS82 is the production of antifungal activity against the mushroom pathogenic fungus *Mycogone perniciosa* but not against the mushroom fungus *Agaricus bisporus*. In this study, the mutant MS82MT19 generated with the EZ-Tn5 transposon system completely lost the antifungal activity against *M. perniciosa*. An open-reading frame named as *PafR* and predicted to code for a sensory box GGDEF/EAL domain protein, was disrupted in MS82MT19. To further confirm the function of this gene, site-directed mutagenesis with insertion of the terminatorless *nptII* cassette into the *PafR* gene was used to generate the mutant MS82SD19. As expected, there was no detectable antifungal activity of mutant MS82SD19 against *M. perniciosa*. These results suggest that the *PafR* gene plays an important role in the production of antifungal activity of *P. fluorescens* strain MS82.

Keywords

Pseudomonas fluorescens, Antifungal Activity, *Mycogone perniciosa*, Mutagenesis

1. Introduction

Mushrooms contain compounds such as minerals, vitamins, proteins and polysaccharides, and are therefore considered as nutritious foods [1] [2]. Commercial mushroom production is around 16 million tons annually worldwide and

more than 75% of these mushrooms are produced in China, where the total value of mushroom production is ranked the fifth in terms of crop value [3]. *Agaricus bisporus* (Lange) Imbach, the white button mushroom, is grown mainly in North America, Europe, the Far East and Australasia, and is the predominant mushroom produced commercially worldwide [4].

The mushroom fungus *A. bisporus* is attacked by a number of fungal and bacterial pathogens that may cause significant production losses. The environmental conditions under which mushrooms are grown are conducive to the propagation of these pathogens and subsequent development of diseases in the mushroom crop. *Mycogone perniciosa* (Magnus) Delacroix is the fungal pathogen causing wet bubble disease on the mushroom *A. bisporus* in commercial production facilities worldwide [5]. In recent years, the morbidity due to this disease was 40% and production was reduced more than 30% [6]. Only a few fungicides, such as prochloraz-Mn and carbendazim, are registered for diseases control in commercial mushroom production [7]. However, because of food safety issues, some of these fungicides are being phased out for mushroom production. Recently, the European community banned all benzimidazole-based fungicides such as carbendazim for control of the mushroom pathogens like *M. perniciosa* [8]. Typically, biologically-based fungicides are ranked as no or low risk pesticides to animals and humans. Therefore, there is a demand for more biologically-based fungicides that may be used in mushroom disease management [9].

The bacterial genus *Pseudomonas* contains more than 100 species, many of which have been used for biocontrol of plant diseases [10]. Strains of *Pseudomonas* spp. are frequently isolated from soils and many of them have been studied for their antimicrobial activities and ability to protect plants [11] [12] [13] [14]. *P. fluorescens* lives in the soil and protects plant health by induction of plant defenses, nutrient cycling and antagonism against pathogens [15]. It is important to understand the molecular mechanisms of biocontrol activity of bacterial strains used for the development of new antibiotics and biological fungicides.

Strain MS82 was preliminarily identified to *P. fluorescens* based on 16S rDNA analysis [16]. The most important feature of strain MS82 is its antifungal activity against the white button mushroom fungal pathogen *M. perniciosa*, but not the mushroom fungus *A. bisporus*. This character indicates that MS82 has potential for development as a biopesticide for mushroom production. This study is to report the association of the *PafR* gene encoding a sensory box GGDEF/EAL domain protein with antifungal activity against the pathogenic fungus *M. perniciosa*.

2. Materials and Methods

2.1. Plasmids, Bacterial Strains and Culture Conditions

The plasmids and bacterial strains used in this study are listed in **Table 1**. Strain MS82 was isolated from the rhizosphere of a soybean plant grown at the R. R.

Table 1. Plasmids and bacterial strains.

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
TransforMax EC100D *pir*$^{+}$	F– *mcr*A Δ(*mrr*-hsdRMS-*mcr*BC) ϕ80d*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G *pir*+(DHFR)	Epicentre
DH5α	*supE*44 Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Tiangen
Pseudomonas fluorescens		
MS82	Wild-type strain	This study
MS82MT19	GGDEF/EAL::Tn5 derivative of MS82; Km^{r}	This study
MS82SD19	GGDEF/EAL::*nptII* derivative of MS82; Km^{r}	This study
Pf0-1	Wild-type strain	Newell *et al.* [17]
Plasmid		
pUCP26	Expression vector for *Pseudomonas*; Tc^{r}	West *et al.* [18]
pBR325	Cloning vector; Cm^{r}, Tc^{r}, Ap^{r}	Prentki *et al.* [19]
pBSL15	Kanamycin resistance gene cassette; Km^{r}	Alexeyev [20]
pGEM-T Easy	Cloning vector; Ap^{r}	Promega
pMT19E1	EZ-Tn5 carrying genomic DNA of MS82MT19 (digested by *Pst* I)); Km^{r}; 15 kb	This study
pMT19E2	EZ-Tn5 carrying genomic DNA of MS82MT19 (digested by *Xba* I); Km^{r}; 24 kb	This study
pUCP19	pUCP26 carrying the sensory box GGDEF/EAL domain gene, Tc^{r}	This study
pMS1	pGEM-T Easy carrying 1-kb PCR product containing the *Sma*I site of the sensory box GGDEF/EAL domain gene; Ap^{r}	This study
pMS2	pBR325 carrying 1-kb PCR product containing the *Sma*I site of the sensory box GGDEF/EAL domain gene; Tc^{r}	This study
pMS3	the *nptII* gene inserted into pMS2; Km^{r}, Ap^{r}	This study

Km^{r}, kanamycin resistance; Tc^{r}, tetracycline resistance; Ap^{r}, ampicillin resistance; Cm^{r}, chloramphenicol resistance.

Foil Plant Science Research Farm, Mississippi State University. Assessment of antifungal activity of *P. fluorescens* MS82 was conducted using plates of potato dextrose agar (PDA) (Difco, BD Biosciences, Detroit, MI). Strain MS82 was grown on Pseudomonas Agar F (MP Biomedicals, Aurora, OH) for detection of fluorescence. Strain MS82 grew at 28˚C on nutrient broth-yeast extract medium (NBY) [21] with kanamycin (50 μg·mL^{-1}) and tetracycline (25 μg·mL^{-1}).

2.2. Multilocus Sequence Typing Analysis

Bacterial genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation. Madison, WI). Multilocus sequence typing (MLST) analysis was conducted as described by Jolley [22]. The seven housekeeping genes (*glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB* and *rpoD*) were amplified from the DNA of strain MS82 using the primers listed in the website http://pubmlst.org/pfluorescens (**Table 2**). The cycling program included a 2

Table 2. Primers used in MLST.

Primer	Sequence (5'to3')	Gene Product	Product length (bp)
glnS_F	ACCAACCCGGCCAAGAAGACCAGG	glutaminyl-tRNA synthetase	501
glnS_R	TGCTTGAGCTTGCGCTTG		
gyrB_F	GGTGGTCGATAACTCCATCG	DNA gyrase subunit B	501
gyrB_R	CGCTGAGGAATGTTGTTGGT		
ileS_F	TTCCCAATGAARGCCGGCCTGCC	isoleucyl-tRNA synthetase	501
ileS_R	GGGGTGGTGGTCCAGATCACG		
nuoD_F	GAAGTCCTGACCTTCCTGC	NADH dehydrogenase subunit D	516
nuoD_R	GAAGAACTCGGCCATCATG		
recA_F	TGGCTGCGGCCCTGGGTCAGATC	recombinase A	600
recA_R	ACCAGGCAGTTGGCGTTCTTGAT		
rpoB_F	TGGCCGGTCGTCACGGTAACA	DNA-directed RNA polymerase subunit beta	477
rpoB_R	CCGAAACGCTGACCACCGAAC		
rpoD_F	CTGATCCAGGAAGGCAACATCGG	DNA-directed RNA polymerase subunit beta	477
rpoD_R	ACTCGTCGAGGAAGGAGCG		

min initial denaturation step at 95˚C, followed by 30 cycles of 95˚C for 1 min, 56˚C for 30 s, 72˚C for 1 min, and final elongation at 72˚C for 10 min. Sequencing reads were assembled using the Lasergene version software package (DNASTar, Inc. Madison, WI). A phylogenetic tree based on the concatenated sequences (3,453 bp) of seven housekeeping gene fragments [*glnS* (501 bp), *gyrB* (492 bp), *ileS* (552 bp), *nuoD* (516 bp), *recA* (435 bp), *rpoB* (477 bp) and *rpoD* (480 bp)] of MS82 was produced using MEGA6 software.

2.3. Bioassays for Antifungal Activity

The antifungal activities of *Pseudomonas fluorescens* MS82 and its mutants in this study were analyzed using PDA plate bioassays as described previously [23]. Briefly, strain MS82 was grown overnight in NBY liquid medium, after which bacterial cells were collected by centrifugation and washed once with sterile distilled water. The cells were suspended in sterile distilled water to an optical density of 0.3 at 420 nm wavelength (approximately 2×10^8 CFU/mL), a drop of bacterial suspension (5 μL) was spotted on the center of each PDA plate, and plates were incubated 3 d at 28˚C. Each plate was then oversprayed with a standardized (OD_{420} = 0.3) conidial suspension of one of the three fungal indicator species: *M. perniciosa* JS01 (collection of Jindi Song), *Trichoderma viride* TX12 (Collection of Shien Lu) and *G. candidum* F-260 (a gift of Dennis Gross). The oversprayed plates were further incubated in the dark at 28˚C for 2 d. To evaluate the antifungal activity of MS82 against the mushroom fungus *A. bisporus* (Strain MS46, isolated from fresh mushroom), two PDA discs (5 mm in diameter) containing the mycelium of *A. bisporus* were placed 4 cm apart onto each of 9-cm PDA plates. A 5-μL aliquot of bacterial cell suspension (OD_{420} = 0.3) was immediately spotted onto the same plate equidistant from the edge of each fungal disc. The inoculated plates were incubated in the dark for three weeks at

25˚C. The inhibitory zones of MS82 and its mutants against the indicator fungi were recorded and compared with each other. Three replicates of the plate bioassays were performed independently.

2.4. Random Mutagenesis Analysis

In order to find the genes associated with antifungal activity, EZ-Tn5 <R6Kγori/KAN-2>Tnp Transposome Kit (Epicentre Biotechnologies, Madison, WI) was used as described by the manufacturer to randomly mutate strain MS82. Strain MS82 was grown in LB broth with shaking (220 rpm) at 28˚C until early log phase (OD_{600} = 0.8 - 1.0). The cells were harvested by centrifugation at 5000 rpm for 6 min at 4˚C, washed twice with the same volume and once with 0.5 volume of 300 mM sucrose, re-centrifuged, and finally re-suspended in 0.01 volumes of 300 mM sucrose [24]. Before electroporation, the bacterial suspension was chilled on ice for 30 min. Aliquots of 80-μL cell suspension were mixed with one μL of EZ-Tn5 < R6Kγori/KAN-2 > Tnp Transposome and transferred to chilled 0.1 cm gap cuvettes. Electroporation was conducted using a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) with the settings of 25 μFD, 200 Ω and 1.8 kV. Immediately after electroporation, the cells were transferred into 3 ml LB broth and shaken (220 rpm) at 28˚C for 2h. The colonies growing on NBY plates supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin were picked up as potential mutants for antifungal plate bioassays. The mutant MS82MT19 that had no visible antifungal activity against *M. perniciosa* was cultured on NBY plates with 50 $\mu g \cdot mL^{-1}$ kanamycin for further study. The Tn5 transposon fragment was amplified by PCR with the primers EzTn5F (5'-TTA CGA AAC ACG GAA ACC-3') and EzTn5R (5'-TCT AAT ACC TGG AAT GCT GTT-3'), provided by the EZ-Tn5 <R6Kγori/KAN-2>Tnp Transposome Kit manufacturer, to confirm the presence of the transposon in the mutant.

2.5. DNA Cloning and Sequence Analysis of the Targeted Genes

Bacterial genomic DNA of mutant MS82MT19 was extracted as described above. The bacterial genomic DNA was digested by restriction enzymes *Pst* I and *Xba* I, self-ligated, and then transformed into electrocompetent *E. coli* strain TransforMax EC100D pir^+ (Epicentre Biotechnologies, Madison, WI) as instructed by the kit to generate plasmids pMT19E1 and pMT19E2 (**Table 1**). Sequencing reactions were performed using the primers KAN-2FP-1 and KAN-2RP-1 supplied in the transposon kit to the flanking genomic DNA fragments of the Tn5 transposon in mutant MS82MT19.

The entire gene (*i.e.*, the *PafR* gene) targeted by transposon in mutant MS82MT19 was sequenced by the Sanger primer walking method [25]. In brief, five pairs of primers were designed and used to sequence the DNA flanking the Tn5 transposon in the plasmid pMT19E2. A consensus sequence of the entire gene region was generated using the Lasergene package. The gene functions were predicted through BLAST analysis against the GenBank database. The web-based software BPROM in the Softberry package was used for promoter

prediction [26].

2.6. Site-Directed Mutagenesis of the *PafR* Gene Targeted in Mutant MS82MT19 and Plasmid Construction for Complementation Assays

Efforts to complement the mutant MS82MT19 with a full-length targeted gene *in trans* were made through introduction of the plasmid pUCP26 harboring the full-length gene [18]. However, the antifungal activity of the mutant could not be restored to the wild type level at all. Therefore, we used insertion of the *nptII* gene into the *PafR* gene to generate a nonpolar site-directed mutation. A 1kb fragment containing the *Sma*I site of the *PafR* gene of the wild-type strain MS82, for which PCR primers containing restriction sites of *Aat*II and *Pst*I were designed (FP-AatII-1: 5'-GAC AGA CGT CTA CCG AGG TCA ACC AGG TTG-3'; RP-PstI-1: 5'-CGA ACT GCA GCG AGG TCG AGT GCT CGA AAA C-3'), was cloned into the pGEM-T Easy vector to produce the plasmid pMS1. The 1 kb fragment was digested by *Aat*II and *Pst*I and then subcloned into pBR325 [19], generating plasmid pMS2 containing tetracycline resistance. A 1.1-kb *Sma*I fragment of plasmid pBSL15 [20], which carries the *nptII* gene without a transcriptional terminator, was inserted into pMS2 at the *Sma*I site to generate plasmid pMS3 containing kanamycin and tetracycline resistance. Plasmid pMS3 was electroporated into strain MS82 for marker exchange mutagenesis as previously described [27], resulting in the generation of the mutant MS82SD19 which has resistance to kanamycin but sensitive to tetracycline. PCR was used to verify the double-crossover marker exchange in the mutant MS82SD19. The antifungal activity of the mutant MS82SD19 against *M. perniciosa* was evaluated with plate bioassays as described above.

3. Results

3.1. Multilocus Sequence Typing Analysis of the Strain MS82

Seven housekeeping gene fragments (*glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB* and *rpoD*) were obtained by PCR amplification. These DNA sequences were uploaded into GenBank with the accession numbers: KU510319 (*glnS*), KU382113 (*gyrB*), KU510314 (*ileS*), KU510315 (*nuoD*), KU510316 (*recA*), KU510317 (*rpoB*) and KU510318 (*rpoD*). The concatenated sequence (3453 bp) of the seven gene fragments [*glnS* (501 bp), *gyrB* (492 bp), *ileS* (552 bp), *nuoD* (516 bp), *recA* (435 bp), *rpoB* (477 bp) and *rpoD* (480 bp)] was generated in silico. A phylogenetic tree based on the concatenated sequences of MS82 was constructed using MEGA6 (**Figure 1**). Strain MS82 was tightly clustered with the *P. fluorescens* strain Pf0-1 with the bootstrap value of 100%. These data suggest that strain MS82 belongs to the bacterial species *P. fluorescens.*

3.2. Antifungal Activity of Strain MS82

Strain MS82 suppressed the mycelial growth of the fungal pathogen *M. perniciosa* with an inhibition zone of 34.17 ± 0.44 mm in the plate bioassay (**Figure**

2(B)). Strain MS82 also inhibited the growth of *T. viride*, and *G. candidum* (Figure 3). Interestingly, strain MS82 did not affect the mycelial growth of the mushroom fungus *A. bisporus* (Figure 2(A)). The antifungal activities of strain Pf0-1, the closest strain to MS82 based on phylogenetic analyses, were tested using the same methods as MS82. The results showed that strain Pf0-1 did not inhibit the growth of *M. perniciosa*, *T. viride*, or *G. candidum* (Figure 3). These results suggest that strain MS82 has an antifungal spectrum different from *P. fluorescens* strain Pf-01 and that its ability to inhibit a variety of fungi may be exploited for biocontrol and development of antibiotics for use in mushroom production.

3.3. Identification of the Mutant MS82MT19

Strain MS82MT19 was obtained from random mutagenesis that completely lost the antifungal activity and was chosen for further genetic analyses (Figure 2(C)). The 16S rRNA sequencing confirmed that t MS82MT19 was a mutant of strain MS82. Two plasmids, pMT19E1 and pMT19E2 (Table 1) carrying the re-

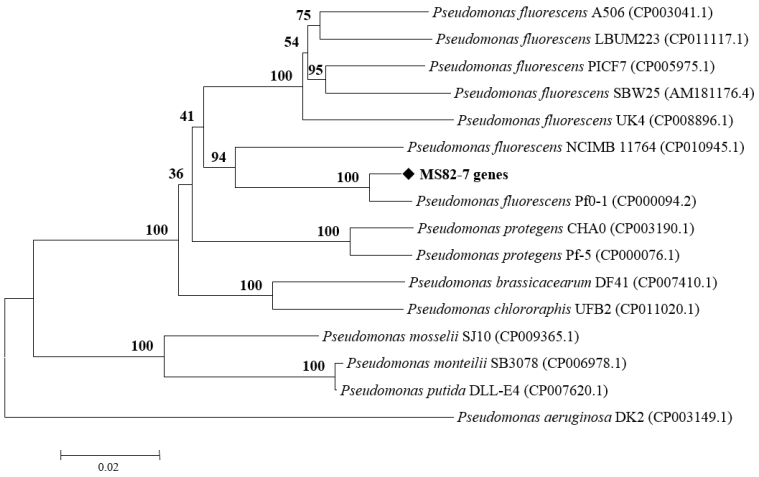

Figure 1. Phylogenetic tree of MS82 and related strains of *Pseudomonas* based on MLST analysis.

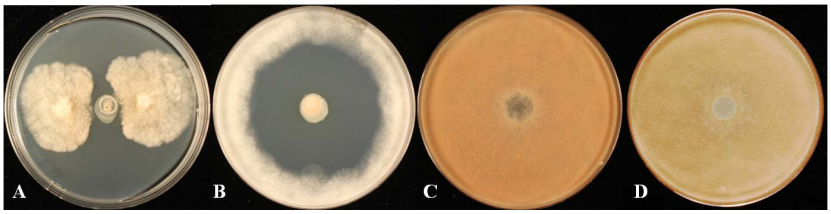

Figure 2. Plate bioassays of antifungal activities of *P. fluorescens* MS82 and its mutants against the fungal indictors *M. perniciosa* and *A. bisporus*. (A) The wild type strain MS82 (5 µl containing 1×10^6 cells) was inoculated onto the potato dextrose agar (PDA) plate simultaneously with *A. bisporus* (two 5 mm PDA discs with mycelia), then incubated in the dark for 3 weeks at 25˚C. (B)-(D) The wild type strain MS82 (B); MS82MT19 (C); and MS82SD19 (D) were each inoculated (5 µl l containing 1×10^6 cells) onto PDA plates and incubated in the dark for 3 d at 28˚C and then *M. perniciosa* was oversprayed on the plates. The oversprayed plates were further incubated in the dark at 28˚C for 2 d.

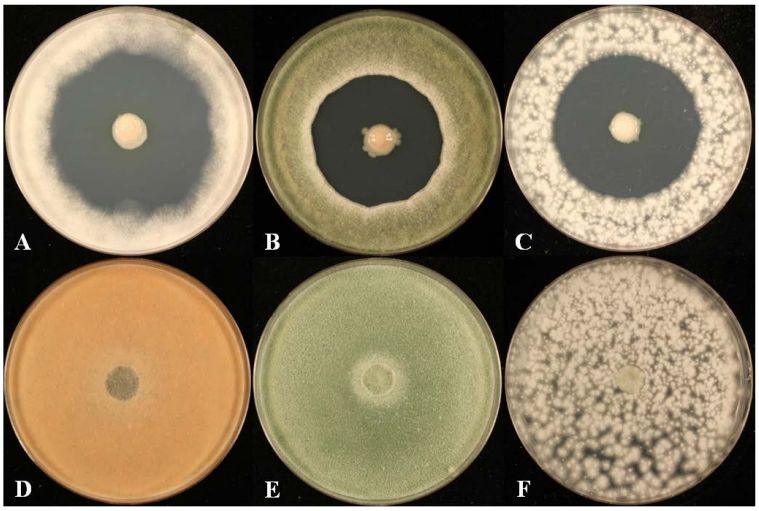

Figure 3. Plate bioassays for antifungal activities of *P. fluorescens* strains MS82 and Pf0-1. One droplet of bacterial suspension (5 µl containing ~10^6 cells) was inoculated onto each potato dextrose agar plate. Plates (A) (B) and (C) were inoculated with MS82; plates (D) (E) and (F) were inoculated with Pf0-1. The plates were incubated in the dark for 2 d at 28˚C before being oversprayed with spore suspension of *M. perniciosa* (A) (D); *T. viride* (B) (E) or *G. candidum* (C) (F); then incubated another 3 d in the dark at 28˚C.

gions flanking the EZ-Tn5 transposon insertion, were produced from MS82MT19 genomic DNA using the plasmid rescue method. The plasmids were digested by *Pst* I and *Xba* I, demonstrating that they carried different lengths of genomic DNA insertions that include the Tn5 transposon. Preliminary BLAST analyses of the flanking regions of the transposon in the two plasmids revealed that the Tn5 transposon carried by the mutant MS82MT19 was inserted into a gene that has a high identity (~98%) at nucleotide level to the putative gene Pfl01_4876 that was predicted to code for a sensory box GGDEF/EAL domain protein of *P. fluorescens* Pf0-1 (GenBank Accession No. CP000094.2).

3.4. Sequence Analysis of the Targeted Gene in Mutant MS82MT19

Sequence analysis showed that the putative proteins encoded by the gene disrupted by the transposon in MS82MT19 shares high identities (99-100%) with sensory box GGDEF/EAL domain proteins of *Pseudomonas*. A 4.5kb flanking DNA region of the transposon, which was inserted in the genome of the mutant MS82MT19, was sequenced from the plasmids pMTE1 and pMTE2 (**Table 1**) by using primer walking and Sanger DNA sequencing. Further analysis revealed that only one open-reading frame of 3849 nt encoding the sensory box GGDEF/EAL domain protein is disrupted by the transposon in MS82MT19. This gene was named as the *PafR* gene (Pseudomonas antifungal regulatory gene) and its sequence was deposited into GenBank with the accession number KU510320. The promoter region (−35 box: ATGCTA and −10 box: GCTTATAGT) was identified 38 nucleotides upstream of the putative start codon of the predicted protein *PafR*. A typical sequence-dependent terminator was not found from the

untranslational 3' end of the gene. The BLAST results revealed that the putative protein of this gene shares 99% amino acid identity to the sensory box GGDEF/EAL domain protein of *P. fluorescens* Pf0-1 (GenBank accession: ABA76613). In addition, the results showed that the transposon in the MS82MT19 genome was inserted between the nucleotides 1393 and 1394 of the sensory box GGDEF/EAL domain gene.

3.5. Characterization of the Role of the Sensory box GGDEF/EAL Domain Gene in Production of the Antifungal Product

The role of sensory box GGDEF/EAL domain *PafR* gene targeted in MS82MT19 in production of antifungal activity was further determined using the site-directed mutagenesis. After 16S RNA DNA sequencing confirmed the identity of the mutant MS82SD19, the insertion of the terminitorless *nptII* cassette in the *PafR* gene was determined by PCR analysis with the primers FP-AatII-1 and RP-PstI-1. As expected, the PCR products were 1.1 kb in size from genomic DNA of the wild type strain MS82 and 2.2 kb containing the *nptII* cassette from both MS82SD19 genomic DNA and plasmid pMS3 (Figure 4), respectively. The

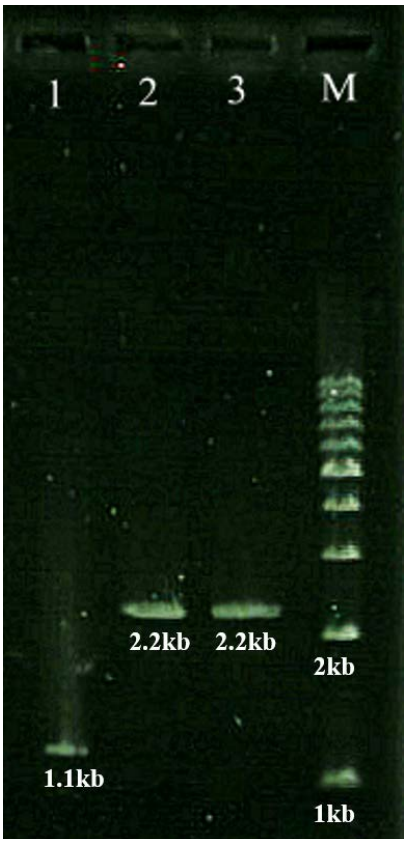

Figure 4. PCR confirmation of site-directed insertional mutation in the mutant MS82SD19. The primers FP-AatII-1 and RP-PstI-1 were used to amplify the fragment of the *PafR* gene. Lane 1: PCR product of the partial *PafR* gene amplified from MS82 (1.1 kb); Lane 2: PCR product of the partial *PafR* gene amplified from plasmid pMS3 (2.2 kb); Lane 3: PCR product of the partial *PafR* gene amplified from mutant MS82SD19 (2.2 kb); Lane M: 1kb ladder.

plate bioassay results revealed that the mutant MS82SD19 containing *nptII* gene did not produce the antifungal activity against *M. perniciosa* (**Figure 2(D)**), which is the same as the phenotype of the mutant MS82MT19. Collectively, the destruction of the *PafR* gene of MS82 strain either by random mutagenesis or by site-directed insertional mutagenesis resulted in the loss of antifungal activity against *M. perniciosa*. These results demonstrate that the *PafR* gene is the key gene in the antifungal activity of MS82 production.

4. Discussion

Pseudomonas strains can be isolated from various environmental niches such as soil and plant rhizospheres. *P. fluorescens* is a soil bacterium and many strains possess plant growth promoting functions [15]. The genome sequences of *Pseudomonas* strains SBW25, Pf0-1 and Pf-5 revealed considerable diversity and their genomic heterogeneity is reminiscent of a species complex [28]. Consequently, a few strains including Pf-5 were grouped as the new species *P. protegens* [29]. The strain MS82, isolated from soybean rhizosphere, exhibits fluorescence on Pseudomonas agar F plates under ultraviolet illumination. Phylogenetic analysis of MLST shows that MS82 is most close to *P. fluorescens* Pf0-1. Collectively the results of this study further confirmed that MS82 belongs to *P. fluorescens*. *P. fluorescens* not only has potential applications in biocontrol and bioremediation but also as a model for studying bacterial survival and fitness in soil [30]. Notably, bioassays for antifungal activity reveled that MS82 has a unique antifungal spectrum that is different from *P. fluorescens* Pf0-1. This is not surprising due to the considerable diversity and genomic heterogeneity in the species [29], but it suggests that further study of the antifungal compounds produced by MS82 is needed.

The sensory box GGDEF/EAL domain is present in many organisms, including *Vibrio*, *Pseudomonas*, *Staphylococcus*, and is involved in regulation of cellular functions [31] [32] [33]. The proteins with GGDEF and EAL domains are c-di-GMP cyclases or phosphdiesterase, respectively [34]. At least one N terminal sensor domain such as GAF or PAS, which regulates activity by an irritant such as oxygen [35], is frequently contained in the GGDEF/EAL domain proteins [36]. The pioneering work by Ross [37] showed the molecule c-di-GMP is an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus*. Another study showed that c-di-GMP was associated with production of cellulose in *Agrobacterium tumefaciens* [38]. In other species, c-di-GMP levels affect transitions from sessile to moving cells and thereby play a role in the change between multicellular biofilm and planktonic motile cell status [34]. The biofilm formation is also initiated by production of some compounds having antifungal activity, such as xantholysins, which are produced by the rhizosphere isolate *P. putida* BW11M1 [39]. To the best of our knowledge, this is the first report of the association of a bacterial gene encoding sensory box GGDEF/EAL domain protein with production of antifungal activities. However, it remains to be determined how the *PafR* gene is involved molecularly in production of antifungal

activity of strain MS82.

In this study, it was determined that the *PafR* gene of strain MS82 contains a GGDEF, an EAL and three PAS domains. Genetic analyses both random mutagenesis and site-directed insertional mutagenesis revealed the crucial role of the sensory box GGDEF/EAL-coding gene of MS82 in antifungal activity of that strain. Unexpectedly, we failed to restore the antifungal activity of the mutant MS82MT19 (*PafR*::miniTn5) by introduction of the full-length *PafR* gene and carried into the mutant by the *Pseudomonas* expression vector pUCP26 [17]. However, nonpolar mutation using the terminatorless *nptII* cassette [40] [41] verified the role of the *PafR* gene in production of antifungal activity. This indicates that the function of the *PafR* gene may also be affected by the other factors, such as expression level of the gene [42] [43]. Nevertheless, the results of this research indicate the *PafR* gene encoding the sensory box GGDEF/EAL domain plays an important role in antifungal activity and provides a crucial clue to understand molecular mechanisms of antifungal activity of MS82. Further work is under way to identify the genes required for production of the antifungal agents of MS82. The studies will provide useful clues for development of MS82 as a biological control agent to manage fungal diseases of mushroom cultivation.

Acknowledgements

This research was supported in part by Natural Science Foundation of Jiangsu Province for Youth (BK20150547), China Agriculture Research System (CARS24) and the US Department of Agriculture, National Institute of Food and Agriculture (MIS-401170).

References

[1] Mattila, P., Suonpaa, K. and Piironen, V. (2000) Functional Properties of Edible Mushrooms. *Nutrition*, **16**, 694-696. https://doi.org/10.1016/s0899-9007(00)00341-5

[2] Kouser, S., Ahmed, M. and Shah, S. (2013) Disease Status and Yield Losses Due to Wet Bubble Disease (*Mycogone perniciosa*) Associated with the Cultivation of White Button Mushroom at Different Mushroom Units of Kashmir Valley. *The Plant Pathology Journal*, **12**, 104-109. https://doi.org/10.3923/ppj.2013.104.109

[3] Zhao, X., Shi, Y., Xie, X. and Li, B. (2013) Screening and Evaluation of Induced Resistance to Brown Blotch Disease in *Pleurotus* spp. by Low Virulence and Avirulent Strains of *Pseudomonas tolaasii*. *Acta Edulis Fungi*, **20**, 96-100.

[4] Chang, S. (1999) World Production of Cultivated Edible and Medicinal Mushrooms in 1997 with Emphasis on *Lentinus edodes* (Berk.) Sing, in China. *International Journal of Medicinal Mushrooms*, **1**, 291-300. https://doi.org/10.1615/intjmedmushr.v1.i4.10

[5] Fletcher, J.T., Jaffe, B., Muthumeenakshi, S., Brown, A.E. and Wright, D.M. (1995) Variations in Isolates of *Mycogone perniciosa* and in Disease Symptoms in *Agaricus bisporus*. *Plant Pathology*, **44**, 130-140. https://doi.org/10.1111/j.1365-3059.1995.tb02725.x

[6] Fan, J., Zhang, H., Zhang, Z., Gong, P., He, W. and Zhu, Y. (2012) Integrated Control Technology of *Mycogone perniciosa* Magn. of *Agaricus bisporus*. *Northern Horticulture*, **6**, 168-170.

[7] Gea, F.J., Tello, J.C. and Navarro, M.J. (2010) Efficacy and Effects on Yield of Different Fungicides for Control of Wet Bubble Disease of Mushroom Caused by the mycoparasite *Mycogone perniciosa*. *Crop Protection*, **29**, 1021-1025. https://doi.org/10.1016/j.cropro.2010.06.006

[8] Regnier, T. and Combrinck, S. (2010) *In vitro* and *in vivo* Screening of Essential Oils for the Control of Wet Bubble Disease of *Agaricus bisporus*. *South African Journal of Botany*, **76**, 681-685. https://doi.org/10.1016/j.sajb.2010.07.018

[9] Cai, S., Wen, Z., Zhang, T., Wen, J. and Xie, B. (2009) Identification of a Bacterium Inhibiting the Mycelial Growth of *Mycogone perniciosa*. *Acta Edulis Fungi*, **16**, 92-94.

[10] Yang, M.M., Mavrodi, D.V., Mavrodi, O.V., Bonsall, R.F., Parejko, J.A., Paulitz T. C., Thomashow, L.S., Yang, H.T., Weller, D.M. and Guo, J.H. (2011) Biological Control of Take-All by Fluorescent *Pseudomonas* spp. from Chinese Wheat Fields. *Phytopathology*, **101**, 1481-1491. https://doi.org/10.1094/phyto-04-11-0096

[11] Manuel, J., Selin, C., Fernando, W.G. and De, K.T. (2012) Stringent Response Mutants of *Pseudomonas chlororaphis* PA23 Exhibit Enhanced Antifungal Activity against *Sclerotinia sclerotiorum in Vitro*. *Microbiology*, **158**, 207-216. https://doi.org/10.1099/mic.0.053082-0

[12] Rella, A., Yang, M.W., Gruber, J. and Montagna, M.T. (2012) *Pseudomonas aeruginosa* Inhibits the Growth of *Cryptococcus* Species. *Mycopathologia*, **173**, 451-461. https://doi.org/10.1007/s11046-011-9494-7

[13] Xu, J., Deng, P., Showmaker, K.C., Wang, H., Baird, S. and Lu, S. (2014) The *pqqC* Gene Is Essential for Antifungal Activity of *Pseudomonas kilonensis* JX22 against *Fusarium oxysporum* f. sp. Lycopersici. *FEMS Microbiology Letters*, **353**, 98-105. https://doi.org/10.1111/1574-6968.12411

[14] Kilani-Feki, O., Khiari, O., Culioli, G., Ortalo-Magné, A., Zouari, N., Blache, Y. and Jaoua, S. (2010) Antifungal Activities of an Endophytic *Pseudomonas fluorescens* Strain Pf1TZ Harbouring Genes from Pyoluteorin and Phenazine Clusters. *Biotechnology Letters*, **32**, 1279-1285. https://doi.org/10.1007/s10529-010-0286-9

[15] Haas, D. and Keel, C. (2003) Regulation of Antibiotic Production in Root-Colonizing *Pseudomonas* spp. and Relevance for Biological Control of Plant Disease. *Annual Review of Phytopathology*, **41**, 117-153. https://doi.org/10.1146/annurev.phyto.41.052002.095656

[16] Ma, L., Qu, S., Wang, X., Deng P., Lin, J., Li, H., Hou, L., Jiang, N., Song, J. and Lu, S. (2016) Identification of an Antifungal Bacterium against Mushroom Pathogen *Trichoderma viride* and Characterization of Genes Associated with Antifungal Activity. *Jiangsu Journal of Agricultural Sciences*, **32**, 528-533.

[17] Newell, P.D., Yoshioka, S., Hvorecny, K.L., Monds, R.D. and O'Toole, G.A. (2011) Systematic Analysis of Diguanylate Cyclases That Promote Biofilm Formation by *Pseudomonas fluorescens* Pf 0-1. *Journal of Bacteriology*, **193**, 4685-4698. https://doi.org/10.1128/JB.05483-11

[18] West, S.E., Schweizer, H.P., Dall, C., Sample, A.K. and Runyenjanecky, L.J. (1994) Construction of Improved *Escherichia-Pseudomonas* Shuttle Vectors Derived from pUC18/19 and Sequence of the Region Required for Their Replication in *Pseudomonas aeruginosa*. *Gene*, **148**, 81-86.

[19] Prentki, P., Karch, F., Iida, S. and Meyer, J. (1981) The Plasmid Cloning Vector pBR325 Contains a 482 Base-Pair-Long Inverted Duplication. *Gene*, **14**, 289-299.

[20] Alexeyev, M.F. (1995) Three Kanamycin Resistance Gene Cassettes with Different Polylinkers. *BioTechniques*, **18**, 52, 54, 56.

[21] Vidaver, A.K. (1967) Synthetic and Complex Media for the Rapid Detection of

Phytopathogenic Pseudomonads: Effect of the Carbon Source. *Journal of Applied Microbiology*, **15**, 1523-1524.

[22] Jolley, K.A. and Maiden, M.C. (2010) BIGSdb: Scalable Analysis of Bacterial Genome Variation at the Population Level. *BMC Bioinformatics*, **11**, 595. https://doi.org/10.1186/1471-2105-11-595

[23] Gu, G., Smith, L., Liu, A. and Lu, S. (2011) Genetic and Biochemical Map for the Biosynthesis of Occidiofungin, an Antifungal Produced by *Burkholderia contaminans* Strain MS14. *Applied and Environmental Microbiology*, **77**, 6189-6198. https://doi.org/10.1128/AEM.00377-11

[24] Choi, K.H., Kumar, A. and Schweizer, H.P. (2006) A 10-min Method for Preparation of Highly Electrocompetent *Pseudomonas aeruginosa* Cells: Application for DNA Fragment Transfer between Chromosomes and Plasmid Transformation. *Journal of Microbiological Methods*, **64**, 391-397.

[25] Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Liu, C.R., Guenthner, D., Bovee, D., Olson, M.V. and Manoil, C. (2003) Comprehensive Transposon Mutant Library of *Pseudomonas aeruginosa*. *PNAS*, **100**, 14339-14344. https://doi.org/10.1073/pnas.2036282100

[26] Solovyev, V., Kosarev, P., Seledsov, I. and Vorobyev, D. (2006) Automatic Annotation of Eukaryotic Genes, Pseudogenes and Promoters. *Genome Biology*, **7**, S10, S11-S12.

[27] Lu, S.E., Scholz-Schroeder, B.K. and Gross, D.C. (2002) Characterization of the sala, syrF and syrG Regulatory Genes Located at the Right Border of the Syringomycin Gene Cluster of *Pseudomonas syringae* pv. *syringae*. *Molecular Plant-Microbe Interactions*, **15**, 43-52. https://doi.org/10.1094/MPMI.2002.15.1.43

[28] Silby, M.W., Cerdeño-Tárraga, A.M., Vernikos, G.S., Giddens, S.R., Jackson, R.W., Preston, G.M., Zhang, X., Moon, C.D., Gehrig, S.M., Godfrey, S.A.C., Knight, C.G., Malone, J.G., Robinson, Z., Spiers, A.J., Harris, S., Challis, G.L., Yaxley, A.M., Harris, D., Seeger, K., Murphy, L., Rutter, S., Squares, R., Quail, M.A., Saunders, E., Mavromatis, K., Brettin, T.S., Bentley, S.D., Hothersall, J., Stephens, E., Thomas, C.M., Parkhill, J., Levy, S.B., Rainey, P.B. and Thomson, N.R. (2009) Genomic and Genetic Analyses of Diversity and Plant Interactions of *Pseudomonas fluorescens*. *Genome Biology*, **10**, R51.51-R51.16. https://doi.org/10.1186/gb-2009-10-5-r51

[29] Ramette, A., Frapolli, M., Saux, F.L., Gruffaz, C., Meyer, J.M., Defago, G., Sutra, L. and Moenne-Loccoz, Y. (2011) *Pseudomonas protegens* sp. nov., Widespread Plant-Protecting Bacteria Producing the Biocontrol Compounds 2, 4-Diacetylphloroglucinol and Pyoluteorin. *Systematic and Applied Microbiology*, **34**, 180-188.

[30] Varivarn, K., Champa, L.A., Silby, M.W. and Robleto, E.A. (2013) Colonization Strategies of *Pseudomonas fluorescens* Pf0-1: Activation of Soil-Specific Genes Important for Diverse and Specific Environments. *BMC Microbiology*, **13**, 92. https://doi.org/10.1186/1471-2180-13-92

[31] Holland, L.M., O'Donnell, S.T., Ryjenkov, D.A., Gomelsky, L., Slater, S.R., Fey, P.D., Gomelsky, M. and O'Gara, J.P. (2010) A Staphylococcal GGDEF Domain Protein Regulates Biofilm Formation Independently of Cyclic Dimeric GMP. *Journal of Bacteriology*, **190**, 5178-5189. https://doi.org/10.1128/JB.00375-08

[32] Newell, P.D., Monds, R.D. and O'Toole, G.A. (2009) LapD Is a bis-(3',5')-cyclic Dimeric GMP-Binding Protein That Regulates Surface Attachment by *Pseudomonas fluorescens* Pf0-1. *PNAS*, **106**, 3461-3466. https://doi.org/10.1073/pnas.0808933106

[33] Beyhan, S., Odell, L.S. and Yildiz, F.H. (2008) Identification and Characterization of

Cyclic Diguanylate Signaling Systems Controlling Rugosity in *Vibrio cholerae*. *Journal of Bacteriology*, **190**, 7392-7405. https://doi.org/10.1128/JB.00564-08

[34] Simm, R., Morr, M., Kader, A., Nimtz, M. and Romling, U. (2004) GGDEF and EAL Domains Inversely Regulate Cyclic di-GMP Levels and Transition from Sessility to Motility. *Molecular Microbiology*, **53**, 1123-1134. https://doi.org/10.1111/j.1365-2958.2004.04206.x

[35] Chang, A.L., Tuckerman, J.R., Gonzalez, G., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M. and Gilles-Gonzalez, M.A. (2001) Phosphodiesterase A1, a Regulator of Cellulose Synthesis in *Acetobacter xylinum*, Is a Heme-Based Sensor. *Biochemistry* **40**, 3420-3426. https://doi.org/10.1021/bi0100236

[36] Galperin, M.Y., Nikolskaya, A.N. and Koonin, E.V. (2001) Novel Domains of the Prokaryotic Two-Component Signal Transduction Systems. *FEMS Microbiology Letters*, **203**, 11-21. https://doi.org/10.1111/j.1574-6968.2001.tb10814.x

[37] Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-ohana, P., Mayer, R., Braun, S., De Vroom, E., Van Der Marel, G.A., Van Boom, J.H. and Benziman, M. (1987) Regulation of Cellulose Synthesis in *Acetobacter xylinum* by Cyclic Diguanylic Acid. *Nature*, **325**, 279-281. https://doi.org/10.1038/325279a0

[38] Amikam, D. and Benziman, M. (1989) Cyclic Diguanylic Acid and Cellulose Synthesis in *Agrobacterium tumefaciens*. *Journal of Bacteriology*, **171**, 6649-6655. https://doi.org/10.1128/jb.171.12.6649-6655.1989

[39] Li, W., Rokni-Zadeh, H., De Vleeschouwer, M., Ghequire, M.G., Sinneve, D., Xie, G., Rozenski, J., Madder, A., Martins, J.C. and De Mot, R. (2013) The Antimicrobial Compound Xantholysin Defines a New Group of *Pseudomonas* Cyclic Lipopeptides. *PloS ONE*, **8**, e62946. https://doi.org/10.1371/journal.pone.0062946

[40] Engl, C., Waite, C.J., McKenna, J.F., Bennett, M.H., Hamann, T. and Buck, M. (2014) Chp8, a Diguanylate Cyclase from *Pseudomonas syringae* pv. *tomato* DC3000, Suppresses the Pathogen-Associated Molecular Pattern Flagellin, Increases Extracellular Polysaccharides and Promotes Plant Immune Evasion. *MBio*, **5**, e01168-14. https://doi.org/10.1128/mBio.01168-14

[41] Chen, K.C., Ravichandran, A., Guerrero, A., Deng, P., Baird S., Smith, L. and Lu, S. (2013) The *Burkholderia contaminans* MS14 *ocfC* Gene Encodes a Xylosyltransferase for Production of the Antifungal Occidiofungin. *Applied and Environmental Microbiology*, **79**, 2899-2905. https://doi.org/10.1128/AEM.00263-13

[42] Luu, R.A., Schneider, B.J., Ho, C.C., Nesteryuk, V., Ngwesse, S.E., Liu, X., Parales, J.V., Ditty, J.L. and Parales, R.E. (2013) Taxis of *Pseudomonas putida* F1 toward Phenylacetic Acid Is Mediated by the Energy Taxis Receptor Aer2. *Applied and Environmental Microbiology*, **79**, 2416-2423. https://doi.org/10.1128/AEM.03895-12

[43] Horbal, L., Fedorenko, V., Bechthold, A. and Luzhetskyy, A. (2013) A Transposon-Based Strategy to Identify the Regulatory Gene Network Responsible for Landomycin E Biosynthesis. *FEMS Microbiology Letters*, **342**, 138-146. https://doi.org/10.1111/1574-6968.12117