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Study of endophytic *Bacillus amyloliquefaciens* CC09 and its antifungal cyclic lipopeptides

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ABSTRACT

Endophytic microbes are considered to be one of the most important bioactive compounds pool with a potential application in both pharmaceutical and agrichemical industries since the observation of taxol that could be synthesized by endophytic fungus *Taxomyces andreanae*. In this study, we reported an endophytic bacterium CC09, which was isolated from healthy *Cinnamomum camphora* leaves, exhibited a potential to produce antifungal metabolites inhibiting the growth of several phytopathogens such as *Glomerella glycines, Rhizoctonia solani* and *Alternaria alternate*. Based on 16S rRNA and *rpoB* genes analysis and physiological and biochemical assays, the strain CC09 was identified as *Bacillusamyloliquefaciens*. The antifungal constitutes in the culture filtrate of strain CC09 were predicted to be iturin family based on PCR amplification of specific genes including *BmyC, ItuC, ItuD, ItuF and ItuR* (encoding Iturin synthethases), *FenD and FenE* (encoding Fengency synthethases) and *SrfC* (encoding Surfactin synthethases), and confirmed by high performance liquid chromatography (HPLC). The broad antifungal spectra of the active constitutes in the metabolites indicated that the endophytic bacterium strain CC09 could be used as a potential biocontrol agent to control plant fungal diseases.

1.INTRODUCTION

Endophytic bacteria colonize the host tissue without causing evident harm or eliciting symptoms of plant diseases, which is different from endosymbionts by not residing in living cells or surrounding by a membrane compartment [1]. Although the mechanisms are not well understood, we do know that many endophytic bacteria have beneficial effects on plants, such as growth promotion, production promotion, disease prevention and inter-cell stress reduction [2,3]. Since Strobel et al. [4] firstly isolated taxol from a *Taxus brevifolia* endophytic fungus *Taxomyces andreanae*, many new antitumor, bactericidal and insecticidal compounds have been isolated and identified from endophytic microorganisms [5]. These traits make the endophytic bacteria a high potential of application on pharmaceutical, agriculture and ecology.

Bacillus, one of the commonly isolated endophytic bacteria, can produce many antifungal and antibacterial compounds with different structures, such as cyclic lipopeptides (CLPs). CLPs, synthesized by non-ribosomal synthetases, are a group of very surface-active amphiphilic compounds with heat-, base- and protease-stable properties [6].

According to the length of fatty acid chain, sub-chain and amino acid substitution groups, CLPs can be divided into three families: surfactin, iturin and fengycin. Among them, iturins and fengycins display a broad antagonistic activity against phytopathogens [7], and surfactins show strong antiviral, antimycoplasma and antibacterial activities, but no fungitoxicity[8]. The biological activity of CLPs is mainly owing to their amphiphilic nature, which made them steadily interact with lipid layers by different mechanisms (altering cell membrane structure and permeability, forming ion-conducting pores) in a dose-dependent way[9]. Besides, the peptidic moieties also play an important role in their specific functions[10]. Given the broad spectrum of antagonistic activity and the ability to promote plants growth, *Bacillus* and its active CLPs are potentially useful as bio-control agents [11].

In this study, we reported an endophytic bacterial strain CC09, which was isolated from *Cinnamomum camphora* leaves and identified as *B. amyloliquefaciens* based on the combination of sequence analysis of 16S rRNA gene and *rpoB* gene, and assays of physiological and biochemical properties. The active compounds of CLPs were detected by PCR amplification with several pairs of primers targeting the CLPs synthetase genes and further confirmed by HPLC analysis.

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2. MATERIALS AND METHODS

2.1 Strains and medium

The strain CC09 was isolated from healthy leaves of *Cinnamomum camphora* growing in a field at Nanjing University, Jiangsu, China on February 2005, according to the described methods[12]. Culture medium used in this study was potato sucrose agar plates (PSA: potato, 200 g; sucrose, 20 g; agar, 15 g; distilled water, 1L)[13]. A similar medium but lacking agar was used as liquid medium (PS).

Test phytopathogens *Glomerella glycines*, *Rhizoctonia solani*, *Alternaria alternate*, *Phytophthora capsici*, *Botrytis cinerea*, *Fusarium graminearum* were maintained in our lab.

2.2 Identification of the strain CC09

Methods used for the identification of strain CC09 were described by Liu et al. (2007), including biochemical and physiological assays, and 16S rRNA sequence analysis with a pair of primers *8f* and *1492r*. Several pairs of primers were used for PCR amplification of *rpoB* gene (Table 1). The PCR mixtures were prepared in 50 μL volumes containing 10×buffer ($MgCl_2$ contained) 5 μL, 10 mM dNTP 1 μL, primers (10 μmol/L) 1 μL, chromosome DNA of strain CC09 1 μL, Taq DNA polymerase 1 μL, double distilled water 40 μL. DNA amplification was performed in a GeneAmp PCR system 2,400 (Perkin Elmer) with an initial denaturation for 2 min at 94 ℃, followed by 25 cycles of denaturation (1 min at 94 ℃), annealing (1 min at 55 ℃), and extension (1 min at 72 °C), plus a final extension for 7 min at 72 °C. The PCR products were purified using small agarose gel DNA recovery kit (Tianwei), sequenced at Ji Tian Nanjing Company, and analyzed by using the BLAST search program in the GenBank to compare with the similar sequences retrieved from the DNA databases. MEGA 4.0 biology software was adopted to construct the 16S rRNA and *rpoB* phylogenetic tree.

2.3 Antifungal activity test of the fermentation broth

After incubated in LB liquid medium for 12 h (OD_{600}=1.2), the strain CC09 was inoculated in a proportion of 1% into a 1 L flask with 250 mL PS medium, and incubated at 37 ℃, 120 rpm for 36 h. The fermentation broth was centrifugated at 10,310×g at room temperature for 10 min to collect the supernatant for antifungal activity assay.

1 ml of the supernatant mixed with 9 ml of the PSA medium (50 ℃) containing ampicillin (400 μg/ml) was poured into a Petri dish (9 cm in diameter). Once the medium had cooled, target fungi discs (7 mm in diameter) taken from the fresh margin of the mycelium were placed in the middle of the Petri dish with the mycelium-side downward, and the dish was incubated at 28 ℃ for 48 h. Triazolone and sterile water were used as the positive and negative controls respectively. The inhibitory activities of the fermentation broth were recorded as the percentage reduction in the growth of the mycelia in comparison with that of mycelia in the control dishes[14]. Each experiment had 3 replicates.

2.4 PCR amplification of the genes encoding CLPs synthetases

Several pairs of primers (Table 1) were used for PCR amplification of the genes that encoded the part of the synthetases of fengycin (*FenD*, *FenE*), iturin (*ItuC*, *ItuD*), bacillomycin (*BmyC*) and surfactin (*SrfC*) in strain CC09[10,11,15]. Same PCR performance was conducted for *B. subtilis* (positive control) and sterile water (negative control). DNA amplification was performed with an initial denaturation for 3 min at 94 ℃, followed by 25 cycles of denaturation (1 min at 94 ℃), annealing (30 s at 55 ℃), and extension (30 s at 72 °C), plus a final extension for 5 min at 72 °C. PCR products were detected by running agarose gel electrophoresis and sequences analysis.

2.5 Determination of the antifungal compounds

The antifungal compounds were isolated and gathered primarily from the broth by macroporous resin (Nan Kai University, Tianjin, China) adsorption. The fermentation broth went through the macroporous resin at a rate of 4 ml/min. Having been adsorbed completely, washed the column with 85% ethanol at the same rate above. Eluant component was collected under reduced pressure with rotary evaporator. The extract was then disposed with silicagel (Marine Chemical Plant, Qingdao) column, the elution system was chloroform-methanol. Then TLC-bio-autography method was employed to guide the separation of the active sections[14] and further purified with Sephadex-LH20 (Pharmacia Biotech, Sweden) chromatography. Furthermore, the structure of the active compounds was analyzed by reverse phase HPLC (Waters Alliance 2695, absorption wavelength 220 nm) analysis coupled with UV (UV Detector: Waters 2996 Photo Diode Array) and MS (Mass Spectrometer: Thermo Finnegan LCQ Deca XP Plus. Electrospray ionization) detection. The results were taken a comparison to the data of standard compounds in the Syngenta natural product dereplication database.

3. RESULTS AND DISCUSSION

3.1 Identification of strain CC09

Endophytic bacteria, living for most of their life cycles inside healthy plant tissues, interact closely with the host plants and always show great antagonistic activities towards bacterial and fungal pathogens and therefore could be potentially used as biological control agents [16,17]. Compared to traditional chemical agents, they are environment friendly, safer and more effective and durable, besides, endophytic bacteria could not only inhibit the growth of phytopathogens, but also are beneficial for their hosts[18].

According to morphologic observation, strain CC09 had the following characteristics: the colony was milk white and opaque, with smooth and moist surface at the primary state and adhering to the medium tightly, but the colony surface became hilly wrinkled in later period and its border transformed into a smooth type, white biofilm formed when cultured statically in liquid medium. Besides, this strain is gram-positive, rod-shaped, sometimes chain-shaped, and spores were produced in the later

Table 1 PCR primers and target genes used in this work.

Primers	Sequences	Target gene	References
8f	AGAGTTTGATCCTGGCTCAG	16S rRNA	[14]
1492r	TACCTTGTTACGACTT	16S rRNA	[14]
907r	CCGTCAATTCCTTTRAGTTT	16S rRNA	[14]
rpoB_r1f	AGGTCAACTAGTTCAGTATGG	*rpoB*	This work
rpoB_r1r	TAATTCAGCAAGCGGGTTCG	*rpoB*	This work
rpoB_r2f	TCAGCTACTTCTTCAACCTC	*rpoB*	This work
rpoB_r2r	TTACCTACGAGAAGGTCTCC	*rpoB*	This work
rpoB_r3f	AGATCACCCGTGATATTCC	*rpoB*	This work
rpoB_r3r	TACTCTTTCGTTACTGCGTC	*rpoB*	This work
FenD1f	TTTGGCAGCAGGAGAAGTTT	*FenD*	[31]
FenD1r	GCTGTCCGTTCTGCTTTTTC	*FenD*	[31]
FenEF	GTTTCATGGCGGCGAGCACG	*FenE*	[31]
FenER	GATTCGCGGGAAGCGGATTGAGC	*FenE*	[31]
Bacc1f	GAAGGACACGGCAGAGAGTC	*BmyC*	[31]
Bacc1r	CGCTGATGACTGTTCATGCT	*BmyC*	[31]
Sur3f	ACAGTATGGAGGCATGGTC	*SrfC*	[31]
Sur3r	TTCCGCCACTTTTTCAGTTT	*SrfC*	[31]
ituF	CTGCCTGCGTATATGATTCCGGC	*ItuC*	[31]
ituR	CCGTGATGATGCCGTTCTTCAATCC	*ItuC*	[31]
ItuD1f	GATGCGATCTCCTTGGATGT	*ItuD*	[31]
ItuD1r	ATCGTCATGTGCTGCTTGAG	*ItuD*	[31]

Table 2 Physiological and biochemical characteristics of strain CC09 and the reference strains of Buchanan and Gibbons and Dong and Cai [19,20].

Item	*B. amyloliquefaciens*	*B. subtilis*	Strain CC09
Shape	Rod or cylinder	Rod or cylinder	Rod
Size	(0.6-0.8μm)×(1.5-3.0μm)	(0.7-0.8μm)×(2.0-3.0μm)	0.75 μm×2.1μm
Endospore	Middle, elliptic column or elliptical	Middle, elliptic column or elliptical	Middle, elliptic column
Gram stain	+	+	+
Glucose acid-producing	+	+	+
Glucose gas-producing	-	-	-
Voges-proskauer test	+	+	+
MR	NA	NA	-
Pectin degradation	NA	NA	+
Arabinose utilization	NA	+	+
Xylose utilization	NA	+	+
Sorbose utilization	NA	+	+
Maltose utilization	NA	+	+
Sucrose utilization	NA	+	+
Fructose utilization	NA	-	+
Citrate utilization	-	+	-
Nitrate reductase	+	+	+
Nitrate gas-producing	NA	+	-
Nitrite gas-producing	NA	+	-
Glucose ferment	+	+	+
Malonate utilization	NA	+	-
pH5.7	+	+	+
55℃	-	-	-
Indole production	-	-	-

"+" represents positive results, "-"represents negative results, "NA" represents data not applicable.

period, which was mid-born and elliptic column or elliptical. These phenomena suggested that strain CC09 was *Bacillus* sp. Based on 16S rRNA gene sequence analysis, strain CC09 was most likely *B. amyloliquefaciens* (Fig. 1), which had a 100% similarity with *B. amyloliquefaciens* (AB681482.1).

In order to accurately distinguish strain CC09 from *B. subtilis* and *B. amyloliquefaciens*, the biochemical and physiological properties of the strain were performed according the characteristics described in Bergey's Manual of Systematic Bacteriology Volume 3 (2009). The data listed in Table 2 indicated that strain CC09 is most likely to be *B. amyloliquefaciens* rather than *B. subtilis*.

As we all know, it is quite difficult to identify *Bacillus* species only based on traditional physiological and biochemical methods, even the 16S rRNA gene analysis[15,21]. Usually, a comparative analysis of the 16S rRNA gene sequence is the most commonly used genotypic method for bacterial identification[22], and strains that show 97% sequence similarity in the 16S rRNA are generally considered to be the same species[23]. However, the high similarity (98.1-99.8%) has been observed for the 16S rRNA gene sequences among different *Bacillus*species, such as *B. subtilis* and *B. amyloliquefaciens*[10]. In order to overcome the limitation of 16S rRNA gene analysis, Palys et al. (2000)[24] and Rooney et al. (2009)[25] reported that the functional genes such as

gyrA, *rpoB*, *purH*, *polC* and *groeL* could be used to distinguish the difference between *B. subtilis* and *B. amyloliquefaciens*. Thus, in this study, we performed a further sequence analysis for the conserved *rpoB* gene encoding the subunit of RNA polymerase. According the phylogenetic analysis, strain CC09 was grouped to *B. amyloliquefaciens* with a 100% similarity (Fig. 2). Combined with physiological and biochemical properties and 16S rRNA and *rpoB* genes sequences, the strain CC09 was determined as *B. amyloliquefaciens*.

3.2 Antifungal activity of the cell-free fermentation broth

The cell-free fermentation broth, which was obtained by centrifugation (10,310 *g* for 10 min) of the culture broth of strain CC09 that was cultured at 28 °C for 48 h, exhibited significant growth inhibition against the test phytopathogens, with which the inhibition rate of 95%, 85% and 80% against *G. glycines*, *R. solani* and *A. alternate* respectively; moderate inhibition rate against *F. graminearum* (50%) and *F. oxysporum* (40%); and weak inhibition rate against *P. capsici* (18%) (Fig. 3).

3.3 Detection of CLPs synthetase genes

Based on PCR amplification (Fig. 4), the chromosome DNA of strain CC09 was amplified with the primer pairs of *FenEF* and *FenER* (target: *FenE*), *Sur3f* and *Sur3r* (target: *SrfC*), *ituF* and *ituR* (target: *ItuC*), *ItuD1f* and ItuD1r (target: *ItuD*), but not with the primer pairs of *Bacc1f* and *Bacc1r* (target: *BmyC*), FenD1f and *FenD1r* (target: *FenD*). This result indicated that stain CC09 at least had the gene clusters that encode the CLPs synthetases of iturins (*ItuD*, *ituR*), fengycins (*FenE*) and surfactins (*SrfC*).

Moreover, sequences analysis showed that the given PCR products had a high homology similarity with *Bacillus* genes that were retrieved from GenBank. This result also indirectly indicated that strain CC09 has the potential to produce three families of CLPs.

Because of the structure difference of CLPs and the diversity of its producing strains, and meanwhile the commonly coexistence of CLPs in the metabolites, it's difficult to identify and purify which family them belong to. The detection of CLPs synthetase gene corresponding to NRPS afford us a easy way to predict the structure of these complex, and then make the isolation and purification process of CLPs much easier.

3.4 Confirmation of the antifungal CLPs

Through X-5 macroporous resin absorption, we obtained about 280 mg of brown extract from 13 L culture broth of strain CC09. And 40 mg final active compounds were then obtained by the further purification procedures.

By comparing the UV absorbance, MS spectrum and retention time with the standard CLPs compounds purchased from Sigma, we proved that the antifungal fraction of the extract contained surfactins, iturins and fengycins, which was consistent with the analysis of CLPs synthetases genes.

B. amyloliquefaciens is a species of bacterium in the genus *Bacillus* that was discovered in soil 1943[26]. According to genomics comparison, *B. amyloliquefaciens* is not only closely related to *B. subtilis* with similarity of 50% genes in the whole genome[27], but also can synthesize several CLPs, such as iturins, fengycins and sufactins[28], which is confirmed by our study based on PCR amplification and sequence analysis of the genes encoding CLPs synthetase as well as HPLC detection. It has been well documented that CLPs exhibit excellent biological activities, which can be used to control of both soil-borne diseases and phyllosphere diseases, such as tomato damping-off disease caused by *R. solani*, melon necrotic leaves disease infected by *P. fusca* [29,30]. However, to our knowledge, *B. amyloliquefaciens* and its active metabolites are rarely reported to be used to control root fungal diseases. Compared to *B. subtilis*, *B. amyloliquefaciens* has at least two advantages [27]: (1) it not only exhibits antimicrobial activities, but also improves plant growth; (2) it employs about 7.5% of genes in the chromosomes, which encode the nonribosomal peptide-synthetases (NRPSs) and polyketide synthetases (PKSs). These characteristics together with the endophytic properties make the strain CC09 of *B. amyloliquefaciens* a potential for agricultural application as an alternative agent of *B. subtilis*.

4. CONCLUSION

Endophytic microbes are considered to be one of the most important bioactive compounds pool with a potential application in both pharmaceutical and agrichemical industries. An endophytic bacterium that was isolated from healthy *Cinnamomum camphora* leaves and identified to be *Bacillus amyloliquefaciens* CC09 based on 16S rRNA and *rpoB* genes analysis and physiological and biochemical assays shows a potential antifungal activity against several phytopathogens such as *Glomerella glycines*, *Rhizoctonia solani* and *Alternaria alternate*. The antifungal mechanism of strain CC09 was probably due to the production of CLPs such as iturins based on PCR amplification of specific genes encoding iturin, fengency and surfactin synthethases. The broad antifungal spectra of the active constitutes in the metabolites and good environmental adaption of strain CC09 made it possible to be developed as a potential biocontrol agent in control of plant fungal diseases.

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