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Screening of Primer Sets for Amplification Cyclic Lipopeptide Gene of *Bacillus* Against Banana Fusarium Wilt

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Abstract Cyclic lipopeptides (CLPs) are a class of natural agricultural antibiotics produced by *Bacillus*, it plays an important role in the biological control using of *Bacillus*. In this study, 20 strains of *Bacillus*, including *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus siamense*, and *Bacillus cereus* were determined the antagonistic ability against to pathogenic fungi such as Foc 4. PCR amplification was performed on 20 strains of *Bacillus* and 7 soil samples, by using the designed 14 pairs of primers for CLPs-related synthetase genes, including Bacillomycin, Fengycins, Iturins, Surfactin etc, screen CLPs primers that can be used for *Bacillus* and soil monitoring. The results showed that: a total of 16 *Bacillus* strains have the antagonistic activity against pathogens, and microscopic observation of the crude lipopeptide extract of fermentation broth of these *Bacillus* strains can destroy the biofilm system and cell wall of Foc4, causing the mycelium and meristems to expand and deform; 12 pairs primers of this study (BamD-F/R, BamA-F/R, FenA-F/R, FenB-F/R, 61FenA-F/R, 61FenB-F/R, ItuA-F/R, ItuB-F/R, ItuC-F/R, ItuD-F/R, SrfAA-F/R, SrfAB-F/R) could effective amplify CLPs gene, and find that all of test *Bacillus* strains has the ItuC gene, there is a positive relationship between the antagonistic ability of *Bacillus* and the diversity and richness of CLPs genes, through PCR amplification of total soil DNA, it was found that the designed CLPs primers were feasible to monitor *Bacillus* in soil environment. It would be used as an important supplement to conventional plate counting and high-throughput sequencing methods, monitor and guide the application of *Bacillus* control soil-borne diseases of plants to provide theoretical support for further field.

Keywords Banana Fusarium Wilt; Antagonistic *Bacillus*; Cyclic lipopeptide antibiotic gene

As a devastating soil-borne banana disease, Banana Fusarium Wilt is a pathological continuous cropping disease caused by infection of race 1 and race 4 of *Fusarium oxysporum* f.sp.cubense. At present, the control techniques of banana Fusarium Wilt include breeding for disease resistance, cultivation management, chemical and biological control, etc. Due to the increasingly serious Fusarium Wilt of banana, farmers are forced to blindly use chemical pesticides in the production process, but the control effect of chemical pesticides is not ideal and easy to cause environmental pollution (Fu et al., 2016). Biological control technology has the characteristics of being environmentally safe and friendly, resistant to pathogens, improving soil quality and promoting crop growth, and has become an important measure to promote sustainable agricultural development (Chen et al., 2015). The main means of biological control technology is to apply microbial agents with antagonistic ability to pathogenic fungi into the soil of the disease area, including *Bacillus* spp., *Trichoderma* spp., *Actinomyces* spp., etc. (Ma, 2012). *Bacillus* has become the preferred object of biological control research and commercial production of biopesticides due to its characteristics of forming spores that are easy to be fermented and stored and relatively stable in biological characteristics, as well as its ability to produce antibacterial active substances at all stages of growth (Bais et al., 2004), and has been widely used in production practice.

It is an important way to control soil-borne diseases by controlling the balance of rhizosphere microorganisms with antagonistic *Bacillus* or its metabolites. The antimicrobial active substances produced by *Bacillus* metabolism play a key role in biological control, the most representative of which are cyclic lipopeptides (CLPs). The antimicrobial peptides mainly include Bacillomycin, Fengycins, Iturins and Surfactin (Cawoy et al., 2015),

which have been proved to be safe for human body and have no pollution to the environment (Chen et al., 2008). In addition, studies have shown that CLPs, among the antibacterial substances produced by *Bacillus*, is the main component that plays an antagonistic role against plant pathogens (Chen et al., 2015), and these substances play an important role in the prevention and control of plant diseases by *Bacillus*.

Increasing attention is being paid to integrated control techniques for soil-borne diseases such as banana fusarium wilt and overcoming the obstacles of continuous cropping. Therefore, monitoring of beneficial microorganisms and natural products as potent biopesticides in soil is of vital importance. For example, a recent study confirmed the presence of iturin, fengycin, and surfactin-type CLPs in lettuce rhizosphere soils (Chowdhury et al., 2015). However, the monitoring of CLPs, which plays a key role in the prevention and control of soil-borne diseases in soil, has been rarely reported. As beneficial microorganisms must be able to colonize in large numbers in rhizosphere soil, beneficial functional genes such as CLPs also need to be maintained in sufficient numbers in rhizosphere soil to play their roles (Tarkka et al., 2008).

In this study, 14 CLPs synthase genes were amplified by PCR using genomic DNA of *Bacillus* with broad spectrum resistance as the template to analyze the coverage of these genes in the tested *Bacillus*, and the feasibility of monitoring CLPs synthase genes in soil was discussed by PCR amplification using genomic DNA of multiple soil sites as the template. Whether it can be used as an important supplement to conventional coating plate counting and high-throughput sequencing methods to lay a foundation for monitoring the content and expression of beneficial microorganisms with this functional gene in soil, and provide theoretical support for comprehensive field prevention and control.

1 Results and Analysis

1.1 Effects of different living bacteria of bacillus on the growth of pathogenic fungi

According to the method of Wang et al. (2018), the antagonistic activity of 20 strains of *Bacillus* BQA2 against 7 pathogenic fungi such as Foc4 was determined (Table 1). Among the tested *Bacillus*, the antagonistic strains of *Fusarium oxysporum* (Foc4-B2, *Fol*), *Anthraxis* (*Colletotrichum gloeosporioides*), *Phytophthora litchis* (*P. litchi*, *P. nicotianae*) and *Rhizopteris* (*P. musarum*) showed antagonistic activity to 80% of the tested pathogens. Except strains BNA, 168, SD20 and GL05, all the other bacillus strains showed certain antagonistic activity against the tested pathogenic fungi, and had good antibacterial broad-spectrum. However, there were significant differences ($P<0.05$) in the antagonistic activity against different pathogenic fungi, and generally showed good antagonistic activity against *Anthrax*, followed by *Fusarium oxysporum* and *Phytophthora nicotiana*, and most of them were *P. musarum* and *P. litchi*. By comparing the diameters of different bacillus strains against pathogenic fungi, it was found that strain QB33 had the best antagonistic effect against Foc4, BC had the best antagonistic effect against *Fol*, QB12 had the best antagonistic effect against *C.g.Mango*, X5 had the best antagonistic effect against *C. g. Litchi*, BQA2 had the best antagonistic effect against *C. g. Litchi*. C200 had the best antagonistic effect on *P. nicotianae*, CL25 had the best antagonistic effect on *P. musarum*, and SS1, which had antagonistic effect on other pathogenic fungi, had no antagonistic effect on *P. litchi*. Through this experiment, the antagonistic activity differences of 20 strains of living *Bacillus* preserved in laboratory against several pathogenic fungi were studied, among which *Bacillus subtilis* and *Bacillus amyloliquefaciens* showed better inhibitory effect.

1.2 Effects of lipopeptide crude extracts from *Bacillus* fermentation broth on pathogen Foc4

Among the tested *Bacillus*, according to the evaluation method of Saravanakumar et al. (2019), the crude extracts of fermentation broth of strains 168, SD20, GL05 and SS1 showed no inhibition against banana fusarium wilt pathogenic fungi, and the inhibition level was evaluated as follows: Strain BC showed a very high inhibitory effect on Foc4 (++++), and strains X5, C200, BQA2, QB12, QB33, QB61, 6051, No.9, CL25, E51 showed a high inhibitory effect on Foc4 (+++). Strains BLG01 and Me showed moderate inhibition to Foc4 (++)

Scanning electron microscopy was used to observe Foc4 treated with control (normal growth) and antagonistic crude lipopeptides of *Bacillus*. It was found that the crude lipopeptides of *Bacillus* could destroy the external structure of *F. oxysporum* and inhibit its growth and development. From the control group, the mycelia surface

was dense, smooth, uniform and full, and the mycelia were complete. The mycelia surface at the edge of the inhibition zone was rough, wrinkled, deformed, and the integrity was destroyed, and the phenomenon of expansion, malformation, and incomplete spore development occurred (Figure 1).

Table 1 Diameter of inhibition zone of different bacillus to pathogenic fungi (cm)

Strains	Foc4-B2	Fol	C.g.Mango	C. g. Litchi	P. litchi	P. nicotianae	P. musarum
BQA2	1.74±0.01abc	1.55±0.10ab	1.50±0.18a	1.86±0.14ab	1.27±0.25a	1.53±0.12cde	1.23±0.12ab
BLG01	1.28±0.03fg	1.43±0.28bcd	1.17±0.15bc	1.84±0.16ab	1.03±0.12abc	1.7±0.16bcd	0.98±0.06cd
C200	1.51±0.01de	1.30±0.05cde	1.13±0.12bc	1.63±0.13c	0.77±0.09bc	2.57±0.26a	0.93±0.09cd
QB12	1.88±0.02ab	1.55±0.05ab	1.55±0.04a	1.71±0.09bc	0.80±0.08bc	2.07±0.21b	1.30±0.04ab
QB33	1.93±0.08a	1.50±0.05ab	1.53±0.21a	1.83±0.08ab	0.73±0.05c	1.70±0.22bcd	1.32±0.13a
QB61	1.28±0.08fg	1.40±0.05bcd	1.33±0.06abc	1.81±0.09ab	0.83±0.12bc	1.67±0.25bcd	1.23±0.09ab
H2	1.70±0.05bc	1.45±0.05bc	1.37±0.12ab	1.93±0.13a	1.03±0.12abc	1.83±0.26bcd	1.25±0.15ab
X5	1.58±0.43cde	1.43±0.03bcd	1.33±0.08abc	1.94±0.11a	1.17±0.12ab	1.77±0.12bcd	1.27±0.15ab
BNA	0.00h	0.00g	0.00d	0.00f	0.00d	0.00f	0.00e
SS1	1.16±0.06g	1.15±0.10ef	1.07±0.09c	1.41±0.09e	0.00d	1.13±0.21e	0.80±0.16d
Me	1.18±0.03g	1.30±0.15cde	1.30±0.21abc	1.71±0.09bc	0.87±0.17abc	1.40±0.16de	0.98±0.08cd
168	0.00h	0.00g	0.00d	0.00f	0.00d	0.00f	0.00e
6051	1.30±0.10fg	1.20±0.05ef	1.27±0.10abc	1.47±0.03de	0.95±0.39abc	1.53±0.12cde	1.30±0.08ab
BC	1.55±0.05cde	1.64±0.01a	1.53±0.23a	1.71±0.09bc	1.10±0.08abc	1.33±0.12de	1.20±0.08ab
TW	1.55±0.10cde	1.28±0.03de	1.25±0.08abc	1.64±0.06c	0.97±0.17abc	1.40±0.08de	1.28±0.08ab
NO.9	1.63±0.02cd	1.30±0.05cde	1.37±0.06ab	1.83±0.13ab	1.17±0.26ab	2.00±0.08bc	1.08±0.08ba
CL25	1.40±0.15ef	1.18±0.03ef	1.40±0.18ab	1.74±0.06bc	1.07±0.17abc	1.50±0.08ecd	1.33±0.05a
E51	1.43±0.18ef	1.10±0.08f	1.33±0.10abc	1.59±0.06cd	1.10±0.08abc	2.17±0.29ab	0.88±0.08cd
SD20	0.00h	0.00g	0.00d	0.00f	0.00d	0.00f	0.00e
GL05	0.00h	0.00g	0.00d	0.00f	0.00d	0.00f	0.00e

Note: Different lowercase letters in the same column represent significant difference ($p<0.05$)

Figure 1 Scanning electron microscopy results of the crude extract of Bacillus fermentation broth against Foc4

Note: A: Control; B,C,D,E,F: Treatment by CLPs

Antagonistic *Bacillus* can secrete some antibacterial substances acting on the biofilm system, destroy the cell structure of pathogenic fungi, so that pathogenic fungi can not grow and reproduce normally, lose its pathogenicity, so as to reduce the occurrence of diseases. Transmission electron microscopy (TEM) observed that pathogen Foc4 in the control group was not affected by antagonism of *Bacillus*, and its cell structure was complete and clear in shape, with complete nucleus, complete organelle types and obvious cell structure boundaries (Figure 2). In the treatment group, the pathogen of banana fusarium wilt was antagonized by crude lipopeptide extracts, and there was no discernible organelle structure. In C, the membrane and cell wall showed a similar phenomenon of "plasma wall separation", and the membrane was obviously incomplete; in D, the membrane disappeared, and the cell wall showed cleavage.

Figure 2 Transmission electron microscope results of the crude extract of *Bacillus* fermentation broth against Foc4
Note: A,B:Control; C,D: Treatment by CLPs

1.3 PCR amplification of lipopeptide antibiotic genes of *Bacillus*

By optimizing the PCR program (Table 3), the optimal PCR reaction program was obtained as follows: pre-denaturation at 94°C for 3 min, denaturation at 95°C for 45 s, annealing at 60°C (ItuA at 65°C) for 45 s, extension at 72°C for 1 min, 30 cycles, and extension at 72°C for 5 min. Using this program to amplify CLPs related synthesis gene sequences. It was found that 12 pairs of designed primers could effectively amplified the CLPs gene of *Bacillus* spp. (BamD-F/R, BamA-F/R, FenA-F/R, FenB-F/R, 61FenA-F/R, 61FenB-F/R, ItuA-F/R, ItuB-F/R, ItuC-F/R, ItuD-F/R, SrfAA-F/R, SrfAB-F/R), the antagonistic *Bacillus* could amplify ItuC gene of the 20 strains of *Bacillus*, 80% could be amplified to BamD, 90% to BamA, 80% to FenA, 90% to FenB, 25% to 61FenA, 45% to 61FenB and 45% to ItuA. 95% of ItuB, 100% of ItuC, 65% of ItuD, 65% of SrfAA and 85% of SrfAB could be amplified. MycF and MycA could not be amplified to the target band, among which ItuC had the highest coverage. BamD, BamA, FenB and ItuA had the highest specificity (Figure 3).

1.4 Gene amplification of lipopeptide antibiotics in *Bacillus* soil

Tiagen Soil DNA Extraction Kit extracted soil DNA for CLPs gene PCR amplification (Figure 3; Figure 4), it was found that lipopeptide antibiotic genes could be amplified to a certain extent in all soil samples. In the tested soil samples, the amplification efficiency and specificity of Hainan red loam were the best, which was related to the application of microbial fertilizer rich in antagonistic bacillus. The primers BamA, FenB, ItuC and SrfAA of Hainan soil samples with the best specificity were selected to send the amplified products for sequencing. The amplified products had high homology with the genes obtained in NCBI. The results of 704 bp PCR showed that the bamA-F/R primer had 99% homology with the gene involved in Bacillomycin biosynthesis. Fenb-F/R primers were 99% homologous to a region of 715 bp PCR products and Fengycin biosynthetic operon. Analysis of the 640 bp PCR product with ituC-F/R primer showed that the primer was 99% homologous to a region of the operon biosynthesized by Iturin. The 816 bp PCR product was analyzed using srfaa-F/R primers and showed 98% homology to a region of SrfAA associated with Surfactin biosynthesis. Bacillomycin, Fengycin, Iturin and Surfactin antibiotic genes were found in soil of many places in China. Through this experiment, the possibility of lipopeptide antibiotic gene amplification in soil can be determined.

Figure 3 PCR products of CLPs genes of 20 strains of *Bacillus*

Note: M: DL2000 DNA Marker; 1~20: BQA2, BLG01, C200, QB12, QB33, H2, Me, BC, TW E51, NO.9, CL25, X5, QB61, 6051, 168, BNA, SS1, SD20, GL05 genomic DNA; 21,22: Negative control (ddH₂O); A~N Primer of CLPs: BamD, BamA, FenA, FenB, 61-FenA, 61-FenB, ItuA, ItuB, ItuC, ItuD, SrfAA, SrfAB, MycF, MycA

Figure 4 PCR products of lipopeptide antibiotic genes in multiple soils

Note: M: DL2000 DNA Marker; 1~14 Primer of CLPs: BamD, BamA, FenA, FenB, 61-FenA, 61-FenB, ItuA, ItuB, ItuC, ItuD, SrfAA, SrfAB, MycF, MycA; A~G: Red soil samples from Hainan, red soil from Jiangxi, red soil from Zhejiang, sandy soil from Hebei, desert soil from Gansu, gray soil from Xinjiang and ddH₂O

2 Discussion

Bacteriostasis proteins, bacteriostasis polypeptides and bacteriostasis lipopeptides are the main bacteriostasis active substances secreted by *Bacillus* (Yang et al., 2015), among which lipopeptide antibiotics represented by Iturins and Fengycins are the important active substances against fungi (Falardeau et al., 2013). These lipopeptide

antibiotics can destroy the cell membrane by interacting with the membrane, leading to leakage of cytoplasm. It can also inhibit the synthesis of glucan, peptidoglycan and chitin, which are important components of cell wall, leading to the obstruction of cell wall formation of pathogenic fungi, cytoplasm leakage and protoplast exposure. This results in the cleavage of pathogenic fungi (such as hyphae, conidia or zoospores) at different life stages (Saravanakumar et al., 2019).

The results of scanning electron microscopy and transmission electron microscopy showed that the antagonistic lipopeptide crude extracts of fermentation broth could cause the mycelia of pathogenic fungi to appear rough surface, shrinkage, malformation, integrity destruction, expansion of meristem nodes, malformation and incomplete spore development. The pathogenic fungi treated with crude extracts showed similar phenomena of "plasmal-wall separation", unclear organelle structure boundary, cell membrane destruction, cell wall lysis and so on. This phenomenon is consistent with the results of Arrebola et al. (2010), indicating that antagonistic bacillus can secrete lipopeptide antibiotics (CLPs) to show the ability to inhibit a variety of pathogenic fungi including banana fusarium wilt. PCR detection of CLPs gene of the tested *Bacillus* showed that the 14 pairs of primers designed had a significant amplification efficiency on the 20 tested bacillus strains. Among them, the species and abundance of lipopeptide antibiotic genes of bacillus with high bacteriostatic ability in the in vivo confrontation test were also relatively high. And crude extracts from fermented liquid of Foc4 *Bacillus* with high inhibition can amplify a certain number of CLPs synthetic gene segments, and can swell to CLPs gene types and abundance, the higher its inhibition also stronger, it shows high pathogenic fungus inhibition and fat gene diversity and richness of peptide antibiotics has positive contact. The results of this study are consistent with those of Cao (2016). The diversity and abundance of lipopeptide antibiotic genes can also be used as a condition for screening biocontrol bacillus strains and predicting their antagonistic potential.

Some studies have found that factors such as temperature, ultraviolet light, acid base and protease have varying degrees of influence on CLPs (Lian et al., 2011), which makes it difficult to obtain CLPs information directly from the complex soil environment. Interestingly, Surfactin had poor inhibitory activity against fungi directly, but synergistically acted with Iturin to induce systemic resistance in plants and exhibited strong inhibitory activity against *F. oxysporum*, as well as lethal action against root-knot nematodes (Meena et al., 2016). Moreover, it is conducive to the colonization of rhizosphere soil and roots of bacteria, which is also the key and prerequisite for the sustained effect of antagonistic bacillus and the successful control of plant pathogenic fungi (Kavitha et al., 2005). Therefore, it is necessary to monitor multiple CLPs synthesis genes. In this study, primers of CLPs related synthetic genes were used for PCR amplification of soil total DNA to directly obtain the information of CLPs related synthetic genes in soil, which is an important antibacterial active substance. The results of genomic DNA amplification in soil samples showed that CLPs synthetic gene was feasible in soil detection, which provided a reference for quantitative or semi-quantitative monitoring of this beneficial functional gene in soil in the future.

3 Materials and Methods

3.1 Tested strains

The tested strains included *Fusarium oxysporum* f.sp. *cubense* Race 4, Foc4, (B2 strain), *Fusarium oxysporum* f.sp. *lycopersici* (Fol), *Colletotrichum gloeosporioides* Pen (C.g. Mango), *Colletotrichum gloeosporioides* Penz (C. g. Litchi), *Peronophthora litchi* (P. litchi), *Phytophthora nicotianae* (P. nicotianae), and *Phyllosticta musarum* (P. musarum) (Table 2). They were isolated or preserved by our laboratory (Laboratory of Microbial Resources Research and Utilization, Institute of Environment and Plant Protection, Chinese Academy of Tropical Agricultural Sciences).

3.2 Media, reagents and instruments

LB medium, PDA medium, laboratory fermentation medium (AYTK medium): sucrose 20 g, soybean meal 10 g, yeast powder 1 g, distilled water 1 L, natural pH.

Sangon®Ezup Column Bacterial DNA Purification Kit, Tiangen®Soil Genomic DNA Extraction Kit, Tiangen®2×Taq PCR Mix, other reagents purchased from Chinese medicine. Bio-Rad T100 PCR instrument, Hitachi

TM4000 Plus desktop scanning electron microscope, HT-7700 transmission electron microscope, VILBER Fusion FX7 Spectra multifunctional imaging system.

Table 2 Antagonistic *Bacillus* in this study

Strains	Source or reference	Classification
BQA2	Wild Forest Soil in Bawangling, Hainan	<i>B. amyloliquefaciens</i>
BLG01	Rhizosphere soil of bananas in Wenchang, Hainan	<i>B. amyloliquefaciens</i>
C200	Mangrove soil in Wenchang, Hainan	<i>B. amyloliquefaciens</i>
QB12	Sandy soil in Dongfang Banqiao Town, Hainan	<i>B. amyloliquefaciens</i>
QB33		<i>B. amyloliquefaciens</i>
QB61		<i>B. subtilin</i>
H2	Mangrove soil in Wenchang, Hainan	<i>B. amyloliquefaciens</i>
X5	Banana Root System, Hainan	<i>B. amyloliquefaciens</i>
SS1	Mouse feces	<i>B. subtilin</i>
BNA	Rhizosphere soil of banana in Danzhou, Hainan	<i>B. subtilin</i>
Me	Banana field soil in Honghe, Yunnan	<i>B. amyloliquefaciens</i>
168	Fujian Academy of Agricultural Sciences	<i>B. subtilin</i>
6051		<i>B. subtilin</i>
BC	Microbial agent of BaoCui	<i>B. siamensis</i>
TW	Microbial agent from TaiWan	<i>B. amyloliquefaciens</i>
NO.9	Rhizosphere soil of banana in Hai'an, Guangdong	<i>B. amyloliquefaciens</i>
CL25	Banana root system in Zhanjiang, Guangdong	<i>B. amyloliquefaciens</i>
E51	Soil of Banana Garden in Dongfang, Hainan	<i>B. amyloliquefaciens</i>
SD20	Molasses	<i>B. cereus</i>
GL05	Soil of Banana Garden in Guilin, Guangxi	<i>B. cereus</i>

3.3 Determination of antagonistic activity of *Bacillus*

According to the antagonistic activity detection method proposed by Wang et al. (2018), the antagonistic activity of active *Bacillus* was measured. For each pathogen fungus, pathogen fungi were obtained by shaking flask or plate culture of PDA liquid, and spore suspension was prepared. Using inverted plate method, spore suspension was added into the PDA medium which was about to solidify and shaken evenly. The final concentration was about 10^6 cfu/mL, and then the spores were inoculated with 0.5 cm *Bacillus* cake, and cultured at 28°C for 3~4 d. The diameter of antagonist circle was observed and recorded.

Referring to the method of Yang et al. (2015), crude lipopeptide substances were extracted from *Bacillus* fermentation broth, and the antagonistic activity of Foc4 was measured. 150 µL crude extract of *Bacillus* fermentation broth with bactericidal filter membrane was added into 0.5 cm hole, and cultured at 28°C for 4~7 d to observe the size of antagonistic circle. Referring to the evaluation method of Saravanakumar et al. (2019), the inhibition level evaluation was as follows: the antagonistic zone less than 1 mm was no inhibition; When the antagonistic zone was less than 2 mm, the inhibition was low (+). The antagonistic zone between 2 and 3 mm was moderate (++). The antagonistic zone between 3 mm and 4 mm showed high inhibition (+++). The inhibition of the antagonistic bands larger than 4 mm was very high (+++). Mycelia on the medium with edge of antagonistic zone of strain X5 were selected for preparation (Chen, 2018), and observed by SCANNING electron microscopy and transmission electron microscopy to analyze its antagonistic mechanism.

3.4 Gene amplification of bacillus lipopeptide antibiotics

Gene sequences of lipopeptide antibiotics such as Bacilin were downloaded from GenBank and compared with the whole genome of tested *Bacillus*. Primers were designed using Primer Premier 5.0 for high-homology segments selected (Table 3). Bacterial DNA extraction kit was used to extract bacillus genomic DNA, and 14 CLPs related synthetic genes were detected by PCR. The reaction system was 20 µL: Mix 10 µL, upstream and downstream primers (10 µmol/L) 0.5 µL each, DNA template 1 µL, and ddH₂O supplement to 20 µL. The PCR reaction sequence was as follows: pre-denaturation at 94°C for 3 min, denaturation at 95°C for 45 s, annealing at

50°C~65°C for 45 s, extension at 72°C for 1 min, 30 cycles, and extension at 72°C for 5 min. After PCR, PCR products were detected by 1.5% agarose gel electrophoresis.

Table 3 Primer information of lipopeptide antibiotics from *Bacillus*

Antibiotic	Primer name	Refseq	Position	Product size (bp)	Sequence (5'-3')
Bacillomycin	BamD-F	AY137375	1227-1247	729	TTCAGGATGCCGTTACACTTG
	BamD-R		1934-1955		GCGTCGTCATATTGGAATAAG
	BamA-F		13269-13289	704	ATCAAGAGCCAGGAAGCGTAT
	BamA-R		13950-13972		GCCGATAGGATGTGTAAGTAGTC
Fengycin	FenA-F	BGC000109	29109-29129	697	GTCTTGTAACGGCACCTGAAC
	FenA-R	5	29782-29803		TGTATTAGATAGCGGAGGAACC
	FenB-F		32280-32299	715	GCTCCATTACGCTCCTCTT
	FenB-R		32973-32994		CCGCATCTCTTCCAATCTTCTG
QB61-fengycin	61-FenA-F	QB61_0195	801-822	1424	GCAGCAATATAACCGCACAGA
	61-FenA-R	6	2224-2245		CAGCATTATCAGAGCAGGAG
	61-FenD-F		1354-1375	809	GAGCAACAGGTTATCCAATCGT
	61-FenD-R		2162-2181		GCATCGTCAGCAGTCATCAC
Iturin	ItuA-F	KY111358	1641-1660	885	TGCCAGACAGTATGAGGCAG
	ItuA-R	AB050629	2506-2525		CATGCCGTATCCACTGTGAC
	ItuB-F		81-100	874	TAAAGCAGCGGATAAAGCGT
	ItuB-R		935-954		AATGGCGACTAACGTATCGG
	ItuC-F		5810-5829	640	CCGTAATCAACCGTCTCGTT
	ItuC-R		6430-6449		GGGTGAGCTGCAACTTCTC
	ItuD-F		112-131	647	GATCGGATCTCCTTGGATGT
	ItuD-R		739-758		ATCGTCATGTGCTGCTTGAG
Surfactin	SrfAA-F	AJ575642	13327-13346	816	GGTTCAGCGGATTACAGATT
	SrfAA-R		14122-14142		GGACGGTTGTAGTAGCCTTCT
	SrfAB-F		19750-19773	586	CTGGAGCAATCATCATATCGTCAT
	SrfAB-R		20313-20335		ATAGAGCGGCACATAATCATAGC
Mycosubtilin	MycF- R	AF184956	2961-2982	843	TCGTCAGGTTGGAATAGGTCTT
	MycF- F		2140-2163		CGGTTAGTGTTATCGCTTATCAAG
	MycA-F		12850-12868	1 036	AGCGGTCGTCGGATGTATG
	MycA-R		13867-13885		AGCAGCGGTCAATCGTTCT

3.5 Gene amplification of lipopeptide antibiotics in soil

Total DNA of soil samples was extracted by Tiangen Soil DNA Extraction Kit, and PCR amplification was performed with the designed specific CLPs primers. The reaction system was 20 μ L: Mix 10 μ L, upstream and downstream primers (10 μ mol/L) 0.5 μ L each, DNA template 1 μ L, and ddH₂O supplement to 20 μ L. The PCR reaction sequence was as follows: pre-denaturation at 94°C for 3 min, denaturation at 95°C for 45 s, annealing at 50°C~65°C for 45 s, extension at 72°C for 1 min, 30 cycles, and extension at 72°C for 5 min. After PCR, PCR products were detected by 1.5% agarose gel electrophoresis.

3.6 Data analysis

The experimental data were expressed as mean $\pm$ standard deviation (X $\pm$ SD). SAS9.2 software was used for statistical analysis of the experimental data. One-way analysis of variance was performed for the data, and the difference significance level was $p<0.05$.

Authors' contributions

DT is the experimental designer and executor of this study. ZZ, YLY and WJ assisted in data analysis and writing the first draft of the paper; GLJ and LZQ participated in experimental design and analysis of experimental results; HJS is the architect and principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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