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Mining, Isolation and Identification of Siderophore Synthesis Gene from *Brevibacillus brevis* GZDF3

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Abstract: Objective of this paper is to excavate the siderophore synthesis gene from *Brevibacillus brevis* GZDF3 and verify its type and antibacterial effects. The method is using genome mining technology to analyze the siderophore synthesis genes and the phylogenetic tree of each synthesis gene was constructed separately. Iron free medium was utilized to induce the synthesis of siderophore and CAS liquid detection method was used for qualitative and quantitative analysis on siderophore. The type of siderophore was preliminaries identified by Arnow and its antibacterial effects were analyzed according to the agar punching method. The results show that a siderophore synthesis gene cluster with 83% similarity to Petrobactin was found in *Brevibacillus brevis* GZDF3 genome. Iron free medium could induce siderophore synthesis and the optimal incubation time cultured in iron free medium was 30 h and 48 h. Antagonistic strain GZDF3 had the capacity to synthesize catechol-type siderophore. Also, GZDF3 had a powerful antibacterial effect on pathogenic fungus *Fusarium oxysporum* of rotted root on *Pinellia ternata*. Therefore, *Brevibacillus brevis* GZDF3 can produce catechol-type siderophore in an iron-deficient culture medium, which was also a main antifungal active substance.

Keywords: *Brevibacillus brevis*, Genome Mining, Petrobactin Siderophore, CAS Detection, Antibacterial

Introduction

Iron is one of the essential trace elements of all living organisms and it participates in many biological metabolism, such as photosynthesis, respiration, oxygen transport, gene regulation and DNA biosynthesis. Iron is an abundant metal, being the fourth most plentiful element in the Earth's crust. The majority of iron in the environment is in the form of insoluble ferric oxide/hydroxide complexes and to limit the growth of pathogens in vivo, serum contains iron-binding proteins such as transferrin and lactoferrin that maintain a very low concentration of free iron ($\sim 10^{-18}$ M). In order to obtain the iron necessary for survival, microbes have evolved many ways to obtain iron and synthetic siderophore is one of the important approaches (Carroll *et al*., 2017; Serrano, 2017).

The siderophores are low molecular weight, high affinity iron chelators that are secreted in response to the iron limitation to scavenge iron and their collection system is the most diverse and broadly distributed iron uptake mechanism for microorganisms (Zawadzka *et al*., 2009). The molecular weight is between 500-1500 Da and the siderophore specific transporter systems import the Fe-siderophore across the cell membrane to the cytoplasm (Fukushima *et al*., 2013). Indeed, there are more than 500 known species of siderophores and siderophores known species of siderophores and siderophores have been classified according to the Fe(III)-coordinating groups: Catecholate, hydroxamaces,

carboxylates or mixed (Arora and Verma, 2017; Barry and Challis, 2009). At present, siderophore also can be classified by their mode of mode biosynthesis, Non-Ribosomal Peptide Synthetase (NRPS)-dependent or NRPS-independent biosynthesis (NIS) (Han *et al*., 2013). The synthesis mechanism of NRPS has been studied deeply, such as Pyoverdine synthesized by *Pseudomonas aeruginosa*, the synthesis of Enterbactin by *Enterobacteriaceae* and more. Although there are limited studies on NIS, there is a definite understanding of its synthesis mechanism, such as Aerobactin synthesized by *Enterobacteriaceae*, Staphyloferrin synthesized in *Staphylococcus aureus*, Desferrioxamine in the *Streptomyces* and more. However, recent studies have found that there is a siderophores with NRPS and NIS in nature, such as Petrobactin, which is synthesized by *Bacillus anthraci* (Hagan *et al*., 2016). *Bacillus anthraci* siderophore Petrobactin is encoded by *asbABCDEF* genes, of which AsbAB belong to the NIS family and the AsbCDE synthetases belong to the NRPS family and a dehydrase (AsbF) (Lee *et al*., 2007).

Studies have shown that under the environment of iron deficiency, *Rhizobium leguminosarum* (Storey *et al*., 2006) and *Bacillus megaterium* can both synthesize Schizokinen. However, *Paenibacillus Ash* strains (Hertlein *et al*., 2014), *Bacillus subtilis* (Grossman *et al*., 1993) and *Bacillus cereus* (Hayrapetyan *et al*., 2016) can synthesize catecholates Bacillibactin. *Bacillus anthracis* and *Bacillus cereus* can not only synthesize Bacillibactin, but also synthesize Petrobactin. The complete Bacillibactin and Petrobactin synthesis gene cluster have been found in the genome of *Bacillus thuringiensis* (Wilson *et al*., 2006; Hollensteiner *et al*., 2016). Bacterial siderophore is not only a wide variety of species, but also a variety of functions. The siderophores in biocontrol bacteria have strong antagonistic effects on a variety of pathogenic fungus. In China, the study of Chen *et al*. (2006) showed that the *Pseudomonas sp.* WCS358r strain had a potent inhibitory effect on the growth of chestnut germs and the germination of conidia, while the deletion mutant JM218 of siderophore had almost no inhibition effect. After adding 200 μmol/L $FeC1_3$ in the medium, inhibition ability of WCS358r on the pathogen was significantly reduced. It was shown that the siderophore produced WCS358r was an important factor to inhibit the growth of the mycelium and the germination of the conidia of the pathogen (Chen *et al*., 2006). The antagonistic experiment of Eucalyptus gray mold also showed that the siderophores of *Pseudomonas* was some other important factor controlling the Eucalyptus gray mold (Ran *et al*., 2005). Yu *et al*. (2017) reported that the siderophore synthesized by BAF.1 strain of *Pseudomonas Syringa* has strong antibacterial activity to *Fusarium spp*. and also has bacteriostasis to other 11 plant pathogenic fungus, which is to inhibit the germination of fungal spores and cause the change of hypha morphology (Yu *et al*., 2017). Sulochana *et al*. (2014) proved that the siderophores purified ferrite from KB culture medium of *Pseudomonas aeruginosa* strain JAS-25 showed strong antagonistic activity to *Fusarium spp*. and *Aspergillus* (Sulochana *et al*., 2014).

Brevibacillus brevis GZDF3 (CGMCC No. 10121) is an antagonistic bacteria (Zhu *et al*., 2017), Gram-positive bacteria, bacillus and spores separated from the rhizosphere soil of *Pinellia ternata* from Journal of Chinese Medicinal Materials in Guizhou province. The results showed that the strain not only had a strong antagonistic effect on the *Pectobacterium carotovorum subsp. carotovorum*, *Fusarium oxysporum* and *Fusarium solani*, but also a variety of pathogenic bacteria such as *Fusarium pepper*, *Curvularia leaf spot of maize* and *Villosiclava virens* and their antibacterial components are worth studying in depth (Shi *et al*., 2015). For this reason, we sequence the genome and found that there was a synthetic genetic cluster in the GZDF3 genome with 83% similarity of *Bacillus anthraci* Petrobactin synthesis, but no Bacillibactin synthesis gene cluster was found in the genome. Presently, no reports on the bacteriostasis of Petrobactin are reported. Therefore, this study will further use bioinformatics to analyze the gene cluster of Petrobactin synthesis of GZDF3 strain, construct the phylogenetic tree of synthetic gene, use the common CAS to detect siderophores and carry out quantitative analysis and bacteriostasis experiment, which lays the foundation for the later development and application.

Materials and Methods

Experimental Materials

Fusarium oxysporum and *Brevibacillus brevis* GZDF3 were isolated and preserved in the Department of Biotechnology, College of Biology and Engineering, Guizhou Medical University. Whole genome sequence of *Brevibacillus brevis* GZDF3 (accession number: LVYG00000000).

Genome Mining and Synthetic Gene Cluster Analysis of Siderophore Synthesis Gene of Brevibacillus brevis GZDF3

The whole genome sequence was used as material and the secondary metabolite prediction software AntiSMASH predicted the secondary metabolite synthesis gene cluster of *Brevibacillus brevis* GZDF3 (LVYG00000000) (Weber *et al*., 2015). ORFfinder and BlastP are further used to analyze gene clusters.

The local database of *Brevibacillus brevis* GZDF3 DNA sequence was constructed by sequence analysis software BioEdit 7.0.9.0. Taking the nucleotide sequence

of *asbABCDEF* gene of *Bacillus anthracis* Petrobactin in NCBI as the leader sequence (GenBank: AE017334.2). Blastn was used to search the DNA sequence database of *Brevibacillus brevis* GZDF3 for mining the synthetic gene. AsbABCDEF used DNAMAN 4.0 for amino acid sequences alignment (Hall, 1999).

Construction of Phylogenetic Tree

Multiple sequence comparison was carried out through the BlastP online search provided by NCBI with the homologous amino acid sequence to the AsbABCFEF strain of *Brevibacillus brevis* GZDF3. The Neighbor-Joining method (NJ) was utilized to construct the phylogenetic tree of six synthetic genes (Kummar *et al*., 2016). *Staphylococcus aureus* (BAX04613.1), *Streptomyces malaysiensis* (ATL83423.1), *Coccidioides immitis* RS (XP_001245522.2), *Streptomyces malaysiensis* (ATL83423.1), *Deinococcus maricopensis* DSM 21211 (ADV68839.1), *Neorhizobium galegae* bv. orientalis (CDZ62393.1) and *Streptomyces clavuligerus* (EFG10332.1) were selected separately as outgroup. The NJ tree was constructed with the programs MEGA 7.0.14. and use the self spreading value (Bootstrap) to test its reliability. The repetition time was 1000 for assessing the evolutionary relationship between different bacteria siderophore AsbABCDEF.

Synthesis and Type Identification of Siderophore from Brevibacillus brevis GZDF3

Synthesis of Siderophore

To screen for siderophore production, GZDF3 was inoculated in modified Sugar-Aspartic acid (SA) medium (20 g/L sucrose, 2.0 g/L asparagine, 0.5 g/L K_2HPO_4 and 0.5 g/L $MgSO_4$) and inoculation amount of 5% in a rotary shaker at 200 rpm and 28°C for 48 h. The supernatant was centrifuged at 12,000 r/min for 10 minutes and filtered with a 0.22 μm microporous membrane to remove bacteria. The production of siderophore was qualitatively determined and expressed as percent siderophore (SU) by CAS liquid detection according to the method described by Schwyn and Neilands (1987) and calculations using the following formula: SU = [(Ar-As)/Ar]×100%. Where Ar is the absorbance of reference and As is the absorbance of the sample at 630 nm. (Guo *et al*., 2016; Schwyn and Neilands, 1987).

Detection of Siderophore by Arnow Method

At first, 1 mL reagent containing nitrite molybdate with 1 mL of 1 M NaOH was added to 1 mL culture filtrate and 1 mL of 0.5 M HCl. The color change of the solution was observed. The catechol solution of 1 mL 100 μM was used as a positive control and water as negative control (Ramasamy *et al*., 2016).

Effect of Culture Time on the Synthesis of Siderophore by Antagonistic Bacteria GZDF3

GZDF3 was activated to prepare bacterial culture medium. The inoculation amount of 5% was connected to 250 mL SA medium at 28°C and 200 r/min cultured for 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h and 60 h respectively. The growth density cell was measured by the spectrophotometer at OD_{600} nm and CAS liquid detection method was used to initially detect the content of synthetic siderophore (Arora and Verma, 2017).

The Antimicrobial Activity of Antagonistic Bacterium GZDF3 and its Synthetic Siderophore to Fusarium oxysporum

Pathogenic fungi: Pathogenic fungi *Fusarium oxysporum* was isolated and preserved in the laboratory. It was inoculated in the PDA plate to activate 7 d and absorbed 1 mL aseptic water drops added to the purified fungal culture plate. The hypha was eluted and added to the 30 mL PDB liquid medium at 28°C and 200 r/min cultured for 24 h and then the mycelium suspension (1×10^8 /mL) was made with aseptic water for reserve.

Preparation of NA culture solution of antagonistic bacteria GZDF3: The seed solution was inoculated into 250 mL NA liquid medium (0.3% Beef Extract, 1% peptone, 0.5% NaCl) according to the inoculation amount of 5% and then was centrifuged at 12000 r/min for 10 min after 48 h on the rocking bed of 28°C and 200 r/min; the supernatant was taken for reserve. Preparation of SA culture solution of antagonistic bacteria GZDF3: The seed solution was inoculated into 250mL SA medium at 28°C and 200 r/min cultured for 48 h and centrifuged at 12000 r/min for 10 min. The supernatant was filtrated by 0.22 μm microporous membrane for filtration and bacteria removal and then reserve.

The agar punching method: The spore suspension was evenly coated on the PDA plate. Each plate was punched with a perforator at 2 cm from the center of the plate (diameter 8 mm) and each plate was hit 4 holes. 150 μL antagonistic NA culture and SA culture medium were added to each two holes respectively and 150 μL of uninoculated NA medium and SA medium were added to the two wells on the other plate. The size of the bacteriostasis was measured after constant temperature culture at 28°C for 1-2 d.

Results

The Mining of Antagonistic Bacteria GZDF3 Siderophore

In the genome of *Brevibacillus brevis* GZDF3, there are multiple clusters of genes encoded by different secondary metabolites, of which cluster 37 is a siderophore gene cluster as showed in (Fig. 1A). And it

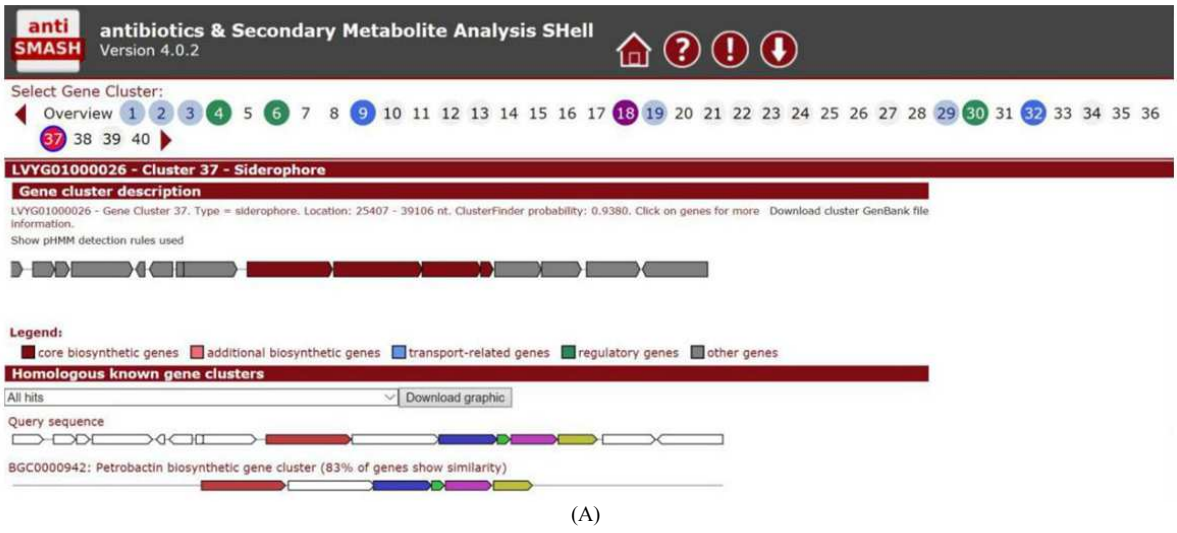
reaches 83% similarity to the biosynthetic gene cluster of *Bacillus anthraci* Petrobactin siderophore. Further analysis of the gene cluster by ORFfinder and BlastP revealed that the gene cluster had 15 ORF. The similarity was 54.66% between ORF8 and the synthetic gene AsbA of *Bacillus anthraci* Petrobactin siderophore, 43.63% between ORF9 and AsbB, 48.17% between ORF10 and AsbC, 51.65% between ORF11 and AsbD, 51.06% between ORF12 and AsbE and 52.14% between ORF13 and AsbE. The synthetic gene a*sbABCDEF* of *Bacillus anthraci* Petrobactin siderophore was used for the local Blast comparison with *Brevibacillus brevis* GZDF3 and it was found that it was corresponding to GENE5689, GENE5690, GENE5691, GENE5692, GENE5693 and GENE5694 of *Brevibacillus brevis* GZDF3 respectively as shown in (Fig. 1B).

Construction of Phylogenetic Tree

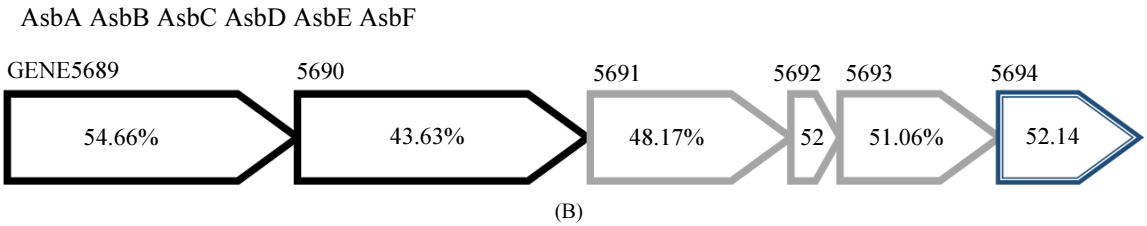
The NJ tree of AsbABCDEF is constructed by MEGA 7.0.14 software (Fig. 2). From the whole, the siderophore AsbABCDEF with different bacteria can be divided into two groups that the *Brevibacillus brevis* GZDF3 and the bacillus of short bacillus are clustered into a class and the *Bacillus anthracis*, *Bacillus thuringiensis* and *Bacillus cereu* are clustered into a class. AsbA, AsbB, AsbD and AsbE of *Brevibacillus brevis* GZDF3 were all clustered into a small branch with *Brevibacillus brevis* NBRC 100599, while AsbC was isolated to one branch; AsbF and *Brevibacillus brevis* ATCC 35690 were clustered into a branch. While on the other group, four synthetase genes of AsbABCD were all that *Bacillus anthraci* and *Bacillus thuringiensis* clustered into one branch and *Bacillus cereus* was isolated to the other branch. AsbE was that *Bacillus anthraci* and *Bacillus cereus* clustered into a branch and *Bacillus thuringiensis* was isolated to a branch. AsbF was that *Bacillus cereus* and *Bacillus thuringiensis* were clustered into a branch and *Bacillus anthracis* was isolated to a branch.

Synthesis and Identification of Siderophore

GZDF3 was cultured on the SA medium for 48 h, as depicted in (Fig. 3A), GZDF3 produced siderophores that are represented by dark red after specific color reaction of CAS detection solution. According to the formula SU = [(Ar-As) /Ar]×100%, the maximum siderophore production was obtained (SU=27.035%) after 48 h of incubation, which indicated that *Brevibacillus brevis* GZDF3 can synthesize siderophore. And using the Arnow's assay, we observed the formation of a red-orange color (Fig. 3B), which indicated that GZDF3 produced the catecholates species siderophore.

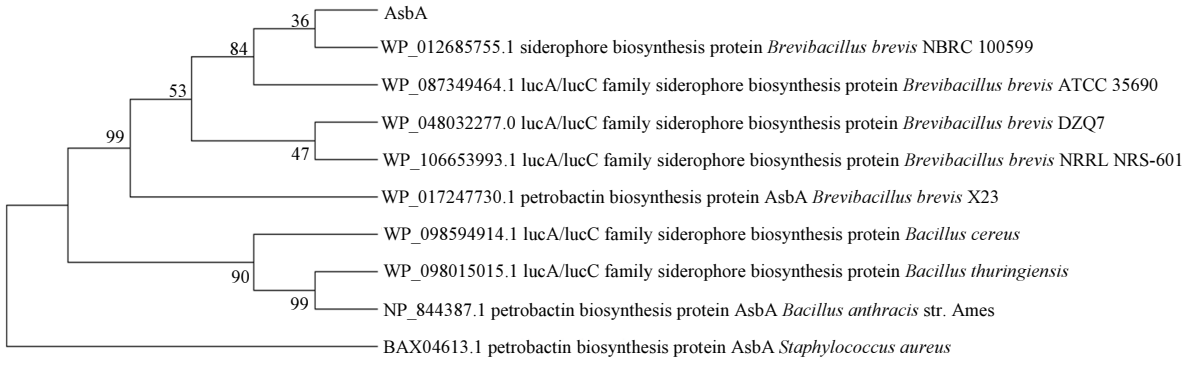


(A)

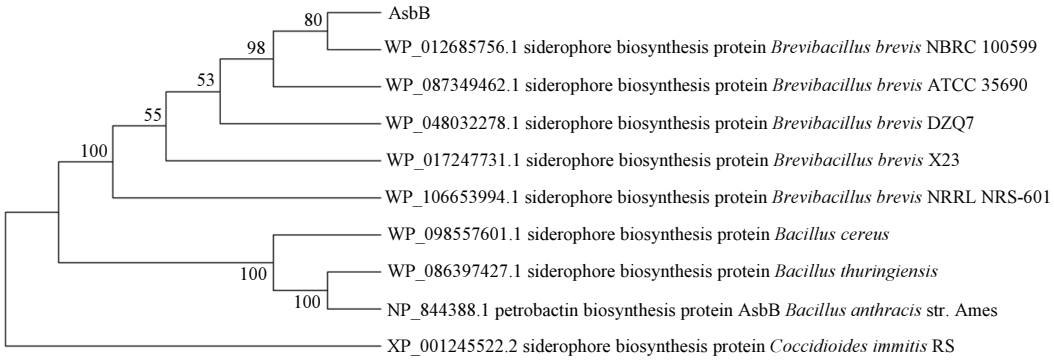


(B)

Fig. 1: (A) Analysis on the secondary metabolites of *Brevibacillus brevis* GZDF3 strain genome; (B) Target gene and synthetic gene of *Brevibacillus brevis* Petrobactin siderophores


AsbA
WP_012685755.1 siderophore biosynthesis protein *Brevibacillus brevis* NBRC 100599
WP_087349464.1 lucA/lucC family siderophore biosynthesis protein *Brevibacillus brevis* ATCC 35690
WP_048032277.0 lucA/lucC family siderophore biosynthesis protein *Brevibacillus brevis* DZQ7
WP_106653993.1 lucA/lucC family siderophore biosynthesis protein *Brevibacillus brevis* NRRL NRS-601
WP_017247730.1 petrobactin biosynthesis protein AsbA *Brevibacillus brevis* X23
WP_098594914.1 lucA/lucC family siderophore biosynthesis protein *Bacillus cereus*
WP_098015015.1 lucA/lucC family siderophore biosynthesis protein *Bacillus thuringiensis*
NP_844387.1 petrobactin biosynthesis protein AsbA *Bacillus anthracis* str. Ames
BAX04613.1 petrobactin biosynthesis protein AsbA *Staphylococcus aureus*
36
84
53
99
47
90
99

(A)


AsbB
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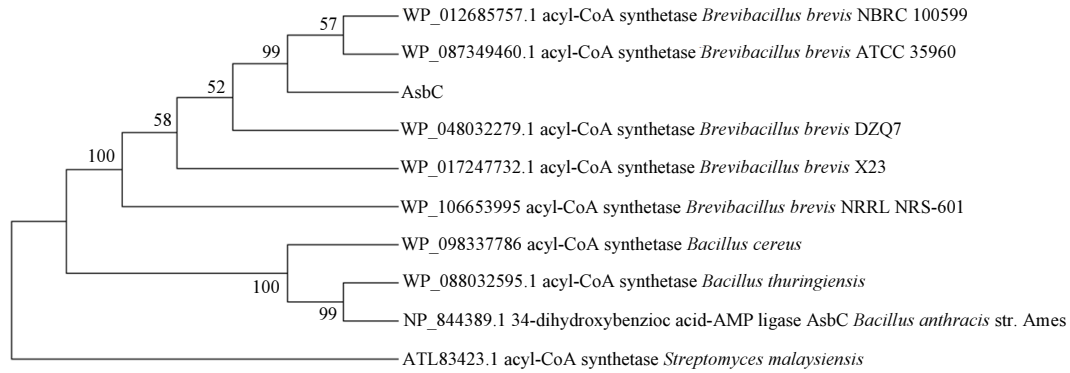
WP_087349462.1 siderophore biosynthesis protein *Brevibacillus brevis* ATCC 35690
WP_048032278.1 siderophore biosynthesis protein *Brevibacillus brevis* DZQ7
WP_017247731.1 siderophore biosynthesis protein *Brevibacillus brevis* X23
WP_106653994.1 siderophore biosynthesis protein *Brevibacillus brevis* NRRL NRS-601
WP_098557601.1 siderophore biosynthesis protein *Bacillus cereus*
WP_086397427.1 siderophore biosynthesis protein *Bacillus thuringiensis*
NP_844388.1 petrobactin biosynthesis protein AsbB *Bacillus anthracis* str. Ames
XP_001245522.2 siderophore biosynthesis protein *Coccidioides immitis* RS
80
98
53
55
100
100
100

(B)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WP_087349460.1 acyl-CoA synthetase *Brevibacillus brevis* ATCC 35960
AsbC
WP_048032279.1 acyl-CoA synthetase *Brevibacillus brevis* DZQ7
WP_017247732.1 acyl-CoA synthetase *Brevibacillus brevis* X23
WP_106653995 acyl-CoA synthetase *Brevibacillus brevis* NRRL NRS-601
WP_098337786 acyl-CoA synthetase *Bacillus cereus*
WP_088032595.1 acyl-CoA synthetase *Bacillus thuringiensis*
NP_844389.1 34-dihydroxybenzioc acid-AMP ligase AsbC *Bacillus anthracis* str. Ames
ATL83423.1 acyl-CoA synthetase *Streptomyces malaysiensis*
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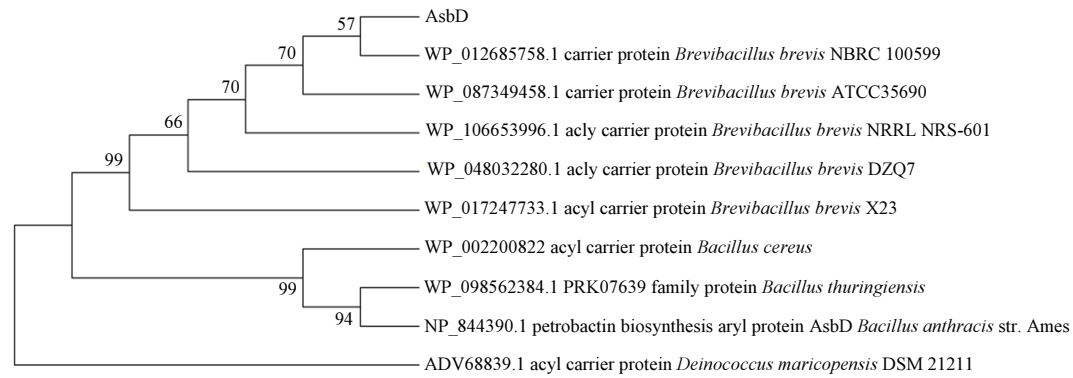
99
52
58
100
100
99

(C)


AsbD
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WP_087349458.1 carrier protein *Brevibacillus brevis* ATCC35690
WP_106653996.1 acly carrier protein *Brevibacillus brevis* NRRL NRS-601
WP_048032280.1 acly carrier protein *Brevibacillus brevis* DZQ7
WP_017247733.1 acyl carrier protein *Brevibacillus brevis* X23
WP_002200822 acyl carrier protein *Bacillus cereus*
WP_098562384.1 PRK07639 family protein *Bacillus thuringiensis*
NP_844390.1 petrobactin biosynthesis aryl protein AsbD *Bacillus anthracis* str. Ames
ADV68839.1 acyl carrier protein *Deinococcus maricopensis* DSM 21211
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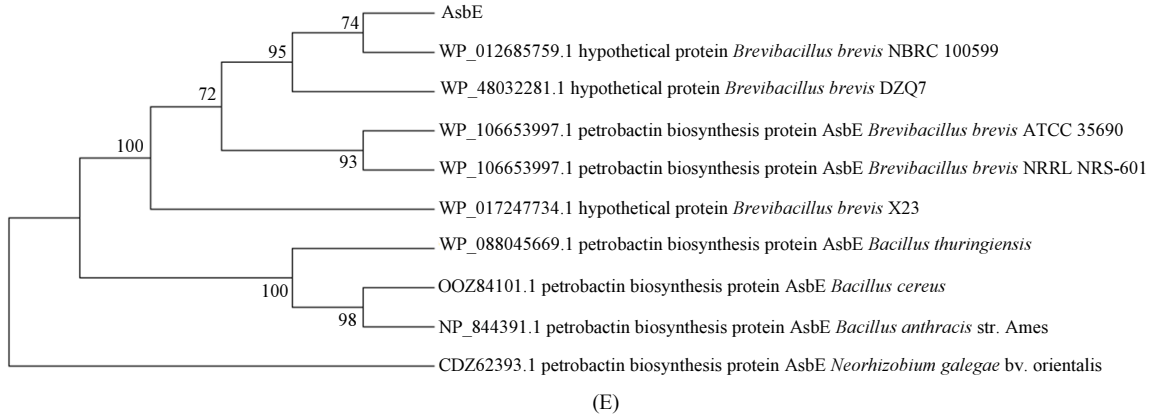
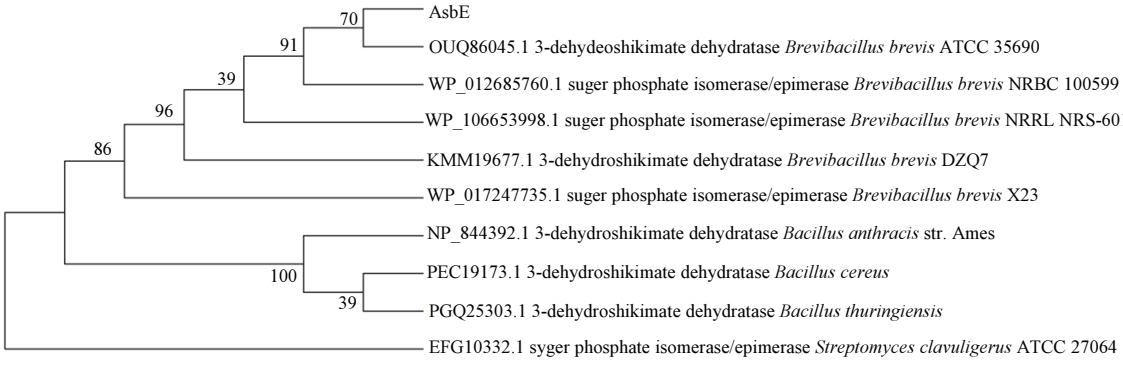
70
70
66
99
99
94

(D)



(E)



(F)

Fig. 2: (A) The Phylogenetic tree of siderophore synthetase AsbA; (B) The Phylogenetic tree of siderophore synthetase AsbB; (C) The Phylogenetic tree of siderophore synthetase AsbC; (D) The Phylogenetic tree of siderophore synthetase AsbD; (E) The Phylogenetic tree of siderophore synthetase AsbE; (F) The Phylogenetic tree of siderophore synthetase AsbF

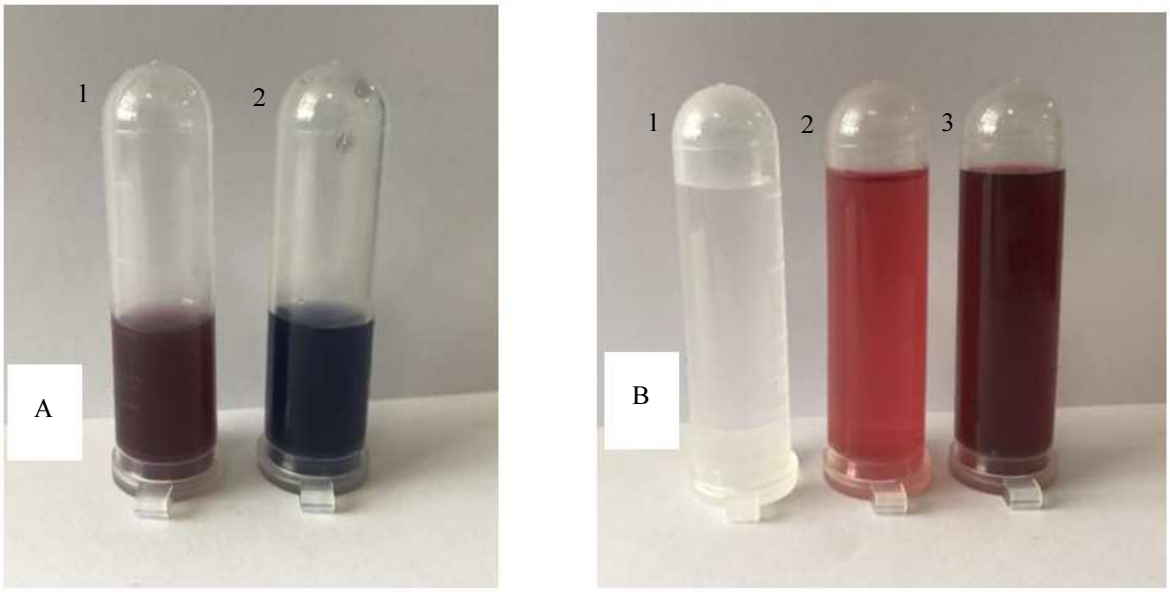


Fig. 3: Synthesis and type detection of siderophore; (A) CAS liquid detection: 1. Iron free fermentation liquid supernatant; 2. Negative; (B) Arnow experiment: 1. Negative; 2. Iron free fermentation liquid supernatant; 3. Catechol solution

Optimum Culture Time for the Synthesis Siderophore of Bacillus Subtilis GZDF3

As shown in (Fig. 4), the synthesis of GZDF3 siderophore in the SA medium was affected by the incubation time and it reached two peaks when the incubation time was 30 h and 48 h. Meanwhile, the activity unit SU of the siderophore was 24.09% and 24.20% at OD_{630} nm respectively and the synthesis of siderophore was not detected during 0-18 h. And the growth curve of *Brevibacillus brevis* GZDF3 cultured in SA medium showed that it was basically consistent with the four growth stages of bacteria at OD_{600} nm.

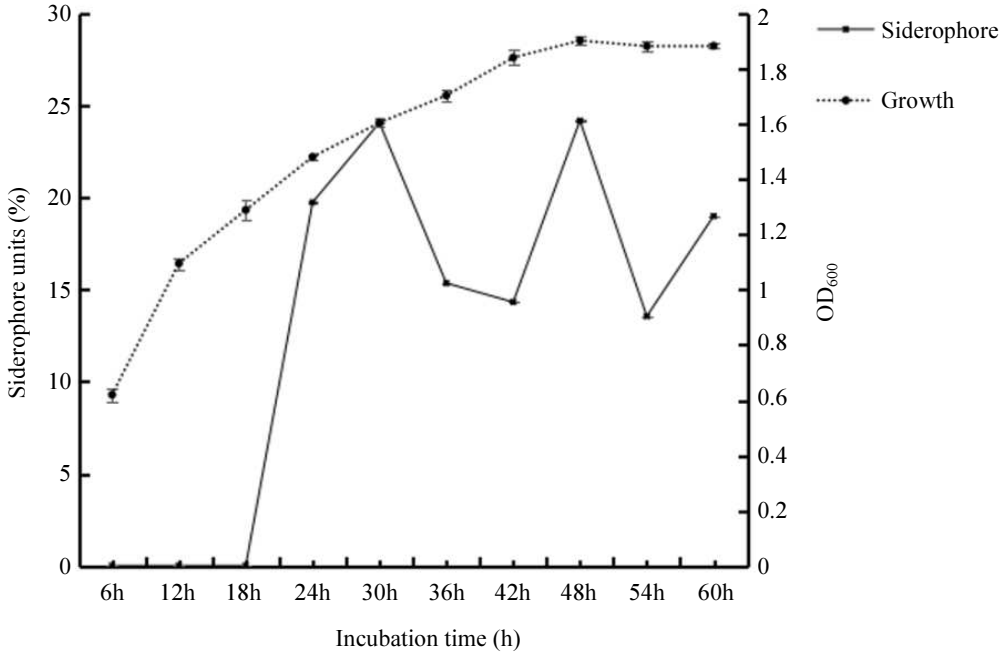


Fig. 4: Variation of siderophore production and cell growth with different incubation time; Error bars represent standard deviations of three replicates

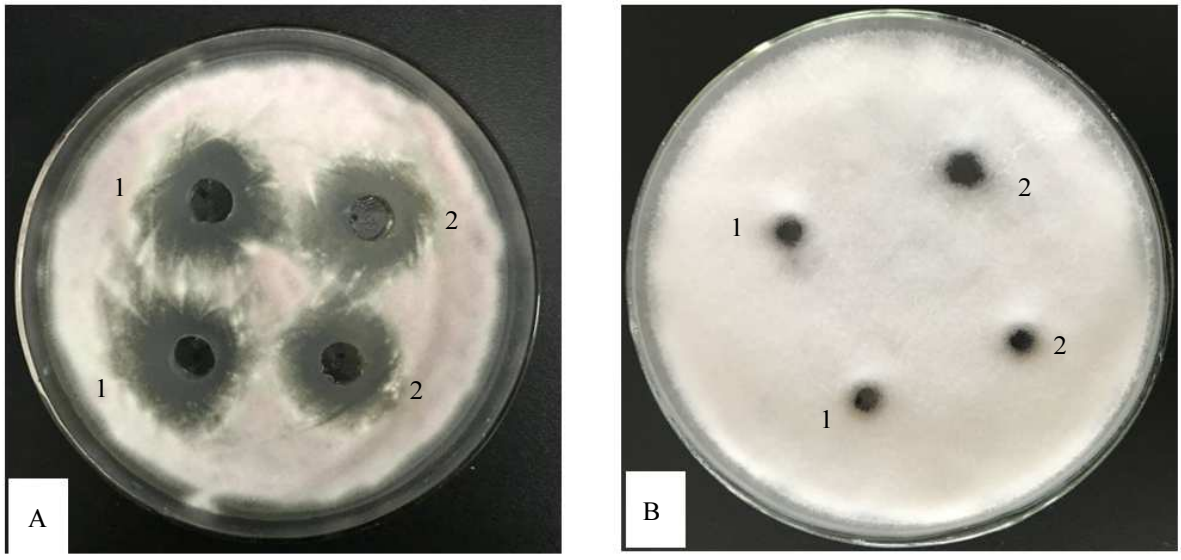


Fig. 5: The antagonistica activity of *Brevibacillus brevis* GZDF3 (A) Fermentation liquid medium: 1. antagonistic NA culture; 2. antagonistic NA culture; (B) Uninoculated liquid medium: 1. NA culture; 2. SA culture

Bacteriostatic Activity of the Siderophore Synthesized by Bacillus Subtilis GZDF3 on Fusarium Oxysporum

As shown in (Fig. 5A1), the GZDF3 has a strong antagonism to *Fusarium oxysporum* that was one of the Pathogenic fungus causing rotted root on *Pinellia ternata* and the inhibition zone was 18-20 mm; while the bacteriostasis of the siderophore fermented and synthesized by iron free medium on *Fusarium oxysporum* was shown in (Fig. 5A2) and the inhibition zone was 15-16 mm. As shown in (Fig. 5B), uninoculated NA and SA liquid medium were none inhibition zone. The results showed that siderophore had obvious bacteriostasis on *Fusarium oxysporum* of Pathogenic bacteria of rotted root on *Pinellia ternata.*

Conclusion

Petrobactin siderophore was first found in *Marinobacter hydrocarbonoclasticus* (Barbeau *et al*., 2002). It was further found that the *Bacillus anthraci* strains and *Bacillus cereus* strains can also synthesize Petrobactin (Wilson *et al*., 2006). For *Bacillus anthracis*, it can synthesize Petrobactin and Bacillibactin. Petrobactin is the main siderophore in *Bacillus anthracis*. Because it is not identified by the innate immune system and it has been found that the Petrobactin is the cardinal factor in the anthrax model, most of which is the mixed type of catechol-carboxylate siderophore (Nusca *et al*., 2012). It appears that Bacillibactin has higher importance in *Bacillus cereus* on insect model experiments (Hayrapetyan *et al*., 2016). In this study, a siderophore synthesis gene cluster with Petrobactin similarity of 83% was discovered, while no Bacillibactin siderophore was found. At present, functions of Petrobactin and Bacillibactin synthetic genes *asbABCDEF* and *dhbBCF* have been researched and reported (Segond *et al*., 2014). Cendrowski *et al*. (2004) reviewed the functions of gene *asbA* by directional gene knockout. Under the condition of iron deficiency, the *asbA* deletion mutant showed a decrease in the siderophore yield and a weaker growth. Moreover, the pathogenicity of *asbA* deletion mutant mice was also significantly reduced (Cendrowski *et al*., 2004). Nusca *et al*. (2012) realized the heterologous expression of Petrobactin siderophore by using citric acid, spermidine and 3, 4-two hydroxybenzoic acid substrates. It is found that the function of AsbA synthase can be compensated by AsbB. The strain deletion of AsbA synthetase with the other five genes can also synthesize part of Petrobactin (Nusca *et al*., 2012). The *asbE* gene deletion cannot synthesize Petrobactin siderophore showed that *asbE* gene was also a key gene in the pathway of Petrobactin siderophore synthesis. The function and structure of *asbB* (Nusca *et al*., 2012), *asbD* (Schmelz *et al*., 2009) and *asbF* (Pfleger *et al*., 2008) in Petrobactin siderophore synthesis gene cluster have been investigated. Domagalski *et al*. (2013) determined the crystal structure of different chorismic acid synthetase DhbC of Bacillibactin siderophore in *Bacillus anthraci*. DhbC participated in the biosynthesis of 2, 3-two hydroxybenzoate (DHB) and methyl naphthoquinone (MK) of the respiratory chain group; the consumption of DhbC led to the deficiency of DHB, which showed the importance of DhbC in siderophore biosynthesis (Domagalski *et al*., 2013). Hertlein *et al*. (2014) studied the deletion of DhbF by gene inactivation mutagenesis and confirmed that *dhb* gene cluster was in charge of the synthesis of Bacillibactin siderophore (Hertlein *et al*., 2014). The study predicted that *Brevibacillus brevis* GZDF3 can synthesize Petrobactin siderophores. Comparison analysis showed that the similarity of GZDF3 strains and *Bacillus anthraci* Petrobactin siderophore synthetase AsbA, AsbB, AsbC, AsbD, AsbE, AsbF were 54.66%, 43.63%, 48.17%, 51.65%, 51.06%, 52.14% respectively. The phylogenetic tree of six siderophores synthesis AsbABCDEF showed that the siderophore synthetase was mainly divided into two groups and the siderophore synthetase of *Brevibacillus brevis* genus was clustered into one branch and the other bacterial sources were gathered into the other branch. Among the different bacterial sources, except that AsbF synthetase was *Bacillus anthraci* clusters alone, AsbE synthase was that *Bacillus anthraci* and *Bacillus cereus* clustered into a branch, the other four synthases was all that *Bacillus anthraci* and *Bacillus thuringiensis* clustered into a branch. It is identified by CAS liquid detection and Arnow's assay that the siderophore synthesized by *Brevibacillus brevis* GZDF3 was catecholates but the type synthesized by *Bacillus anthraci* was a mixture of catechol-carboxylate.

Siderophore in biocontrol bacteria has strong antagonism against various pathogenic fungus, while the inhibition on Petrobactin siderophore is not yet available. Li *et al*. (2014) found that *Bacillus amylus* SQR9 has a broad-spectrum antifungal activity. When six fungal pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani* were found, five synthetic genes of Bacillibactin siderophores in the strains all express up-regulation. While in the mutant strains with deficiency of Bacillibactin synthetic gene, the antifungal activity decreased or even no antibacterial activity. It indicates that Bacillibactin siderophore plays a major role in the control of fungal diseases (Li *et al*., 2014). *Bacillus anthracis* Petrobactin siderophore is closely related to the virulence of *Bacillus anthracis* (Cendrowski *et al*., 2004) and the Petrobactin siderophore is a NRPS-NIS mixed. However, there is no report on the bacteriostasis of Petrobactin siderophore in the biocontrol bacteria. In this study, the GZDF3 strain used iron free medium to

promote the synthesis of siderophores and its supernatant showed strong antagonistic activity to *Fusarium oxysporum*. Based on this, we believe that the synthesis regulation and bacteriostasis activity of *Brevibacillus brevis* GZDF3 siderophore are worthy of further studys.

This study preliminaries revealed that the mining and identification of *Brevibacillus brevis* GZDF3 to Petrobactin siderophore and the strong bacteriostasis to Pathogenic fungi *Fusarium oxysporum*, which provides the basis for further separation and purification and functional verification, especially in the prevention and control of fungi in Guizhou medicinal herbs. Meanwhile, it has great significance to the protection and further development and application of traditional Chinese medicinal materials.

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Author's Contributions

Miaomiao Sheng and Huake Jia: Contributed to the planning and implementation of this study as well as interpretation of article preparation and drafted the manuscript.

Xiaomai Tao, Lina Zeng, Tingting Zhang, Zuquan Hu and Zhu Zeng: Coordinated the data-analysis and assisted in the writing of the manuscript.

Hongmei Liu: Contributed to the planning and implementation of research work and revising the article.

Conflict of Interest

The authors declare that they have no competing interests. The corresponding author affirms that all of the authors have read and approved the manuscript.

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