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Analysis of the Ketosynthase Genes in *Streptomyces* and Its Implications for Preventing Reinvestigation of Polyketides with Bioactivities

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Abstract

Cucumber wilt by *Fusarium oxysporium* f sp. *cucumarinum* is one of the most important soil-borne diseases. Among control strategies for plant soil-borne pathogen, biocontrol systems eliminate neither pathogen nor disease but bring them into natural balance. Polyketides form the largest part of the known natural products, and most of them come from actinomycetes especially streptomyces. Analysis for the ketosynthase genes in streptomyces can implicate new polyketides. In the course of the screening for producers of polyketides with antifungal activities, 117 strains were isolated. The screening program was performed by means of PCR amplification using degenerated primers corresponding to type II ketosynthase (KS) genes. Among 117 isolates, 33 isolates showed antifungal activities and 17 isolates showed positive amplification signal. Antagonism of the 17 isolates against *Fusarium oxysporium* f sp. c*ucumarinum*, *Bacillus subtilis and Erwinia carotovora* subsp. *carotovora* in vitro were analyzed. The diversities of KS-II gene from the 17 isolates were abundant based on phylogenetic tree analysis. The 17 isolates were divided into 6 clades based on KS-II gene sequence. The results showed that different isolates which belong to the same species present different antagonism activities and also the different streptomyces species showed different bioactivities. Among 17 isolates, isolates DQ1, DQ23, GAN1, HVG60 and HVG71 have the potential ability to produce new type polyketides. This method may not only prevent reinvestigation to find bioactive molecules described previously, but also alleviate some of the biases introduced by using conventional cultivation techniques.

Keywords: diversity, *Streptomyces*, polyketide, ketoacyl-synthase (KS), antifungus

1. Introduction

Plant soil-borne disease by pathogen fungi is one of the most stubborn diseases. Cucumber wilt by *Fusarium oxysporium* f sp. *cucumarinum* is one of the most important soil-borne diseases. Among control strategies on plant soil-borne pathogen, Chemical control made a great contribution to suppressing occurrence of soil-borne disease and reducing its damage. But due to the negative effect of chemical control, for example, environment pollution, the security of human and livestock, new control strategies were needed to be discussed. Crop rotation, graft cultivation and biological control are effective approaches, but crop rotation and graft cultivation mean extensive fields and much more financial investment. While biocontrol systems do not eliminate neither pathogen nor disease but bring them into natural balance. Polyketides which exhibit various biological activities are structural diverse secondary metabolites from microorganisms, and is the largest part of the known natural products (Metsa-Ketela et al., 1999). Polyketides have attracted great attention since these compounds have been widely applied as antibacterial drugs in medicine. Recently, some of polyketides such as validamycin, venturicidin, trichodermin, nikkomycin showed fine activities against plant pathogen fungi and have been widely applied as important biocontrol products (Rhodes et al., 1961; Bago et al., 1996; Yang et al., 2010; Qian et al., 2011). Most of polyketides have been isolated from *Streptomyces*, so *Streptomyces* play a key role due to their ability to produce numerous different polyketides. And the genome analyses of *Streptomyces* spp. demonstrated that the isolates possess many PKS genes with unknown functions (Bentley et al., 2002; Ikeda et al., 2003). Thus, *Streptomyces* seem to have the possibility to produce hitherto unidentified polyketides against plant pathogen fungi.

In the procedure of polyketides biosynthesis, the condensation of acyl-thioester molecules which is catalyzed by polyketide synthase (PKS) has a similar mechanism comparing with fatty acid biosynthesis. Three types of PKSs have been known to be significantly different from each other in their structures and functions (Katz and Donadio, 1993). This work focused on type II minimal PKS. Recent molecular approaches have successfully revealed the presence of large numbers of cryptic PKS gene in the genome of *Streptomyces* spp. Since the presence of novel PKS genes in organisms provides good clues for production of novel polyketides, the sequencing of PKS genes is often used as a screening method for the discovery of novel polyketides (Ayuso et al., 2005; McAlpine et al., 2005; Banskota et al., 2006). The objective of this work was to assess the use of a PCR-based technique in the preliminary screening for microorganisms which were as producer of potential novel polyketide against plant pathogen fungi.

2. Materials and Methods

2.1 Actinomyces Isolates

Three different soil samples collected from northeast of China were used to isolate A*ctinomyces* strains. *Actinomyces* strains were isolated by serial dilution method. Based on colonies morphology and color, potential *Actinomyces* colonies were streaked in further isolation and purification. The isolates NEC-782, NEC-738, NEC-576, NEC-223 came from China General Microbiological Culture Collection (CGMCC).

2.2 Antifungal Assays in Vitro

Antagonism of the isolates against fungus was carried out *in vitro.* Mycelia-disks were prepared from growing margin of cultures of A*ctionmyces* isolates and placed on the centre of potato dextrose agar (PDA) plate. Meanwhile, fungal mycelia-disks of *F. oxysporium* f sp. c*ucumarinum* were placed on sides around the PDA plate at 30 mm distance to the mycelia-disk of the isolate. These plates were incubated at 25°C for 96 h. Antagonism of the actinomyces isolates against fungi was determined according to the presence of zones of growth inhibition. The isolates which showed activities against fungi were selected and transferred to corresponding isolation medium slants for further investigation.

2.3 Degenerate Primers

To clone the putative KS homologues of the type II PKS involving biosynthesis of polyketides, a PCR-based protocol was used. According to the relatively high DNA sequence identity of the type II KS in A*ctinomyces*, the degenerating primers were designed after aligning DNA sequences of eleven KS gene from *Actinomadura verrucosospora* (AB019690), *S. argillaceus* (X89899), *S. tendae* (AJ630301), *S. fradiae* (X87093), *S. murayamaensis* (AY228175), *S. coelicol*orA3(2) (X63449), *S. violaceoruber* (X16144), *S. rimosus* (DQ143963), *S. glaucescens* (X15312), *S. venezuelae* (AF126429) and *S. antibioticus* (AJ632203). A portion of PKS- II gene was amplified using the degenerate primers named KS-P1 (5′-TCWCVCCBATCRYSRTSGCSTG-3′) and KS-P2 (5′-GCMGATSGCICCSAGBGAGTG-3′).

2.4 Genomic DNA Extraction and PCR Reactions

Genomic DNA of the isolates which showed activities against fungi were extracted as previously described (Hopwood et al., 1985). The procedure of PCR was performed in a 20 μl reaction mixture containing 10×LA Buffer 3 μl, 200 μmol/l dNTP 2 μl, 1.5 mM $MgCl_2$ 1.5 μl, 1μmol/l of each primer 1.5 μl, 2.5 U Taq DNA polymerase 0.5 μl (TaKaRa Biotechnology (Dalian) Co., Ltd, Japan) and approximately 100 ng of genomic DNA 1.5 μl. PCR conditions were as follows: 5 min at 95°C followed by 40 cycles for 1 min at 95°C, 1 min at 65°C, and 2 min at 72°C. The annealing temperature was optimized to 65°C while the remaining parameters kept identical. A 5-min extension step at 72°C was applied at the end of the PCR program.

The amplification fragments were cloned into the pMD19-T vector (TaKaRa Biotechnology (Dalian) Co., Ltd.), which was followed by nucleotide sequence analysis. The sequence homology search was carried out using BLAST in NCBI, and all the sequences were submitted to GenBank. DNA sequences obtained in this work were deposited in GenBank under accession numbers shown in Table 1. Neighbor-Joining trees were generated using MEGA 4.1by the Tajima-Nei distance model.

2.5 Identification of Actinomyces Isolates

Identification of isolates which gave positive amplification signal was carried out by morphological, chemical and molecular methods. Morphological characterization was based on the description of Shiring and Gotlieb (Shiring & Gottlieb, 1966). Cell-wall amino acid analysis was performed with the methods of Becker (Nozawa et al., 2007). Genomic DNA extraction was done as described previously (Tripathi & Rawal, 1998). And the complete 16S rDNA gene was amplified with a pair of universal primers A

(5′>AGAGTTTGATCCTGGCTCAG<3′) and B (5′>AAGGAGGTGATCCAGCCGCA<3′). After sequencing the PCR products, similar sequences were searched in GenBank by BLAST.

2.6 Antibacterial Assays in Vitro

Antibacterial assays of the isolates which gave positive amplification signal were determined. Sterilized nutrient agar (60 ml) were heated up to completely melt and then gradually cooling to 50℃, rapidly mixed with 10 ml of the suspension of *E. carotovora* subsp. *carotovora* and *B. subtilis* (10^8 cell/ml) respectively before pouring the plates (200 mm × 150 mm) and then solidified. Mycelia-disks of isolates were put on the centre of plates respectively. All of plates were incubated at 28℃ and 37℃ for 24 h respectively, and antagonism was determined by measuring the diameter of inhibition zones. The corresponding medium instead of the mycelia-disk of the isolates was used as control. The entire experiment was repeated three times.

3. Results

3.1 Isolation of the Actinomyces

A total of 117 *actinomyces* isolates were randomly selected and isolated basing on their colony morphology and color. All isolates were identified as A*ctionmyces* according to morphological, physiological characteristics (data not shown).

Antifungal assays *in vitro*

33 *actinomyces* isolates including the isolates from CGMCC showed antifungal activity against *F. oxysporium* f sp. c*ucumarinum*. Among of them, 6 isolates which the diameter of inhibition zones against fungi were more 10mm were showed in figure 1. The 6 isolates included isolates H86, T12-24, NEC-576, NEC-782, HVG60 and HVG71.

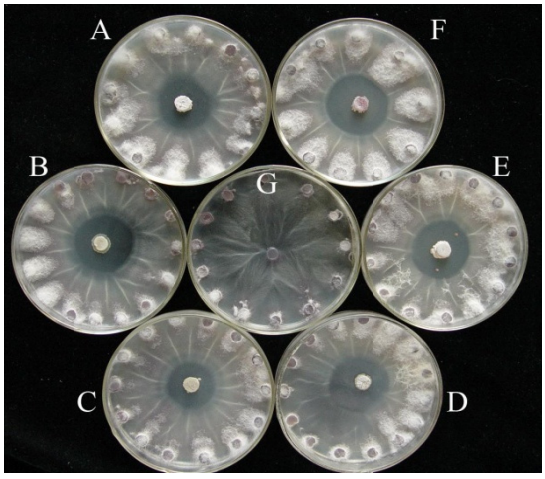

Figure 1. Antagonism of six isolates which the diameter of inhibition zones against *F. oxysporium* f sp. c*ucumarinum* were more 10mm (capital A indicate the isolate H86; capital B indicate the isolate T12-24; capital C indicate the isolate HVG60; capital D indicate the isolate NEC-782; capital E indicate the isolate NEC-576; capital F: the isolate HVG71; capital G: the corresponding medium was used as control)

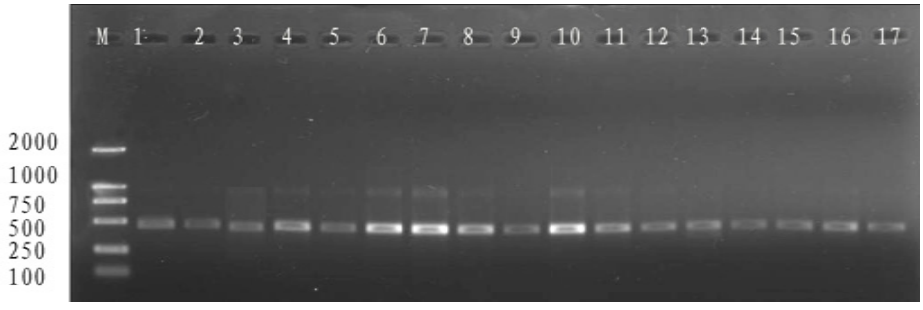

Figure 2. The amplification of DNA fragments of expected size with DNA from 17 isolates by the designed primer pairs (line M: Marker DL2,000; line 1-17: The PCR products of similar size with DNA isolated from the isolates DQ1, DQ7, DQ23, GAN1, G30, G32, GT1H, H86, HVG60, HVG71, NEC-782, NEC-738, NEC-576, NEC-223, T12-24, T12-209, and YL-1)

3.2 PCR Production and Analysis of Nucleotide Sequence

Among 33 *Actinomyces* isolates, DQ1, DQ7, DQ23, GAN1, G30, G32, GT1H, H86, HVG60, HVG71, NEC-782, NEC-738, NEC-576, NEC-223, T12-24, T12-209, and YL-1 gave positive amplification signals (Figure 2), the amplification of DNA fragments of expected size (~470bp) with DNA from the 17 isolates were obtained in the standard PCR protocol. All sequences from the 17 isolates were accordance with DNA sequence homology with the KS-II gene after performing BLAST search. And all of sequences were deposited in the target domain of KS-II gene comparing with known sequences in databanks. The KS-II sequences were deposited in the GenBank database and the accession number were showed in the table 1.

Table 1. The nearest phylogenetic neighbor, similarity and bioactivities based on 16S rDNA and KS gene sequence from 17 isolates

Clades	Name, metabolites, bioactivity *	Isolates No.	Accession No. #	Nearest phylogenetic neighbor △	Bioactivities ▲	★	◇
A	*S. violaceoruber* Granaticin with activity against G+ bacteria	DQ7	FJ620886	*S. aurantiacus* (99%)	++	+	-
		G30	FJ878809	*S. fradiae* (100%)	++	+	-
		H86	FJ620888	*S. fradiae* (100%)	+++	+	-
		NEC-223	EU285547	*S. venezuelae* (99%)	+	++	+
		T12-24	EU382732	*S. fradiae* (99%)	++++	+	-
		T12-209	GQ495635	*S. fradiae* (100%)	+	+	-
		YL-1	GQ495638	*S. fradiae* (99%)	+	+	-
B	*S. halstedi* carbomycin and deltamycins	NEC -782	EU252154	*S. ambofaciens* (99%)	++++	+	+
		NEC -738	EU250989	*S. glaucescens* (100%)	+	+	+
C	*S. anulatus* dihydroabikoviromycin with antifungal activity	HVG60	FJ878805	*S. olivochromogenes* (99%)	+++	-	-
D	*S. collinus* collinomycin with activities against G+ bacteria	GT1H	FJ878810	*S. rishiriensis (99%)*	++	+	-
		NEC -576	EU285546	*S. antibioticus* (99%)	++++	+	-
E	*S. hachijoensis, S. griseus* subsp. *Formicus*, Trichomycin and hedamycin	DQ1	FJ878801	*S. fradiae* (99%)	+	+	-
F	Independent clade	DQ23	FJ878802	*S. graminearus* (99%)	+	++	-
		G32	FJ620892	*S. fradiae* (99%)	+	+	+
		GAN1	FJ878808	*S. fradiae* (99%)	+	+	+
		HVG71	FJ620889	*S. fradiae* (99%)	++++	+	-

*: Name of the closest match type strains on KS sequence and metabolites and bioactivity of the closest match type strains; #:Accession No. for KS genes in NCBI; △: Nearest phylogenetic neighbor and its (%) similarity based on 16S rDNA sequence; ▲: Bioactivities against *F. oxysporium f sp. cucumarinum*; ★: Bioactivities against *B. subtilis*; ◇: Bioactivities against *E. carotovora* subsp. *carotovora*

+ indicate diameter of zones of growth inhibition was less 5 mm; ++ indicate diameter of zones of growth inhibition was between 5-10 mm; +++ indicate diameter of zones of growth inhibition was between 10-15 mm; ++++ indicate diameter of zones of growth inhibition was more 15 mm; - indicate no antagonism against microorganism

Phylogenetic trees was constructed based on KS gene sequences from the 17 isolates and the nearest phylogenetic neighbour in databanks in the Genbank. Phylogenetic relationship of the KS genes from the 17 isolates and known homological KS sequences in databanks were sorted to six different clades, showing the abundant diversity among the 17 streptomyces isolates (Figure 3). Among these clades, each clade had at least one characteristic KS gene sequence different from others. Figure 3. Phylogenetic relationships of the KS sequences from 17 isolates and the nearest phylogenetic neighbor. Neighbor-Joining trees were generated using MEGA 4.1by using the Tajima-Nei distance model.

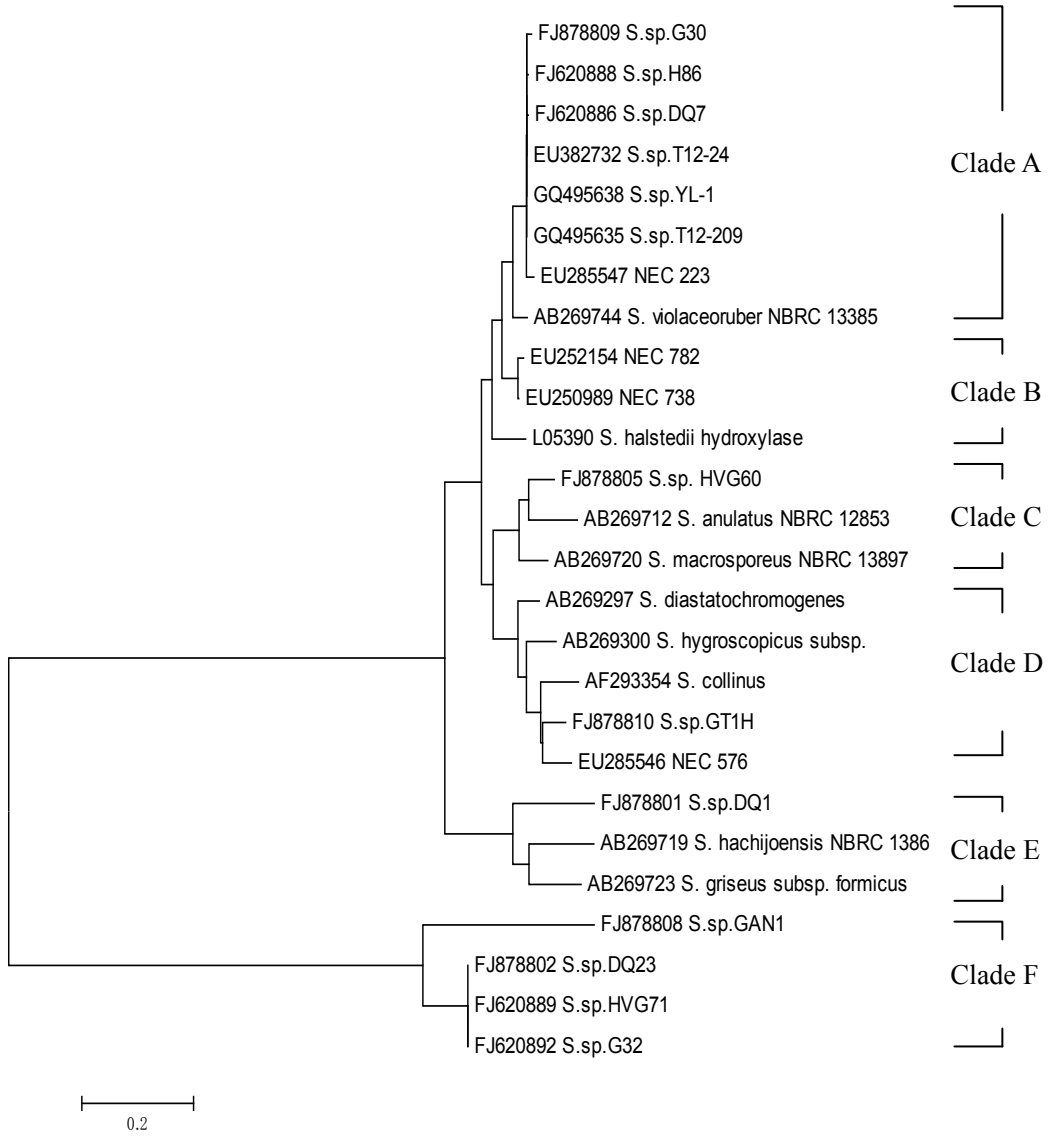

Figure 3. Phylogenetic relationships of the KS sequences from 17 isolates and the nearest phylogenetic neighbor. Neighbor-Joining trees were generated using MEGA 4.1by using the Tajima-Nei distance model

According to the morphology, culture characteristics and 16S rDNA gene sequences, 17 isolates were belong to *Streptomyces* (Table 1). The antagonism of 17 streptomyces isolates which gave positive signal was indicated that isolates GAN1, G32, NEC-738, NEC-782, NEC-223 showed antagonism against *E. carotovora* subsp. *carotovora* on nutrient agar, isolates DQ1, DQ7, DQ23, GAN1, G30, G32, GT1H, H86, HVG71, NEC-782, NEC-738, NEC-576, NEC-223, T12-24, T12-209, and YL-1 inhibited growth of *B. subtilis* (Table 1).

The results showed that the KS sequences from isolates DQ7, G30, H86, NEC223, T12-24, T12-209 and YL-1 showed sequence homology with *S. violaceoruber* (Figure 3, clade A), and *S. violaceoruber* type strain could produce granaticin which showed the activities against gram positive bacteria. The isolates NEC 782, NEC 738 and *S. halstedii* were classified to clade B, and *S. halstedii* type strain can produce carbomycin and deltamycins. Isolates HVG60 and *S. anulatus* were formed a small clade C, and *S. anulatus* type strain could produce antifungal compound dihydroabikoviromycin. The KS sequences from isolates GT1H, NEC576 and *S. collinus* formed clade D, and *S. collinus* type strain can produce collinomycin which showed activities against positive bacteria (Zhang & Yao 2005). The KS sequences from isolate DQ1 showed the sequence homology with *S. hachijoensis* and *S. griseus* subsp. *formicu*s and they formed clade E. *S. hachijoensis* could produce trichomycin belonging to type I polyketides, and *S. griseus* subsp. *formicu*s could produce hedamycin belonging to type II

polyketides (Hosoya et al., 1955; Javidpour et al., 2011). The KS sequences from isolates DQ23, G32, HVG71 and GAN1 were formed the clade F which was independent clade from other KS genes, especially isolate GAN1 formed an unattached branch.

According to the morphology, culture characteristics and 16S rDNA gene sequences, isolates DQ1, GAN1, G30, G32, H86, HVG71, T12-24, T12-209, and YL-1 were all belong to *S. fradiae* (Table 1). Polyketides that *S. fradiae* produced were abundant such as tylosin, B-587 and B-62169. tylosin, B-587 and B-62169, however, which show antagonism against bacteria, don't show antifungal activity. Moreover, isolates DQ1, GAN1, G30, G32, H86, HVG71, T12-24, T12-209, and YL-1 presented antagonism against gram positive bacteria, the results were consistent with the fact that polyketides produced by *S. fradiae* show antagonism against gram positive bacteria by literature. Meanwhile, *S. fradiae* could produce fradicin which showed antifungal activity, but fradicin was not belong to polyketide (Cundliffe et al., 2001; Zhang & Yao, 2005). So the isolates DQ1, GAN1, G30, G32, H86, HVG71, T12-24, T12-209, and YL-1 were capable of producing antifungal compound which may not belong to polyketides. On the basis of phylogenetic tree, although species were different, isolates DQ7, G30, H86, NEC223, T12-24, T12-209 and YL-1 had the potential ability which could produce the similar polyketide. During synthesis process of type II polyketides, these isolates may have a similar keto-group reductive mechanism with *S. violaceoruber*. *S. anulatus* type strain produce dihydroabikoviromycin showing antifungal activity, but dihydroabikoviromycin does not belong to type II polyketide (Holmalahti et al., 1998). Isolate HVG60 showed strong antagonism against *F. oxysporium* f sp. c*ucumarinum*, however, it didn't showed any activity against *B. subtilis and E. carotovora* subsp. *carotovora.* Isolate HVG60 is belong to *S. olivochromogenes* which can produce oleandomycin, chromomycin A3 and succinimycin Oleandomycin, chromomycin A3 and succinimycin show antagonism against bacteria and do not show inhibiting activity to fungi. So the antifungal compound from isolate HVG60 is very interesting and isolate HVG60 should be studied in the future. Synthesis mechanism of compounds against positive bacteria from isolates GT1H and NEC576 are similar to collinomycin belong to analog of type II polyketide (Zhang & Yao, 2005; Wu et al., 2008). Isolate GT1H is belong to *S. rishiriensis* which produce mycotrienin, coumermycin A1 and AT265. Mycotrienin shows antagonism against fungi, and coumermycin A1 and AT265 show antagonism against bacteria. Mycotrienin is belong to type I polyketides, coumermycin A1 is belong to type II polyketides, and AT265 is belong to nucleoside antibiotics (Claridge et al., 1984). NEC576 is *S. antibioticus* which could produce actinomycin which belong to type II polyketides (Alekhova and Novozhilova, 2001).

Isolate DQ1 showed the sequence homology with *S. hachijoensis* which could produce trichomycin belonging to type I polyketides and *S. griseus* subsp. *formicu*s which could produce hedamycin belonging to type II polyketides (Javidpour er al., 2011), isolate DQ1 may produce two kind of polyketides or new polyketides with similar cyclic mechanism to type II polyketides, so isolate DQ1 will be a potential isolate producing new type polyketides. Morphology, culture characteristics and 16S rDNA gene sequences of isolates DQ1, GAN1, G30, G32, H86, HVG71, T12-24, T12-209, and YL-1 showed similarity with *S. fradiae,* but KS gene sequence from these isolates were different and formed 3 different clades (clade A, E, F) in the phylogenetic tree. The results showed that these isolates had the potential ability which produce different polyketides and may be different subspecies of *S. fradiae.* The results also indicated that different isolates which were belong to the same specie presented different antagonism activities, and isolates were belong to different species had the similar KS-II gene sequence. Basing on the phylogenetic tree, isolates DQ23, HVG71, G32 and GAN1 had obvious different keto-group reductive mechanism comparing with the other isolates, and which maybe have the potentiality to produce new type polyketides. Isolates HVG71, G32 and GAN1 showed similar activity against *B. subtilis*. But isolate HVG71 showed obvious different antagonism against *F. oxysporium* f sp. c*ucumarinum.* Isolate DQ23 showed similarity with *S. graminearus* basing on 16SrDNA sequence, morphological and physiological characteristics. Chemical structure of antifungal metabolites from *S. graminearus* is unknown. So isolates DQ23 and HVG71 will be potential isolates producing new type polyketides. Isolate GAN1 formed an unattached branch and showed its unique KS gene sequence. Purification and structure analysis for metabolite from isolates DQ1, DQ23, GAN1, HVG60 and HVG71 will be for further study.

3. Discussion

Analysis of the ketosynthase genes in streptomyces can implicate new polyketides discovery This method may not only prevent reinvestigation to find bioactive molecules described previously, but also alleviate some of the biases introduced by using conventional cultivation techniques. The most importantly, this method is hoped to apply in screening the KS-related sequence from the culture-independent microorganisms. In the latter context, redesigning the primers to take account of the specific codon usage of the organism would likely improve primer selectivity and efficiency in different taxa. The standard PCR method, described here, enables fast insight into

the KS arsenal in *streptomyces*. The method described in this work is faster and can be easily used in setting out pilot studies for new polyketides. In brief, PCR approach enabled us to fish out new KS genes and even more to target different KS genes within culture-dependent and culture-independent organism.

Many *streptomyces* containing PKS gene clusters and much more bacteria had long been recognized as an important source of bioactive molecules (Hertweck, et al., 2007; Hopwood, 2009b). *Streptomyces* cultures for novel industrially important products and pharmaceuticals have been screened for decades. However, the rate of discovery has not kept pace with resource expenditure and advances in technology and has even declined in recent years (Strohl, 2000). This unfortunate circumstance arises because the strains and molecules have been described previously, and these strains are being rediscovered and re-described. Better methods for the more efficient identification and screening of *streptomyces* cultures and soils containing potentially interesting secondary metabolites are clearly needed (Metsa-Ketela et al., 2002; Wawrik et al., 2005; Hisayuki and Shigeaki, 2006). Molecular screening strategies can be used to complement drug discovery programs and breathe new life into underutilized culture collections and culture-independent potential resource. Once the DNA is obtained, it can be screened with multiple probes, the result is that provide a complete metabolic profile of the isolates. Specific microorganisms can be targeted, or removed from the screening, while reducing the number of the isolates which needs to be analyzed, enabling an increased emphasis on the fermentation conditions of fewer isolates and increasing the probability of obtaining novel compounds.

To investigate the metabolic potential of the *actinomyces* strains in further, a standard PCR approach employing degenerate primers to target the type II KS genes was developed. The amplifications of DNA fragments of expected size with DNA from different *streptomyces* were obtained in the standard PCR protocol. The results suggested that the type II of KS fragments from *streptomyces* species and even from other genes could be amplified by the designed primers. Among 17 *streptomyces* isolates which gave a positive amplification signal, the isolates DQ1, DQ23 and HVG71 had the potential which produce new type polyketides against plant pathogen fungi. These results suggested that the presence of these amplification fragments could be a good indicator of antagonism, and indicated that this approach was useful to assess the presence of isolates with potential antagonism.

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