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Full Length Research Paper

Isolation and identification of two marine-derived *Streptomyces* from marine mud of coast and offshore Zhuhai, and bioactive potential for plant pathogenic fungi

Long Jianyou, Xia Jianrong and Chen Yongheng*

Department of Environmental Engineering, School of Environmental Science and Engineering, Guangzhou University, University City west outer ring road 230, Guangzhou, Guangdong 510006, P. R. China.

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Two different actinomycete strains (XAS585 and XAS588), producing antifungal substances, were isolated from the marine mud of coast and offshore Zhuhai city, Guangdong province, China. On the basis of micro-morphology, physiological and chemotaxonomic characterization and phylogenetic similarity of 16S rDNA gene sequences, the strains XAS585 and XAS588 were identified as *Streptomyces roseobiolascens* XAS585 and *Streptomyces roseofulvus* XAS588, respectively. Antifungal profiles of the ethyl acetate extracts of the two strains fermented broth (FBE) were evaluated against the test phytopathogenic fungi; the results showed that the FBE of *S. roseobiolascens* XAS585 exhibited strong antifungal activity against mycelia growth of all test fungi at 150 µg/ml, while the FBE of *S. roseobiolascens* XAS588 also exhibited strong antifungal activity, except *Sclerotinia scleroitiorum*, *Valsa mali* and *Cercospora sorghi* at 150 µg/ml. In addition, the FBE of both strains at 100 µg/ml showed inhibitory effect against the condina germination of *Alternaria alternate*, *Exserlhilum turcicum* and *Bipolaris sorokiniana*. This study showed that the two marine-derived *Streptomyces* may provide potent sources for antifungal metabolites.

Key words: Actinomycete, phytopathogenic fungi, antimicrobial activity, marine mud, *Streptomyces roseobiolascens* XAS585*, Streptomyces roseofulvus* XAS588.

INTRODUCTION

Actinomycetes are an important group of microorganisms due to their ability to produce a wide array of secondary metabolites. Especially, the genus *Streptomyces* were the largest antibiotic-producing actinomycetes (Imada et al., 2005; Raja et al., 2010). For the past half century, majority of antibiotics and antitumor drugs have come from terrestrial actinomycetes; however, discoveries of new pharmaceuticals from terrestrial actinomycetes have trailed off dramatically in recent years. Therewith, actinomyctetes from marine environments have aroused the keen interest of researchers (Maldonado et al., 2005; Ward and Bora, 2006; Kozue Anzai et al., 2008). The marine environment has several peculiar characteristics that are not seen in terrestrial areas with high hydrostatic pressure, high concentration of salts and low concentration of organic matter. Microorganisms living in the marine environment are obviously expected to be different from terrestrial ones. It is reported that marine actinomycetes not only have several new species, but also have plenty novel structures with potent bioactivities (Takizawa et al., 1993; Lin and Liu, 2010). According to incomplete statistics, the number of novel compounds isolated from marine actinomycetes in the 21th century is more than twice of the last century. Research results showed that marine actinomycetes would become another important microorganism resource for pharmaceutical industries, like terrestrial actinomycetes (Berdy,

*Corresponding author. E-mail: longjianyou@gmail.com. Tel: +86 135 70339968.

trove with an abundant resource of marine actinomycetes. From certain marine actinomycete isolates, many bioactive molecules with useful properties such as antimicrobial agents (Macherla et al., 2005; Manam et al., 2005) and antitumor compounds (Maskey et al., 2004; Kwon et al., 2006) have been discovered. These novel metabolites discovered in marine actinomycetes provide new way for crop protection and drug development (Zhao et al., 2009).

This study focused on the actinomycetes from marine sediments within the neritic zone in the sea area round Zhuhai city of Guangdong province of China, which is located in the north of South China Sea. In a previous screening study, 32 actinomycete isolates were obtained from marine mud samples *in vitro* and two strains exhibited strong antifungal activities in preliminary bioactive screening. This paper deals with the taxonomy and antimicrobial profiles of the ethyl acetate extracts of these two actinomycete strains *in vitro*, and explored the capacity of antiphytopathogenic fungi.

MATERIALS AND METHODS

Collection of marine sediment samples

Marine sediment samples were collected using a piston core sediment sampler from the neritic zone in the Zhuhai coast. All the samples were immediately stored at -20℃ until the isolation of sediment samples in the laboratory.

Isolation of marine actinomycete

To isolate the marine actinomycete, aliquots (0.1 ml) of serially diluted samples were heated to 55℃ for 30 min in order to eliminate bacterial growth, and were inoculated by spread Petri plate onto starch casein agar (SCA) (composition: soluble starch, 10 g; K_2HPO_4, 2 g; KNO_3, 2 g; casein, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; filtered sea water, 1000 ml and pH, 7.0 ± 0.1). The medium was supplemented with cyclohexamide at 25 µg/ml to minimize contamination with fungi and 10 µg/ml nalidixic acid to minimize contaminant growth respectively (Takizawa et al., 1993; Ravel et al., 1998). The plates were incubated for two weeks under the dark at 28 ± 1℃ and then the colonies with a tough or powdery texture, dry or folded appearance and branching filaments with or without aerial mycelia (Mincer et al., 2002) were subcultured and transferred to the SCA plates or slants without the antibiotic substance. Until further use, the slants were kept in cold room at 4℃ (Das et al., 2008).

Morphological and physiological characteristics of antinomycete isolates

Firstly, the two strains were inoculated on SCA medium and incubated for 14 days in an incubator under the dark at 28 ± 1℃. The micro-morphology and sporulation were observed by optics microscopy (Olympus, Japan). Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (US National Bureau of Standard 1976). The studies on physiological characteristics of the two marine actinomycete isolates were carried out following the methods recommended by the International Streptomyces Projects (ISP), Shirling et al. (1966) and Waksman and Henrici, (1943), and the utilization of carbon sources was tested according to the growth condition on the plates containing different sugar sources.

DNA extraction, PCR amplification and sequence analysis

The two strains were grown on 100 ml of SCA liquid medium under constant shaking at 28 ± 1℃ for 2 days. Spore was obtained by centrifugation and filteration. Extraction of genomic DNA of the strain was performed according to the method described by Lee et al. (2003).

PCR amplification of 16S rDNA was performed using both universal and genus specific primers: forward primer (5'-CGGAGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAAGGAGGTGATCCAGCCGCA-3'). The reaction mixture was prepared in a total volume of 50 µl containing 10×PCR reaction buffer (5 µl), $MgCl_2$ (2 mM), Taq DNA polymerase (TAKARA) (1.5 U), dNTP (200 µM), forward primer (2 µM), reverse primer (2 µM) and template DNA (2 µl). The PCR programme used was an initial denaturation at 94℃ for 3 min followed by 30 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ for 10 min. The PCR product was detected by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide (EB) staining. Then, the PCR product was submitted to Shanghai Biological Technology Co. Ltd. (Shanghai, China) for sequence analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 software (Kumar et al., 2004). The 16S rDNA sequence was aligned using the CLUSTAL X1.83 (Thompson et al., 1994) against the corresponding nucleotide sequences of representatives of the genus bacterial retrieved from GenBank. Evolutionary distance matrices were generated and a phylogenetic tree was inferred by the neighbor-joining method. Tree topologies were evaluated by bootstrap analysis based on 1000 re-samplings of the neighbor-joining data set (Galeano and Martinez, 2007; Garrity et al., 2007; Lin and Liu, 2010).

Preparation of the fermented broth extracts (FBE) of two actinomycete stains

Pure cultures of the strains were transferred aseptically and individually into the seed medium [GYP medium: glucose (10.0 g), peptone (4.0 g), yeast extract (1.0 g), sterile distillate water (1000 ml)]. After 24 h incubation, the seed culture at a rate of 10% (v/v) was inoculated into the production medium [GS medium: soluble starch (20.0 g), KNO_3 (1.0 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.001 g), sterile distillate water (1000 ml)]. The fermentation was carried out at 28 ± 1℃ for 7 days under agitation at 180 rpm. After 7 days, the flasks were harvested and culture filtrates were extracted three times with equal volume of ethyl acetate, and the merged solvent extracts were concentrated to dryness in vacuum to yield a crude residue. Similar protocol was followed for all the three strains. The residues (10 mg) were then dissolved in dimethyl sulphoxide (DMSO) and diluted serially by sterilized water, and the extracts thus obtained were used for antifungal activity against the test fungi.

Inhibition of mycelial growth

The suppression of fermented broth extracts (FBE) against the eight phytopathogenic fungi were measured using a growth rate method. 80% carbendazin wettable powder (Zhejiang Guo sheng

nong wang kai fa Co. Ltd., China) dissolved with sterile water was used as a positive control. DMSO and sterile water were used as the solvent controls. A serial dilution of FBE and the positive control

was mixed with the molten PDA in the conical flasks, and then poured into Petri dishes. Once the agar had solidified, 6 mm diameter agar plug with plant pathogen fungi culture was inversely planted in the centre of each Petri dish. The plates were incubated at 27 ± 1℃ for 72 h and the diameter of the inhibition zones of the test fungi around each well was measured. Each experiment was carried out in triplicate. The inhibitory effect was calculated using the following equation (Yin et al. 2010):

$IR (\%) = (D_c – D_t / D_c) \times 100$

Where, IR = inhibitory rate (%), D_c = diameter of negative control (mm) and D_t = diameter of treatment or positive control (mm).

Inhibition of conidia germination

The conidia of the three phytopathogenic fungi were suspended and diluted in 1% glucose solution to 1×10^5 /ml. This solution (20 µl/well) was added to 96-well cell culture plate and mixed with a serial dilution of FBE (20 µl/well). The plate was incubated at 25±1℃ in the dark for 5 h. Spores were considered to have germinated when the length of germ tube was equal to or more than that of the spore itself (Vicedo et al., 2006). The germination rate and germination inhibitory effect were calculated using the following equations:

$GR (\%) = (N_{gc} \div N_{tc}) \times 100$

$IR (\%) = [(GR_c - GR_t) \div GR_c] \times 100$

Where GR = germination rate (%), N_{gc} = number of germinated conidia, N_{tc} = number of total conidia, IR = germination inhibitory rate (%), GR_t = germination rate of the samples (%) and GR_c = germination rate of the control (%).

Statistical analysis

Data are presented as mean ± SE (standard error). Significance of difference between different treatment groups was tested using one-way analysis of variance (ANOVA) and significant results were compared with Duncan's multiple range tests using SPSS 13.0 software. Homogeneity of variance was assessed by residual plots. For all the tests, the significance was determined at the level of $P<0.05$.

RESULTS AND DISCUSSION

Identification and classification of the two actinomycetes

A total of 32 marine actinomycetes were readily isolated from the sea sediment samples on SCA medium using dilution plate technique, two of which exhibited strong antifungal activities, namely XAS585 and XAS588. The cultural, micromorphological, physiological and biochemical characteristics of the two strains are shown in Table 1.

On the SCA medium, the color of the aerial medium was ash for XAS585, blue for XAS588, while the color of substrate mycelium appeared ash and dark blue for the two actinomycetes, respectively. No pigmentation was observed in the SCA medium of the two strains. It is reported that gray and ash color series of actinomycetes are the dominant forms in the soils (Rizk et al., 2007; Kavitha, et al., 2010) as well as in marine environments (Remya and Vijayakumar, 2008).

Sporophore morphology of the two strains grown on SCA medium for 7 days showed long and spiral pattern. Both strain XAS585 and XAS588 had the ability to secrete enzymes such as gelatinase, amylase and proteinase, but did not produce cellulose and nitrate redutase. Carbohydrate utilization test play a prominent role in the taxonomic characterization of actinomycete strains (Pridham and Gottlieb, 1948). Strain XAS588 efficiently utilized the carbon sources such as glucose, sucrose, xylose, maltose, maduraose, lactose, mannose and fructose. Inositol, sorbose and rhamnose were not assimilated by the strain XAS585 which exhibited positive response to H_2S production and melanoid pigmentation on tyrosine agar, while only melanoid pigmentation was observed to be positive for the strain XAS588. Both strains grew well from 10 to 40℃.

16S rDNA sequences of the two actinomycetes were submitted to GeneBank (www.ncbi.nlm.nih.gov), the GeneBank accession numbers of strain XAS588 and XAS585 were GQ395243 and GQ395240, respectively. Alignment of 16S rDNA sequences of the strain XAS588 and strain XAS585 with GeneBank database showed that sequence similarities of both strains were significantly high (99%). The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Figure 1.

On the basis of the morphological and physiological characterizations and 16S rDNA sequence analysis, the strain XAS585 and XAS588 were preliminarily identified as *Streptomyces roseobiolascens* XAS585 and *Streptomyces roseofulvus* XAS588, respectively.

Antifungal activity of the two actinomycete strains

The fermentation broth of *S. roseobiolascens* XAS585 and *S. roseofulvus* XAS588 were collected from four days old medium. The ethyl acetate extracts of the fermentation broth of two strains (FBE) showed more inhibitory effect against mycelial growth of the eight test phytopathogenic fungi including *Botrytis cinerea*, *Alternaria alternate, Fusarium* f. *cucumerinum, Colletotrichum gloeosporioides, Bipolaris sorokiniana, Sclerotinia scleroitiorum*, *Valsa mali* and *Cercospora sorghi.* In the mycelial growth test, the FBE of *S. roseobiolascens* XAS585 exhibited more inhibitory effect against *Botrytis cinerea, Fusarium* f.*cucumerinum, Colletotrichum gloeosporioides, Bipolaris sorokiniana, Sclerotinia scleroitiorum* and *Cercospora sorghi* at the concentration of 150 µg/ml than the positive control (carbendazin) at 250 µg/ml; especially, the inhibitory rate of FBE of *S. roseobiolascens* XAS585 was more than

90% against *Bipolaris sorokiniana* at 150 µg/ml. The FBE of *S. roseofulvus* XAS588 also showed more inhibitory effect against the medium growth of the test fungi, except

Table 1. Cultural, physiological and biochemical characteristics of the two actinomycete strains (XAS588 and XAS585).

Parameter	XAS588	XAS585
Cultural characteristic		
Aerial mass color	Blue	Ash
Reverse side pigment	Dark blue	Ash
Soluble pigment	No	No
Micro-morphology		
Substrate mycelium	Leather-like	Irregular
Aerial mycelium	No diaphragm	No diaphragm
Spore chain morphology	Long, spiral	Long, spiral
Physiological characteristic		
Gelatinase	+	+
Proteinase	+	+
Amylase	+	+
Cellulose	-	-
Nitrate reductase	-	-
Use of carbon source		
Dextrose	G	G
Sucrose	G	G
Xylose	G	G
Maltose	G	G
Maduraose	G	G
Inositol	N	N
Lactose	G	G
Sorbose	N	N
Mannose	G	G
Arabinose	N	G
Rhamnose	N	N
Fructose	G	G
Biochemical characters		
H_2S production	-	+
Melanoma production	+	+
Growth at		
10℃	G	G
20℃	G	G
30℃	G	G
40℃	G	G
50℃	G	N
pH6	N	N
pH6.5	G	G
pH7	G	G
pH7.5	G	G
pH8	G	G

+, Positive result; -, negative result; G, growth; N, no growth.

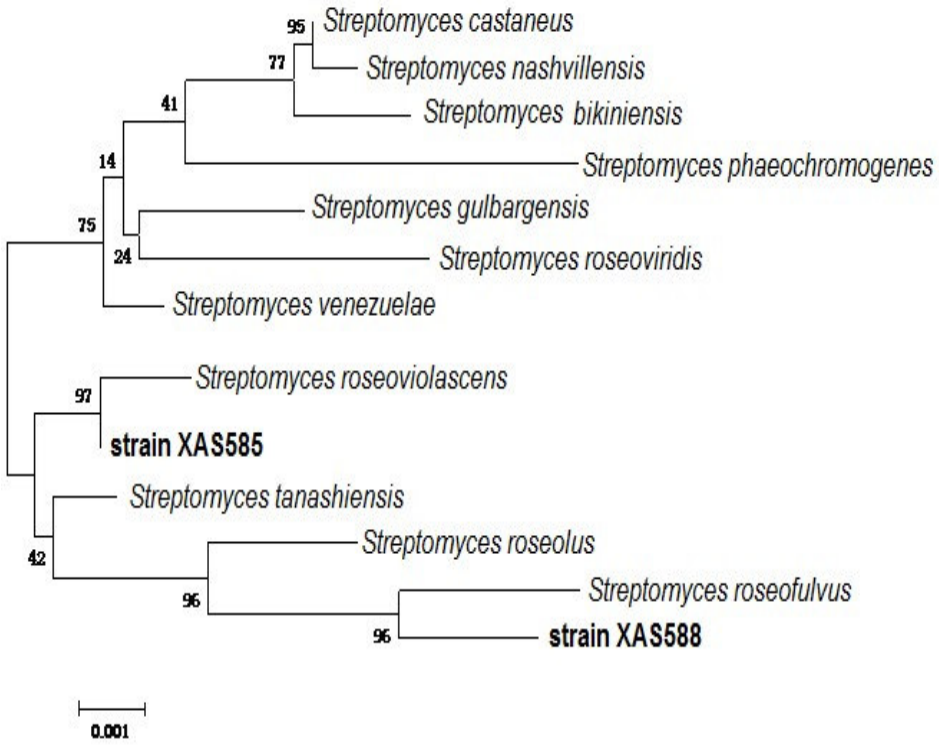

Figure 1. Phylogenetic tree showing the position of strains XAS585 and XAS588 on the basis of 16S rDNA gene sequence analysis. Number sat nodes are bootstrap values (%) based on neighbour-joining analysis of 1000 resampled datasets. The scale bar indicates the number of nucleotide substitutions per site.

Table 2. Inhibition of ethyl acetate extracts of the two actinomycete fermentation broths against eight phytopathogenic fungi.

Plant pathogen	Inhibition ratio (%) ± SE		
	XAS588	XAS585	Carbendazin
Botrytis cinerea	84.2±0.3^{a}	76.3±0.2^{b}	74.2±0.1^{a}
Alternaria alternata	81.9±0.3^{a}	70.6±0.2^{c}	71.9±0.3^{a}
Fusarium f.*cucumerinum*	87.5±0.1^{a}	78.7±0.1^{b}	67.5±0.2^{b}
Colletotrichum gloeosporioides	92.6±0.3^{a}	78.7±0.3^{b}	72.6±0.3^{a}
Bipolaris sorokiniana	90.1±0.2^{a}	90.9±0.1^{a}	70.7±0.2^{a}
Sclerotinia scleroitiorum	6.1±0.3^{c}	75.1±0.2^{b}	66.5±0.1^{b}
Valsa mali	54.7±0.2^{b}	70.8±0.1^{c}	64.2±0.2^{b}
Cercospora sorghi	38.1±0.1^{c}	75.3±0.2^{b}	58.1±0.6^{c}

The ethyl acetate extracts concentrations of the two strains fermented broth were 150 µg/ml, while the concentration of carbendazin was 250 µg/ml.
Values with different letters in the same column were significantly different ($P<0.05$) according to Duncan's multiple range test.

were more than 90% against *B. sorokiniana* and *C. gloeosporioides* at 150 µg/ml (Table 2). In the conidia germination test, the FBE of both strains showed inhibitory effect against the conidia germination of *Alternaria alternate*, *Exserlhilum turcicum* and *B. sorokiniana* at the concentration of 100 µg/ml, which was equal to the positive control, carbendazin at 200 µg/ml (Table 3). The results showed that the two marine-derived actinomycetes produced bioactive metabolites that antagonized many plant pathogenic fungi.

Screening bioactive molecules-metabolizing actinomycetes from marine environments has been considered to be a promising option. In this study, the two strains (XAS585 and XAS588) isolated from marine mud were identified as *S. roseobiolascens* XAS585 and *S. roseofulvus* XAS588 based on the results of the morpho-

Table 3. Inhibition effect of ethyl acetate extracts of the two actinomycete fermentation broths against the conidia germination of three phytopathogenic fungi.

Sample	Inhibition ratio (%) ± SE		
	Bipolaris sorokiniana	***Exserlhilum turcicum***	***Alternaria alternata***
XAS588	94.8±0.31[a]	88.2±0.22[a]	78.7±0.20[a]
XAS585	92.9±0.27[a]	81.7±0.23[b]	76.3±0.19[a]
Carbendazin	93.2±0.27[a]	85.7±0.15[a]	79.3±0.14[a]

The ethyl acetate extracts concentrations of two strains fermented broth were 100 µg/ml, while the concentration of carbendazin was 200 µg/ml.
Values with different letters in the same column were significantly different (P<0.05) according to Duncan's multiple range test.

logical, physiological characterizations and 16S rDNA gene sequence analysis. The ethyl acetate extracts of the two strains fermented broth were equivalent or better than the carbendazin against some phyto-pathogenic fungi *in vitro*. In comparison with synthetic chemical fungicides, natural products are readily biodegradable and not likely to cause resistance (Yin et al., 2010). This study revealed that *S. roseobiolascens* XAS585 and *S. roseofulvus* XAS588 are promising microorganisms that produce antimicrobial compounds. The isolation and purification of the two strains metabolites are underway in our laboratory.

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