Taxonomy Characteristics and Chemical Constituents of Strain H41-51

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Abstract

The strain H41-51 was identified as Streptomyces antibioticus according to morphological, cultural, and physiological and biochemical characteristics in combination with 16S rDNA sequence and phylogentic tree analysis of the strain, and the 16S rDNA accession number in Genbank was KU756253. Four cyclic dipeptide compounds, named cyclo (Phe-Ala), cyclo (R-Pro-S-Phe), cyclo (S-Pro-S-Phe) and cyclo (D-Phe-L-Ile) (1-4), were isolated from the fermentation broth of strain H41-51. The structures of the cyclic dipeptides were determined by spectroscopic methods including NMR and MS as well as comparison with the data of literatures.

Keywords

Streptomyces Antibioticus, Taxonomy Characteristics, Cyclic Dipeptide.

1. Introduction

In order to discover new natural products, research has moved towards the study of microorganisms in ocean [1]. After three decades of research in marine microbiology, we now know that important bioactive metabolites were discovered by the genera of marine-derived actinobacteria. In addition, approximately 45%-55% of the known 10,000 antibitics were from Streptomyces [2]. The taxonomy characteristic of actinomycetes is important to the further study on active chemical constituents [3].

In this paper, we report the identification according to morphological, cultural, and physiological and biochemical characteristics in combination with 16S rDNA sequence and phylogentic tree analysis of the marine-derived strain H41-51 as *Streptomyces antibioticus*. *Streptomyces antibioticus* H41-51 was found to produce four cyclic dipeptides as cyclo (Phe-Ala), cyclo (*R*-Pro-*S*-Phe), cyclo (*S*-Pro-*S*-Phe) and cyclo (D-Phe-L-Ile) (1-4).

2. Materials and Methods

2.1 General

The strain, *streptomyces antibioticus* (strain H41-51), was isolated from sea sediment at mangrove site in South China Sea, Zhapo, Yangjiang, Guangdong province. The strain has been deposited under 4℃ in a test tube with sandy soil at the Institute of Plant Protection, Guangdong Academy of Agricultural Science. The morphological properties were observed with an optical microscope (AXIOROP biomicroscope, Saisi Corporation, Germany) and a JEOL JSM-5600 scanning electron microscope (JEOL Ltd., Tokyo, Japan). ESI-MS spectra were obtained on a Finnigan LCQ Advantage MAX mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). NMR spectra were recorded on a Bruker 300 MHz spectrometer (Bruker Corporation, Fallanden, Switzerland) and the chemical shifts were given in ppm referred to residual $CHCl_3$ as 7.25 ppm (^1H) and to $CDCl_3$ as 77.0 ppm (^{13}C). The fermentation was performed with a fermentor (Type C 20-3, B. Braun Biotech International, Germany) in 30L. DNA extraction was carried out according to bacteria genomic DNA extraction.

2.2 Taxonomy of the producing strain

2.2.1 Cultural characteristics

The cultural characteristics of strain H41-51 were performed after 21 days incubation at 28℃. The characteristics of each culture including growth, aerial mycelium and reverse side color, soluble pigment growth were observed on seven kinds of media following as Gause's No. 1 agar, glucose-asparagine agar, starch agar, glucose-yeast-extract agar, patato stock, Czapek's agar and Kjeldahl agar. The color names used in this study were based on Manual of Determinative Streptomyces [4].

2.2.2 Morphological characteristics

The strain was grown on Gause's No. 1 agar at 28℃ for 7 days and examined under optical and scanning electron microscope.

2.2.3 Physiological and biochemical characterizations

The physiological characterizations including the whole cell analysis, starch hydrolysis, milk peptonization and coagulation, gelatin liquefaction, melanoid pigment on peptone-yeast extract-iron agar, cellulose hydrolysis and ability to utilize nine kinds of carbon sources of the strain were performed by the methods described in *Manual of Determinative Streptomyces*[4].

2.2.4 16S rDNA sequence

Genomic DNA was isolated following the procedure of bacterial genomic DNA extraction. The strain was cultured in 30 ml of YMG medium without $CaCO_3$ for 32 h at 28℃ and 150 rpm. 16S rDNA was amplified by PCR using the forward primer 27F

(5'-AGAGTTTGATCATGGCTCAG-3') and reverse primer 1492R (5'- AAGGA GGTGA TCCA GCCGCA-3'). PCR process was initial denaturation at 94℃ for 3 minutes, denaturation at 94℃ for 30 seconds, annealing at 56℃ for 30 seconds and extension at 72℃ for 100 seconds, repeating 34 cycles of the four steps. Sequencing was performed by the company of biotechnology of Aoke Dingsheng in Beijing. The 16S rDNA gene sequences of related taxa were obtained from GenBank and edited using the Blast search program. Multiple alignments were performed with the CLUSTALW 1.8 program. Evolutionary distances were calculated using the Kimura two-parameter model. Phylogenetic tree was constructed using the neighbor-joining, maximum-likelihood, and minimum evolution methods in the Mega2 program with bootstrap values based on 1000 replications.

2.3 Fermentation

A loopful of the strain mature slant culture was inoculated to each of three 1000 ml Erlenmeyer flasks containing 350 ml of sterile seed medium consisted of corn starch 2.5%, yeast powder 3.5%, sea water crystal 0.3%, $CaCO_3$ 0.15%, KNO_3 0.1%, $MgSO_4$ 0.06%, K_2HPO_4 0.09%, $FeSO_4$ 0.002%, pH 7.0~7.2 adjusted prior to sterilisation and cultured for 36 h at 28 ℃ with reciprocal shaking at 150 rpm. The content was inoculated to 20 liters of sterile fermentation medium identical to that above. The fermentation was carried out at 28℃ for four days, with aeration of 18 L/min, agitation of 300 rpm.

2.4 Isolation

The culture broth was filtered with the aid of filter. The filtrate was extracted with ethyl acetate for three times. The extract of ethyl acetate was passed through a column of sephadex LH 20. The column was washed with chloroform-methanol (1:1). The fraction-6 was purified using semi-preparative HPLC on a reverse-phased ODS silica gel column using isocratic elution in methanol-water (60:40) at a flow rate of 3 ml/min. Four cyclic dipeptides as cyclo (Phe-Ala), cyclo (*R*-Pro-*S*-Phe), cyclo (*S*-Pro-*S*-Phe) and cyclo (D-Phe-L-Ile) (1-4) were successively eluted.

3. Results

3.1 Taxonomy

The aerial mecylia grew abundantly on the Gause's No. 1 agar medium and potato stock, but scantly on the glucose-asperagine agar, starch agar and glucose-yeast-extract agar. The color of aerial

mecylia was grayish white to dark gray, and the basal mecylia was yellow to brownish yellow color. The soluble pigments generated by the strain easily diffused on potato stock medium, but did not diffuse on Gause's No. 1 agar, glucose-asparagine agar and starch agar media. The cultural characteristics of strain H41-51 are shown in Table 1.

Table 1. Cultural characteristics of strain H41-51

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Gause's No.1 agar	Good	Abundant; grey	Brownish yellow	None
Glucose-asparagine agar	Poor	Poor; grey	Stramineous yellow	None
Starch agar	Poor	Poor; grayish white	Marigold yellow	None
Glucose-yeast-extract agar	Poor	Poor; ivory	Pomegranate caylx yellow	None
Czapek's agar	Moderate	Moderate; grey	Pumpkin yellow	None
Kjeldahl agar	Moderate	Moderate; ivory	Lemon yellow	None
Potato stock	Good	Abundant; ivory	Brown	Brown

The branching type of spores was verticillate (Fig. 1). The mature sporophores were shown by electron microscopy to be monoverticillate with 15 spores in each chain and the spores were cylindrical and measure 1~1.3 × 0.5~0.7 μm in size. The surface was smooth. There was no fragmentation of hyphae or formation of spores in the substrate mycelium (Fig. 2).

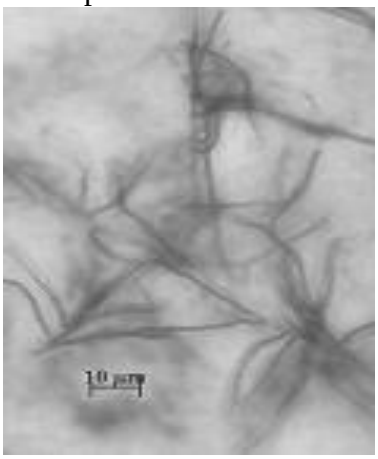

Fig. 1 The spore hypha morphology of strain H41-51 indicated with optical microscope (400 ×)

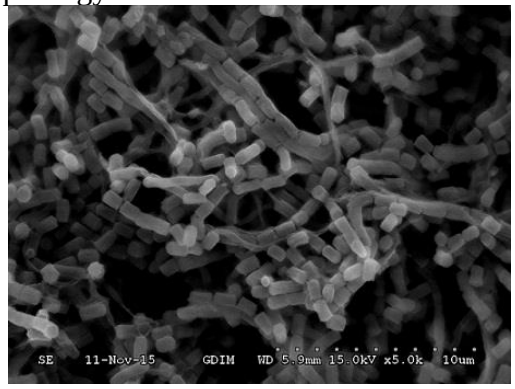

Fig. 2 The spore chain morphology of strain H41-51 indicated with scanning electron micrograph (5000 ×)

Analysis of whole cell hydrolysates showed the presence of L-DAP, glycine, alanine, L-aspartic acid, ribose, glucose, and the cell wall of the strain was classified accordingly as type Ⅰ. The physiological

properties of the strain are shown in Table 2. The strain was positive for milk peptonization, gelatin liquefaction, and starch hydrolysis and for production of melanoid pigment on peptone-yeast extract-iron agar, but negative for milk coagulation and cellulose hydrolysis. The strain could utilize sucrose, inosite, mannitol, galactose, rhamnose and arabinose.

Table 2. Physiological properties of strain H41-51

		Carbon utilization	
Starch hydrolysis	positive	Sucrose	+
Milk coagulation	negative	Inosite	+
Milk peptonization	positive	Mannitol	+
Melanin production	positive	Galactose	+
Gelatin liquefaction	positive	Rhamnose	+
cellucose hydrolysis	negative	Arabinose	+
		Fructose	-
		Xylose	-

+ : utilization; - : no utilization

Analysis of 16S rDNA sequence and phylogenetic tree (Fig. 3) showed that the strain was most closely related to *S.* antibioticus, sharing 16S rDNA similarity of 99%. In combination with the taxonomic characteristics described above, the strain H41-51 was authenticated to be *Streptomyces antibioticus* and the Genbank accession number was KU756253.

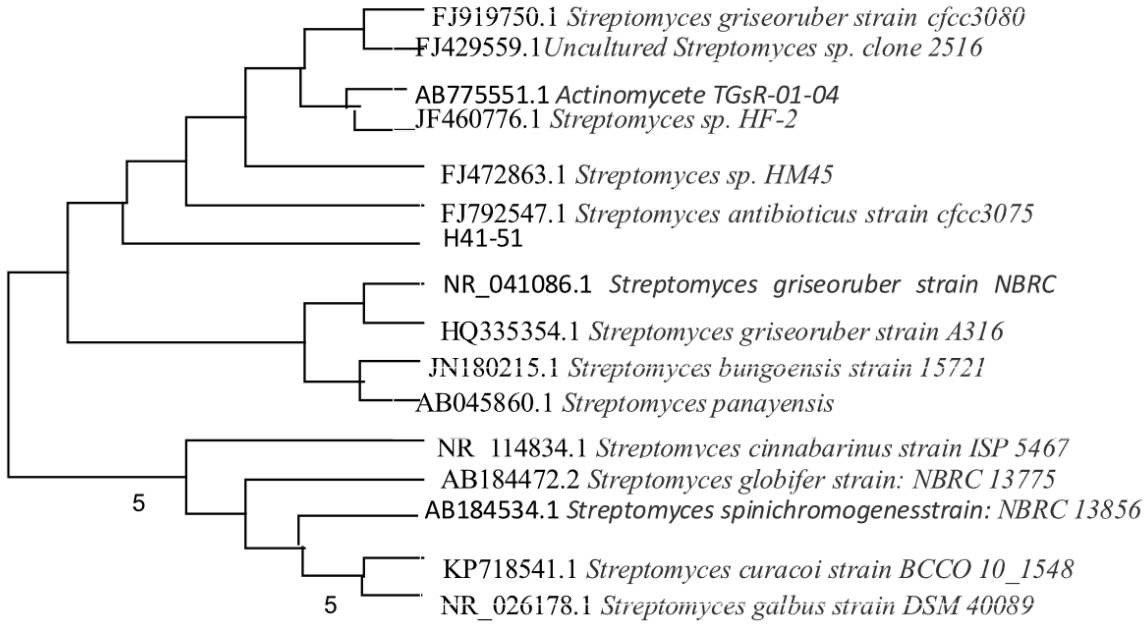

Fig. 3 Neighbor-joining tree based on 16S rDNA gene sequences showing the position of strain H41-51 and related taxa.

3.2 Structure elucidation

Compounds 1-4 (Fig. 4) were all obtained as colorless needles. The ESI-MS gave quasi-molecular ion peak at *m/z* 219.3 $[M+H]^+$, 245.3 $[M+H]^+$, 267.3 $[M+Na]^+$ and 261.3 $[M+H]^+$ respectively. The ^1H and ^{13}C chemical shifts are shown in tables 3, 4 and 5 respectively. And the comparisons with literatures [5-8] confirmed that the isolated compounds are cyclo (Phe-Ala), cyclo (*R*-Pro-*S*-Phe), cyclo (*S*-Pro-*S*-Phe) and cyclo (D-Phe-L-Ile) (1-4).

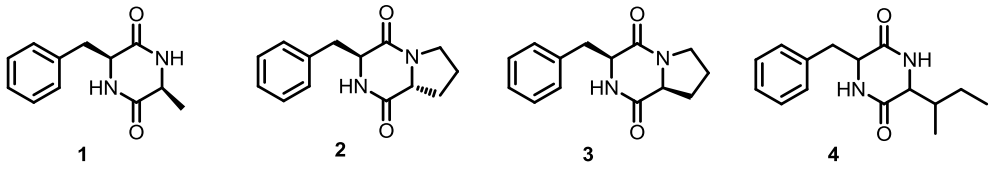

Fig. 4 The structures of compounds 1-4

Table 3. ^{1}H-NMR (300 MHz, $CDCl_3$) data of compounds 1 and 2

1		2	
No.	^{1}H	No.	^{1}H
1	7.95 (1H, br. s)	3	3.40-3.62 (2H, m)
3	4.11 (1H, m)	4	1.74-1.94 (2H, m)
4	8.04 (1H, br. s)	5	1.74-2.15 (2H, m)
6	3.56 (1H, m)	6	2.81 (1H, dd, *J* = 9.9, 6.3 Hz)
7	2.82 (1H, dd, *J* = 13.8, 4.8 Hz); 3.08 (1H, dd, *J* = 13.8, 4.8 Hz)	NH	7.02 (1H, br. s)
9	7.10 (1H, m)	9	4.30 (1H, m)
10	7.14 (1H, m)	10	3.18 (1H, dd, *J* = 13.8, 6.0 Hz); 3.06 (1H, dd, *J* = 13.8, 4.2 Hz)
11	7.18 (1H, m)	1'	7.19-7.30 (5H, m)
12	7.20 (1H, m)		
13	7.22 (1H, m)		
14	0.41 (3H, d, *J* = 7.2 Hz)		

Table 4. ^{1}H-NMR (300 MHz, $CDCl_3$) data of compounds 3 and 4

3		4	
No.	^{1}H	No.	^{1}H
3	3.52-3.64 (2H, m)	NH	6.07 (1H, s)
4	1.85-2.03 (2H, m)	3	3.93 (1H, m)
5	1.85-2.03 (1H, m); 2.30 (1H, m)	4	2.01 (1H, m)
6	4.05 (1H, t, *J* = 7.8 Hz)	5	0.98 (2H, d, *J* = 3.6 Hz)
NH	6.08 (1H, s)	6	0.87 (3H,t, *J* = 3.6 Hz)
9	4.29 (1H, dd, *J* = 9.9, 2.4 Hz)	7	1.25 (3H, m)
10	2.83 (1H, dd, *J* = 14.4, 9.9 Hz); 3.52-3.64 (1H, m)	NH	6.32 (1H, s)
1'	7.20-7.35 (5H, m)	10	4.26 (1H, m)
		11	3.40 (1H, dd, *J* = 6.9, 4.5 Hz); 2.96 (1H, dd, *J* = 6.9, 4.5 Hz)
		2'	7.21 (2H, m)
		3'	7.34 (2H, m)
		4'	7.29 (1H, m)

Table 5. ^{13}C-NMR (75 MHz, $CDCl_3$) data of compounds 1-4

1		2		3		4	
No.	^{13}C	No.	^{13}C	No.	^{13}C	No.	^{13}C
2	165.9	1	165.3	1	165.2	1	167.6
3	55.4	3	45.4	3	45.5	3	56.2
5	167.8	4	21.8	4	22.5	4	38.3
6	49.8	5	29.0	5	28.4	5	23.7
8	126.7	6	57.9	6	56.4	6	11.9
9	130.4	7	170.3	7	169.8	7	15.5
10	128.1	9	59.0	9	59.2	8	167.1
11	136.1	10	40.5	10	36.9	10	60.3
12	128.1	11	135.0	1'	135.9	11	40.5
13	130.4	12	130.1	2'	129.3	1'	135.3
14	19.7	13	129.0	3'	129.3	2'	129.4
		14	127.9	4'	127.6	3'	129.8
						4'	127.9

4. Conclusion

Based on morphological, cultural, physiological characteristics and 16S rDNA sequence and phylogenetic tree analysis of strain H41-51, the strain was identified as the species *Streptomyces antibioticus*. Four cyclic dipeptide compounds, named cyclo (Phe-Ala), cyclo (*R*-Pro-*S*-Phe), cyclo (*S*-Pro-*S*-Phe) and cyclo (D-Phe-L-Ile) (1-4), were isolated from the fermentation broth of the strain *Streptomyces antibioticus* H41-51.

Acknowledgements

This work was supported by the Grant (No. 81383320) of National Natural Science Foundation of China.

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