ISSN : 0974 - 7508 Volume 8 Issue 2

Trade Science Inc.

Natural Products

An Indian Journal

Short Communication

NPAIJ, 8(2), 2012 [50-52]

1,6-dihydrophenazine producing actinomycete *Nocardiopsis* sp. DS14-1 isolated from the deep sea sediment

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Received: 30th November, 2011 ; Accepted: 30th December, 2011

ABSTRACT

The microorganisms dwelling in deep sea are suggested to be evolutionally diverse from those living in normal soil and atmosphere. To find new useful microorganism, we performed the isolation of microorganisms from the deep sea sediments at 4991 m depth. As a result, we found red pigment producing actinomycete strain DS14-1. The 16S rDNA analysis on DS14-1 indicated that DS14-1 belonged to genus *Nocardiopsis*. The pure pigment was isolated from the agar culture of strain DS14-1 and identified as 1,6-dihydrophenazine, by NMR and MS spectra.

KEYWORDS

Deep sea sediment;
Actinomycete;
Nocardiopsis;
1,6-dihydrophenazine.

INTRODUCTION

Actinomycetes mainly living in soils produce wide variety of secondary metabolites and have been regarded as valuable sources of drug discovery. Approximately two-thirds of naturally occurring antibiotics are derived from actinomycetes[1]. Recently, actinomycetes isolated from marine environments including deep sea sediments are paid much attention as a new promising biological source. Actinomycetes belonging to *Nocardiopsis* genus have been reported as producers of bioactive compounds isolation from *Nocardiopsis*[2-6]. However, a few reports have been published regarding *Nocardiopsis* isolated from deep sea sediments. *Nocardiopsis* sp. 7326 which produced cold-adapted a-amylase was obtained from the 900 m depth sediment of Pydz Bay, Atlantic[7]. Cyclic peptide producing strain, *Nocardiopsis* sp. M0349, was isolated from the 3000 m depth sediment of Clarion-Clipperton Fracture Zone, Mid-pacific[8]. In present report, we isolated red pigment producing actinomycete *Nocardiopsis* sp. DS14-1 from the 4991 m depth sediment from the Godzilla Megamullion in the Philippine Sea. Here we describe isolation and identification of red pigment as 1,6-dihydrophenazine from the acetone extract of *Nocardiopsis* sp. DS14-1.

Soil samples were collected from the West Hipbone Rise of the Godzilla Megamullion[9] in the Philip-

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pine Sea. Sampling was performed with the submersible Shinkai 6500 on its dive #1146 (16° 00.03', N 139° 06.82' E; 4991 m depth) during the YK09-05 cruise of R/V Yokosuka. Sediments were collected using conical tubes and stored at -80 °C until usage. Approximately 1 g of each soil was spread over the TCG agar medium (3 g of tryptone, 5 g of casitone, 4 g of glucose, 20 g of agar, in 1 L of artificial seawater). The inoculated agar plates were incubated at 30 °C for 7 days. In order to obtain actinomycetes, bacterial colonies which form aerial hyphae and spores were selectively isolated by observation. Each bacterial strain was successively cultivated for purification. As a result, 10 strains were isolated with TCG medium. Among 10 strains, the strain named DS14-1 showed obvious production of red pigments when it grew on ISP2 agar medium (4 g of yeast extract, 10 g of malt extract, 4 g of glucose, 20 g of agar in 1 L of distilled water) or TCG agar medium.

The identification of DS14-1 was performed by direct sequencing of amplified 16S rDNA sequence using PCR[10]. The phylogenetic tree was constructed with neighbor joining method using the multiple-alignment program ClustalX[11]. As shown in Figure 1, it is indicated that the genetic position of DS14-1 was located in the genus of *Nocardiopsis*. The strain DS14-1 was closely related to alkaliphile *Nocardiopsis dassonvillei* with the high identity of 99 %.

To obtain the pigment, DS14-1 was cultured using 400 mL of ISP2 agar medium. The agar culture was extracted with acetone. The acetone extract was subjected to column chromatography using hydrophobic resin CHP20P (Mitsubishi Chemical Co.) eluted with 20% aqueous MeOH, 60% aqueous MeOH, and 100%MeOH. The 60% MeOH fraction was subjected to HPLC purification with gradient elution mode. The condition is following: ODS column (Nomura Chemical Co., Develosil ODS-MG5, 4.6×250 mm), flow rate 1mL/min with linear gradient elution of 5% MeCN to 50% MeCN containing 0.05% TFA during 20 min, with detection of UV absorbance at 360 nm.

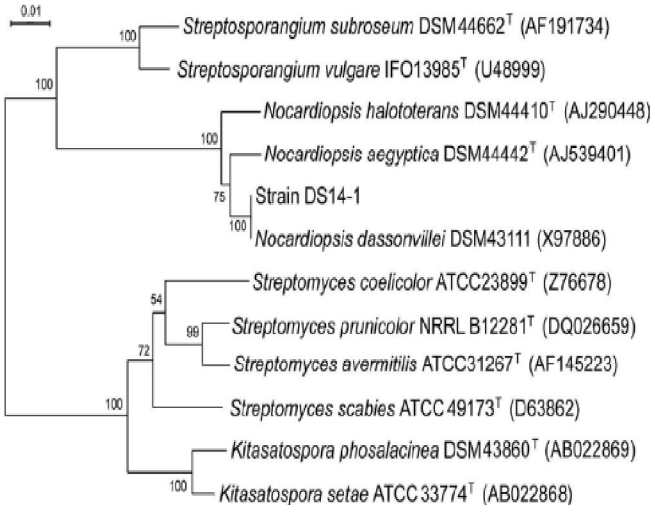

Figure 1 : Phylogenetic position of the strain DS14-1

To elucidate the chemical structure, we attempted analyses using NMR spectroscopy (JEOL, ECA-600) on the pigment. The pigment was dissolved in the solvent mix ($CDCl_3$/CF_3COOD 9:1). The ^1H NMR spectrum indicated the presence of 3 protons at 7.65, 8.09, and 8.73 ppm (TABLE 1). The ^{13}C NMR spectrum, HMBC, and HMQC analysis revealed the existence of three-substituted benzene ring. The downfield shifted chemical shifts of carbon of position 1 and 6 (150.0 ppm) indicated the presence of hydroxyl residue (TABLE 1). The molecular formula was determined by the high resolution ESI-MS as $C_{12}H_8N_2O_2$ (in positive mode, ion peak at 213.06277 for $C_{12}H_9N_2O_2$, mmu -3.63). The compound was identified as 1,6-dihydrophenazine (Figure 2 and TABLE 1) by the comparison with the literature data[12]. The production yield of 1,6-dihydrophenazine was determined by HPLC analysis as approximately 0.16 μg per 1 mg of dry cell

TABLE 1 : Chemical shift values of 1,6-dihydrophenazine in $CDCl_3$/CF_3COOD (9:1)

Position	δH (ppm)	Coupling constant	δC (ppm)
1, 6			150.0
2, 7	7.65	d, 7.6 Hz	117.0
3, 8	8.17	dd, 7.6, 8.9 Hz	138.0
4, 9	8.09	d, 8.9 Hz	116.2
4a, 9a			136.0
10a, 5a			131.0

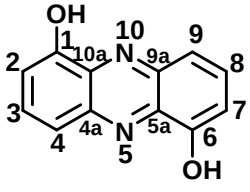

Figure 2 : Chemical structure of 1,6-dihydrophenazine

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weight when DS14-1 was cultivated for 12 days at 30 °C using ISP2 agar medium.

So far, there have been several reports about isolation of *Nocardiopsis* which produced useful substances from deep sea. These multiple reports supported the possibility that there may be broad distribution of *Nocardiopsis* in the deep sea sediments. The genetically close related strain *Nocardiopsis dassonvillei* DSM 43111 (Figure 1) was reported to produce promising tyrosine kinase inhibitor, K-252a[13]. Tsujibo et al. reported that *N. dassonvillei* OPC-15 intracellularly accumulated phenazines including 1,6-dihydrophenazine, 1,6-dihydrophenazine 5-monooxide, 1,6-dihydrophenazine 5,10-dioxide[12]. In present study, we isolated other bacterial strains which appear to be actinomycetes, and further screening to search for new antibiotic is ongoing.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The 16S rDNA sequence of *Nocardiopsis* sp. DS14-1 was deposited in DDBJ database under the accession number AB609587.

ACKNOWLEDGEMENTS

We thank the captain and crew of R/V Yokosuka as well as the Shinkai operation team for the sample recovery. This study was supported by Research fund of the Salt Science Research Foundation (SSRF). It was also supported in part by Grants-in-aids (No. 20880014, 19340148 and 21540477) for Scientific Research from the Japan Society for the Promotion of Science.

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