JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 25-30

Metabolic Characteristics of the Atlantic Deep-sea Derived Fungus *Cladosporium* sp. FA1-2 in Extreme Environments

Peihai Li[1#], Yaqin Fan[1#], Hao Chen[1*], Yaxi Chao[1], Fengmei Wang[2], Suqi Wu[1], Xiaomei Zhang[2] Ning Du[1] and Junhui Chen[1]

[1]Key Lab of Marine Bioactive Substances, First Institute of Oceanography, SOA, China.
[2]Technical center, Shandong Entry-Exit Inspection and Quarantine Bureau, PR China.

(Received: 16 February 2015; accepted: 20 April 2015)

The objective of our research was to explore metabolic characteristics of the deep-sea fungi in extreme environment. The study revealed a rather low population density of cultivable fungi in deep-sea sediments. Sixteen compounds, including nine cyclic dipeptides (1-3, 8, 9, 12, and 14-16), were obtained from the fermentation broth of the *Cladosporium* sp. FA1-2 derived from the Atlantic Ocean. The deep-sea fungal metabolic characteristics in low temperature and oligotrophic medium were reported here for the first time. The characteristics of metabolites were connected with the adaptive mechanism of deep-sea fungi and could be used as a reference to study the secondary metabolites of fungi residing in extreme environments.

Key word: extreme environments; metabolic characterization; deep-sea fungus; *Cladosporium* sp..

Extreme environments of low temperature, limited nutrition, darkness, high pressure and high salinity are undoubtedly striking characteristics of deep-sea environment. Fungi are known to adapt to certain extreme environments[1], and many novel fungi were isolated from deep-sea extreme environment. The deep-sea fungi, one of the most ecologically important groups of deep-sea microorganisms, have been studied since the isolation of deep-sea fungi was first reported approximately 50 years ago [2]. A few analyses of clone libraries based on DNA and cDNA already indicated that fungi appeared to be the dominant eukaryotes of deep-sea subsurface sediments[3].

In recent decades, researchers have paid increased attention to secondary metabolites of deep-sea fungi. As described in previous reviews, 76% of deep-sea natural products possessed biological activity, over one half of which exhibited signicant cytotoxicity towards a range of human cancer cell lines[4]. With multidrug resistance rising to a critical point[5], it is imperative that the search for new chemical entities moved into uncharted waters.

The secondary metabolites of marine fungi were affected by different culture conditions such as nutritional factors, temperature, pressure, salts, saccharides, pH, trace elements, and heavy metals[6-8]. Under favourable culture conditions, a large number of new, biologically active and potentially useful secondary metabolites were found from the culture of deep-sea derived fungi. Fifteen new depsidone-based analogues were isolated from the fermentation broth of a deep-sea fungus *Spiromastix* sp. in rice medium at 25°C [9]. Five new fungal hybrid polyketides were isolated from the deep-sea derived fungus *Cladosporium sphaerospermum* in rice medium at 28°C [10]. But,

* To whom all correspondence should be addressed.
Tel.: +86-(532)88963855; Fax: +86-(532) 8896385;
E-mail: hchen@fio.org.cn

how are the metabolites of fungi in deep-sea extreme environment? As far as we all know, there is little research on the metabolites of deep-sea fungi in the extreme environment.

To explore metabolic characteristics of deep-sea fungi in extreme environment, we simulated the low temperature and oligotrophic condition to culture the Atlantic deep-sea derived *Cladosporium* sp. FA1-2, which was representative strain isolated from the deep-sea matrix below about 3, 000m in Atlantic Ocean. Sixteen simple compounds including nine cyclic dipeptides were isolated. The obvious metabolic characteristics may be linked to the adaptive mechanism of deep-sea *Cladosporium* fungi and could be used as an inspiration to discover secondary metabolites of fungi residing in extreme environments.

MATERIALS AND METHODS

General experimental procedures

^{1}H NMR and ^{13}C NMR spectra were recorded using a JEOL JNM-ECP 600 spectrometer. Mass spectra were determined on an Agilent Technologies G1969A mass spectrometer. Analytical HPLC system (HITACHI, Japan) consisted of Organizer, UV Detector L-2400, Pump L-2130 and software Hitachi Model D-2000 Elite using a C18 column (YMC-pack ODS-A, 150×4.6 mml.D, S-5 ¼M, 12 nm, 1 mL/min). Semipreparative HPLC was operated on the same system using a Prep RP-18 column (YMC-pack ODS-A, 250×10 mml.D, S-5 ¼M, 12 nm, 2.5 mL/min) with UV detection. Commercially available Si gel (200–300 mesh, Qingdao Haiyang Chemical Co.), Lobar LiChroprep RP-18 (40–63 mm, Merck), and Sephadex LH-20 (Pharmacia) were used for open column chromatography.

Fungal material

Seventeen sites were sampled in Atlantic Ocean in November 2012 in the depth of about 3,000 m. The deep-sea sediment samples were collected 0.5 cm below the surface of the sediments by box corer (60×60×40cm) and placed directly in sealed sterile polythene bags. Then sediment samples were kept frozen until they were used. For the isolation of cultivatable fungi, 5g (fresh weight) of each sediment sample were homogenized in 30 ml sterile seawater. The homogenates were diluted (1: 10, 1: 100 and 1: 1000) using sterilized seawater. About 0.1 ml from each dilution of the sample suspension was spreaded on MEA (Malt extract, 17 g; Peptone, 3 g; Agar, 20 g; sea water, 1 L) medium. Three replicates for each medium were prepared. Plates were incubated at 4! (the average temperature of the sediment in deep sea) and monitored daily for 30 days to allow development of slow-growing colonies. Each fungal morphotype in each plate was isolated in pure culture and maintained for further use. The Fungal strains were identified according to morphological traits and 18S rDNA sequence analysis. The nuclear small-subunit genes were amplified from genomic DNA using pairs of primers: NS1/NS8. The strains were assigned on the basis of compiled partial 18S rDNA sequence comparisons with FASTA and BLASTN algorithms against sequences in the GenBank databases. A phylogenetic tree was constructed by the neighbour-joining method using the Molecular Evolutionary Genetics Analysis package (MEGA version 4.0), and the bootstrap analysis was performed with 1000 repetitions. All the isolates' partial 18S rDNA sequences obtained from the deep-sea fungi have been submitted to and deposited at GenBank database. The pure strains were deposited in the Research Center for Marine Ecology, the First Institute of Oceanography, SOA, Qingdao, PR China.

Fermentation and Preparation of Extract

For chemical investigation, initially 11 fungal strains were prepared on MEA slants and stored at 4!. The *Cladosporium* fungal strain FA1-2 was cultivated statically in liquid 25% Malt Extract Broth medium (MEB: Malt extract, 3 g; Peptone, 0.6 g; filtered sea water, 1 L; pH 6), After 50 days of fermentation at 4!, the fermentation broth was extracted with EtOAc, while the dried mycelium was smashed in pulp refiner, and immersed in acetone/water (4:1, v/v) for 2 hour under ultrasonical agitation. The acetone/water extraction was evaporated *in vacuo* and then extracted with EtOAc for four times. The combined extraction (14 g) was stored at 4°C.

Isolation and identification of fungal secondary metabolites

The crude extracts of *Cladosporium* sp. FA1-2 were fractionated by silica gel chromatography eluting with a stepped gradient elution of petroleum ether (60-90!)/EtOAc and

Dichloromethane/MeOH. Then the fractions were separated by C-18 ODS column using a stepped gradient elution of MeOH/H2O and Sephadex LH-20 columns with MeOH. The compounds were further purified by semi-preparative HPLC. The purified compounds were identified by their spectroscopic properties (^{13}C and ^{1}H NMR and HRESIMS).

RESULTS

Identification of fungi strains

By spread-plate technique, a total of eleven fungal strains were cultured from eight of the seventeen deep-sea sediment samples collected in Atlantic Ocean (Fig 1). No fungi were isolated from the samples collected at sites S9-S17. Phylogenetic analysis based on 18S ribosomal DNA (rDNA) sequences showed that eleven strains were assigned to *Cladosporium* (7 strains), *Penicillum* (3 strains), *Aureobasidium* (1 strain), respectively (Fig 2). The accession numbers are KF741226 and from KF776910 to KF776919. For strain FA1-2(1675bases), the accession number is KF776910. Fungal strain FA1-2 resides in the genus *Cladosporium*, family *Dematiaceae*, order *Miniliales*, class *Hyphomycetes*, subphylum *Deuteromycota*, Kingdom *Fungi*. As the representive of fungal strains derived from deep-sea sediment samples, the *Cladosporium* sp. FA1-2 was studied.

Isolation and identification of fungal secondary metabolites

Sixteen compounds (Fig 3) were isolated from extract of *Cladosporium* sp. FA1-2 and identified by their spectroscopic properties (^{13}C and ^{1}H NMR and ESI-MS) (the data is seen in supporting information). Compounds 1-3, 8, 9, 12, 14-16 were cyclic dipeptides; compounds 4 and 5 were aromatics; compound 6 was pyrone; compounds 7, 11 and 13 were alkaloids; and compound 10 was ergosterol.

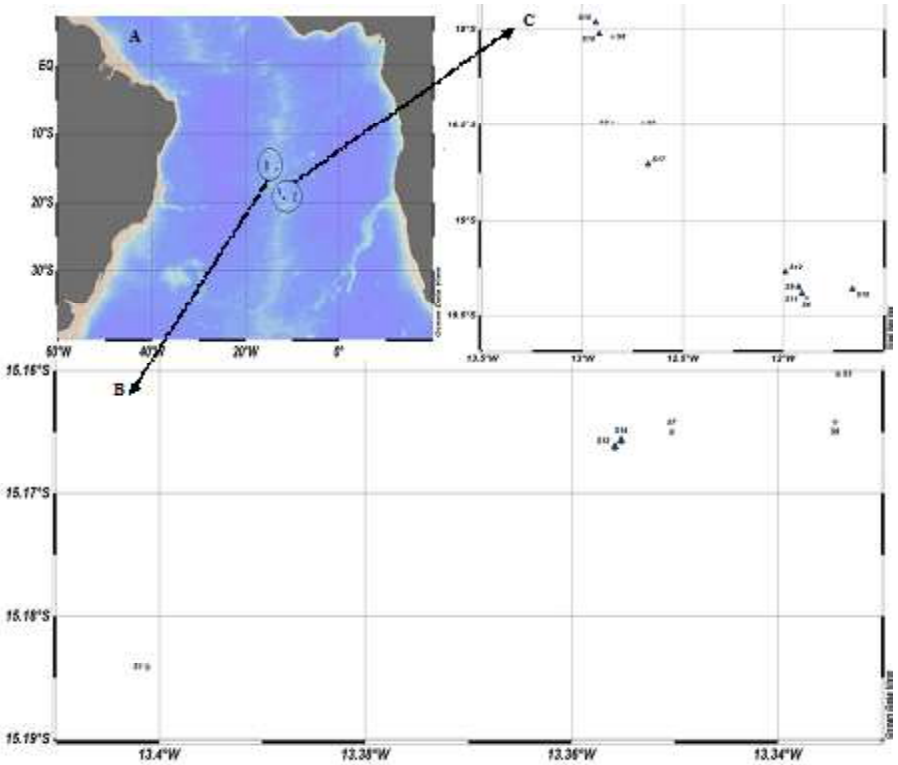

Fig. 1. Map of Collection sites in Atlantic Ocean. The strains FA1-2 / FA1-3, FB8, FA8-3, FA10-2 / FA10-3, FA16/FA16-3, FA9, FA6-2 and A7 come from the sites S1 (13°24'47"W, 15°11'3"S, 2859m), S2 (12°51'16"W, 18°29'14"S, 3224m), S3 (13°20'37"W, 15°9'37"S, 3032m), S4 (11°53'7"W, 19°24'19"S, 2401m), S5 (12°42'17"W, 18°29'16"S, 2152m), S6 (13°20'46"W, 15°9'51"S, 2854m), S7 (13°21'18"W, 15°9'54"S, 2790m) and S8 (12°51'13"W, 18°2'20"S, 3312m), respectively. No fungi were isolated from the samples collected at sites S9-S17.

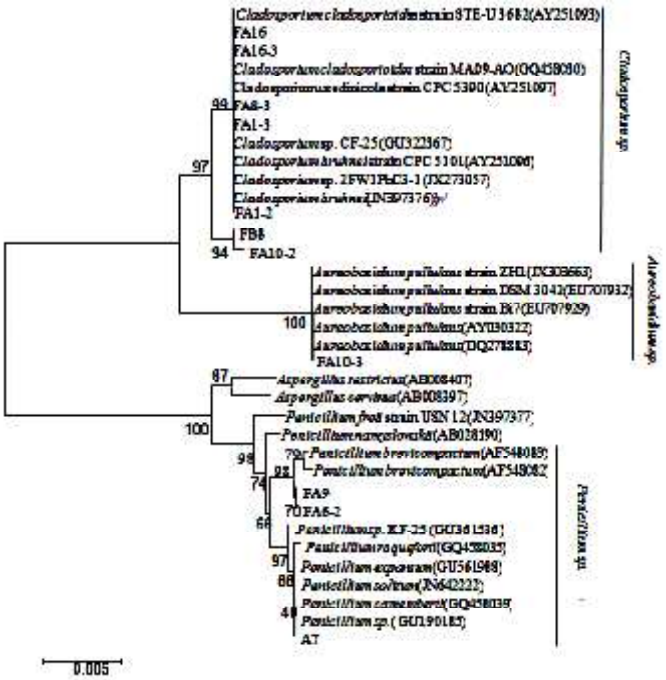

Fig. 2. Phylogenetic tree of 11 deep-sea derived fungal strain. Phylogenetic tree shows the relationships of fungi based on 18S rRNA gene partial sequences isolated from the deep-sea fungi compared with sequences of the same gene of known fungal genera and species derived from the database Genbank. The tree was constructed using the Neighbor-Joining method, and it is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Phylogenetic analyses were conducted in MEGA4

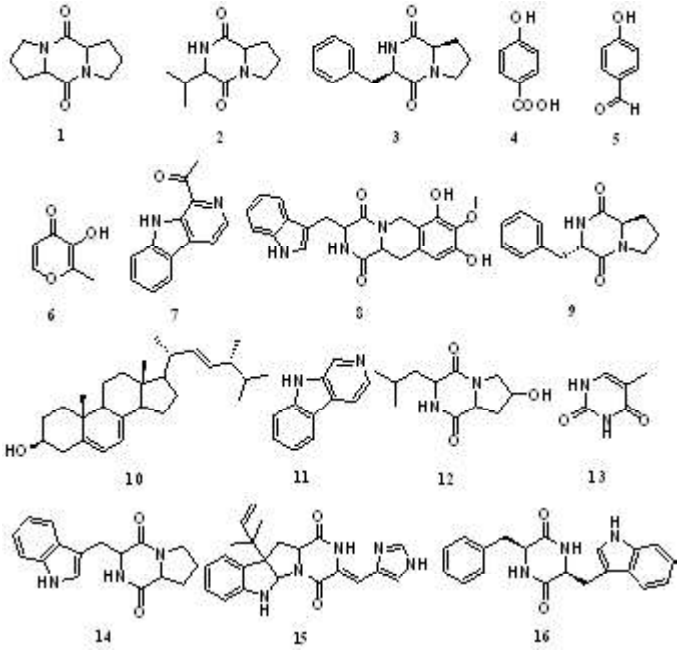

Fig. 3. Chemical structures of compounds 1-16

DISCUSSION

Although the deep sea has extreme environment such as low temperature, limited nutrition, darkness and high pressure, there are always microbes from sea surface to 11,034 m deep-sea trench, even 800 m deep in abysmal deposit[11]. In this context, eleven culturable fungal strains (7 *Cladosporium*, 3 *Penicillium* and 1 *Aureobasidium*) were obtained from eight of the seventeen deep-sea sediment samples collected below about 3,000m in Atlantic Ocean (Fig 1). Nearly 50% of samples appeared not to contain culturable fungi under the experimental conditions used. Compared with hundreds of marine fungi isolated from soil, mangrove plant, seaweed and sediment near the shore of ocean[12-14] the density of the culturable fungi in the deep-sea sediments below about 3,000m in Atlantic Ocean was found to be low.

To adapt to the extreme environment, marine microorganisms have evolved a distinct survival mechanism different from the terrestrial microorganisms [8]. We speculated metabolic characteristics of deep-sea fungi in extreme environment might be connected with the adaptive mechanism. Sixteen compounds were isolated from the deep-sea derived fungal strain FA1-2 at low temperature and oligotrophic condition that imitated the deep-sea extreme environment. All compound structures were simple, and most compounds were cyclic dipeptides. These showed the characteristics of secondary metabolites derived from deep-sea fungi in extreme environment. The cyclic dipeptides resulted from the condensation of two amino acids that were essential for life. The nonribosomal peptide synthases and cyclodipeptides synthases that synthesize diketopiperazine had been defined[15-17]. Cyclic dipeptides had simple structure and biosynthetic pathway, and might be used as the basic survival nutrients of fungi in deep-sea extreme environment. This may be helpful for fungi to save energy and resources to survive. Cyclic dipeptide is the smallest cyclic peptide in nature and has a variety of biological activities[18]. Several cyclic dipeptides (compound 2, 3 and 9) exhibited antifouling and antibacterial activities[19-20]. The bioactivities of cyclic dipeptides may exert an important impact on microbial biofilms, protein and nucleic acid of which the adaptive mechanism had been demonstrated in the extreme environment[21]. Now it is widely recognized that microbial secondary metabolites have important ecological functions and are not merely artifacts of laboratory culture or metabolic waste products[22]. Cyclic dipeptides also played an important role in regulatory mechanism of quorum sensing as signal molecules[23-25]. Microorganisms had a symbiosis and cooperated to cope with the extreme environments in the deep sea. The characteristics of secondary metabolites were favorable for each microorganism to survive in the deep-sea sediments. However, the specific role in adaptive mechanism was not yet clear and needed further research.

In conclusion, this is the first report of a comprehensive study of the deep-sea fungal metabolic characteristics in extreme environments. The deep sea is a mysterious world, its research is even more difficult than the deep space exploration. In a dark, poor nutrition and high pressure environment, organisms are how to survive? Especially, microorganisms are how to adapt to the extreme environment? What is the function of fungi in deep-sea ecosystem? How to answer these problems is facing great challenges to researchers. The research of microbial adaptive mechanisms in deep-sea extreme environment will deepen our understanding of deep-sea microbes and also will promote development of deep-sea microbial resources. In future, the deep-sea conditions will be simulated more accurately and further study will determine the specific role of cyclic dipeptides in fungal adaptive mechanism.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (2015CB755906) the Hi-Tech Research and Development Program of China (2013AA092901-2), the China Ocean Mineral Resources Research and Development Association (DY125-15-T-07) the Public Welfare Project of SOA (201205027) and the Basic Scientific Fund for National Public Research Institutes of China (GY0213G12).

REFERENCES

1. Gunde C N, Plemenitas A. Ecology and molecular adaptation of the halophilic black yeast Hortaea werneckii. *Rev. Environ. Sci. Biotechnol.*, 2006; **5**: 323–331
2. Yuriko N, Takahiko N. Fungal diversity in deep-sea extreme environments. *Fungal Ecology*, 2012; **5**: 463-471.
3. Edgcomb V P, Beaudoin D, Gast R, Biddle J F, Teske A. Marine subsurface eukaryotes: the fungal majority. *Environ. Microbiol.*, 2011; **13**: 172–183.
4. Danielle S. Deep-sea natural products. *Nat. Prod. Rep.*, 2008; **25**: 1131–1166.
5. Danielle S, Wei L. Recent advances in deep-sea natural products. *Nat. Prod. Rep.*, 2014; 31: 999-1 025.
6. Bugni T S, Ireland C M. Marine derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod. Rep*., 2004; **21**:143–163.
7. Yang L H, Miao L, Lee O O, Li X, Xiong H, Pang K L, Lilian V, Qian P Y. Effect of culture conditions on antifouling compound production of a sponge-associated fungus. *Appl. Microbiol. Biotechnol*., 2007; **74**: 1221–1231.
8. Huang J, Lu C, Qian X, Huang Y, Zheng Z, Shen Y. Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi. *Acta. Oceanol. Sin*., 2011; **30**(3): 118-123.
9. Niu S, Liu D, Hu X, Peter P, Shao Z, Lin W. Spiromastixones A”O, Antibacterial Chlorodepsidones from a Deep-Sea-Derived Spiromastix sp. Fungus. *J. Nat. Prod.*, 2014; **77**: 1021-1030.
10. Wu G, Sun X, Yu G, Wang W, Zhu T, Gu Q, Li D. Cladosins A”E, Hybrid Polyketides from a Deep-Sea-Derived Fungus, Cladosporium sphaerospermum. *Journal of Natural Products*, 2014; **77**: 270-275.
11. Peter W, Ian M, John R P. Geomicrobiology of deep, low organic carbon sediments in the Woodlark Basin, Pacific Ocean. *FEMS Microbiology Ecology*, 2002; **42**: 59-70.
12. Schmit, J P, Shearer C A. A checklist of mangrove associated fungi, their geographical distribution and known host plants. *Mycotaxon*, 2003; **80**: 423-477.
13. Siti A A, Nazura Z, Jones E B G. Biodiversity of marine fungi in Malaysian mangroves. *Botanica Marina*, 2010; **53**: 545–554.
14. Jariya S, Orathai S, Jones E B G, Souwalak P. Diversity of higher marine fungi at Hat Khanom-Mu Ko Thale Tai National Park, Southern Thailand. *Songklanakarin J. Sci. Technol*., 2011; **33**(1): 15-22.
15. Sattely E S, Fischbach M A, Walsh C T. Total biosynthesis: In vitro reconstitution of polyketide and nonribosomal peptide pathways. *Nat. Prod. Rep.*, 2008; **25**(4): 757-793.
16. Gondry M, Sauguet L, Belin P, et al. Cyclodipeptide synthases are a family of tRNA-dependent peptide bond-forming enzymes. *Nature Chemical Biology*, 2009; **5**(6): 414-420.
17. Strieker M, Tanovic A, Marahiel M A. Nonribosomal peptide synthetases: Structures and dynamics. *Current Opinion in Structural Biology*, 2010; **20**(2): 234-240.
18. Lu X, Xu D. Review on researching development of cyclic dipeptied bioactivity. *China Journal of Bioinformatics*, 2011; **9**(4): 289-291.
19. Han X. Screening of cytotoxic mangrove endophytic fungi and Study on its Secondary Metabolites of Two Selected Strains. *Master thesis of Ocean University of China*. 2009.
20. Shu-Hua Qi, Ying Xu, Hai-Rong Xiong, Pei-Yuan Qian, Si Zhang. Antifouling and antibacterial compounds from a marine fungus Cladosporium sp. F14. *World J. Microbiol Biotechnol*. 2009; **25**: 399–406.
21. Xi F, Zheng T, Jiao N, Zhang Y. A preliminary analysis of mechanism of deep-sea microorganisms diversity. *Advance in earth sciences*, 2004; **19**(1): 38 “46. (in Chinese)
22. Paul R J, Philip G W, Oh D C, Lisa Z, William F. Species-Specific Secondary Metabolite Production in Marine Actinomycetes of the Genus *Salinispora*. *Appl. Environ. Microbiol.*, 2007; **73**: 1 146-1 152.
23. Holden MT, Ram Chhabra S, de Nys R, et a1. Quorum sensing cross talkÿisolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram—negative bacteriaÿ*Mol Microbiol*, 1999; **33**(6): 1 254-1 266.
24. Zhang L H. Dang Y H. Quorum sensing and signal interference: diverse implicationsÿ*Mol Microbiol*, 2004; **53**(61): 1563-1571.
25. Mitova M, Tommonaro G, Hentschel U, et a1. Exocellular cyclic dipeptides from a ruegeria strain associated with cell cultures of *Suberites domuncula*. *Mar Biotechnol*, 2004; **6**(1): 95-103.