Deep Ocean Water Research, 15(3), 117–124, 2015

原著論文

Phenotypic Characterization of a Microbe Producing Substances for Oxidative Stress Resistance Isolated from the Deep Seawater in Izu-Akazawa, Japan

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Abstract

In this study, we tried to isolate microbes from a bag filter (BF) which was used to remove suspended solids from deep seawater (DSW) at a DSW pumping station in Izu-Akazawa, Shizuoka prefecture, Japan. As a result, 941 strains were isolated and only 10 strains from all the isolates showed a resistant effect against oxidative stress. The one strain which showed the highest ability of oxidative stress resistance among 10 strains was called strain "No. 586", and its 16S rRNA sequence analysis was performed. As a result, the homology of strain No. 586 matched *Pseudoalteromonas denitrificans* JCM21248 at 98.07%. After comparing the phenotypic characteristics of strain No. 586 and the type strain, it was shown that there were differences in the growth temperature and the color of the colony between the two strains. Furthermore, the resistant effect of strain No. 586 against oxidative stress was remarkably higher than that of the type strain.

Key Words: Deep seawater, Fibroblast, *Pseudoalteromonas denitrificans*, Oxidative stress resistance

1. Introduction

Recently, the search for sources of beneficial substances produced by microbes has been extended from the terrestrial environment to the marine environment (Taga, 2002). Due to the unique features of the marine environment such as high salinity, low temperature and high hydrostatic pressure, microbes from the marine environment are expected to produce beneficial substances. Although there have been a number of reports on beneficial substances isolated from surface seawater (SSW), marine sediment and marine life (Namikoshi, 2011), DSW remains as an attractive source for novel microbes. Generally, DSW is collected from the sea at a depth that is deeper than 200 m. There are various differences seen when comparing environments in DSW and SSW. DSW has characteristics such as lower temperatures, a richness in inorganic nutrients and fewer microbes compared with SSW (Takahashi, 2006). There are few reports on microbes in the DSW environment (Imada, 2009), however microbes from the DSW environment are interesting from the viewpoint of applied microbiology. According to the report of Yada *et al.* (2003), various microbial communities exist in DSW. Moreover, it was reported that beneficial substance-producing actinomyces were isolated from DSW by Igarashi *et al.* (2005). From these reports, DSW is expected to contain microbes producing various beneficial substances. Therefore, we tried to search for microbes producing various beneficial substances in DSW of Izu-Akazawa collected from the sea at the depth of 800 m.

In this study, the ability of oxidative stress resistance (Martorell *et al.*, 2011) was investigated as a beneficial

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effect. It is known that oxidative stress greatly influences the health and life span of humans. Oxidative stress induces significant damage in normal cells. It is known that oxidative stress can lead to diseases such as Alzheimer's and cardiomyopathy (Harman, 1956; Calabrese *et al.*, 2005; Schriner *et al.*, 2005; Dai *et al.*, 2010; Mao *et al.*, 2012). Furthermore, it was reported that oxidative stress did not only influence the interior of the body but also the exterior of the human body by inducing wrinkles and a slackening of the skin (Morita *et al.*, 2013; Masaki *et al.*, 1995; Valencia *et al.*, 2008). Until now, antioxidants have usually been applied to resist the influence of oxidative stress (Atalay *et al.*, 2006; Devasagayam *et al.*, 2004). According to Suganuma *et al.* (2010) and Inui *et al.* (2008), antioxidants inhibited the decomposition and reduction of collagen. For example, vitamin E is well-known antioxidant which is often used as an antioxidant for many foods. Vitamin E can scavenge some species of free radicals and protect foods from oxidation. However, as antioxidants oxidize themselves through oxidative stress, it is very difficult to maintain their antioxidant activity. Furthermore, it was reported that the effect cannot be increased, even when a concentration of the antioxidant is higher (Herbert *et al.*, 2006). We tried searching for substances inducing oxidative stress resistance in microbes living in DSW. Indeed, it was recently reported by Martorell *et al.* (2011) that there were substances different from antioxidants inducing oxidative stress resistance founded in cocoa.

Above all, this study aimed to isolate microbes that produce substances for oxidative stress resistance by screening the cell viability of human fibroblasts.

2. Materials and methods

2.1 Isolation of microbes

The BF was used for the one-month in order to remove detritus from DSW in a DSW pumping station of the DHC Corporation in Izu-Akazawa, Shizuoka prefecture, Japan. Each the BF was sent to our laboratory every month from October in 2010 to March in 2012. The BF was cut into square as in the shape (2 cm × 2 cm) in a clean environment and was then dispersed in 20 mL of DSW which had been sterilized at 121℃ for 15 min. Afterwards, 0.1 mL of this suspension was spread on various kinds of agar media described in section 2.2 for the isolation of microbes, and then they were incubated for 7 days at various temperatures from 5 to 27℃. Finally, isolated strains were obtained by collecting colonies appeared on the media.

2.2 Culture media for isolation of microbes

Various kinds of agar media were used. These media (PYBG, Tsutsui *et al.*, 2006; ZoBell 2216E, Oppenheimer and ZoBell, 1952; YPD, Fujii *et al.*, 1996) were used for the isolation of marine microbes or yeasts. They were diluted 10 to 20 times as needed. Each medium contained 50 μg/mL cycloheximide (Sigma) and 20 μg/mL nalidixic acid (Wako). These isolates were cultured in the medium which contained 0.1% glucose and 0.5% Bacto-peptone (Difco) in DSW.

2.3 Sample for evaluations

In order to obtain beneficial microbes, isolates were cultured separately for 7 days at 5 to 27℃ with shaking (160 rpm) in 20 mL of the medium in a 100 mL-Erlenmeyer flask with baffles. The supernatant of each isolate was collected by centrifugation and then evaluated by the method described in section 2.5.

2.4 Cell cultures

Fibroblasts (NB1RGB, Riken BRC) and B16 mouse melanoma (4A5, Riken BRC) were cultured in Eagle's MEM. Two lines of cancer cells, HepG2 (Riken BRC, human hepatocellular carcinoma) and Caco-2 (Riken BRC, human adenocarcinoma derived from colon) were cultured in Dulbecuo's modified Eagle medium (Nissui; DMEM). The concentration of fetal bovine serum (FBS) and *t*-butyl hydroperoxide (70%, Wako; *t*-BuOOH) were determined in consideration for each cell condition.

These methods were closely shown in section 2.5 and section 2.7.1.

2.5 Screening of active strains

For the screening of active strains, NB1RGB cultured in Eagle's MEM containing 10% FBS in 96-well plates (Iwaki) were used. The cells were cultured in the Eagle's MEM containing 10% FBS for one day previously. After the culture medium was exchanged to Eagle's MEM containing 0.5% FBS which included the supernatant from each active strain at a final concentration of 5%, and the cells were further cultured for 2 days. The cells then were treated with *t*-BuOOH at a final concentration of 800 μM for 4 h at 37℃. After removal of the medium by pipetting, the cells were rinsed with PBS (−) (Nissui). The cells were filled with fresh Eagle's MEM containing 10% FBS, and cell viability was evaluated according to the method of MTT reduction (Yamada *et al.*, 2008). The control group was examined by the same method of the test group except that it was treated without *t*-BuOOH. Active strains which showed the activity of more than 50% in the calculation of the following formula were selected. The strain which showed the highest activity was sent to next examinations in order to investigate characteristics of microbe. All results are presented as average ± standard deviation (SD) at three experiments in one trial. Statistical differences were measured using the Student's *t*-test with significance set at 5%.

$$\text{Activity}(\%)=\frac{\text{Cell viability of test groups}}{\text{Cell viability of control groups}}\times 100$$

2.6 16S-rDNA sequence analysis of the strain which showed the highest activity

The strain selected in section 2.5 was cultured in 20 mL of ZoBell 2216E medium diluted 10 times in a 100 mL-Erlenmeyer flask with baffles for 7 days (160 rpm, 5℃). After cultivation, the DNA of the strain was extracted, isolated and then purified. To identify the species of the strain, its DNA sequence was subjected to the BLAST search on NCBI (http://www.ncbi.nlm.nih/BLAST/) according to the method of Imada *et al.* (2014).

2.7 Characteristics of the strain which showed the highest activity

2.7.1 Comparison of oxidative stress resistance activities with the strain and the type strain

The type strain *P. denitrificans* JCM21248 (Riken BRC), which was similar to the strain (No. 586) in the present study, was obtained for the comparison of oxidative stress resistant activities. NB1RGB cultured in Eagle's MEM containing 5% FBS in 96-well plates for one day were further cultured for 2 days in the same medium containing the culture supernatant from the strain or the type strain at a final concentration of 5%. After cultivation for 2 days, the medium was changed to Eagle's MEM containing 0.5% FBS. After treatment with 1.6 mM *t*-BuOOH for 4 h, the cells were used to investigate the activity according to the method in section 2.5. 4A5 cultured in Eagle's MEM containing 10% FBS in 96-well plates for one day were further cultured for 2 days in the same medium containing the culture supernatant from the strain or the type strain at a final concentration of 5%. After cultivation for 2 days, 4A5 were treated by the same method as described above. HepG2 and Caco-2 were cultured in DMEM containing 5% FBS in 96-well plates for one day. Both cells were treated by the same method as described above. All results are presented as average ± SD at six experiments in one trial (NB1RGB and 4A5) or three experiments in one trial (HepG2 and Caco-2). Statistical differences were measured using the Student's *t*-test with significance set at 5%.

2.7.2 Comparison of phenotypic characteristics of the strain and the type strain

The strain and the type strain was incubated for 7 days at 5℃ in a PYBG medium diluted 20 times to investigate their Gram-staining and growth temperature. A pH range which allows the growth of each strain was examined by cultivating both strains for 7 days on a rotary shaker (160 rpm) in a PYBG liquid medium

diluted 20 times. Furthermore, seawater concentrations for cell growth were also investigated using a ZoBell 2216E medium prepared with various concentrations of Herbst's artificial seawater (Yumoto, 2003). Both strains were cultured at 5℃ except during the research on growth temperature. The effect of antioxidant activity was examined by using 1,1-diphenyl-2-picrilhydrazyl (97%, Tokyo-Kasei; DPPH) according to the method of Urabe *et al.* (2008).

3. Results

3.1 *Isolation of active strains from BF of DSW in Izu-Akazawa*

In this study, 941 strains of microbes were isolated from BF from Izu-Akazawa under various culture conditions. The activities of oxidative stress resistance in the culture supernatants of various isolates were examined as described in Materials and methods. As a result, 10 strains which demonstrated remarkable activity were obtained. These 10 strains were obtained by incubation at temperatures ranging from 5 to 20℃ but any active strain was not obtained at 27℃ (Tables 1, 2). Strain No. 586 which showed the highest activity in all active strains was further investigated.

Table 1. The number of isolated strains and oxidative stress resistant strains from BF in Izu-Akazawa, Japan.

Culture temperature (℃)	Isolated strains	Active strains	Appearance rate (%)
5	144	2	1.4
15	294	4	1.4
20	292	4	1.4
27	211	0	0
Total	941	10	1.1

Table 2. Active strains isolated from BF in Izu-Akazawa, Japan.

Active strain No.	Activity of oxidative stress resistance (%)*
Blank**	30.0 ± 11.2
NC***	28.8 ± 12.6
586	99.1 ± 2.5
962	91.2 ± 8.5
817	83.2 ± 3.3
896	80.1 ± 0.6
485	69.6 ± 2.0
1071	68.3 ± 8.2
506	66.2 ± 5.6
752	65.5 ± 0.7
596	62.2 ± 2.4
563	56.0 ± 3.8

*Activity of oxidative stress resistance was shown in section 2.5. Data were presented as means ± standard deviation. **Blank is Eagle's MEM containing 0.5% FBS. ***Negative control (NC) is supernatant of non-inoculation culture medium.

3.2 *Identification of strain No. 586*

Strain No. 586 which showed highest activity in this study was identified by sequencing 16S rRNA gene. The determined sequence of strain No. 586 showed a prominent homology of 98.07% with the corresponding sequence of *Pseudoaletromonas denitrificans* JCM21248 (Riken BRC) as the type strain (Table 3).

3.3 *Comparison of the activities of strain No. 586 and the type strain*

Strain No. 586 was compared with the type strain on the activities of oxidative stress resistance. As a result, it was shown that strain No. 586 had higher activity than the type strain in NB1RGB test (Figure 1). However, neither of the strains showed any activity in using 4A5, HepG2 and Caco-2 tests (Data not shown).

3.4 *Comparison of phenotypic characteristics of strain No. 586 and the type strain*

The phenotypic characteristics of strain No. 586 and the type strain were compared (Table 4). The temperature of the upper growth limit of strain No. 586 was 15℃, while that of the type strain was 20℃. The colony of strain No. 586 on an agar medium was purple, how-

Table 3. Identification of strain No. 586.

Related species	Homology (%)	Matched bases in sequence alignment
Pseudoalteromonas denitrificans	98.07	1,318/1,344
P. marinigluthinosa	94.23	1,322/1,403
P. arctica	93.94	1,318/1,403

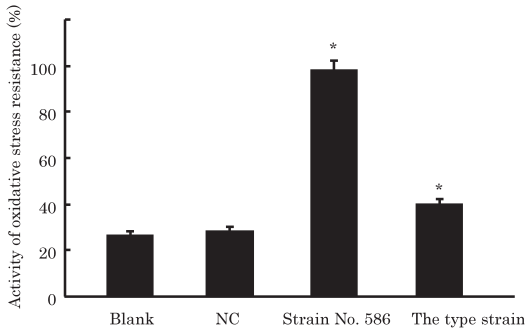

Fig. 1. Comparison of the oxidative stress resistance activities of strain No. 586 and the type strain (*P. denitrificans* JCM21248) in NB1RGB test. Blank is Eagle's MEM containing 0.5% FBS. Negative control (NC) is supernatant of non-inoculation culture medium. Error bars indicate standard deviation among six experiments in one trial. *$p<0.05$ compared with blank.

Table 4. Phenotypic characteristics of strain No. 586 and the type strain*.

Characteristics	Strain No. 586	The type strain
Activity of oxidative stress resistance (%) **	97.7 ± 4.5	39.7 ± 2.3
Color of colony	Purple	Red
Growth temperature (℃)		
5	+ + + ***	+ + +
10	+ +	+ + +
15	+	+ + +
20	—	+ +
27	—	—
Gram-staining	Negative	Negative
pH range for growth	5–10	5–10
Seawater concentration for growth (%v/v)	75–100	75–100

Pseudoalteromonas denitrificans* JCM21248. **Activity of oxidative stress resistance was shown in section 2.5. Data were presented as means ± standard deviation. * + + +: good growth, + +: moderate growth, +: poor growth, —: no growth.

ever that of the type strain was red. So, it was shown that pigments produced by both strains were different. While, the concentrations of seawater and the pH ranges for cell growth were the same for both strains. Either strain did not show antioxidative activity when assayed by the DPPH method.

4. Discussion

In this study, we tried to isolate microbes at various temperatures from DSW. As a result, 941 strains of microbes were isolated from BF soaked in the DSW of Izu-Akazawa, Japan. In the number of isolated microbes, there were little difference by the culture temperature (5, 15, 20 and 27℃). And so, it was found that the number of microbes isolated from the each temperature was almost the same. This result was supported by the report of Yada *et al.* (2003) of which there were a lot of known and unknown microbes in DSW. All of 10 isolates as active strains were isolated at various temperatures from 5 to 20℃, although no strain was isolated at the temperature of 27℃. It seemed that there were few species of microbes isolated at 27℃, whereas there were various species of microbes isolated at other temperatures. As a result, it might be able to obtain active strains at culture temperature from 5 to 20℃. However, further investigation is needed to identify the species of the strain.

Strain No. 586 showed the highest activity of oxidative stress resistance in this study was related to *Pseudoalteromonas denitrificans* with a sequence homology of 98.07%. Therefore, phenotypic characteristics of strain No. 586 and the type strain were compared. As a result, there were some differences in phenotypic characteristics on the upper limit of growth temperature and the color of colonies between strain No. 586 and the type strain. Furthermore, the activity of oxidative stress resistance of strain No. 586 was remarkably higher than that of the type strain. The activity of strain No. 586 was 97.7 ± 4.5%, while that of the type strain was the only 39.7 ± 2.3%. From these results, it seemed that strain No. 586 would be different from *Pseudoalteromonas denitrificans*. It was reported that novel marine bacteria which was related to *P. denitrificans* was isolated from the seawater of the depth at 320 m on Cape Muroto offing (Kochi, Japan) by Yada *et al.* (2008). They reported that the microbe produced a purple pigment called violacein, whereas no oxidative stress resistance was reported. So it seemed that this research is the first report on substances of oxidative stress resistance produced by

marine microbe which was related with *P. denitrificans*.

These substances produced by strain No. 586 were not simple antioxidative substance but unique substances which induced the ability of oxidative stress resistance of NB1RGB. They did not show strong antioxidants activity when assayed by the DPPH method. Furthermore they were not effective to various cancer cells, 4A5, HepG2 and Caco-2. The effect induced by substances from strain No. 586 was observed only in NB1RGB cells. However, it may be better for applications to human that substances produced by strain No. 586 could not show the ability of oxidative stress resistance on various cancer cells in this study. This is because it was known that some anticancer agents generated free radical to kill cancer cell (Davis *et al.*, 1999). If substances produced by strain No. 586 induce the ability of oxidative stress resistance in cancer cells, such anticancer agents may lose the ability to kill cancer cells. In any case, it seemed that the identification of chemical structures of these substances is expected. In the future, it is necessary to investigate the activity of the oxidative stress resistance induced by substances produced by strain No. 586 with normal cell, except for NB1RGB. As a result of this study, we succeeded in the isolation of microbes having wide temperature ranges for cell growth from DSW. Until now, there were only a few reports about microbes isolated from DSW. We believe that DSW will be a search source for beneficial microbes because some active microbes, including strain No. 586, were obtained in this study. Furthermore, it was suggested that substances produced by strain No. 586 showed the highest activity on NB1RGB inducing the ability of resistance against oxidative stress. It seemed that this research is the first report on substances of oxidative stress resistance produced by a microbe in marine environments.

Acknowledgement

We thank Mr. Masahiro Suzuki of DHC Co. for performing the test with HepG2.

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(Received 27 September 2014;
Accepted 7 December 2014)

伊豆赤沢海洋深層水から分離した酸化ストレス抵抗性関与物質を産生する微生物の特徴

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要　旨

本研究では伊豆赤沢の海洋深層水から懸濁物を濾過するバック状フィルター（以後，BF）を入手して微生物の分離を試みた．その結果，941株の微生物が分離され，そのうち10株が酸化ストレス抵抗性を示した．そこでこれらの分離株の中でも最も高い抵抗性を示した株（No. 586株と命名）について，16S rRNAの塩基配列解析から，種の同定を行ったところ，*Pseudoalteromonas denitrificans* JCM21248の標準菌株と98.07％の相同性が得られた．次にNo. 586株と標準菌株の表現型を比較した結果，生育温度とコロニーの色調に差異が見られた．さらに，No. 586株の酸化ストレス抵抗性は標準菌株に比べて顕著に高いことが判明した．

キーワード：海洋深層水，線維芽細胞，*Pseudoalteromonas denitrificans*，酸化ストレス抵抗性