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Cloning, Expression and Bioinformatics Analysis of Dioxygenase Gene from Antarctica Sea-Ice Strain *Shewanella* sp. NJ49

Wang Yi-bin[1], Liu Fang-ming[1], Liang Qiang[1], Zhang Xiu-fang[2], Zheng Zhou[1], Zang Jia-ye[1] and Miao Jin-lai[1]*

[1]First Institute of Oceanography, State Oceanic Administration, Qingdao - 266061, China.
[2] Qingdao Hiser Hospital, Qingdao - 266033, China.

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In the biodegradation of aromatic compound pollution, dioxygenase is a key enzyme of many bacterial pathways. A naphthalene dioxygenase gene was identified and cloned from the Antarctic psychrophilic bacteria *Shewanella* sp.NJ49; the gene sequence, structure, functions, and expression have analyzed. A full-length sequence (924 bp) of dioxygenase gene was obtained, and the amino acid sequence of dioxygenase gene from *Shewanella* sp.NJ49 was similar to the catechol 2,3-dioxygenase (C23O) from *Pseudomonas mendocina*. The homologic identity was 99%. The expression level of dioxygenase gene by qRT-PCR in different habitats showed that NJ49 had the highest expression level when temperature was 15°C, naphthalene concentration was 50 mg/L, and the gene was under UV-B radiation for 4 h.

Key words: Antarctica microorganisms; Dioxygenase gene; PAHs degradation; Bioinformatics.

The problems of large-scale petroleum hydrocarbon spills and petroleum hydrocarbon pollutants in the ocean have been increasingly serious, and petroleum hydrocarbon has become a major pollutant in ocean ecosystems. Microorganisms' degradation and bioremediation are the main mechanism of petroleum hydrocarbon and polycyclic aromatic hydrocarbons (PAHs) degradation; thus, bioremediation technologies to degrade environmental pollution have developed rapidly in recent years. Much has been learned about the biodegradation of pollution related to the enzymes of microorganisms and the corresponding enzymatic reactions (Yen *et al*, 1988; Miyazaki *et al*, 1997; Bosch *et al*, 1999). During the biodegradation process of various aromatic compounds, such as benzene, biphenyl, and polycyclic aromatic hydrocarbons, the rate of benzene ring degradation depends on the cell's capability of enzyme production. Dioxygenase is a key enzyme of many bacterial pathways for the degradation of aromatic compounds, where by the reaction rate of polycyclic aromatic hydrocarbon biodegradation is controlled by the oxidation process of benzene ring (Merimaa *et al*, 2006).

Some scholars have researched the biodegradation process of aromatic compounds, involving oxygenase and oxygenase gene (Merimaa *et al*, 2006; Gibson *et al*, 2000; Bugg, 2003; Liu *et al*, 2008; Bosch *et al*, 2000). The oxygenase and the structure and function of oxygenase gene in cold-adapted microorganisms, however, remain largely unknown. In preliminary studies, four psychrophilic bacteria that could degrade petroleum hydrocarbons and polycyclic aromatic hydrocarbons with high efficiency at low-temperatures (0°C-10°C) have been isolated and identified from the Antarctic region. Researchers then measured the growth curves and degradation curves of these bacteria and analyzed the influence of environmental factors to simulate the sea-ice environment during growth and degradation (Liu *et al*, 2010; Wang *et al*, 2011, 2011, 2014). Although

* To whom all correspondence should be addressed.
Tel: +86+532-88967430, fax: +86+532-88967959;
E-mail: miaojinlai@fio.org.cn

the basic work has been completed, the properties and the characterization of the genes of dioxygenase need to be further explored, since this enzyme played a key catalytic role during the biodegradation process.

In this paper, a naphthalene dioxygenase gene was identified and cloned from the Antarctic cold-adapted strain *Shewanella* sp.NJ49; the gene sequence, structure, functions, and expression were analyzed by several methods, including genomic walking, real-time quantitative polymerase chain reaction (PCR), and bioinformatics analysis. We then evaluate the low-temperature PAH-degradation mechanisms of psychrophilic bacteria, using the molecular characteristics of the key enzyme. Finally, we lay the foundations for the system establishment of efficient artificial PAHs biodegradation and a deeper exploration of the functions of the dioxygenase gene.

MATERIALS AND METHODS

Bacterial strain

Bacterial strain that named *Shewanella* sp.NJ49 was isolated and purified from the Antarctic sea-ice, which was collected during the Chinese 19th Antarctic Science Exploration in 2002-2003. The optimum growth temperature of NJ49 was between 10°C to 15°C, and it can degrade petroleum hydrocarbon and PAHs efficiently at low-temperatures (Liu *et al*, 2010; Wang *et al*, 2011). This bacterium was deposited in the low-temperature strain library of Key Laboratory of Marine Bioactive Substances, SOA.

Reagents, culture media and methods

Mineral medium (MMC) (Wilson, 2001): NaCl 24 g/L, $MgSO_4 \cdot 7H_2O$ 7.0 g/L, NH_4NO_3 1.0 g/L, KCl 0.7 g/L, KH_2PO_4 2.0 g/L, Na_2HPO_4 3.0 g/L, pH 7.4; Microelements media: $CaCl_2$ 0.02 mg/L, $FeCl_3 \cdot 6H_2O$ 0.5 mg/L, $CuSO_4$ 0.005 mg/L, $MnCl_2 \cdot 4H_2O$ 0.005 mg/L, $ZnSO_4 \cdot 7H_2O$ 0.1 mg/L. The screening media were supplemented by some microelements and naphthalene. Microelements and naphthalene were sterilized using 0.2 μm microfiltration membranes (Wang *et al*, 2014).

We used a genomic walking kit, which included four restriction endonucleases (*DraI*, *EcoRV*, *PvuII*, and *StuI*) (Clontech Co., Ltd, USA), genomic walking adaptor, rTaq DNA polymerase, T4 NDA ligase, pMDTM-18 T vector and PCR reagents (TaKaRa Co. Dalian, China), a genomic DNA extraction kit, a DNA gel extraction kit (TIANGEN Biotech Co., Ltd), and pGEM-T Easy kit (Promega Biotech Co., Ltd).

PCR thermal cycler: PCT-200 (MJ Research Inc. USA); Real-time quantitative PCR: ABI 7000 (USA).

Extraction and sequencing of genomic DNA

The catechol-2,3-dioxygenase gene was amplified by PCR from the chromosomal DNA of Shewanella bacteria NJ49 with the degenerate primers DioF and DioR. A genomic DNA extraction kit was used to extracting DNA of *Shewanella* sp.NJ49, the PCR amplification conditions were as follows: initial denaturing 5 min at 95°C; 30 cycles of denaturantion 45 s at 95°C, annealing 1 min at 40°C, extension 1min at 72°C, a final extension of 5 min at 72°C (Pini *et al*, 2007). The amplified DNA fragment which after PCR was separated on 1.5 % agarose gel, eluted from the gel and purified using the DNA gel extraction kit. The purified PCR product was connected to the cloning plasmid pMDTM-18 T vector and then transformed into *E.coli* DH5a, which amplified the DNA inserted fragment by universal sequencing primers and selected the positive recombinant. The gene's sequence was made by GenScript (Nanjing) Co., Ltd, and the obtained sequence was compared by analysis of the NCBI database BLAST.

Genome walking and cloning of *Shewanella* sp.NJ49 dioxygenase gene

Primary PCR of Genome Walking

Two gene specific sense and antisense primers (Gsp) were designed for the 3' end and 5' end amplification, respectively (Tab.1) according to the previously known sequence of the catechol-2, 3-dioxygenase fragment. Degenerate walker primers (Ap1 and Ap2) were provided by the GenomeWalker Kit. Genomic DNA was digested with each of the restriction enzymes *Dra I*, *EcoR V*, *Pvu II* and *Stu I* respectively and incubated at 37°C overnight. Then genomic DNA was ligated to GenomeWalker Adaptor to construct four Genomewalker libraries as template for PCR reaction. The amplification reaction was performed in a volume of 50μL with 10×Advantage 2 PCR buffer, 10mM dNTPs, 10μM degenerate walker primer Ap1 and gene specific primer Gsp1, 40μL deionized H2O, 1μL 50×Advantage 2 Polymerase Mix and 1μL template.

Secondary PCR of Genome Walking

The PCR product was diluted 50-fold and 1μL was used as a DNA template for secondary PCR containing10μM degenerate walker primer Ap2 and gene specific primer Gsp2 in a volume of 50μL.

The upstream primer and downstream primer of the first- and second-round PCR that named U348Gsp1, D280Gsp1, U313Gsp2, and D329Gsp2 (The gene's sequence was made by GenScript (Nanjing) Co., Ltd), and the gene walker primers of dioxygenase gene that named Gsp1c and Gsp2c were designed based on the cDNA sequence of dioxygenase gene that was cloned from the cDNA library of *Shewanella* sp.NJ49. The primers AP1 and AP2 that were used for the genomic walking kit were the primers of first- and second- round PCR (Guo *et a*l, 2006).

Expression of gene by real-time quantitative PCR

The experiments were divided into three groups, and different temperature, naphthalene concentrations, and UV-B radiation levels were disposed in the MMC medium for each group. In the first group, the temperature gradient was disposed as 0°C, 5°C, 10°C, 15°C, and 20°C; the naphthalene concentration was 50 mg/L; the culture time was 2 days. In the second group, the naphthalene concentration gradient was disposed as 0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L; the culture time was 2 days and the temperature was 15°C. In the third group, we disposed UV-B radiation gradient as 0.5 h, 1 h, 2 h, 4 h, and 6 h; the temperature was 15°C, and the naphthalene concentration was 50 mg/L.

The reversed transcription-polymerase chain reaction was adopted to obtain cDNA from NJ49. The real-time PCR reaction was performed in 20 μL volumes (Margesin, 2000), which contains approximately 10 μL of SYBR (Takara), 0.4 μL of each primers (10 μmol/L), 2 μL of cDNA, and 7.2 μL of deionized water. Meanwhile a negtive control without templates was set in order to compare with the other samples and verify the absence of any contamination by genomic DNA. The PCR amplification was performed under the following conditions: 30 s at 95°C, 5 s at 95°C, 10 s at 55°C, and extension of 30 s at 72°C, repeated for 40 cycles. At the end of the RT-PCR a melting curve analysis was performed by a final step that consists of the measurement of the SYBR Green I signal intensities during a 0.3 °C temperature increment every 10 s from 60°C to 95°C. The control groups were introduced simultaneously. Specially, the experiments evaluating the stability of the housekeeping genes and the gene expression assays with the 16S rRNA gene were performed in triplicate (Quiroz *et al*, 2010), so that quantification could be performed in triplicate Real-Time PCR runs. The resulting data were processed by the $2^{-\Delta\Delta CT}$ method (Livak *et al*, 2001) using SPSS 17.0 software (SPSS Inc. Chicago, USA).

RESULTS AND DISCUSSION

Cloning of NJ49 dioxygenase gene and full sequence

Amplification and cloning of NJ49 dioxygenase gene fragment occurred with degenerate primers DioF and DioR. Agarose gel electrophoresis analysis of the products amplified by primers DioF and DioR detected a target fragment approximately 800 bp. The positive clone was selected and sequenced, and then the sequencing results were comparatively analyzed in GenBank by the BLAST programs. Finally a fragment sequence about 796 bp of dioxygenase gene was obtained. After splicing the dioxygenase gene fragment sequence, an upstream sequence and a downstream sequence by the BioEdit and the ClustalX, a full-length sequence was obtained.

Bioinformatics analysis of NJ49 dioxygenase gene

The obtained amino acid sequences of NJ49 dioxygenase gene were compared with other sequences available in GenBank (Fig.1): the amino acid sequence of dioxygenase gene from *Shewanella* sp.NJ49 was similar to the catechol 2,3-dioxygenase (C23O) that reported in *Pseudomonas mendocina* (AAW81669), *Planococcus* sp.S5 (ADV39941), *Stenotrophomonas maltophilia* (ABS54524), *Achromobacter* sp.BP3 (ACF20629), and *Acinetobacter* sp.YAA (BAA23555) (Merimaa *et al*, 2006; Wojcieszynska *et al*, 2011; Hupert-Kocurek *et al*, 2012).

Using the Neighbor Joining method, we characterized the phylogenetic relationship between strain NJ49 and other related reference species, based on the dioxygenase gene sequence analyses (Fig.2). As that shown in the figure, this

phylogenetic tree was divided into two separate branches, where *Shewanella* sp.NJ49 and *Pseudomonas mendocina* (Merimaa *et al*, 2006) had the closest evolutionary relationship and a homologic identity of 99%.

There are many strains of *Pseudomonas* could degradated PAHs, like *Pseudomonas spp*. (Whyte *et al*, 1997), *P. mendocina* (Merimaa *et al*, 2006), and *Pseudomonas* sp. ND6 (Jiang *et al*, 2004). Such a high homology of gene sequences indicates a recent common genetic origin of all these enzymes and horizontal transfer of the gene that coded C23O

Table 1. The primers and sequences of dioxygenase fragments and full-length gene clone

Primers	Primers Sequences(5'-3')
DioF	52 -TGATCGAGATGGACCGTGACG-32
DioR	52 -TCAGGTCAGCACGGTCATGAA-32
Ts2s	5'-AAYAGAGCTCAYGARYTRGGTCAYAAG-3'
Ts2smod	5'-AAYAGAGCTCAYGARITIGGICAYAAR-3'
Deg1re	5'-GTGGAATTCGCRTGRTGRTGRTGRTCIGARTG-3'
Deg1re2	5'-GTGGAATTCGCRTGRTGRTGRTCRCTRTG-3'
16S	F:GACATCCACAGAAGAGACCAGAGAR:CCCACACATTTCACAACACGA
Gspc1	5'-AAATGGTGCTGGCGTAAGAGT-3'
Gspc2	5'-AAGACGCCACCGCTAATGT-3'
U348Gsp1	5'-ACACTCATCGCCCAAGCGTTGAGAATA-3'
D280Gsp1	5'-ACTCCAGAGGAAAGGCAAACGAACCTG-3'
U313Gsp2	5'-GAAGCTCCAGGTTCGTTTGCCTTTCC-3'
D329Gsp2	5'-AACGCTTGGGCGATGAGTGTTGTCCT-3'

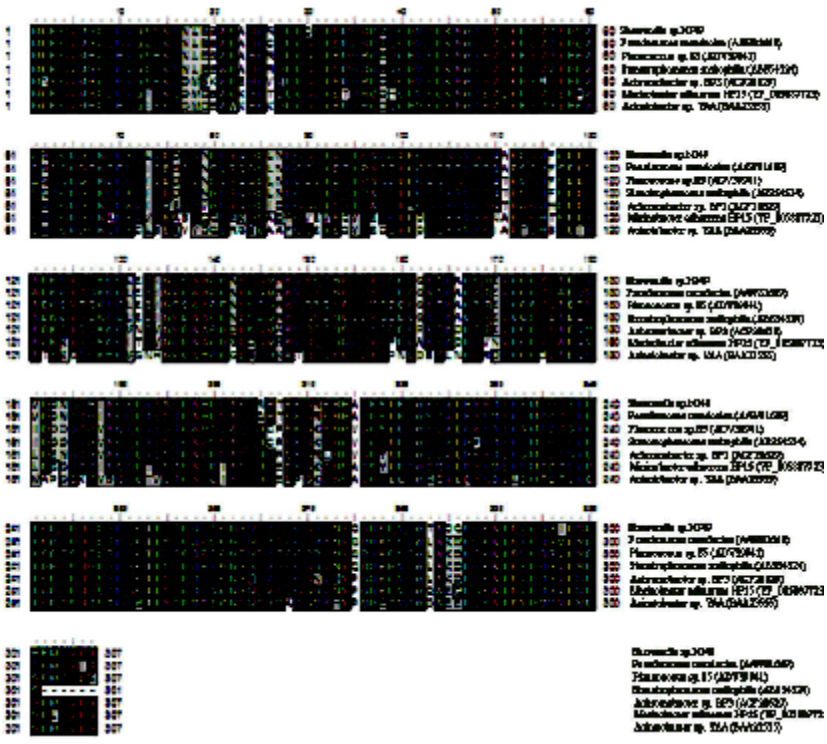

Fig.1 Amino acid sequence alignment of the dioxygenase from Antarctic psychrophile *Shewanella* sp. NJ49 with other sequences in GenBank

in many microorganisms. However, the optimum growth temperature of *Pseudomonas mendocina* was above 30°C (Heinaru *et al*, 2000), and the C23O from *Planococcus* sp.S5 was optimally active at 60°C (Hupert-Kocurek *et al*, 2012), all of that were much higher than NJ49. So NJ49 has obvious advantages at low-temperature.

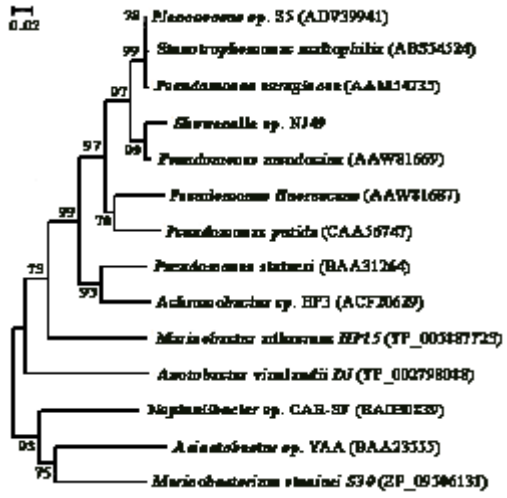

Fig.2 Phylogenetic tree of *Shewanella* sp. NJ49 based on dioxygenase gene sequences

The bootstrap values (%) are given at the nodes to which they apply, and the scales bars represent 0.02 substitutions/site, the data-set was bootstrapped 1000 times. The accession numbers are in parentheses.

Real-time PCR analysis of dioxygenase gene expression

Under the conditions of different temperatures and the naphthalene concentration was 50 mg/L, the expression of the dioxygenase gene increased with temperature increases (Fig. 3); when the temperature was 0 °C, 5 °C, or 10 °C, the relative expression of the gene was 1.5 times, 2.2 times, or 2.6 times greater, respectively. Maximum relative expression of 2.8 times occurred at 15 °C. However, the expression decreased rapidly as temperature increased beyond 15 °C, and expression reached the lowest level when above 20 °C.

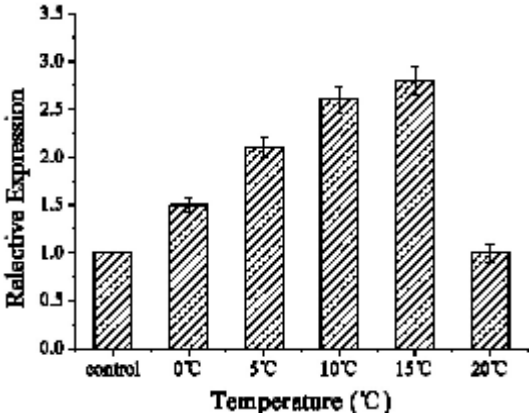

Fig.3 Gene expression levels of Antarctic psychrophile *Shewanella* sp. NJ49 dioxygenase gene with temperature gradient analyzed by qRT-PCR

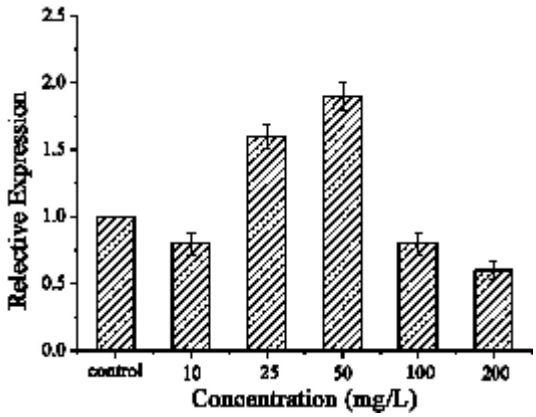

Fig.4 Gene expression levels of Antarctic psychrophile *Shewanella* sp. NJ49 dioxygenase gene cultured with naphthalene concentration gradient

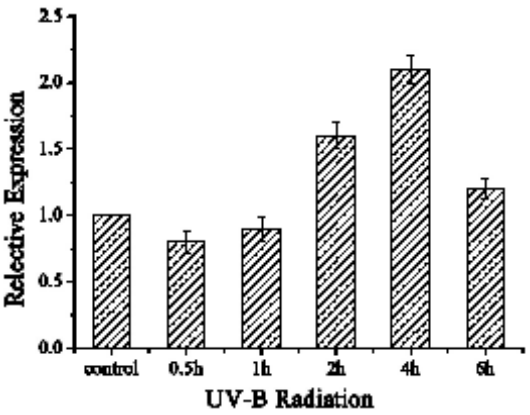

Fig.5 Gene expression levels of Antarctic psychrophilic bacteria *Shewanella* sp. NJ49 dioxygenase gene with UV-B radiation for 0.5h, 1h, 2h, 4h and 6h analyzed by qRT-PCR

Figure 4 shows the expression levels of *Shewanella* sp. NJ49 dioxygenase gene that cultured with a naphthalene concentration gradient. The relative expression lower was than that of a control group when the naphthalene concentration was 10 mg/L, and that were 1.6 times,1.9 times when the naphthalene concentration were 25 mg/L and 50 mg/L, the expression decreased greatly when the naphthalene concentration exceeds 100 mg/L. This was because naphthalene might be a stimulus of the bacterium NJ49 in a certain range of concentrations, the activity of dioxygenase gene was not activated when concentration was low, but it was inhibited by a higher concentration.

From figure 5, expression levels of *Shewanella* sp. NJ49 dioxygenase gene with UV-B radiation for 0.5 h, 1 h, 2 h, 4 h and 6 h analyzed by qRT-PCR, the relative expression was lower than that of a control group when the UV-B radiation for 0.5 h and 1 h, the expression of the dioxygenase gene was increased with UV-B radiation for 2 h and 4 h, and the relative expression were 1.6 times and 2.1 times. But the relative expression was only 1.1 times with UV-B radiation for 6 h.

Illumination radiation varies greatly in Antarctic region, and radiation time will inevitably affect the metabolic processes of microorganisms. It is confirmed that UV-B is obviously harmful to microorganisms, and the harmful aims are protein, DNA, and so on (Miao *et al*, 2004).Through the UV-B radiation experiment, we learned that UV-B suppressed the expression of the dioxygenase gene. Initially, the expression of dioxygenase decreased when NJ49 was irradiated by UV radiation, and the expression level of dioxygenase gene increased gradually with the extension of UV-B radiation time. Finally, the level of dioxygenase gene expression was inhibited and decreased with time increasing by UV-B radiation.

In summary the gene expression experiments of the temperature gradient that NJ49 had the highest expression level when the temperature was 15 °C, the results were consistent with the results of the NJ49 PAH-degradation experiments at low temperatures. According to our previous research (Liu *et al*,2010; Wang *et al*, 2011&2014), *Shewanella* sp.NJ49 was a psychrophilic bacterium that grew most rapidly at 10°C, and it can hardly grow at temperatures exceeding 20°C; therefore, the dioxygenase gene expressed rarely at high temperatures. Thus, the catechol 2,3-dioxygenase of *Shewanella* sp.NJ49 belongs to cold-adapted enzymes which have highly effective even at low temperatures.

An ever-increasing number of aromatic hydrocarbons-degradation genes (generally from genus *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Bacillus*, *Burkholderia*, and *Rhodococcus*) (Wojcieszynska *et al*, 2011) have been cloned and separated, and the sequences, structure, composition, functions, expression, and regulation of catechol 2,3-dioxygenases gene have been studied thoroughly. Many researchers have characterized the various pathways of aromatic hydrocarbon biodegradation. Microbial degradation of aromatic hydrocarbons proceeds generally via the *ortho* fission catalyzed by catechol 2,3-dioxygenases and the reaction beginning of the oxidation of benzene ring, while dioxygenase cleaves the aromatic ring of the substrate outside the two hydroxyl groups and produces 2-hydroxymuconate semialdehyde, and the intermediate products enter the TCA cycle (Hayaishi *et al*, 1955; Merimaa *et al*, 2006; Gibson *et al*, 2000; Senda *et al*, 1996; Hugo *et al*, 1998; Bugg, 2003). The crystal structure of 2,3-dioxygenase monomer consists of two domains containing repetition of ±-² module (Hatta *et al*, 2003; Kim *et al*, 2005).

According to previous research results, dioxygenase is a homotetramer and contains catalytically essential Fe(II). The reaction proceeded by an ordered bi-unit mechanism. First, catechol bound to the enzyme, and then the compound bound to dioxygen to form a tertiary complex. This aromatic ring was cleaved to produce 2-hydroxymuconate semialdehyde (Senda *et al*, 1996; Hugo *et al*, 1998; Bugg, 2003).

To date, the studies on the biodegradation and bioremediation of PAHs have been mostly focusing on areas of those strains which can degrade efficiently at normal temperature, but the pollution and bioremediation in cold environments is an important problem which should not be ignored.The degradation of aromatic hydrocarbons in cold waters depends on enzymes functioning at low temperatures. Most of the bacteria could not grow at low temperatures, even hardly to degrade aromatic hydrocarbons

effectively. Therefore the cold-adapted bacteria were needed. Only limited work has occurred in this field. So the discovery of biodegradation in the environment with the lower temperature optima is very important for understanding the biochemical and evolutionary basis of cold bioremediation of bacteria, which was useful for the degradation of environmental pollution.

CONCLUSIONS

In this article, the gene of low-temperature degrading enzyme from an aromatic hydrocarbon degradation bacterium *Shewanella* sp.NJ49 that isolated from Antarctic sec-ice was studied. After cloning and splicing fragment sequences, a full-length sequence of dioxygenase gene was obtained, according to the results of phylogenetic tree and amino acid sequence analysis in GenBank, the amino acid sequence of dioxygenase gene from *Shewanella* sp.NJ49 was similar to the catechol 2,3-dioxygenase from *Pseudomonas mendocina*, and the homologic identity was 99%. The expression level of dioxygenase gene by qRT-PCR in different habitats showed that NJ49 had the highest expression level when the temperature was 15 °C, naphthalene concentration was 50 mg/L and under UV-B radiation for 4 h. These results of dioxygenase gene expression experiments were consistent with previous NJ49 PAH-degradation experiments.

Through this research, we gained some understanding of the structure and function of the dioxygenase gene from Antarctic psychrophilic bacterium *Shewanella* sp.NJ49, aiding scientist in the in-depth study of bioremediation mechanisms that degrade the aromatic hydrocarbon pollution in cold waters with using Antarctic cold-adapted bacteria.

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