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Molecular Cloning and Characterization of the *alkB* gene of *Shewanella* sp. NJ49 from Antarctic Sea Ice

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A psychrophilic bacterium *Shewanella* sp. NJ49 isolated from Antarctic sea-ice is capable of utilizing diesel fuel as the sole carbon and energy source. The gene encoding alkane monooxygenase (*alkB*) was obtained by using a genome walking technique in this strain, and revealed an open reading frame (ORF) of 1176 bp encoding a protein of 392 amino acid residues. Amino acid sequence alignment showed that its genetic relationship is close (99% identity) with *Rhodococcus erythropolis* PR4 (accession no. YP 002764193.1), *Rhodococcus erythropolis* SK121 (accession no. ZP 04388098.1) and *Rhodococcus* sp. Q15 (accession no. AAK97448.1). The *alkB* gene expression variations under different temperatures, various concentrations of n-Hexadecane, different handling time of UV-B radiation and high salinity were quantitatively analyzed by fluorescent quantitative real-time PCR (qRT-PCR). Finally the optimized expression levels were obtained, respectively. These results indicate that *alkB* gene as a functional gene likely play a crucial role in *Shewanella* sp. NJ49 degrading petroleum hydrocarbon under Antarctic extreme environments.

Key words*:* Antarctic bacterium; *Shewanella* sp. NJ49; Hydrocarbon degradation; Alkane.

In the Antarctic, although petroleum exploitation is not permitted, anthropogenic hydrocarbon contamination deriving from tourism, research and fishing activities is increasing more and more, especially for the accidents of tanker spills and tanker sinking[4,25]. Hydrocarbons of oil origin are serious environmental pollutants due to their persistence and high toxicity in biological systems including the marine habitats. Once released into marine environment hydrocarbons are partially degraded by endemic microbial communities, the most toxic and refractory fraction settle into sediments, resulting in damages to marine ecosystems.

In recent years, the biodegradation of petroleum at low temperature in Antarctic has received considerable attention. Numerous psychrophilic and psychrotolerant petroleum-degrading bacteria have been isolated from Antarctic seas and their degradation potential investigated, such as *Gelidisbacter*, *Psychroserpens*, *Actinomycetales*, *Sphingomonas* and *Halomonas*[3, 20, 35, 37]. So far, lots of research about Antarctic petroleum-degrading bacteria has focused on the characterization of strains, but relatively little is known about the genetic characteristics of their alkane degradative systems. The alkane monooxygenase (*alkB*) plays an important role in the process of degrading petroleum hydrocarbon of Antarctic microorganism[5,19]. Terminal alkane hydroxylation,

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which can catalyze the initial terminal oxidation of the alkane substrate to a 1-alkanol, is the initial step and the alkane substrate is catalyzed by a multi-hydroxylase families consisting of and two soluble proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) or a single-component monooxygenase[6,30]. Monooxygenases can either introduce one O atom to a carbonyl group or introduce oxygen on a C-H (hydroxylases)[5]. Some gene fragments of alkane monooxygenase (*alkB*) from Antarctic sea were analyzed[11, 21] but the full-length *alkB* sequence from Antarctic sea bacteria has not been reported.

Antarctic psychrophilic bacterium *Shewanella* sp. NJ49, capable of utilizing diesel as the sole carbon and energy source under the temperature of 0-20°C was screened from Antarctic sea-ice[13]. In this study, the gene encoding alkane monooxygenase (*alkB*) was cloned from NJ49 by GenomeWalker technology. Furthermore, we also researched and analyzed the expression levels of NJ49 alkB gene with various treatment conditions by fluorescent quantitative real-time PCR (qRT-PCR).

MATERIALS AND METHODS

Bacteria materials and Cultivation

The psychrophilic petroleum hydrocarbon-degrading bacterium strain NJ49 was isolated from the floating ice sample near the Zhongshan Research Station of Antarctica (69.8 S, 77.8 E) during the 18th Antarctic expedition of China (2001-2002) and designated as *Shewanella* sp. NJ49 [13]. The strain was inoculated in MMC (NaCl, 24 g; $MgSO_4 \cdot 7H_2O$, 7.0 g; NH_4NO_3, 1.0 g; KCl, 0.7 g; KH_2PO4, 2.0 g; Na_2HPO_4, 3.0 g; distilled water, 1.0 L; pH 7.4) liquid culture medium supplemented n-Hexadecane as the sole carbon and energy source under the condition of 8°C, 120 r/min for 2d. Seawater was filtered and sterilized previously by autoclaving and the salinity was 28‰.

Cloning of alkane monooxygenase fragment

Genomic DNA was extracted from NJ49 with the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Beijing, China). The primers used in this study are shown in Table 1. Two forward primers Ts2s, Ts2smod and two reverse primers Deg1re, Deg1re2 were designed to amplify alkB monooxygenase gene fragments by PCR respectively[21]. Two successive PCR rounds are required to obtain partial *alkB* gene. The first PCR amplification conditions are as follows: initial temperature at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min, with one additional extension 72°C for 7 min. With 1ul of the first PCR round product as template, then the second PCR round was carried out with the same conditions of the first PCR round. The expected DNA band was excised from agarose (1.0%, w/v) gel and purified by TIANgel Midi Purification kit (Tiangen Biotech Co., Beijing, China) according to the supplier's instructions. DNA fragments were cloned into PMD18-T Vector (TaKaRa Biotech Co., Dalian, China) and finally sequenced (GenScript Biotech Co., Nanjing, China).

Genome Walking PCR

Genomic DNA was digested with each of the restriction enzymes Dra I, EcoR V, Pvu II and Stu I separately and incubated at 37°C overnight. Each digestion was checked on a 1% agar gel and purified[12]. Then genomic DNA was ligated to GenomeWalker Adaptor to construct four Genomewalker libraries. Based on the previously known sequence of the *alkB* gene fragment, two specific sense primers and two specific antisense primers (Table 1) were designed for the 3' end and 5' end amplification, respectively. Primer Ap1 and Ap2 were provided by the GenomeWalker Kit.

With the primers U318 Gsp1, D245 Gsp1 and Ap1 for the primary PCR amplification, fetching the primary PCR products of four Genomewalker libraries and diluting 50-fold as templates for the secondary PCR[1]. In a similar way, the primers U266 Gsp2, D284 Gsp2 and Ap2 were used for the amplification of expected sequence. The thermal cycling conditions according to the instructions of Genome Walker Kit (Clontech Biotech Co., Mountain View, CA, USA). 5 ul of the primary PCR products were electrophoresed in 1.5% agarose gel[24]. The following steps include PCR products extraction, purification, cloning and sequencing.

Sequence analysis

BLAST searches were performed on the separated 5' and 3' end sequences of NJ49 to identify with the other known sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Then the *alkB* sequence was linked in accordance with DNAStar

7.1 (DNASTAR Inc., USA). Moreover, the amino acid sequence of the *alkB* gene was analyzed using the Six Frame Translation of Sequence system (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html). The theoretical molecular weight (Mw) and isoelectric point (PI) of protein were computed by ExPASy Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). The multiple sequence alignments of alkane monooxygenase were performed by the program ClustalX and Bioedit[27,38]. Furthermoreÿa phylogenetic tree was constructed using the program Mega 4.0 software[26] .

Real-time Fluorescent Quantitive PCR (qRT-PCR)

Total RNAs were extracted with 1 ml of TRIzol Reagent (Invitrogen, USA) from NJ49. The isolated RNA was eluted with 50 ul RNase-free water (Solarbio), and quality was analyzed on 1% agarose gel electrophoresis. The concentrations and quality of RNA were measured with a Nanodrop Spectrophotometer (ND 2000c, USA). Generally, A260/A280 ratio was between 1.8 and 2.0 [7]. Then the cDNA was obtained by total RNA reverse transcription reaction in a 20 µl mixture with MMLV-reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

mRNA quantification was carried out by a two step RT-PCR protocol[23]. Real-time fluorescent quantitative PCR was implemented with 10ul of 2×SYBR Premix Ex TaqTM a! (TaKaRa Biotech Co., Dalian, China), 0.4 µl 10uM of each primer, 2 µl of diluted cDNA mix and 7.2 µl dH_2O in 20 µl final volume by ABI StepOnePlus™ Real-time PCR System (Applied-Biosystems, USA). The alkB primers and the 16S primers that were used as an internal control to calibrate the cDNA templates were listed in Table 1.The amplifying conditions as follows: 95°C for 30s, then 95°C for 5 s, 55°C for 10s, and 72°C for 30s, for 40 cycles. An amplification curve and a melting curve (by heating from 60°C to 95°C with temperature steps of 0.3°C) were checked to verify the results[17]. Meanwhile a negative control without templates was set to compare with the other samples and verify the absence of any contamination by genomic DNA. The experiments also evaluated the stability of the housekeeping genes and the gene expression assays with the 16S rRNA gene were performed in triplicate[23]. Furthermore, the data were analyzed with the comparative Ct (2-””CT) approach[14] and processed using SPSS17.0 Data processing system software.

RESULTS

Cloning and sequencing of alkB gene

Four combinations of primers (Ts2smod-Deg1re; Ts2smod-Deg1re2; Ts2s-Deg1re; Ts2s-Deg1re2) were applied respectively for the PCR amplification of the alkane monooxygenase gene (*alkB*) fragment and finally an expected fragment of 548 bp length was obtained. Meanwhile, two *alkB* gene fragments of 1289 and 588 bp were acquired by genome walking technique with the

Table 1. Primers sequence used in PCR

Primers	Primers Sequences(5'-3')
Partial cDNA cloning	
Ts2s	5'-AAYAGAGCTCAYGARYTRGGTCAYAAG-3'
Ts2smod	5'-AAYAGAGCTCAYGARITIGGICAYAAR-3'
Deg1re	5'-GTGGAATTCGCRTGRTGRTGRTGRTCIGARTG-3'
Deg1re2	5'-GTGGAATTCGCRTGRTGRTGRTCRCTRTG-3'
Genome walking PCR	
U318Gsp1	5'-GCGACACCGAACAGAACAACCGACAT-3'
U266Gsp2	5'-TGATGCTCCAGTGCGTGGTGCCCAAA-3'
D245Gsp1	5'-GGCACCACGCACTGGAGCATCAAGAA-3'
D284Gsp2	5'-GCCTGGCCCATGTCGGTTGTTCTGTT-3'
qReal-time PCR	
alkB F	5'-TCGTCGGGCGGATTCTT-3'
alkB R	5'-CGGATGGAATGTCTTCTGGTG-3'
16S F	5'-GACATCCACAGAAGAGACCAGAGA-3'
16S R	5'-CCCACACATTTCACAACACGA-3'

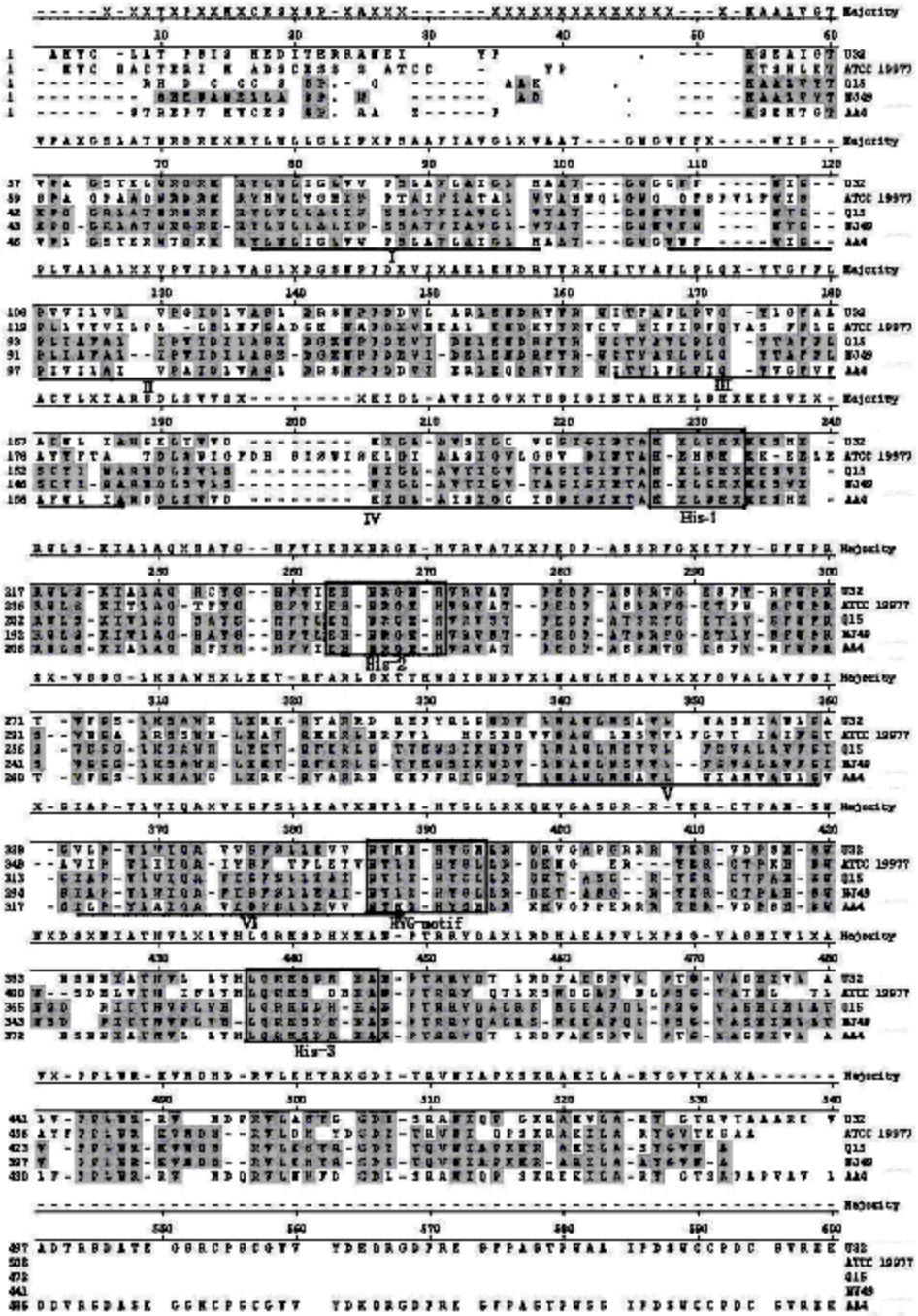

Fig. 1. Amino acid Sequence alignment of the alkane monooxygenase from *Shewanella* sp. NJ49 with other sequences from *Amycolatopsis mediterranei* U32 (accession no. YP_003768535.1), *Mycobacterium abscessus* ATCC 19977 (accession no. YP_001704327), *Rhodococcus* sp. Q15 (accession no. AAK97448.1), *Streptomyces* sp. AA4 (accession no. ZP_05483348.1) in Genebank were analyzed by ClustalX program and DNAStar software. The three conserved histidine boxes of the eight-histidine motif (HIS-1, HIS-2, and HIS-3) and the additional histidine motif NYXEHYGL (HYG motif) are framed and marked. The locations of the six putative transmembrane helices in the *alkB* sequence from *Shewanella* sp.NJ49 are underlined

two specific sense primers and two specific antisense primers. In addition, ORF finder analysis showed that the sequence of *alkB* gene contained an open reading frame of 1176bp encoding a 392 amino-acid protein with a predicted molecular weight of 44.3 kDa and a theoretical pI of 8.89, which was submitted to GenBank under accession number HQ214630.

Sequence analysis

After the comparison among the deduced amino acid sequence and the other known sequences from the NCBI, the results revealed the *alkB* of *Shewanella* sp. NJ49 was homologous to the putative alkane-1-monooxygenase (*alkB*1) of *Rhodococcus* sp. Q15 (AAK97448.1), *alkB*1 gene of *Rhodococcus erythropolis* SK121 (ZP_04388098.1) and *alkB*1 gene of *Rhodococcus erythropolis* PR4 (YP_002764193.1). Moreover, the deduced amino acid sequence of *Shewanella* sp. NJ49 were all greater than 93% identity compared with the known sequences of other bacteria such as *Mycobacterium gilvum* PYR-GCK (YP_001134633.1, 93%) and *Rhodococcus equi* 103S (YP_004008018.1, 96%) etc.. The amino acid sequence alignment of *Shewanella* sp. NJ49 with the other bacteria is shown in Fig. 1. Interestingly, the *alkB* of *Shewanella* sp. NJ49 and the majority of other alkane monooxygenases hold the homologous positions and lengths of the six transmembrane helices that were conserved and originally introduced in *P. Putida* GPo1 *AlkB*[31]. A phylogenetic analysis was performed using the deduced amino acid *alkB* sequences of 18 various genera of bacteria that were reported in Genbank (Fig. 2). The phylogenetic tree can be devided into two major branches from the phylogenetic relationship, designated I and II. In clade I, *Shewanella* sp. NJ49 (HQ214630) showed closest relationship with *Rhodococcus*[2, 21, 32, 36], which is capable of degrading hydrocarbon from the polar oceanic ecosystem according to the previous reports. In addition, *Alcanivorax*[21] and *Burkholderia*[35] belong to clade II were relatively distant from *Shewanella* sp. NJ49, however, they were regarded as the most important degrading bacteria of ocean.

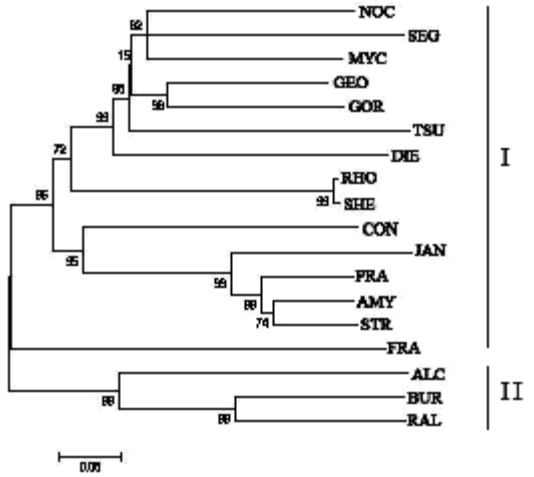

Fig. 2. Phylogenetic tree of bacterial *alkB* sequences. Branch lengths are drawn to scale and the numbers at respective nodes indicate the bootstrap values in percentage (Scale bar, 0.05 substitutions per amino acid site). Species and sequence accessions (GenBank) are as follows: *Alcanivorax dieselolei* (ALC), AAT91722.2; *Amycolatopsis mediterranei* U32 (AMY) YP_003768535.1; *Burkholderia* sp. 383 (BUR) YP_368326.1; *Conexibacter woesei* DSM 14684 (CON) YP_003397515.1; *Dietzia* sp. E1 (DIE) ACN62569.1; *Frankia* sp. EuI1c (FRA)YP_004016090.1; *Geobacillus* sp. MH-1 (GEO) ACR55689.1; *Gordonia* sp. TF6 (GOR) BAD67020.1; *Janibacter* sp. HTCC2649 (JAN) ZP_00996652.1; *Mycobacterium chubuense* NBB4 (MYC) ACZ65961.1; *Nocardia farcinica* IFM 10152 (NOC) YP_120833.1; *Prauserella rugosa* (PRA) CAB51024.2; *Ralstonia pickettii* 12J (RAL) YP_001892637.1; *Rhodococcus* sp. Q15 (RHO) AAK97448.1; *Segniliparus rotundus* DSM 44985 (SEG) YP_003658078.1; *Shewanella* sp. NJ49 (SHE) ADO21492.1; *Streptomyces* sp. AA4 (STR) ZP_05483348.1; *Tsukamurella paurometabola* DSM 20162 (TSU) YP_003647687.1

Effects of temperature on expression of alkB gene

Strain NJ49 was cultured in MMC fluid culture medium with 20 mg/L n-Hexadecane at 0°C, 5°C, 10°C, 15°C and 20°C for 2 days. The results of qRT-PCR revealed obvious difference among the five temperature gradient groups in the gene expression levels of *alkB* gene (Fig. 3). The target genes demonstrated a characteristic bell-shape expression rhythm. Initially, the expression level was gradually increasing from 0°C to 10°C with a value of 1.4-fold, 2.5-fold, 3.9-fold, respectively. Meanwhile, the *alkB* gene of NJ49 at 15°C can reach the highest point of the expression level quickly with a value of 6.1-fold. However, it declined at 20°C sharply and the expression level of 20°C sample was lower than all other samples.

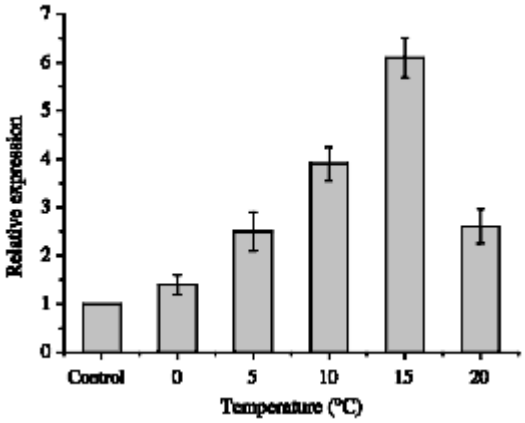

Fig. 3. Gene expression levels of *Shewanella* sp. NJ49 monooxygenase with temperature gradient 0°C, 5°C, 10°C, 15°C and 20°C analyzed by qRT-PCR

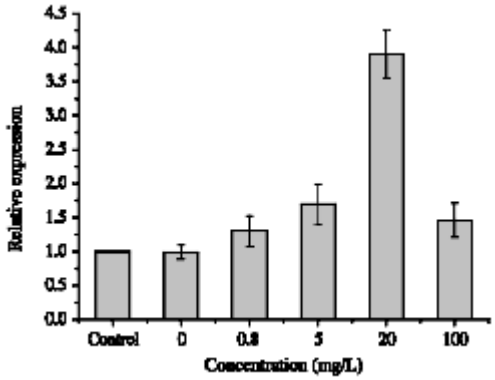

Fig. 4.Gene expression levels of *Shewanella* sp. NJ49 monooxygenase with concentration gradient 0 mg/L, 0.8 mg/L, 5 mg/L, 20mg /L and 100 mg/L n-Hexadecane analyzed by qRT-PCR

Effects of n-Hexadecane concentration on expression of alkB gene

Strain NJ49 was cultivated in MMC fluid culture medium with 0 mg/L, 0.8 mg/L, 5 mg/L, 20 mg/L and 100 mg/L n-Hexadecane at 15°C for 2 days. The target gene expression levels of four samples which were added n-Hexadecane is higher obviously along with the increasing concentration of n-Hexadecane than the control gene with no n-Hexadecane (Fig. 4). Originally, the expression level increased 1.3-fold and 1.7-fold corresponding to the n-Hexadecane concentration of 0.8 mg/L and 5 mg/L, respectively. Especially, the highest point of expression is the sample of 20 mg/L n-Hexadecane with a maximum value of 3.9-fold. Afterwards its expression level declined with a certain extent.

Effects of high salinity on expression of alkB gene

Strain NJ49 was cultured in MMC fluid culture medium with 20 mg/L n-Hexadecane at 15°C containing 90‰ NaCl for 2h, 4h, 6h, 12h and 24h. The result of qRT-PCR revealed that the expression levels of *alkB* gene were restrained as the extension of the processed time with high salinity (Fig. 5). After 6h treatment in MMC liquid culture medium of 90‰ NaCl, the expression levels decreased 1.2 fold compared with the sample of 2h treatment and increased 2.3 fold compared with the sample of 24h treatment.

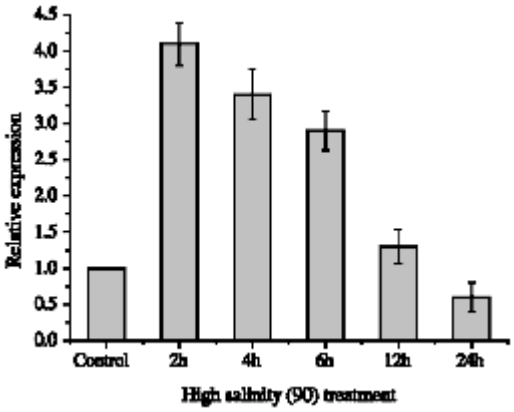

Fig. 5. Gene expression levels of *Shewanella* sp. NJ49 monooxygenase with high salinity (90‰ NaCl) for 2h, 4h, 6h, 12h and 24h analyzed by qRT-PCR

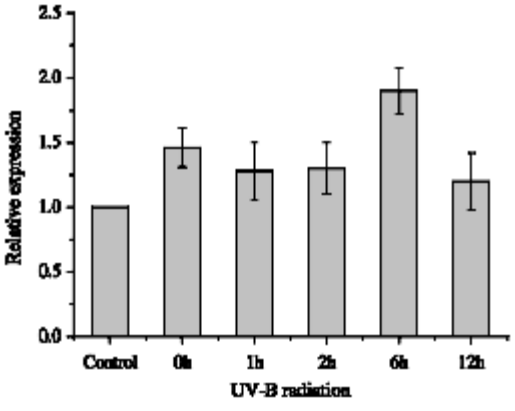

Fig. 6. Gene expression levels of *Shewanella* sp. NJ49 monooxygenase with UV-B (65 ¼w cm^{-2}) radiation for 0h, 1h, 2h, 6h and 12h analyzed by qRT-PCR

Effects of UV-B radiation on expression of alkB gene

Strain NJ49 was cultured in MMC fluid culture medium with 20 mg/L n-Hexadecane at 15°C under UV-B radiation reaching 65 μw cm^{-2} irradiance for 0h, 1h, 2h, 6h and 12h respectively (Fig. 6). The result of qRT-PCR revealed that the expression levels of *alkB* gene both decreased to 1.3 fold after 1h and 2h UV-B radiation treatment compared with the sample of no ultraviolet radiation. However, it increased to 1.9 fold and decreased to 1.2 fold after 6h and 12h UV-B radiation treatment, respectively.

DISCUSSIONS

Alkane monooxygenases (Alk) are the key enzymes for alkane degradation. Despite the studies reporting the detection of *alk* genes by isolation and phylogenetic identification of indigenous cold-adapted degrading microorganisms and the characterization of communities with hydrocarbon degradation capacity[11, 21, 22], information on the DNA code of these alk genes in polar environments remains scarce. In this study, the sequences of alkane monooxygenase of *Shewanella* sp. NJ49 was cloned and has the high identity with *alkB1* of Gram-positive bacterium *Rhodococcus* who is reported as an important and predominant alkane-degradative bacterium in both pristine and contaminated polar areas[33]. In addition, *Rhodococcus* has been reported that it is capable of assimilating and degrading C12–C32 *n*-alkanes at low temperatures[32]. The multiple sequence alignment revealed that *alkB* of *Shewanella* sp. NJ49 was well-conserved with and belongs to the alkane degradative system.

Alkane monooxygenase can catalyze a wider range of alkanes than the other monooxygenases and have diverse sequences among Gram-negative and Gram-positive isolates[10, 29]. About the structure of the alkane degradative system in bacteria, *Shewanella* sp. NJ49 showed amino acid sequence identities of 99% (the most high identity) to *Rhodococcus,* and that there are relatively more conserved sequences of alkane monooxygenase in various bacteria. We conclude that the structure of *alkB* of *Shewanella* sp. NJ49 is similiar to *Rhodococcus* strains, which may possess at least four alkane mono-oxygenase gene homologs (*alkB1*, *alkB2*, *alkB3* and *alkB4*)[34]. In addition, there may be several characteristic features of three histidine boxes (HIS1, HE[L/M]XHK; HIS2, EHXXGHH and HIS3, LQRH[S/A]DHHA) (Fig. 1). An additional histidine box HYG motif (NYXEHYG[L/M]) which is well-conserved and be taken as characteristic feature for bacterial alkane monooxygenases as HIS3 box[31, 34]. Moreover, the HIS3 box is longer than the HIS 1 and HIS 2 and holds completely conserved sequences in all alkane monooxygenases, however, it displays unlike conserved sequences in other hydrocarbon monooxygenases. The definite and specific structure need to further research. The degenerate primers may be applied to other Antarctic bacteria to amplify alkane monooxygenase fragments.

Biodegradation processes of organic pollutants in Antarctic sea ice are more difficult to achieve than those in most temperate oceans. An understanding of the community structure and how it is affected by factors which may be limiting hydrocarbon degradation will allow us to better understand contaminated sites and thus allow better optimisation of site remediation activities[22]. In this present study, we obtained the optimized conditions of *alkB* gene expression with regard to temperature, concentration of n-hexadecane, high salinity and UV-B radiation by qRT-PCR analysis, however, these conditions are variable and unstable owing to seasonal climatic change, the melt and drifting of Antarctic iceberg, and ocean current variation and so on[18, 28]. The optimized temperature and concentration of n-hexadecane show that *Shewanella* sp. NJ49 is a psychrophilic bacterium according to the classical definitions of psychrophiles and psychrotrophs[16] and its relative high degradative ability for petroleum hydrocarbon of high concentration. We deduced that it may be the results of acclimatization in the process of isolation and cultivation under artificial conditions after collecting from Antarctic.

In addition, UV radiation is also very important for Antarctic organisms such as algas, bacteria and phytoplankton etc.[8, 9]. UV radiation is increasing obviously in the Antarctic regions as the aggravation of depletion of stratospheric ozone and has an effect on various vital movements and physiological properties of Antarctic organisms including DNA damage, impairment of

photosynthesis, respiration, protein synthesis and other metabolic functions[15]. As showed in Fig. 6, the expression levels of *alkB* gene decreased initially compared with the sample of 0h UV-B radiation. However, it increased to 1.9 fold after 6h UV-B radiation and decreased to 1.2 fold after 12h UV-B radiation. The result indicates that *alkB* gene of *Shewanella* sp. NJ49 can expression normally with certain intensity of UV-B radiation because of the defense systems and adaptability by itself for Antarctic extreme environment, although the expression levels can be restrained originally.

CONCLUSIONS

In this study, the *alkB* gene of *Shewanella* sp. NJ49 from Antarctic sea ice was cloned and its molecular characterization was analyzed. The highest expression level of the newly recognized *alkB* gene was obtained at 15°C and under 20 mg/L n-Hexadecane. Meanwhile the *alkB* gene can resist UV-B radiation to a certain extent and be restrained by high salinity containing 90‰ NaCl. The results will allow us to better understand the genetic characteristics of low temperature alkane degradative system and aid in the development of bioremediation schemes for low temperature marine environment.

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