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Identification of Regulatory Sequences and Expression Analysis of *OmpR* Gene Under Different Stress Conditions in the Antarctic Bacterium *Psychrobacter* sp. G

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Abstract An *OmpR* gene, named *OmpR*503, was cloned from the Antarctic psychrotrophic bacterium *Psychrobacter* sp. G according to its genomic draft. The deduced amino acid sequences of *OmpR*503 were highly conserved with other known protein members of *OmpR* family. qRT-PCR analysis showed that the expression of *OmpR*503 gene was significantly enhanced by high salinity (90, 120). The expression of *OmpR*503 gene was also significantly increased at low temperature (0, 10 °C), whereas depressed at high temperature (30 °C). When the strain was subjected to combined stress (0 °C with a salinity of 90), the expression of *OmpR*503 gene was increased significantly, which was up to 3.0-fold. In Antarctica, freezing tolerance of psychrotrophic bacteria is often accompanied by tolerance to osmotic stress caused by a lack of free water, thus the cold inducibility of *OmpR*503 gene might help the strain adapt to the harsh environment more efficiently.

Introduction

Signal transduction systems through protein phosphorylation play an important role in bacteria's response to complex environmental changes. The simplest form of these systems includes two protein components, a sensor His-kinase and a response regulator [12, 23]. The sensor, which is often located in the cytoplasmic membrane, monitors the environmental changes and relays the information to the response regulator in the cytoplasm by His-Asp phosphorelay [17, 28]. The response regulator, in turn, mediates changes in gene expression of the sensor [23]. This is the type of the so called two-component signal transduction system.

The *EnvZ*/*OmpR* system is a typical two-component His-Asp phosphorelay signal transduction system in *Escherichia coli* [10]. EnvZ is a transmembrane osmosensor which exhibits the kinase activity toward *OmpR* and the phosphatase activity toward phosphorylated *OmpR* (*OmpR*-P) [2, 9]. *OmpR* is a transcriptional activator protein which binds to the promoter regions of both the OmpC and OmpF [3, 8, 22]. *OmpF* and *OmpC* are two membrane porin proteins which serve as passive diffusion pores across the outer membrane. The two membrane porin proteins are reciprocally regulated by *OmpR* at the transcriptional level [32]. *ompF* is preferentially expressed at low osmolarity and *ompC* at high osmolarity [21]. Yoshida [32] proposed that osmotic signals regulated the levels of *OmpR*-P by modulating the ratio of kinase to phosphatase activity of EnvZ.

OmpR is a two domain response regulator, including an N-terminal receiver or phosphorylation domain and a C-terminal DNA-binding domain, the two domains are joined by a linker region [13, 19, 29]. The level of phosphorylated *OmpR* (*OmpR*-P) appears to be a determining factor for *ompC* and *ompF* regulation [5, 13, 32].

Antarctic ecosystems are cold, low-nutrient environments that experience large temperature fluctuations. A sudden temperature downshift is one of the pivotal environmental stress factors encountered by bacteria in the polar regions of the Earth [17, 25]. In addition, sea ice freezes and thaws during different seasons, which in turn affects the salinity of the offshore seawater due to the

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fluctuation of free water [15]. Accordingly, they have developed adaptative mechanisms that successfully enable them to thrive in this harsh environment [25]. The Antarctic bacterium *Psychrobacter* sp. G was isolated from the surface (0–20 cm) water samples in the waters off southwestern King George Island, one of the South Shetland Islands and preserved in our laboratory.

Previous studies mainly focused on the structure and functional mechanism of *OmpR* in *E.coli* [1, 11, 20, 26]. Only recently, a few studies were performed on the transcriptional analysis of *OmpR* gene under stress treatments [7, 33]. Gao et al. [7] found that *OmpR* gene was upregulated dramatically with the increase of medium osmolarity in *Yersinia pestis*. However, information on this aspect was still very insufficient. To our knowledge, no study on *OmpR* in Antarctic psychrotrophic bacterium have been conducted. In this study, we investigated the expression of *OmpR*503 gene under different osmotic conditions by qRT-PCR. In addition, freezing tolerance of bacteria in Antarctic is often accompanied by tolerance to dehydration caused by a lack of free water at low temperatures, thus we also investigated its expression characters at different temperature and the combined stress of the two factors. This study will be helpful to further clarify the mechanism of the *EnvZ*/*OmpR* two-component signal transduction systems.

Materials and Methods

Strain, Medium, and Growth Conditions

Antarctic bacterium *Psychrobacter* sp. G was isolated and preserved in our laboratory [14]. *E. coli* DH5α (D9057, TaKaRa, Dalian, China) and Vector pMD18-T (D101A, TaKaRa, Dalian, China) were used in gene cloning.

The culture medium used to culture *Psychrobacter* sp. G were Luria–Bertani broth. NaCl was used as osmotic agent in high osmolarity media. The component of the culture mediums and the growth conditions of *Psychrobacter* sp. G were as previously described [27].

Cloning of *OmpR*503 Gene

The genomic DNA of *Psychrobacter* sp. G was isolated using a Genomic DNA Prep Kit (DP302, Tiangen Biotech, Beijing, China), following the manufacturer's instructions. In order to obtain the full-length *OmpR* gene, nucleotide sequences from −500 to −300 bp upstream of the initiation codon ATG and 1–200 bp downstream of the termination codon TAA were used to design the primers according to the genomic draft of *Psychrobacter* sp. G (data not published). Primers were designed by Primer premiers 5.0 program and designated as *OmpR*503-F and *OmpR*503-R (Table 1).

The target gene was amplified by PCR programed for 35 cycles (95 °C for 1 min; 47 °C for 30 s; 72 °C for 1 min 30 s). The target fragments were excised, and purified by agarose gel DNA fragment recovery kit (DP209-2, Tiangen Biotech, Beijing, China). The purified fragments were subcloned into pMD-18T vector and sequenced subsequently (Sunny Biotechnology, Shanghai, China).

Bioinformatics Analysis of *OmpR*503

The regulatory sequences, i.e., −10 region, −35 region, ribosomal binding site (RBS), and open reading frame (ORF) were analyzed using the Softberry (http://linux1.softberry.com/berry.phtml) [24] and the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) [4]. The deduced amino acid sequences of amplified *OmpR* gene were compared and aligned with the protein database in GenBank (http://www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST). Theoretical isoelectronic point (pI) and molecular weight (Mw) of *OmpR*503 were given by ExPASy Compute pI/Mw tool (http://au.expasy.org/tools/pi_tool.html). Multiple sequences alignments were performed with ClustalW multiple alignment program (DNASTAR Inc., USA).

Stress Treatments

The optimal growth temperature and salinity of *Psychrobacter* sp. G were 20 °C and 45, respectively [27]. *Psychrobacter* sp. G was initially cultured at its optimal growth condition. As OD_{600} of the culture reached 0.5, the stress treatments were carried out as follows: (1) In temperature stress treatments, cultures were then kept in different temperatures for different times (0, 10, and 30 °C for 2, 6, and 12 h, respectively). (2) In osmotic stress treatments, cultures were centrifuged at 8,000×*g* for 5 min at 20 °C. The pellets were collected, and the same volume of medium with a final salinity of 0, 15, 90, and 120 were added, respectively. Strains were resuspended thoroughly

Table 1 Primers used in present study

Primer	Nucleotide sequences (5′–3′)
*OmpR*503-F	TGTGCATTTGTCGAGGTT
*OmpR*503-R	TTGCGCCCGTTGTA
*OmpR*503-QF	ATTCACCCGTCGTTA
*OmpR*503-QR	ACTCCGTCGTCAAAA
GAPDH-QF	AGTCAGGCACATTTAGCG
GAPDH-QR	GGCATAGCCCCATTCATT

by gentle inverting. The cultures were then kept in 20 °C for 2, 6, and 12 h, respectively. (3) In the combined stress treatments, cultures were then kept in the conditions as follows: a final salinity of 15 at 0 °C; a final salinity of 15 at 30 °C; a final salinity of 90 at 0 °C and a final salinity of 90 at 30 °C, respectively. *Psychrobacter* sp. G cultured under the optimal conditions (20 °C with a salinity of 45) was used as control for qRT-PCR analysis.

Expression Analysis of *OmpR*503 Gene by qRT-PCR

Total RNA was extracted using the RNAiso Plus Kit (D9108D, TaKaRa, Dalian, China), following the manufacturer's instructions. The purity of RNA was evaluated measuring absorption at 260 nm and the ratio of absorption at 260:280 nm (A260/A280).

The PrimeScript RT reagent Kit (DRR037A, TaKaRa, Dalian, China) was used to perform the reverse transcription of RNA. The cDNA for qRT-PCR was prepared using 500 ng of total RNA and Random 6 primers.

SYBR PrimeScriptTM RT-PCR Kit (DRR041A, TaKaRa, Dalian, China) was used to perform the qRT-PCR analysis. The primers targeted against sequences of the *OmpR*503 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes of *Psychrobacter* sp. G named *OmpR*503-QF, *OmpR*503-QR, *GAPDH*-QF, and *GAPDH*-QR are listed in Table 1. qRT-PCR was performed on Stratagene Mx3000P qPCR System for 40 cycles (95 °C for 5 s; 44 °C for 15 s; 72 °C for 20 s). All reactions were carried out in three duplicates. Quantification of mRNA was based on Ct (threshold cycle) values. The Ct value of the *OmpR*503 gene was normalized using the Ct value corresponding to GAPDH gene. The efficiency of each qRT-PCR was also calculated. Data analysis was executed using comparative Ct ($2^{-\Delta\Delta Ct}$) method [6, 16]. Data obtained from qRT-PCR analysis were subjected to analysis of variance to determine differences in the mean values among the treatments. Significance was concluded at $P < 0.05$, highly significance was concluded at $P < 0.01$. Statistical analysis was performed by SPSS 13.0.

Results

Bioinformatics Analysis of *OmpR*503 Gene

The full-length nucleotide sequences of *OmpR*503 gene is elaborated in Fig. 1. The ORF of *OmpR*503 gene was 765 bp in length and encoded a protein consisting of 254 amino acid residues with a molecular mass of 28.6 kDa and a calculated pI of 5.1. The nucleotide sequences and deduced amino acid sequences of *OmpR*503 gene were submitted to GenBank with an accession number of JQ317931.

The putative promoter sequences −35 region (5′-TTGCCA-3′) and −10 region (5′-TGCTCAAAT-3′) were identified. A typical RBS (5′-GAGG-3′) was 8-bp upstream from the initiation codon (Fig. 1).

The deduced amino acid sequences of *OmpR*503 from *Psychrobacter* sp. G were compared with other known representative bacterial *OmpR* family proteins (Fig. 2). Multiple sequences alignment revealed that the deduced amino acid sequences of *OmpR*503 gene were highly conserved with other known *OmpR* family proteins. *OmpR*503 had two distinctive domains: the N-terminal receiver domain (residues 1–128) and the C-terminal DNA-binding domain (residues 144–254). The two domains were connected by a loop consisting of 15 amino acid residues (residues 129–143). Five α-helices and five β-sheets, designated as α1–α5 and β1–β5, were alternately arranged in the N-terminal receiver domain of *OmpR*503. Phospho accepting residue Asp62 was located in β3. Two conserved amino acid residues Thr90 and Tyr109, participated in phosphor-transfer from kinase to the Asp, were located in β4 and β5, respectively. The C-terminal DNA-binding domain contains three α-helices and six β-sheets, designated as α1–α3 and β1–β6, respectively. Two helices, α2 and α3, and a loop consisting of 11 residues between them created the HTH DNA-binding motif, were also found (Fig. 2).

Expression Characters of *OmpR*503 in Response to Osmolarity Stress

Temporal expression of *OmpR*503 gene in response to salinity is shown in Fig 3. qRT-PCR analysis showed that the expression of *OmpR*503 gene was not affected by low salinity (0, 15) treatments. When the strain was exposed to high salinity (90, 120), the expression of *OmpR*503 gene was significantly increased. When the salinity of culture media was 90, the expression of *OmpR*503 gene was increased immediately after treatment, and maintained at a high level during the next 12 h. The highest expression was reached after 2 h treatment with a value of 2.6-fold compared to control. However, when the salinity of culture media was up to 120, the expression of *OmpR*503 gene was increased gradually and reached its maximum of 3.0-fold compared to control after 12 h treatment.

Expression Characters of *OmpR*503 Gene in Response to Temperature Stress

The expression characters of *OmpR*503 gene in response to the temperature stress are shown in Fig. 4. qRT-PCR analysis showed that the expression of *OmpR*503 gene was also enhanced by low temperature (0, 10 °C). When the strain was cultured at 0 °C, the expression of *OmpR*503 gene was significantly enhanced after 6 and 12 h

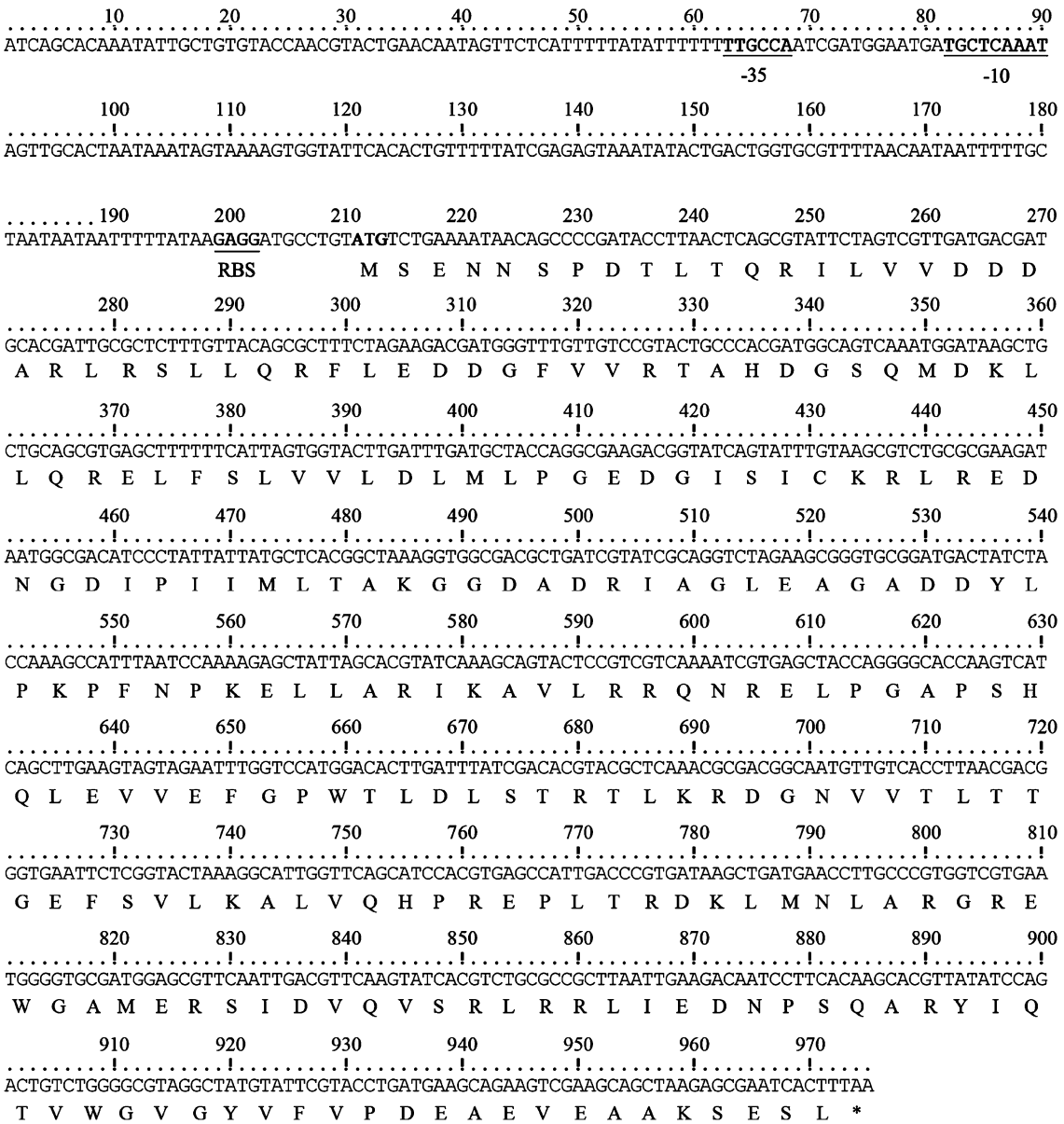

Fig. 1 Sequence analysis of *OmpR*503 gene and its regulatory sequences. The putative promoter elements (−35 region and −10 region) and RBS are in *boldface* and *underlined*. The initiation codon ATG is in *boldface*

treatments, the highest expression was reached after 6 h treatment with a value of 3.7-fold compared to control. The results also showed that the expression of *OmpR*503 gene was inhibited by high temperature (30 °C).

Expression Characters of *OmpR*503 Gene in Response to Combined Stress of Temperature and Osmolarity.

The expression characters of *OmpR*503 gene in response to the combined stress of temperature and osmolarity are shown in Fig. 5. Consistent with our expectation, the expression of *OmpR*503 gene was increased significantly when *Psychrobacter* sp. G was treated at 0 °C with a salinity of 90 from its optimal growth condition. The maximum was reached with a value of 3.0-fold compared to control after 6 h treatment. What is worth paying attention to was that its expression was inhibited significantly under all the other three combined stress treatments.

Discussion

In the present study, an *OmpR* gene named *OmpR*503 was cloned from *Psychrobacter* sp. G according to its genomic draft. Multiple sequences alignment revealed that the deduced amino acid sequence of OmpR503 is highly conserved and similar with other known *OmpR* family members (Fig. 2). Two helices (α2 and α3) and 11 residues

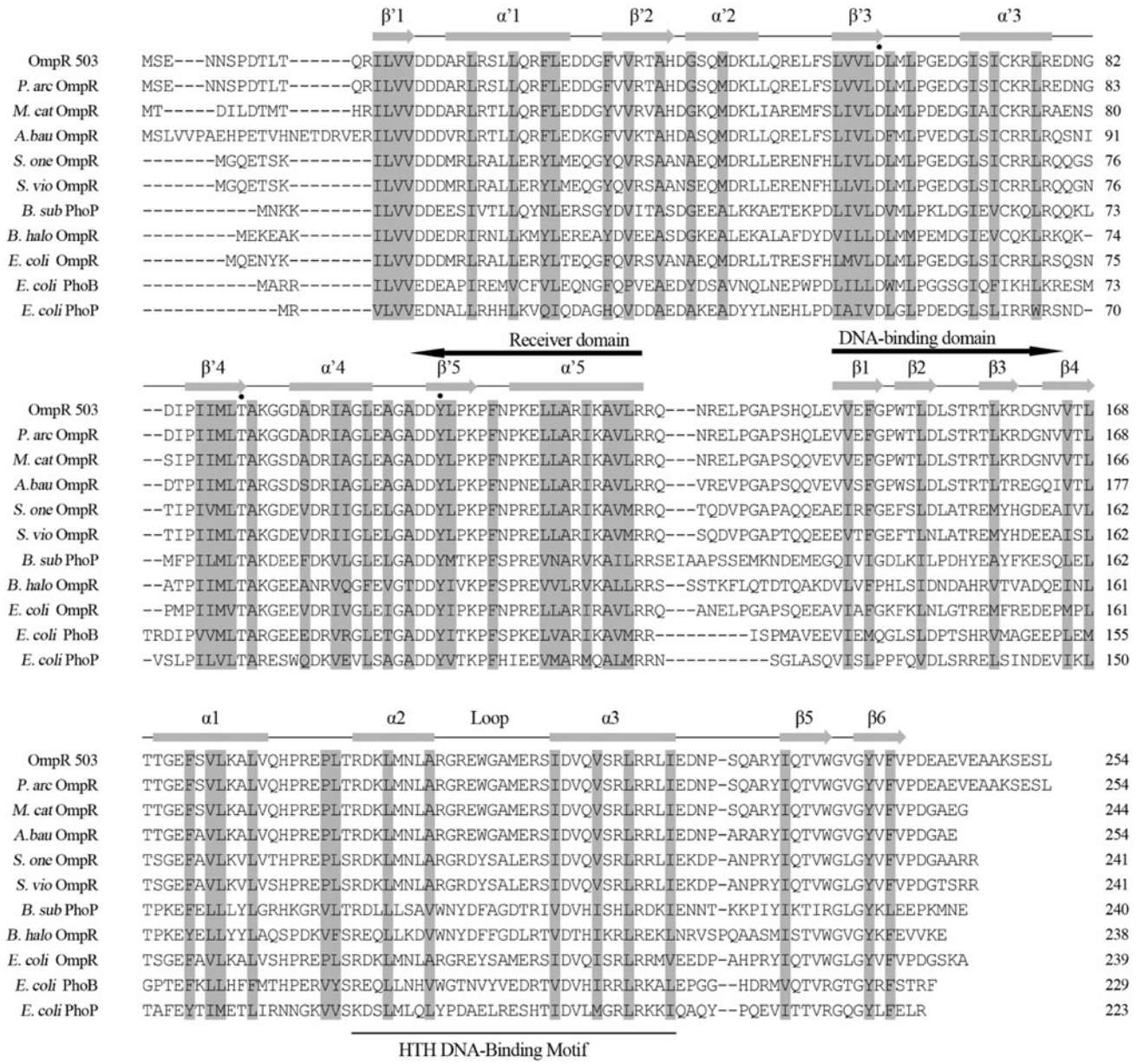

Fig. 2 Amino acid sequences alignment of *OmpR*503 with other representative bacterial *OmpR* family members. *OmpR* family proteins used in this study were as following: *Psychrobacter arcticus OmpR* (YP_263434), *Moraxella catarrhalis OmpR* (YP_003626227), *Acinetobacter baumannii OmpR* (YP_001705954), *Shewanella oneidensis OmpR* (NP_720149), *S. violacea OmpR* (YP_003554910), *Bacillus subtilis* PhoP (CAA47908), *B. halodurans OmpR* (NP_242446), *E. coli OmpR* (ACB15477), *E. coli* PhoB (ACJ50526), and *E. coli* PhoP (ADN45906). The secondary structures are indicated above these sequences. Hydrophobic residues conserved among the aligned members are *shaded*. Phospho accepting residue Asp and conserved Thr/Ser and Tyr residues that participated in phospho-transfer from kinase to the Asp in the receiver domain are indicated by a *black spot* above. The loop between α2 and α3 is also indicated above the sequences

between them created a structure similar to the helix-turn-helix (HTH) motif, suggesting it belonged to the family of winged HTH DNA-binding proteins [11]. Previous studies showed that α3 was a DNA-recognition helix, and four well-conserved hydrophobic residues (Ile208, Val212, Leu215 and Ile219) in it created a hydrophobic face, which was involved in the interaction between *OmpR* and DNA [18, 21]. The loop formed by 11 residues (RGREWGAMERS) between α2 and α3 was considered to be involved in an interaction with RNA polymerase (Fig. 2) [21]. Phospho-accepting residue Asp62 and the conserved residues Thr90 and Tyr109 that participated in phospho-transfer from kinase to the Asp in the receiver domain were highly conserved among *OmpR* family members (Fig. 2) [31].

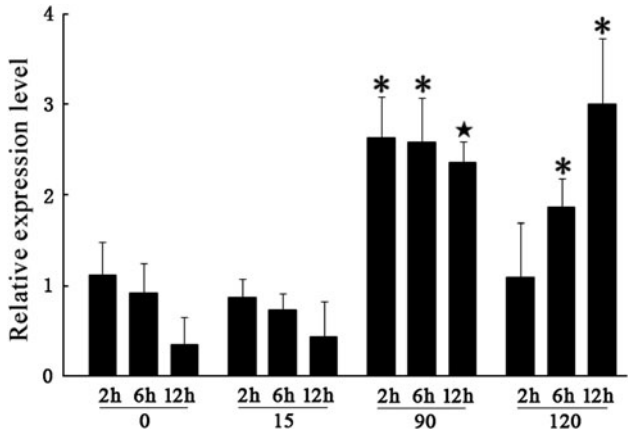

Fig. 3 Real-time quantitative PCR analysis of *OmpR*503 gene expression in response to osmolarity stress. *Standard error bars*: $*P < 0.05$; $^{\star}P < 0.01$

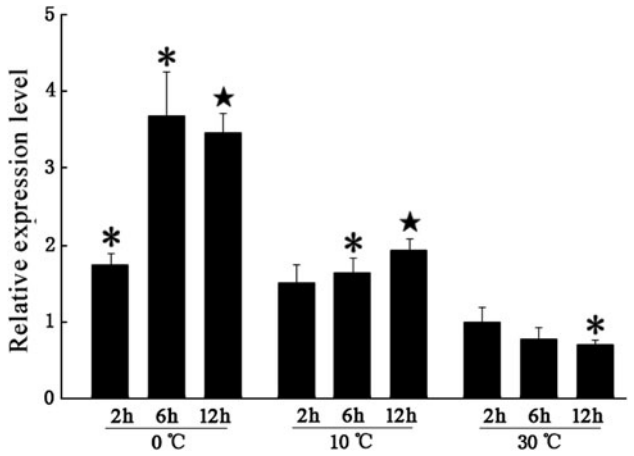

Fig. 4 Real-time quantitative PCR analysis of *OmpR*503 gene expression in response to temperature stress. *Standard error bars*: $*P < 0.05$; $^{\star}P < 0.01$

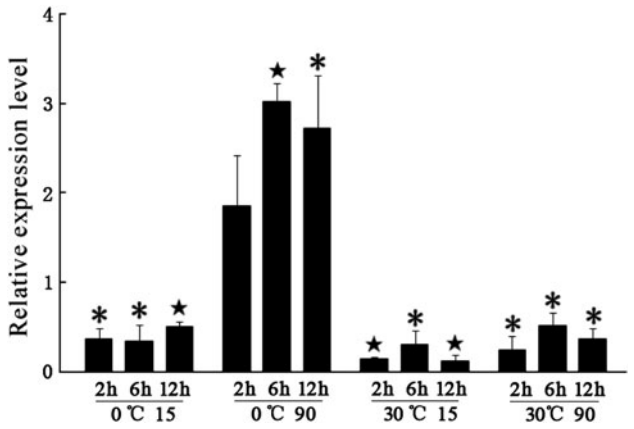

Fig. 5 Real-time quantitative PCR analysis of *OmpR*503 gene expression in response to combined stress of temperature and osmolarity. *Standard error bars*: $*P < 0.05$; $^{\star}P < 0.01$

Previous studies mainly focused on the structure and functional mechanism of *OmpR* in *E. coli*, such as the crystal structure of *OmpR*; the relationship between *EnvZ*, *OmpR*, *OmpF*, and *OmpC* [1, 11, 20]. Our results were in accordance with the recent two studies, which revealed that the adaption action performed not only on the phosphorylation of *OmpR* but also on its expression. The results showed that the expression of *OmpR*503 gene was enhanced by high salinity (90, 120), while low salinity (0, 15) had no significant effects on its expression (Fig. 3), which is in accordance with Gao et al. (2011), [7] who found that *OmpR* gene was up-regulated dramatically with the increase of medium osmolarity in *Yersinia pestis*. The explanation might be as follows: when the strain was shifted from its optimal salinity to an environment with high salinity, more *OmpR*-P was needed to activate the expression of *OmpC*, and then the expression of *OmpR* gene was activated. However, when the medium osmolarity was lower than before, *OmpR*-P already existed in the cytoplasm was enough to activate the expression of OmpF.

In Antarctica, seawater freezes and thaws in different seasons which in turn affects the salinity of seawater where *Psychrobacter* sp. G inhabits [14]. Accordingly, the effect of temperature as well as the combined effects of temperature and osmolarity on the expression of *OmpR*503 gene was also investigated. The results showed that the expression of *OmpR*503 gene was also enhanced by low temperature (0, 10 °C), and was inhibited by high temperature (30 °C) (Fig. 4). Furthermore, its expression was increased significantly when *Psychrobacter* sp. G was treated at 0 °C with a salinity of 90. Its expression was inhibited significantly under all the other three combined stress treatments (Fig. 5). Antarctic ecosystems are permanently cold and low-nutrient environments, with large temperature fluctuations. Freezing tolerance is often accompanied by tolerance to the osmotic stress caused by a lack of free water [25, 30]. In such an environment, cold inducible *OmpR* might help the strain adapt to the harsh condition more efficiently.

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