

CspE is Overproduced by Temperature Downshift in the *Acinetobacter johnsonii* DBP-3

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Abstract The denitrifying bacterium *Acinetobacter johnsonii* strain DBP-3 which was capable of removing phosphate, nitrate, and ammoniacal salt is psychrotolerant, whereas, the cold shock response mechanisms or the cold shock proteins (Csps) was unclear. In this article, the optimal growth temperature (25 °C) and cold shock temperature (7.5 °C) were determined firstly by an Arrhenius plot of the growth of the strain DBP-3. Then, among the seven cold shock-like protein genes which were cloned and identified referenced by *A. johnsonii* SH046 genome, qRT-PCR and shotgun-LTQ mass spectrometry showed that Csp3 and Csp4 were overexpressed under cold shock condition. Furthermore, Western blotting confirmed the result with the antibodies against Csp3 and Csp4 prepared by ourselves. Finally, the phylogenetic analysis showed that the similarity percent between Csp3 and Csp4 was 76.85 %, and Csp3 and Csp4 belonged to CspE family. The results indicated that CspE is overproduced by temperature downshift and may play an important role in the psychrotolerant process of strain DBP-3.

Dan Su and Linlin Hao contributed equally to this work.

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Introduction

Microorganisms are adapted for optimum functioning in their normal physiological environments. Any extreme change in the environmental conditions from the optimum inflicts a stress on an organism [20, 21]. Most bacteria are able to tolerate small changes in an environmental parameter and can adapt over the time scale of minutes, hours, or days [7]. Morita [17], Bower and Daeschel [1] indicated that psychrotolerant organisms are able to grow at low temperatures with much lower growth rates, although they have growth optima in the range of mesophilic organisms (20–40 °C). Previously, *A. johnsonii* strain DBP-3, isolated by Li [14] from a eutrophic water body through low temperature-oriented enrichment culture, is able to grow and perform denitrification under both aerobic and anoxic conditions, also capable of growing satisfactorily at 10–30 °C, furthermore it was noted that it can grow at 5 °C. *Acinetobacter johnsonii*, as a psychrotolerant gram-negative bacterium, plays an important role in environmental engineering and contamination control on account of its peculiar metabolic and physiological features under cold shock conditions [13, 14, 27]. Cold shock proteins, composed of about 70 amino acids [19], are, by far, the most strongly induced proteins at low temperatures [4]. Data have shown that the expression of the cold shock proteins (Csps) reaches a maximum level during the phase of the cold shock adaptation, the so-called acclimation [26]. However, the researches on Csps of *A. johnsonii* have not been reported yet, so in this paper for better understanding the mechanisms of cold adaption for the psychrotolerant denitrifying bacterium *A. johnsonii* DBP-3, the *Csp* genes were searched using bioinformatics methods and cloned. The expression characteristics at transcriptional level of these *Csps* under cold shock conditions were

investigated using qRT-PCR. Furthermore, SDS-PAGE, mass spectrometry, and Western blotting were performed to detect the CspE induced under cold shock conditions at translational level. This work may contribute to further studies focusing on the application of *A. johnsonii* at low temperature.

Materials and Methods

Bacterial Strain, Growth Characteristics, and Cold Shock Treatment

Acinetobacter johnsonii strain DBP-3 which was deposited in the China General Microbiological Culture Collection Center (CGMCC 4753) was cultured at 25 °C in the denitrification medium under shaking [14]. The growth kinetics of DBP-3 was investigated by measuring the OD₆₀₀ of the 1 % inoculated cultures at different temperatures (5, 7.5, 10, 12.5, 15, 25, 30, 37, and 40 °C). An Arrhenius plot of the growth of strain DBP-3 was established for setting the cold shock temperature purpose as described previously [16]. For cold shock experiment, *A. johnsonii* cells were grown to the mid-exponential phase (OD₆₀₀ 0.6–0.8) at 25 °C in denitrification medium, after which 25 ml of the culture was incubated in 100 ml of pre-cooled (7.5 °C) fresh medium for 1 h and 3 h. Additionally, another 25 ml of the culture was incubated in 100 ml of non-cooled (25 °C) medium to use as control for qRT-PCR, SDS-PAGE, and Western blotting.

Screening and Identification of *Acinetobacter johnsonii* Cold Shock Proteins

According to the highly conserved RNA-binding motifs, i.e. RNP1 and RNP2 possessed by almost all cold shock proteins of microorganisms [8], seven CspE were found in *A. johnsonii*, then the genes were located in the genome of *A. johnsonii* SH046 (NZ_ACPL000000000) [18], and further checked in GenBank by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The seven gene fragments were amplified by PCR using primers in Table S 1 in the supplementary material and sequenced by Comate Bioscience Co., Ltd (Changchun, China).

SDS-PAGE and Protein Identification by Shotgun-LTQ Mass Spectrometry

A. johnsonii DBP-3 samples (the bacteria in the cold shock treated group and the control) were harvested at 1 h and 3 h post cold shock, and washed for four times with PBS, then the pellets were re-suspended in Triton X-100 lysis buffer. Cells were lysed using sonication (45 W, sonics VCX750,

Vedeng, 4 times for 15 s with intermediate ice cooling) and centrifuged at 21,000×*g* for 30 min at 4 °C, and the supernatant was separated by SDS-PAGE using 15 % acrylamide gel. After Coomassie staining, the revealed differential band (1 h after cold shock) was excised and identified by shotgun-LTQ mass spectrometry at Shanghai Applied Protein Technology Co., Ltd (Shanghai, China).

Expression Analysis of the Csp Genes by qRT-PCR

Total RNA of the treatment and control samples were extracted using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instruction. The Prime-scriptTM RT reagent kit (TaKaRa, Dalian, China) was used to perform the reverse transcription of RNA into cDNA. cDNA samples were then used as templates for qRT-PCR using SYBR[®] Premix *Ex Taq*TM (Tli RNaseH Plus) (Takara, Dalian, China) according to the manufacturer's recommendations. Primers for qRT-PCR are listed in Table S 1 in the supplementary material. qRT-PCR was performed on the Bio-Rad iQ5 Multicolor Real-Time PCR detection system for 40 cycles (95 °C for 10 s; 50 °C for 15 s; and 72 °C for 30 s). All tests were conducted in three independent biological replicates. Quantification of mRNA was based on the threshold cycle (Ct) values. The Ct values of the seven Csp genes were normalized by the Ct value of the *16 s rRNA* gene. Data analysis was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [15]. Data obtained from qRT-PCR analysis were subjected to variance analysis to evaluate differences in the mean values among the treatments. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Preparation of Polyclonal Antiserum

In order to produce the antibodies against Csp3 and Csp4 in *A. johnsonii*, Csp3 and Csp4 genes were amplified and cloned into the pET32a expression vector, separately. The recombinant pET32aCsp3/Csp4 were transformed into the *E. coli* BL21 (DE3) to produce the N-terminal trx-his-tag recombinant Csp3 and Csp4 proteins which were over-produced by inducing with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Proteins were then purified using HisPurTM Ni-NTA Resin (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The purified proteins were mixed with Freund's complete adjuvant (Sigma, USA) and injected subcutaneously to rabbits in multiple places as described by Vaitukaitis [24]. Rabbits were boosted four times at 3-week intervals, and bleeding was done 10 days after. All experimental works with rabbits were approved by the Animal Care and Ethics Committee of Jilin University, China.

Western Blotting Analysis

For identifying the expressed proteins in strain DBP-3 under cold shock treatment, Western blotting was performed using rabbit antiserum (1:500) with antibodies against Csp3 and Csp4 as the primary antibodies and Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies as the secondary antibodies (1:5000, Boster, China), respectively. Western blotting was visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Amino Acid Sequences Analysis

The complete deduced protein sequences of the Csp3 and Csp4, together with the Csps of the strains possessing similar function with strain DBP-3 and representative bacterium selected from GenBank, were used for the multiple sequence alignment and phylogenetic tree construction. Multiple sequence alignments were performed using the ClustalX program [3]. The phylogenetic tree was constructed using the program Mega 6.0 [23]. Phylogenetic analysis was carried out by applying the neighbor-joining and maximum-likelihood algorithms to ensure coherency of the clusters formed. The bootstrapping supports for the trees were calculated from a sample of 1000 replicates.

Results

Growth Characteristics of *A. johnsonii* Strain DBP-3 at Different Temperatures

Growth kinetics was performed at nine temperatures for studying the growth characteristics of *A. johnsonii* strain DBP-3. As can be seen in Fig. 1a and b, the optimal and the minimal temperature for the growth of *A. johnsonii* were 25 °C and below 5 °C, respectively. The growth rate significantly decreased at 40, 15, 10, 7.5, and 5 °C than at 25 °C.

According to the Arrhenius plot (Fig. 1c), the temperatures over which *A. johnsonii* can grow were divided into the following ranges by the inflection points of 30, 15, and 10 °C: an optimum sub-range from 15 to 30 °C, within which the $\ln(k)$ value remain unchanged and suggested that the growth under the influence of the temperature from 15 to 30 °C is about the same; a cold shock sub-range below the optimum which exhibited two distinct slopes separated by the critical temperature 10 °C, within each slope the activation energy of growth is constant; and a heat shock sub-range (above 30 °C). A temperature of 7.5 °C in the higher slope of cold shock sub-range, which

has a significant impact on the growth, was chosen for the cold shock experiments.

The Identification of *Csps* Genes in the *Acinetobacter johnsonii* DBP-3

Based on the genomic data of *A. johnsonii* SH046 (NCBI accession No. NZ ACPL00000000), seven *Csps* (temporarily named *Csp1* to *Csp7*) which encode the proteins including the conserved nucleic acid-binding motifs RNP1 and RNP2 were found. These seven genes were amplified by PCR as shown in Fig. 2 and sequenced.

Csp3 and Csp4 were Overproduced by the Temperature Downshift in the *Acinetobacter johnsonii* DBP-3

In order to investigate the expression characteristics of *Csps* responding to cold shock treatment, the protein expression patterns of whole-cell lysate from *A. johnsonii* with and without cold shock treatment were compared using SDS-PAGE. As indicated in Fig. 3a, a protein band of approximately 7 kDa was present in the lysate of the cold shock treated cells and appeared not to be present in the lysate of the untreated cells. For the ~7 kDa protein band, eight groups of peptide fragments of different proteins were detected by shotgun-LTQ mass spectrometry (Table 1), one group of peptide fragments (GPNVAVNI-VAIL) was belonged to cold shock protein whose molecular size was about 7 kDa, which matched with the band size. Homologous analysis of sequences showed that they were Csp3 and Csp4 who shared high homology with CspE. Interestingly, qRT-PCR results showed that the expressions of *Csp3* and *Csp4* were significantly enhanced in the cold shock treatment group, which had an almost 400 fold change ($P < 0.05$), and no significant difference was observed on the other *Csps* (Fig. 3b, c). Additionally, Western blotting results showed that Csp3 and Csp4 were both overexpressed at low temperature (7.5 °C) while no significant band was observed in the normal-temperature cells (Fig. 4), which further confirmed that Csp3 and Csp4 were overproduced at 7.5 °C than at 25 °C in *A. johnsonii* DBP-3.

Furthermore, deduced amino acid sequences of Csp3 and Csp4 of DBP-3, together with the Csps of the strains possessing similar function with strain DBP-3 and representative bacterium were selected to perform sequence alignment and constructed a phylogenetic tree. Multiple sequence alignment revealed that the amino acid sequences of Csp3 and Csp4 were homologous with the amino acid sequences of other known Csps. Besides, Csp3 and Csp4 also possessed RNP1 and RNP2 motifs that are known to be involved in binding to single-stranded nucleic acids (Fig. 5)

Fig. 1 The growth characteristics of *A. johnsonii*. The growth curves of *A. johnsonii* strain DBP-3 at various temperatures which were 5–12.5 °C (a) and 15–40 °C (b). The means and standard deviations of the data showed in the figure were from triplicate experiments. c An Arrhenius plot of the relationship between growth rate (k) and temperature (kelvins) for *A. johnsonii* DBP-3

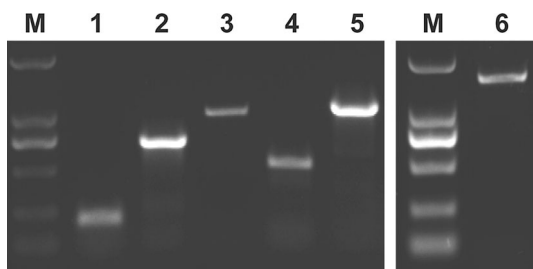
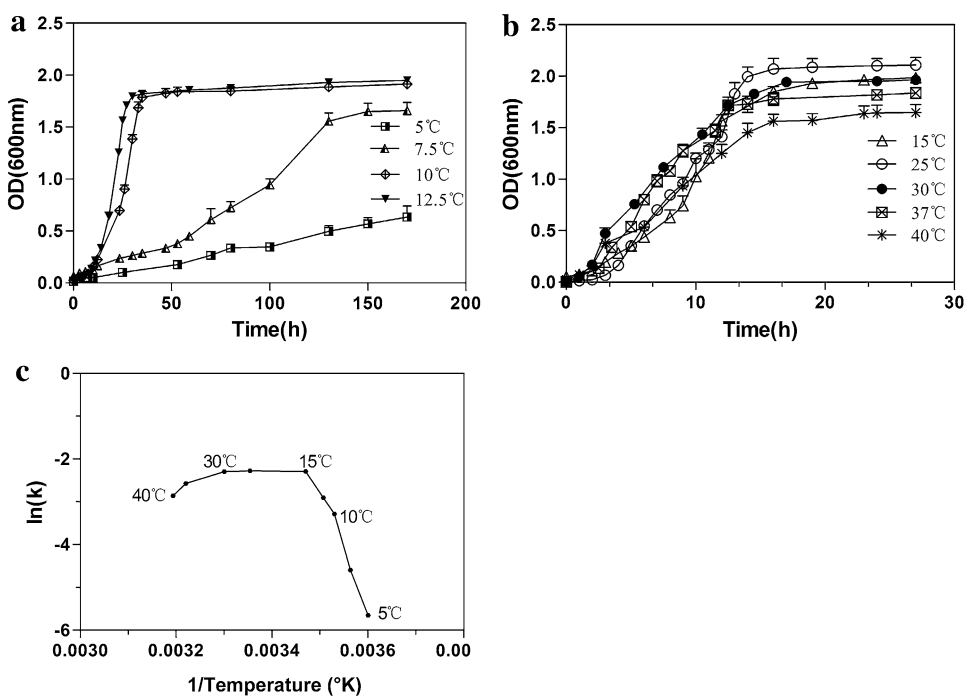


Fig. 2 Identification of the seven *Csp* genes. Lane M, DL 2000 DNA marker (from top to bottom 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, and 100 bp); Lane 1–5, PCR products of the applied *Csp1*, *Csp2*, *Csp3*, *Csp4*, and *Csp6* genes; Lane 6, PCR product of the applied *Csp5* and *Csp7* genes (the positions of *Csp5* and *Csp7* genes are close together in the genome of *A. johnsonii*, so the forward primer was located at the upstream of *Csp5* gene and the reverse one was at the downstream of *Csp7* gene)

[12]. Phylogenetic analysis showed that Csp3 and Csp4 had a close relationship and they were relatively close to *Pseudomonas* sp. Csp5 but were relatively distant from *Listeria*.sp and *Bacillus* sp. Csp6, moreover they had a relatively closer relationship to CspE of different strains, which revealed that Csp3 and Csp4 belong to CspE (Fig. 6).

Discussions

Bacteria respond to the decrease of temperature in a specific manner. The synthesis of most cellular proteins is inhibited after a decrease in temperature [10]. Psychrotolerant microorganisms are usually the organisms most

frequently found in cold environments, perhaps because they may have better nutritional adaptability [25] or due to horizontal gene transfer from mesophiles [2]. In this article, the psychrotolerant denitrifying bacterium *A. johnsonii* DBP-3 grew well at 15–30 °C, and 7.5 °C was chosen as the cold shock temperature according to the Arrhenius plot.

Cold shock proteins, composed of about 70 amino acids [19], are the most strongly induced proteins at low temperatures [4]. The first cold shock protein was identified in 1987 [5, 10], expression of the cold shock proteins reaches a maximum level during the phase of the cold shock adaptation, the so-called acclimation [26]. Most of the Csp5 share the conserved RNPI and RNP2 motifs which are known to be involved in the binding with the single-stranded nucleic acids [9, 12, 22]. Furthermore, a multiple-deletion analysis showed that Csp5 play important roles not only during cold shock adaptation but also during the stationary phase or under nutrient stress [6].

In this article, Csp3 and Csp4 were detected to be overproduced by temperature downshift in strain DBP-3 by LTQ mass spectrometry, qRT-PCR, and Western blotting. The overall DNA sequence similarity percent between Csp3 and Csp4 was about 76.85 %, while the similarity percent of peptides between Csp3 and Csp4 was 84.51 %. Thus, in Western blotting, the antibodies of the two proteins reacting with each other was a possible situation, and could be explained by the phylogenetic analysis, which revealed that Csp3 and Csp4 had a close relationship with each other, and belonged to CspE as well. These results indicated that CspE may play an important role in the

Fig. 3 ~7 kDa proteins of *A. johnsonii* overexpressed at cold shock treatment cells. **a** The comparison of proteins produced from the non-cold-shocked culture (*lane 1*) and from the cold-shocked culture for 1 h (*lane 2*) and 3 h (*lane 3*) of *A. johnsonii* DBP-3 in SDS-PAGE, the protein standards were shown in lane M. **b** qRT-PCR analysis of *Csp1*, *Csp2*, *Csp5*, *Csp6*, *Csp7* mRNA expressions in response to the temperature downshift of the *A. johnsonii* DBP-3. **c** qRT-PCR analysis of *Csp3* and *Csp4* mRNA expressions in response to the temperature downshift of the *A. johnsonii* DBP-3. Standard error bars are shown. *** $P < 0.001$ compared with the control group

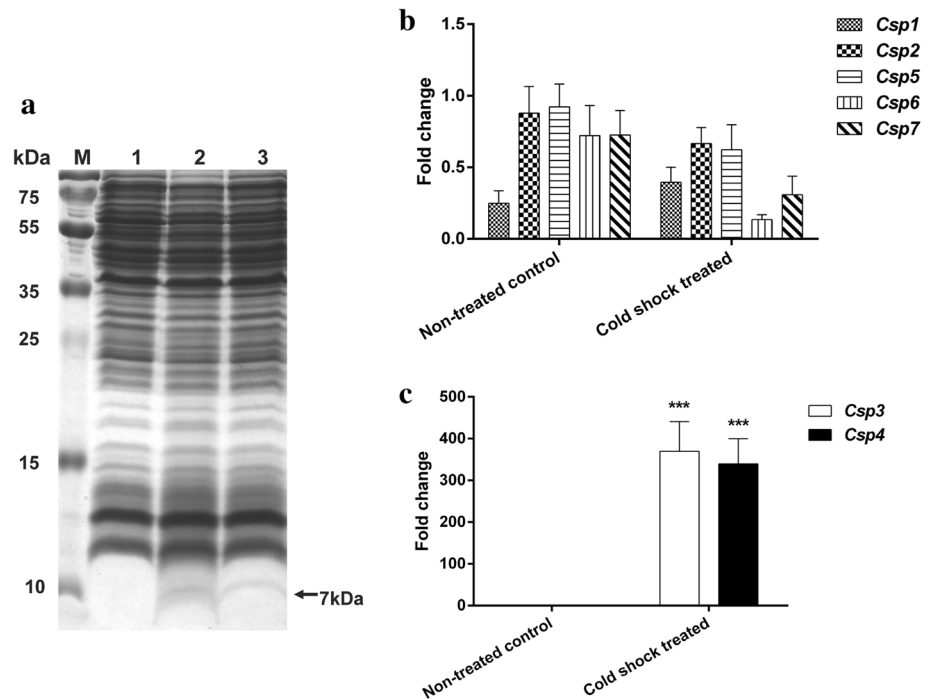


Table 1 The reference information of the peptide fragments detected with shotgun-LTQ from the protein band of approximately 7 kDa showed in the lysates of the cold shock treated cells

Group	Sequence	Protein name	GenBank	Overlap rate
1	K.NVQGAWINEQK.V K.SFEDAIQTGIK.V	Uncharacterized protein	WP_004978694.1	34.85 %
2	K.GQTLQDPFLNSLR.K R.IPVSIFLVNGIK.L	RNA-binding protein Hfq	WP_004984873.1 EEY95749.1	14 % 14 %
3	R.IVEINTTVSQDQVIATLSER.G	Uncharacterized protein	WP_004281879.1	31.25 %
4	K.EAVGFLVTIK.E	Dihydropolyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	WP_004695562.1	2.5 %
5	K.GPNAVNIVAL.- ! K.GPNAVNIVAL.-	Cold shock protein	WP_004980161.1 WP_004693381.1	14 %
6	R.FLEAVAR.Q	ATP synthase subunit C	WP_000424060.1	8.64 %
7	R.INAQGQGSANPIASNATAEGR.E	Uncharacterized protein	WP_004984284.1	9.72 %
8	R.LVSTAGTGYFYTTTK.N	50S ribosomal protein L33	WP_003654787.1	29.41 %

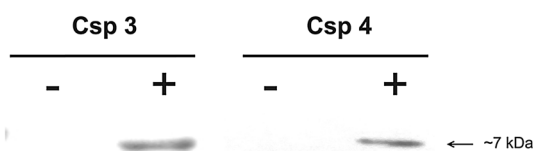


Fig. 4 Analysis of the effect of cold shock stress on the expression of Csp3 and Csp4 proteins by Western blotting. Each lane contains equal amounts of total protein. Samples were obtained from the control (–) and cold shock treated (+) *A. johnsonii* DBP-3 cells, and reacted with the antibodies against Csp3 (*left*) and Csp4 (*right*)

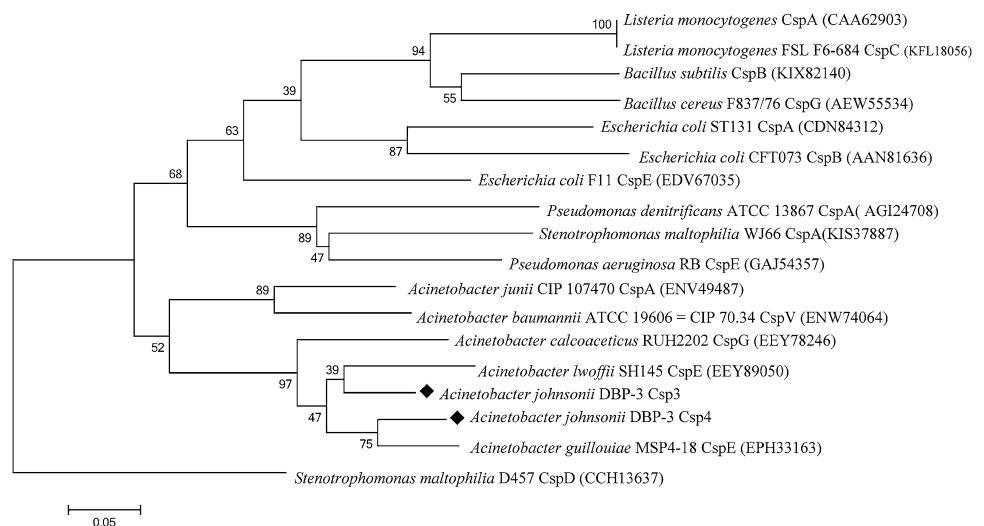
psychrotolerant mechanism of *A. johnsonii* DBP-3, which were in accordance with the findings of Kandasamy [11] who indicated that *Enterobacter ludwigii* PAS1 adapts quickly to low temperatures by a constitutive expression of the potentially cryoprotective CspE. Nevertheless, the understanding of the cold shock response in strain DBP-3 is still in infancy, further studies on the mechanisms employed by *A. johnsonii* DBP-3 to acclimate low temperature such as the interactions of different Csps, quantitative differences in Csps expression at the translational

Fig. 5 Amino acid sequence alignment of Csps of different strains. Sequence alignment of Csp3 and Csp4 of DBP-3 and the Csps of the strains possessing similar function with strain DBP-3 and representative bacterium was performed. Conserved nucleic acid-binding sequence motifs, the RNA-binding motifs RNP1 and RNP2, are labeled

<i>ajo</i> Csp3	.MSTT..VTGTVKWFNETKGFGLIQESGP.DVFAHFREIASSGFKTLHEGQQVVSIEQGGKGPNAVNIVAL..	aa
<i>ajo</i> Csp4	.MSNSNVVKGTVKWFNETKGFGLIQESGP.DVFAHFSEIANSGFKTLFEGQQVVSIAQGGKGPNAVNIVAI..	71
<i>aju</i> CspA	.MSNTA..TGTGTVKWFNETKGFGLIHADSGQ.DVFAHFSEIQNSGFKVLHEGQRVSVTAQGGKGPQATGITVVAQ..	71
<i>alw</i> CspE	.MSTT..VTGTVKWFNETKGFGLIQESGP.DVFAHFKEISSSGFKTLFEGQRVSVVDGQKGPNAVNIVAL..	69
<i>aba</i> CspE	.MSNT...TGTGTVKWFNETKGFGLIQDEGK.DVFAHFSEIQTGGFKVLLLEGQRVQVFTVQGGKGPQASNITIVTN	70
<i>aca</i> CspG	.MSNT...ANGTVKWFNETKGFGLIQESGP.DVFAHFKEIASSGFKTLYEGQKVTFNIAQGGKGPQASAVNIPE..	69
<i>eco</i> CspA	.MSGK...MTGIVKWFNADKGFGLIPDDGSKDVFVHFSAIQNDGKSLDEGQKVSFTIESGAKGPAAGNVTSL..	70
<i>eco</i> CspB	.MSNK...MTGLVKWFNADKGFGLISPDGSKDVFVHFSAIQNDNFRTLFEQGVKTVSVESGAKGPAANVITD..	71
<i>eco</i> CspE	.MS.K...IKGNVWFNESAEGFGLIPEDGSKDVFVHFSAIQNTNGFKLAEGRVFEITNGAKGPAANVIAL..	69
<i>lmo</i> CspA	MNMEQ...GTVKWFNAEKGFGLIERENG.DVVFVHFSAIQDGFKSLDEGQAVTFDVEEGQRGPAANVQKA..	68
<i>lmo</i> CspC	..MEQ...GTVKWFNAEKGFGLIERENG.DVVFVHFSAIQDGFKSLDEGQAVTFDVEEGQRGPAANVQKA..	66
<i>bsl</i> CspB	..MLE...GKVKWFNESAEGFGLIEVEGQD.DVVFVHFSAIQGEGFKTLEEGQAVSFIVEGNRGPQANVTKEA..	67
<i>bce</i> CspG	..MTLT...GKVKWFNESAEGFGLIEVEGQD.DVVFVHFSAITDGFKSLDEGQEVSEVEEDGNRGPQAKNVVKL..	67
<i>pma</i> CspA	.MSDR...QTGTVKWFNADKGFGLIPESGP.DLFVHFRAIQGTGFKTLQEGQKVFIAVQGGKGMQADQVQAV..	69
<i>pma</i> CspD	.MP....NGTVKWFNADKGFGLISPDGSAQVFAHFSAINSKGFRLSEGRQVTVYDVTGQPKGAQASNITPAE..	68

RNP1 RNP2

Fig. 6 Phylogenetic tree of Csps of different strains. Phylogenetic tree of Csp3 and Csp4 of DBP-3 and the Csps of the strains possessing similar function with strain DBP-3 and representative bacterium constructed using the program Mega 6.0. Numbers at each branch indicate the percentage of times a node was supported in 1000 bootstrap pseudo replications by neighbor joining. Accession numbers are shown in parentheses



level under different conditions, and regulatory elements of Csps are needed to clarify.

In conclusion, the optimal growth temperature of *A. johnsonii* was 25 °C and the cold shock temperature was below 7.5 °C. *Csp3* and *Csp4* who belonged to CspE were overexpressed significantly under the cold shock treatment.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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