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Research Article 研究报告

Roles of three cold shock proteins in the psychrophilic seafood bacterium *Shewanella baltica*

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Abstract: [Objective] *Shewanella baltica* is a specific spoilage microorganism commonly detected in refrigerated seafood at the end of shelf life, while the knowledge about the roles of cold shock proteins (CSPs) in this bacterium is limited. This study aims to unravel the roles of three *csp* genes in *S. baltica* SB-19. **[Methods]** BEAST was employed to study the molecular evolutionary of *csp* genes in food-derived microorganisms of *Gammaproteobacteria*, and qPCR to determine the expression patterns of the three *csp* genes in *S. baltica* SB-19. The strains with *csp* gene knockout were constructed, and their growth rates, quorum sensing, spoilage ability were analyzed under different temperatures and environmental stresses. Finally, three strains with heterologous expression of *csp* were constructed, and their responses to different temperatures and environmental stresses were examined. **[Results]** Three *csp* genes, *cspC*, *cspD*, and *cspG*, were identified in *S. baltica*. All the *cspD* genes of *Gammaproteobacteria* formed a monophyletic clade and segregated from other *csp* genes approximately 1 109.6 million years ago (MYA). The *cspC* and *cspG* of *S. baltica* segregated approximately 858.8 MYA. Both *cspC* and *cspG* were involved in the response to cold shock, whereas *cspD* did not. The knockout and overexpression experiments revealed that *cspG* was essential for *S. baltica* survival at low temperature and played a universal role in the response to environmental stress, and *cspC* aided the bacterial response to stress. The *cspD* gene was associated with bacterial growth. Furthermore, the result of the experiments with inoculation of knockout strains in fish juice indicated that both *cspC* and *cspG* were associated with the spoilage ability of *S. baltica* below room temperature. **[Conclusion]** The three *csp* genes play different roles in *S. baltica*. The findings pave new ways for the research on the cold adaptation and spoilage mechanism of spoilage microorganisms.

Keywords: *Shewanella baltica*; cold shock protein; evolution; cold shock; stress tolerance; spoilage ability

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海产品嗜冷菌波罗的海希瓦氏菌中 3 个冷激蛋白功能分析

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摘　要：【目的】波罗的海希瓦氏菌是冷藏海产品中常见的腐败菌，而该菌中关于冷激蛋白的功能研究尚未见报道。本研究从分子生物学角度分析波罗的海希瓦氏菌中 3 个冷激蛋白各自的功能。【方法】采用 BEAST 软件分析 γ-变形菌纲中部分食源性微生物的冷激蛋白进化时间，接着利用实时荧光定量 PCR 方法检测波罗的海希瓦氏菌 3 个冷激蛋白基因的表达规律，进而构建 3 个冷激蛋白的基因敲除株，分析敲除株在不同温度和不同环境胁迫条件下的生长状况、群体感应现象以及致腐能力，最后构建 3 个冷激蛋白的异源表达菌株并分析它们在不同温度和不同环境胁迫条件下的生长状况。【结果】波罗的海希瓦氏菌中鉴定到 3 个冷激蛋白，分别为 *cspC*、*cspD*、*cspG*。所有 γ-变形菌纲的 *cspD* 基因单独聚成一支，并于 1 109.6 百万年前与其他 *csp* 基因相分离，波罗的海希瓦氏菌的 *cspC* 和 *cspG* 在 858.8 百万年前互相分开。*cspG* 基因是波罗的海希瓦氏菌低温生存的必需基因，且广泛响应环境胁迫条件；*cspC* 基因对 *cspG* 基因功能的实施起辅助作用；*cspD* 不响应冷激，但却会随生长阶段的变化而发生变化。此外，*cspC* 基因和 *cspG* 基因在低温条件下与细菌的致腐能力相关。【结论】波罗的海希瓦氏菌 3 个冷激蛋白基因各有不同，且 *cspC* 基因和 *cspG* 基因与该菌致腐能力有关，这为今后研究腐败菌的冷适应和致腐机制提供了新思路。

关键词：波罗的海希瓦氏菌；冷激蛋白；进化；冷激；压力耐受；致腐能力

Shewanella baltica, belonging to *Shewanellaceae*, *Alteromonadales*, *Gammaproteobacteria*, is a specific spoilage organism (SSO) commonly found in large yellow croaker, Danish marine fish, trout, shrimp, and mussel during cold storage[1-2]. Via its strong spoilage ability, it can reduce trimethylamine N-oxide (TMAO) in seafood to trimethylamine (TMA) and produce biogenic amines and hydrogen sulfide with offensive flavors and odors, thus accelerating the deterioration of seafood[3-4]. Its low temperature tolerance is the main factor underlying the ability of *S. baltica* to act as an SSO[5].

Many organisms exhibit a cold shock response to temperature downshifts, with boosted expression of cold-induced proteins (Cips) and transient repression of non-Cips. Some types of Cips have been identified, such as cold shock protein (Csp), exoribonuclease, RNA helicase csdA, PNPase, and RNaseR, and Csps are the main Cips produced by bacteria under cold shock conditions[6]. Csps are highly structurally

conserved proteins with typical structural units of cold shock domains (CSDs) that are present in organisms ranging from bacteria to vertebrates[7]. Csps were originally found in bacteria and are directly or indirectly involved in transcription and translation as RNA chaperones during the cold shock response and cold-adapted growth stages[8]. At present, Csps in some bacteria have been well studied, such as 9 Csps in *Escherichia coli*, 3 Csps in *Bacillus*, 5 Csps in *Lactococcus lactis*, and 3 Csps in *Lactococcus plantarum*[6,8]. However, not all Csps are cold shock-inducible proteins. Among the 9 *E. coli* Csps, 4 are highly induced by cold shock (CspA, CspB, CspG, and CspI) and 1 by nutrient stress (CspD), 2 exhibit constitutive expression at physiological temperature (CspC and CspE), and 2 remain uncharacterized (CspF and CspH)[9]. In addition, Csps are associated with bacterial stress responses, such as desiccation, osmosis, oxidation, pH, and ethanol, which are independent of cold induction[10-11]. Therefore, comparing the functions of different Csps can be helpful for understanding the adaptation of bacteria to low temperature and other stressful conditions.

The cold adaptation mechanism of *Shewanella* has been studied. In *Shewanella oneidensis*, two Csps (So1648 and So2787) participate in the cold shock response, with only So1648 acting at very low temperature[12], which was shown by Maillot et al. to be a DnaK (Hsp70) that allow *S. oneidensis* to survive at low temperatures[13]. *Shewanella putrefaciens*, another well-known SSO and cold-resistant microorganism of refrigerated marine fish, has physiological properties and spoilage capacity very similar to those of *S. baltica*[14]. *S. putrefaciens* adapts to low temperature by increasing its total unsaturated liquid content[15], and 3 Csps have been identified *in silico* in this bacterium, but these have not been tested *in vivo* or *in vitro*[16]. In *S. baltica*, genome-wide gene expression changes under cold stress were assessed and verified by transcriptional sequencing and q-PCR, and 855 differentially expressed genes were screened, including *cspC*, *dnaK*, *rpoE*, *rpoS*, *rpoD*, *hupA*, *fliA*, *flgF*, etc.[5].

As a psychrophilic bacterium, *S. baltica* can grow even at temperatures close to 0 °C. To efficiently inhibit its growth in refrigerated seafoods, a thorough understanding of the cold adaptation mechanism of *S. baltica* in various stressful environments during food storage is needed. In this study, 3 *csp* genes were identified in the *S. baltica* SB-19 strain based on local basic local alignment search tool (BLAST), and the most recent common ancestor of *csp* genes in the *Gammaproteobacteria* class was estimated by phylogenetic analyses. Then, the functions of 3 *csp* genes were verified by gene knockout (KO) in *S. baltica* and overexpression of CSP in *E. coli*. The results of this study may contribute to a more comprehensive characterization of the molecular basis of cold tolerance and spoilage ability in *S. baltica*.

1 Material and Methods

1.1 Preparation of materials

The *S. baltica* SB-19 strain was isolated from the large yellow croaker at the end of refrigerated shelf life and identified in our previous report[17]. Live-farmed large yellow croaker was purchased from Hangzhou Aquatic Market, transported to the laboratory within two hours and kept alive. The process of sterile fish juice was performed as previously described[17]. All primer synthesis and DNA sequencing procedures were performed by Sangon Biotech (Shanghai) Co., Ltd.

1.2 Estimation of the *csp* origin time

The genome of *S. baltica* SB-19 strain had been sequenced, assembled, and annotated in our previous report[17]. Its genomic protein sequences were used to construct a BLAST database, and the threshold for local BLAST alignment was set to *E* value>1e-3 and identity>50%. The *csp* gene sequences of the *Gammaproteobacteria* class (NMDCX0000207) were aligned by MAFFT v7.221 with G-INS-I strategy, and the best-fit nucleotide substitution models were analyzed by ProtTest v3.4[18]. The origin time of *csp* genes was estimated by BEAST v2.6, and the parameters were set as Yu et al.[7] proposed: a GTR model

with four gamma category counts for nucleotide substitution, a clock model with a relaxed clock log normal, and a Yule model as a tree prior were run for 100 million generations and sampled every 10 000 Markov chain Monte Carlo (MCMC) generations, discarding 10 million generations as burn-in. The effective sample size (ESS) values were estimated by Tracer v1.7.2, the maximum clade credibility (MCC) tree was built by TreeAnnotator with a 10% burn-in and a 0.5 posterior probability limit, and the annotated trees were visualized by FigTree v1.4.4. The corresponding Csp protein sequences were downloaded from the NCBI database, and the RNP1 and RNP2 domains were obtained after alignment with MEGA X v10.2.2.

1.3 Prediction of protein structure and analysis of protein-protein interaction

The CSP protein sequences of *S. baltica* were submitted to SWISS-MODEL (https://swissmodel.expasy.org/) for predicting 3D structure, which were quality assessed in the SAVES 6.0 (https://saves.mbi.ucla.edu/) using Verify3D and PROCHECK. The protein-protein interaction with CSP was predicted by STRING (https://cn.string-db.org/) with a full STRING network type, using the *S. baltica* OS155 strain available in the database as a reference strain.

1.4 Determination of gene expression levels

The *S. baltica* SB-19 strain was cultured in LB medium, incubated at 30 °C, and sampled at 6, 12, 18, and 24 h. For the cold stress treatment, cells of the *S. baltica* SB-19 strain at the end of the exponential growth phase were subjected to temperature shifts down to 20, 10, and 4 °C for 120 min. Total RNA was extracted with TRIzol reagent (Invitrogen), digested with DNase (Promega), and reverse transcribed into cDNA. To analyze gene expression levels, real-time quantitative PCR (qPCR) was performed using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The 16S rRNA gene was employed as the housekeeping gene (NMDCX0000208). The relative expression levels of genes were calculated as previously described[19].

1.5 Construction of KO strains

The genomic DNA of the *S. baltica* SB-19 strain was extracted using the MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa), and upstream and downstream sequences of the target genes were amplified from the genomic DNA. The pKD3 plasmid was extracted using the MiniBEST Plasmid Purification Kit (TaKaRa), and the chloramphenicol (Cm) acetyltransferase gene cassette was amplified from the plasmid. The three PCR fragments were joined using nested PCR. The recovered *S. baltica* SB-19 strain carrying pKD46 plasmid was inoculated in LB medium containing ampicillin (Amp) and 4% L-arabinose for 4 h with shaking, followed by cold shock in ice for 20 min, and then washed with 10 glycerol. The joined PCR fragment was transferred into suspension cells by electroporation. Under the stress of Cm and L-arabinose, the pKD46 plasmid was removed from the transformed cells. Subsequently, the pCP20 plasmid was transferred into the above transformants, and the FRT-flanked sequence was lost under the pressure of Amp, thus a KO strain of the target gene was obtained[17].

1.6 Evaluation of the growth rates of the wild-type (WT) and KO strains

To generate growth curves, the wild-type (WT) strain of *S. baltica* SB-19 and the KO strains were cultured with shaking at 30, 15, and 4 °C. At different growth stages, 1 mL of treated cells was subjected to serial 10-fold dilutions and then incubated on plate count agar (PCA) plates to count the total viable count (TVC) as previously described[17], and the growth dynamics were modified based on the Gompertz model as described by Qian et al.[20]:

$$\lg N_t = \lg N_0 + \lg\left(\frac{N_{\max}}{N_0}\right)\times \exp\left\{-\exp\left[\frac{(\mu_{\max}\times 2.718)}{\lg\left(\frac{\mu_{\max}}{N_0}\right)}\times(\lambda - t)+1\right]\right\}$$

where N_0, N_t, and N_{max} represent the original

bacterial count, the bacterial count at t hour of incubation, and the maximum bacterial count, respectively; μ_{max} denotes the maximum growth rate; λ denotes the lag phase time; and t denotes the incubation time.

1.7 Stress resistance assays for the WT and KO strains

The WT and KO strains were incubated with shaking at 30 °C until the initial stationary phase (OD_{600} of 1.8). For salt stress treatments, 1‰ (*V*/*V*) cells were inoculated into LB medium containing certain concentrations of NaCl (2%, 3%, and 4%). For nutrient deprivation stress treatments, 1‰ (*V*/*V*) cells were inoculated into LB medium at dilutions of 1/2, 1/4, and 1/8. For heavy metal stress treatments, 1‰ (*V*/*V*) cells were inoculated into LB medium with a range of concentrations of $CuCl_2$ (1.0, 2.0, and 3.0 mmol/L). For sterilizing stress treatments, 1‰ (*V*/*V*) cells were inoculated into LB medium with a range of concentrations of NaClO (0.1%, 0.15%, and 0.2%). All the treated cells were cultured with shaking at 30 °C for 24 h, and cell density was assessed by OD_{600} values. All the above detailed experimental steps have been described previously[21].

1.8 Assessment of the quorum-sensing characteristics of the WT and KO strains

The WT and KO strains were incubated with shaking at 30 °C. To extract the autoinducers of diketopiperazines (DKPs), the supernatants of the strains were extracted and mixed with chloroform. DKPs were extracted and identified by gas chromatography-mass spectrometry (GC-MS, 7890/5975, Agilent). Autoinducer-2 (AI-2) activity was assayed using the *Vibrio harveyi* BB170 and *V. harveyi* MM17 strains, and luminescence was measured by a luminometer (Perkin-Elmer Victor X). To detect biofilms, cells of the WT and KO strains were transferred to 24-well cell culture plates at 30 °C for 24 h and subsequently assessed by the crystal violet method. Extracellular protease activity was assessed using Petri dishes containing tryptone soya agar (TSA) and skim milk. Cells of the WT and KO strains were incubated at 30 °C for 24 h, and then the diameters of the diffusion zones were measured. All the above detailed experimental steps have been described previously[17].

1.9 Spoilage capability analysis of the WT and KO strains

The WT and KO strains were incubated with shaking at 30 °C until the initial stationary phase (OD_{600} of 1.8). Then 1 mL of the bacterial suspension was transferred to 100 mL of sterilized fish juice and incubated at 30 °C, 15 °C, and 4 °C for 24, 72, and 144 h, respectively. The total volatile basic nitrogen (TVB-N) content in the fish juice samples was measured using a Kjeltec 8400 automatic nitrogen determination apparatus (Foss), and the TMA content in the fish juice samples was determined spectrophotometrically by colorimetric formation of picric acid salt[22].

1.10 Construction of overexpressing Csp in *Escherichia coli*

The coding region of the target gene was amplified from the genomic DNA of the *S. baltica* SB-19 strain (NMDCX0000208), and then it was cloned into the prokaryotic expression vector pET21a. The recombinant plasmid (pET21a-CSP) was expressed in the *E. coli* BL21 strain with IPTG induction[23]. Recombinant proteins were purified with Ni-NTA agarose (Qiagen) and subjected to SDS-PAGE. Subsequently, proteins were transferred onto a polyvinylidene fluoride membrane using the wet method. The anti-6×-His tag mouse monoclonal antibody (Invitrogen) was used as the primary antibody (1:2 000), and rabbit anti-mouse IgG HRP antibody (Invitrogen) was used as the secondary antibody (1:5 000)[24].

1.11 Stress resistance assays of *Escherichia coli* overexpressing Csp

The *E. coli* BL21 strains carrying the pET21a-CSP recombinant plasmid were incubated at 37 °C with shaking until the initial stationary phase (OD_{600} of 1.8). Then, 1‰ (*V*/*V*) cells were inoculated into LB medium with 1 mmol/L IPTG for 4 h; subsequently, the cells were subjected to cold stress at 4 °C, NaCl treatment, nutrient deprivation, $CuCl_2$ treatment, and NaClO treatment as described in section 2.6. Strains under

all treatments except cold stress were subjected to shacking cultivation at 37 °C for 18 h, and all the bacterial densities were assessed by measuring the OD_{600} values. The *E. coli* BL21 strain harboring the empty vector of pET21a was used as a control.

1.12 Statistical analyses

All analyses were performed in triplicate, and the results are reported as the means and standard deviations. All data were statistically analyzed using one-way analysis of variance (ANOVA), and the separation of means was performed by Tukey's multiple range test implemented using SPSS software. Differences were considered significant at P value<0.05.

2 Results and Analyses

2.1 Identification and *in silico* analysis of Csps in the *Shewanella baltica* SB-19 strain

In this study, only three potential Csps of the *S. baltica* SB-19 strain were screened by performing local BLAST with *E. coli* and other *Shewanella* species. To explore the characteristics of these three *csp* genes, the timing of *csp* evolution in the *Gammaproteobacteria* class was estimated (Figure 1). The *sb1551* gene of the *S. baltica* SB-19 strain was clustered with all *cspD* genes, so it was named *cspD*. Since the nomenclature of the *csp* gene is not based on its sequence or function[25], the names of *sb2351* and *sb2603* were consistent with the closest *csp* genes of *Shewanella hafniensis*, *cspC* and *cspG*, respectively. The conserved motifs of RNP1 and RNP2 were also found in CspC, CspD, and CspG of the *S. baltica* SB-19 strain (Figure 1), suggesting that these three Csps might also be structurally conserved proteins.

All the *cspD* genes formed a monophyletic branch and were segregated from other *csp* genes in the *Gammaproteobacteria* class approximately 1 109.6 million years ago (MYA) (Figure 1), suggesting that *cspD* genes might be functionally conserved and distinct from other *csp* genes, and that *csp* genes in the *Gammaproteobacteria* class originated at least 1 109.6 MYA. The *cspC* and *cspG* genes of the *S. baltica* SB-19 strain had a relatively short divergence time, and segregated approximately 858.8 MYA, indicating that they might have redundant and overlapping functions.

The molecular structures of CspC, CspD, and CspG were predicted on the SWISS-MODEL website. The best sequence similarity and model quality of the three cold shock proteins were all based on the 3i2z A-chain. Then their models were assessed by Verify3D for the residues with averaged 3D-1D score≥0.1. The values of CspC and CspD were 82.20% and 92.97%, respectively, above the 80% threshold, and the residues in most favored regions for both were 92.7% and 92.3%, respectively, indicating that the protein models of CspC and CspD were qualified. However, the CspG was only 66.18% assessed by Verify3D, suggesting that this protein structure failed to obtain a qualified model on SWISS-MODEL website.

Protein-protein interacting with CspC were Polynucleotide phosphorylase (PNPase) and deoxyuridine 5′-triphosphate nucleotidohydrolase (DUT) (Figure 2A). For CspD, in addition to DUT, PtsN and cystathionine beta-synthase (CBS) domain containing protein interacted with it (Figure 2B). CspG only interacted with DUT (Figure 2C).

2.2 Both the *cspC* and *cspG* genes responded to cold shock, while the *cspD* gene did not

To determine the roles of these three *csp* genes in the *S. baltica*, their expression levels were determined by qPCR under temperature downshifts and in different bacterial growth phases. The expression levels of the *cspC* and *cspG* genes increased with decreasing ambient temperature. Under cold shock stress at 4 °C, the expression levels of the *cspC* and *cspG* genes increased 2.9-fold and 14.6-fold, respectively, compared with that at the normal culture temperature (Figure 3A, 3C), indicating that these two genes are cold-inducible genes. In contrast, the expression level of the *cspD* gene decreased continuously with decreasing of culture temperature; under cold

shock stress conditions at 4 °C, the gene expression level was only 0.18-fold that under normal culture temperature (Figure 3B), suggesting that the *cspD* gene was not a cold shock-inducible gene and that its function was independent of cold shock. Based on this finding, we next examined whether the three *csp* genes of the *S. baltica* were dependent on growth time.

Cells of the *S. baltica* SB-19 strain were grown in culture medium to the lag, log, and stationary phases. With the extension of culture time, the expression level of the *cspC* gene decreased slowly, and it was only 0.77-fold that in the lag phase (Figure 3D). For the *cspG* gene, the

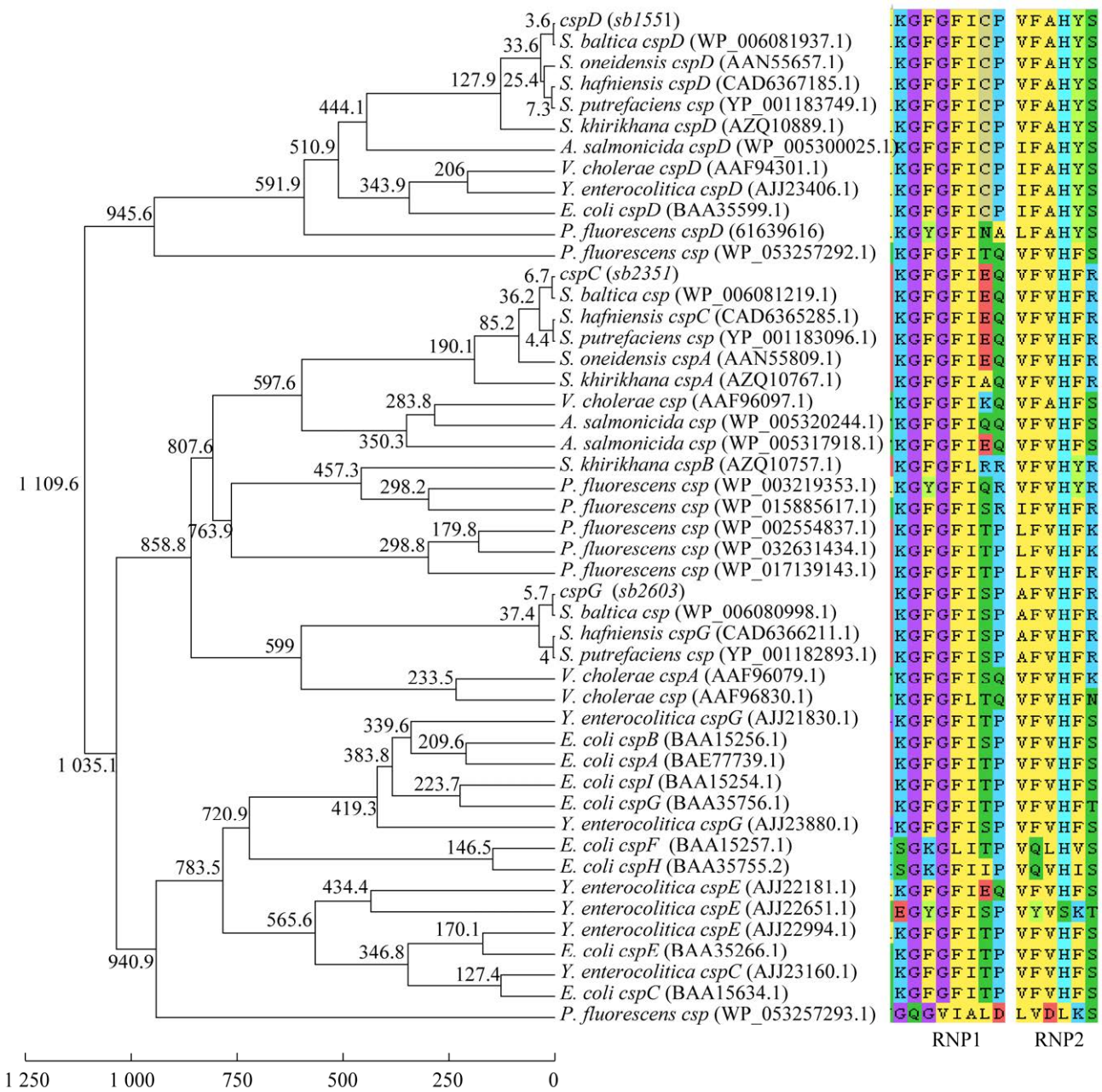

Figure 1 Phylogenetic group of representative Csps in the *Gammaproteobacteria* class. Branch lengths are given as million years ago (MYA). Conserved RNP motifs of Csps are labeled in colored.

expression level decreased significantly at the early growth stage and was only 0.032-fold that in the lag phase after 6 h of culture, followed by a low expression level until the end of culture (Figure 3F). Surprisingly, the expression level of the *cspD* gene increased with increasing culture time and was 2.35-fold higher than that in the lag phase at the end of the growth stage (Figure 3E). Therefore, the *cspD* gene of the *S. baltica* might be associated with the growth phase.

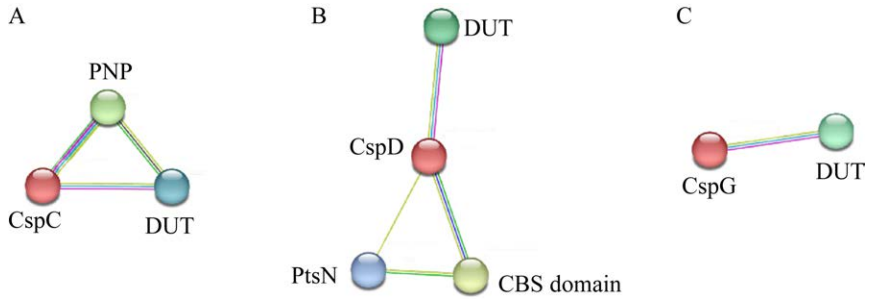

Figure 2 Protein-protein interaction of three CSPs in the *Shewanella baltica*. Panels A, B, and C represent CspC, CspD, and CspG, respectively.

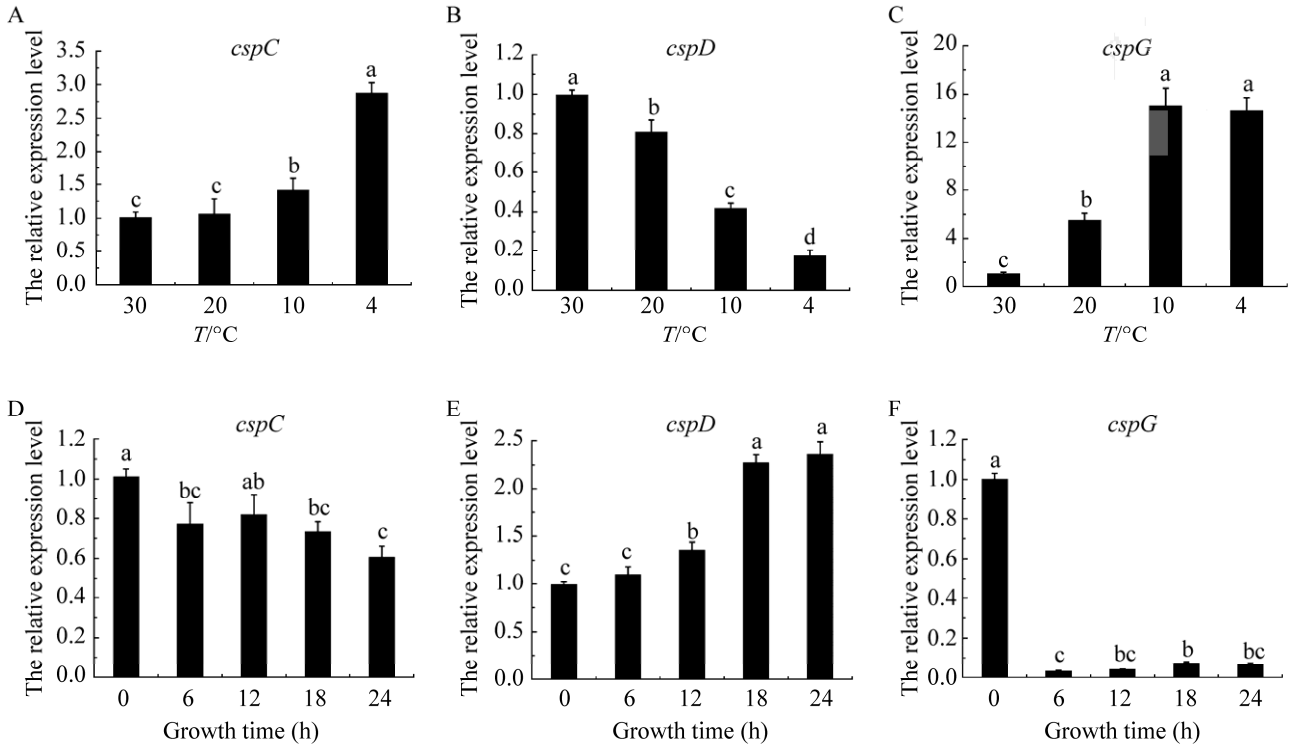

Figure 3 Gene expression levels determined by qPCR. Panels A, B, and C represent the relative expression levels of the *cspC*, *cspD*, and *cspG* genes in *Shewanella baltica* under different temperatures, respectively. Panels D, E, and F represent the relative expression levels of the *cspC*, *cspD*, and *cspG* genes in *S. baltica* at different growth stages, respectively. Error bars represent the standard deviation of sample means. Sample means with different lowercase letters indicate significant differences (P<0.05).

2.3 Deletion of the *cspC* gene or *cspG* gene affected the growth of the *Shewanella baltica* at low temperature

To elucidate the functions of the three *csp* genes of the *S. baltica*, the knockout strains Δ*cspC*, Δ*cspD*, and Δ*cspG* were generated. The WT strain of *S. baltica* grew rapidly at 30 °C and reached the stationary phase in approximately 20 h, with a maximum growth rate (μ_{max}) and maximum counts of 0.184 6 and (8.35±0.13) $\log_{10}$ CFU/mL, respectively. The growth rates and maximum cell count of the other three KO strains were similar to those of the WT strain, with no significant differences between them (Figure 4A).

When the temperature dropped to 15 °C, the growth rate of the WT strain slowed down, and the μ_{max} decreased to 0.059 1, but the maximum count still reached (8.49±0.17) $\log_{10}$ CFU/mL. Compared with the WT strain, the Δ*cspC* and Δ*cspG* strains had slower growth rates, and their maximum counts in the stationary phase were (7.98±0.14) $\log_{10}$ CFU/mL and (7.07±0.16) $\log_{10}$ CFU/mL, respectively (Figure 4B). Under the low-temperature culture condition (4 °C), the growth rate of the WT strain dropped sharply, with a μ_{max} of only 0.027 8, but its maximum count in the stationary phase was (8.21±0.13) $\log_{10}$ CFU/mL, which was close to those at 30 °C and 15 °C. Compared with the WT strain, the Δ*cspC* strain showed a much slower growth rate, with a μ_{max} and maximum count of 0.013 5 and (6.98±0.12) $\log_{10}$ CFU/mL, respectively. The Δ*cspG* strain exhibited a cold-sensitive phenotype characterized by a sustained decrease in cell density at 4 ℃ (Figure 4C), suggesting that the *cspG* gene is essential for *S. baltica* survival at low temperature. Surprisingly, the μ_{max} and maximum count of the Δ*cspD* strain were close to those of the WT strain, regardless of the variation in culture temperature.

2.4 Three *csp* genes of the *Shewanella baltica* SB-19 strain responded to environmental stresses but not to quorum sensing

To investigate whether three *S. baltica csp* genes were regulated by other abiotic stresses, KO strains were exposed to various treatments. Under salt stress, a low concentration of 2% slightly increased the cell density of all strains, and the cell density then gradually decreased with increasing NaCl concentration (Figure 5A). The densities were not significantly different between the WT and Δ*cspD* strains in all NaCl concentration tests; the Δ*cspC* and Δ*cspG* strains had significantly lower densities than the WT and Δ*cspD* strains at 3% and 4% NaCl (P<0.05), indicating that the *cspC* and *cspG* genes are involved in the adaptation to osmotic stress, unlike *cspD*.

In the nutrient deprivation test, the cell densities of all strains gradually decreased with the reduction in nutrient levels (Figure 5B), and the Δ*cspG* strain had the lowest cell density under the

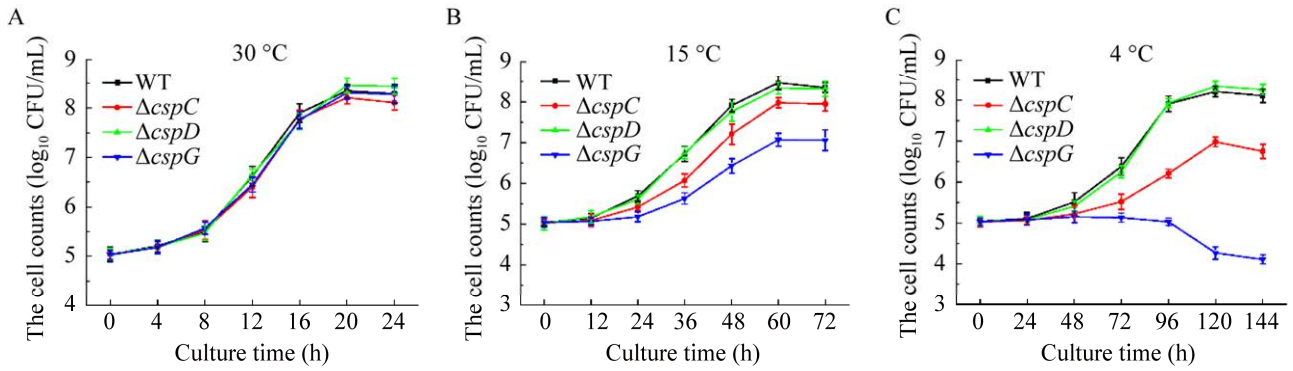

Figure 4 Growth curve of WT and three KO strains Δ*cspC*, Δ*cspD*, and Δ*cspG* under different temperatures. Panels A, B, and C represent strains grown at 30, 15, and 4 °C, respectively. Error bars represent the standard deviation of sample means.

same nutrient-deficient conditions. Unlike that under the NaCl treatment, the cell density of the Δ*cspD* strain was 76.6%, 54.0%, and 44.1% that of the WT strain under 1/2, 1/4, and 1/8 LB content, respectively. Furthermore, the expression level of the *cspD* gene increased with increasing culture time (Figure 3B), suggesting that the *cspD* gene was growth phase-dependent and associated with starvation stress. Surprisingly, the cell density of the Δ*cspC* strain was barely affected under nutrient deficiency, with no significant differences between the Δ*cspC* and WT strains at nutrient levels of 1/2 and 1/8. Therefore, both *cspD* and *cspG* genes responded to nutrient stresses, while the *cspC* gene did not.

Heavy metals induce intracellular reactive oxygen species (ROS) production and antioxidant enzyme activities, and copper-induced oxidative stress exhibits a stronger intensity than that induced by zinc, silver, arsenic, and cadmium[26], and such oxidative stress further leads to *csp* gene expression[27]. In this study, the addition of 1.0 mmol/L $CuCl_2$ to the medium had no effect on the cell density of all strains and even increased the cell density slightly (Figure 5C). Under 2.0 and 3.0 mmol/L $CuCl_2$ treatments, the cell density of the Δ*cspG* strain was significantly lower than those of the WT, Δ*cspC*, and Δ*cspD* strains (P<0.05), but there was no significant difference between the cell densities of the Δ*cspC* and Δ*cspD* strains. Under 3.0 mmol/L $CuCl_2$, compared with the WT strain, the cell density of the Δ*cspC*, Δ*cspD*, and Δ*cspG* strains decreased to 80.2%, 77.3%, and 35.0%, respectively, indicating that *cspG* was the major gene responsible for resistance to heavy metal copper ions in *S. baltica*.

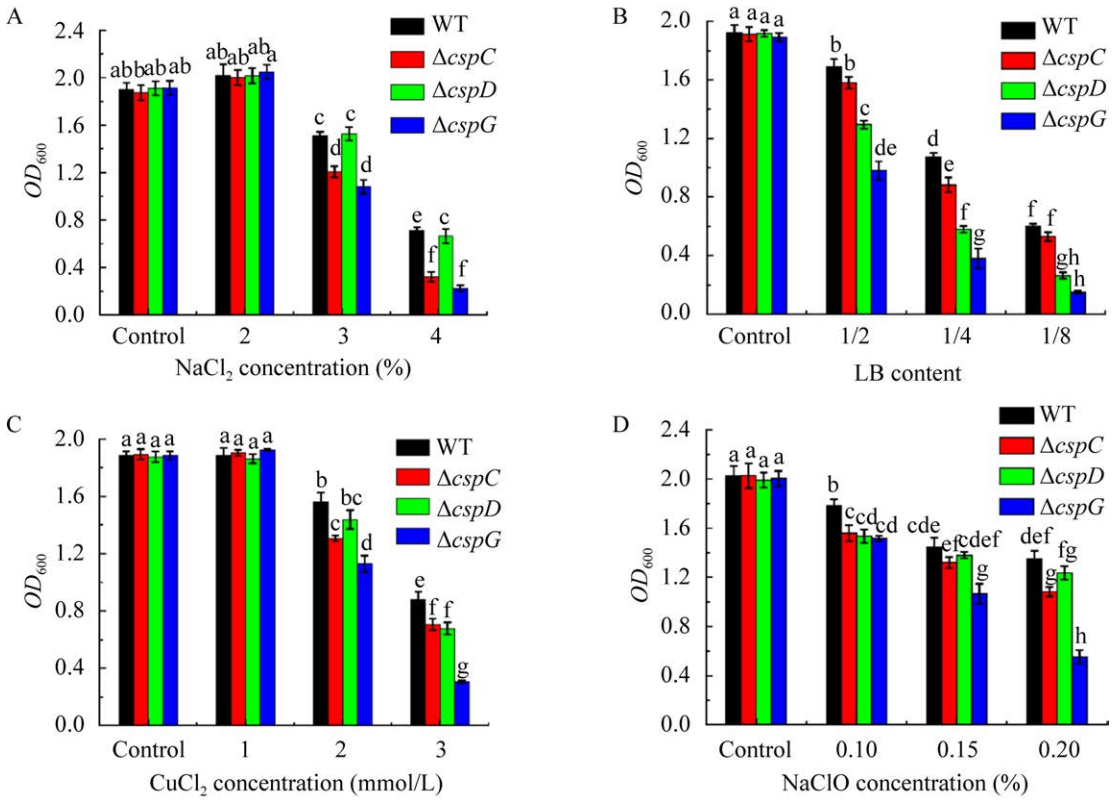

Figure 5 Growth analysis of WT and KO strains under environmental stresses. Panels A, B, C, and D represent strains grown under salt, nutritional deprivation, heavy metal, and disinfectant stresses, respectively. Error bars represent the standard deviation of sample means. Sample means with different lowercase letters indicate significant differences (P<0.05).

The *S. baltica* SB-19 strain was sensitive to the disinfectant sodium hypochlorite (NaClO), and the cell density of all strains decreased with increasing NaClO concentration (Figure 5D). At 0.1% NaClO, the growth of the WT strain was inhibited, and the cell density of three KO strains was lower than that of the WT strain. When the concentration was increased to 0.15% or 0.20%, the cell density of the Δ*cspG* strain was significantly lower than that of the other three strains (P<0.05), indicating that the *cspG* gene was critical for eliminating oxidative stress in *S. baltica*. Overall, the *cspG* gene played a universal role in the stress response, the *cspC* gene aided *S. baltica* in responding to osmotic, heavy metal, and disinfectant stresses, and the *cspD* gene was associated with bacterial growth and resistance to nutrient deprivation stress.

Upon incubation at 30 °C, the quorum sensing autoinducers of DKPs and AI-2 secreted by WT and the three KO strains showed the same trend of increasing and then decreasing within 24 h, and the contents of DKPs and AI-2 were not significantly different among these four strains at the same time point (Figure 6A, 6B), indicating that the deletion of the *csp* gene did not affect the secretion of quorum sensing autoinducers in the *S. baltica* SB-19 strain. After incubation at 30 °C for 24 h, the Δ*cspC* strain had slightly lower biofilm and spot diameters than the other three strains, but there was no significant difference between them (Figure 6C, 6D), further suggesting that the deletion of the *csp* gene did not affect quorum sensing in the *S. baltica* SB-19 strain.

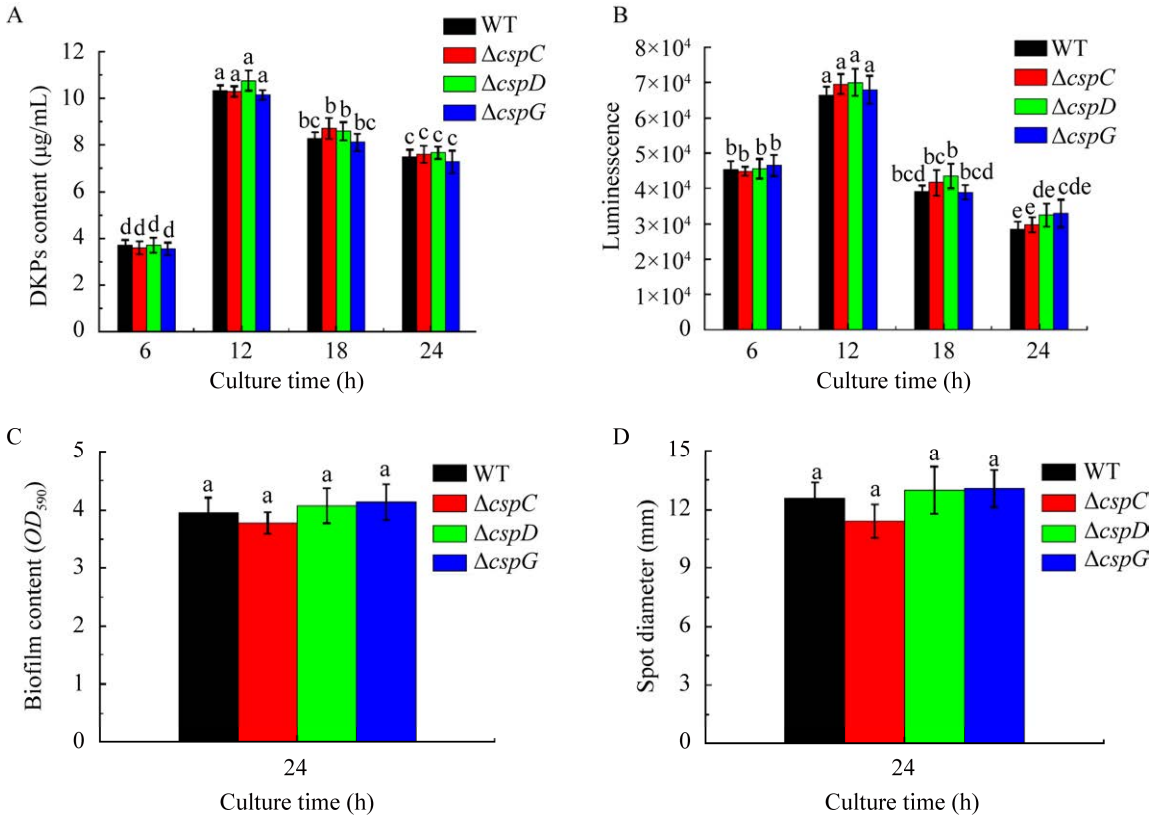

Figure 6 Quorum sensing characteristics of the WT and KO strains. A: DKPs content within 24 h. B: AI-2 content within 24 h. C: Biofilms formed for 24 h. D: Extracellular protease activity for 24 h. Error bars represent the standard deviation of sample means. Sample means with different lowercase letters indicate significant differences (P<0.05).

2.5 The *cspC* and *cspG* genes affect the spoilage capacity of *Shewanella baltica* at temperatures below room temperature

To investigate whether the three cold shock proteins can also affect the spoilage ability of *S. baltica* under other temperature conditions, TVB-N and TMA in sterilized fish juice inoculated with three KO strains were examined in this section under three incubation conditions of 4, 15, and 30 °C. After 144 h of incubation at 4 °C, the TVB-N contents in fish juice inoculated with the WT and Δ*cspD* strains were close, and the TVB-N content in fish juice inoculated with Δ*cspC* strain [(46.4±5.5) mg/100 mL] was significantly lower than those in the WT and Δ*cspD* strains, whereas the TVB-N content in fish juice inoculated with the Δ*cspG* strain [(27.4±3.8) mg/100 mL] was significantly lower than that in the Δ*cspC* strain (P<0.05) (Figure 7A); at this time point, the TMA content in fish juice was similar to the TVB-N content, and the contents in samples inoculated with the Δ*cspC* and Δ*cspG* strains were significantly lower than samples inoculated with the WT and Δ*cspD* strains (P<0.05) (Figure 7B). This result confirmed that both *cspC* and *cspG* affect the spoilage ability of *S. baltica* at 4 °C.

When the temperature was increased to 15 °C and samples were incubated for 72 h, the TVB-N content in fish juice inoculated with the Δ*cspC* and Δ*cspG* strains was (46.6±3.1) mg/100 mL and (33.4±2.1) mg/100 mL, respectively, both of which were significantly lower than those of the WT and Δ*cspD* strains (P<0.05) (Figure 7C); at this time point, the TMA content in fish juice inoculated with the Δ*cspC* strain [(48.4±6.4) mg/100 mL] was also lower than those of the WT and Δ*cspD* strains, while the fish juice inoculated with the Δ*cspG* strain had the lowest TMA content [(38.6±5.4) mg/100 mL] (Figure 7D), suggesting that only the deletion of *cspG* gene at 15 °C below room temperature causes *S. baltica* to exhibit significantly lower spoilage capacity.

After incubation at 30 °C for 24 h, the TVB-N content and TMA content of fish juice inoculated with the WT strain were not significantly different from those of fish juice inoculated with other three KO strains (Figure 7E, 7F), indicating that three genes of *cspC*, *cspD*, and *cspG* were not associated with the spoilage ability of the *S. baltica* at higher than room temperature.

2.6 Heterologous expression of CSP enhanced environmental stress tolerance in *Escherichia coli*

To further validate the functions of *S. baltica* proteins CspC, CspD, and CspG, they were heterologously expressed in *E. coli* (Figure 8A), and then these strains were exposed to various stress conditions. There was no significant difference in the growth rates at 37 °C (Figure 8B), indicating that CSP overexpression did not affect growth under normal conditions. Under cold stress at 4 °C, the growth rate of *E. coli* was severely slowed, but the cell counts of CspC- and CspG-overexpressing strains were significantly greater than those of strains harboring the empty vector and CspD at 72 h of incubation (P<0.05) (Figure 8C). At the end of cold stress incubation, the cell counts of the CspG-overexpressing strain were significantly greater than those of the CspC-overexpressing strain, which in turn were significantly greater than those of the empty vector strain (P<0.05), confirming that the CspC and CspG proteins of *S. baltica* could contribute to the survival of *E. coli* in a cold environment.

Then, *E. coli* strains overexpressing CspC, CspD, and CspG were subjected to four stress conditions: salt stress, nutrient deprivation, heavy metal stress, and disinfectant stress. Under 3% NaCl stress, the cell densities of the CspC- and CspG-overexpressing strains were significantly higher than those of the CspD overexpression and empty vector strains (P<0.05) (Figure 8D). When the NaCl concentration increased to 4%, the cell density of the CspG overexpression strain was significantly higher than that of the CspC-overexpressing strain, which in turn was significantly higher than that of the other two strains (P<0.05). There was no significant difference in cell density between the CspD-overexpressing and empty vector strains under all the salt stress conditions tested.

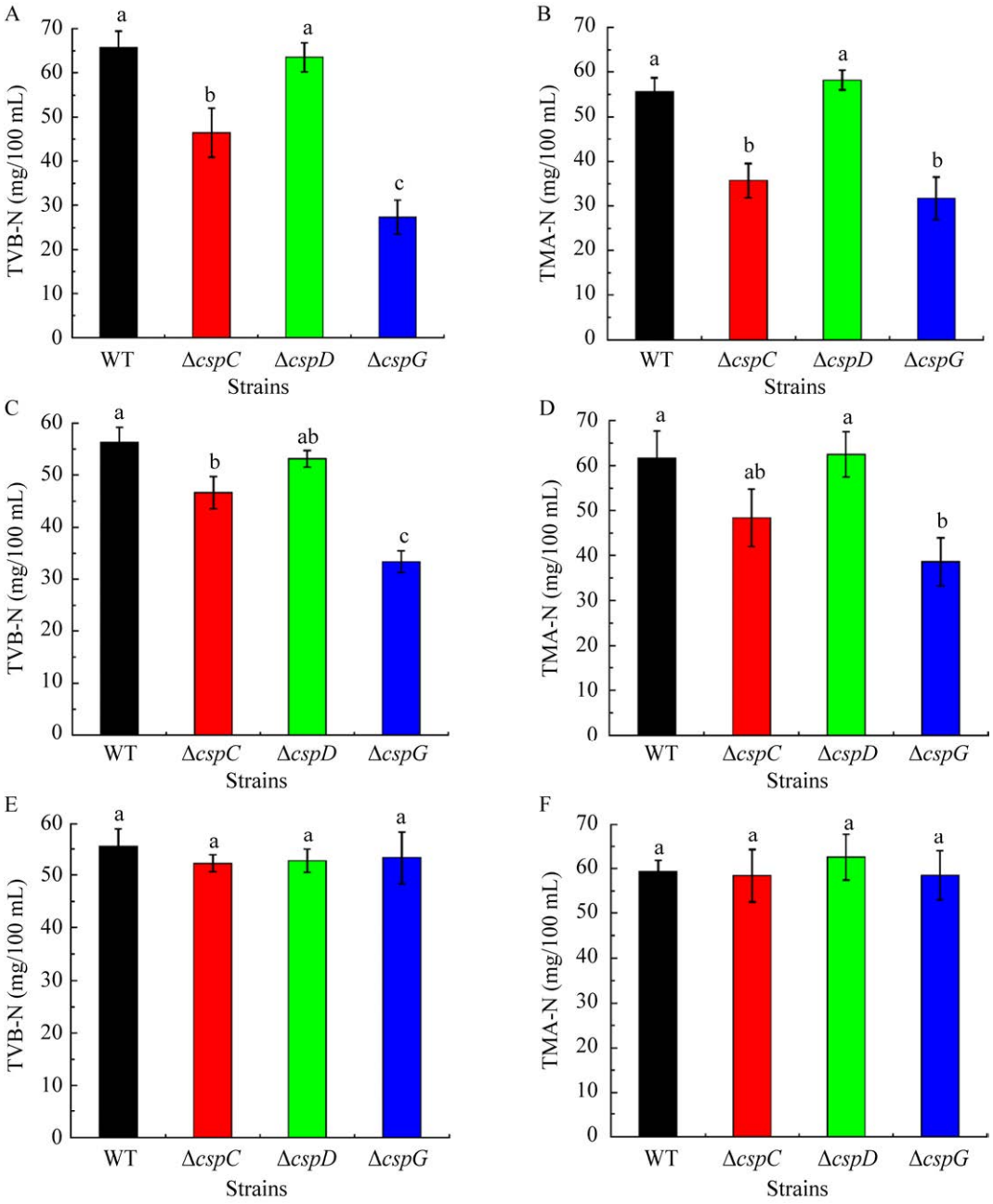

Figure 7 Spoilage ability of the WT and KO strains in fish juice. Panels A, C, and E represent the TVB-N content in fish juice at 4 ℃ for 144 h, 15 ℃ for 72 h, and 30 ℃ for 24 h, respectively. Panels B, D, and F represent the TMA content in fish juice at 4 ℃ for 144 h, 15 ℃ for 72 h, and 30 ℃ for 24 h, respectively. Error bars represent the standard deviation of sample means. Sample means with different lowercase letters indicate significant differences (P<0.05).

When the LB content was reduced by half, the cell densities of the CspD and CspG overexpression strains were higher than those of the CspC-overexpressing and empty vector strains; when it was reduced to a quarter of the original value, the cell density of the CspD-overexpressing strain was significantly higher than those of other three strains (Figure 8E), indicating that overexpression of the CspD protein helped the bacteria survive under nutrient deficiency.

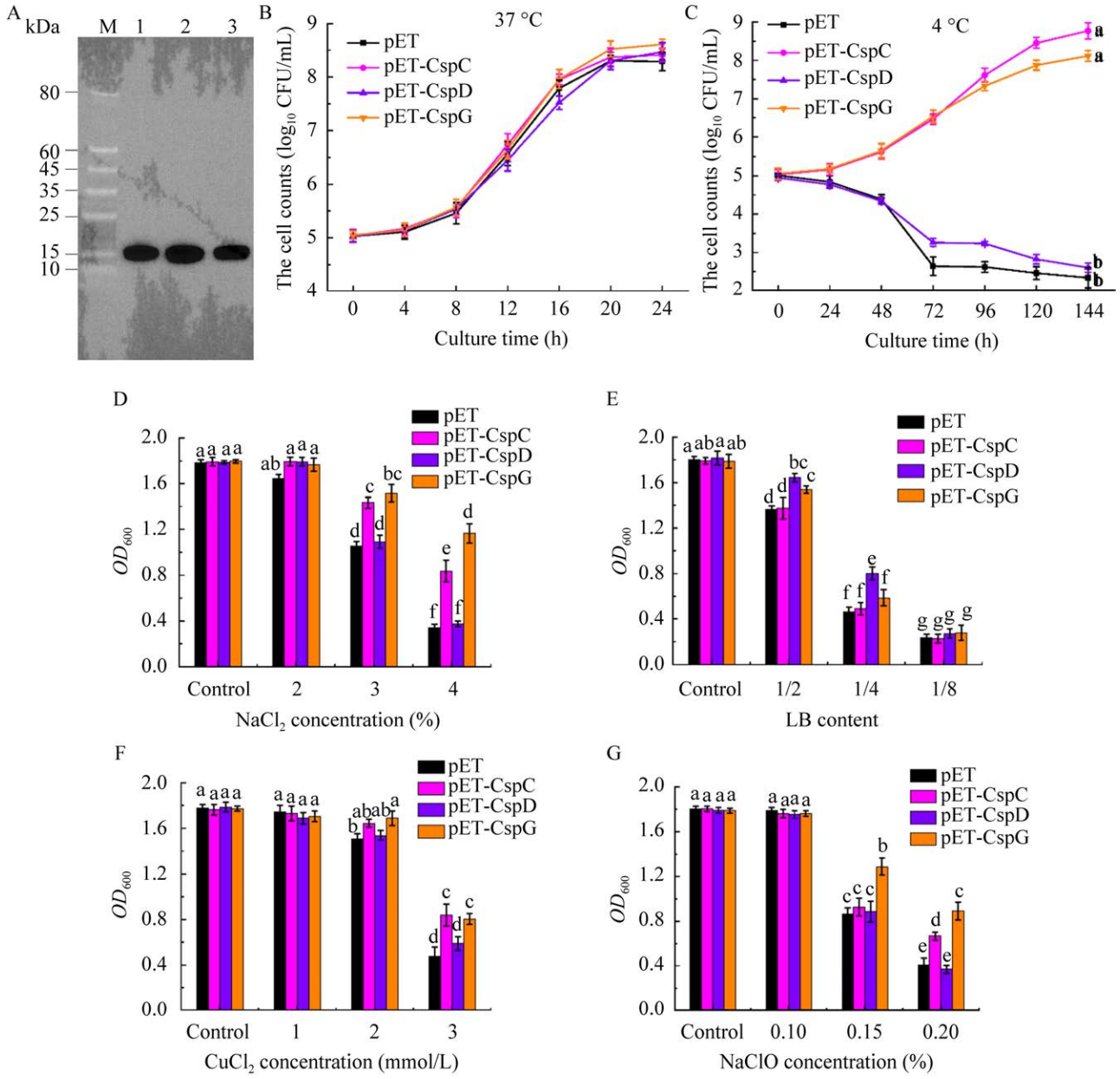

Figure 8 Heterologous expression of CSPs in *Escherichia coli*. A: Expression of CspC, CspD, and CspG in *E. coli* assessed by SDS-PAGE. M: Protein molecular weight marker; 1: Overexpressed CspC protein after purification; 2: Overexpressed CspD protein after purification; 3: Overexpressed CspG protein after purification. B: Growth curve of transformed *E. coli* at 37 ℃. pET: *E. coli* cells harboring the empty vector pET21a; pET-CspC: *E. coli* cells harboring the overexpression vector pET21a-CspC; pET-CspD: *E. coli* cells harboring the overexpression vector pET21a-CspD; pET-CspG: *E. coli* cells harboring the overexpression vector pET21a-CspG. C: Growth analysis of transformed *E. coli* at 4 ℃. D: Growth analysis of transformed *E. coli* under salt treatment. E: Growth analysis of transformed *E. coli* under nutritional deprivation. F: Growth analysis of transformed *E. coli* under heavy metal treatment. G: Growth analysis of transformed *E. coli* under disinfectant treatment. Error bars represent the standard deviation of sample means. Sample means with different lowercase letters indicate significant differences (P<0.05).

Under 2 mmol/L copper stress, only the cell density of the CspG-overexpressing strain was higher than that of the empty vector strain. When the copper concentration was increased to 3 mmol/L, the cell densities of the CspC- and CspG-overexpressing strains were higher than those of the CspD-overexpressing and empty vector strains (Figure 8F), indicating that the CspC and CspG proteins contribute to bacterial resistance to heavy metal (copper) stress.

Sodium hypochlorite at 0.1% had no inhibitory effect on *E. coli*. When its concentration was increased to 0.15%, the bacterial density of all the strains decreased dramatically, while the density of the CspG-overexpression strain was significantly higher than that of the other three strains (P<0.05), but there was no significant difference among the densities of the latter. The bacterial inhibition effect was more obvious when the concentration of sodium hypochlorite was raised to 2%, and the densities of strains harboring the empty vector and CspD were only 20.7%–22.8% that of the control group, while the densities of the CspC overexpression and CspG overexpression strains were 37.0% and 49.8% of the control group, respectively (Figure 8G). This indicated that the CspC and CspG proteins also contributed to bacterial resistance to disinfectants.

3 Discussion

S. baltica is a psychrophilic seafood bacterium whose *cspC*, *cspD*, and *cspG* genes are highly homologous to those of other *Shewanella* species (Figure 1). In addition, these three *csp* genes encode protein sequences with conserved RNP1 (K/S-G-F/K/Y-G-F/L-I-X-X) and RNP2 (V/L/I-F/Q-V/A/L-H-X-S/T/R) structural domains that functioned to bind single-stranded DNA and RNA, which has been confirmed in *E. coli*, *B. subtilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Yersinia pestis*, *Haemophilus influenzae*, and *Listeria monocytogenes*[8].

Some researchers have suggested that there is a regular clock associated with the evolution of biological macromolecules, that is, the rate of molecular evolution is approximately constant[28]. In this study, we selected some food microorganisms from the *Gammaproteobacteria* class, such as *Aeromonas salmonicida*, *Pseudomonas fluorescens*, *Vibrio cholerae*, and *Yersinia enterocolitica*, and estimated the origin time of their *csp* genes. Yu et al. calculated the molecular clock of 89 *csp* gene sequences in 26 bacterial taxa and found that the origin time of *csp* genes in the *Gammaproteobacteria* class was approximately 1 000–1 500 MYA[7], which was consistent with our determined value of 1 109.6 MYA.

All *cspD* genes of the *Gammaproteobacteria* class aggregated individually (Figure 1). The *cspD* gene of *E. coli* is not induced by cold shock, but it was found to be active during the stationary growth phase[29]; the *cspD* gene of *S. baltica* also showed the same expression pattern as *E. coli* during the cold shock and growth periods (Figure 3), suggesting that *cspD* is a functionally conserved gene.

Protein-protein interaction results showed that all CspC, CspD, and CspG interacted with DUT (Figure 2). dUTP pyrophosphatase, encoded by the DUT gene, catalyzes the hydrolysis of deoxyuridine triphosphate (dUTP) into deoxyuridine monophosphate (dUMP) and inorganic pyrophosphate (PPi) to eliminate DNA breakage and cell death caused by dUTP incorporation during DNA replication[30]. In addition to DUT, CspC also interacted with PNPase, an exoribonuclease that catalyzed the processive phosphorolytic degradation of RNA from the 3′-end[31], suggesting CspC and CspG played a crucial role in promoting bacterial growth at low temperature by maintaining normal nucleic acid metabolism. Proteins that interacted with CspD were more complex and also included PtsN and CBS domain containing protein, the former acting as a switch regulating carbon and nitrogen metabolism[32], and the latter catalyzing cysteine biosynthesis to produce hydrogen sulfide[33],

speculating that CspD might function differently from CspC and CspG.

During cold stress incubation at 4 ℃, the cell density of the Δ*cspG* strain decreased continuously, and the growth rate of the Δ*cspC* strain was dramatically downshifted, while the Δ*cspD* strain showed little change (Figure 4C), indicating that the deletion of *cspC* gene could be partially compensated by *cspG*, but not vice versa. In addition, heterologous expression of the *cspG* or *cspC* gene of *S. baltica* in *E. coli* showed that the overexpression strains were better adapted to low-temperature environments and reproduced at 4 ℃ (Figure 8C). In *E. coli*, the *cspA*, *cspB*, *cspG*, and *cspI* genes responded to cold shock, while other *csp* genes are not involved in this process[8]. In *B. subtilis*, deletion of *cspC* or *cspD* did not affect cellular growth at low temperature[34]. Therefore, *S. baltica cspG* was the major cold shock gene that partially compensated for the lack of *cspC*, while the *cspD* gene was not associated with cold shock.

Stress tolerance and cellular robustness are vital characteristics for microbial to survival in complex and variable environments. CSP proteins help cells to overcome multiple stresses, including nutrient deficiency, bacteriocins, UV light, osmotic stress, and cleaning and disinfectant chemical stresses[10]. In this study, the cell density of the Δ*cspG* strain was always the lowest under salt, nutrient deprivation, heavy metal, and disinfectant stresses, indicating that the *cspG* gene played a universal role in the stress response (Figure 5A–5D), similar to the *E. coli cspA* gene. The latter was not only the main gene involved in the response to cold shock stress, but also involved in multi-stress tolerance, including to nutrient deficiency[35]. Although the density of the Δ*cspC* strain was significantly reduced under salt stress, it was only slightly reduced under heavy metal and disinfectant stresses, and not significantly changed under nutrient deprivation. In addition, *E. coli* with heterologous expression of *S. baltica cspG* gene showed greater tolerance to environmental stress than that with the *cspC* gene (Figure 8D and 8G), suggesting that the *cspC* gene was a functional accessory gene to *cspG* in *S. baltica*. This is similar to the finding in *Clostridium botulinum* ATCC 3502, whose *cspB* and *cspC* genes, but not *cspA*, are important for pH, salinity, and ethanol stress responses[11]. Compared with the WT strain, the Δ*cspD* strain showed significantly lower cell density under nutrient deprivation but was not responsive to salt and disinfectant stresses, and the expression level of this gene increased with cell growth (Figure 3B), indicating that the *cspD* gene is a growth-related gene. The same is true for the function of the *cspD* gene in *E. coli* and enteropathogenic *Yersinia*[8,25]. Based on the structurally conserved features of the *cspD* gene (Figure 1), we hypothesized that the acquisition of nutritional and growth-related functions by *cspD* occurred prior to differentiation from other *csp* genes.

S. baltica exhibits quorum sensing at high cell densities, secretes two signaling molecules (AI-2 and DKPs), forms biofilms, and produces extracellular enzymes[17]. Under cold shock at 4 °C and 10 °C, *Vibrio parahaemolyticus* still could form biofilm[36]. In *L. monocytogenes*, deletion of single (Δ*cspA*) or multiple (Δ*cspAB*, Δ*cspAD*, and Δ*cspABD*) *csp* genes led to reduced biofilm formation[37]. In the tobacco isolate *Ralstonia solanacearum* CQPS-1, the *cspD3* gene was not essential for normal growth, cold shock adaptation, and biofilm formation, but KO of this gene led to increased swimming motility[38]. However, the three KO strains in this study exhibited no significant difference in quorum sensing from the WT strain (Figure 6), suggesting that the relationship between *csp* genes and biofilm formation in different bacteria was not the same, which further indicated that although the function of *csp* genes is conserved, the regulatory pathways associated with *csp* differed among bacteria.

Proteins in aquatic products decompose into nitrogenous substances such as ammonia and amines, namely TVB-N, during spoilage. Marine fish are rich in TMAO, which are deoxidized and reduced to odorous TMA by spoilage bacteria.

Therefore, the TVB-N and TMA contents have become important indicators for identifying fish spoilage[39]. At 4 °C, compared with WT and Δ*cspD* strains, the rate of spoilage of fish juice inoculated with Δ*cspC* and Δ*cspG* strains were slower rate and lower degree (Figure 7A and 7B), indicating that *cspC* and *cspG* promote the spoilage ability of *S. baltica* at low temperatures. As the temperature increased to 15 °C, the deletion of *cspC* appeared to be unimportant for the spoilage ability of *S. baltica*, but *cspG* still showed a significant spoilage correlation (Figure 7C and 7D). When the temperature was increased to 30 °C, both *cspC* and *cspG* did not have any relationship with spoilage in *S. baltica* (Figure 7E and 7F), reflecting that these two genes are temperature-dependent functional genes that might contribute to the correct folding and expression of spoilage-associated proteins (e.g., torT, cysM, and trxB) at low temperature[40], leading to spoilage of aquatic products at low temperature.

4 Conclusion

In this study, three cold shock proteins, cpsC, cspD, and cspG, were screened and identified in the psychrophilic bacterium *S. baltica*. The *csp* genes of food microorganisms in the *Gammaproteobacteria* class were analyzed, and all the *cspD* genes clustered individually into a single branch and segregated 1 109.6 MYA. Combined with the results of KO and overexpression, these results showed that the *cspD* gene is a structurally conserved gene and is insensitive to cold shock and environmental stresses, such as salt, heavy metals, disinfectants stresses, but is associated with the bacterial growth stage and nutritional deprivation. The *cspC* and *cspG* genes of *S. baltica* segregated from the other *cps* genes in the *Gammaproteobacteria* class at approximately 858.8 MYA, responding to cold shock and environmental stresses; these genes were dominated by the *cspG* gene, followed by the *cspC* gene. Moreover, the *cspC* and *cspG* genes were also associated with the spoilage ability of *S. baltica* under cold storage. This study provides a theoretical basis for the elucidation of the cold adaptation mechanism of psychrophilic bacteria and the further development of seafood cold storage and preservation.

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