Genomic and transcriptomic analyses reveal distinct biological functions for cold shock proteins (VpaCspA and VpaCspD) in Vibrio parahaemolyticus CHN25 during low-temperature survival

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Abstract

Background: Vibrio parahaemolyticus causes serious seafood-borne gastroenteritis and death in humans. Raw seafood is often subjected to post-harvest processing and low-temperature storage. To date, very little information is available regarding the biological functions of cold shock proteins (CSPs) in the low-temperature survival of the bacterium. In this study, we determined the complete genome sequence of V. parahaemolyticus CHN25 (serotype: O5:KU5T). The two main CSP-encoding genes (VpacspA and VpacspD) were deleted from the bacterial genome, and comparative transcriptomic analysis between the mutant and wild-type strains was performed to dissect the possible molecular mechanisms that underlie low-temperature adaptation by V. parahaemolyticus.

Results: The 5,443,401-bp V. parahaemolyticus CHN25 genome (45.2% G+C) consisted of two circular chromosomes and three plasmids with 4,724 predicted protein-encoding genes. One dual-gene and two single-gene deletion mutants were generated for VpacspA and VpacspD by homologous recombination. The growth of the ΔVpacspA mutant was strongly inhibited at 10°C, whereas the VpacspD gene deletion strongly stimulated bacterial growth at this low temperature compared with the wild-type strain. The complementary phenotypes were observed in the reverse mutants (ΔVpacspA-com, and ΔVpacspD-com). The transcriptome data revealed that 12.4% of the expressed genes in V. parahaemolyticus CHN25 were significantly altered in the ΔVpacspA mutant when it was grown at 10°C. These included genes that were involved in amino acid degradation, secretion systems, sulphur metabolism and glycerophospholipid metabolism along with ATP-binding cassette transporters. However, a low temperature elicited significant expression changes for 10.0% of the genes in the ΔVpacspD mutant, including those involved in the phosphotransferase system and in the metabolism of nitrogen and amino acids. The major metabolic pathways that were altered by the dual-gene deletion mutant (ΔVpacspAD) radically differed from those that were altered by single-gene mutants. Comparison of the transcriptome profiles further revealed numerous differentially expressed genes that were shared among the three mutants and regulators that were specifically, coordinately or antagonistically modulated by VpaCspA and VpaCspD.

Our data also revealed several possible molecular coping strategies for low-temperature adaptation by the bacterium. (Continued on next page)
Background

*Vibrio parahaemolyticus* naturally occurs in marine, estuarine and aquaculture environments worldwide and causes serious seafood-borne gastroenteritis and death in humans, particularly when raw, undercooked or mishandled seafood is consumed [1, 2]. *V. parahaemolyticus* was initially identified in 1950 in Osaka, Japan, where an outbreak of acute gastroenteritis that caused the consumption of semidried juvenile sardines sickened 272 people and killed 20 [3]. To date, over eighty *V. parahaemolyticus* serotypes have been described based on the somatic (O) and capsular (K) antigens [1]. Of these serotypes, complete genome sequences have been published for three *V. parahaemolyticus* strains—RIMD2210633 (serotype: O3:K6) [4], BB22OP (serotype: O4:K8) [5] and UCM-V493 (serotype: O2:K28) strains [6]. Additionally, two complete and multiple draft genome sequences for the *V. parahaemolyticus* strains are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genome/) and online (http://www.genomesonline.org) [7–9].

*V. parahaemolyticus* is a gram-negative bacterium that is frequently isolated from raw seafood [2]. Seafood is often subjected to post-harvest processing and low-temperature storage, during which the bacterium is challenged to survive under detrimental cold conditions. Previous studies have indicated that the temperature decrease elicits complex cold shock responses in food-related bacteria (e.g., lactic acid bacteria, food spoilage bacteria and food-borne pathogens), such as the regulation of uptake or synthesis of compatible solutes, DNA supercoiling modifications, membrane fluidity maintenance, and cold shock protein (CSP) production (for a review, see [10]).

CSPs comprise a group of low-molecular-weight proteins of approximately 7 kDa. CSP families that contain between two and nine members have been identified in food-related bacteria and several food-borne pathogens, including *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas fragi* [11]. In *E. coli*, the CSP family contains nine members (A–I), of which CspA is a well characterised RNA chaperone that reduces low temperature-associated increases in RNA secondary folding [10]. Although CSPs share a high degree of sequence similarity (>45%) with two conserved RNA-binding motifs, it is surprising that not all CSP members are cold-inducible, which implies that they may function in different cellular processes [11]. CspD in *E. coli* reportedly plays a negative regulatory role in chromosomal replication in nutrient-depleted cells [12]. Recent studies have indicated that the MqsR/MqsA toxin/antitoxin pair directly regulates CspD, which may be involved in toxicity and biofilm formation in *E. coli* [13].

Despite its significance in human health and in the aquaculture industry, the molecular mechanisms that underlie the low-temperature survival of *V. parahaemolyticus* remain largely unknown. Previous studies have revealed three *E. coli* CSP homologues in *V. parahaemolyticus*, including CspA, CspD and the cold shock DNA-binding domain-containing protein [14]. The cspA gene was up-regulated at the transcriptional level by over 30-fold after *V. parahaemolyticus* was treated for 60 min at 10 °C, a temperature below which bacterial growth was arrested [14]. However, the genes that encoded the other two homologues were undetectable by DNA microarray and real-time reverse transcription PCR (qRT-PCR) [14], which suggested that CspA could be a major CSP in *V. parahaemolyticus* during low-temperature growth. This study is the first to sequence, assemble and annotate the complete genome of *V. parahaemolyticus* CHN25 (serotype: O5:KUT), which has recently been isolated and characterised [15–17]. We constructed one dual-gene and two single-gene deletion mutants of the two main *V. parahaemolyticus* CHN25 CSPs (designated as VpaCspA and VpaCspD) and determined the global-level gene expression profiles of the mutant and wild-type strains by Illumina RNA-Sequencing. These data will refine our grasp of the molecular mechanisms that underlie the low-temperature adaptation of the most common seafood-borne pathogen worldwide.

Results and discussion

Genomic features of *V. parahaemolyticus* CHN25

The complete genome sequence of *V. parahaemolyticus* CHN25 was determined by 454-pyrosequencing (see Methods). It consisted of two circular chromosomes that
contained 3,416,467 bp and 1,843,316 bp (see Additional file 1: Figure S1). The genome also contained three plasmids (92,495 bp, 83,481 bp and 7,642 bp), all of which were absent from the other known V. parahaemolyticus genomes (see Additional file 2: Figure S2). The complete V. parahaemolyticus CHN25 genome contained 5,443,401 bp with a 45.2% G + C content; 4,724 protein-encoding genes were predicted, of which approximately 34.8% encoded hypothetical proteins with unknown functions in public databases. Additionally, 9 rRNA operons and 55 ribosomal protein-encoding genes, 107 tRNA genes, and 30 pseudogenes were identified and annotated.

In marked contrast to the other known V. parahaemolyticus genomes, an integrative and conjugative element (ICEVpaChn1) was identified in the CHN25 genome. The 89.9-kb element (VpaChn25_2302 to Chn25_2378) contained sulfamethoxazole and streptomycin resistance genes. Mating assays demonstrated the active self-transmissibility of the antibiotic resistance from V. parahaemolyticus CHN25 to E. coli MG1655 [15]. Five prophage gene clusters that ranged from 6.5 to 36.6 kb were identified in the CHN25 genome, and they displayed high degrees of sequence identity with Vibrio phage martha 12B12 (GenBank accession no. HQ316581), Vibrio phage VPUSM 8 (GenBank accession no. KF361475), Vibrio phage henriette 12B8 (GenBank accession no. HQ316582), and Vibrio phage N4 [18]. Additionally, five insertion sequences (ISs) were detected in the genome, including ISShrf9 (Tn3), ISVa1L, ISVpa3 (IS5) and ISVsa3 (IS91); the latter existed as two copies in the genome, which suggested that it was probably active. We concluded that the V. parahaemolyticus CHN25 genome has undergone major rearrangements associated with mobile genetic elements.

Consistent with the other V. parahaemolyticus genomes, most of the genes that encoded enzymes for the predicted central metabolic pathways were present in the CHN25 strain, including those required for glycolysis, oxidative phosphorylation and tricarboxylic acid cycle (TCA). Additionally, the CHN25 genome also contained high degrees for three restriction and modification (R-M) systems (types I, II and IV) and four DNA repair systems (base excision repair, nucleotide excision repair, mismatch repair and homologous recombination), most of which were present in several other V. parahaemolyticus strains. The high frequency of the horizontal gene transfer in the CHN25 strain (i.e., ICEVpaChn1) may have led the bacteria to hijack the R-M and DNA repair mechanisms to generate genetic diversity without losing genomic stability [19].

Construction of the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of V. parahaemolyticus CHN25

To investigate the low-temperature adaptation that was mediated by the predicted CSPs in V. parahaemolyticus CHN25, we constructed a deletion mutant of the VpacspA gene. The upstream and downstream sequences (approximately 0.5 kb) that flanked the VpacspA gene were obtained by PCR and cloned into a suicide vector, pDS132, to yield the recombinant vector, pDS132 + ΔVpacspA. The inserted 1,041-bp sequence was confirmed by DNA sequencing (data not shown). The recombinant vector was transformed into E. coli (GenBank accession no. KF361475), and the chloramphenicol-resistant transformant was obtained and conjugated with V. parahaemolyticus CHN25. Positive exconjugants were obtained using the two-step allelic exchange method (see the Methods section) and validated by PCR. DNA sequencing of the PCR product further confirmed the in-frame deletion of the 213-bp sequence of the VpacspA gene from the V. parahaemolyticus CHN25 genome (data not shown).

Similarly, the VpacspD gene that encoded a cold shock-like protein was deleted from the bacterial genome using the aforementioned method. The ΔVpacspD mutant with a 219-bp in-frame deletion was confirmed by DNA sequencing (data not shown). Furthermore, the VpacspD gene was also successfully deleted from the ΔVpacspA mutant, yielding a dual-gene deletion mutant of ΔVpacspAD (data not shown). The genome-level transcriptome data provided direct evidence of the successful construction of the three mutants, in which expression of the corresponding VpacspA or VpacspD genes was undetectable (see below).

Survival of the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants at 10 °C

To gain insights into the possible effects of the CSP-associated gene deletions on V. parahaemolyticus CHN25 low-temperature survival, we determined growth curves for the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants, which were grown in LB broth (3% NaCl, pH 8.5) at 37 °C or 10 °C. As shown in Fig. 1 (A), no apparent differences in growth were observed between the mutant and wild-type strains at 37 °C, which was an optimal growth temperature. However, the ΔVpacspA mutant showed a longer lag phase (>30 h) and grew more slowly compared with the wild-type strain at 10 °C (Fig. 1b), demonstrating that VpaCspA was a crucial CSP in V. parahaemolyticus CHN25 low-temperature survival. Although VpaCspD was identified as one of the three homologues of the E. coli CSPs [14], the VpacspD gene deletion unexpectedly stimulated mutant growth at 10 °C in our study, which was notably faster than the wild-type strain (Fig. 1b), indicating that VpaCspD likely functioned as a low-temperature bacterial growth inhibitor. A BLAST analysis revealed that the VpaCspD sequence shared a 70% amino acid identity with CspD in E. coli (EcCspD) (Fig. 2), which has been proposed to function as a novel inhibitor of DNA replication in nutrient-depleted cells [12]. Unlike EcCspD, its null mutant grew well over a 15 to 42 °C
temperature range with no detectable morphological changes. Our data indicated that VpacspD also functioned as a low-temperature induced-CSP (see below). Because only three CSP-associated genes were identified in V. parahaemolyticus and because VpaCspD only displayed a 48% amino acid sequence identity with VpaCspA (Fig. 2), VpaCspD may have evolved to gain different biological functions. Interestingly, the ΔVpacspAD mutant also grew poorly at low temperature compared to the wild-type strain (Fig. 1b), indicating that the phenotype of the VpacspA gene deletion dominated that of the VpacspD gene (see below).

Construction of the reverse mutants ΔVpacspA-com and ΔVpacspD-com and complementary phenotypes at 10 °C
The cspA gene was amplified from the genomic DNA of V. parahaemolyticus CHN25 by PCR, and cloned into the expression vector pMMB207, which yielded the recombinant vector pMMB207-VpacspA. The inserted 213-bp sequence was confirmed by DNA sequencing (data not shown). This recombinant vector was then electrotransformed into the ΔVpacspA mutant competent cells, and generated the reverse mutant ΔVpacspA-com (see the Methods section). Similarly, the recombinant vector pMMB207-VpacspD carrying the 240-bp cspD gene was also constructed, and electrotransformed into the ΔVpacspD mutant, yielding the reverse mutant ΔVpacspD-com. The growth curves for the reverse mutants ΔVpacspA-com and ΔVpacspD-com were also determined, which were incubated in LB broth (3% NaCl, 5 μg/mL chloramphenicol, pH 8.5) at 37 °C or 10 °C (Fig. 3). Consistent with the results in Fig. 1a, no obvious difference in growth at 37 °C was observed among the wild type, the mutants ΔVpacspA and ΔVpacspD, and the reverse mutants ΔVpacspA-com and ΔVpacspD-com (Fig. 3a). However, at 10 °C, the reverse mutants displayed similar growth phenotype as the wild type (Fig. 3b), demonstrating that the distinct phenotypes of the mutants ΔVpacspA and ΔVpacspD were indeed resulted from the VpacspA and VpacspD gene deletions in V. parahaemolyticus CHN25.

Fig. 1 Growth of V. parahaemolyticus CHN25 and the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants in LB broth (3% NaCl, pH 8.5) at 37 °C (a) and 10 °C (b).

Fig. 2 A multi-sequence alignment of the CSPs from V. parahaemolyticus CHN25 and E. coli. The numbers above the alignments indicate the relative positions of the entirely aligned sequences. Identical and conserved (>50% of the sequences) amino acid residues are highlighted in black and grey, respectively; the consensus sequence is shown below the alignment. The RNA-binding motifs (RNP-1 and RNP-2) are boxed. The EcCspA and EcCspD sequences were derived from E. coli JM83 (Yamanaka et al. [12]), while the VpaCspA (VpaChn25A_0413) and VpaCspD (VpaChn25_1036) sequences were obtained from V. parahaemolyticus CHN25 in this study.
Transcriptome profiles for the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants at 10 °C

To further investigate the VpaCspA- and VpaCspD-mediated low-temperature survival of V. parahaemolyticus CHN25, we determined global-level gene expression profiles for the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants that were grown at 10 °C, where distinct growth phenotypes were evident. Based on the complete genome sequence of V. parahaemolyticus CHN25, this analysis revealed numerous differentially expressed genes (DEGs) in the mutants, indicating that VpaCspA and VpaCspD likely functioned as master or global regulators in low-temperature bacterial growth. Five hundred seventy-two genes were significantly altered in the ΔVpacspA mutant compared with the wild-type strain; these genes represented approximately 12.4% of the expressed genes in V. parahaemolyticus CHN25. Of these, 263 genes showed higher transcriptional levels (fold change ≥ 2.0), while 309 genes were down-regulated (fold change ≤ 0.5). The altered genes in the ΔVpacspA mutant were grouped into eighty-three gene functional catalogues that were identified in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (data not shown). The ΔVpacspD gene deletion elicited 10% of the differentially expressed genes in the bacterium, including 242 up-regulated and 219 down-regulated genes that were grouped into seventy-six gene functional catalogues (data not shown). Additionally, the expression of 352 and 289 genes was up- and down-regulated, respectively, in the dual-gene deletion mutant (ΔVpacspAD), which accounted for 13.9% of the expressed genes; they were grouped into seventy-four gene functional catalogues (data not shown). A complete list of the DEGs for the three mutants is available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE65998. To validate the transcriptome data, we examined ten representative genes for each of the three mutants by qRT-PCR. The resulting data were correlated with data from the Illumina RNA-Sequencing analysis, and there was no statistically significant difference between the two datasets (P = 0.982) (Table 1).

The major low-temperature survival-associated metabolic pathways that were altered in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants

Major altered metabolic pathways in the ΔVpacspA mutant

Based on the gene set enrichment analysis (GSEA) of the transcriptome data against the KEGG database, the following seven significantly altered metabolic pathways were identified in the ΔVpacspA mutant at 10 °C: valine, leucine and isoleucine degradation; the propanoate, ascorbate and aldarate, sulphur, and glycerophospholipid metabolic pathways; ATP-binding cassette (ABC) transporters; and bacterial secretion systems (Table 2). Of these, the DEGs that were linked to valine, leucine and isoleucine degradation as well as propanoate metabolism were up-regulated (2.1029- to 8.5787-fold), which may have resulted in increases in acetyl-CoA and propanoyl-CoA and subsequent entry into the TCA and pyruvate metabolic cycles, respectively, by the ΔVpacspA mutant. For the other five altered metabolic pathways, most of the DEGs were down-regulated in the ΔVpacspA mutant, which was directly related to its remarkable low-temperature growth inhibition. For example, the expression of twenty-four genes that were linked to ABC transporters was up-regulated (2.1029- to 8.5787-fold), which may have resulted in increases in acetyl-CoA and propanoyl-CoA and subsequent entry into the TCA and pyruvate metabolic cycles, respectively, by the ΔVpacspA mutant.

For the other five altered metabolic pathways, most of the DEGs were down-regulated in the ΔVpacspA mutant, which was directly related to its remarkable low-temperature growth inhibition. For example, the expression of twenty-four genes that were linked to ABC transporters was reduced (0.4917- to 0.1424-fold); they included the glycine betaine (GB)/proline, oligopeptide, iron(III) and zinc ABC transporters. This indicated the positive regulation of these ABC transporters by VpaCspA during low-temperature V. parahaemolyticus CHN25 survival.

Bacterial secretion systems play important roles in virulence, symbiosis, interbacterial interactions, and environmental stress [20]. The genes that encoded components of the four secretion system types (T1SS, T2SS,
T3SS1 and T6SS2) were identified in the *V. parahaemolyticus* CHN25 genome. Of these, eleven genes were differentially expressed in the Δ*VpacspA* mutant at the low temperature. Activation of the *tolC* gene, which encodes an outer membrane protein of T1SS, has been reported in *Psychrobacter cryohalolentis* K5 during growth at sub-zero temperatures [21]. In this study, *tolC* gene expression (VpaChn25_1399) was down-regulated (0.4446-fold) in the Δ*VpacspA* mutant, indicating that VpaCspA positively regulated low-temperature *tolC*

| Locus / gene in *V. parahaemolyticus* CHN25 | Description of encoded protein | Fold change RNA-Seq | Fold change RT-PCR |
|--------------------------------------------|-------------------------------|----------------------|--------------------|
| Δ*pacspA* mutant                           |                               |                      |                    |
| VpaChn25A_0188                             | Hypothetical protein          | 13.41                | 10.21              |
| VpaChn25_1642                              | Tricarboxylic transport TctC  | 10.04                | 10.35              |
| VpaChn25_1640                              | GntR family transcriptional regulator | 4.24                | 2.49               |
| VpaChn25_0561                              | Aldehyde dehydrogenase       | 7.05                 | 5.24               |
| VpaChn25_0068                              | Hyperosmotically inducible periplasmic protein | 3.03                | 2.33               |
| VpaChn25A_1398                             | L-threonine 3-dehydrogenase   | 0.07                 | 0.03               |
| VpaChn25_1399                              | 2-amino-3-ketobutyrate CoA ligase | 0.07                | 0.03               |
| VpaChn25_2988                              | Amino acid ABC transporter substrate-binding protein | 0.14                | 0.09               |
| VpaChn25A_0149                             | Transcriptional regulator CpxR | 0.22                | 0.12               |
| VpaChn25A_0568                             | Transcriptional regulator Betl | 0.48                | 0.32               |
| Δ*pacspD* mutant                           |                               |                      |                    |
| VpaChn25_1716                              | Glycine betaine transporter periplasmic subunit | 16.04               | 19.13              |
| VpaChn25A_0188                             | Hypothetical protein          | 12.6                 | 11.11              |
| VpaChn25_1642                              | Tricarboxylic transport TctC  | 7.95                 | 7.13               |
| VpaChn25A_0561                             | Aldehyde dehydrogenase       | 4.08                 | 3.23               |
| VpaChn25_1640                              | GntR family transcriptional regulator | 2.29                | 2.62               |
| VpaChn25_2248                              | Glycerol uptake facilitator protein GlpF | 0.04                | 0.02               |
| VpaChn25A_1398                             | L-threonine 3-dehydrogenase   | 0.05                 | 0.03               |
| VpaChn25_1399                              | 2-amino-3-ketobutyrate CoA ligase | 0.048               | 0.021              |
| VpaChn25_2988                              | Amino acid ABC transporter substrate-binding protein | 0.2                 | 0.14               |
| VpaChn25A_0149                             | Transcriptional regulator CpxR | 0.3                 | 0.25               |
| Δ*pacspAD* mutant                          |                               |                      |                    |
| VpaChn25A_1312                             | PTS system fructose-specific transporter subunit IIABC | 8.77                | 21.54              |
| VpaChn25A_0303                             | PTS system fructose-specific transporter subunit IIA | 8.07                | 19.04              |
| VpaChn25A_1313                             | Mannose-6-phosphate isomerase | 5.17                | 16.82              |
| VpaChn25_0669                              | Trehalose-6-phosphate hydrolase | 4.51                | 9.77               |
| VpaChn25_0561                              | Aldehyde dehydrogenase       | 2.97                 | 4.1                |
| VpaChn25_2248                              | Glycerol uptake facilitator protein GlpF | 0.033               | 0.039              |
| VpaChn25_2249                              | Glycerol kinase              | 0.04                 | 0.07               |
| VpaChn25_0112                              | Phosphoenolpyruvate carboxykinase | 0.17                | 0.22               |
| VpaChn25_2988                              | Amino acid ABC transporter substrate-binding protein | 0.31                | 0.43               |
| VpaChn25A_0149                             | Transcriptional regulator CpxR | 0.42                | 0.46               |
### Table 2: Major altered metabolic pathways in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of *V. parahaemolyticus* CHN25 grown at the low temperature

| Changed metabolic pathway | Locus / Gene       | Fold change | Description of encoded protein                        |
|--------------------------|--------------------|-------------|-------------------------------------------------------|
| Valine, leucine and isoleucine degradation | VpaChn25A_0480     | 2.4983      | Acetyl-CoA acetyltransferase                           |
|                          | VpaChn25A_0554     | 3.0843      | Hydroxymethylglutaryl-CoA lyase                       |
|                          | VpaChn25A_0555     | 3.2361      | Acyl-CoA carboxylase alpha chain                      |
|                          | VpaChn25A_0556     | 2.4995      | Enoyl-CoA hydratase/isomerase                         |
|                          | VpaChn25A_0557     | 2.662       | Acyl-CoA carboxyltransferase beta chain               |
|                          | VpaChn25A_0558     | 2.216       | Acyl-CoA dehydrogenase                               |
|                          | VpaChn25A_0560     | 2.8734      | Acyl-CoA thiolase                                     |
|                          | VpaChn25A_0561     | 7.0543      | Aldehyde dehydrogenase                               |
|                          | VpaChn25A_0565     | 7.3736      | 3-hydroxyisobutyrate dehydrogenase                    |
|                          | VpaChn25A_1033     | 7.4779      | Methylmalonate-semialdehyde dehydrogenase            |
|                          | VpaChn25A_1036     | 2.1029      | Acyl-CoA dehydrogenase                               |
| Propanoate metabolism   | VpaChn25_1376      | 6.0834      | 4-aminobutyrate aminotransferase                     |
|                          | VpaChn25_1635      | 6.3526      | PrpE protein                                         |
|                          | VpaChn25_1638      | 8.5787      | Methylcitrate synthase                               |
|                          | VpaChn25_1639      | 6.7065      | 2-methylisocitrate lyase                             |
|                          | VpaChn25_2798      | 3.959       | Acetyl-CoA synthetase                                 |
| ABC transporters        | VpaChn25A_0128     | 0.4376      | ABC transporter substrate-binding protein             |
|                          | VpaChn25A_0130     | 0.428       | ABC transporter ATP-binding protein                   |
|                          | VpaChn25A_0533     | 3.1521      | High-affinity branched-chain amino acid transport ATP-binding protein |
|                          | VpaChn25A_0535     | 3.9903      | ABC transporter membrane spanning protein            |
|                          | VpaChn25A_0536     | 5.0642      | High-affinity branched-chain amino acid transport permease |
|                          | VpaChn25A_0537     | 6.8975      | Hypothetical protein                                 |
|                          | VpaChn25A_0538     | 4.7093      | High-affinity branched-chain amino acid transport ATP-binding protein |
|                          | VpaChn25A_0571     | 0.3015      | Glycine betaine-binding ABC transporter              |
|                          | VpaChn25A_0572     | 0.3358      | Permease                                             |
|                          | VpaChn25A_0573     | 0.3801      | ABC transporter ATP-binding protein                   |
|                          | VpaChn25A_0595     | 0.4067      | Ribose ABC transporter permease                      |
|                          | VpaChn25A_0604     | 0.406       | Iron (III) ABC transporter periplasmic iron-compound-binding protein |
|                          | VpaChn25A_1325     | 0.2076      | Iron (III) ABC transporter ATP-binding protein       |
|                          | VpaChn25A_1326     | 0.2262      | Iron (III) ABC transporter periplasmic iron-compound-binding protein |
|                          | VpaChn25A_1327     | 0.3019      | Iron-hydroxamate transporter permease subunit        |
|                          | VpaChn25A_1333     | 2.1634      | Transport protein                                     |
|                          | VpaChn25A_1544     | 0.2026      | Iron-dicitrate transporter substrate-binding subunit  |
|                          | VpaChn25_0306      | 0.4761      | Thiamine transporter membrane protein                 |
|                          | VpaChn25_0363      | 2.1743      | ABC transporter substrate binding protein            |
|                          | VpaChn25_0846      | 0.4129      | Zinc ABC transporter permease                        |
|                          | VpaChn25_0848      | 0.4556      | Zinc ABC transporter periplasmic zinc-binding protein |
|                          | VpaChn25_1344      | 0.2076      | Oligopeptide ABC transporter ATP-binding protein     |
|                          | VpaChn25_1345      | 0.2716      | Oligopeptide ABC transporter ATP-binding protein     |
|                          | VpaChn25_1346      | 0.2355      | Oligopeptide ABC transporter permease                |
|                          | VpaChn25_1347      | 0.3245      | Oligopeptide ABC transporter permease                |
Table 2 Major altered metabolic pathways in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of V. parahaemolyticus CHN25 grown at the low temperature (Continued)

| Gene/Pathway                                      | Log2 Ratio | Function                                                                 |
|--------------------------------------------------|------------|--------------------------------------------------------------------------|
| VpaChn25_1348                                    | 0.3723     | Oligopeptide ABC transporter periplasmic oligopeptide-binding protein    |
| VpaChn25_1613                                    | 2.4584     | Amino acid ABC transporter substrate-binding protein                      |
| VpaChn25_1714                                    | 0.4917     | Glycine/betaine/proline ABC transporter                                  |
| VpaChn25_1913                                    | 2.222      | Hypothetical protein                                                      |
| VpaChn25_2428                                    | 0.4529     | Iron (III) ABC transporter permease                                       |
| VpaChn25_2429                                    | 0.3234     | Iron (III) ABC transporter periplasmic iron-compound-binding protein     |
| VpaChn25_2987                                    | 0.2357     | Amino acid ABC transporter permease                                       |
| VpaChn25_2988                                    | 0.1424     | Amino acid ABC transporter substrate-binding protein                      |
| VpaChn25A_0952                                   | 4.5459     | Hypothetical protein                                                      |
| VpaChn25A_0954                                   | 6.1398     | ClpA / B-type chaperone                                                   |
| VpaChn25A_0966                                   | 6.0405     | Hypothetical protein                                                      |
| VpaChn25A_0969                                   | 7.0563     | Hypothetical protein                                                      |
| VpaChn25_1662                                    | 0.3404     | Type III secretion system protein                                         |
| VpaChn25_1663                                    | 0.2481     | Type III secretion system protein                                         |
| VpaChn25_1664                                    | 0.2099     | Translocation protein in type III secretion                              |
| VpaChn25_1665                                    | 0.2746     | Translocation protein in type III secretion                              |
| VpaChn25_1666                                    | 0.2109     | Translocation protein in type III secretion                              |
| VpaChn25_1887                                    | 0.4446     | Outer membrane protein TolC                                              |
| VpaChn25A_0230                                   | 0.1973     | PTS system ascorbate-specific transporter subunit IIC                    |
| VpaChn25A_0231                                   | 0.1668     | Sugar phosphotransferase component II B                                  |
| VpaChn25A_0232                                   | 0.1096     | Phosphotransferase enzyme II, A component                                |
| VpaChn25_0788                                    | 0.2523     | Cysteine synthase A                                                      |
| VpaChn25_0937                                    | 3.0238     | Cysteine synthase / cystathionine beta-synthase family protein            |
| VpaChn25_1397                                    | 2.7136     | Homoserine O-succinyltransferase                                          |
| VpaChn25_2650                                    | 0.4424     | Phosphoadenosine phosphosulfate reductase                                |
| VpaChn25_2651                                    | 0.4934     | Sulfite reductase subunit beta                                           |
| VpaChn25_2652                                    | 0.2017     | Sulfite reductase (NADPH) flavoprotein subunit alpha                      |
| VpaChn25_2692                                    | 2.1317     | Cystathionine gamma-synthase                                              |
| VpaChn25A_0425                                   | 0.4957     | Diacylglycerol kinase                                                     |
| VpaChn25A_0732                                   | 2.2688     | Outer membrane phospholipase A                                           |
| VpaChn25_0642                                    | 0.4375     | Phosphatidylglycerophosphatase A                                         |
| VpaChn25_0885                                    | 0.3795     | Surfactin synthetase                                                     |
| VpaChn25_2245                                    | 0.2175     | Glycerophosphodiester phosphodiesterase                                 |
| VpaChn25_2251                                    | 0.2013     | Glycerol-3-phosphate dehydrogenase                                       |

ΔVpacspD mutant

| Gene/Pathway                                      | Log2 Ratio | Function                                                                 |
|--------------------------------------------------|------------|--------------------------------------------------------------------------|
| VpaChn25A_0230                                   | 0.2003     | PTS system ascorbate-specific transporter subunit IIC                    |
| VpaChn25A_0231                                   | 0.1886     | Sugar phosphotransferase component II B                                  |
| VpaChn25A_0232                                   | 0.1253     | Phosphotransferase enzyme II, A component                                |
| VpaChn25A_0303                                   | 12.8135    | PTS system fructose-specific transporter subunit IIA                     |
| VpaChn25A_1196                                   | 0.3193     | Mannitol-specific PTS system enzyme II component                          |
| VpaChn25A_1309                                   | 3.4141     | PTS system fructose-specific transporter subunit II B                    |
| VpaChn25A_1312                                   | 4.9223     | PTS system fructose-specific transporter subunit IIABC                    |
| VpaChn25_0668                                    | 0.4181     | PTS system trehalose (maltose)-specific transporter subunits IIABC        |
### Table 2 Major altered metabolic pathways in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of *V. parahaemolyticus* CHN25 grown at the low temperature (Continued)

| Pathway | Gene | Fold Change |
|---------|------|-------------|
| Alanine, aspartate and glutamate metabolism | VpaChn25_0370 | 3.4733 | Adenylosuccinate synthase |
| | VpaChn25_0104 | 2.2225 | Glutamine synthetase |
| | VpaChn25_0345 | 0.3501 | Glucosamine-fructose-6-phosphate aminotransferase |
| | VpaChn25_0436 | 2.1782 | Glutamate synthase subunit beta |
| | VpaChn25_0437 | 2.0222 | Glutamate synthase subunit alpha |
| | VpaChn25_1375 | 3.4554 | Succinate-semialdehyde dehydrogenase |
| | VpaChn25_1376 | 3.0672 | 4-aminobutylate aminotransferase |
| | VpaChn25_2552 | 0.4804 | Glutaminase |
| | VpaChn25_2583 | 2.0986 | Aspartate carbamoyltransferase |
| | VpaChn25_2584 | 2.7419 | Aspartate carbamoyltransferase |
| | VpaChn25_2581 | 0.4744 | Aspartate ammonia-lyase |
| Arginine and proline metabolism | VpaChn25_1371 | 2.2205 | Aldehyde dehydrogenase |
| | VpaChn25_1372 | 2.779 | Oxidoreductase |
| | VpaChn25_1373 | 3.3592 | Carbon-nitrogen hydrolase |
| | VpaChn25_1374 | 3.206 | Hypothetical protein |
| | VpaChn25_2581 | 3.3917 | Arginine deiminase |
| | VpaChn25_2719 | 2.8035 | Succinylglutamic semialdehyde dehydrogenase |
| | VpaChn25_2720 | 3.9099 | Arginine/ornithine succinyltransferase |
| | VpaChn25_2721 | 2.1486 | Bifunctional N-succinylaminopimelate-aminotransferase/acyetylornithine transaminase protein |
| Valine, leucine and isoleucine degradation | VpaChn25A_0480 | 3.4643 | Acetyl-CoA acetyltransferase |
| | VpaChn25A_0555 | 2.023 | Acyl-CoA carboxylase alpha chain |
| | VpaChn25A_0557 | 2.0591 | Acyl-CoA carboxyltransferase beta chain |
| | VpaChn25A_0561 | 4.081 | Aldehyde dehydrogenase |
| | VpaChn25A_0565 | 3.2953 | 3-hydroxyisobutyrate dehydrogenase |
| | VpaChn25A_1038 | 0.4859 | Enol-CoA hydratase / isomerase |
| | VpaChn25_0020 | 2.6571 | Multifunctional fatty acid oxidation complex subunit alpha |
| | VpaChn25_2076 | 0.4816 | 3-ketoacyl-CoA thiolase |
| Propanoate metabolism | VpaChn25_1635 | 2.316 | PrpE protein |
| | VpaChn25_1638 | 4.6552 | Methylcitrate synthase |
| | VpaChn25_1639 | 3.654 | 2-methylisocitrate lyase |
| | VpaChn25_2433 | 3.0153 | Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase |
| | VpaChn25_2798 | 2.1035 | Acetyl-CoA synthetase |
| Nitrogen metabolism | VpaChn25A_0296 | 4.3124 | Oxidoreductase protein |
| | VpaChn25A_0917 | 3.7442 | Nitrite reductase large subunit |
| | VpaChn25A_1392 | 0.4447 | Carbonic anhydrase |
| | VpaChn25_1817 | 9.4056 | Cytochrome c552 |
| | VpaChn25_2448 | 0.2912 | Carbonic anhydrase |
| ΔVpacspAD mutant TCA | VpaChn25_0313 | 0.2603 | Malate dehydrogenase |
| | VpaChn25_0837 | 0.1445 | Type II citrate synthase |
| | VpaChn25_0838 | 0.2716 | Succinate dehydrogenase cytochrome b556 large membrane subunit |
Table 2 Major altered metabolic pathways in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of *V. parahaemolyticus* CHN25 grown at the low temperature (Continued)

| Gene                | Expression fold change | Function                                      |
|---------------------|------------------------|-----------------------------------------------|
| VpaChn25_0840       | 0.402                  | Succinate dehydrogenase flavoprotein subunit  |
| VpaChn25_0841       | 0.355                  | Succinate dehydrogenase iron-sulfur subunit   |
| VpaChn25_0843       | 0.3915                 | Dihydrolipoamide succinyltransferase          |
| VpaChn25_0844       | 0.3535                 | Succinyl-CoA synthetase subunit beta           |
| VpaChn25_0845       | 0.3009                 | Succinyl-CoA synthetase subunit alpha          |
| VpaChn25_1035       | 0.4921                 | Isocitrate dehydrogenase                      |
| VpaChn25_2762       | 0.478                  | Fumarate reductase flavoprotein subunit       |
| VpaChn25_2763       | 0.4836                 | Fumarate reductase iron-sulfur subunit        |
| VpaChn25_2764       | 0.4898                 | Fumarate reductase subunit C                  |
| VpaChn25_2765       | 0.3058                 | Fumarate reductase subunit D                  |
| **PTS**             |                        |                                               |
| VpaChn25A_0230      | 0.3036                 | PTS system ascorbate-specific transporter subunit IIC |
| VpaChn25A_0231      | 0.2665                 | Sugar phosphotransferase component II B       |
| VpaChn25A_0232      | 0.232                  | Phosphotransferase enzyme II, A component     |
| VpaChn25A_0303      | 8.0738                 | PTS system fructose-specific transporter subunit IIA |
| VpaChn25A_1312      | 8.7743                 | PTS system fructose-specific transporter subunit IIABC |
| VpaChn25A_0356      | 0.3818                 | PTS system mannitol-specific transporter subunit IIABC |
| VpaChn25A_0232      | 0.2665                 | Sugar phosphotransferase component II B       |
| VpaChn25A_0231      | 0.232                  | Phosphotransferase enzyme II, A component     |
| VpaChn25A_0232      | 0.2665                 | Sugar phosphotransferase component II B       |
| **Butanoate metabolism** |                      |                                               |
| VpaChn25A_0528      | 2.6111                 | Acetoacetyl-CoA synthetase                    |
| VpaChn25A_0560      | 2.8181                 | Acyl-CoA thiolase                             |
| **Fructose and mannose metabolism** |                |                                               |
| VpaChn25A_1313      | 5.1663                 | Mannose-6-phosphate isomerase                 |
| VpaChn25A_0355      | 0.3626                 | Mannitol-1-phosphate S-dehydrogenase          |
| **Pyruvate metabolism** |                      |                                               |
| VpaChn25A_0307      | 2.3933                 | Hypothetical protein                          |
| VpaChn25A_0367      | 0.321                  | Phosphoenolpyruvate synthase                  |
| VpaChn25A_0934      | 0.4136                 | D-lactate dehydrogenase                       |
| VpaChn25_0112       | 0.1659                 | Phosphoenolpyruvate carboxykinase             |
| VpaChn25_1693       | 2.2321                 | Aldehyde dehydrogenase                       |
| VpaChn25_1927       | 0.408                  | Pyruvate kinase II                           |
| **Oxidative phosphorylation** |                |                                               |
| VpaChn25A_0546      | 2.1237                 | Cytochrome BD2 subunit II                     |
| VpaChn25S_1519      | 0.4848                 | Cytochrome c oxidase subunit CcoP             |
| VpaChn25S_1521      | 0.4651                 | cbb3-type cytochrome c oxidase subunit II     |
| **Cysteine and methionine metabolism** |                |                                               |
| VpaChn25S_0576      | 0.293                  | Homocysteine synthase                         |
| VpaChn25S_0788      | 0.2948                 | Cysteine synthase A                           |
| VpaChn25S_0937      | 2.3269                 | Cysteine synthase/cystathionine beta-synthase family protein |
| VpaChn25S_1880      | 2.8826                 | S-methyltetrahydropteroylglutamate-homocysteine |
| VpaChn25S_2471      | 0.4433                 | S-ribosylhomocysteine                         |
| VpaChn25S_2646      | 2.2961                 | Aspartate kinase                              |
| **Arginine and proline metabolism** |                |                                               |
| VpaChn25A_1611      | 0.4145                 | Bifunctional proline dehydrogenase/pyrrol-5-carboxylate dehydrogenase |
| VpaChn25S_1332      | 0.1954                 | Hydroxyproline-2-epimerase                    |
| VpaChn25S_1335      | 0.3527                 | Ornithine cyclodeaminase                      |
| VpaChn25S_1544      | 0.3291                 | NAD-glutamate dehydrogenase                  |
| VpaChn25S_2719      | 0.3969                 | Succinylglutamic semialdehyde dehydrogenase   |
| VpaChn25S_2720      | 0.482                  | Arginine /ornithine succinyltransferase      |
Table 2 Major altered metabolic pathways in the ΔVpacspA, ΔVpacspD and ΔVpacspAD mutants of V. parahaemolyticus CHN25 grown at the low temperature (Continued)

| Pathway                                      | VpaChn25_2721 | VpaChn25_0345 | VpaChn25_0438 | VpaChn25_0439 | VpaChn25_1114 | VpaChn25_2022 | VpaChn25_2721 | VpaChn25_0345 | VpaChn25_0438 | VpaChn25_0439 | VpaChn25_1114 | VpaChn25_2022 |
|----------------------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Alanine, aspartate and glutamate metabolism  | 0.2687        | 0.2743        | 2.461         | 2.6963        | 0.407         | 0.4771        | 0.2687        | 0.2743        | 2.461         | 2.6963        | 0.407         | 0.4771        |

VpaChn25, chromosome 1; VpaChn25A, chromosome 2

during low-temperature survival by V. parahaemolyticus will provide important insights on this topic.

Major altered metabolic pathways in the ΔVpacspD mutant

Based on the GESA-KEGG analysis, the following six significantly altered metabolic pathways were identified in the ΔVpacspD mutant at 10 °C: the phosphotransferase system (PTS); alanine, aspartate and glutamate metabolism; arginine and proline metabolism; the propanoate and nitrogen metabolic pathways; valine, leucine and isoleucine degradation.

Consistent with its active low-temperature growth phenotype, several DEGs that were linked to PTS, to nitrogen, arginine and proline metabolism and to alanine, aspartate and glutamate metabolism were significantly up-regulated in the ΔVpacspD mutant. A major barrier to protein function at low temperatures is the inability to maintain sufficient flexibility so that it can increase its interactions with substrates to reduce its required activation energy [23]. In arginine and proline metabolism, all eight DEGs were up-regulated in the ΔVpacspD mutant. For example, expression of an arginine deiminase (VpaChn25_2581) and an arginine/ornithine succinyltransferase (VpaChn25_2720), which are required to convert L-arginine to L-citrulline and then to N2-succinyl-L-arginine, were up-regulated by 3.3917- and 3.9099-fold, respectively. Arginines are structurally stabilizing factors that contain side chains that form salt bridges and hydrogen bonds [24]. Our data indicated that a low-temperature decrease in L-arginine in the ΔVpacspD mutant may have promoted increased protein flexibility. Moreover, the abundance of proline residues is related to increased protein stability due to the rigidity of the N-Cα bond [23]. In this study, a decrease in proline resulted from up-regulated proline metabolism-associated enzymes may have also enhanced protein flexibility in the ΔVpacspD mutant. To our knowledge, these genes have not been previously linked to low-temperature survival.

Expression of a glutamine synthetase (VpaChn25_0104), which catalyses L-glutamate to L-glutamine, was up-regulated in the alanine, aspartate and glutamate metabolic pathways. However, the genes that encoded a glutaminase (VpaChn25_2552) and a glucosamine-fructose-6-phosphate aminotransferase (VpaChn25_0345), which convert L-glutamine to L-glutamate and then to D-glucosamine, showed opposite expression profiles, which suggested a decrease in L-glutamate accumulation in the ΔVpacspD mutant. This was also suppressed in the psychrophilic proteins of Vibrio salmonicida [25].

Unexpectedly, the comparative transcriptome analysis revealed very few genes that were up-regulated in ΔVpacspD but down-regulated in the ΔVpacspA mutant, indicating that these genes were specifically and negatively governed by VpaCspD. Additionally, in the ΔVpacspA mutant, VpacspD gene expression was increased (2.5073-fold) at the low temperature, which was validated by qRT-PCR analysis, but no significant change in VpacspA gene expression was observed in the ΔVpacspD mutant. The results indicated that VpaCspD was inhibited by VpaCspA at low temperatures, which was consistent with the growth phenotypes described above.

Major altered metabolic pathways in the ΔVpacspAD mutant

Similarly, the GESA-KEGG analysis revealed the following nine significantly changed metabolic pathways in the ΔVpacspAD mutant at 10 °C: TCA; PTS; butanoate metabolism; fructose and mannose metabolism; the pyruvate and cysteine and methionine metabolic pathways; arginine and proline metabolism; alanine, aspartate and glutamate metabolism; oxidative phosphorylation. Interestingly, these altered metabolic pathways were different from those that were induced in the ΔVpacspA mutant, although both mutants demonstrated the slower-growth phenotype at the low temperature. Most of the DEGs that were linked to TCA, oxidative phosphorylation, and pyruvate metabolism were inhibited in the ΔVpacspAD mutant, which may explain its slower growth at this low temperature. The down-regulated central metabolic pathways were also observed in other bacteria that were grown at a low temperature [26].
Similar to the \( \Delta VpacspD \) mutant, the alanine, aspartate and glutamate metabolic pathways, PTS, and the arginine and proline metabolic pathways were also significantly changed in the \( \Delta VpacspAD \) mutant. However, distinct expression patterns were detected in the two mutants. For example, in contrast to the \( \Delta VpacspD \) mutant, all seven DEGs that were involved in arginine and proline metabolism were down-regulated (0.482- to 0.1954-fold) in the \( \Delta VpacspAD \) mutant. Additionally, the phosphoenolpyruvate-dependent PTS is a major sugar transport multi-component system in bacteria, by which multiple sugars are transported into bacteria, concomitantly phosphorylated, and fed into glycolysis [27]. In this study, expression of the genes that encoded the cellobiose- and trehalose specific transporter subunits (VpaChn25_2566 and Chn25_0668) also displayed opposite patterns between the \( \Delta VpacspD \) and \( \Delta VpacspAD \) mutants. These results highlighted the antagonistic regulatory effects by VpaCspA and VpaCspD on low-temperature survival of \( V.\ parahaemolyticus \) CHN25.

In cysteine and methionine metabolism, a homocysteine synthase (VpaChn25_0676) and \( S \)-ribosylhomocysteine synthase (VpaChn25_2471), which are involved in converting O-acetyl-L-homoserine and \( S \)-ribosyl-L-homocysteine to L-homocysteine, were down-regulated (0.2930- and 0.4433-fold, respectively) in the \( \Delta VpacspAD \) mutant. However, a 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (VpaChn25_1880) that catalyses L-homocysteine to L-methionine was up-regulated (2.8826-fold). These results suggest the attenuation of L-homocysteine in the \( \Delta VpacspAD \) mutant, which may reduce interference by L-homocysteine with amino acid metabolic and translation processes at low temperatures [28].

**Differentially expressed regulators (DERs) that are involved in the low-temperature survival of the \( \Delta VpacspA, \Delta VpacspD \) and \( \Delta VpacspAD \) mutants**

The \( V.\ parahaemolyticus \) CHN25 genome contains approximately two hundred and seventy-two genes that encode putative transcriptional or response regulators, which represent approximately 5.8% of all protein-encoding genes in the bacterium. Changes in the expression of transcription factors, especially the master regulators, can modulate global regulatory networks that, in some cases, are essential for bacterial adaptation to changing environments [13]. In this study, the genome-level transcriptome data revealed thirty, thirty-three and thirty-six DERs in the \( \Delta VpacspA, \Delta VpacspD \) and \( \Delta VpacspAD \) mutants at 10 °C, respectively (see Additional file 3: Table S1). They globally or specifically regulate various cellular processes, including cold-temperature survival in bacteria, by regulating transcriptional or response regulators that are involved in DNA-binding, LysR-type transcriptional regulators, and GntR, AraC/XylS, ArsR, LuxR, and DeoR regulator families.

Of these regulators, several directly regulate gene expression in response to environmental signals in other bacteria. For example, a recombination regulator, RecX (VpaChn25_2483), which regulates DNA recombination and protects the cell from ionising radiation and UV-irradiation in \( E.\ coli \) [29], was notably down-regulated (0.2654-fold) in the \( \Delta VpacspA \) mutant; this indicates the positive regulation of RecX by VpaCspA in \( V.\ parahaemolyticus \) CHN25 at low temperatures. Interestingly, a transcriptional regulator, BetI (VpaChn25A_0568), was also inhibited in the \( \Delta VpacspA \) mutant (0.4784-fold), which negatively regulated the betI and betIBA genes that governed GB synthesis from choline in \( E.\ coli \) [30]. Moreover, two genes (VpaChn25_1793 and Chn25_1442), which encode the osmotically inducible betaine-choline-carnitine transporters (BCCTs) that mediate the acquisition of preformed GB [31], were also down-regulated in the \( \Delta VpacspA \) mutant. These data indicate that VpaCspA may stimulate an accumulation of cellular GB that adjusts the hydration level of the bacterial cell cytoplasm at low temperatures [32, 33]. Additionally, expression of an important transcriptional regulator, PdhR (VpaChn25_2454), which belongs to the GntR family of transcriptional regulators, was repressed (0.3122-fold) in the \( \Delta VpacspA \) mutant. PdhR regulates central metabolism by controlling transcription of the components that form the pyruvate dehydrogenase complex [34].

Among the DERs that were elicited in the \( \Delta VpacspD \) mutant, two regulators (a response regulator (VpaChn25_1251) and a MerR family transcriptional regulator (VpaChn25A_1361)) were up-regulated in the \( \Delta VpacspD \) mutant at low temperatures (2.891- and 2.8939-fold, respectively). The latter regulates gene transcription in response to different environmental signals, including signals from heavy metal ions, organic compounds, and oxidative stress [35]. Approximately 56.5% of the DERs in the \( \Delta VpacspD \) mutant were down-regulated, of which half were exclusively expressed in the \( \Delta VpacspD \) mutant (e.g., the two-component response regulator (VpaChn25A_1000) and sigma-E factor negative regulatory protein RseA (VpaChn25_2510)) [36].

Transcriptome data comparisons revealed mosaic DER expression profiles in the \( VpacspA \) mutant. Interestingly, three regulators of T3SS1 gene expression were inhibited in both \( \Delta VpacspD \) and \( \Delta VpacspAD \) cells at the low temperature. These included ExsA (VpaChn25_1689) and ExsE (VpaChn25_1692), which belonged to the ExsACDE regulatory cascade, and a T3SS1 regulator (VpaChn25_1651), which was indicative of positive regulation of T3SS1 by VpaCspD at low temperatures; this function was similar to that of VpaCspA. Likewise, expression of the UhpC regulator (VpaChn25A_0772),
a membrane-bound sensor for external glucose-6-phosphate in *E. coli* [37], was also decreased in the two mutants. UhpC was reported to negatively modulate a YE0480 gene in *Yersinia enterocolitica*, which encoded a homologue of the FhaC accessory protein; FhaC was strongly expressed at 10 °C but not at 37 °C in *Bordetella pertussis* [38].

Interestingly, three DERs were detected in all three mutants, and the other five were synchronously induced in both Δ*VpacspA* and Δ*VpacspD* cells, indicating either similar regulatory functions that were shared between *Vpa*CspA and *Vpa*CspD or *Vpa*CspA/D-independent regulation in *V. parahaemolyticus* CHN25 at low temperatures. The molecular responses of bacteria to external environmental signals are complex, but two-component signal transduction systems reportedly play important roles in low-temperature adaptation by several bacteria [39–41]. In this study, the expression of a cytosolic response regulator, CpxR (*VpaChn25A_0149*), which belongs to the two-component Cpx-envelope stress system [42], was repressed in the three mutants. The Cpx system responds to a broad range of environmental stimuli (e.g., pH, salt, metals, lipids and misfolded proteins) that cause perturbation of the envelope [43]. In this study, our data showed positive regulation of CpxR by both *Vpa*CspA and *Vpa*CspD, which may have protected envelope-bound proteins from low-temperature damage.

Taken together, our transcriptome data revealed a complex molecular regulatory network that was specifically, coordinately or antagonistically modulated by *Vpa*CspA and *Vpa*CspD during low-temperature adaptation by *V. parahaemolyticus*. Numerous regulators, which act as activators or repressors in response to multiple environmental stressors in bacteria, were also elicited in the three mutants. A future in-depth regulatory network analysis will improve our understanding of low-temperature adaptation mechanisms in *V. parahaemolyticus*.

### Possible low-temperature adaptation mechanisms that are mediated by *Vpa*CspA and *Vpa*CspD in *V. parahaemolyticus* CHN25

The most common strategy that has been adopted by bacteria to survive a low-temperature environment is the accumulation of compatible solutes (e.g., GB, choline, carnitine, and mannitol) by uptake or biosynthesis [11]. In this study, a similar low-temperature strategy by *V. parahaemolyticus* CHN25 was observed (Fig. 4). For example, seven genes that were associated with GB biosynthesis, BCCT and GB-binding ABC transporters were significantly inhibited in the Δ*VpacspA* mutant, which indicated that *Vpa*CspA likely stimulated cellular GB accumulation to adjust the hydration level of the cytoplasm and to protect the bacterium from low-temperature damage.

Interestingly, in this study, the glycerophospholipid metabolism-associated *glpDFKQ* genes were more strongly inhibited in Δ*VpacspAD* than in the Δ*VpacspA* or Δ*VpacspD* mutants, which indicated a coordinated low-temperature activation of the genes by *Vpa*CspA and *Vpa*CspD. For example, expression of the *glpF* gene (*VpaChn25_2248*), which encodes a glycerol uptake facilitator and functions in substrate equilibration between the extracellular and intracellular spaces [44], was down-regulated in Δ*VpacspA* (0.2794-fold), strongly
suppressed in VpacspD (0.0457-fold), and suppressed in VpacspAD (0.0337-fold). Similarly, the glpQ and glpD genes encode a glycerophosphodiester phosphodiesterase (VpaChn25_2245) and a glycerol-3-phosphate dehydrogenase (VpaChn25_2251), and they catalyse sn-glycerol-3-phosphocholine to choline and sn-glycerol-3-phosphate (G3P) and G3P to dihydroxyacetone phosphate (DHAP), respectively. Expression of the glpQ and glpD genes was also more strongly inhibited in VpacspAD (0.0787- and 0.0636-fold, respectively) than in VpacspA (0.2175- and 0.2013-fold, respectively) or VpacspD (0.1011- and 0.0302-fold, respectively), indicating a positively superposed regulation of the choline biosynthesis genes by VpaCspA and VpaCspD; this may have resulted in an increase in cellular compatible solutes to maintain cell membrane integrity at low temperatures. However, the decreased DHAP indirectly led to increased biofilm formation and contributed to several survival advantages under various environmental and energy insults in several other bacteria [45, 46]. Moreover, the glpK gene, which encodes a glyceral kinase (VpaChn25_2249) that catalyses glyceral to G3P, showed similar expression profiles in all three mutants, which probably resulted in attenuated cellular G3P accumulation at low temperatures. G3P has been reported to mediate catabolite repression through adenylate cyclase inhibition, which leads to decreases in 3’-5’-cyclic adenosine monophosphate (cAMP) and inactivation of the cAMP receptor protein (CRP); CRP is a global regulator that participates in sugar metabolism and plays an important role in cold adaptation by E. coli [44].

Protective roles for trehalose in response to low-temperature, heat and osmotic stressors have been reported, including prevention of the denaturation and aggregation of specific proteins, in vivo activity as a free radical scavenger, and stabilisation of cell membrane fluidity [47]. In this study, expression of the trehalose (maltose)-specific transporter subunit II BC components (VpaChn25_0668) was down-regulated in the VpacspA (0.2935-fold) and VpacspD (0.4181-fold) mutants, indicating the positive regulation of trehalose-specific transport by VpaCspA and VpaCspD to promote bacterial adaptation to a low-temperature environment. Nevertheless, the gene showed an opposite expression pattern in the VpacspAD mutant (3.948-fold), which implied unknown regulatory mechanisms in the VpacspAD mutant by which trehalose was transported.

Biofilm formation is related to bacterial survival in various environments. It has been reported that type IV pili (TFP) played an important role in the biofilm formation of V. parahaemolyticus [48]. In this study, the complete genome sequence analysis revealed a mannose-sensitive hemagglutinin gene cluster (mshHJKLMN) that was required for TFP formation in V. parahaemolyticus CHN25. Interestingly, the msh gene cluster was significantly down-regulated in the VpacspA mutant, which indicated a positive regulation of TFP by VpaCspA. The enhanced biofilm formation likely increased the persistence of V. parahaemolyticus in the aquatic environment by enhancing low-temperature colonisation of environmental surfaces [49].

Additionally, our transcriptome data also revealed several other molecular mechanisms that facilitated the low-temperature survival of V. parahaemolyticus CHN25 (Fig. 4). For example, VpaCspD negatively regulated arginine and proline metabolism, which likely resulted in increased cellular protein flexibility and stability so that efficient functionality could be maintained at the low temperature.

Conclusions
This study is the first to describe the complete 5,443,401-bp genome sequence (45.2% G + C) of V. parahaemolyticus CHN25 (serotype: O5:KUT), which consists of two circular chromosomes and three plasmids with 4,724 predicted protein-encoding genes. One dual-gene and two single-gene deletion mutants of the main CSPs, VpaCspA and VpaCspD, in V. parahaemolyticus CHN25 were successfully constructed. Our data demonstrated that VpaCspA was a primary CSP in the bacterium, whereas VpaCspD functioned as a growth inhibitor at 10 °C. Moreover, VpacspD gene expression was negatively regulated by VpaCspA. A global-level transcriptomic analysis revealed distinct gene expression profiles among the three mutants. Approximately 12.4% of the expressed genes in V. parahaemolyticus CHN25 were significantly altered in the VpacspA mutant at 10 °C, including those involved in amino acid degradation, ABC transporters, secretion systems, sulphur metabolism and glycerophospholipid metabolism. The low temperature elicited significant changes in expression of 10.0% of the genes from the VpacspD mutant, including genes that were involved in the phosphotransferase system and in nitrogen and amino acid metabolism. The following major altered metabolic pathways in the VpacspAD mutant radically differed from those in the single-gene mutants at 10 °C: TCA; PTS; butanoate metabolism; fructose and mannose metabolism; pyruvate, cysteine and methionine metabolism; arginine and proline metabolism; alanine, aspartate and glutamate metabolism; and oxidative phosphorylation. The transcriptome profile comparisons further revealed numerous DEGs that were shared among the three mutants and DEGs that were specifically, coordinately and or antagonistically mediated by VpaCspA and VpaCspD at a low temperature. V. parahaemolyticus appears to have evolved several molecular strategies with a complex gene regulation network for coping with cold-induced damage. The results from this study improve our
understanding of the genetic basis for low-temperature survival of the most common seafood-borne pathogens worldwide.

**Methods**

**Bacterial strains, plasmids and culture conditions**

*Escherichia coli* DH5α λpir (BEINUO Biotech (Shanghai) CO., LD. Shanghai, China) was used as a host strain for DNA cloning. The pDS132 plasmid [50] (a kind gift from Professor Dominique Schneider) was used as a suicide vector to construct the gene deletion mutants. *E. coli* β2155 λpir [51] (a kind gift from Professor Weicheng Bei) was used as a donor strain in the conjugation experiments. The pMMB207 plasmid [52] (Biovector Science Lab, Inc., Beijing, China) was used as an expression vector to construct the reverse mutants. *V. parahaemolyticus* CHN25 was isolated and characterised by Song et al. [15], Sun et al. [16] and He et al. [17] and modified for mutant construction by Sun et al. (unpublished). The bacterium was positive for the *tdh* gene but contained no toxic *tdh* and *trh* genes [15]. The *E. coli* strains were routinely incubated in Luria-Bertani (LB) medium (1% NaCl, pH 7.2) [53] at 37 °C, and the *V. parahaemolyticus* strains were grown in LB medium (3% NaCl, pH 8.5). The diaminopimelic acid (DAP) auxotrophic *E. coli* strains were grown in LB medium that contained 0.3 mM DAP. Cells that were grown on the mating plates were transferred onto LB plates (3% NaCl, pH 8.5) without chloramphenicol and incubated overnight; serial dilutions were spread onto the plates. Exconjugants with successful plate mating assays were performed using the heat-shock method [52]. Positive transformants were screened by colony PCR. The recombinant plasmid, pDS132 + ΔVpacspA, was subsequently prepared and transformed into DAP auxotroph *E. coli* β2155 competent cells. Plate mating assays were performed using *E. coli* β2155 (pDS132 + ΔVpacspA) as the donor and modified *V. parahaemolyticus* CHN25 as the recipient, as previously described [15, 50]. Mating was performed at 37 °C for 12 h on LB plates (1.5% NaCl, pH 7.2) that contained 0.3 mM DAP. Cells that were grown on the mating plates were transferred onto LB plates (3% NaCl, pH 8.5) that contained 5 μg/mL chloramphenicol, which enabled the optimal growth of *V. parahaemolyticus* CHN25.

**Genome sequencing, assembly, gene functional annotation, and comparative genome analysis**

Whole-genome sequencing of *V. parahaemolyticus* CHN25 was performed at the Chinese National Human Genome Centre (Shanghai, China) using the Genome Sequencer FLX (GS-FLX) system (Roche, Mannheim, Germany), which yielded 177,497 reads with a genome sequencing depth of 22-fold. The sequencing reads were assembled using the Newbler V2.3 software [54]. Gap closure was performed by primer walking and combinatorial PCR as previously described [55]. The final genome assembly was performed using the Phred-Phrap-Consed software packages [56]. Protein-coding genes were predicted by the EasyGene software [57], and functional assignments were inferred based on standalone Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) searches against the SWISS-PROT, GenBank, Clusters of Orthologous Groups of proteins (COGs) [58], and Pfam databases [59]. The rRNA genes were annotated using the FGenesB tool (http://softberry.com/), and tRNA genes were detected using the tRNAscan-SE programme [60]. IS elements were identified using the IS Finder [VC41]. Prophage-associated genes were predicted using Prophage finder (http://phast.wishartlab.com/). The clustered regularly interspaced short palindromic repeats (CRISPRs) were identified using the CRISPRFinder [61]. Potential virulence factors were detected using the Virulence Factor database (http://www.mgc.ac.cn/VFs/). Whole genome sequence alignments were performed using MUMmer3.2.3 software (http://www.tigr.org/software/mummer/) [62].

**Deletion of the VpacspA and VpacspD genes in *V. parahaemolyticus* CHN25**

Genomic DNA was prepared using the Biospin Bacteria DNA Extraction Kit (BIOER Technology, Hangzhou, China). Plasmid DNA was isolated using the TaKaRa MiniBEST Plasmid Purification Kit Version 3.0 (Japan TaKaRa BIO, Dalian Company, China). A markerless deletion mutant of the *VpacspA* gene was constructed by homologous recombination (Philippe et al. 2004). Based on the *VpacspA* gene sequence (213 bp, assigned to VpaChn25A_0413) of the *V. parahaemolyticus* CHN25 genome, primer pairs were designed (cspa-up-F/cspa-up-R and cspa-down-F/cspa-down-R) to target the upstream (528 bp) and downstream (513 bp) sequences, respectively, of the *VpacspA* gene (see Additional file 4: Table S2). The amplified PCR products were individually digested with corresponding restriction endonucleases (TaKaRa), purified, and ligated into the pDS132 XbaI and SacI cloning sites as previously described [50, 63]. The ligated DNA was transformed into *E. coli* DH5α λpir competent cells using the heat-shock method [52]. Positive transformants were screened by colony PCR. The recombinant plasmid, pDS132 + ΔVpacspA, was subsequently prepared and transformed into DAP auxotroph *E. coli* β2155 competent cells. Plate mating assays were performed using *E. coli* β2155 (pDS132 + ΔVpacspA) as the donor and modified *V. parahaemolyticus* CHN25 as the recipient, as previously described [15, 50]. Mating was performed at 37 °C for 12 h on LB plates (1.5% NaCl, pH 7.2) that contained 0.3 mM DAP. Cells that were grown on the mating plates were transferred onto LB plates (3% NaCl, pH 8.5) that contained 5 μg/mL chloramphenicol, which enabled the optimal growth of *V. parahaemolyticus* CHN25. Transconjugants were then inoculated into LB broth (3% NaCl, pH 8.5) without chloramphenicol and incubated overnight; serial dilutions were spread onto the selective LB agar plates, which were supplemented with 10% (wt/vol) sucrose. Exconjugants with successful double crossover deletions of the *VpacspA* gene were screened by colony PCR using the cspa-up-exF and cspa-down-exR primer pair and confirmed by DNA sequencing. The 219-bp VpacspD gene (VpaChn25_1036) deletion was carried out using the method described
above with the primer designs listed in Additional file 4: Table S2. Furthermore, the \(Vpacs\)D gene was also deleted from the \(\Delta Vpacs\)A mutant to create the dual-gene deleted \(\Delta VpacsAD\) mutant.

**Construction of the reverse mutants of the \(Vpacs\)A and \(Vpacs\)D genes in \(V.\ parahaemolyticus\) CHN25**

The \(Vpacs\)A gene was amplified from the genomic DNA of \(V.\ parahaemolyticus\) CHN25 by PCR with the cspA-com-F and -R primers (Additional file 4: Table S2). The PCR product was, digested with corresponding restriction endonucleases (TaKaRa), purified, and ligated into the expression vector pMMB207 at the SacI and XbaI cloning sites. The ligated DNA was transformed into \(E.\ coli\) DH5\(\alpha\) and positive transformants were screened as described above. The recombinant plasmid pMMB207 + \(Vpacs\)A was then prepared and transformed into the \(\Delta Vpacs\)A mutant by electroporation. The competent cells of the \(\Delta Vpacs\)A mutant was prepared according to the method Hamashima et al. \[64\] with minor modification. Briefly, the \(\Delta Vpacs\)A mutant was inoculated into 5 mL Mueller-Hinton Broth (MHB, 3% NaCl, pH7.0) (Beijing Land Bridge Technology Co., Beijing, China) and incubated at 37 °C. The overnight culture was then collected by centrifugation at 2,700 g for 4 min, 4°C, and the cell pellet was suspended and washed with cooled EP buffer (272 mM sucrose, 1 mM MgCl₂, 7 mM KH₂PO₄-Na₂HPO₄, pH 7.4) for three times. The washed cells were finally suspended with 8 mL cooled EP buffer, and 200-μL aliquots of the cells were stored at −80 °C. The electrotransformation was performed according to the method \[64\]. Briefly, 1 μg DNA of the plasmid pMMB207 + \(Vpacs\)A was added into 200 μL competent cells of the \(\Delta Vpacs\)A mutant, and incubated on ice for 15 min. The electrotransformation was performed at 25 ms, 1.5 kV, 100 Ω, 25 μF conditions using the Gene Pluser XCell (Bio-Rad, USA). The electrotransformation mixture was incubated at 37 °C overnight. The positive electrotransformant (\(\Delta Vpacs\)A-com mutant) were screened by colony PCR with primers cspA-com-FR and \(tlh\)-FR, and confirmed by DNA sequencing analysis. Similarly, the reverse mutant \(\Delta Vpacs\)D-com was also constructed with the cspD-com-F and -R primers (Table S2) using the same methods described above.

**Illumina RNA sequencing**

Bacterial cells were cultured at 10 °C until they reached their logarithmic growth phase and were collected by centrifugation. Total RNA was prepared using the RNeasy Protect Bacteria Mini Kit (QIAGEN Biotech Co. Ltd., Hilden, Germany) and QIAGEN RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocols. The DNA was removed from the samples with the RNase-Free DNase Set (QIAGEN). Three independently prepared RNA samples were used in each Illumina RNA-sequencing experiment. A wild-type strain that was cultured under identical conditions was used as the control.

The sequencing library construction and Illumina sequencing were conducted at Shanghai Biotechnology Co., Ltd. (Shanghai, China) according to the TrueSeq™RNA Sample Preparation Guide (Illumina, San Diego, CA, USA). The abundant 16S and 23S rRNA were depleted using the Ribo-Zero rRNA Removal Kit (EpiCentre Biotechnologies, Madison, WI, USA). First-strand cDNA was synthesised using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY, USA) with random hexamer primers. AMPure XP Beads (Beckman Coulter, Beverly, MA, USA) were used to isolate double-stranded cDNA that was synthesised with the Second Strand Master Mix (Invitrogen). The cDNA fragments underwent an end-repair process to convert the overhangs into blunt ends. A single “A” nucleotide was added to the 3’ ends of each blunt fragment to prevent them from ligating to one another during the adapter ligation reaction. The adapters (data not shown) with corresponding single “T” nucleotides on their 3’ ends were ligated, and PCR reactions were performed to enrich the DNA fragments that contained adapter molecules on both ends. Prepared sequencing libraries were quantified with a Qubit® 2.0 Fluorometer (Invitrogen) and validated using the Agilent High-Sensitivity DNA assay on the Agilent Bioanalyser 2100 system (Agilent Technologies, Santa Clara, CA, USA). Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TrueSeq PE Cluster Kit v3-cBot-HS (Illumina). Sequencing was conducted using an Illumina HiSeq 2500 platform, which generated 2 × 100-bp paired-end reads. High quality reads that passed the Illumina quality filters were used for sequence analyses.

**Data analysis**

Quality filtration of raw RNA-seq data were performed using the FASTX-Toolkit version 0.0.13 (http://hanon-lab.cshl.edu/fastx_toolkit/index.html) to remove the sequencing adapters, identical and low-quality reads, and ribosomal RNA sequences. The resulting clean reads were aligned to the \(V.\ parahaemolyticus\) CHN25 genome using the Bowtie2 version 2.0.5 software (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Gene transcriptional abundance of assembling transcripts was estimated according to the reads per kilobase of exon model per million mapped reads (RPKM) method described by Mortazavi et al. \[65\]. The fold-change was
determined for each gene by calculating the ratio of the RPKM values between the sample and the control. The genes with criteria fold-changes $\geq 2.0$ or $\leq 0.5$ and $p$-values $<0.05$ relative to the control were defined as DEGs. These DEGs were used for GSEA against the KEGG database (http://www.genome.jp/kegg/), and significantly changed metabolic pathways were identified when the enrichment test $p$-value fell below 0.05, which was validated by ebioService (http://sas.ebioservice.com/portal/root/molnet_shbh/index.jsp) (Shanghai Biotechnology Co., Ltd., Shanghai, China) [16].

Real-time reverse transcription PCR

Selected DEGs and significantly enriched genes in the transcriptome-sequencing analysis were validated by qRT-PCR. Oligonucleotide primers were designed using the Primer 5.0 software (http://www.premierbiosoft.com/) (see Additional file 5: Table S3) and synthesised by Shanghai Sangon Biological Engineering Technology Services Co., Ltd. (Shanghai, China). The conditions that were utilised to grow the cells for the qRT-PCR analysis were identical to those used for Illumina RNA sequencing. The qRT-PCR reactions were performed as previously described [16]. Primer specification was confirmed by agarose gel electrophoresis and melting curve analyses, and qRT-PCR amplification efficiencies (E) were analysed using the Applied Biosystems 7500 software programme (Applied Biosystems, Foster City, CA, USA). The relative expression ratio ($R$) of the target gene was calculated based on $E$ and the crossing point (CP) deviation of the sample versus the control, and it was expressed relative to the reference gene using the delta-delta threshold cycle ($C_{T}$) method as previously described by Pfaffl [66]. The 16S rRNA gene was used as the reference gene, as previously described [67]. All determinants were performed in triplicate.

Additional files

Additional file 1: Figure S1. Circular maps of the $V$. parahaemolyticus CHN25 chromosomes. (a) and (b) represent the larger and smaller chromosomes of $V$. parahaemolyticus CHN25, respectively. Each circle in the grey lines, except for the two innermost circles, illustrates specific features on the plus (outer region) and minus (inner region) strands. The lines and boxes in the three outermost circles are coloured according to the COG categories. The circles indicate the following from the outside inwards: first circle, predicted protein-coding genes; second circle, GC-skew (values above zero in red, values below zero in blue); third circle, GC content. (TIF 22070 kb)

Additional file 2: Figure S2. Circular maps of the $V$. parahaemolyticus CHN25 plasmids. (a)-(c): each circle in the grey lines, except for the two innermost circles, illustrates specific features on the plus (outer region) and minus (inner region) strands. Lines and boxes in the three outermost circles are coloured according to the COG categories. The circles indicate the following from the outside inwards: first circle, predicted protein-coding genes; second circle, GC-skew (values above zero in red, values below zero in blue); third circle, GC content. (TIF 15096 kb)

Additional file 3: Table S1. The DERs in the $\Delta VpacsPA$, $\Delta VpacsPD$ and $\Delta VpacsPD$ mutants of $V$. parahaemolyticus CHN25 at low temperatures. (DOC 96 kb)

Additional file 4: Table S2. Oligonucleotide primers for mutant construction used in this study. (DOC 48 kb)

Additional file 5: Table S3. Oligonucleotide primers used for the RT-PCR analysis in this study. (DOC 59 kb)

Abbreviations

ABC: ATP-binding cassette; BCCTs: Betaine-choline-carnitine transporters; BLAST: basic local alignment search tool; cAMP: 3’-5’-cyclic adenosine monophosphate; COGs: clusters of orthologous groups; CP: crossing point; CRISPRs: clustered regularly interspaced short palindromic repeats; CRP: cAMP receptor protein; CSPs: Cold shock proteins; DAP: Diaminopimelic acid; DEGs: Differentially expressed genes; DHA: Dihydroxyacetone phosphate; GB: Glycine betaine; G3P: Sn-glycerol-3-phosphate; GSEA: Gene set enrichment analysis; ISs: Insertion sequences; M: Restriction and modification; KEGG: Kyoto encyclopedia of genes and genomes; Pts: Phosphotransferase system; TCA: Tricarboxylic acid cycle; TFP: Type IV pili; TSS: Secretion system types

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Availability of data and materials

The annotated complete genome sequence of $V$. parahaemolyticus CHN25 has been deposited in the GenBank database under the accession numbers: CP010883, C25C1.sqn C25C1 CP010884, C25C2.sqn C25C2 CP010885, C25P1.sqn C25P1 CP010886, C25P2.sqn C25P2 CP010886 and C25P3.sqn C25P3 CP010887. The RNA-Seq data have been deposited in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE65998.

Authors’ contributions

CZ, BS, TL, HZ, FS, MY, WB, XP, QS, LX and LC participated in the design and discussion of the study. BS performed genome gap closure, gene annotation and comparative genome analysis. HZ directed the sequencing and comparative genome analysis. WG and WH assisted the genome sequencing and gene annotation. CZ carried out the major experiments in gene deletion mutant and transcriptomic analysis. YW constructed gene reverse mutants and analyzed their growth curves. CZ, BS, TL and LC analyzed the data. LC wrote the manuscript, and HZ, QS and LX revised it for important improvement. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Ethics approval and consent to participate

Not applicable.

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