CHAPTER 6

Expression Pattern of General Stress-Response and Virulence Genes in *A. hydrophila* under Various Stress Conditions
6.1. Introduction

_Aeromonas hydrophila_ possesses intrinsic characteristics such as resistance to antibiotics and chlorine, ability to survive at low temperature, ability to form biofilm on food surfaces, and production of virulence factors like aerolysin that contribute to its significance as a food-borne pathogen (Elhariry, 2011). A large array of genes involved in metabolic fitness and virulence make _A. hydrophila_ adaptive to diverse environmental conditions and hosts. This is why it is generally referred as “jack-of-all-trades” (Seshadri et al., 2006). The control of _A. hydrophila_ during production and storage of food products is one of the vital steps in public protection against its infection. In addition, the environmental conditions that _A. hydrophila_ may encounter in foods could influence its virulence potential. As the exposure of bacteria to various stresses often leads to increased virulence (Wesche et al., 2009), there is a need to understand the influence of different stresses on the expression of virulence and stress responsive genes in _A. hydrophila_.

Though there are few reports regarding the influence of environmental factors like temperature, salinity and pH on the survival and pathogenicity of _A. hydrophila_ (Pianetti et al., 2008; Vivekanandhan et al., 2003), a knowledge void exists regarding the impact of intervention strategies, food matrices, and processing environment–related conditions on the expression of general stress-response and virulence genes in _A. hydrophila_. Till date, there are no reports regarding the effect of stress on the expression of stress-response and virulence genes in _A. hydrophila_.

Most of the earlier studies on other food-borne pathogens have reported effect of various stresses on the stress responsive and virulence genes of the exponentially growing cells. These logarithmically growing cells are physiologically distinct from cells found in...
natural and production environments (Navarro Llorens et al., 2010). The cells in the food environment are generally in stressed conditions similar to that of stationary phase-like state. The microorganism encounters various stress conditions like nutrient replenishment, nutrient starvation, acid shock, alkaline shock, cold shock and heat shock during various stages of food production, processing and preservation, and in natural and host environments.

A number of studies have reported the expression of various housekeeping, stress response and virulence genes of different bacterial species under diverse stress conditions. These include the genes known to be involved in housekeeping functions, like the genes encoding primary sigma factor (rpoD) which keep essential genes and pathways operational, and glyceraldehyde-3-phosphate dehydrogenase (gapA), general stress regulatory genes rpoS and uspA and virulence genes. The aim of this study was to determine the impact of various food-related stress events (nutrient replenishment, nutrient starvation, pH 4 and pH 9, temperature 8 °C and 37 °C) on the expression of housekeeping (rpoD and gapA), general stress response (rpoS and uspA) and virulence (aer) genes in stationary phase A. hydrophila CECT 839T (reference and sequenced strain) and A. hydrophila A331 (food isolate) using quantitative real-time PCR (RT-qPCR). The expression of different genes in two different A. hydrophila strains, CECT 839T (milk isolate) and A331 (chicken isolate), under various stress conditions was compared.

6.2. Materials and methods

6.2.1. Bacterial strains and growth conditions

For the gene expression studies, two strains of A. hydrophila were used: the type strain CECT 839T (milk isolate; obtained from Dr. Valérie Leclère, Université des
Sciences et Technologies de Lille USTL, Villeneuve d'Ascq cedex, France) and other strain A331 (chicken isolate; identified as *A. hydrophila* by 16S rRNA and *rpoD* gene sequencing) (Chapter 2). Stocks of *A. hydrophila* strains were maintained in tryptic soy broth (TSB; Hi-Media, India) containing 20% glycerol at -80 °C. Cultures were also maintained at 4 °C on tryptic soy agar (TSA; Hi-Media, India) plates.

### 6.2.2. Stress treatment

The impact of six treatments (acid/ alkaline shock, cold/ heat stress, and nutrient deprivation/ replenishment) was assessed in stationary phase of both the *A. hydrophila* strains (CECT 839^T^ and A331) (Figure 6.1). In brief, a loopful of culture from TSA slant was grown for 18 h (30 °C, 150 rpm) in 25 ml TSB broth. For each biological replicate of both the strains, a 1:100 dilution in 100 ml of TSB in a 250-ml flask was incubated for 18 h at 30 °C, 150 rpm separately. Both the cultures were divided into seven groups of 5-ml aliquots each representing one control and six treatments (nutrition replenishment, nutrition deprivation, cold-shock, heat-shock, acid-shock and alkaline-shock). All the tubes were centrifuged (22 °C for 10 minutes at 12,000 x g) and resuspended in the spent media adjusted to different conditions.

Nutritionally replenished (NR) and deprived (ND) cells were resuspended in 5 ml of pre-tempered (30 °C) fresh TSB (pH 7.2) and 0.85% saline, respectively. For acid and alkaline shocks, cells were resuspended in 5 ml of spent TSB media adjusted to pH 4 using 1N HCl and pH 9 using 1N NaOH, respectively. For cold shock and heat stress, cells were resuspended in 5 ml of pre-tempered spent TSB media (temperature adjusted to 8 °C and 37 °C), respectively. All the cells exposed to different treatments were incubated for 30 minutes under defined conditions on shaker incubator at 150 rpm.
Control cells were resuspended in the original 5 ml of spent TSB maintained at 30 °C. Post-incubation, samples were taken for the lethality assessment and RNA isolation.

**Figure 6.1.** Flow chart for stress treatment and lethality assessment

**6.2.3. Lethality assessment**

For lethality assessment, 1 ml of culture was taken out at 0 min from control, and 30 min from the control and six treatment conditions, serially diluted, and pour-plated onto TSA. Enumeration of colony forming units (CFU) was performed after incubation at 30 °C for 24 h and comparisons were made between control and treatment samples.

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6.2.4. RNA isolation

Four ml of culture from control and treatment samples was transferred to microcentrifuge tubes and centrifuged at 10,000 x g for 5 min. To stabilize RNA transcripts, cell pellets were resuspended in 1 ml of RNA protect bacteria reagent (Qiagen, GmbH, Hilden, Germany). All samples were stored at -80 °C until RNA extraction was carried out. Total RNA was isolated using Triazol method (Tri-Reagent-RT, Molecular Research Center Inc., Cincinnati, Ohio, USA) according to the manufacturer’s instructions. Briefly, cells were lysed by suspending in 10 mM Tris-Cl-1 mM EDTA buffer (pH 8.0 containing lysozyme 2 mg/ml) and then thawed in liquid nitrogen. One ml Tri-reagent-RT was added to the sample and vortexed for 10 min. Fifty microlitres of bromoanisole was added to the mixture, incubated at room temperature for 10 min and centrifuged at 10,000 x g for 10 min at 4 °C. The upper aqueous phase was mixed with equal volume of isopropanol and the samples were centrifuged at 12,500 x g for 15 min at 4 °C. The RNA pellet was washed twice with 800 µl of chilled 70% ethanol, and then air-dried, and the RNA was resuspended in RNase-free milliQ water and stored at -80 °C until used. RNA quality and quantity was determined for individual preparations using non-denaturing agarose gel electrophoresis and by spectrophotometric analysis (Eppendorf, Germany) at OD_{260nm} and ratio of OD_{260:280nm} and OD_{260:230nm}. The RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions to remove any contaminating genomic DNA. Complete removal of genomic DNA from the DNase-treated samples was checked by performing real-time PCR.
6.2.5. cDNA preparation

About 1µg of DNase-treated total RNA was subjected to cDNA synthesis using DyNAmo™ cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the supplier’s directions. The reverse transcription reaction was performed sequentially for 10 min at 25 °C, for 60 min at 37 °C, and for 5 min at 85 °C. All the cDNA preparations were stored at -20 °C.

6.2.6. Primer design

Complete sequences for the genes of interest were obtained from the PubMed database (accession number NC_008570.1). The gene-specific primers for the housekeeping, general stress response and virulence genes (Table 6.1) were designed using integrated DNA technologies Primer Quest software (www.idtdna.com/site). The amplicon sizes ranged between 110 to 190 bp. Melting temperatures of the primers were designed for 60 °C, with a melting temperature difference of less than 2 °C for each primer pair. Primers were obtained from Metabion International (Germany) (Table 6.2).

Table 6.1. Selected A. hydrophila genes related to ancillary housekeeping functions, stress response and virulence for quantitative real-time PCR analysis

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping genes</td>
<td>gapA, rpoD, 16S rRNA</td>
</tr>
<tr>
<td>General stress-response &amp; global regulators</td>
<td>rpoS, uspA</td>
</tr>
<tr>
<td>Virulence genes</td>
<td>aer</td>
</tr>
</tbody>
</table>
Table 6.2. Primers for quantitative real-time PCR analysis of various housekeeping, stress-response and virulence genes in *A. hydrophila* CECT 839^T^ and A331 following various food-environment related stresses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA Forward</td>
<td>TGGCCTTGACATGTCTGGAATCCT</td>
</tr>
<tr>
<td>16S rRNA Reverse</td>
<td>ACCATTACGTGCTGGCAACAAAGG</td>
</tr>
<tr>
<td>gapA Forward</td>
<td>TGCTCAAGGTGATGCAGGACAAGT</td>
</tr>
<tr>
<td>gapA Reverse</td>
<td>AGTTGTGATGAGGTTCTGGTCGT</td>
</tr>
<tr>
<td>rpoD Forward</td>
<td>AACAAGCTCAACCGTATCTCCCGT</td>
</tr>
<tr>
<td>rpoD Reverse</td>
<td>TGGATATGGGCTCTTTGGCGATCT</td>
</tr>
<tr>
<td>rpoS Forward</td>
<td>TCCGATGAACTGATGGCACACCTGA</td>
</tr>
<tr>
<td>rpoS Reverse</td>
<td>GCAAAATTGGACHTCGATCATGCGCT</td>
</tr>
<tr>
<td>uspA Forward</td>
<td>CGCTCAAAATCACCACAGATCAAC</td>
</tr>
<tr>
<td>uspA Reverse</td>
<td>TATCGACATCGACAACGTGAAG</td>
</tr>
<tr>
<td>aerA Forward</td>
<td>TTGCATAAACCTGGACCCCTGAC</td>
</tr>
<tr>
<td>aerA Reverse</td>
<td>TCTTGGACCAGTTGATGGCAGTAT</td>
</tr>
</tbody>
</table>

6.2.7. Quantitative real-time PCR (RT-qPCR) analysis

The SYBR green RT-qPCR assay was performed using amplification master cycler ep *realplex* (Eppendorf, Germany) and DyNAmo Flash SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) at 94 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s and extension at 72 °C for 20 s. Two microlitres of the template was amplified in a 20 µl reaction volume containing primers at a final concentration of 0.5 µM, 10 µl of 2X DyNAmo Flash SYBR Green
Master Mix, and water. Following amplification, threshold cycle (C\textsubscript{T}) values for each target genes over different stress conditions for each A. hydrophila strain were determined and a melting curve analysis of PCR products was performed to ensure the specificity of the PCR. The relative expression ratios of the target genes over different stress conditions for each A. hydrophila strain were calculated according to the Pfaffl method using 16S rRNA levels as the reference (Pfaffl, 2001). As 16S rRNA gene was not affected by various stress treatments, it was selected as the calibrating gene. All treatments were compared to control cDNA derived from stationary phase cells. The relative expression ratios were calculated using the REST-MCS version 2 software (http://www.gene-quantification.de/rest-mcs.html). The RT-qPCR was performed with three biological and three technical replicates. Only genes with a relative signal log\textsubscript{2} ratio value above 1.0 or below −1.0 were considered to be significant.

6.3. Results and Discussion

6.3.1. Effect of stress on survival of A. hydrophila cells

The effect of different stresses on the survival of A. hydrophila CECT 839\textsuperscript{T} and A331 was studied. Cell counts of the control CECT 839\textsuperscript{T} and A331 strains were 3.1 x 10\textsuperscript{9} and 4.2 x 10\textsuperscript{9} CFU/ml, respectively at 0 min. No significant differences (P > 0.05) in CFUs were observed between control and all treatment cells for both the strains after 30 min (Fig. 6.2). Results indicate that the viability of both A. hydrophila CECT 839\textsuperscript{T} and A331 cells were not significantly affected by the treatment conditions and the cells were under sub-lethal stress. Similar observations were reported in CFUs of control and treatment (nutritional replenished, acid-shocked and cold-shocked) cells of E. coli O157:H7 (Allen et al., 2008).
6.3.2. Gene expression after nutrient replenishment for 30 min

The housekeeping genes (rpoD and gapA) were significantly up-regulated by 6.2- and 1.3-fold in A. hydrophila CECT 839T and 5.5- and 1.2-fold in A. hydrophila A331, respectively on resuspension of 18 h old A. hydrophila cells into fresh TSB (Fig. 6.3). RpoD (σ70) is a 70 kDa "housekeeping" or primary sigma factor that recognizes promoters of the growth-related and housekeeping genes expressed in the exponential phase of bacterial growth and transcribes most genes in the growing cells. Glyceraldehyde-3-phosphate dehydrogenase (gapA) is a housekeeping gene that catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate during glycolysis. The increased expressions of these genes show that the cells are actively growing after nutrient replenishment. A. hydrophila strains may be experiencing the "feast" aspect of bacterial life on resuspension of the stationary phase cells into fresh TSB. Similar trend was
observed in *E. coli* O157:H7 following nutrient replenishment with significant up-regulation of 52% genes, including genes involved in DNA replication and maintenance (*dnaE, hupB, mutH, mutL* and *mutS*) and housekeeping genes (*dnaA, rpoA, tufA, gapA* and *narH*) involved in protein synthesis and carbon and amino acid metabolism (Allen *et al.*, 2008).

![Figure 6.3](image)

**Figure 6.3.** Gene expression analysis of *rpoS, aer, uspA, rpoD* and *gapA* genes in *A. hydrophila* CECT 839T and A331 in response to nutrient replenishment for 30 min

RpoS (σ^S^), a 38 kDa alternative sigma factor, widely present in many gram-negative bacteria, is a general stress response regulator and regulates different stationary phase and stress response genes. Significant induction of *rpoS* gene was observed in CECT 839T (1.7-fold); whereas, it was marginally expressed in A331 (0.7-fold) (Fig. 6.3) indicating that different strains of *A. hydrophila* may have different abilities to cope with the stress. Though it is well documented that *rpoS* plays an important role in stationary
phase, its role in exponential phase cells has been shown lately (Dong et al., 2008). RpoS is likely to play a fine-tuning regulatory role in early exponential phase to adjust gene expression in the preparation for any potential stress, distinct from its role of active protection for cell survival in stationary phase cells (Battesti et al., 2011). It is possible that stationary phase *A. hydrophila* cells may undergo considerable physiological reprogramming dominated initially by stress response induction to adapt to a nutrient rich environment.

The expression of *uspA* gene in CECT 839T and A331 was significantly up-regulated by 5.5- and 2.6-fold, respectively on resuspension of 18 h old *A. hydrophila* cells into fresh TSB (Fig. 6.3). UspA, a member of universal stress proteins (Usps), is reported to be highly expressed in response to a large number of stresses like heat, substrate starvation for glucose or phosphate, upon entry to stationary phase in rich medium, exposure to antimicrobial agents and oxidative stress (Kvint et al., 2003). Allen et al. (2008) have also observed induction of *uspA* gene by 2.8-fold on resuspension of stationary phase *E. coli* O157:H7 cells into fresh TSB.

Aerolysin (*aer*) is a major virulence factor of *Aeromonas* and possesses both haemolytic and enterotoxic activity. The *aer* gene was up-regulated by 4.0- and 0.7-fold in *A. hydrophila* CECT 839T and A331, respectively (Fig. 6.3) demonstrating that the ability to produce aerolysin may vary in different strains of *A. hydrophila*. Expression of *aer* gene was significantly induced in *A. hydrophila* CECT 839T (milk isolate) as compared to A331 (chicken isolate) following nutrient replenishment. It is possible that media stimulation resulting from fresh TSB or the induced physiological switch from stationary phase to exponential growth may contribute to induction of virulence genes. Similar significant inductions of virulence (*stx1* and *hly*) genes have been reported in *E. coli* O157:H7.
following media stimulation (Allen et al., 2008). Up-regulation of numerous genes associated with attachment, virulence, oxidative stress, antimicrobial resistance and DNA repair was observed in *E. coli* O157:H7 on exposure to carbohydrates rich lysates of lettuce leaves (Kyle et al., 2010).

### 6.3.3. Gene expression after nutrient deprivation for 30 min

Significant up-regulation of *gapA* gene by 1.9- and 1.6-fold was observed in *A. hydrophila* CECT 839T and A331, respectively. The *rpoD* gene was significantly up-regulated by 2.0- fold in CECT 839T; whereas, it was slightly induced in A331 (0.8-fold) (Fig. 6.4). There are no reports on the expression of these genes under nutrient deprivation in *Aeromonas* species. However, our results contradict the observation of Allen et al. (2010), who showed attenuation of house-keeping genes (*dnaA*, *rpoA*, *tufA*) in *E. coli* O157:H7 cells subjected to nutrient deprivation.

Significant up-regulation of *rpoS* gene by 2.3- and 1.5-fold was observed in CECT 839T and A331, respectively on resuspension of 18 h cells into normal saline (Fig. 6.4). RpoS controls up to 10% of the *E. coli* genes and prepare the cell for survival in stress conditions (Weber et al., 2005). Induction of *rpoS* gene by 1.2-fold was observed in microarray analysis of *E. coli* O157:H7 cells subjected to nutrient deprivation (Allen et al., 2010). A mild induction of *rpoS* gene may have a significant impact on survival of the microorganism in the food chain (Vasudevan and Venkitanarayanan, 2006).

The *uspA* gene was up-regulated by 3.3- and 2.2-fold in *A. hydrophila* CECT 839T and A331, respectively (Fig. 6.4). Synthesis of UspA (*uspA*) and its paralogs: UspC (*yecG*), UspD (*yiiT*), UspE (*ydaA*), and UspG (*ybdQ*) is reported to be induced by
starvation for glucose or phosphate and upon entry to stationary phase in rich medium (Siegele, 2005).

![Figure 6.4](image)

**Figure 6.4.** Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839<sup>T</sup> and A331 in response to nutrient deprivation for 30 min.

The expression of *aer* gene was significantly induced in CECT 839<sup>T</sup> (1.9-fold), whereas, it was marginally expressed in A331 (0.4-fold) after nutrient deprivation for 30 min (Fig. 6.4). Strain-dependent variation in the expression of aerolysin was observed in *A. hydrophila* following nutrient deprivation. There are no reports of expression of aerolysin under nutrient deprivation in *Aeromonas* species. However, induction of Shiga toxin (*stx*) due to starvation has been reported in *E. coli* O157:H7 (Leenanon et al., 2003). Allen *et al.* (2010) have reported the induction of virulence genes (*hlyB*, *katP* and *stx2b*) in *E. coli* O157:H7 following nutrient deprivation.
Overall, the expression of $rpoD$, $uspA$ and $aer$ genes was more in both the A. *hydrophila* strains under nutrient replenishment condition as compared to nutrient deprivation; whereas higher expressions of $gapA$ and $rpoS$ genes were observed in nutrient deprivation as compared to nutrient replenishment.

6.3.4. Gene expression after cold shock (8 °C) for 30 min

*A. hydrophila* may be exposed to cold shock and long-term refrigeration during prolonged storage of foods. Thus, the impact of cold shock on *A. hydrophila* stress and virulence physiology was studied. Significant induction of housekeeping ($rpoD$ and $gapA$) genes was observed in *A. hydrophila* CECT 839$^T$ and A331 cells following cold-shock (8 °C) for 30 min (Fig. 6.5). In only cold stress, the induction of $rpoD$ gene was more in A331 as compared to CECT 839$^T$; whereas, in all other studied stresses higher induction of $rpoD$ gene was observed in CECT 839$^T$. Allen *et al.* (2008) observed induction of housekeeping gene $dnaA$ in *E. coli* O157:H7 cells subjected to cold shock; whereas, $gapA$ and $tufA$ genes were attenuated.

The $rpoS$ gene was significantly induced in *A. hydrophila* CECT 839$^T$ (1.9-fold); whereas, it was marginally induced in A331 (0.9-fold) (Fig. 6.5). The secondary structure of the $rpoS$ transcript may be altered due to the decreased temperature and thus resulting in a stabilized molecule that leads to increased levels of $\sigma^S$ (Hengge-Aronis, 2002). Our results agree with Allen *et al.* (2008) who observed up-regulation of $rpoS$ gene by 6.2-fold in *E. coli* O157:H7 cells following cold shock (7.5 °C) for 15 min. The $uspA$ gene was insignificantly induced in both *A. hydrophila* CECT 839$^T$ (0.9-fold) and A331 (0.5-fold) following cold shock. Nystrom and Neidhardt (1993) have also reported that synthesis of UspA is not induced in *E. coli* due to cold shock.
In the present study, no significant expression of \textit{aer} gene was observed in \textit{A. hydrophila} CECT 839\(^T\) (0.6-fold) and A331 (0.2-fold) under cold shock at 8 °C for 30 min (Fig. 6.5). This is the first study regarding the expression of various genes following cold shock in \textit{Aeromonas} species. Inter-strain variability on virulence (\textit{aer}) and stress response (\textit{rpoS} and \textit{uspA}) gene transcription levels was observed indicating that the virulence and stress gene transcriptions of the two strains are not equally affected by the cold shock. Similar inter-strain variation in the expression of virulence and stress response genes has been reported in \textit{C. jejuni} following cold shock (Poli \textit{et al.}, 2012).
6.3.5. Gene expression after heat shock (37 °C) for 30 min

*A. hydrophila* is exposed to variations in temperature during its survival in food processing conditions and when inside the human body. Many virulence genes in the food-borne pathogens are regulated by the shift in temperature. Hence, the gene expression profile of housekeeping, stress response and virulence genes in *A. hydrophila* CECT 839\(^T\) and A331 cells subjected to heat shock (37 °C) for 30 min was studied. The housekeeping (*rpoD* and *gapA*) genes were marginally induced in *A. hydrophila* CECT 839\(^T\) and A331 with exception of 1.1-fold induction of *gapA* gene in CECT 839\(^T\) (Fig. 6.6). Significant induction of various genes involved in the uptake and utilization of amino acids, carbohydrates, and iron was observed in *E. coli* K-12 at human body temperature (37 °C) compared to 23 °C (White-Ziegler *et al.*, 2007).

![Gene expression analysis](image)

**Figure 6.6.** Gene expression analysis of *rpoS, aer, uspA, rpoD* and *gapA* genes in *A. hydrophila* CECT 839\(^T\) and A331 in response to heat stress (37 °C) for 30 min
No significant induction of \textit{rpoS} and \textit{uspA} genes in \textit{A. hydrophila} CECT 839$^T$ and A331 cells was observed in response to heat shock (37 $^\circ$C) for 30 min (Fig. 6.6). However, significant up-regulation of \textit{uspA} gene was observed in \textit{Salmonella} Typhimurium following temperature upshift from 30 $^\circ$C to 37 $^\circ$C indicating that growth at and transition to higher temperatures may be stressful to the organism (Liu \textit{et al.}, 2007). Significant induction of \textit{aer} gene in CECT 839$^T$ (1.3-fold) was observed following heat shock (37 $^\circ$C) for 30 min; whereas, it was marginally induced in A331 (0.2-fold) (Fig. 6.6). Strain dependent variation in the expression of virulence and stress response genes was observed in \textit{A. hydrophila} strains following temperature up-shift.

\textbf{6.3.6. Gene expression after acid shock (pH 4) for 30 min}

Food-borne pathogens encounter organic and inorganic acids in foods or in the gastrointestinal tract and cells of the host. The housekeeping genes (\textit{gapA} and \textit{rpoD}) were marginally induced in \textit{A. hydrophila} CECT 839$^T$ and A331 with exception of 1.3-fold induction of \textit{gapA} gene in CECT 839$^T$ (Fig. 6.7) indicating that minimum level of RNA transcription and carbon metabolism is occurring in \textit{A. hydrophila} strains following acid shock.

No significant induction of \textit{rpoS} gene was observed in \textit{A. hydrophila} CECT 839$^T$ and A331 following acid shock (pH 4) for 30 min (Fig. 6.7). Lee \textit{et al.} (1995) reported the role of RpoS in survival of virulent \textit{Salmonella} Typhimurium in acid stress. Marginal induction of \textit{rpoS} gene (0.6-fold) was observed in \textit{Shewanella oneidensis} following acid shock (Leaphart \textit{et al.}, 2006).
Figure 6.7. Gene expression analysis of $rpoS$, $aer$, $uspA$, $rpoD$ and $gapA$ genes in A. hydrophila CECT 839\textsuperscript{T} and A331 in response to acid stress (pH 4) for 30 min.

The acid-resistance response is complex and involves many regulators. Moreover, RpoS-dependent genes have been shown to play a major role in acid resistance (Battesti et al., 2011). Though the pathway of induction of RpoS in cells exposed to low pH is not fully understood, stabilization of RpoS by the two-component PhoPQ system may play an important role in its induction (Bougdour et al., 2008). Induction of 48 acid shock proteins, including inter-lapping proteins RpoS, Fur, PhoP and OmpR, were observed in S. Typhimurium stationary phase cells during acid adaptation and shock. These proteins, which are under the control of multiple, overlapping regulatory systems, protect the cell against acid and perhaps other environmental stresses (Wesche et al., 2009). Significant induction of $uspA$ gene was observed in A. hydrophila CECT 839\textsuperscript{T} (1.4-fold) and A331.
(2.4-fold) in response to acid shock (pH 4) for 30 min (Fig. 6.7). The synthesis of UspA protein is induced in *E. coli* in response to growth inhibition caused by various factors, including acidic shock (Liu *et al.*, 2007). As compared to other stresses, only in acid stress, the induction of *uspA* gene was more in A331 than CECT 839<sup>T</sup>. A protein belonging to universal stress protein family was induced by 0.2-fold in *S. oneidensis* subjected to acid shock (pH 4) for 30 min (Leaphart *et al.*, 2006).

Insignificant induction of *aer* gene was observed in *A. hydrophila* CECT 839<sup>T</sup> and A331 following acid shock (pH 4) for 30 min (Fig. 6.7). House *et al.* (2009) have also reported no significant change in the Shiga toxin production (both secreted and periplasmic extracts) in *E. coli* O157:H7 following acute acid stress treatment.

### 6.3.7. Gene expression after alkaline shock (pH 9) for 30 min

Food-borne pathogens are exposed to alkaline conditions in natural environments, human body (pancreatic duct just below the pylorus) and food industry. Many detergents and chemical sanitizers, such as caustic soda (NaOH) and ammonium compounds, are used to clean food processing facilities and food contact surfaces. Studies of bacterial response to alkaline pH have been less extensive to date compared with those of acidic pH.

The expression of housekeeping genes (*rpoD* and *gapA*) was down-regulated by 0.5 to 4.2 fold in *A. hydrophila* CECT 839<sup>T</sup> and A331 subjected to alkaline shock (pH 9) for 30 min (Fig. 6.8). Marginal attenuation (-0.752) of *gapA* gene has been reported in *E. coli* K-12 following alkaline stress (pH 8.7) (Maurer *et al.*, 2005). Global regulators and stress response genes (*rpoS* and *uspA*) were insignificantly attenuated in CECT 839<sup>T</sup> and A331 following alkaline stress (Fig. 6.8) indicating insignificant/no role of these genes in adaptation to alkaline stress in *A. hydrophila*.
Figure 6.8. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T^ and A331 in response to alkaline stress (pH 9) for 30 min.

There are no reports on the expression of these studied genes under alkaline stress in *Aeromonas* species. Leaphart *et al.* (2006) have also reported insignificant down-regulation of *rpoS* and universal stress protein in *S. oneidensis* following alkaline stress for 30 min. Similar to *E. coli*, *S. oneidensis* appears to modulate its transcriptome in response to an alkaline environment primarily by affecting the expression of genes involved in central intermediary metabolism (particularly assimilative sulfur metabolism), sulfate transport, and Na^+^/H^+^ antiporter systems (Leaphart *et al.*, 2006). Attenuation of *aer* gene by 1.3- and 0.6-fold was observed in *A. hydrophila* CECT 839^T^ and A331, respectively (Fig. 6.8).
Overall, the trend of expression of all the studied genes under all the stress conditions in both *A. hydrophila* 839\(^T\) and A331 strains remained the same. However, inter-strain variability in the virulence and stress response gene transcription levels was observed among *A. hydrophila* 839\(^T\) and A331 strains. More pronounced up-regulation or down-regulation for most of the genes was observed in *A. hydrophila* 839\(^T\) as compared to A331 for all the stress conditions. These variations indicate the physiological differences among these strains in encountering various stresses. These observations are consistent with previous report where strain-dependent differences in general stress-response and virulence gene expression was observed in *Enterococcus faecalis* (Lenz et al., 2010).

### 6.4. Conclusions

Quantitative real time-PCR analysis was successfully used to examine the effect of food-environment related stresses on the expression of housekeeping, stress-response regulator and aerolysin genes in *A. hydrophila* CECT 839\(^T\) and A331. Strain-dependent differences in the level of expression of different genes under various stress conditions were observed among *A. hydrophila* CECT 839\(^T\) and A331 strains indicating genetic heterogeneity within the species. The study also indicated that different strains of *A. hydrophila* have different abilities to cope with stress and induce aerolysin production. Overall, nutrient replenishment and deprivation significantly induced the expression of housekeeping (*rpoD* and *gapA*), general stress regulators (*uspA* and *rpoS*) and virulence gene (*aer*) indicating their importance in regulating the survival and virulence of *A. hydrophila* under these stress conditions. RpoS was significantly induced in cold shock indicating its role in cold shock adaptation. Significant induction of *aer* gene in *A. hydrophila* CECT 839\(^T\) during an increase in temperature (37 °C) signify that this strain
may be potentially pathogenic to humans. Acid stress significantly induced the expression of *uspA* gene, which may regulate various genes required for its survival. However, alkaline stress showed significant down-regulation of all the studied genes. Induction of different stress regulators under various stress conditions suggests that initial stress events may prepare a cell for surviving and sustaining any subsequent stress in a better manner. To the best of our knowledge, this is the first study to investigate the gene expression of virulence and stress response genes in *A. hydrophila* strains as affected by food environmental parameters. However, further studies are required to better understand these adaptive responses in the context of pathogen persistence and virulence in the food.