Effects of stressors on the expression of virulence factors and cell survival in Clostridium perfringens

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Abstract

Clostridium perfringens is a normal inhabitant of the gastro-intestinal tract of humans and animals and is also commonly found in soil. *C. perfringens* causes gas gangrene and food-borne illnesses, and is characterized by its ability to produce numerous extracellular toxins and enzymes, including α-toxin (*plc*), θ-toxin (*pfoA*), κ-toxin (*colA*), α-clostripain, and others which are responsible for its pathogenicity. Since *C. perfringens* is thought to be constantly exposed to various stressful conditions when in the host and even more so in the extra-host environment, this study examined the effect of various environmental stressors, such as oxidative stressors (aeration and H_2O_2), extreme temperatures, acid and alkaline conditions and bile acid, on the expression of major virulence-related genes by using Northern blot analysis. We found that expression of the *plc* gene (encoding the key virulence factor, alpha-toxin) was greatly increased at 55℃ even though the cells were in a completely non-culturable state at that temperature. The *pfoA* gene (encoding a virulence factor, perfringolysin O) was up-regulated by 5- and 15-min oxidative stress by aeration. These results showed that *C. perfringens* is capable of sensing, responding to, and adapting to various kinds of stressors, and that *C. perfringens* modulates its virulence gene expression in response to various stressful environmental changes. This work may help to clarify the behavior of *C. perfringens* during infection in humans and animals, which are highly stressful environments for the bacterium. Understanding the mechanisms of survival by further studying the global response to stress conditions would be an effective way to develop methods to control life-threatening infections of *C. perfringens*.

Key words *Clostridium perfringens*, stress response, virulence, transcription

Introduction

Clostridium perfringens is a Gram-positive anaerobic spore-forming bacterium that is widely distributed in nature [1]. It is commonly found in the environment (in soil and sewage) and in the intestines of animals and humans as a member of the normal flora [1)2)]. *C. perfringens* is known to be the most prolific toxin-producing member of the clostridial group. Toxins produced by *C. perfringens* are responsible for a wide variety of lethal human and veterinary diseases, such as clostridial myonecrosis (known as gas gangrene), food poisoning, and necrotic enteritis [3)].

Gas gangrene is the most important disease caused by this organism. The pathogenesis of the disease is as follows: *C. perfringens* enters the body through an injury or surgical wound and grows rapidly in the host tissues, simultaneously producing toxins and enzymes that cause extensive necrosis,

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gas production, and massive destruction of the host tissues [4]. Death may occur due to systemic toxemia and shock. These toxins include the alpha-toxin (phospholipase C, which is responsible for membrane damage by hydrolyzing phospholipids and formation of gangrene lesion), theta-toxin (or perfringolysin O, a member of the cholesterol-dependent cytolysin family that causes pore-forming and complete hemolysis of red blood cells), kappa-toxin (or collagenase, which hydrolyzes collagen molecules and results in loss of tissue integrity), and alpha-clostripian (cysteine protease, which degrades host proteins into amino acids that would aid bacterial survival in the host), which are encoded by the *plc*, *pfoA*, *colA*, and *ccp* genes, respectively [5].

C. perfringens are commonly exposed to potential stressors (not only in the host but also in their natural environment and in food processing and cooking) such as oxidative agents, extreme temperatures, pH changes (acid or alkaline), various osmotic pressures, and enteric bile acids. These environmental factors would affect bacterial growth, survival, and toxin production, since the ability of bacterium to sense and respond quickly to such stressors is essential for survival [6].

Bacteria have evolved various defense mechanisms to face the challenges of changing environments and to survive under stressors [7]. One of these mechanisms is the regulation of protein synthesis, which is important for enabling bacteria to respond and adapt to a rapidly changing environment. Several studies have reported that various stressors induced virulence genes in multiple pathogens [7-9].

We considered that it would be of interest to identify the environmental conditions affecting virulence gene expression and the mechanism by which various stressors lead to changes in gene expression. This could help in clarifying the ability of *C. perfringens* to cause such a wide range of diseases by surviving under harsh conditions, and ultimately in the design of new treatment options. While most previous studies have focused on the identification of virulence factors, the regulation of their expression, and their impact on the pathogenicity of *C. perfringens* [10-12], there have been no studies examining the effects of environmental stressors on virulence gene expression and survival in *C. perfringens*. Thus the aims of this study were (1) to evaluate the effect of various environmental stressors on the expression of major virulence genes (*pfoA*, *plc*, *colA*, *ccp*) as well as the secondary virulence regulator, VR-RNA, and (2) to examine any changes in the cell viability and morphology of *C. perfringens* after being exposed to various stressors.

Materials and Methods

Strains, media, and culture conditions

C. perfringens strain 13 [13] was cultured from -80 ℃ stock for 16-20 hrs at 37 ℃ in anaerobic conditions on Brain Heart Infusion (BHI) blood agar plates. Cells were transferred to 5 ml of GAM (Gifu Anaerobic Medium; Nissui, Japan) broth and incubated for 4 hr in a 37 ℃ water bath to obtain a starter culture. One milliliter of the starter culture was inoculated into 20 ml fresh GAM broth and incubated for 2 hrs at 37 ℃ to reach the early exponential growth phase. This 2-hr culture was used for testing the effect of various stressors and the survivability of *C. perfringens*. BHI agar plates were used for the colony count analysis.

Stress conditions used

The exponentially-growing cell culture was divided into two tubes, one for the control condition (without stressor) and the other for the stressed condition, and then both tubes were kept at 37 ℃ for 10 min. Oxidative stress was generated either by exposure to air by vigorously shaking the culture in a 37 ℃ incubator at 200 rpm for 5, 15, 30 or 60 min, or by exposing cells to H_2O_2 at a concentration of 200, 400, or 600μM for 15 min. For bile acid stress, the culture was supplemented with bile salt LP0055 (Oxoid, Ltd. Basingstoke, UK) at a concentration of 0.05%, 0.1% or 0.5%. An osmotic shock was simulated by adding NaCl at a concentration of 0.4, 0.6, 0.8 or 1.2 M. Incubation under acidic condition was achieved by lowering the pH to 4.2, 3.8, 3.3 or 2.6 by adding 50, 75, 100 or 150 mM HCl, respectively, while, under the alkaline condition, the pH was increased to 7.5, 8.5, 9.1 or 9.5 by adding 50, 75, 90, or 100 mM NaOH, respectively. Finally, temperature-shift stress was applied by transferring the 37 ℃ culture to an atmosphere of 25 ℃, 42 ℃, 50 ℃ or 55 ℃ and incubating for 15 min.

Cell viability and morphological testing

Cell viability under stressed conditions was evaluated by plating the bacterial suspensions after

exposure to various stressors on BHI agar plates with x10 serial dilutions. After incubation under anaerobic conditions at 37℃ for 16 to 20 hr, the number of visible colonies from appropriate dilutions was counted. All experiments were independently repeated more than three times. The morphological examination was performed on smears obtained from the cultures subjected to normal as well as some stressors using a Biozero BZ-8000 microscope (Keyence, Osaka).

Growth curves of *C. perfringens*

Growth curves of *C. perfringens* were determined in GAM broth at 37℃ over a period of 6 hrs. One percent of an overnight culture was inoculated into GAM broth, and the growth was monitored by measuring the optical density at 600 nm every hr.

Northern hybridization.

Total RNA was extracted from the control and stressed *C. perfringens* cells as previously described [11]. Northern hybridization was performed using an Alkphos-direct kit and CDP-star Chemiluminescence Reagent (GE Healthcare, Piscataway, NJ). DNA probes were generated by PCR from genomic DNA of *C. perfringens* strain 13 with the appropriate primer sets (Table 1) and labeled with alkaline phosphatase according to the manufacturer's instructions. All Northern hybridization experiments were performed at least three times, and the representative data were shown in figures, after the reproducibility of the multiple experiments was confirmed.

Statistical analysis

Analysis of variance (ANOVA) with Dunnett post-hoc analysis was used to determine significance in the cell viability experiments. Significance was taken as $P < 0.05$.

Results

Effect of oxidative stress by aeration on the expression of major virulence genes and cell viability

The expression of virulence genes was compared between non-stressed (control) and stressed *C. perfringens* cells. The transcription level of *pfoA* was up-regulated by aeration at 5 and 15 min (Fig. 1A),

Table 1. Oligonucleotides used in this study

Primer	Sequence (5' to 3')
pfoA-F	CAAGCTGCTTTCAAAGCTCT
pfoA-R	CCCTTAGAATACTCTGTAGA
plc-F	AGTGTACGCTTGGGATGGAA
plc-R	TTTCCTGGGTTGTCCATTTC
colA-F	GGATATGATGCTAAAAACACTGAGTTCTAT
colA-R	CCTGATGAATTTTTTCCACCAAA
ccp-F	TGGGAAAAGTGAATTTCCAG
ccp-R	CCAATGTGGTATTGCTTGTC
vrr-F	TGAAACATACAAAAAGGATT
vrr-R	TACAATTATGGAATATGCAA

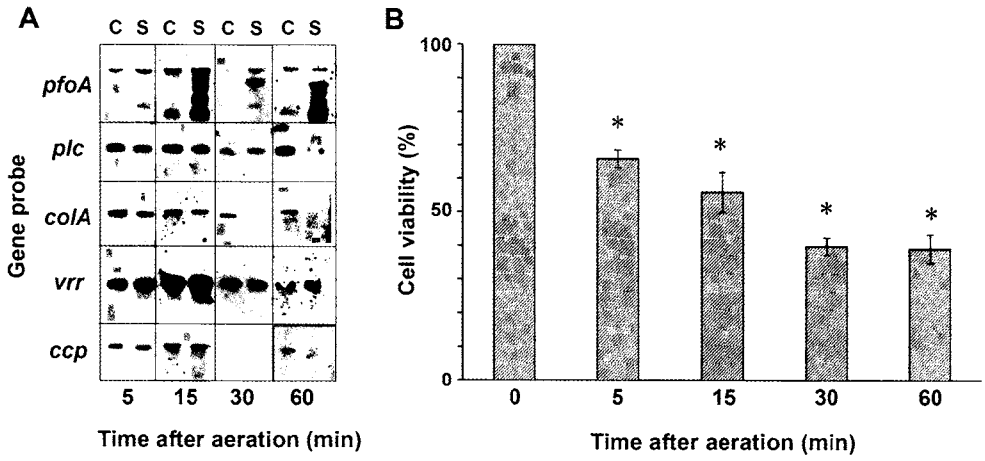

Fig. 1. Effect of oxidative stress by aeration. (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to aeration stress for 5, 15, 30 or 60 min compared to the control cells. Ten micrograms of total RNA was loaded in each lane (the loading was confirmed in ethidium-bromide-stained gel). The gene probes used for hybridization are indicated on the left. The letter C indicates the control condition and the letter S indicates the stress condition. The representative data is shown from three reproducible data. (B) The viability (%) of the control and stressed *C. perfringens* after exposure to air for different amounts of time. Data represent the mean ± SD, n = 3. The significance of the differences of cell viability was checked with ANOVA ($P < 0.05$). * $P < 0.05$ compared with control cells by Dunnett's post-hoc analysis.

and then returned to the same level as in the control cells at 30 and 60 min. Under the stressed condition, the expression of the *plc* gene showed no significant difference at 5, 15, and 30 min; but the expression was not seen at 60 min. In the case of the *colA* gene, the aeration did not affect *colA* expression at 5 and 15 min, but the stress had a negative effect on *colA* expression at 30 and 60 min. The expression of *ccp* under the stressed condition did not differ among the time points. When compared to the other genes examined above, only the *vrr* gene showed the same transcription between the stressed and control states over all the time intervals. Thus, virulence genes in the presence of aeration showed variable levels and timing of expression.

To examine the effect of oxidative aeration stress on cell viability, a colony count experiment was carried out (Fig. 1B). The results indicated that a little less than half of the *C. perfringens* cells were able to survive even after the exposure to air (Fig. 1B), indicating that *C. perfringens* might be fairly tolerant to an oxidative condition even though it is classified into strictly anaerobic bacteria.

Effect of oxidative stress by hydrogen peroxide on the expression of major virulence genes and cell viability

Application of oxidative stress using different concentrations of H_2O_2 showed that the expressions of *pfoA*, *colA*, and *ccp* were maintained at the same levels as in the control cells at an H_2O_2 concentration of 0.2 mM, and then their expressions decreased as the concentration of H_2O_2 increased. But for *plc* and *vrr*, the level of expression was maintained with no significant difference from the control level at H_2O_2 concentrations of 0.2 and 0.4 mM, while it decreased at an H_2O_2 concentration of 0.6 mM (Fig. 2A). Colony count experiments indicated that viability of *C. perfringens* cells decreased by increasing the concentration of H_2O_2, with the deaths varying from 15% at 0.2 mM to 74% at the last concentration of 0.6 mM (Fig. 2B). These results also support that certain proportions of *C. perfringens* cells can resist the oxidative stress mediated by H_2O_2.

Effect of temperature

C. perfringens cells cultured at 37 ℃ were immediately exposed to temperatures of 25 ℃, 42 ℃, 50 ℃ and 55 ℃ for 15 min, and then the total RNA was extracted and Northern experiments were carried out (Fig. 3A). The results revealed that all examined gene expressions showed no change at 25 ℃, except that *ccp* was over-expressed and *pfoA* showed a slight decrease at that temperature. But at 42 ℃, the expressions of all the genes except *colA* slightly decreased. When the temperature was shifted up to 50 ℃, the expression of all examined genes decreased. The most surprising result was that the *plc* gene (encoding a key virulence factor in the infection of *C. perfringens*) showed a significant expression

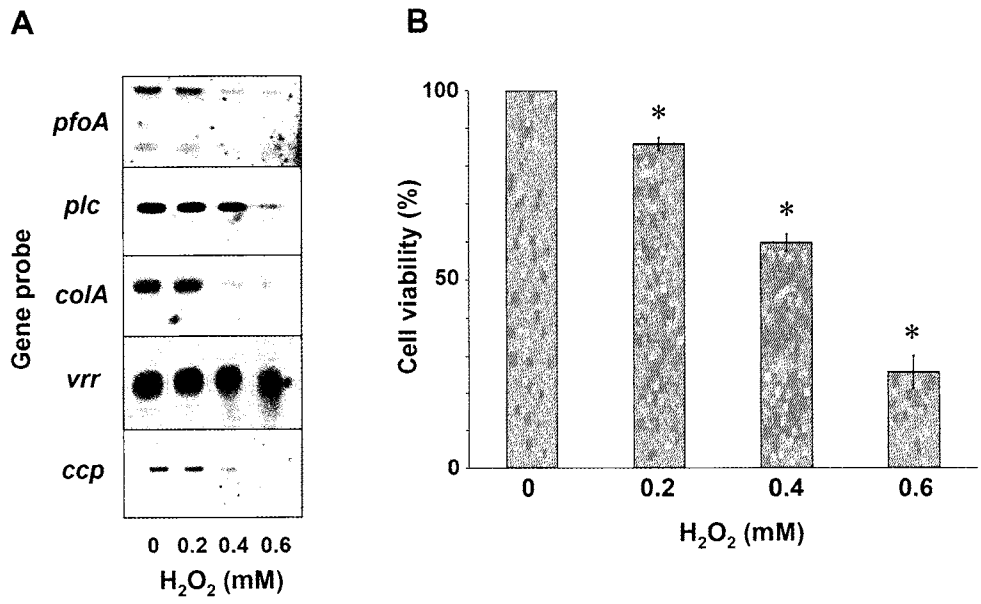

Fig. 2. Effect of oxidative stress by H_2O_2. (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to different concentrations of H_2O_2 for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of control and stressed *C. perfringens* after exposure to H_2O_2 at different concentrations. Data represented and analyzed as same as Fig. 1.

level at 55℃, while the mRNAs from other genes were totally missing at this temperature (Fig. 3A).

Concerning the viability of *C. perfringens* cells under different temperatures, the results showed that the viability increased by ~ 2.5 fold at 50℃ compared to that in the control (at 37℃), but the viability decreased at a lower temperature of 25℃ (Fig. 3B). Microscopic observation of cells grown at 50℃ showed no apparent morphological changes compared with control cells at 37℃, while many aggregations of cells were observed in the culture grown at 55℃ (data not shown). In spite of this, some cells seemed to still be alive and to maintain their normal shape, even though they were totally non-culturable on the plates.

Effect of bile acid

To determine whether or not bile acid has an effect on the expression of virulence genes, *C. perfringens* was exposed to different concentrations of bile

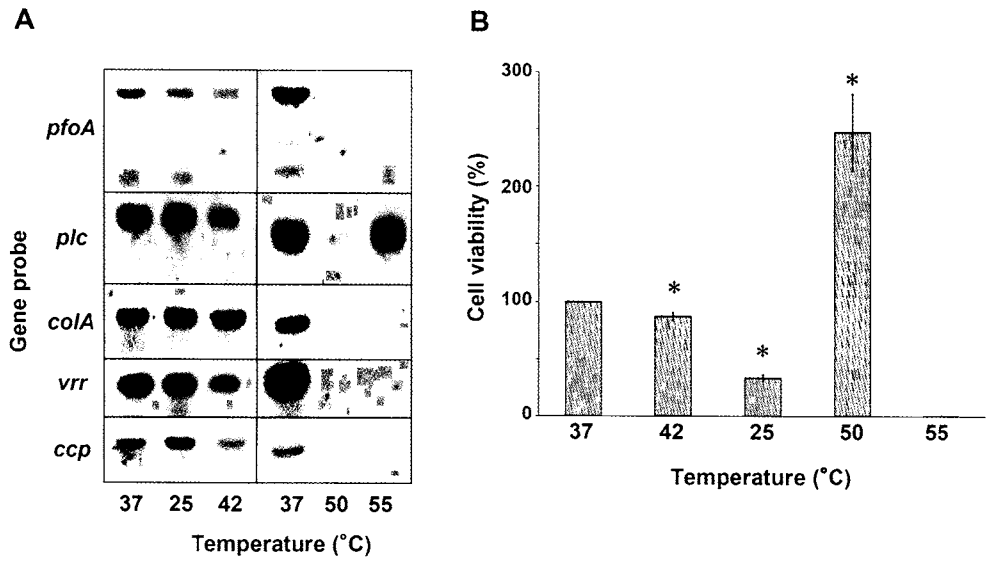

Fig. 3. Effect of temperature. (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to different amounts of temperature stress for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of the *C. perfringens* cells under the control condition and the stress condition after culture at various temperatures. Data represented and analyzed as same as Fig. 1.

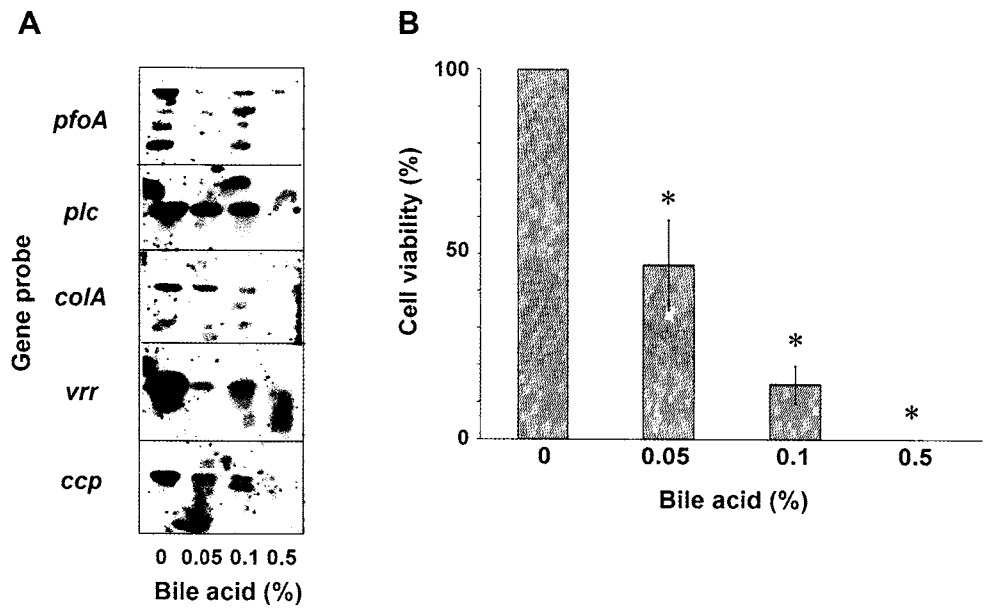

Fig. 4. Effect of bile acid. (A) Northern blot analysis of total RNA from C. perfringens isolated after exposure of cells to different concentrations of bile acid for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of the *C. perfringens* cells under the control condition and the stress condition after culture at various concentrations of bile acid. Data represented and analyzed as same as Fig. 1.

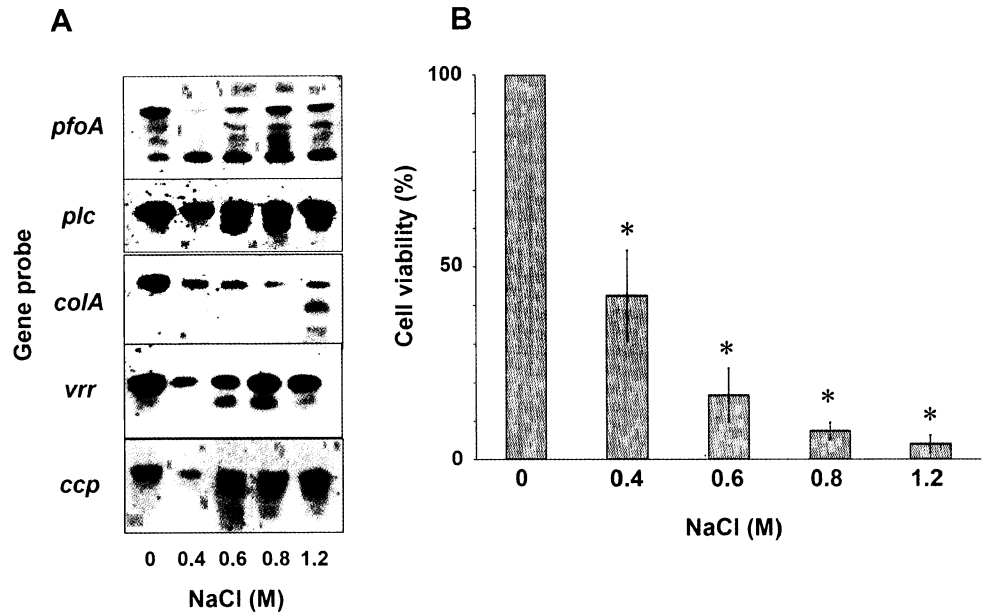

Fig. 5. Effect of osmolarity. (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to different concentrations of NaCl for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of the *C. perfringens* cells under the control condition and the stress condition after culture at various concentrations of NaCl. Data represented and analyzed as same as Fig. 1.

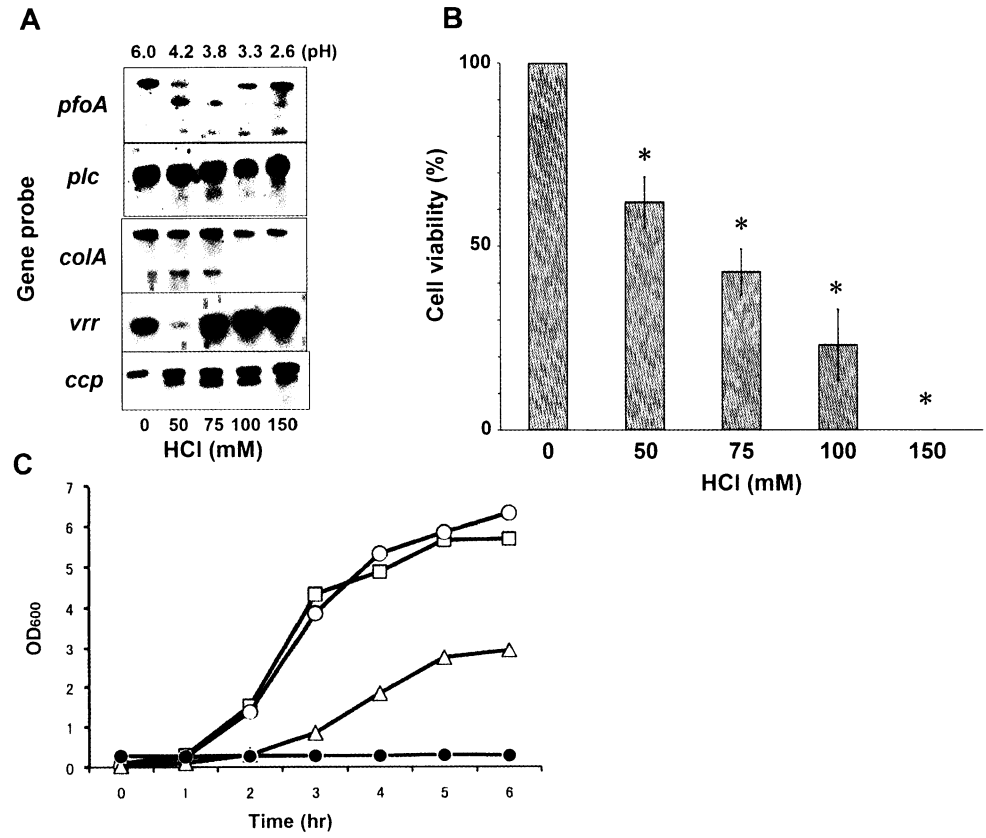

Fig. 6. Effect of acidic condition. (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to different concentrations of HCl stress for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of the *C. perfringens* cells under the control condition and the stress condition after culture at various concentrations of HCl. Data represented and analyzed as same as Fig. 1. (C) Growth curves of *C. perfringens* under different pH. Control, square; pH 6, open circle; pH 5, triangle; pH 4, closed circle.

(0.05%, 0.1% and 0.5%) for 15 min. Northern blot analysis showed that the gene expressions of *pfoA*, *colA*, *ccp* and *vrr* gradually decreased with increasing concentration of bile acid. On the other hand, *plc* maintained its expression level until 0.1% (Fig. 4A). Bile acid also affected the viability of cells by decreasing the colony count with increasing concentration of bile acid (Fig. 4B).

Effect of osmolarity

Salt was used to create the high osmotic condition, and the resulting effects of osmotic stress on virulence gene expression and viability of *C. perfringens* are presented in Fig. 5A and 5B. High osmolarity altered the virulence gene expression in a manner dependent on the concentration of NaCl. The remarkable result was that all examined genes showed a decrease in expression at the lower concentration of NaCl (0.4 M), while at higher concentrations (0.6, 0.8 and 1.2 M), their transcription levels increased again to levels similar to those in the control experiment, with the exception of *colA*, whose expression decreased at all concentrations tested. Also, cell viability decreased with increasing concentration of NaCl (Fig. 5B). Microscopic examination showed no morphological difference between control and cells with 1.2 M NaCl (data not shown), indicating that *C. perfringens* can resist a high osmotic condition of 7.3% (1.2 M) NaCl.

Effect of acidic condition

Since *C. perfringens* is a potential food-borne gastro-intestinal pathogen, and thus would be exposed to highly acid environments in the stomach, we checked the effect of acid on virulence gene expression. Neither *plc* nor *ccp* showed any detectable difference in expression under the different concentrations of HCl tested here. There was an obvious decrease in the expression of *pfoA*, *colA*, and *vrr* at pH 4.2 (50 mM HCl), while the expression levels of these genes increased again at higher concentrations of HCl (75, 100, 150 mM) (Fig. 6A). On the other hand, the colony count decreased by increasing concentration of HCl, but no colony was seen at 150 mM HCl (Fig. 6B). Under microscopic observation, cell aggregation occurred at 150 mM HCl (data not shown), while all examined genes were expressed under the same condition. The growth curves of *C. perfringens* under various starting pH values indicated that cells could not grow at pH 4 (Fig. 6C). These data indicated that the viability and culturability of *C. perfringens* cells were greatly affected by low pH, but, interestingly, the cells under low pH still could express several virulence-related genes at a level indistinguishable from the control cells.

Effect of alkaline condition

The effect of alkaline condition was examined by adding different concentrations of NaOH (50, 75, 90, and 100 mM). The expression of all examined genes decreased quite rapidly as pH increased (Fig. 7A). However, the colony count gradually decreased by increasing concentration of NaOH (Fig. 7B).

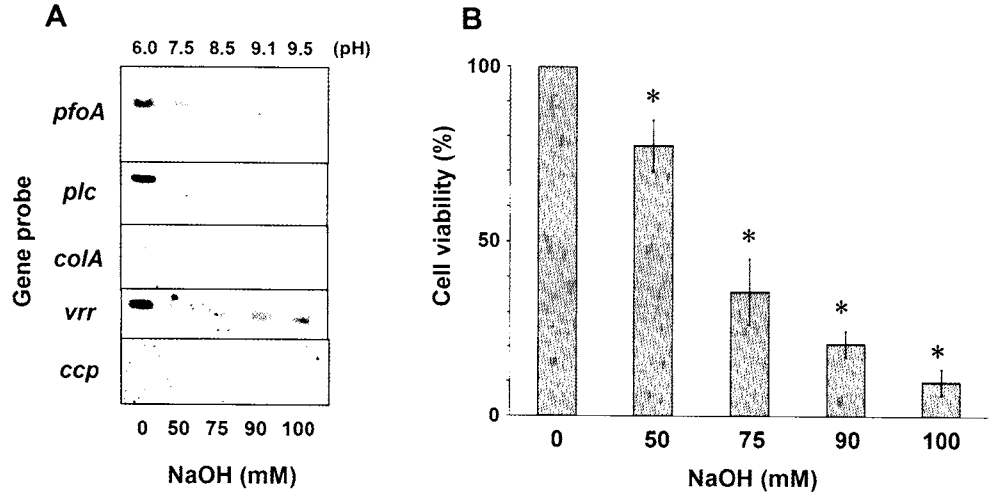

Fig. 7. Effect of alkaline condition (A) Northern blot analysis of total RNA from *C. perfringens* isolated after exposure of cells to different concentrations of NaOH for 15 min compared to the control. Total RNA was analyzed as same as Fig. 1. (B) The viability (%) of the *C. perfringens* cells under the control condition and the stressed condition after culture at various concentrations of NaOH Data represented and analyzed as same as Fig. 1.

Discussion

In this study, a wide range of environmental stressors were tested for their effects on the expression of the most important virulence genes in *C. perfringens*. These virulence factors are important in the establishment of infection and the progression of a myonecrotic disease, gas gangrene. The results of the present study demonstrated that some environmental stressors might influence the expression of virulence genes in *C. perfringens*, and that the virulence genes were differentially expressed in response to different stressors.

Since *C. perfringens* is classified as a strictly anaerobic bacterium, we were interested in checking the effect of oxidative stress either by aeration or H_2O_2 on the expression of major virulence genes. Major findings obtained from this study indicated that oxidative stress by aeration increased the expression of *pfoA* (perfringolysin O), the second most important virulence factor in infection with *C. perfringens*. There was no significant difference in other major virulence genes tested in this study, such as *plc*, *colA*, or *ccp* during the short periods of exposure to oxygen (5-15 min). These facts are in agreement with the results observed in *Porphyromonas gingivalis* [14]. Also, modification or alteration of the expression patterns of virulence factors by oxidative stress has been reported in other bacteria, such as *Salmonella typhimurium* [15]. This study provides evidence that the expression of *pfoA* increased after exposure to atmospheric oxygen. Regarding the cell survival under oxidative stress, the viability was reduced and the colony count decreased with increasing duration of stress application, which was similar to the previously reported responses of other bacteria, such as *Clostridium acetobutylicum* and thermophilic *Streptococci* [16][17].

Previous studies have shown that the response of *C. perfringens* cells to oxidative stress depends on certain compounds present in the medium [18]. In this study, the growth of *C. perfringens* cells slightly decreased after aeration but showed complete recovery to the control level (data not shown). This might have been due to the bacterium immediately ceasing its metabolic activity and resuming only after restoring its anaerobic conditions [19]. These results might also confirm the observation that *C. acetobutylicum* ceased its metabolic activity in the presence of oxygen [20].

The virulence genes tested in this study were differentially expressed in response to oxidative condition by H_2O_2. These results are in good accordance with the previous studies by Chang et al.[21][22] and Palma et al.[23]. Moreover, the viability of *C. perfringens* decreased with the increase in the concentration of H_2O_2, but the viability of the cells was not totally lost. These results indicated that although *C. perfringens* is regarded as a strict anaerobe, it could still survive under exposure to air and H_2O_2.

The most striking result of this study, which has not previously been shown in *C. perfringens*, was that the expression of the *plc* gene (encoding alpha-toxin, the key virulence factor in the pathogenesis of gas gangrene) increased at 55 ℃, although the other virulence genes were not expressed at all and the cells were totally non-culturable at that high temperature. These results indicated that *C. perfringens* might form viable-but-not-culturable (VBNC) cells under high temperature stress. In a recent study, heat stress induced various genes involved in the adhesion and survival of *S. typhimurium* in the host [24]. Also, TDH gene production increased after thermal stress at 47 ℃ in *Vibrio parahaemolyticus* [25]. Meanwhile, the most suitable temperature for expression of *pfoA* was 37 ℃, while *pfoA* expression decreased at 25 ℃ and 42 ℃, and *pfoA* was not expressed at all at 50 ℃ or 55 ℃. These results are in good agreement with the results of Karlsson et al., which described that the expressions of toxin A and B in *Clostridium difficile* were heat-dependent, with maximum expressions at 37 ℃ and minimum expressions at 22 ℃ and 42 ℃ [26]. In contrast, the *ccp* gene (encoding alpha-clostripian) showed higher expression at 25 ℃ than at 37 ℃.

The second remarkable result under temperature stress was that the viability and proliferation of cells greatly increased at 50 ℃ when compared with those of the control at 37 ℃. Meanwhile, in *Yersinia pestis*, incubation of cells at 50 ℃ for 10 min killed the majority of cells *in vitro*, while the incubation at 45 ℃ for 10 min had little effect on their viability [27]. Furthermore, a previous study indicated that when a culture of *Campylobacter jejuni* was shifted from 42 ℃ to 55 ℃ for 3 min, its viability was reduced to 40% [28].

In our study the heat stress did not significantly affect the cell morphology, except for the observation

of many aggregated cells at 55 ℃. These results are in agreement with the results shown by Chifiriuc et al. [25]. On the other hand, previous studies in *C. perfringens* demonstrated that cold shock at 15 ℃ led to morphological changes, with cells becoming more elongated than those at 37 ℃ [29].

Bile salts act as detergents and have strong antibacterial activity. Since the bile salts concentration in the lumen of the mammalian intestine ranges from 0.2% to 2% [30], the bile salts are expected to play an important role in the host defense against bacterial infection. However, the bacterium may combat this defense by its virulence response using bile as a signal for the control of virulence genes, as described previously [31]. In *C. perfringens*, the expression of the genes *pfoA*, *colA*, *ccp* and *vrr* gradually decreased by increasing the concentration of bile acid. On the other hand, *plc* maintained its expression until a bile acid concentration of 0.5% (Fig. 4A). Compared with their effects on other enteric pathogens, bile acids more negatively affected the production of virulence factors in *C. perfringens*. In *Vibrio cholerae*, 0.2% to 0.4% crude bile has been shown to reduce the production of the major virulence factors (cholera toxin and toxin co-regulated pili) [32]. In contrast, Hang and Mekalanos [30] demonstrated in *V. cholerae* that bile acids could induce *in vitro* cholera toxin expression under conditions more close to the physiological conditions. In *Escherichia coli*, bile salt induced the expression of specific stress-responsive genes [33]. Recently, Gotoh et al. reported that crude bile is a potent host-derived inducer of TDH (thermostable direct hemolysin) and type three secretion system 2-related protein production in *V. parahaemolyticus* [34]. Regarding the cell viability of *C. perfringens*, it significantly decreased as the bile concentrations increased. This is in agreement with the previously reported results that bile salt at concentrations of 0.1% and 0.2% reduced the growth rate of *Enterococcus faecalis* (10-fold and 30-fold reductions in the growth rate, respectively), and that incubation with 0.3% bile salts led to a 1000-fold decrease of the viable cells [35]. In contrast, Pace et al. demonstrated that the addition of bile or deoxycholic acid to estuarine water-cultured bacteria led to an increase in the direct viable count and colony counts among the virulent strains of *V. parahaemolyticus* [36].

We were interested in studying salt stress in *C. perfringens*, since salt is one of the most commonly used practical methods for food preservation. Our results showed altered expression of various virulence genes in response to salt stress, and the most surprising result was that all of the genes tested showed a decrease in expression at an NaCl concentration of 0.4 M (the lowest concentration in this experiment), which is similar to the estimated osmolarity of more than 0.3 M NaCl in the intestinal lumen [34]. The expressions of these genes then increased again at higher NaCl concentrations, except in the case of *colA*, which was low at all concentrations of NaCl.

Osmotic stresses have been studied extensively in a wide range of bacteria, including *Helicobacter pylori* [37][38], in which increasing salt concentrations led to an increase in the expression of its virulence factors, CagA and VacA, and their responses to osmotic stress differed from one strain to another. In contrast, Gong et al. showed that high osmolarity increased the level of expression of *prgI* and *sipB* virulence genes in *Salmonella* [39]. In *L. monocytogenes*, listeriolysin O (LLO) activity was regulated by sodium chloride [40], and in *Staphylococcus aureus*, addition of NaCl to the growth medium led to suppression of the expression of virulence genes, such as *spa*, *hla* and *tst* [9]. On the other hand, the viability of *C. perfringens* cells decreased with increasing concentration of NaCl in our experiments, which is consistent with the previous observation that osmotic shock (400 mM NaCl) for 15 min in *C. acetobutylicum* lead to 66% cell survival [16].

Our data showed that a high concentration of NaCl (1.2 M) had no effect on the morphology of *C. perfringens* cells, which differed from the results of previous reports [25][38], in which osmotic stress induced significant changes in the cell morphology of *H. pylori* and *V. parahaemolyticus*.

The study of acidic stress is of particular interest because food-borne bacteria are exposed to extremely low pH in the stomach, and thus adaptation to acid stress might be required for the development of intestinal infectious diseases. Moreover, in food preservation, acids are often used to control the bacterial survival in foods.

Our study suggested that acidic condition changed the expression of the virulence genes, and the response to acidic conditions differed among

genes. The *plc* and *ccp* genes did not show any difference in their expression under various pH levels, indicating that exposure to sublethal acid stress does not always lead to the induction of virulence genes. Among the other genes tested, *pfoA*, *colA* and *vrr* showed unexpected decreases in expression at 50 mM HCl (pH 4.2), the lowest concentration in this study. Our present results are consistent with previous observations, which showed that acid stress could influence the virulence capacity of *L. monocytogenes* by inducing or reducing the expression levels of virulence and virulence-associated genes [41]. On the other hand, Sue et al. showed that the expression of the *inlA* and *bsh* virulence genes of *L. monocytogenes* was significantly increased within 5 or 15 min after acid stress (pH 4.5) [42].

In *C. perfringens*, acidic condition affected the viability of cells, and decreasing viability with increased concentration of acid was observed. These results are in agreement with the results of Zotta et al. [17] and Bonomo et al. [43], who reported that acid stress reduced the number of viable cells. It was also demonstrated that, in order to maintain viability, the bacterial cells should maintain their pH, which depends on the glucose availability in the medium [44].

Microscopic analysis of cells after addition of 150 mM HCl to GAM broth for 15 min was associated with morphological changes of cell aggregation, while all virulence genes were still expressed at pH 2.6. This finding is consistent with previous research demonstrating that *C. jejuni* showed morphological changes due to acid stress [45]. It has also been reported that exposure of *C. jejuni* to acid stress led to the formation of a coccoid cell that was viable but non-culturable [46].

The Northern blot analysis in this study showed that the expression of most virulence genes of *C. perfringens* was repressed by alkaline stress, which implies that *C. perfringens* could not respond well to the alkaline condition regarding to the expression of virulence factors. However recently, Giotis et al. reported the repression of a large number of proteins along with the synthesis of 8 novel proteins in response to alkaline stress in *L. monocytogenes* 10403 strain [47]. Moreover, an earlier study by Bingham et al. in *E. coli* showed that alkaline stress led to induction of a novel gene locus (*alx*) [48].

Conclusion

In this study, we found that expression of the *plc* gene (encoding the key virulence factor, alpha-toxin of *C. perfringens*) was greatly increased at 55 ℃ even though the cells were in a completely non-culturable state at that temperature. The *pfoA* gene (encoding a virulence factor, perfringolysin O in *C. perfringens*) was up-regulated by 5- and 15-min oxidative stress by aeration. These results showed that *C. perfringens* is capable of sensing, responding to, and adapting to various kinds of stressors, and that *C. perfringens* modulates its virulence gene expression in response to various stressful environmental changes. This modulation might be essential for *in vivo* survival and the achievement of infection in the human body. Based on our results, further studies will be needed to elucidate the relationship between the stress response and the pathogenesis of the anaerobic flesh-eater, *C. perfringens*.

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References

1) McDonel JL. *Clostridium perfringens* toxins (Type A, B, C, D, E). Pharmacol Ther 10; 617-655, 1980

2) Hatheway CL. Toxigenic clostridia. Clin Microbiol Rev 3; 66-98, 1990

3) Petit L, Gibert M, Popoff MR. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol 7; 104-110, 1999

4) Rood JI. Virulence genes of *Clostridium perfringens*. Annu Rev Microbiol 52; 333-360, 1998

5) Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S, Hayashi H. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc Natl Acad Sci USA 99; 996-1001, 2002

6) Van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek 82; 187-216, 2002

7) Abee T, Wouters JA. Microbial stress response in minimal processing. Int J Food Microbiol 50; 65-91, 1999

8) Mekalanos JJ. Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol 174; 1-7, 1992

9) Chan PF, Foster SJ. The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. Microbiol 144; 2469-2479, 1998

10) Shimizu T, Ba-Thein W, Tamaki M, Hayashi H. The *virR* gene, a member of a class of two-component response regulators, regulates the production of perfringolysin O, collagenase, and

hemagglutinin in *Clostridium perfringens*. J Bacteriol 176; 1616-1623, 1994

11) Ba-Thein W, Lyristis M, Ohtani K, Nisbet IT, Hayashi H, Rood JI, Shimizu T. The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J Bacteriol 178; 2514-2520, 1996

12) Ohtani K, Hirakawa H, Tashiro K, Yoshizawa S, Kuhara S, Shimizu T. Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*. Anaerobe 16; 258-264, 2010

13) Mahony DE, Moore TI. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. Can J Microbiol 22; 953-959, 1976

14) Meuric V, Gracieux P, Tamanai-Shacoori Z, Perez-Chaparro J, Bonnaure-Mallet M. Expression patterns of genes induced by oxidative stress in *Porphyromonas gingivalis*. Oral Microbiol Immunol 23; 308-314, 2008

15) Jones BD, Falkow S. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect Immun 62; 3745-3752, 1994

16) Hillmann F, Fischer RJ, Bahl HJ. The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein. Arch Microbiol 185; 270-276, 2006

17) Zotta T, Ricciardi A, Ciocia F, Rossano R, Parente E. Diversity of stress responses in dairy thermophilic *streptococci*. Int J Food Microbiol 124; 34-42, 2008

18) Trinh S, Briolat V, Reysset G. Growth response of *Clostridium perfringens* to oxidative stress. Anaerobe 6; 233-240, 2000

19) Hillmann F, Döring C, Riebe O, Ehrenreich A, Fischer RJ, Bahl H. The role of PerR in O_2-affected gene expression of *Clostridium acetobutylicum*. J Bacteriol 191; 6082-6093, 2009

20) O'Brien RW, Morris JG. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J Gen Microbiol 68; 307-318, 1971

21) Chang W, Small DA, Toghrol F, Bentley WE. Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide. BMC Genomics. 6; 115, 2005

22) Chang W, Small, DA, Toghrol F, Bentley WE. Global transcriptome analysis of *Staphylococcus aureus* response to hydrogen peroxide. J Bacteriol 188; 1648-1659, 2006

23) Palma M, DeLuca D, Worgall S, Quadri LE. Transcriptome analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. J Bacteriol 186; 248-252, 2004

24) Sirsat SA, Burkholder KM, Muthaiyan A, Dowd SE, Bhunia AK, Ricke SC. Effect of sublethal heat stress on Salmpnella Typhimurium virulence. J Appl Microbiol 110; 813-822, 2011

25) Chifiriuc MCB, Pircalabioru G, Dinu S, Iordache C, Israil A, Lazar V. The effects of heat and osmotic stress on *Vibrio parahaemolyticus* survival rates, cellular morphology and TDH production. Int J Appl Biol Pharm Technol 1; 1084-1091, 2010

26) Karlsson S, Dupuy B, Mukherjee K, Norin E, Burman LG, Akerlund T. Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. Infect Immun 71; 1784-1793, 2003

27) Han Y, Zhou D, Pang X, Zhang L, Song Y, Tong Z, Bao J, Dai E. Wang J, Guo Z, Zhai J, Du Z, Wang X, Wang J, Huang P, Yang R. DNA microarray analysis of the heat-and cold-shock stimulons in *Yersinia pestis*. Microb Infect 7; 335-348, 2005

28) Mihaljevic RR, Sikic M, Klancnik A, Brumini G, Mozina SS, Abram M. Environmental stress factors affecting survival and virulence of *Campylobacter jejuni*. Microb Pathog 43; 120-125, 2007

29) De Jong AEI, Rombouts FM, Beumer RR. Behaviour of *Clostridium perfringens* at low temperatures. Int J Food Microbiol 97; 71-80, 2004

30) Hung DT, Mekalanos JJ. Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independent manner. Proc Natl Acad Sci USA 102; 3028-3033, 2005

31) Prouty AM, Brodsky IE, Manos J, Belas R, Falkow S, Gunn JS. Transcriptional regulation of *Salmonella* enterica serovar Typhimurium genes by bile. FEMS Immunol Med Microbiol 41; 177-185, 2004

32) Gupta S, Chowdhury R. Bile affects production of virulence factors and motility of *Vibrio cholerae*. Infect Immun 65; 1131-1134, 1997

33) Bernstein C, Bernstein H, Payne CM, Beard SE, Schneider J. Bile salt activation of stress response promoters in *Escherichia coli*. Curr Microbiol 39?; 68-72, 1999

34) Gotoh K, Kodama T, Hiyoshi H, Izutsu K, Park KS, Dryselius R, Akeda Y, Honda T, Iida T. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. PLoS One 5; e13365, 2010

35) Rincé A, Le Breton Y, Verneuil N, Giard J, Hartke A, Auffray Y. Physiological and molecular aspects of bile salt response in *Enterococcus faecalis*. Int J Food Microbiol 88; 207-213, 2003

36) Pace JL, Chai TJ, Rossi HA, Jiang X. Effect of bile on *Vibrio parahaemolyticus*. Appl Environ Microbiol 63; 2372-2377, 1997

37) Loh JT, Torres VJ, Cover TL. Regulation of *Helicobacter pylori cagA* expression in response to salt. Cancer Res 67; 4709-4715, 2007

38) Gancz H, Jones KR, Merrell DS. Sodium chloride affects *Helicobacter pylori* growth and gene expression. J Bacteriol 190; 4100-4105, 2008

39) Gong H, Su J, Bai Y, Miao L, Kim K, Yang Y, Liu F, Lu S. Characterization of the expression of *Salmonella* Type III secretion system factor PrgI, SipA, SipB, SopE2, SpaO, and SptP in cultures and in mice. BMC Microbiol 9; 73, 2009

40) Myers ER, Dallmier AW, Martin SE. Sodium chloride, potassium chloride, and virulence in *Listeria monocytogenes*. Appl Environ Microbiol 59; 2082-2086, 1993

41) Werbrouck H, Vermeulen A, Van Coillie E, Messens W, Herman L, Devlieghere F, Uyttendaele M. Influence of acid stress on survival, expression of virulence genes and invasion capacity into Caco-2 cells of *Listeria monocytogenes* strains of different origins. Int J Food Microbiol 134; 140-146, 2009

42) Sue D, Fink D, Wiedmann M, Boor KJ. SigmaB-dependent gene induction and expression in *Listeria monocytogenes* during

osmotic and acid stress conditions simulating the intestinal environment. Microbiology 150; 3843-3855, 2004.

43) Boromo MG, Sico MA, Grieco S, Salzano G. Fluorescent differential display analysis of *Lactobacillus sakei* strains under stress conditions. J Microbiol Methods 82; 28-35, 2010

44) Shabala L, Budde B, Ross T, Siegumfeldt H, McMeekin T. Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. Int J Food Microbiol 75; 89-97, 2002

45) Shaheen BW, Miller ME, Oyarzabal OA. In vitro survival at low pH and acid adaptation response of *Campylobacter jejuni* and *Campylobacter coli*. J Food Safety 27; 326-343, 2007

46) Rollins DM, Colwell RR. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl Environ Microbiol 52; 531-538, 1986

47) Giotis ES, Muthaiyan A, Blair IS, Wilkinson BJ, McDowell DA. (2008). Genomic and proteomic analysis of the Alkali-Tolerance Response (AlTR) in *Listeria monocytogenes* 10403S. BMC Microbiol 8; 102, 2008

48) Bingham RJ, Hall KS, Slonczewski JL. Alkaline induction of a novel gene locus, *alx*, in *Escherichia coli*. J Bacteriol 172; 2184-2186, 1990