Canadian Journal of Microbiology

Unveiling the acid stress response of clinical genotype *Vibrio vulnificus* isolated from the marine environments of Mangaluru coast, India

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2018-0700.R1
Manuscript Type:	Article
Date Submitted by the Author:	16-Mar-2019
Complete List of Authors:	DSouza, Caroline; Nitte University, Nitte University Centre for Science Education & Research Prabhakar Alva, Prathiksha ; Nitte University, Nitte University Centre for Science Education & Research Karanth Padyana, Anupama; Nitte University, Nitte University Centre for Science Education & Research Karunasagar, Iddya; Nitte University, Nitte University Centre for Science Education & Research Karunasagar, Indrani; Nitte University, Nitte University Centre for Science Education & Research Kumar Ballamoole, Krishna; Nitte University, Nitte University Centre for Science Education & Research
Keyword:	<i>Vibrio vulnificus</i> , stress response, acid tolerance, lysine dependent response
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

**Unveiling the acid stress response of clinical genotype *Vibrio vulnificus* isolated from
the marine environments of Mangaluru coast, India**

**Caroline D’Souza¹, Prathiksha Prabhakar Alva¹, Anupama Karanth Padyana¹,
Iddya Karunasagar², Indrani Karunasagar¹, Ballamoole Krishna Kumar^{1*}**

¹Division of Infectious Diseases, Nitte University centre for Science Education and
Research, Nitte (Deemed to be University), Deralakatte, Mangaluru-575018, Karnataka,
India

²Nitte (Deemed to be University), University Enclave, Medical Sciences Complex,
Deralakatte, Mangaluru-575018, Karnataka, India

***Address for correspondence**

Ballamoole Krishna Kumar
Assistant Professor
Division of Infectious Diseases
Nitte University Centre for Science Education and Research
Nitte University, Mangaluru-575018, India
Ph:+91-824-2204292
Email: krishnakumarb@nitte.edu.in; findkkumar@gmail.com

Abstract

Gastric acidity is one of the earliest host defences faced by ingested organisms, and successful pathogens need to overcome this hurdle. The objective of this study was the systematic assessment of acid stress response of *Vibrio vulnificus* isolated from coastal regions of Mangaluru. Acid shock experiments were carried out at pH 4.0 and pH 4.5 with different experimental conditions expected to produce a varied acid response. Exposure to mild acid before the acid shock was favourable to the bacteria but was dependent on cell population and pH of the media and was independent of the strains tested. Lysine dependent acid response was demonstrated with reference to the previously identified lysine decarboxylase system. Additionally, the results showed that inoculation into the oyster provided some level of protection against acid stress. Increased expression of Lysine/Cadaverine related genes were observed upon the addition of ground oyster which was accurately determined by quantitative real-time PCR proving the phenomenon observed. The potential role of ornithine was analysed with regard to acid stress, but no change in the survival pattern was observed. These findings highlight the physiology of bacteria in acid stress.

Keywords: *Vibrio vulnificus*; stress response; acid tolerance; lysine dependent response

Introduction

Vibrio vulnificus is an opportunistic human pathogen that is highly lethal and is responsible for the majority of reported seafood-related deaths (Oliver 2013). *V. vulnificus* is associated with molluscan seafood especially oysters, and consumption of raw oysters by immunocompromised individuals leads to septicaemia in most of the cases (Phillips et al. 2017). The organism is also able to cause severe wound infection when an open wound is exposed to seawater harbouring the bacteria during recreational activities or fishing (Strom and Paranjpye 2000). As *V. vulnificus* is a prominent invasive bacterium, it can penetrate the intestinal wall and enter the blood stream resulting in life threatening primary septicaemia with an average mortality rate exceeding 50% in immunocompromised individual (Horseman et al. 2011). The rapidity in the progression of *V. vulnificus* infection implies that bacteria have several mechanisms to quickly overcome the host immune responses (Jones et al. 2009). Survival of *V. vulnificus* during the passage through the acid environment of the stomach as well as during its transient residence in the macrophage phagosome depends on its ability to tolerate or resist low pH and successful counteraction of this initial resistance becomes one of the virulence determinants of the bacteria.

Most of the enterobacteriaceae members seem to be intrinsically more resistant to lethal acidic pH. *Escherichia coli* and *Shigella flexneri* adapted to stationary phase can persist at pH 2.0 to pH 3.0 for several hours (Hersh et al. 1996). *Salmonella* species can survive at pH 2.5 for at least 2 h (Gorden et al. 1993). However, both clinical as well as environmental strains of *V. vulnificus* were found to be acid sensitive at pH 4.0 and pH 3.0 as the viability reduced several folds just within 15 minutes of acid exposure indicating that during passage through gut, protection by ingested food may explain survival in this environment (Koo et al. 2000a). Since survival in an acidic environment is multi-factorial and highly dependent on the experimental

design adopted, connecting all the data and drawing conclusions on responsible factors is difficult. Survival highly correlates with the exposure to mild sublethal acidic pH before the acid shock (Koutsoumanis et al. 2004; Lianou et al. 2016). Conditions such as growth phase, media used, pH, inhibitory substances in the spent media and cell density affects the outcome of the experiment (Chen et al. 2004; Chiang et al. 2012; Cui et al. 2001; Gorden et al. 1993; Alvarez et al. 2009). Most of the bacteria survive when they are inoculated into certain types of food (Waterman and Small 1998). Previous reports on the acid response of *V. vulnificus* have revealed that lysine decarboxylase enzyme takes part in the acid resistance. Among various acid resistance systems, lysine decarboxylase is one of the mechanisms in organisms like *E. coli*, *S. typhimurium* and *V. cholerae* (Merrell et al. 1999; Alvarez et al. 2010; Soksawatmekhin et al. 2004). Bacteria decarboxylate lysine into highly basic amine cadaverine with the help of lysine decarboxylase which is then secreted to the media for a new substrate (Kanjee et al. 2013). The system requires lysine as a substrate for the induction of enzyme which suggests that organisms survive extreme acidic pH depending upon the amino acids present (Neely et al. 1996; Park et al. 1996). cadBA, an antiporter of the lysine /cadaverine plays a significant role during acid stress in several organisms including *S. typhimurium* and *V. cholerae* (Park et al. 1996; Merrell et al. 2000). cadBA operon partially identical to that of *V. cholerae* was identified in *V. vulnificus*, and mutational analysis revealed its role in acid tolerance response (Rhee et al. 2002). The system was further explored by the identification of transcriptional regulator CadC & AphB and finding its role in acid resistance mechanism (Rhee et al. 2004; Rhee et al. 2005; Rhee et al. 2006).

V. vulnificus shows a high degree of genetic diversity which is directly linked with the pathogenic potential of the organism. Most of the clinical isolates belong to clinical (C) genotype, and most environmental strains belong to E genotype (Jones et al. 2009). Although C

and E genotype correlates with the isolation source, there are reports of environmental strains possessing the C genotype (Sangeetha et al. 2017). The disparity of genotype distribution has led to uncertainty in virulence potential of the strains. Herein we characterize the environmental isolates of Indian origin belonging to C genotype in terms of their ability to resist the acid stress with various parameters to obtain a conjoint overview of acid response. Lysine decarboxylase as a potential contributor to counteract the acid stress was explored in these strains. Level of lysine decarboxylase related genes in the microenvironment of oyster was analyzed, and induction of the lysine decarboxylase was explored during the lysine dependent response. Whether all the related genes of the system works in coordination to protect the bacteria from acid stress was analyzed to get an overall view of the functioning of the system.

Materials and Methods

Bacterial strains and culture conditions

All *V. vulnificus* isolates used in this study were revived from stock culture preserved at -80°C (Sanyo, Japan) in 30% glycerol broth. These strains were grown in 5 mL of Luria-Bertani (LB) broth (HiMedia Laboratories Pvt. Ltd, India) at 37°C overnight with shaking (100 rpm). Four environmental strains and one clinical strain belonging to clinical genotype are evaluated in this study. All the strains belong to biotype 1 and carry *vvhA* and *rtx* genes. E4010, E3715, E3716 and E3717 are environmental strains, isolated from oyster collected along the coast of Mangaluru. *Vibrio vulnificus* K4633 is a clinical strain isolated from the patient with septicaemia, generously provided by Dr Jessica L Jones, US Food and Drug Administration. All the experiments were done using E4010 where as to compare the acid tolerance response among *V. vulnificus* strains all the above mentioned strains were included.

Growth kinetics of *V. vulnificus* in different pH

V. vulnificus E4010 was examined for growth at different pH values. First 100 mL of the LB broth was acidified with 1N HCl to pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 respectively. 100µl of the late logarithmic culture was inoculated into the broth with different pH and incubated at 37°C for 8h with shaking (100 rpm). Optical density (OD) was measured every half an hour during the incubation time spectrophotometrically at 600 nm (Bio spectrophotometer Eppendorf, Germany).

Preparation of acid-adapted cells

For the acid adaptation treatment, one mL of the late exponential phase culture was suspended in 99 mL of the LB broth (pH 5.0). Bacterial cells were then grown overnight at 37°C with shaking (100 rpm) to obtain the stationary phase cultures ($OD_{600} = 1.8$). *V. vulnificus* grown in a similar condition in un-acidified LB broth (pH 7.0) served as the unadapted cell. Both adapted as well as unadapted cells were further challenged for acid the shock.

Acid challenge studies

LB broth acidified to pH 4.0 and pH 4.5 served as a media for acid challenge studies. One mL of the acid-adapted and un-adapted cells were collected by centrifugation 6000 X g and washed twice with phosphate buffer saline containing 3% NaCl (PBS-3). Then, the cells were suspended in 50 mL of acid challenge medium at an initial population of 10^8 CFU/mL at 37°C with shaking (100 rpm). To evaluate the effect of cell density on bacterial survival, acid-adapted cells were serially diluted in PBS-3 to obtain cell density ranging from 1×10^8 cells/mL to 1×10^5 cells/mL. Cells were then subjected for the acid challenge in acid challenge medium (pH 4.0 and pH 4.5) at 37°C with shaking (100 rpm). The viability of the test organism challenged at pH 4.0 and pH 4.5 was determined every 10 min and 0.5 h respectively. Samples were diluted with LB broth and

plated onto LB agar. The viable colonies were enumerated after 18h of incubation at 37°C and expressed as CFU/mL.

The acid challenge of *V. vulnificus* cells inoculated onto the oyster meat

The ability of bacteria to survive in acid when inoculated onto a food source was tested by using oyster. One mL of the stationary phase cells were collected by centrifugation at 6000 X g and washed with PBS-3. Later cells were suspended in 400 µl PBS-3 and inoculated 0.1 g of the homogenized oyster. Bacteria were allowed to adhere on the surface of the meat particle for 10 min at room temperature. The inoculated oyster was then placed into 50 ml of LB broth acidified to pH 4.0 and pH 4.5 and incubated at 37°C with shaking (100 rpm) for 3 h. Washed bacterial cells were also directly challenged for the acid shock which served as control. Un-inoculated ground oyster was subjected to acid challenge studies to check for the presence of native *V. vulnificus*. The viability of the test organism was calculated by withdrawing 100µl of aliquot every 10 min those challenged at pH 4.0 and 0.5 h those challenged at pH 4.5 and plating them onto LB agar. Colonies were enumerated after overnight (18 h) of incubation at 37°C.

The acid challenge of *V. vulnificus* during the amino dependent acid response

The assay for the lysine-dependent response was performed in minimal broth (pH 4.5) supplemented with the amino acid (1mM L-Lysine or 1mM L-Ornithine) which served as acid challenge media. Before the challenge assay bacterial cells were grown overnight in minimal broth (pH 6.0) supplemented with or without respective amino acid (1mM) and LB broth (pH 6.0) separately at 37°C with shaking (100 rpm). The overnight stationary phase cultures (OD₆₀₀=1.8) grown in different condition were separately diluted 1:50 into acid challenge media to achieve the final bacterial concentration of 1X10⁸ CFU/mL. Flasks were then incubated at 37°C with slow shaking (100 rpm). To check the effect of polyamines in the induction of ornithine

decarboxylase bacteria were grown overnight in minimal broth supplemented putrescine, spermidine, spermine (0.1 mM each) and ornithine(1mM) followed by a challenge assay as mentioned earlier. Samples were taken every half an hour until 3 h, plated onto LB agar and incubated at 37°C for 18 h after which viable bacterial counts were determined.

RNA extraction and DNase treatment

RNA extraction was done from the samples subjected to adaptive condition before lysine dependent acid challenge. Bacterial cells were grown overnight with or without lysine (1 mM) and LB broth (pH 6.0) at 37°C with shaking (100 rpm). One mL of the stationary phase (OD₆₀₀=1.8) bacterial cells were collected by centrifugation at 6000 X g for 10 min at 4°C and then subjected for RNA extraction. To assess the *cadBA* operon levels upon the addition of oyster, *V. vulnificus* was inoculated to the ground oyster and then subjected to acid challenge studies at pH 4.5. RNA was extracted at 0.5 h of acid challenge experiment. Extraction of RNA was done using the RNeasy mini kit (Qiagen, Germany). DNase treatment was carried out as per manufactures protocol (Thermo Fisher Scientific Inc, USA). RNA concentrations were normalised to 50 ng/µl and stored at -80°C until use.

Gene expression analysis by real-time PCR

The quantitative real-time PCR (qPCR) assay was performed using iTaq™ Universal SYBR Green one-step kit (BioRad, USA) which has the advantages of cDNA synthesis and PCR amplification in a single step. Primers were designed using reference sequence of *V. vulnificus* with sequence accession number AF324470 & NC_004460.2. Details of primers are provided in Table S1. The appropriate concentrations of primers for all genes were determined, and the optimized concentrations were used in the subsequent step. Analysis of the melt curve was performed to check the amplification of untargeted regions. The efficiency of amplification of

primers used was validated by amplifying the serially diluted cDNA. The slope of the graph was almost equal to 0 confirming the equal amplification efficiency of primers. Relative quantification of all the genes was calculated using the $2^{-\Delta\Delta C_t}$ formula (Levac and Schmittgen 2001). RpoA gene was used to standardize the relative expression of the target gene. The real-time PCR master mix (10 μ l) contained 5 μ l of 2 X universal SYBR green master mix, 200nM of forward and reverse primers, 0.125 μ l of reverse transcriptase, 1 μ l of template RNA and volume was adjusted with molecular grade water. The reaction was performed in real-time PCR (CFX96 Touch™ Real-time PCR detection system, BioRad, USA) with cDNA synthesis at 50°C for 10 min, initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 Sec, primer annealing at 55°C for 20 Sec and final extension and elongation at 72°C for 20 Sec. Data acquisition was performed by Bio-Rad CFX Manager at the end of each elongation step.

Statistical analysis

Factorial ANOVA was performed with the Bonferroni correction for the statistical analysis of data using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

Results and Discussion

Survival of *V. vulnificus* E4010 under different acidic conditions

The first step in addressing acid tolerance response of an environmental strain of *V. vulnificus* E4010, the organism was grown in LB broth with varying pH levels at 37° C (pH 1.0 to pH 7.0). As illustrated in Fig. 1., *V. vulnificus* E4010 grows between the pH range of 5.0 to 7.0 and no growth was observed under acidic conditions with a pH value of 4.0 or lower. *V. vulnificus* previously tested for their ability to survive at pH 2.0, and pH 3.0 showed complete loss of viability within 1.7 min and 18 min respectively (Koo et al. 2000b). *V. vulnificus* at pH 5.0

showed a growth lag extended to 1 h followed by an exponential phase. No lag phase was observed in pH 6.0 and pH 7.0, though bacteria at pH 6.0 had lower cell density at the end of 8 h. It has been reported previously that *V. vulnificus* grows best at neutral pH. *V. vulnificus* environmental strain MP-2 showed proliferation in nutrient broth at pH value above 6.2 whereas it did not show any growth at pH 5.2 (Chase et al. 2011; Wang et al. 2004). MO6-24 (clinical strain) and ATCC 27562 (environmental strain) showed static growth at pH 5.0 with 1 log reduction at the end of 10 h without expansion of the population. However, in contrast to the previous observation, *V. vulnificus* E4010 analysed in this study showed proliferation at pH 5.0 despite of initial lag phase. This disparity may be due to the pH acclimation of the strain in their natural environment, and also cell density plays a vital role in the bacterial ability to bypass the lag. Based on the results obtained, pH 4.0 and pH 4.5 were chosen as experimental pH studies with acid shock.

The adapted strain of *V. vulnificus* E4010 survives better during acidic shock at pH 4.5

Acid tolerance response has been described previously in many organisms, and most of them were able to tolerate the lethal shock if cells are adapted to growth at a mild acidic pH before placing them in a low pH environment (Chiang et al. 2014; Foster et al. 1995; Merrel et al. 2002). To determine whether the acid adaptation of *V. vulnificus* E4010 would affect the subsequent survival at pH 4.0 and pH 4.5, *V. vulnificus* E4010 was exposed to a mild acid condition at pH 5.0 overnight and challenged at pH 4.5. Fig. 2. illustrates the effect of acid adaptation on the survival of *V. vulnificus* E4010 during acid shock at pH 4.0 and pH 4.5. It is observed that exposure to a mild acid condition enhanced the survival of *V. vulnificus* E4010 at pH 4.5. Acid-adapted *V. vulnificus* E4010 survived up to 40 minutes of exposure at pH 4.0 while unadapted cells survived for only for 20 minutes. At pH 4.5, though bacterial counts declined

about four log units, survival was observed for over 3 h (<0.001). Survival of pathogenic microorganisms under acid stress conditions is often linked to the expression of an adaptive stress response that enhances their ability to withstand harsh environmental conditions. Acid adaptation has been shown to help microorganisms tolerate lethal acid shock (Chiang et al. 2014; Foster et al. 1995; Merrel et al. 2002). Acid adaptation would help the pathogens to maintain a higher intracellular pH, synthesise specific acid shock proteins (ASPs), chaperones, and decarboxylases which would aid coping up with acid stress. (Dahl et al. 2014; Lund et al. 2014; Tetteh and Beuchat 2003). Stationary phase also provides some protection as all the stress-related proteins are upregulated (Navarro et al. 2010) in this phase. Studies indicate that bacterial resistance to acid increases with the increase in the adaptive time provided (Chiang et al. 2012). The results presented here indicate that *V. vulnificus* E4010 can mount an acid tolerance response if adapted to mild acid before the acid shock. These results were consistent with previous observation where *V. vulnificus* without acid adaptation was found to be sensitive at pH 4.0 as well as pH 3.0 with several log reduction whereas acid-adapted cells were able to survive nearly 1 hour at pH 4.0 (Bang et al. 2004; Koo et al. 2000a).

Comparison of adaptive acid tolerance of *Vibrio vulnificus* strains

While the C genotype is associated with clinical strain, many isolates from environmental strains also have displayed C genotype. These findings have further created an interest to investigate whether environmental strains with C genotype have the equal ability to resist acid stress as that of clinical strains. To analyse the strain-dependent acid tolerance response, five strains of *V. vulnificus* were acid-adapted at pH 5.0, overnight. The survival of *V. vulnificus* after exposure at pH 4.5 was studied at every half an hour until 3 h. Examination of four environmental strains and one clinical strain revealed poor resistance to acidic stress. Bactericidal activity of acidic pH

was considerable with 5 log reduction in the bacterial counts irrespective of the strains (Table 2). Since all the strains almost equally responded to the acidic stress, it can be suggested that acid survival is not influenced by the isolation source. Previous studies have revealed that environmental and clinical strains of *V. vulnificus* tested in simulated gastric fluid and acidified Tryptic soy broth at pH 3.0 showed no difference in the survival. When the same strains were tested at pH 4.0, Oyster isolate showed greater resistance. pH 2.0 proved lethal with no strain dependent selectivity for the survival (Koo et al. 2000a, 2000b). Hence there is no clear association between acid resistances of *V. vulnificus* with regard to their isolation source. Role of genotype in the acid survival could not be explained as all the strains belonged to the C genotype.

Cell density-dependent acid survival

Survival of the bacterium at various cell densities was separately checked at pH 4.0 as well as pH 4.5. No particular trend in cell density-dependent survival or sensitivity was observed at pH 4.0. Bacteria at different cell densities were equally susceptible for the acid stress as indicated by no difference in duration of survival after the acid shock. When the experiment was carried out at pH 4.5, there was a clear density-dependent acid resistance observed (<0.001), as bacteria at higher cell density survived better than bacteria at low cell number (Fig. 3). In contrast to this *E. coli* shows density-dependent sensitivity, the bacterial cells at higher density were more prone to acid stress (Datta et al. 1999). However, this phenomenon is mostly present in the strains that are highly acid resistant and absent in *Listeria* and other Gram-positive bacteria. Cell density-dependent acid sensitivity phenotype in *E. coli* has been attributed to limited availability of amino acid at higher cell density, and any role of diffusible substance to this pattern has been ruled out (Cui et al. 2001). Survival assays later reported in *E. coli* revealed more than two order

magnitude of survival in higher cell density. This acid resistance phenotype at higher cell density was explained by the involvement of acid fitness genes encoding putative membrane proteins that take part in the in the acid resistance only at higher cell density (Mates et al. 2007). Larger cell populations influencing the acid adaptation was also found in *Streptococcus mutants* (Li et al. 2001). The role of cell density in acid stress might be related to infectious dose variation in the bacteria. *Vibrio cholerae*, non-typhoidal *Salmonella* and *Shigella flexneri* have an infectious dose of 10^8 , 10^5 , 10^2 respectively among which *V. cholerae* proved to be more acid sensitive (Blaser and Newman. 1982; Cash et al. 1974; DuPont et al. 1989). The pattern of survival observed in this study reflects the need of higher cell density for the effective crossover of the acid barrier of the stomach. Hence the role of the cell population in response to acid stress can be viewed from many dimensions and difference in the cell density-dependent acid sensitivity/resistance with respect to strains observed is not clear. Further studies are required to elucidate the exact basis for the survival pattern observed in this study.

***V. vulnificus* E4010 requires external lysine supplement to tolerate acid stress**

Putrescine, spermidine, spermines are widely distributed polyamines that act as key mediators during the acid stress. Microbial pathogens either synthesize polyamines by decarboxylation of precursor amino acid or they uptake exogenous polyamine. Decarboxylation of amino acid to its respective amine and CO_2 is the strategy used by most of the Gram-negative organisms to neutralize the acidic environment (Shah et al. 2008). Arginine and glutamate system are extensively studied in *E. coli* and shown to be the most effective system in ameliorating the acidic stress (Lin et al. 1996; Iyer et al. 2003). *V. vulnificus* seems to use Lysine decarboxylase as its strategy to combat the acid stress by breaking lysine to cadaverine a highly basic amine. Mutational analysis of *cadA* (lysine decarboxylase) and *cadBA* (lysine/cadaverine antiporter)

have revealed that these gene products are essential in the survival of *V. vulnificus* during acid shock. *cadC* acts as a positive regulator thereby taking part in acid response (Rhee et al. 2002). To gain further insight, in this study lysine-dependent acid response was explored and also the potential role of ornithine was studied. Initially, acid-adapted cells were grown in complex medium (LB broth) were added to the experimental broth (LB broth acidified to pH 4.5) supplemented with lysine and ornithine separately. Results obtained suggests that adaptive growth in complex media at pH 6.0 was not sufficient to rescue the bacteria during the subsequent acid challenge in minimal broth supplemented with the amino acid at pH 4.5. Subsequent attempt to identify the induction of decarboxylase enzyme, lysine and ornithine were supplemented to the adaptive medium (Minimal broth supplemented with 1mM lysine and ornithine separately). The result showed significant rescue was observed in response to lysine supplementation in both clinical as well as environmental strains (<0.001), whereas no response was elicited in response to ornithine. Even though cell count decreased over a period of incubation time, it sustained its growth for 3 h whereas control cells without the induction were diminished within 90 mins (Fig. 4.). Most of the decarboxylase enzymes are induced by their amino acid substrate. Lysine decarboxylase is readily induced in minimal medium supplemented with lysine and peak activity is observed at pH6.0 (He et al. 2017). These factors also determine the induction of *cadBA* (Neely and Olson 1996; Park et al. 1996).

Ornithine has been noted to be one of the low pH-inducible amino acids playing a role in successfully rescuing from acid stress in *Salmonella Typhimurium* (Viala et al. 2011). In the present study no survival was observed with regard to ornithine; hence polyamine mediated induction was tried supplementing the adaptive broth with putrescine spermidine spermines and ornithine followed by studying the survival in experimental media pH 4.5 supplemented with

ornithine. Ornithine decarboxylase induction occurs only in the anoxic condition and activity is peak at neutral pH; thus no rescue was observed and hence the role of ornithine decarboxylase could not be established in *V. vulnificus* acid response. (Kashiwagi et al. 1991; Viala et al. 2011). Induction of lysine decarboxylase in relation to polyamine was not analysed.

Relative expression of the genes involved in the Lysine dependent response

In the context of the clear demonstration of lysine-dependent survival, transcriptional activity of genes contributing to lysine decarboxylation system *cadA* (Lysine decarboxylase), *cadB* (Lysine/Cadaverine antiporter), *cadC* (Positive regulator of *cadBA* operon) *aphB* (Transcriptional regulator of *cadC*) was analyzed. Relative expression levels of all the genes were studied in the adaptive media used for the induction of enzyme (Bacterial cells were grown overnight in minimal broth supplemented with or without lysine at pH6.0 and bacterial cell grown overnight in LB broth at pH 6.0) before the acid shock. Fold change in all the transcript levels was logarithmically expressed (Love et al. 2014). The result showed a striking difference in the expression levels of all the genes in the different media used. Transcriptional levels of all the genes tested were upregulated several folds in the media supplemented with lysine (<0.001), whereas those grown in minimal broth without lysine supplement and grown in LB broth showed minimal expression. The mRNA transcripts of *cadA* & *cadC* were 3.5 fold lower in minimal broth without lysine supplement compared to the expression level in LB broth (Fig. 5.). Reason for this could be because of the availability of lysine in the form of tryptone. Result demonstrated the need for lysine and acidic condition during the adaptive growth phase for the expression of all the genes related to lysine decarboxylation system. Relative expression ratio showed 5.7 fold greater amplification of *cadA* than *cadB* and followed the trend that was observed *Vibrio parahaemolyticus* which showed 10 times higher *cadA* mRNA transcripts than

cadB (Tanaka et al. 2008). Though *cadBA* is transcribed as a single transcriptional unit difference in the expression level observed could be because of translational variation (Lim et al. 2011). In addition, transcriptional regulators (*cadC* & *aphB*) had expression level much higher than that of *cadA* and *cadB*. Overall expression ratio followed the pattern 5: 1: 24: 27 corresponding to *cadA* : *cadB* : *cadC* : *aphB*. In *V. vulnificus* lysine decarboxylation is effective in protecting the bacteria from acid stress as reported earlier. Along with the decarboxylase enzyme, it also requires membrane lysine cadaverine antiporter to deliver amino acid and decarboxylated product from the cytoplasm (Rhee et al. 2002). *cadC* is found to be a positive regulator of *cadBA* operon activates it in a pH-dependent manner (Rhee et al. 2005). Later, LysR family transcriptional regulator *AphB* was identified which directly binds to the promoter of *cadC* and activates the expression (Rhee et al. 2006). The study showed the coordinated and coupled expression of all these gene products is required in the lysine-dependent survival. Lysine decarboxylase activity previously analysed in *V. vulnificus* showed that transcription occurs in the acidic pH and the result obtained in this study confirmed the phenomenon observed.

Survival of *V.vulnificus* inoculated on to the ground oyster

V. vulnificus present naturally in the coastal environment and concentrates especially in the oyster. Consumption raw oyster leads to septicaemia leading to high mortality (Strom and Paranjpye 2000). Assessment of acid tolerance response in the food system makes it more relevant representing the natural scenario. Experiments were undertaken to examine the effect of ground oyster on the survival of bacteria challenged at pH 4.0 and pH 4.5 for 3 h at 37^o C. *V. vulnificus* displayed significantly higher survival rates when inoculated into the food (<0.001). Bacteria have shown sustainability for more than 3 hours at pH 4.5 and more than 1h at pH 4.0 (fig. 6.). Addition of ground oyster to the experimental media has not affected the pH; thus

376 increase in the survival due to the rise in the pH upon the addition of oyster has been ruled out.
377 The acid response of clinical as well as oyster isolate when checked in the simulated gastric fluid
378 at pH 4.0, oyster isolate gave better survival which was consistent with the response in tryptic
379 soy broth containing 2% NaCl (TSB-2) acidified to pH 4.0 (Koo et al. 2000a, 2000b). Reduction
380 in the synergistic effect of heating, thermoradiation and pH in the oyster and fish homogenate
381 has been previously reported (Ama et al. 1994). The protein content of the food allows the
382 bacteria to survive an extremely acidic environment either by providing a microenvironment
383 with higher pH or by encapsulation of the bacteria from acid shock (Waterman and Small 1998).
384 With the understanding of the survival on the addition of ground oyster, the expression of the
385 *cadBA* operon was analyzed. The result demonstrated that there was several fold increase in the
386 relative expression level of the genes involved in the lysine decarboxylation system. The
387 expression pattern of *cadA* (Lysine decarboxylase), *cadB* (Lysine/Cadaverine antiporter), *cadC*
388 (Positive regulator of *cadBA* operon) *aphB* (Transcriptional regulator of *cadC*) showed fold
389 change level of 6.17, 4.35, 1.88 and 1.82 respectively (Table S3). Lysine decarboxylase activity
390 previously analysed in *V. vulnificus* showed that transcription occurs in the acidic pH (Rhee et al.
391 2002). The requirement of lysine for the activation of lysine-dependent pathways has been
392 reported in *E.coli*, *S. Typhimurium* and *V. parahaemolyticus* (Neely and Olson 1996; Park et al.
393 1996; Tanaka et al. 2008). Lysine decarboxylase induction in the natural scenario in *V. vulnificus*
394 can be due to the availability of amino acids present in the oyster. This part of the study needs to
395 be further clarified by exploring the content of the oyster which provides a suitable
396 microenvironment to keep the lysine decarboxylase related genes at a higher level. Overall
397 expression pattern of lysine decarboxylase system remained close to the tendency observed
398 during the lysine dependent response. Altogether information obtained in this study suggests

protection to gut acid when *V. vulnificus* is ingested along with oyster. The precise role of oyster meat that has led to survival has to be further studied.

Conclusion

The ability of the bacteria to withstand adverse acidic environment is the presumptive determinant of the virulence potential of the bacteria. To overcome the acid stress in the host, bacteria have adapted various strategies to survive and multiply in stress condition. Acid adaptation provided a significant survival ratio indicating bacteria's ability to mount the acid resistance. These findings highlight the behaviour of bacteria subjected to mild acid during food processing thereby showing the necessity to consider the phenomenon of acid tolerance response for microbial food safety. Polyamine mediated protection against acid stress was explored, and significant rescue was observed with the supplement of lysine reflecting the role of lysine decarboxylase in ameliorating acidic stress by converting precursor amino acid into its corresponding amines. Induction of lysine decarboxylase requires adaptive growth phase supplemented with lysine for the expression. An interesting observation in the aspect of lysine decarboxylase system revealed the higher expression of cadBA related genes in the presence of oyster, reflecting the protective aspect of the food against the acidity of the stomach. In summary, ability to withstand acid shock when inoculated to the food surface, lysine-dependent acid survival and ability to mount acid tolerance response when acid-adapted reflects the different need of the bacteria to withstand the acid stress.

Acknowledgement: The authors are grateful to the Directorate of Minorities, Government of Karnataka (DOM/FELLOWSHIP/CR-10/2017-18) as well as Science and Engineering Research Board, Department of Science and Technology, Government of India (ECR/2017/000721) for the partial financial support for carrying out this study.

Reference

- Alvarez-Ordenez, A., Fernandez, A., Bernardo, A. and Lopez, M. 2009. Comparison of acids on the induction of an acid tolerance response in *Salmonella typhimurium*, consequences for food safety. Meat. Sci. 81(1): 65-70.
- Alvarez-Ordenez, A., Fernandez, A., Bernardo, A. and Lopez, M. 2010. Arginine and lysine decarboxylases and the acid tolerance response of *Salmonella Typhimurium*. Int. J. Food. Microbiol. 136(1): 278-282.
- Ama, A.A., Hamdy, M.K. and Toledo, R.T. 1994. Effect of heating, pH and thermoradiation on inactivation of *Vibrio vulnificus*. Food. Microbiol. 11(3): 215-227.
- Bang, W. and Drake, M.A. 2005. Acid adaptation of *Vibrio vulnificus* and subsequent impact on stress tolerance. Food. Microbiol. 22(4): 301-309.
- Blaser, M.J. and Newman, L.S. 1982. A review of human salmonellosis: I. Infective dose. Rev. Infect. Dis. 4(6): 1096-1106.
- Cash, R.A., Music, S.I., Libonati, J.P., Snyder, M.J., Wenzel, R.P. and Hornick, R.B. 1974. Response of man to infection with *Vibrio cholerae*. I. Clinical, serologic, and bacteriologic responses to a known inoculum. J. Infect. Dis. 129(1): 45-52.

- 442 Chase, E. and Harwood, V.J. 2011. Comparison of the Effects of Environmental Parameters on
443 Growth Rates of *Vibrio vulnificus* Biotypes I, II and III by Culture and Quantitative PCR.
444 Appl. Environ. Microbiol. 77(12): 4200-4207.
- 445 Chen, Y., Liming, S.H. and Bhagwat, A.A. 2004. Occurrence of inhibitory compounds in spent
446 growth media that interfere with acid-tolerance mechanisms of enteric pathogens. Int. J.
447 Food. Microbiol. 91(2): 175-183.
- 448 Chiang, M.L., Chen, H.C., Wu, C. and Chen, M.J. 2014. Effect of acid adaptation on the
449 environmental stress tolerance of three strains of *Vibrio parahaemolyticus*. Foodborne.
450 Pathog. Dis. 11(4): 287-294.
- 451 Chiang, M.L., Chou, C.C., Chen, H.C., Tseng, Y.T. and Chen, M.J. 2012. Adaptive acid
452 tolerance response of *Vibrio parahaemolyticus* as affected by acid adaptation conditions,
453 growth phase, and bacterial strains. Foodborne. Pathog. Dis. 9(8): 734-740.
- 454 Cui, S., Meng, J. and Bhagwat, A.A. 2001. Availability of Glutamate and Arginine during Acid
455 Challenge Determines Cell Density-Dependent Survival Phenotype of *Escherichia coli*
456 Strains. Appl. Environ. Microbiol. 67(10): 4914-4918.
- 457 Dahl, J.U., Koldewey, P., Salmon, L., Horowitz, S., Bardwell, J.C. and Jakob, U. 2015. HdeB
458 functions as an acid-protective chaperone in bacteria. J. Biol. Chem. 290(1): 65-75.
- 459 Datta, A.R. and Benjamin, M.M. 1999. Cell density dependent acid sensitivity in stationary
460 phase cultures of enterohemorrhagic *Escherichia coli* O157: H7. FEMS. Microbiol. Lett.
461 181(2): 289-295.
- 462 DuPont, H.L., Levine, M.M., Hornick, R.B. and Formal, S.B. 1989. Inoculum size in shigellosis
463 and implications for expected mode of transmission. J. Infect. Dis. 159(6): 1126-1128.

- 464 Foster, J.W. 1995. Low pH adaptation and the acid tolerance response of *Salmonella*
465 *typhimurium*. Crit. Rev. Microbiol. 21(4): 215-237.
- 466 Gorden, J. and Small, P. 1993. Acid resistance in enteric bacteria. Infect. Immun. 61(1): 364-
467 367.
- 468 He, A., Penix, S.R., Basting, P.J., Griffith, J.M., Creamer, K.E., Camperchioli, D., Clark, M.W.,
469 Gonzales, A.S., Erazo, J.S.C., George, N.S. and Bhagwat, A.A. 2017. Acid evolution of
470 *Escherichia coli* K-12 eliminates amino acid decarboxylases and reregulates catabolism.
471 Appl. Environ. Microbiol. 83(12): e00442-17.
- 472 Hersh, B.M., Farooq, F.T., Barstad, D.N., Blankenhorn, D.L. and Slonczewski, J.L. 1996. A
473 glutamate-dependent acid resistance gene in *Escherichia coli*. J. Bacteriol. 178(13):
474 3978-3981.
- 475 Horseman, M.A. and Surani, S. 2011. A comprehensive review of *Vibrio vulnificus*: an
476 important cause of severe sepsis and skin and soft-tissue infection. Int. J. Infect. Dis.
477 15(3) e157-166.
- 478 Iyer, R., Williams, C. and Miller, C. 2003. Arginine-agmatine antiporter in extreme acid
479 resistance in *Escherichia coli*. J. Bacteriol. 185(22): 6556-6561.
- 480 Jones, M.K. and Oliver, J.D. 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect. Immun.
481 77(5): 1723-1733.
- 482 Kanjee, U. and Houry, W.A., 2013. Mechanisms of acid resistance in *Escherichia coli*. Annu.
483 Rev. Microbiol. 67: 65-81.
- 484 Kashiwagi, K., Suzuki, T., Suzuki, F., Furuchi, T., Kobayashi, H. and Igarashi, K. 1991.
485 Coexistence of the genes for putrescine transport protein and ornithine decarboxylase at
486 16 min on *Escherichia coli* chromosome. J. Biol. Chem. 266(31): 20922-20927.

- 487 Koo, J., DePaola, A. and Marshall, D.L. 2000a. Effect of simulated gastric fluid and bile on
488 survival of *Vibrio vulnificus* and *Vibrio vulnificus* phage. J. Food. Prot. 63(12): 1665-
489 1669.
- 490 Koo, J., DePaola, A. and Marshall, D.L. 2000b. Impact of acid on survival of *Vibrio vulnificus*
491 and *Vibrio vulnificus* phage. J. Food. Prot. 63(8): 1049-1052.
- 492 Koutsoumanis, K.P. and Sofos, J.N. 2004. Comparative acid stress response of *Listeria*
493 *monocytogenes*, *Escherichia coli* O157: H7 and *Salmonella Typhimurium* after
494 habituation at different pH conditions. Lett. Appl. Microbiol. 38(4): 321-326.
- 495 Li, Y.H., Hanna, M.N., Svensater, G., Ellen, R.P. and Cvitkovitch, D.G. 2001. Cell density
496 modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms.
497 J. Bacteriol. 183(23): 6875-6884.
- 498 Lianou, A., Nychas, G.J.E. and Koutsoumanis, K.P. 2017. Variability in the adaptive acid
499 tolerance response phenotype of *Salmonella enterica* strains. Food. Microbiol. 62: 99-
500 105.
- 501 Lim, H.N., Lee, Y. and Hussein, R. 2011. Fundamental relationship between operon organization
502 and gene expression. Proc. Natl. Acad. Sci. 108(26): 10626-10631.
- 503 Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N. and Foster, J.W. 1996. Mechanisms
504 of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol.
505 62(9): 3094-3100.
- 506 Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time
507 quantitative PCR and the 2- $\Delta\Delta CT$ method. Methods. 25(4): 402-408.
- 508 Love, M.I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion
509 for RNA-seq data with DESeq2. Genome. Biol. 15(12): 550.

- 510 Lund, P., Tramonti, A. and De Biase, D. 2014. Coping with low pH: molecular strategies in
511 neutralophilic bacteria. FEMS. Microbiol. Rev. 38(6): 1091-1125.
- 512 Mates, A.K., Sayed, A.K. and Foster, J.W. 2007. Products of the *Escherichia coli* acid fitness
513 island attenuate metabolite stress at extremely low pH and mediate a cell density-
514 dependent acid resistance. J. Bacteriol. 189(7): 2759-2768.
- 515 Merrell, D.S. and Camilli, A. 1999. The *cadA* gene of *Vibrio cholerae* is induced during
516 infection and plays a role in acid tolerance. Mol. Microbiol. 34(4): 836-849.
- 517 Merrell, D.S. and Camilli, A. 2000. Regulation of *Vibrio cholerae* genes required for acid
518 tolerance by a member of the "ToxR-like" family of transcriptional regulators. J.
519 Bacteriol. 182(19): 5342-5350.
- 520 Merrell, D.S. and Camilli, A. 2002. Acid tolerance of gastrointestinal pathogens. Curr. Opin.
521 Microbiol. 5(1): 51-55.
- 522 Navarro Llorens, J.M., Tormo, A. and Martinez-Garcia, E. 2010. Stationary phase in gram-
523 negative bacteria. FEMS. Microbiol. Rev. 34(4): 476-495.
- 524 Neely, M.N. and Olson, E.R. 1996. Kinetics of expression of the *Escherichia coli* cad operon as
525 a function of pH and lysine. J. Bacteriol. 178(18): 5522-5528.
- 526 Oliver, J.D. 2013. *Vibrio vulnificus*: death on the half shell. A personal journey with the
527 pathogen and its ecology. Microb. Ecol. 65(4): 793-799.
- 528 Park, Y.K., Bearson, B., Bang, S.H., Bang, I.S. and Foster, J.W. 1996. Internal pH crisis, lysine
529 decarboxylase and the acid tolerance response of *Salmonella typhimurium*. Mol.
530 Microbiol. 20(3): 605-611.
- 531 Phillips, K.E. and Satchell, K.J. 2017. *Vibrio vulnificus*: From oyster colonist to human
532 pathogen. PLoS. Pathog. 13(1): e1006053.

- 533 Rhee, J.E., Rhee, J.H., Ryu, P.Y., and Choi, S.H. 2002. Identification of the *cadBA* operon
534 from *Vibrio vulnificus* and its influence on survival to acid stress. FEMS. Microbiol. Lett.
535 208(2): 245-251.
- 536 Rhee, J.E., Ju, H.M., Park, U., Park, B.C. and Choi, S.H. 2004. Identification of the *Vibrio*
537 *vulnificus cadC* and evaluation of its role in acid tolerance. J. Microbiol. Biotechnol.
538 14(5): 1093-1098.
- 539 Rhee, J.E., Kim, K.S. and Choi, S.H. 2005. CadC activates pH-dependent expression of the
540 *Vibrio vulnificus cadBA* operon at a distance through direct binding to an upstream
541 region. J. Bacteriol. 187(22): 7870-7875.
- 542 Rhee, J.E., Jeong, H.G., Lee, J.H. and Choi, S.H. 2006. AphB influences acid tolerance of *Vibrio*
543 *vulnificus* by activating expression of the positive regulator CadC. J. Bacteriol. 188(18):
544 6490-6497.
- 545 Sangeetha, M.S., Shekar, M. and Venugopal, M.N. 2017. Occurrence of clinical genotype *Vibrio*
546 *vulnificus* in clam samples in Mangalore, Southwest coast of India. J. Food. Sci. Technol.
547 54(3): 786-791.
- 548 Shah, P. and Swiatlo, E. 2008. A multifaceted role for polyamines in bacterial pathogens. Mol.
549 Microbiol. 68(1): 4-16.
- 550 Soksawatmaekhin, W., Kuraishi, A., Sakata, K., Kashiwagi, K. and Igarashi, K. 2004. Excretion
551 and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*.
552 Mol. Microbiol. 51(5) 1401-1412.
- 553 Strom, M.S. and Paranjpye, R.N. 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*.
554 Microbes. Infect. 2(2): 177-188.

- 555 Tanaka, Y., Kimura, B., Takahashi, H., Watanabe, T., Obata, H., Kai, A., Morozumi, S. and
556 Fujii, T. 2008. Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of
557 transcription and role in acid resistance. J. Appl. Microbiol. 104(5): 1283-1293.
- 558 Tetteh, G.L. and Beuchat, L.R. 2003. Exposure of *Shigella flexneri* to acid stress and heat shock
559 enhances acid tolerance. Food. Microbiol. 20(2): 179-185.
- 560 Viala, J.P., Meresse, S., Pocachard, B., Guilhaon, A.A., Aussel, L. and Barras, F. 2011. Sensing
561 and adaptation to low pH mediated by inducible amino acid decarboxylases in
562 *Salmonella*. PLoS. One. 6(7): e22397.
- 563 Wang, Y. and Gu, J. 2005. Influence of temperature, salinity and pH on the growth of
564 environmental *Aeromonas* and *Vibrio* species isolated from Mai Po and the Inner Deep
565 Bay Nature Reserve Ramsar Site of Hong Kong. J. Basic. Microbiol. 45(1): 83-93.
- 566 Waterman, S.R. and Small, P.L.C. 1998. Acid-sensitive enteric pathogens are protected from
567 killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain
568 solid food sources. Appl. Environ. Microbiol. 64(10): 3882-3886.
- 569
- 570
- 571
- 572
- 573
- 574
- 575
- 576
- 577

Figure legends

Fig.1. Effects of different pH on the growth of *V. vulnificus* in laboratory condition. Bacterial cell density was measured spectrophotometrically at 600 nm.

Fig. 2. Survival of *V. vulnificus* E4010 in pH 4.0 (A) and pH 4.5(B). Bars represent the standard error from three replication.

Fig. 3. Effect of cell density of *V. vulnificus* E4010 on survival at pH 4.0 (A) and pH 4.5 (B). Bars represent the standard error from three replication

Fig. 4. Lysine dependent acid survival: *V. vulnificus* adapted in minimal broth supplemented with lysine at pH6.0 and challenged in minimal broth at pH 4.5 supplemented with lysine (●) *V. vulnificus* adapted in LB broth at pH6.0 and exposed to minimal broth at pH 4.5 supplemented with lysine (■) *V. vulnificus* grown without lysine supplement in minimal broth and exposed to minimal broth at pH 4.5 with lysine supplement (▲). *V. vulnificus* grown without lysine supplement in minimal broth at pH 6.0 and exposed to minimal broth at pH 4.5 without lysine supplement (▼)

Fig. 5. Schematic representation of lysine decarboxylation system-related genes and their relative expression in the media (MB or LB) acidified to pH6 supplemented with or without lysine grown for 12 hrs at 37⁰ C.

Fig. 6. Survival of *V. vulnificus* E4010 inoculated on to oyster meat in pH 4.0(A) and pH 4.5(B). Bars represent the standard error from three replications. *V. vulnificus* has grown overnight to obtain stationary phase at pH 7.0 and approximately 10^7 - 10^8 CFU/mL cells were then subjected for acid challenge studies. Bacteria's were inoculated onto the surface of oyster and then subjected for acid challenge (●) Bacteria inoculated to the acid challenge media directly were considered as the control sample (○)

Draft

Table 1: Adaptive acid tolerance of different *V. vulnificus* strains

Strain	Mean % of survival ^a
E4010	<u>0.0045+0.3</u>
E3715	<u>0.0005+0.2</u>
E3716	<u>0.009+0.1</u>
E3717	<u>0.0025+0.2</u>
K4633	0.0023±0.01

Survival percentage of bacterial strains after 1 h of acid exposure at pH 4.5

V. vulnificus cells were grown overnight to obtain the stationary phase culture. Acid challenge assays were performed in the LB broth acidified to pH 4.5 with the final bacterial concentration of 1×10^7 CFU/ml at 0 h. Surviving percentage was obtained at 1 h of acid exposure by considering the cells at 0 h as 100%.

640 **Supplementary information**

641 Table S1: Primers used in the study

642 Table S2: Standardization of the experimental protocol for the acid challenge studies

643 Table S3: Expression level of *cadBA* related genes upon the addition of ground oyster

Draft

Fig.1. Effects of different pH on the growth of *V. vulnificus* in laboratory condition. Bacterial cell density was measured spectrophotometrically at 600 nm.

91x53mm (300 x 300 DPI)

Fig. 2. Survival of *V. vulnificus* E4010 in pH 4.0 (A) and pH 4.5(B). Bars represent the standard error from three replication.

157x58mm (300 x 300 DPI)

Fig. 3. Effect of cell density of *V. vulnificus* E4010 on survival at pH 4.0 (A) and pH 4.5 (B). Bars represent the standard error from three replication

73x26mm (300 x 300 DPI)

Fig. 4. Lysine dependent acid survival: *V. vulnificus* adapted in minimal broth supplemented with lysine at pH6.0 and challenged in minimal broth at pH 4.5 supplemented with lysine (●) *V. vulnificus* adapted in LB broth at pH6.0 and exposed to minimal broth at pH 4.5 supplemented with lysine (■) *V. vulnificus* grown without lysine supplement in minimal broth and exposed to minimal broth at pH 4.5 with lysine supplement (△). *V. vulnificus* grown without lysine supplement in minimal broth at pH 6.0 and exposed to minimal broth at pH 4.5 without lysine supplement (▽)

81x58mm (300 x 300 DPI)

Fig. 5. Schematic representation of lysine decarboxylation system-related genes and their relative expression in the media (MB or LB) acidified to pH6 supplemented with or without lysine grown for 12 hrs at 37 C.

88x59mm (300 x 300 DPI)

Fig. 6. Survival of *V. vulnificus* E4010 inoculated on to oyster meat in pH 4.0(A) and pH 4.5(B). Bars represent the standard error from three replications. *V. vulnificus* has grown overnight to obtain stationary phase at pH 7.0 and approximately 107-108 CFU/mL cells were then subjected for acid challenge studies. Bacteria's were inoculated onto the surface of oyster and then subjected for acid challenge (●) Bacteria inoculated to the acid challenge media directly were considered as the control sample (○)

72x26mm (300 x 300 DPI)