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Viable but Non-Culturable State (VBNC) of *Escherichia coli* Related to EnvZ under the Effect of pH, Starvation and Osmotic Stress in Sea Water

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Abstract

When exposed extreme environmental conditions such as sea water, bacteria have been shown different survival strategy for continue their life. One of this strategy known as viable but nonculturable (VBNC) state which is very important for nondifferiation bacteria. VBNC cells cause serious human health problems. Little is known, however, about the genetic mechanisms underlying the VBNC state. Under different environmental conditions, porins are important in the survival strategy of bacteria. EnvZ/OmpR work together as regulators of *ompF* and *ompC* gene expression. It is known that the EnvZ system has a role in VBNC state. In this study we tried to find out the viability of EnvZ, OmpC and OmpF mutant *E. coli* under stress effect of osmolarity, pH and starvation. Bacteria were suspended in filtered-autoclaved sea water microcosms and numbers determined over 25 day incubation periods by plate count (PC), direct viable count (DVC) and count of cells capable of respiration (RCC). As regard to results, alkaline pH affected *E. coli* more than acidic pH, which led to decline in number. On the contrary glycine betaine addition to sea water protected *E. coli* porin mutants and also reduced the death rate of bacteria. Under the effect of pH, osmotic stress and starvation stress, wild type *E. coli* and porin mutants entered a dormant state or became VBNC with the exception of MSZ31 (*env*Z mutant) *E.coli* cells which did not enter the VBNC state under the three tested stress conditions. This study is the first report to demonstrate that *E. coli* could not enter the VBNC state in the lack of EnvZ product under the stress of osmolarity, pH and starvation and the relationship between EnvZ and VBNC state are not affected by pH, osmolarity and starvation.

K e y w o r d s: *Escherichia coli*, EnvZ, porins, sea water, VBNC

Introduction

The survival of bacteria in non-optimal environments depends on their ability to regulate and control their metabolism in such a way that they survive suboptimal conditions. In the aquatic environment, various factors including temperature (Özkanca, 1993), starvation stress (Kjelleberg and Hermannson, 1987), osmolarity (Munro *et al*., 1989), pH (Rozen and Belkin, 2001), predation by protozoa (McCambridge and McMeekin, 1983), bacteriophage infection (Gurijala and Alexander, 1990), exposure to visible and UV light (Davies and Evison, 1991) affect the survival of enteric bacteria. Induction of physiological and morphological changes in *Escherichia coli* greatly increases its chances of survival in the aquatic environment. Many enteric bacteria have been shown to enter a viable but non-culturable (VBNC) state in response to such conditions (Barcina *et al.*, 1997; Colwell and Gray 2000). Most microbiologists accept that the change to a VBNC state is an adaptive response to adverse environmental conditions in non-spore forming bacteria which cannot undergo a differentiation process.

The outer membrane consists predominately of phospholipids, lipopolysaccharide and outer membrane proteins (Koebnik *et al.*, 2000). The outer membrane is the first important barrier between Gram-negative bacteria and the surrounding environment. Outer membrane proteins allow for the passive diffusion of small charged and uncharged molecules into bacterial cells. The uptake of nutrients from impoverished environments or under stress is vital to the survival of bacterial cells. The major outer membrane proteins in *E. coli* are OmpF and OmpC and are regulated at the level of transcription by OmpR which

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responds primarily to changes in osmolarity (Nikaido and Vaara 1985; Forst and Inouye 1988). OmpF is a non-specific porin which allows the uptake of small molecular weight nutrients and has what is widely regarded as an indispensable function. This type of porin is widespread among enteric bacteria and is often one of the most abundant porins in the outer membrane of rapidly growing cells. Changes in its concentration in response to environmental stimuli therefore can have major consequences for the survival and growth of the bacteria cell. Changes in the relative amounts of omps (outer membrane proteins) because of natural environmental conditions affect *E. coli* survival (Özkanca and Flint 2002). Lack of porin synthesis through the action of the regulatory proteins OmpR, may cause a serious survival problem for *Escherichia coli* in sea water. Although OmpC porin concentrations are higher in the outer membrane of *E. coli* cells when challenged with high concentrations of acid, OmpC does not appear in cells which have been adapted for growth in alkaline environments. Also, OmpF porin concentrations are higher in alkaline environment than in acid environment (Sato *et al.*, 2000; Sainz *et al.*, 2005; Darcan, 2005; Darcan *et al.*, 2009). These authors concluded that some of the pathogenic potential as well as the survival of *E. coli* in acid environments was likely to be due to the relative concentrations of porins, especially OmpC, OmpF and OmpX, in the outer membrane.

Darcan *et al.* (2003) showed that mutants with defective or absent omps or with related mutations, such as those that inactivate the products of the *envZ* and *ompR* genes, survived poorly compared to the wild-type strain. They suggested that these proteins were important in determining the entry of *E. coli* into the survival state. The omps (especially OmpC and OmpF), the OmpR regulator protein and the EnvZ sensor protein play an important role in the membrane transport of solutes into *E. coli* and some other Gram-negative bacteria (Sato *et al.*, 2000). However, the role of porin proteins in the survival of enteric bacteria in natural environments and their changes during bacterial starvation, changes in osmolarity and pH and during the entry into the viable but non-culturable (VBNC) state still need to be clarified. The pores created in the outer membranes by the porin proteins allow hydrophilic substrate molecules to enter the cell (Nikaido and Vaara, 1985). Hence, they are important molecules for the uptake and transport of substrate scavenged from the aquatic environment. A reduction in the types and concentrations of porin proteins or mutations in the sensory and regulatory mechanisms associated with porins may lead to serious survival problems for aquatic bacteria. It has previously been shown that the concentrations of outer membrane porins changed as bacteria entered the VBNC state and adapted to be able to survive stress conditions (Özkanca *et al.*, 2002).

The VBNC state prevents cells growing on media normally used for their cultivation but such cells retain respiration ability and presumably their pathogenic potential (Sylvester *et al.*, 2001). Any changes which affect the ability of *E. coli* to grow on agar plates has serious consequences when this bacterium is being use as an indicator of faecal pollution. It is of great concern that changes in environmental conditions can alter the ability of this organism to produce colonies when incubated on non-selective or selective media because the methodologies legally accepted for detecting *E. coli* rely on culturing methods. The aquatic environment has been shown to have serious effects on the ability of *E. coli* and other enteric bacteria and their ability to survive in a VBNC state is possible as the respiring cell count is always higher than the viable plate count.

In this study we tried to understand the effect of osmolarity, pH and starvation stress on the relation between VBNC and osmosensor EnvZ and porins. Moreover, the experiments reported here were carried out in order to investigate the survival of porin-deficient mutants under the effect of pH, starvation and osmolarity in filtered-autoclaved sea water. This may help in understanding the role played by omps in the survival of *E. coli* in sea water environments.

Experimental

Material and Methods

Bacterial strains and culture conditions. The *E. coli* strains used in this study are shown in Table I. These were grown in 5 ml nutrient broth at 37°C. After 24 h incubation the culture was harvested by centrifugation, washed twice in filtered-autoclaved Black Sea water and resuspended to a final concentration of approximately 5×10^8 colony forming units (cfu) ml^{-1}.

Sea water was collected from Black Sea coastal area in Samsun, Turkey, filtered through a Whatman No 1 filter and autoclaved at 121°C for 15 min. Flasks (250 ml) containing filtered autoclaved sea water (100 ml) were inoculated with 1 ml of resuspended bacterial culture to give a final bacterial concentration in the microcosms of 5×10^6 cfu ml^{-1}.

Where appropriate, flasks were amended with a final concentration of 1 mM glycine betaine as an osmoprotectant. pH of the microcosms was adjusted to pH 8.3 by the addition of 5 mM N-Tris (hydroxymethyl)-methylglycine (Tricine, Serva), to pH 7.2 by the addition of 5 mM 3-(N-morpholino) propane sulfonic acid (MOPS, Sigma) and to pH 5.5 by the addition of 5 mM 2-(N-morpholino) ethanesulfonic

Table I
Escherichia coli strains used in this study

Strain	Genotype	Reference
W3110	Wild type	Sato *et al*. (2000)
MH1160	MC4100 *ompR101*	Hall and Silhavy (1981)
MSZ31	W3110 *envZ*::*Kan*	Sato *et al*. (2000)
MKCF36	MH20 (*ompF-lacZ*)*16-21*(Hyb) *ompF*$^-$ *ompC*::*Kan*	Sato *et al*. (2000)
MH621	MH20 (*ompF-lacZ*)*16-21*(Hyb) *ompF*$^-$ *ompC*$^+$	Ferrario *et al*. (1995)
MKC505	MH20 (*ompF-lacZ*)*16-21*(Hyb) *ompF*$^+$ *ompC*::*Kan*	Sato *et al*. (2000)

acid monohydrate (MES, Serva). All the microcosms were wrapped in aluminium foil to exclude light and incubated at 37°C for up to 25 days.

Plate counts. The culturability of *E. coli* was assessed by estimation of a viable count using a standard surface spread plate technique. All microcosms were sampled immediately after incubation and at regular intervals thereafter. Samples were serially diluted in quarter strength Ringers' solution and aliquots (100 µl) spread on triplicate nutrient agar plates. Bacteria were incubated for 36 hours at 37°C and were counted as colony forming units after this period of incubation.

Direct viable count. The method of Kogure *et al.* (1979) was used to estimate the direct viable count. Nucleopore track-etch membrane (25 mm diameter, pore size 0.1 µm, Whatman) filters were used for epifluorescent microscope counts. Filters were immersed in a solution of Sudan Black B for 48 h. Stained filters, supported on a Swinnex 25 mm diameter filter discs (Millipore, Watford, Herts, UK) were washed with double distilled water before passage of the sample. After filtration, 0.5 ml acridine orange (0.01% w/v) in 6.6 mM phosphate buffer, pH 6.7) was dropped onto the filters for few minutes. The filters were air dried and cut into pieces. One piece covered with immersion oil and a cover slip and examined using a fluorescence microscope (Nikon Eclipse E 600) under UV light. The number of bacteria was determined in each square of eyepiece graticule. Bacteria were counted from 20 random fields from the central point to edge.

Respiring cell count. 5-Cyano-2,3 ditolyl tetrazolium chloride (CTC) was used to obtain a respiring cell count in the samples using the method of Rodriguez *et al*. (1992). 1 ml sample was transferred to the test tube then CTC was added to tubes and final concentration adjusted to 2 mM. The test tubes were wrapped with aluminium folio and shaked 160 rpm at 37°C for 3 hours. After that, 0.5% of formaldehyde was added to tubes for fixation in order to stop bacterial activity. After filtration (Nuclepore track-etched membrane), 0.5 ml DAPİ (final concentration 15 µg/ml) was dropped onto the filters for 10 minutes. The filters were dried in the air and counted under the epifluorescence microscopy with appropriate an optical standard filter combination for visualization of the dyes. The respiring cell counts were examined CTC formazan crystals by using blue light excitation filter, then the filter set was changed to a UV excitation filter set to determine total cell counts by DAPI. Bacteria were counted from 20 random fields from the central point to edge.

Expression of count data. All count data were expressed as mean of log counts from four replicate experiments. The gradients of the log linear survival curves are expressed as k d^{-1} and the time needed for a two log drop in number is expressed as t_{99} value.

Results

Incubating the various *E. coli* strains used in this study in the dark and in the presence and absence of glycine betaine or changing pH should enable a further insight into the behaviour of enteric bacteria in response to changing environmental conditions from a rich environment such as nutrient broth to an impoverished environment such as filtered-autoclaved sea water. Determination of the numbers of bacteria surviving under each condition by viable plate count, direct viable count and respiring cell count will enable the determination of the entry into the VBNC state for these strains under these conditions. The difference in counts between the viable plate count and the respiring cell count is taken to mean that there is a proportion of the bacterial population which cannot grow on non-selective medium despite still being capable of respiration. The use of strains deficient in a number of omps and the mechanism for regulating changes in porin concentrations in the outer membrane will also highlight the importance of the outer membrane in the survival process of bacteria, the relation between EnvZ and VBNC state.

The effect of starvation stress on porin-deficient *E. coli.* Wild-type *E. coli* W3110 and porin-deficient mutants were incubated in filter-autoclaved sea water for 25 days at 37°C. Plate counts were carried out every 5 days during this period (Fig. 1). Respiring cell count and direct viable count were carried out every 5 days started after 15 days of incubation. Table II

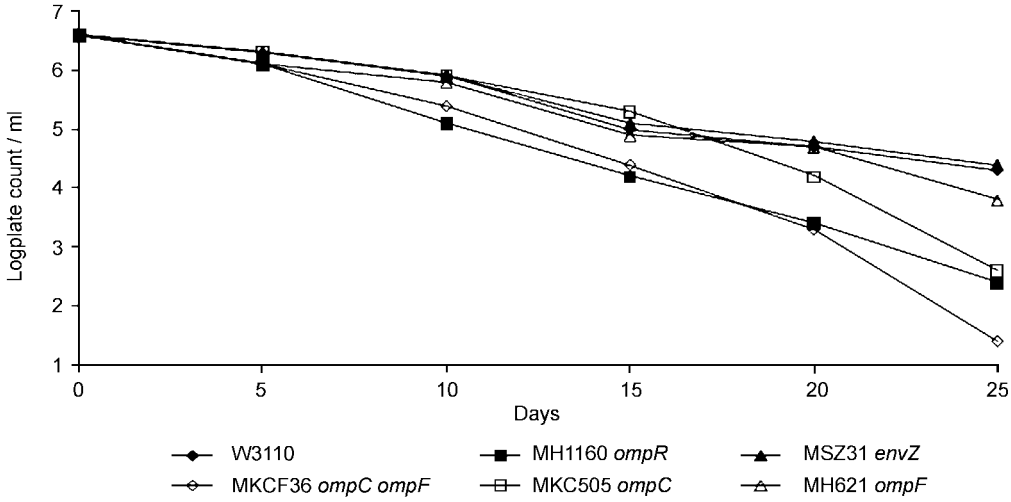

Fig. 1. Survival of *Escherichia coli* in filtered autoclaved sea water.

shows the k and t_{99} values calculated for this experiment and Table III shows the values for the various methods of plate counting after 25 d incubation. After 25 days of incubation, the wild type strain had reduced in viable count by 2.3 logs compared to the initial count. The decline in numbers was log linear with a k value of –0.0983 d^{-1} and a t_{99} of 20.3 d. *E. coli* MSZ31, the strain deficient in *envZ* gene survived for exactly the same length of time (t_{99}=21.4 d). The porin-deficient mutants MH621 (*ompF*$^-$), MKC505 (*ompC*$^-$) and MKCF36 (*ompF*$^-$ and *ompC*$^-$ double mutant) had t_{99} values of 18.3, 13.0 and 9.9 d respectively (Table II). *OmpR* regulator mutations resulted in 4.2 log reduction in cell number with a t_{99} value of 11.7 d. The lack of omps seriously affects *E. coli* survival. In addition to determination of viability through

Table II
The effect of starvation on the viable count of *Escherichia coli* in filtered-autoclaved sea water in the presence and absence of the osmoprotectant glycine betaine

Strain	Absence of glycine betaine		Presence of glycine betaine	
	k	t_{99}	k	t_{99}
W3110 (wild type)	–0.0983	20.3	–0.092	21.7
MH1160 (*ompR*$^-$)	–0.1714	11.7	–0.1331	15.0
MSZ31(*envZ*$^-$)	–0.0931	21.4	–0.1069	18.7
MKCF36 (*ompF*$^-$*ompC*$^-$)	–0.2023	9.9	–0.1069	18.7
MKC505 (*ompC*$^-$)	–0.1537	13.0	–0.1034	19.3
MH621 (*ompF*$^-$)	–0.1091	18.3	–0.0983	20.3

Table III
The effects of starvation in the presence and absence of glycine betaine on the survival of *Escherichia coli* in filtered-autoclaved sea water by a variety of enumeration methods after 25 days of incubation (Initial bacterial count is approximately 6.6 cfu/ml)

Strain	Viable plate count (VC)		Direct viable count (DVC)		Respiring cell count (RCC)		Total cell count (TC)	
	+ GB	– GB	+ GB	– GB	+ GB	– GB	+ GB	– GB
W3110 (wild type)	4.4 ± 0.06	4.3 ± 0.10	4.5 ± 0.06	4.4 ± 0.10	5.2 ± 0.03	4.8 ± 0.05	6.0 ± 0.02	5.9 ± 0.04
MH1160 (*ompR*$^-$)	3.6 ± 0.10	2.4 ± 0.07	3.7 ± 0.10	2.5 ± 0.10	4.6 ± 0.07	4.5 ± 0.03	5.7 ± 0.09	5.6 ± 0.06
MSZ31 (*envZ*$^-$)	4.1 ± 0.03	4.4 ± 0.04	4.2 ± 0.05	4.5 ± 0.04	4.1 ± 0.07	4.4 ± 0.06	5.9 ± 0.03	5.8 ± 0.02
MKCF36(*ompF*$^-$*C*$^-$)	3.7 ± 0.04	1.4 ± 0.30	3.9 ± 0.06	1.6 ± 0.40	4.8 ± 0.01	4.1 ± 0.02	5.6 ± 0.09	5.6 ± 0.06
MKC505 (*ompC*$^-$)	4.1 ± 0.08	2.6 ± 0.08	4.2 ± 0.09	2.8 ± 0.05	4.9 ± 0.07	4.5 ± 0.04	5.8 ± 0.04	5.8 ± 0.03
MH621 (*ompF*$^-$)	4.3 ± 0.06	3.8 ± 0.10	4.4 ± 0.06	3.9 ± 0.10	4.9 ± 0.06	4.7 ± 0.07	5.9 ± 0.03	5.8 ± 0.07

All counts are expressed as mean log count per ml ± standard deviation of 4 replicates.

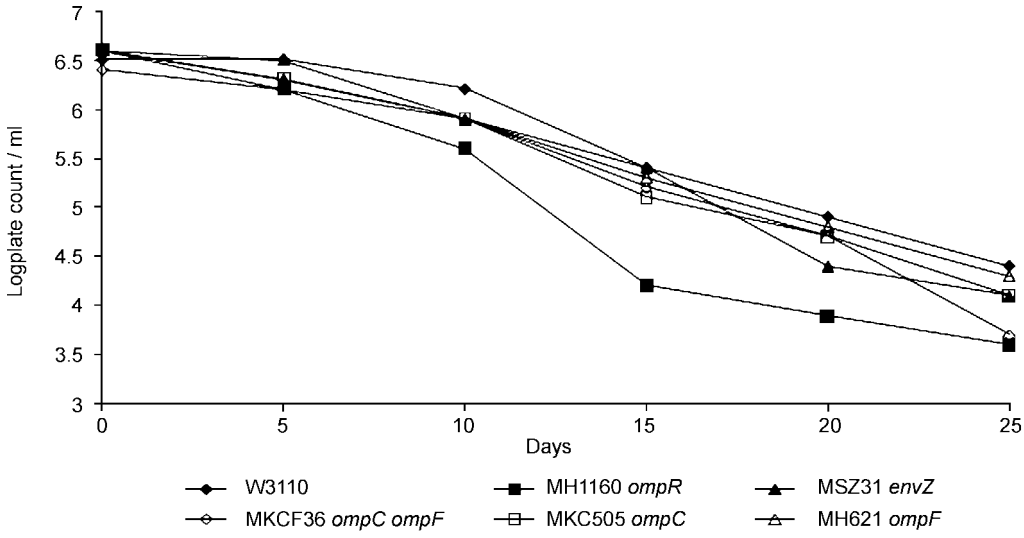

Fig. 2. Survival of *Escherichia coli* in filtered autoclaved sea water in the presence of glycine betaine.

the determination of viable cells able to form colonies using a plate count technique, respiring cell count and direct viable count were also carried out as a measure of possible entry into a VBNC state in filtered-autoclaved sea water microcosms. The total cell count did not change in any of the samples as there was no significant difference between the final total counts in any sample. The respiring cell count also revealed similar values in all cases although the direct viable count and viable plate count values are markedly different. The decline in direct viable count mirror those of the plate count and suggest that although cells are still capable of respiration they cannot grow on nutrient agar plates (a characteristic of the VBNC state). Interestingly, there was no difference between plate count and respiring cell count in *envZ* mutant cells.

The effect of osmolarity on VBNC related to EnvZ/OmpR phosphorelay system. Glycine betaine is often used as an osmoprotectant and was added to filtered-autoclaved sea water to investigate the effect of addition of an osmoprotectant on survival of porin-deficient mutants in sea water. The addition of glycine betaine to sea water microcosm led to an increase in the survival time of the wild type *E. coli* W3110 from a t_{99} value of 20.3 d than 21.7 d (Fig. 2 and Table III). This was a small put reproducible increase in survival in the presence of the osmoprotectant. With the exception of the *envZ* mutant, glycine betaine led to an increase in the survival times for all the omp mutants tested. Notable the survival of the *ompF*/*ompC* double mutant was increased from a t_{99} value of 9.9 d in the absence of the osmoprotectant to 18.7 d in the presence of it. As in the absence of glycine betaine, the total cell count remained relatively constant throughout the 25 day incubation period (Table III). An interesting result obtained here that the addition

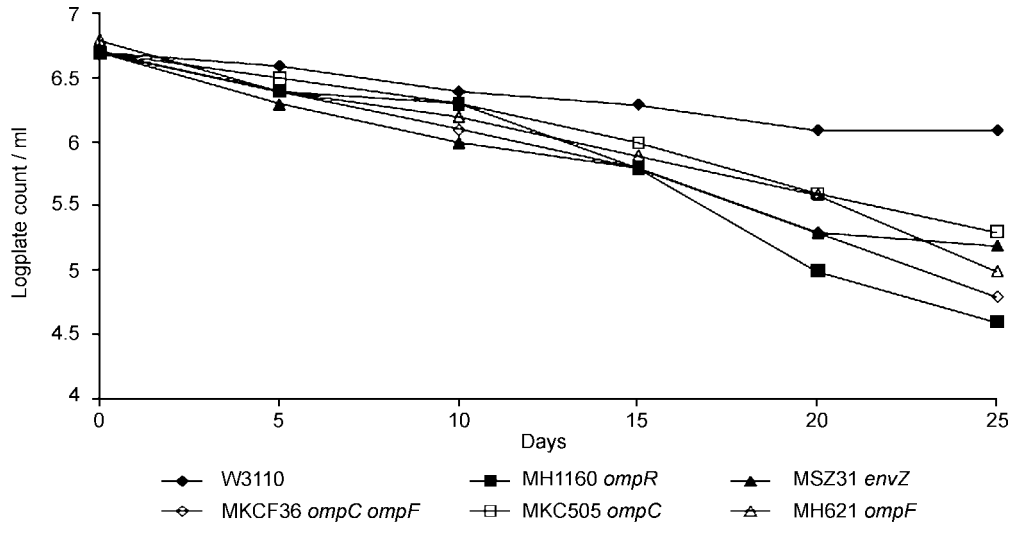

Fig. 3. Survival of *Escherichia coli* in filtered autoclaved sea water at pH 5.5.

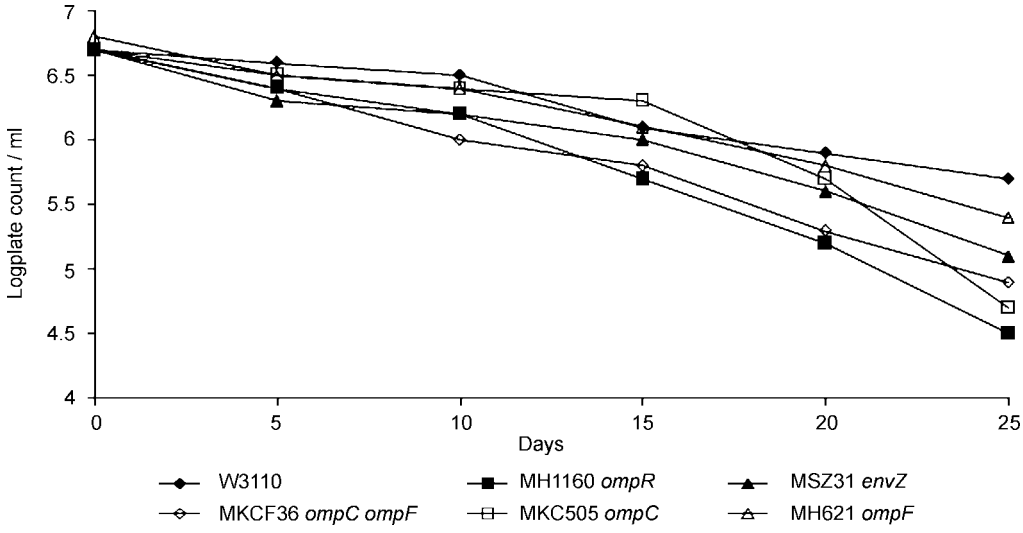

Fig. 4. Survival of *Escherichia coli* in filtered autoclaved sea water at pH 7.2.

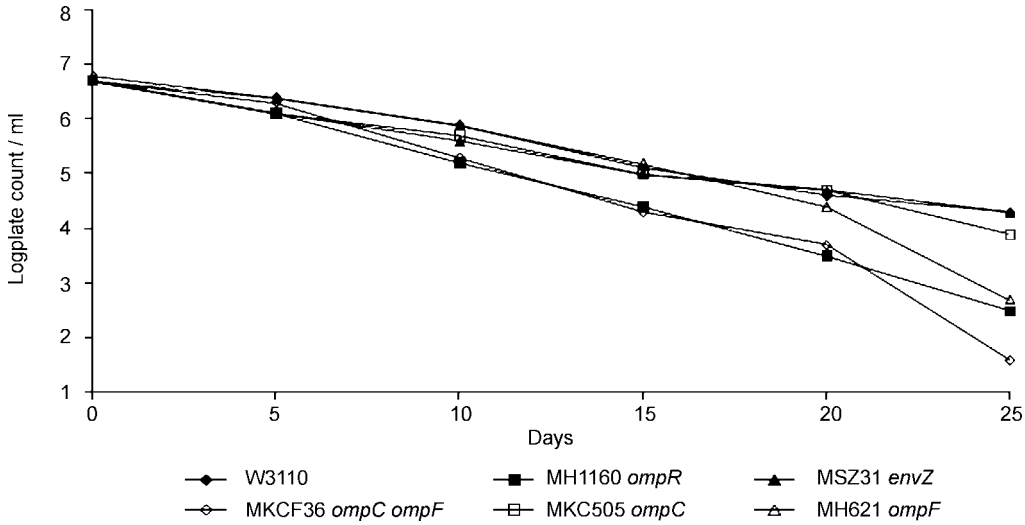

Fig. 5. Survival of *Escherichia coli* in filtered autoclaved sea water at pH 8.3.

of glycine betaine to *envZ* mutant *E. coli* environment resulted in increase survival on the contrary to without addition. The respiring cell count was also little different in the presence or absence of glycine betaine and the better survival is down to the increased viable count on agar plates. The addition of glycine betaine must encourage cells to remain more active in the presence of the salinity of the sea and a lower number of cells enter a VBNC state. In the case of *envZ* mutation, the addition of osmoprotectants did not cause any

Table IV
The effect of different pH adjustments on the survival of *Escherichia coli* in filtered-autoclaved sea water microcosms. k = days^{-1}; t_{99} = days

Strain	pH 5.5		pH 7.2		pH 8.3	
	k	t_{99}	k	t_{99}	k	t_{99}
W3110 wild type	–0.0263	76.0	–0.0429	46.6	–0.104	19.2
MH1160 (*ompR*$^-$)	–0.0869	23.0	–0.0863	23.2	–0.1691	11.8
MSZ31 (*envZ*$^-$)	–0.0611	32.7	–0.0589	33.9	–0.096	20.8
MKCF36 (*ompF*$^-$*ompC*$^-$)	–0.0749	26.7	–0.0714	28.0	–0.196	10.2
MKC505 (*ompC*$^-$)	–0.0669	29.9	–0.0537	37.2	–0.1554	12.8
MH621 (*ompF*$^-$)	–0.0571	35.0	–0.0714	28.0	–0.108	18.5

Table V
Effect of pH on the survival of *Escherichia coli* in filtered-autoclaved sea water by different methods of enumeration after 25 days
(Initial bacterial count is approximately 6.6 cfu/ml)

Strain	pH 5.5				pH 7.2				pH 8.3			
	VC	DVC	RCC	TC	VC	DVC	RCC	TC	VC	DVC	RCC	TC
W3110 (wild type)	6.1 ± 0.06	6.2 ± 0.03	6.3 ± 0.02	6.4 ± 0.01	5.7 ± 0.1	5.7 ± 0.07	6.0 ± 0.05	6.4 ± 0.06	4.3 ± 0.2	4.4 ± 0.1	5.1 ± 0.1	5.8 ± 0.1
MH1160 (*ompR*$^-$)	4.6 ± 0.10	4.6 ± 0.10	5.5 ± 0.07	6.3 ± 0.10	4.5 ± 0.2	4.6 ± 0.05	5.5 ± 0.05	6.3 ± 0.06	2.5 ± 0.02	2.4 ± 0.20	4.4 ± 0.1	5.6 ± 0.1
MSZ31 (*envZ*$^-$)	5.2 ± 0.2	5.3 ± 0.2	5.4 ± 0.3	6.2 ± 0.04	5.1 ± 0.05	5.2 ± 0.2	5.3 ± 0.2	5.9 ± 0.08	4.3 ± 0.09	4.4 ± 0.1	4.3 ± 0.09	5.9 ± 0.07
MKCF36 (*ompF*$^-$*C*$^-$)	4.8 ± 0.10	4.8 ± 0.07	5.0 ± 0.20	6.2 ± 0.04	4.9 ± 0.10	4.9 ± 0.10	5.2 ± 0.08	6.2 ± 0.02	1.6 ± 0.20	1.8 ± 0.10	4.2 ± 0.08	5.8 ± 0.03
MKC505 (*ompC*$^-$)	5.0 ± 0.2	5.1 ± 0.10	5.4 ± 0.10	6.4 ± 0.05	5.4 ± 0.07	5.4 ± 0.10	5.7 ± 0.09	6.2 ± 0.03	2.7 ± 0.10	2.7 ± 0.10	4.7 ± 0.08	5.8 ± 0.04
MH621 (*ompF*$^-$)	5.3 ± 0.06	5.3 ± 0.09	5.6 ± 0.09	6.3 ± 0.04	4.7 ± 0.07	4.7 ± 0.05	5.6 ± 0.05	6.1 ± 0.02	3.9 ± 0.05	3.9 ± 0.06	4.8 ± 0.05	5.8 ± 0.04

changes in RCC and plate counts. The rate of VBNC was changed in *envZ* mutant *E. coli*.

The effect of different pH values on survival of porin-deficient *Escherichia coli.* The effects of pH amendments on the survival of the various mutants were made by adjusting the pH of the sea water to three pH values between 5.5 and 8.3 using buffers. The normal pH of Black Sea water is around pH 8.3 and salt concentration is 0.16%. The results in Figures 3–5 for pH 5.5, 7.2 and 8.3, respectively, show a log linear decline in survival with time and Tables IV and V show the effects of changing the pH on the t_{99} and respiring cell count values. As in the previous experiments, the decline in bacterial numbers is mainly log linear in the semi-log plots.

At pH 5.5 the wild type survives extremely well with a t_{99} value of 76 d (Fig. 3) and the plate count and respiring cell count are very similar values (Table V). The survival of the various mutants flows that seen in the other experiments with the *envZ* mutant surviving better than the mutants with defective or absent outer membrane proteins or a defective regulatory system.

As the pH was increased from 5.5, the survival times for the wild type declined with t_{99} value decreasing to 46.6 d at pH 7.2 and to 19.2 d at pH 8.3. The survival times for all the other mutant strains also declined as the pH became more alkaline. The relative survival times were virtually the same at all the pH values with the *ompR*$^-$ and the *ompF*/*ompC* double mutants showing shorter survival times (Figs 4, 5 and Table IV).

With all the mutant strains, there was a much greater similarity in the plate viable count and the respiring cell count in the samples incubated at pH 5.5 for 25 d. This suggests that at this acidic pH although survival as measured through growth on agar plates is apparently little impaired compared to other pH values tested very few of the cells enter the VBNC state and ultimately this will have much greater effect on the long term survival of the bacterial cells (Table VI). The greatest differences between the respiring cell count and the plate counts are at the alkaline pH suggesting that entry into the VBNC state is faster at an alkaline pH than at an acidic or neutral one.

Discussion

Several important factors have an effect on the survival of enteric bacteria in marine environments. These include the availability of nutrients, the changes in osmolarity and the pH to which the bacteria are subjected ant the physical stress imposed on the bacteria by exposure to sunlight and photo-sensitizers (Kömerik and Wilson, 2002; Kussovski *et al.*, 2001; Sinton *et al.*, 2002). Many of these stresses

have been shown to lead to the formation of dormant bacteria or to the establishment of the VBNC state in the bacteria (Özkanca *et al.*, 2002). This state leads to apparent loss of viability in bacteria because they cease to grow and produce colonies on agar media. However, the cells may still retain the ability to carry out respiration as observed by direct microscopy and can, therefore, often be resuscitated given the appropriate optimal conditions.

The outer membrane is the first important barrier between Gram-negative bacteria and the surrounding environment. Even changes in the relative amounts of omps because of natural environmental conditions affect the survival of *E. coli* (Özkanca and Flint, 2002). The outer membrane proteins, OmpC and OmpF, the OmpR regulator protein and the EnvZ sensor protein play important roles in transport of solutes across the outer membrane of *E. coli* and some other Gram-negative bacteria (Sato *et al.*, 2000). However, the role of porin proteins and their changes during bacterial starvation and the entry of enteric bacteria into dormancy and survival in natural aquatic environments still need to be clarified. The OmpR regulator protein has a function in the regulation of OmpC and OmpF synthesis. Deletion of the *ompR* gene *E. coli* MH1160 produces a mutant which is unable to regulate the synthesis of other outer membrane proteins in response to changes in environmental conditions.

As the major outer membrane proteins, OmpF and OmpC, respond primarily to changes in osmolarity and mutants which cannot regulate the relative amounts of these two porins in the outer membrane will be impaired in their ability to survive the change in osmolarity which an enteric bacterium, such as *E. coli*, would experience when discharged into a marine environment (Nikaido and Vaara 1985; Forst and Inouye 1988). EnvZ/OmpR system is known to be two-component phosphorelay system and to regulate the synthesis of the OmpF and OmpC porin proteins through a series of kinase/phosphatase catalysed cascade reactions (Russo and Silhavy, 1991). The EnvZ protein is an environmental sensor that is bound to the inner membrane and has kinase and phosphatase activities (Forst *et al.*, 1987; Igo and Silhavy, 1988). OmpR is a cytoplasmic DNA-binding protein that is needed to activate the transcription of OmpC and OmpF (Aiba *et al.*, 1989). Osmotic signals regulate the level of the cellular concentration of OmpR-P by modulating the ratio of kinase to phosphatase activity through the bifunctional enzymatic activities of EnvZ.

Starvation stress is one of the important factors that affect survival of enteric bacteria in sea water. As these coliform organisms are routinely used as indicators to evaluate the level of faecal contamination of water, it is important to understand how their survival may be affected by changes in environmental conditions. Any process that reduces the ability of these bacteria to grow on agar plates, still widely regarded as a true measure of viability, could have serious consequences for human health especially with the increasing use of bathing beaches round the world.

There have been some previous studies on the involvement of porin protein expression in relation to the survival of bacteria under starvation stress (Özkanca, 1993; Liu and Ferenci, 2001; Darcan *et al.*, 2003, Darcan 2005). The changes in the OmpF and OmpC porin proteins linked to changes in bacterial growth rate have been investigated (Liu and Ferenci 1998). In this present study, the impact of deletion or impairment of the function of the genes responsible for OmpF and OmpC synthesis and control on the survival of *E. coli* in filtered-autoclaved sea water microcosms was investigated and showed that porin proteins played an important role for the survival of *E. coli* in sea water under starvation stress. This study was carried out for 25 days of incubation in filtered-autoclaved sea water under starvation stress.

In filtered-autoclaved sea water, survival was dependent upon the effects of salinity on the bacteria. The wild type organism survived for 20.3 d in the absence of the osmoprotectant glycine betaine and for a reproducible slight increase in survival of 21.7 d in the presence of the osmoprotectant. Bacteria produce and store some osmoprotectants under osmotic stress in sea water environments (D'Souza-Ault *et al.*, 1993). It has been shown that in the presence of glycine betaine *E. coli* do not lose the ability to produce colonies as quickly in sea water due to osmotic stress. The addition of glycine betaine to stressed cultures increases the proportion of the population capable of producing colonies after exposure to stress in sea water (Roth *et al*., 1988). All the outer membrane protein mutants survived for a shorter period of time with the double *ompF*/*ompC* mutant and the organism deficient in the regulatory protein OmpR always surviving for the shortest periods of time in the microcosms not amended with glycine betaine. The *ompF* mutant and the *envZ* mutant usually survived for time not very different from the wild-type. Both these organisms showed that these genes are less important than the *ompC* gene and the regulatory gene in determining how well the organism survived in sea water. In general, the addition of glycine betaine to sea water led to an increase in colony counts compared to the control, especially for the *ompC-ompF* double mutant which almost doubled the time taken for a two log drop in numbers from 9.9 d to 18.7 d. Comparison between the numbers of cells capable of respiration and those capable of producing colonies on nutrient agar plates showed a much closer comparison between

the samples in microcosms amended with glycine betaine than in the unamended microcosms. This indicates that entry into the VBNC state is being slowed considerably with the addition of the osmoprotectant and cells have changed their mode of response to stress. The normal response seen in the unamended microcosms is for the plate count to drop rapidly and for the respiring cell count to remain relatively high suggesting that a much larger proportion of the population is still capable of producing colonies than actually does. In the presence of the osmoprotectant the response to stress is changed and the counts are virtually identical in all strains. This shows that the VBNC state is a response to stress and that alleviation of some of the effects of stress by the addition here of an osmoprotectant does protect cells from damage and so prevents entry into the VBNC state. One possibility shown from this work is that the authorities should consider the routine addition of glycine betaine to samples collected from sea water in order to minimize the loss in colony forming *E. coli* before samples are tested.

The effect of amending the pH to impose a further stress on the cells was also studied in sea water. It has been reported that pH could be one of the more important factors which affect the survival of *E. coli* in sea water (Dawe and Penrose, 1978; Rozen and Belkin, 2001). The pH range of the Black Sea is usually between 8.1 and 8.3. In this study, all the *E. coli* strains survived for longer at pH 5.5 than at pH 7.2 or 8.3 with the t_{99} value changing from 76 d at pH 5.5 to 19.2 d at pH 8.3 for the wild type. It seems that the survival of *E. coli* was more dependent on pH than the osmolarity of sea water. The *ompF*/*ompC* double mutant and the *ompR* regulatory mutant both showed the lowest survival at all pHs studied but did show some increase in survival in the acidic samples. A lack in the environmental sensor protein, EnvZ, may not cause a serious survival problem for *E. coli* in sea water (wild type 20.3 day and *envZ* mutant 21.4 day in sea water; wild type 19.2 and *envZ* mutant 20.8 day with the t_{99} value at pH 8.3). But when the sea water pH was 5.5 and 7.2, *envZ* mutation caused a serious survival problem for *E. coli*. While survival of wild type *E. coli* was 76 days at pH 5.5 and 46.6 at pH 7.2, it was 32.7 days at pH 5.5 and 33.9 days at pH 7.2 for *envZ* mutant cells with the t_{99} values. It seem that EnvZ is much more important at acidic pH than in alkaline pH sea water

Kaeriyama *et al.* (2006) found that growth was diminished by the deletion of ompC and ompF in high osmolar alkaline media with pHs above 8.0. Such growth reduction was not observed at near neutral pH under hypo- and hyperosmosis. Thus, it was suggested that OmpC and OmpF are required for adaptation to hyperosmosis at pHs above 8.0 but not acidic media. Similar results were reported by Wang *et al.* (2007). It has been reported that disrupted *ompC* causes osmosis sensitivity of *E. coli* in alkaline medium. A porin double mutant *E. coli* (*ompC ompF*) was effected by alkaline pH stronger than at acidic and neutral pH (Table IV–V).

As with the decrease in stress shown in response to the osmoprotectant and osmolarity, there was a much closer correlation between respiring cell counts and plate viable counts in the acid amended samples compared to the more alkaline samples where there was a rapid decline in viable count compared to the decline in ability to show respiration activity. This again supports the idea that alleviation of stress reduces entry into the VBNC state.

The measurement of the respiring cell count is an important technique that can be used to determine the number of cells capable of respiration but unable to form colonies in aquatic environments. In this study plate viable counts and respiring cell counts were carried out at the same sampling time in order to investigate any relationship between the survival of wild-type and porin-deficient mutants under starvation, osmotic and pH stress. Results from the determination of the respiring cell counts suggested that there was a still a population of cells capable of respiring but not capable of growth on agar plates. These results showed that *E. coli* and porin-deficient mutants entered the viable but nonculturable state at different rates according to the stress conditions to which the cells were subjected. Previously, Darcan *et al*. (2003) had showed that the *env*Z mutation could prevent *E. coli* cells entering the VBNC state. The loss of the EnvZ protein has no effect on survival as determined by the plate count, but it could prevent the organism sensing the changes in the environment through which entry into the survival state is triggered. Here, in all cases, the respiring cell count and the plate viable count were not different for *E. coli* MSZ31 *envZ*$^-$. Again this implies that these organisms cannot sense stress changes in the environment and therefore cannot enter a VBNC state. All the other organisms including the wild type show a significant difference between the plate count and respiring cell count under some conditions with the respiring count always being higher suggesting that there are cells in a population capable of respiration but not growth. The highest number of cells entering the viable but nonculturable state was detected with the porin double mutant *E. coli* MKCF36 (*ompC ompF*). A similar loss of porin proteins in *S. typhimurium* LT2 cells subjected to oxidation stress also led to loss of porin proteins, loss of viability and entry into VBNC state (Özkanca *et al*., 2002). Muela *et al.* (2008) also reported that changes in *E. coli* OMPs under environmental conditions

effecting viable but non culturable state. Moreover, Asakura *et al.* (2008) suggested that outer membrane protein W (OmpW) expression was induced in VBNC state in *E. coli* O157:H7.

Finally, the outer membrane is the first important barrier between Gram-negative bacteria and their surrounding environment. EnvZ acts as an osmosensor to monitor the changes in the external osmolarity and modifies OmpR activity by phosphorylation and dephosphorylation (Forst *et al*., 1989). OmpR is a transcriptional activator of both *ompC* and *ompF* genes (Tsui *et al*., 1988). Therefore the effect of starvation, osmolarity and pH stresses on the EnvZ/OmpR system is important in terms of the survival of *E. coli* in sea water. Here, we have shown that there were some changes in terms of colony counts, direct counts and respiring cell counts of *E. coli* wild type and porin deficient mutants in response to changes in the external environment. The most obvious result is that the *envZ* mutant survived for longer in terms of plate counts but ultimately would disappear from culture first as it cannot sense the environmental changes and respond by entering the VBNC state. pH, starvation and osmolarity did not affect the interrelation between EnvZ and VBNC state of *E. coli*. While all tested porin mutants *E. coli* strains entered VBNC state this was not the case for *envZ* mutants.

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Literature

Aiba H., F. Nakasai, S. Mizushima and T. Mizuno. 1989. Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR in *Escherichia coli*. *J. Biol. Chem.* 264: 14090–14094.

Barcina I., P. Lebaron and J. Vives-Rego. 1997. Survival of allochthonous bacteria in aquatic systems: a biological approach. *FEMS Microbiol. Ecol.* 23: 1–9.

Colwell R.R. and D.J. Gray. 2000. Nonculturable Microorganisms in the Environment. American Society for Microbiology, Washington, DC, USA.

Darcan C., R. Özkanca and K.P. Flint. 2003. Survival of non-specific porin-deficient mutants of *Escherichia coli* in Black Sea water. *Letts. App. Microbiol.* 37: 380–385.

Darcan C., R. Özkanca and Ö. İdil. 2009. The role of RpoS, H-NS and AcP on the pH-dependent OmpC and OmpF porin expressions of *Escherichia coli* at different pH. *African Journal of Biotechnology* 8: 1845–1854.

Darcan C. 2005. An investigation on the effect of pH, starvation and osmotic stress on outer membrane porin protein synthesis level of *Escherichia coli* in the black sea water. Ph. D. Thesis, Deparment of Biology, Ondokuz Mayis University, 180 p.

Dawe L.L. and W.E. Penrose. 1978. "Bactericidal'' property of seawater: death or debilition? *Appl. Environ. Microbiol.* 35: 829–833.

Davies C.M. and L.M. Evison. 1991. Sunlight and survival of enteric bacteria in natural waters. *J. Appl. Bacteriol.* 70: 265–279

D'Souza-Ault M., L.T. Smith and G.M. Smith. 1993. Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. *Appl. Environ. Microbiol.* 59: 473–478.

Ferrario M., B.R. Ernsting, D.W. Borstm, D.E. Wiese, R.M. Blumenthal and R.G. Matthews. 1995. The leucine-responsive regulatory protein of *Escherichia coli* negatively regulates transcription of *ompC* and *micF* and positively regulates translation of *ompF*. *J. Bacteriol*. 177: 103–113.

Forst S. and M. Inouye. 1988. Environmentally regulated gene expression for membrane proteins in *Escherichia coli. Annu. Rev. Cell Biol.* 4: 21–42.

Forst S., D. Comeau, S. Norioka and M. Inouye. 1987. Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. *J. Biol. Chem.* 262: 16433–16438.

Forst S., J. Delgado and M. Inouye. 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates the expression of the *ompF* and *ompC* genes of *Escherichia coli*. *Proc. Natl. Acad. Sci.* 86: 6052–6056.

Gurijala K.R. and M. Alexander. 1990. Explanation for the decline of bacteria introduced into lake waters. *Micro. Ecol.* 20: 231–244

Hall M.N. and T.J. Silhavy. 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K-12. *J. Mol. Biol.* 146: 23–43.

Igo M.M. and T.J. Silhavy. 1988. EnvZ, a transmembrane environmental sensor of *Escherichia coli* K12, is phophorylated *in vitro*. *J. Bacteriol.* 170: 5971–5973.

Kaeriyama M., K.Machida, A. Kitakaze, H. Wang, Q. Lao, T. Fukamachi, H. Saito and H. Kobayashi. 2006. OmpC and OmpF are required for growth under hyperosmotic stress above pH 8 in *Escherichia coli*. *Letts. Appl. Microbiol.* 42: 196–201.

Kjelleberg S. and M. Hermannsson. 1987. Short-term responses to energy fluctuation by marine heterotrophic bacteria. In "Microbes in the sea" Ed Mitchell, R., and Sleigh, A. pp. 203–219. Chichester: John Wiley.

Koebnik R., K.P. Locher and P. Van Gelder. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol. Microbiol.* 37: 239–253.

Kömerik N. and M. Wilson. 2002. Factors influencing the susceptibility of gram-negative bacteria to toluidine blue O-mediated lethal photosensitization. *J. Appl. Microbiol.* 92: 618–623.

Kogure K., U. Simidu and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* 25: 415–420.

Kussovski V.K., A.E. Hristov and T.S. Radoucheva. 2001. Proflavine-mediated inactivation of *Salmonella dublin* exposed to visible sunlight in natural fresh water. *Microbios* 105: 119–125.

Liu X. and T. Ferenci. 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in *Escherichia coli*. *J. Bacteriol.* 180: 3917–3922

Liu X. and T. Ferenci. 2001. An analysis of multifactorial influences on the transcriptional control of *ompF* and *ompC* porin expression under nutrient limitation. *Microbiology* 147: 2981–2989.

McCambridge J. and T.A. McMeekin. 1983. Effect of solar radiation and predacious micro-organisms on survival of faecal and other bacteria. *Appl. Environ. Microbiol.* 41: 1083–1087.

Muela A., C. Seco, E. Camafeita, I. Arana, M. Orruńo, J.A. López and I. Barcina. 2008. Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. *FEMS Microbiol. Ecol*. 64 (1), 28–36

Munro P.M., J.M. Gauthier, A. Breittemayer and J. Bongiovanni. 1989. Influence of osmoregulation process on starvation survival of *Escherichia coli* in sea water. *Appl. Environ. Microbiol.* 55: 2017–2024.

Nikaido H. and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* 49: 1–32.

Özkanca R. 1993. Survival and physiological status of *Escherichia coli* in lake water under different nutrient conditions. Ph. D. Thesis. Department of Biological Sciences, University of Warwick, England.

Özkanca R. and K.P. Flint. 2002. The effect of starvation stress on the porin protein expression of *Escherichia coli* in lake water. *Lett. Appl. Microbiol.* 35: 533–537.

Özkanca R., N. Şahin, K. Iţik, E. Kariptaş and K.P. Flint. 2002. The effect of toluidine blue on the survival, dormancy and outer membrane porin proteins (OmpC and OmpF) of *Salmonella typhimurium* LT2 in seawater. *J. Appl. Microbiol.* 92: 1097–1104.

Rodriguez G.G., D. Phipps, K. Ishiguro and H.F. Ridgway. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58: 1801–1808.

Roth W.G., M.P. Leckie and D.N. Dietzler. 1988. Restoration of colony-forming activity in osmotically stressed *Escherichia coli* by betaine. *Appl. Environ. Microbiol.* 54: 3142–3146.

Rozen Y. and S. Belkin. 2001. Survival of enteric bacteria in seawater. *FEMS Microbiol. Rev.* 25: 513–529.

Russo F.D. and T.J. Silhavy. 1991. EnvZ controls the concentration of phosphorylated OmpR to mediate osmoregulation of the porin genes. *J. Mol. Biol.* 222: 567–580.

Sainz T., J. Perez, J. Villaseca, U. Hernandez, C. Eslava, G. Mendoza and C. Wache. 2005. Survival to different acid challenges and outer membrane profiles of pathogenic *Escherichia coli* strains isolated from pozol, a Mexican typical maize fermented food. *Int. J. Food Microbiol.* 105: 357–367.

Sato M, K. Machida, E. Arikado, H. Saito, T. Kakegawa and H. Kobayashi. 2000. Expression of outer membrane proteins in *Escherichia coli* growing at acid pH. *Appl. Environ. Microbiol.* 66: 943–947.

Sinton L.W., C.H. Hall, P.A. Lynch and R.J. Davies-Colley. 2002. Sunlight inactivation of faecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. *Appl. Environ. Microbiol.* 68: 1122–1131.

Sylvester D.M., R. Taylor and T.R. Lattann. 2001. Viable but nonculturable bacteria: a public health threat? *Infect. Dis. Rev.* 3: 70–82.

Tsui P., V. Helu and M. Freundlich. 1988. Altered osmoregulation of *ompF* in integration host factor mutants of *Escherichia coli*. *J. Bacteriol.* 170: 4950–4953.

Wang Y., L. Wang, Y. Sun, Y. Chen, L. Zhu, L. Guo, B. Luo and H. Wang. 2007. Disrupted ompC causes osmosis sensitivity of *Escherichia coli* in alkaline medium. *J. Genetics and Genomics* 34: 1131–1138.