Survival of enteric bacteria in seawater

Yael Rozen, Shimshon Belkin *

Environmental Sciences, Fredy and Nadine Herrmann Graduate School of Applied Science, Hebrew University of Jerusalem, Jerusalem 91904, Israel

Accepted 19 June 2001
First published online 16 July 2001

Abstract

Enteric bacteria exposed to the marine environment simultaneously encounter a variety of abiotic and biotic challenges. Among the former, light appears to be critical in affecting seawater survival; previous growth history plays a major part in preadaptation of the cells, and stationary phase cells are generally more resistant than exponentially growing ones. Predation, mostly by protozoa, is probably the most significant biotic factor. Using *Escherichia coli* as a model, a surprisingly small number of genes was found that, when mutated, significantly affect seawater sensitivity of this bacterium. Most prominent among those is *rpoS*, which was also dominant among genes induced upon transfer to seawater. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Enteric bacterium; Seawater survival; Stress response; Viable but nonculturable; Wastewater; *Escherichia coli*

Contents

1. Introduction .......................................................... 513
2. Terms and abbreviations ................................................. 513
3. Viability and colony formation ............................................ 513
4. Abiotic factors affecting survival ........................................... 516
   4.1. Light ........................................................... 516
   4.2. Salinity ........................................................... 517
   4.3. pH .............................................................. 518
   4.4. Nutrient deprivation ............................................. 518
   4.5. Temperature ..................................................... 518
   4.6. Hydrostatic pressure .......................................... 520
   4.7. Sedimentation ................................................... 520
   4.8. Previous growth history and stress cross-protection ............... 521
5. Biotic factors ...................................................... 521
   5.1. Grazing and competition ......................................... 521
   5.2. Bacteriophages ................................................. 522
   5.3. Antibiotics and toxins ......................................... 522
6. Molecular mechanisms involved in seawater survival .................. 522
   6.1. Effect of specific mutations .................................... 523
   6.2. Genes induced upon seawater exposure .......................... 524
7. Concluding remarks ................................................. 524
Acknowledgements ...................................................... 525
References ............................................................. 525

* Corresponding author. Tel.: +972 (2) 658 4192; Fax: +972 (2) 658 5559. E-mail address: shimshon@vms.huji.ac.il (S. Belkin).

0168-6445/01/$20.00 © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S 0 1 6 8 - 6 4 4 5 ( 0 1 ) 0 0 0 6 5 - 1
1. Introduction

The factors controlling the survival of enteric bacteria in the marine environment have intrigued scientists for decades. Driven by obvious public health concerns as well as by broader attempts to understand bacterial responses to environmental stress, numerous studies have explored the fate of Escherichia coli and other enteric bacteria following their exposure to seawater.

Many of these efforts were motivated by the need to properly evaluate the risk posed by such microorganisms when released into the sea, either to the health of bathers in recreational waters or to the safety of fisheries or marine agriculture. Consequently, colony formation-based coliform die-off rates were often the main parameter used to characterize the bacterial responses, under a variety of biotic and abiotic test conditions. Only in recent years is there an increasing number of reports attempting to assay additional viability criteria, as well as to preliminarily explore the molecular mechanisms involved and their genetic regulation.

Over the years there have been very few publications summarizing available information in this field [1,2]; the most recent of these dates to the mid-80s and focuses mainly on the different viability states of enteric bacteria. The aim of the present review is therefore to partially remedy this situation by providing an updated compilation of the relevant scientific literature. For this purpose, following a discussion of the intricacies of colony formation and other modes of viability testing upon seawater exposure, we shall review the main biotic and abiotic factors reported to affect the sensitivity and survival of enteric bacteria in the sea; we shall then proceed to summarize the relatively scant available information concerning the molecular control of these effects, and point at future directions which will need to be undertaken.

2. Terms and abbreviations

CFU, colony forming unit, also referred to as ‘culturable cells’, viable counts; CTC, 5-cyano-2,3-ditolyltetrazolium chloride, a dye for direct (microscopic) enumeration of respiring cells; DVC, direct viable count, a direct (microscopic) enumeration of substrate-responsive cells; TC, total count, all microscopically countable cells, direct count of cells retaining cellular integrity usually stained by nucleic acid stains such as DAPI (4',6-diamidino-2-phenylindole) or acridine orange; VBNC, viable but non-culturable, cells that retain some form of viability, but can no longer divide in or on media that usually support their growth.

3. Viability and colony formation

As pointed out above, in many of the investigations into the survival of E. coli and other enteric bacteria in the sea, colony formation ability was used as the main or only viability parameter. Roszak and Colwell [3], reviewing survival strategies of microorganisms, and Grimes et al. [2] highlighted the limitations of this approach. The ‘viable but nonculturable’ (VBNC) state concept was introduced to describe cells that remain metabolically active but are unable to divide in or on nutritional media that normally support their growth. This state was demonstrated for many enteric bacterial species during seawater incubation in the dark [4–10] and in the light [11,12]. Gauthier [13], in his review of the environmental parameters associated with the VBNC state, discusses the effects of temperature, radiation, osmolarity and organic matter concentration as possible triggers for the VBNC state. He points out that, as in other cases, the multi-stress situation in seawater, along with the cells’ previous history, plays an important role in their possible entry into VBNC status.

Since marine bacterial pollution is routinely monitored by culturing methods, the findings that enteric VBNC bacteria in seawater can be resuscitated [14,15] or retain their pathogenicity [6] are significant. Other studies, however, demonstrated loss of pathogenicity concomitantly with culturability [8] or failed to resuscitate VBNC cells of several species using various methods [16,17]. The latter reports, according to their authors, cast a doubt on the validity of the entire VBNC concept.

It is thus clear that interpretation of seawater viability studies depends, to a large extent, upon the diagnostic methodologies adopted. Furthermore, the results are also bound to be influenced by the specific conditions during and even before the actual experiments, including the bacterial strains used, their growth history, and the media they were allowed to grow in and/or form colonies on. Other complications may arise from different parameters monitored or units of measurements used. For these reasons, comparing results of different experiments may be problematic. Nevertheless, while summarizing available information, we shall also try to point at emerging general trends and patterns.

It is broadly accepted that the first ability enteric bacterial cells lose in seawater is the capacity to form colonies on a solid medium. Later [7,9,18–21], or in some cases in parallel [4,5,9,16,17], the viability represented as direct viable count (DVC) is lost. Total counts seem to remain almost constant during all reported experiments [4,5,7,9,16–19,21].

Long-term experiments (7–300 days) in which various viability parameters were measured revealed additional changes in the state of the cell with incubation time in seawater. Garcia-Lara et al. [18] reported on E. coli 536, previously grown at 30°C in a minimal medium (M9G), which was transferred to sterile seawater (20°C) for...
30 days. Most of the cells (a 4 log reduction) lost their colony forming ability, but maintained constant total counts (TC). Cellular DNA concentrations increased significantly during the first 5 days, and [3H]thymidine incorporation rates kept increasing for 13 days, following which both parameters were maintained at a stable level. Electron transfer activity and cell-attached exoproteolytic activity remained constant during the entire experiment, while cell-free exoproteolytic activity was drastically decreased [18].

Another experiment [5,9] measured multicellular parameters in Salmonella typhimurium cells during incubation in sterile artificial seawater (20°C) in the dark for 19 days. Colony forming ability, enumerated as colony forming units (CFU), was lost first and decreased by 6 logs within the course of the experiment. Cells showing metabolic activity in the presence of nutrients (DVC) decreased in concentration to an undetectable level (more than a 4 log reduction) in 4 days. Real respiration (with no nutrient addition) and potential respiration (after nutrient addition), both assayed by 5-cyano-2,3-ditolyltetrazolium chloride (CTC) reduction, decreased by 3 logs within 10 and 13 days, respectively. The other three parameters measured – cellular integrity (TC), genomic integrity (cells the DNA content of which was equal or superior to one equivalent genome) and membrane permeability – remained constant during the entire experiment [5,9].

Somewhat different results were obtained in a long-term experiment (294 days) in which the decay of five stationary phase enteric bacterial species (E. coli, Klebsiella pneumoniae, Enterococcus faecalis, Enterobacter aerogenes, and Salmonella choleraesuis) in artificial seawater microcosms (20°C) was monitored using different methods. Plate counts (CFU) and DVC declined gradually and in parallel, while TC remained constant during the entire experiment, indicating cell membrane integrity. Respiratory activity was affected to various extents in the different species [17].

In similar experiments conducted by Smith et al. [21] at the polar environment of McMurdo station in Antarctica, the survival of four stationary phase enteric bacteria (E. coli, S. typhimurium, Yersinia enterocolitica and E. faecalis) in artificial seawater microcosms (20°C) was monitored using different methods. Plate counts (CFU) and DVC declined gradually and in parallel, while TC remained constant during the entire experiment, indicating cell membrane integrity. Respiratory activity was decreased by 2-3 logs during the same period. [21]

The effect of sterilized seawater (22°C) on exponentially growing S. typhimurium was monitored during 32 days [20]. In that period, less than a 10-fold decrease was observed in all cell count parameters studied – TC, DVC and CFU. Cellular carbohydrates, lipids, proteins and DNA gradually decreased to a level of 5–25% at day 32; RNA content did not decrease significantly [20]. Morphological changes were in the following order: cell size reduction, cell wall damage, a wrinkling of the outer membrane, degenerative cellular forms, loss of outer membrane, and compression of nuclear region into the center of the cell. Hydrophobicity and adherence to eukaryotic cells (Hep2) decreased with seawater incubation time while susceptibility to phagocytosis (peritoneal macrophages) increased. All of the above correlated with the virulence of S. typhimurium as tested on mice: mortality of infected animals (as recorded at day 7) decreased from 80% at the first day to 30% by the 16th day [20].

As can be expected, the ability to form colonies on solid media is largely dependent on the medium used. Morinigo et al. [22] defined sublethal cellular injury as the loss of ability to form colonies on selective media, while retaining it on less selective ones. This phenomenon was observed in E. coli, Streptococcus faecalis and Salmonella spp., all of which formed fewer colonies on selective media (Endo agar, KF agar, and xylose-lysine-deoxycholate agar, respectively) than on trypticase soy agar [22]. In a different study [23], the survival of a marine E. coli isolate in a dialysis bag submerged in seawater was examined. While CFUs enumerated on seawater-based nutrient agar remained constant for 6 days, on standard Levine EMB agar the counts declined to zero by the fifth day. The same protective effect of the recovery medium salinity was observed by Gauthier et al. [24], who found that the highest recovery rate of E. coli K12 incubated in seawater was on a nonselective medium (nutrient agar) supplemented with sodium chloride (15 g l⁻¹).

Assuming that enzymatic activities in VBNC cells may still be detectable, disparities may be expected between colony-based counts and fecal coliform enumeration assays based on enzymatic activities. While in studies monitoring sewage pollution in marine waters the results were well correlated [25,26], when enzyme activity of pure cultures was compared to their colony forming ability such discrepancies were indeed recorded [19,27,28]. Two studies described β-D-galactosidase activity in seawater even when no culturable cells could be detected [27,28]. In one of these an environmental E. coli isolate was tested for culturability, viability and β-D-galactosidase activity for 85 days in seawater. Culturability decreased to zero after 60 days while viability (2-(p-iodophenyl)-3-(p-nitrophenoxy)-5-phenyltetrazolium (INT) chloride reduction) decreased by 1–2 logs during the same period. β-D-Galactosidase activity, after an initial increase, remained constant for the duration of the experiment, even when the number of culturable cells reached zero [27]. β-D-Galactosidase activity as an indication of viability was also measured by Pompey et al. [28] using E. coli H10407. Once again, a decrease in culturable cells was observed while enzymatic activity remained constant. When the experiment was repeated under visible light exposure, β-D-galactosidase activity was also affected, although it was still detectable when no colony forming cells remained.

The same E. coli strain was used by Fiksdal et al. [19],
who measured its 4-methylumbelliferyl heptanoate hydro-lase activity in seawater. During the first 16 days CFUs decreased by 2-3 logs while enzyme activity appeared to increase.

Another possible indication of viability was provided for E. coli strains JM83 and JM101 that were shown to maintain plasmids (pBR322 and pUC8, respectively) at a high copy number for at least 21 days in artificial seawater microcosms, even though both strains lost their ability to form colonies on a solid medium [29].

Resuscitation of VBNC cells was demonstrated using several methods. The restoration of colony forming ability by betaine was achieved in osmotically stressed E. coli CA8000 [14]. The cells were exposed to 0.8 M NaCl in a minimal medium, reducing CFU counts by 75% within 2 h. The addition of betaine (2 mM) restored colony forming ability in about 80% of the cells within a similar time period. Colony forming ability could still be restored after 24 h in NaCl, even in the presence of the protein synthesis inhibitor chloramphenicol. The latter observation indicates that the betaine-driven increase in CFU was not due to cryptic growth of culturable cells [14]. A different resuscitation strategy was employed by Roszak et al. [15]: Salmonella enteritidis C1 cells were revived 4 days after the nonculturable state was achieved, by a 25 h incubation in full strength veal infusion broth followed by colony forma-tion on veal infusion agar.

The question whether E. coli can actually enter a viable but nonculturable state was critically examined by Bogosian et al. [16] using stationary phase strain W3110 cells in water and soil. In sterile artificial seawater, total counts were constant during the 56 days of the experiment; at the same time, viability decreased by 3 or 5 logs (at 20°C and 37°C, respectively) regardless of the parameter monitored (CFU, DVC or growth in liquid medium). Attempts to resuscitate these VBNC cells by various means including incubation in different media, a shift to a low temperature, and an osmotic adaptation, did not succeed [16].

Another study attempted to test the hypothesis that the presence of culturable cells may be required for the recovery of the nonculturable ones. Cells of different strains (E. coli, K. pneumoniae, E. faecalis, E. aerogenes, and S. chole-rascus) were incubated in artificial seawater for nearly 300 days. Resuscitation was attempted by nutrient addition or a temperature shift (20°C to 4°C or to 37°C) both in the presence and in the absence of culturable cells. At all time points tested, only cells of the culturable strains could be recovered [17].

In their study of the potential pathogenicity of stationary phase S. typhimurium C52 VBNC (driven by sterile artificial seawater and UV-C stress) cells, Caro et al. [8] claimed the concomitant loss of culturability and pathogenicity (tested in a mouse model), independent of the level of viability. Cell viability as determined by respira-tory activity (CTC reduction), cytoplasmic membrane (fluorescent staining) and genomic integrity (TC) remained unchanged, but metabolic activity (DVC) declined together with culturability and could not be distinguished from the latter. In contrast, Pommepuy et al. [6] demonstrated pathogenic effects of E. coli H10407 cells induced to the VBNC state by a combined seawater and light exposure. Enteropathogenicity was demonstrated by a rabbit intesti-nal loop assay that was confirmed by GM1-ELIZA, a sensitive assay for enterotoxin production. As DVC counts were almost constant during the experiments, and no cul-turable cells could be detected, it was interpreted that the entire population produced the enterotoxin rather than a few active cells [6].

A general conclusion that may be drawn from many of the studies described above is that the hostile situation encountered by enteric bacteria in seawater promotes the loss of colony forming ability while maintaining different aspects of viability. As already pointed out in the past, their fate can thus be considered a special case of the VBNC phenomenon [13]. While it is difficult to point out which, if any, of the several stress factors in this environment may be more important, it is tempting to find similarities between this situation and the responses to starvation, often a major player in the entry into the VBNC condition [13].

4. Abiotic factors affecting survival

4.1. Light

Survival of enteric bacteria in the sea is greatly affected both by UV and visible light. In fact, light is considered to be the single most important contributor to bacterial die-off in the sea [30-33], although its effects are restricted to shallow depths [33]. A depth-dependent effect was also implicated in the importance of the radiation wavelength [34,35].

The toxic effect of light on E. coli in seawater was demonstrated to cause a rapid decrease of colony forming ability [9,12,32,36]. The UV-B (280-320 nm) portion of the solar spectrum is the most bactericidal, causing direct photosynthetic DNA damage [37]. At higher wavelengths, photochemical mechanisms become more important, usually acting through photosensitizers and tending to be more injurious in the presence of oxygen [33].

Fecal microorganisms differed in their sensitivity to light in seawater [33-35], and greater sunlight exposure was required to inactivate enterococci compared to fecal coliforms; the latter group was also found to be more sensitive to light inactivation than fecal bacteriophages (somatic coliphages, F- DNA phages, F- RNA phages and Bacillus fragilis phages) [35].

Light was also demonstrated to exert a significant neg-ative effect on culturability in an in situ experiment, where natural sewage populations of E. coli and enterococci where placed 1-1.5 m deep in seawater for 7 days. The
effect was pronounced when batch culture cells were used, but not in diffusion chambers [38].

In a different study, E. coli and S. typhimurium were exposed to light (approx. 1 MJ m⁻² h⁻¹) in seawater for 4 and 10 h, respectively. Total counts were not affected, while DVC and CFU in both strains decreased by 4-5 logs [12]. Pommepuy et al. [6] demonstrated that following a 26 h exposure to natural sunlight in seawater, E. coli cells showed stable DVC and TC counts, but CFUs decreased from 2 × 10⁶ ml⁻¹ to an undetectable level. Somewhat different effects were obtained under artificial visible light (40 klux); E. coli H10407 concentrations were unaffected when measured as TC, while DVC and CFU exhibited approx. 1.5 and 4 log reduction, respectively, within a 40 h experiment [9]. Similar results were presented [9] for E. coli exposed to visible light (125 W m⁻²) for the same duration: no decrease in TC, approx. a 1 log decrease in DVC and a 6 log decrease in CFU. In non-illuminated bacteria, only a 1.5 log decrease in the CFU was measured, with no effect on either TC or DVC [9].

Kapuscinski and Mitchell [40] showed sublethal injuries to an E. coli isolate incubated in autoclaved filtered seawater exposed to sunlight. The injuries were apparent during the recovery stage on complete (lactose nutrient agar) media, and were not observed in the dark. Addition of either pyruvate or catalase to the minimal medium restored colony forming ability to the levels of the complete medium, implying at least a partial involvement of peroxide damage.

The involvement of direct photooxidative processes in the toxicity of visible light to E. coli was pointed out by Gourmelon et al. [11]. The effect was significantly lower under anaerobic conditions, and was also alleviated in the presence of a hydroxyl radical scavenger (thiourea), an iron chelator (desferrioxamine B), or catalase.

The role of reactive oxygen species in the toxic effect of visible light on E. coli in seawater was also studied by the use of mutants, defective in specific genes involved in antioxidant defense systems. Exposure to artificial light (0.9 mmol photons m⁻² s⁻¹) for 50–100 h highlighted the importance of a functional rpoS for cell survival as determined by colony counts [41]. RpoS is a σ factor involved in various environmental stresses and its presence was shown to have a protective effect on stationary phase E. coli in seawater [42,43]. The RpoS protective effect against light was evident only in stationary phase cells, and a mutation in the rpoS gene caused a CFU decrease of about 5 logs compared to the wild-type. In the stationary phase, mutants defective in genes involved in resistance to hydrogen peroxide (katE katG (catalases), dps (DNA binding protein) and katE katG dps) were all more sensitive to light (1–2 logs) than their corresponding wild-types [41]. In exponentially growing cells, a mutation in oxyR, the regulatory gene of the adaptive response to H₂O₂, led to an increased sensitivity to light (approx. 1 log) [41], further suggesting that the deleterious effects may be associated with H₂O₂ production. However, the increased sensitivity of the rpoS mutant suggested that rpoS-dependent protection against deleterious effects may be independent of H₂O₂ removal [41]. Possible involvement of superoxide radicals was indicated by an enhanced sensitivity of the double superoxide dismutase mutation (sodA sodB); individually, each of these mutations had no effect. Other mutants the seawater survival of which was not affected in the light were the double mutants otsA otsB (trehalose synthesis), fpg uvrA, and recA (DNA repair), xthA (exo-nuclease III), and katE. Interestingly, a mutation in hemA (glutamyl-tRNA reductase), involved in δ-aminolevulinic acid formation from glutamate, had a protective effect [39].

A certain mitigation of the hazardous effect of solar radiation may be provided by dissolved organic material [44], chlorophyll, and particulate matter [45,46]. Such an effect is more pronounced in relatively eutrophic coastal and estuarine areas, and is particularly relevant for UV-B radiation (280–320 nm) owing to selective absorption of shorter wavelengths. Consequently, bacteria in such areas are primarily exposed to visible light (400–775 nm) and, to a lesser extent, to UV-A (320–400 nm) radiation.

An environmental condition that was found to enhance the bactericidal effects of visible light was salinity, suggesting a synergistic effect of the combined stress factors. It was proposed [39,41] that the salinity-driven sensitization is mediated by endogenous components, such as protoporphyrin or ubiquinone biosynthesis intermediates.

4.2. Salinity

When released into the sea, enteric bacteria are subjected to an immediate osmotic upshock, and their ability to overcome this by means of several osmoregulatory systems could largely influence their subsequent survival in the marine environment [24,47].

Survival of E. coli in seawater/distilled water mixtures at different ratios (0, 25, 50, 75 and 100% seawater) for 48 h showed an optimal survival (74%) at 25% seawater. Survival in the end members – distilled water and seawater – was 60% and 8%, respectively [48]. A generally similar effect was reported by Anderson et al. [49], who monitored the survival of an E. coli isolate for 8 days in seawater at selected salinities (1, 1.5, 2.5, and 3%): decreasing salinity was accompanied by increasing survival. Similar results were obtained when NaCl solutions were used instead of seawater [48].

The interactive effects of salinity (sea salts, 3.7%), light (sunlight, approx. 300 W m⁻²) and the presence of organic matter (100 mg l⁻¹ glucose) were studied by Troussellier et al. [9]. Salinity was found to increase E. coli sensitivity (CFU) only in the presence of light, regardless of the presence of organic matter. In the same study it was also shown that an osmotic upshock combined with nutrient deprivation inhibited several transmembrane transport
systems within 60 min in different E. coli strains [9]. During a much longer incubation (30 days), Flatau et al. [50] found different accumulation patterns for histidine (constant accumulation) and maltose (increased during the first 2 weeks and then slowly decreased).

Upon an osmotic upshift, bacterial cells accumulate or synthesize specific osmoprotectant molecules, in order to equalize osmotic pressure and avoid drastic loss of water from the cytoplasm [51]. The accumulation or synthesis of such molecules (trehalose, glycine betaine, glutamic acid) in Salmonella manhattan and S. typhimurium in estuarine waters (3.5% salinity, 20°C) was detected by H-NMR. S. manhattan accumulated trehalose, and an unidentified substance, whereas S. typhimurium accumulated mostly glycine betaine [9]. Trehalose synthesis was also observed for wastewater-borne S. manhattan in oligotrophic seawater [52].

Gluconate added to filter-sterilized seawater was shown to enhance E. coli MC4100 culturability. The effect appeared to be logarithmically correlated to glutamate concentration; glycine betaine uptake and its protective effect were both enhanced in the presence of glutamate [53].

The essential role of osmoregulatory mechanisms in enteric bacterial survival in seawater was demonstrated in several studies. Munro et al. [47,54] and Gauthier et al. [24] found that cells preadapted to high osmolarity are highly resistant to seawater. This, however, depended upon the preadaptation media. Among the induced systems that were shown to be of value in conferring a protective effect for growing E. coli MC4100 were potassium transport, glycine betaine synthesis or transport, trehalose synthesis [47] and glutamate accumulation [55]. The protective advantage of glycine betaine in nutrient-free seawater seemed to vary according to different reports [47,56].

The resistance of E. coli grown in saline media (M9, 0.5 M NaCl) to seawater was suppressed by an osmotic downshift that resulted in a loss of several osmolytes previously accumulated [55]. An osmotic downshift (distilled water or wastewater) increased loss of culturability [55,57].

The survival of non-adapted E. coli cells was not affected by impaired K⁺ transport or glycine betaine uptake, while trehalose synthesis ability was apparently important [47].

4.3. pH

Seawater pH normally ranges between 7.5 and 8.5, and is influenced by temperature, pressure, and the photosynthetic and respiratory activities of microorganisms [58]. An acidic pH (5) was found to be most favorable for E. coli survival (in the 5.0–9.0 range) in both seawater and NaCl solutions, and sensitivity increased with the increase in pH [48]. The authors concluded that seawater pH, normally around 8, contributes to the deleterious effects on E. coli survival.

4.4. Nutrient deprivation

It is a little recognized fact that in the presence of sufficient nutrient levels, E. coli can grow in seawater almost as well as it does in rich laboratory media. In fact, it has been shown that in nutrient-enriched seawater E. coli competed successfully with five marine bacterial isolates [59]. Lopez-Torres et al. [60], in their study of K. pneumoniae and E. coli in membrane diffusion chambers at different coastal areas in Puerto Rico, showed increased survival of E. coli and to a lesser extent of K. pneumoniae (respiring cells) in a site receiving rum distillery effluents. The density of E. coli declined immediately after the effluent discharge stopped, suggesting that the organic load improved their survival [60].

Regularly, however, the amounts of inorganic and organic nutrients in seawater are dramatically lower than those of laboratory media or wastewaters, and bacteria released into the marine environment have to contend with starvation conditions. This was already shown by Carlucci and Pramer [48], who demonstrated that E. coli survival improved with increasing nutrient concentrations, both organic and inorganic. The effect of organics was pronounced when peptone and sewage volatile solids were added, but not glucose. The effect of sewage volatile solids was more pronounced in filter-sterilized seawater, indicating a possible competition for available nutrients with indigenous marine microflora.

Similar results were obtained by Troussellier et al. [9], who studied the combined effects of salinity, light and nutrient availability. When only nutrient deprivation (glucose) was imposed, a small energy charge decrease was observed but CFU and transport abilities were maintained. Combined with the other stress factors, nutrient deprivation led to the inactivation of membrane transport and to a pronounced reduction in energy charge.

Our own results (Rozen and Belkin, unpublished) indicate that while the presence of nutrients actually allows E. coli to grow in seawater, their absence does not necessarily affect the survival (colony formation) of non-growing cells. In fact, cells which were induced to grow in nutrient-enriched seawater were more susceptible to various mutations (kdpABC trkA, kdpABC trkE (potassium transport), nhaA (Na⁺/H⁺ antiporter), fns (histone-like protein), sodA sodB and rpoE (σE)) that had no effect in unamended seawater (see Table 1).

4.5. Temperature

Seawater temperature is another obvious shock confronted by microorganisms the optimal growth of which occurs around 37°C [61]. However, the optimal temperature for survival is not necessarily the same as the one for growth. Indeed, most reports indicate enhanced stability of E. coli at lower temperatures. This was shown by Carlucci and Pramer [62] in a 48-h experiment in natural
seawater (5–40°C), and by Vasoncelos and Swartz [63] in a
6-day experiment in diffusion chambers exposed to tem-
peratures ranging from 8.9°C to 14.5°C.
A similar tendency was reported by Lessard and Sie-
burth [38], who measured the viability (CFU) of natural
sewage populations of E. coli and enterococci in diffusion
chambers from February to August, with experimental
temperatures ranging from 0°C to 20°C. Though other
environmental changes may have contributed to bacterial
survival, it was significantly higher at the lower tempera-
sures.
Short-term experiments (6 h) measuring survival (TC,
DVC, CFU) of Salmonella montevideo in filtered seawater
exposed to visible light (1.03–1.12 MJ m−2 h−1) at three
different temperatures (5°C, 15°C, 25°C), showed no
change in any of the parameters tested, and no differences
among the three temperatures possibly due to the short
time span of the experiment [12].
4.6. Hydrostatic pressure
The release of wastewaters into deep waters was sug-
gested as a solution for immediate public health hazards
cased by near offshore disposal. While the die-off rate of
fecal microorganisms in offshore conditions was empha-
sized in many studies, their resistance and fate under deep-
sea conditions were studied to a much lesser extent.
The response of E. coli to elevated hydrostatic pressure
was described at both the whole cell physiology and pro-
teins levels. A pressure upshift to 546 atm dramatically
inhibited E. coli W3110 growth measured as optical den-
sity, CFU or TC (epifluorescence direct counts) [64]. A
linear decrease in the rate of protein synthesis was ob-
served with increasing pressure up to 1092 atm, where it
essentially ceased. Increased pressure also caused the mod-
ulation of synthesis of specific proteins. Two-dimensional
gels revealed the induction of 55 proteins; 11 of them were
shown to be heat-shock proteins and four were cold-shock
proteins [64].
Under simulated deep-sea conditions (seawater at low
temperature (4°C) and high hydrostatic pressure (up to
1000 atm) [65]) the survival of pure cultures of four human
enteric bacteria as well as a sewage bacterial population
was found to be species-dependent. Following a 12-day
incubation at different hydrostatic pressures, the survival
in some cases (Clostridium perfringens, Vibrio parahaemo-
lyticus and an aerobic sewage bacterial population) was
negatively affected by the pressure, while E. coli and S.
faecalis exhibited higher survival rates at 250 and 500 atm
than at 1 or 1000 atm [65].
It should be noted that the effects reported for hydro-
static pressure (even at 1000 atm) were variable and ap-
parently species-specific. The dumping of raw or partially
mixed sewage into deep waters, therefore, does not guar-
antee a reduction in the health risks inherent in waste-
water-borne microorganisms.
4.7. Sedimentation
Several studies, focusing on the occurrence of coliforms,
fecal coliforms or enterococci in estuarine water and coast-
al sediments, revealed that greater numbers (10- or a 100-
fold higher) of these organisms are found in the sediments
than in the water above them [66,67]. This phenomenon
had been the impetus for detailed studies determining that
these organisms indeed survive better in sediments than in
the water [66,68], and for studies attempting to reveal the
factors involved in the protective effect of the sediment
[66,67,69,70].
A longer survival time of E. coli in sediments than in
seawater was demonstrated in several cases, and was at-
tributed to the higher organic matter content of the sedi-
ment [66,68–70]. One of the compounds shown to be
present was glycine betaine [71]; indeed, marine E. coli
isolates were shown to accumulate glycine betaine from
autoclaved estuarine sediments when mixed with a mini-
mal medium, a phenomenon that was not observed with-
out the sediment [70]. Based on these results, a study try-
ing to assess the protective effect of glycine betaine on E.
coli survival in three media (seawater, low- and high-or-
ganic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
ganic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
ganic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
ganic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
ganic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
media, the addition of glycine betaine had a protective
effect on the survival of E. coli survival in three media (seawater, low- and high-or-
organic carbon sediments) was conducted [56]. In all three
519
FEMSRE 725 5-12-01
mined after 2 and 6 days of incubation) when exposed during the lag phase, lower sensitivity in the exponential phase and a minimal one during the stationary phase [76]. In the light, the difference between exponential and stationary phase cells was much less pronounced [9]. For anaerobically grown cells, a different pattern was observed after 1 or 2 days of exposure, but not after 6 days [76].

In general, cells grown under anaerobic conditions were found to be much more sensitive than aerobically grown cells [73,76], but this effect was not observed when TC was the parameter measured [73]. This correlates well with the possible involvement of reactive oxygen species in the stress imposed by seawater, and hence with the significance of the adaptation to oxidative conditions.

In order to determine the nature of the protection acquired in the preadaptation phase, E. coli MC4100 cells were exposed to five different types of stress for up to 4 h: thermal (48°C), oxidative (H2O2, 1 mM), acid (pH 5.1), osmotic (0.5 M NaCl) and starvation (a nutrient-deficient buffer). The cells were then incubated in seawater (22–25°C) for 6 days. All pretreatments led to a better resistance to seawater compared to non-treated cells [42]. In a different report, a 44°C pretreatment was found to decrease such resistance (CFU), as did growth at pH 6 or 8 compared to pH 7 [73].

Munro et al. [42] concluded that the relative adaptive importance of the various environmental conditions may be ranked as follows: osmotic > starvation > oxidative > acidic > thermal. Apart from osmotic stress, all others induced higher resistance to seawater in an rpoS+ strain than in the rpoS mutant, suggesting that the cross-protection endowed by oxidative, acidic, thermal and nutritional stresses was rpoS-dependent [42].

There was an increased resistance to seawater of cells preadapted to high osmolarity, by 0.5 M NaCl [42,47,73] or by 0.8 M saccharose [47]. The preacquired capabilities for K+ transport, glycine betaine synthesis or transport, and trehalose synthesis, helped to increase the ability of E. coli to survive in seawater [47]. A negative effect on resistance was reported to be exerted by a hypoosmotic shock prior to seawater exposure [55,57].

Another pretreatment variable tested was medium composition. Gauthier et al. [73] reported significant differences between media only after more than 7 days, but with no correlation to the richness or complexity of the medium. An additional adaptive parameter tested by Gauthier et al. [73] was phosphate concentration: low phosphate grown cells, in which alkaline phosphatase synthesis was induced, were shown to remain culturable after longer periods in seawater.

5. Biotic factors

Although mostly very poor in nutrient concentrations, the marine environment is nevertheless inhabited by relatively diverse biological populations that may affect the fate of enteric bacteria entering the system. Indeed, the observations that the numbers of enteric bacteria decreased in natural seawater more than in sterile seawater [24,77] certainly suggest the involvement of biological processes. Support for this hypothesis was provided by Le Guyader et al. [78], who monitored the viability (CFU) of an E. coli culture for 13 days in seawater and sediment. In the presence of seawater indigenous flora, E. coli concentrations decreased earlier and at a faster rate compared to sterile conditions.

Among the major biological mechanisms that have been implicated in affecting enteric bacterial concentrations in seawater are predation [7,77,79–85], competition [59,78,79,86] and bacteriophages [82,87].

5.1. Grazing and competition

Various studies indicate that the main predators of bacteria in the marine environment are protozoa [7,77,84,85]. Using filters with different pore sizes and antibiotics to suppress indigenous bacterial activity, it was shown that bacterial competition, antagonism, and even bacterial predation were relatively unimportant in coliform removal. It was also shown that the reduction in E. coli populations paralleled an increase in the number of protozoa [84]. In the same vein, the survival of fecal coliforms was significantly improved in the presence of cyclohexamide, a compound expected to inhibit eukaryotic organisms. Interestingly, Clostridium perfringens and fecal streptococci were not affected by the addition of this inhibitor, which could be explained either by differential resistance of protozoan species to cyclohexamide or by selective predation on the different bacterial species [7].

Another demonstration of the role protozoan predation plays in enteric bacterial disappearance from marine waters was provided by Gonzalez et al. [77], who enumerated E. coli and E. faecalis for 5 days in natural and filtered (0.2 µm) seawater. Both CFU and direct counts decreased significantly (3–5 logs) in the presence of natural microbiota, an effect that was attributed to protist activity.

Mitchell et al. [81] demonstrated that seawater samples plated on E. coli lawns on solid agar resulted in bacterial colonies surrounded by clearing zones; these were attributed to bacteria utilizing the cell wall of E. coli as a carbon source. Also observed were plaques, possibly caused by obligate parasites such as Bdellovibrio.

Mitchell and Nevo [80] isolated from seawater a Pseudomonas sp. capable of utilizing Flavobacterium capsular polysaccharide as its sole carbon source. They also isolated bacteria capable of growth on capsules of Azotobacter, Rhizobium, Arthrobacter, and the cell wall of E. coli. Activity of the isolated Pseudomonas against living cells of Arthrobacter and E. coli was tested by joint growth in artificial seawater with 0.1% peptone. The authors concluded that growth of both ‘terrestrial’ bacteria was mark-
edly suppressed by the marine bacterium, though this was based on optical density rather than on a more accurate enumerating method.

The role of competition with marine bacteria has been investigated in several studies [59,81]. Experiments measuring \textit{E. coli} survival in autoclaved seawater with different sizes of marine microbial inocula showed a clear effect on \textit{E. coli} CFU counts. Within 7 days, the presence of 10^7 marine bacteria ml^{-1} decreased the \textit{E. coli} population by about 4 logs, while in autoclaved seawater almost no decrease was detected [81]. Furthermore, when a mixed marine bacterial/\textit{E. coli} culture was reinoculated with \textit{E. coli} after 7 days, its elimination from the medium proceeded rapidly with no lag phase. The enhanced removal rates suggested to the authors the existence of microflora parasitic or lytic to \textit{E. coli} [81].

Another approach that was taken in order to assess the role of competitive elimination of Enterobacteriaceae from seawater was based on selecting and isolating organisms defined as successful competitors under various dilution rates or different limiting nutrient concentrations in chemostat experiments [59]. \textit{E. coli} was found to be a successful competitor in rich media but a very poor one under the low nutrient concentrations characterizing natural seawater [59].

5.2. Bacteriophages

Many studies detected coliphages in marine waters subject to sewage contamination [35,82,87–90]. It was also shown that phages detected in seawater were active against \textit{E. coli}, \textit{Aerobacter aerogenes}, and \textit{Serratia marinorubra} [87]. The presence of coliphages was investigated in relation to their usage as indicators for fecal contamination [90–92], and in many cases a positive correlation between fecal phages, enteric viruses, and other pathogens has been recorded [88,91–94]. Nevertheless, the presence of enteric bacterial infectious phages does not necessarily indicate their actual activity in removing coliforms from marine water.

Carlucci and Pramer [87] have shown that bacteriophages were effective in reducing \textit{E. coli} population sizes only under nutrient-rich conditions, suggesting a very minor role for bacteriophages, if any, under natural conditions. This was corroborated by Penon et al. [95], who found no significant deleterious effect of the seawater fraction passing 0.2-μm filters (containing bacteriophages but not bacteria or protozoa) on the survival of fecal bacteria in seawater. The 0.2–2-μm fraction, in contrast, had a significant effect.

5.3. Antibiotics and toxins

Several studies from the 40s through the 60s suggested that the deleterious effects of seawater on enteric bacteria that could not be explained by other factors known at the time, were caused by toxic substances believed to be antibiotics produced by microorganisms [79,96–98]. Similarly, there was also a possible indication of the negative effects of an algal toxin [87].

All reported attempts to find an antibiotic effect of seawater on enteric bacteria yielded no evidence that such compounds are produced under natural conditions and contribute to the elimination of their targets from seawater.

6. Molecular mechanisms involved in seawater survival

Many of the studies quoted to this point share a similar characteristic: colony formation, and to a lesser extent other viability criteria, serve as a practically sole parameter in determining the factors controlling the sensitivity or survival of \textit{E. coli} and other enteric bacteria in seawater. It appears as if a substantial gap exists between these reports and the enormous wealth of information presently available on bacterial physiology, biochemistry and molecular biology. This gap is especially pronounced in the latter category; many of the molecular responses of \textit{E. coli} and \textit{Salmonella} to the environmental parameters mentioned above (salinity, starvation, pH etc.) were studied in great detail, but very few attempts were reported to make use of these data in respect to bacterial survival in seawater. These reports, most of them from the last decade, will be summarized below, along with a few recent findings from our own laboratory.

6.1. Effect of specific mutations

Past attention to the molecular mechanisms determining survival of enteric bacteria such as \textit{E. coli} in artificial seawater or sterile seawater in the dark revealed very few genes crucial to seawater survival. Only six mutations were shown to have a significant effect. The most dominant among them was in \textit{rpoS} [9,39,41–43]. The \textit{rpoS} mutation was shown to be significant were \textit{otsA} [47], \textit{relA}, \textit{spoT} (but only on top of a \textit{relA} deletion [43]) and one or both membrane porins \textit{ompC} and \textit{ompF} [99].

As already noted, the most dominant gene for seawater survival of the above list was \textit{rpoS} [9,39,41–43]. An \textit{rpoS} mutation was shown to decrease seawater survival (CFU) of stationary phase \textit{E. coli} cells by approx. 3 logs over 8 days [43]. The \textit{rpoS} (σ^c) transcription factor controls the expression of a large number of genes involved in cellular responses to a diverse number of stresses, including starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative damage, and transition to stationary phase. A list of over 50 genes under the control of \textit{rpoS} has been compiled [101]. It normally acts as a positive regulator, but some genes are under its negative control. The synthesis and accumulation of σ^c were shown to be
Table 1
Effect of specific mutations on *E. coli* seawater survival and on growth in nutrient-supplemented seawater

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant mutation</th>
<th>Survival in the dark&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival in the light&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Survival in the dark after preadaptation</th>
<th>Growth&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRA-5 [107]</td>
<td>kdpABC</td>
<td>− [47]. [Rozen]</td>
<td>− [47]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TK110 [107]</td>
<td>kdpABC, kefB (trkB)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TK121 [107]</td>
<td>kdpABC, kefC (trkC)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TK133 [107]</td>
<td>kdpABC, trkA</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TK142 [107]</td>
<td>kdpABC, trkE</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TK2205 [47]</td>
<td>kdpABC, trkA, trkD</td>
<td>− [47]</td>
<td>+ [47]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TK401 [107]</td>
<td>kdpABC, trkA, trkD</td>
<td>− [Rozen]</td>
<td>+ [47]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TK2240 [47]</td>
<td>trkA, trkD</td>
<td>− [47]</td>
<td>+ [47]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PLB3360 [108]</td>
<td>envZ (ompF)</td>
<td>+ [99]</td>
<td>+ [99]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PLB3361 [108]</td>
<td>envZ (ompC)</td>
<td>+ [99]</td>
<td>+ [99]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SG477 [109]</td>
<td>envZ (ompC)</td>
<td>− [Rozen]</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SM26 [99]</td>
<td>envZ (ompF, ompC)</td>
<td>+ [99]</td>
<td>+ [99]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SG480 [109]</td>
<td>envZ (ompF, ompC)</td>
<td>− [Rozen]</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UE14 [110]</td>
<td>treA</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK332 [111]</td>
<td>nhaA</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TA15D2 [112]</td>
<td>hns</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>J2 [113]</td>
<td>osmB</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FF4169 [104]</td>
<td>otsA</td>
<td>+ [47]</td>
<td>+ [47]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RO22 [105]</td>
<td>otsAB</td>
<td>− [41]</td>
<td>+ [47]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EF947 [114]</td>
<td>proP, proU</td>
<td>− [47]</td>
<td>+ [47]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RH90 [115]</td>
<td>rpoS</td>
<td>+ [42, 43]</td>
<td>+ [42]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>QC2410 [116]</td>
<td>rpoS</td>
<td>+ [Rozen]</td>
<td>+ [41]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UM122 [117]</td>
<td>rpoS</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA8306 [118]</td>
<td>cyaA</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA8445 [119]</td>
<td>cyaA, crp, rpoL</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA8445-1 [119]</td>
<td>crp, rpoL</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CF1652 [120]</td>
<td>relA</td>
<td>+ [43]</td>
<td>+ [43]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CF1693 [120]</td>
<td>relA, spoT</td>
<td>+ [43]</td>
<td>+ [43]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2408 [116]</td>
<td>dps</td>
<td>−</td>
<td>+ [41]</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2445 [121]</td>
<td>fur</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>UM120 [117]</td>
<td>katE</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2476 [116]</td>
<td>katE, katG</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>QC2463 [116]</td>
<td>katE, katG, dps</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>QC2473 [122]</td>
<td>soxR</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2474 [122]</td>
<td>soxS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2477 [123]</td>
<td>sodA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2478 [123]</td>
<td>sodB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2479 [123]</td>
<td>sodA, sodB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2482 [116]</td>
<td>sodA, sodB</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2589 [122]</td>
<td>sodA, sodB, zwf</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC2431 [116]</td>
<td>oxyR</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SASX41B [39]</td>
<td>hemA</td>
<td>Protective effect [39]</td>
<td>+ [41]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HB101 [39]</td>
<td>recA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DM805 [124]</td>
<td>lexA (SOS repressed)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>QC227 [39]</td>
<td>fpg, uvrA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BW295 [39]</td>
<td>xhpA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CAG0333 [125]</td>
<td>rpoH (groE constitutive)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>AB1899 [126]</td>
<td>lon</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CAG22188 [127]</td>
<td>rpoE</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TN3151 [128]</td>
<td>uspA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CLG302 [75]</td>
<td>phoA</td>
<td>− [75]</td>
<td>+ [75]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W2961 [129]</td>
<td>phoE</td>
<td>+ [100]</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K10 [130]</td>
<td>pit</td>
<td>− [100]</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C10a [130]</td>
<td>pit, pstA</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C75a [131]</td>
<td>pit, phoR</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C75b [132]</td>
<td>pit, phoS</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C112a [132]</td>
<td>pit, phoT</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>, significant effect of the mutation; <sup>b</sup>, no effect; <sup>c</sup>, mutation not tested.
controlled at the transcription, translation, holoenzyme complex formation and proteolysis levels. Transcriptional control of rpoS involves guanosine 3',5'-bispyrophosphate (ppGpp) and polyphosphate as positive regulators, and the cAMP receptor protein–cAMP complex (CRP–cAMP) as a negative one [101].

Munro et al. [43] have demonstrated that in addition to rpoS, a relA deletion and the double relA spoT mutation also affected seawater survival of E. coli. The absence of these two genes from stationary phase E. coli was shown to cause an approx. 4 log decrease in E. coli colony formation ability over 8 days. RelA and SpoT are enzymes, ppGpp synthetase I and II respectively, that are responsible for the synthesis of ppGpp [102]. Among the multiple effects shown to be caused by the increase in intracellular ppGpp concentration [102] is an enhancement of rpoS expression [103]. The hypothesis that the intracellular level of ppGpp could affect seawater survival due to lower rpoS expression was suggested [43], but was not experimentally tested.

An additional gene shown to be important in determining E. coli’s sensitivity to seawater was otsA [47]: within an 8-day experiment, colony formation ability of the mutant (E. coli FF4169) decreased 10–100-fold compared to the wild-type. The otsA gene codes for trehalose-6-phosphate synthase, an enzyme that catalyzes the synthesis of the osmoprotectant trehalose; it is transcriptionally activated both by osmotic stress [104] and by growth into the stationary phase [105]. Not surprisingly, otsA is RpoS-controlled [105,106], and it is not unlikely that at least part of the deleterious effect of the rpoS mutation on E. coli seawater survival is due to the absence of trehalose.

Mutants lacking OmpC, OmpF or both (E. coli strain PLB3261, PLB3260 and SBM26, respectively) also seemed to be impaired in their seawater survival. The relative significance of the two porins depended upon pregrowth conditions: ompF was more dominant following exponential growth in low osmolarity, while ompC appeared to be more important for survival following exponential growth in high osmolarity [99]. It should be noted that standard deviations in these experiments were large, and not all differences appeared significant. In a different experiment, other E. coli strains lacking OmpC or both OmpC and OmpF (strain SG477 and SG480, respectively) were pre-grown to stationary phase in minimal medium. No effect on survival of either mutation was observed during a 7-day seawater exposure (Table 1).

Another group of genes shown to be important for seawater survival when previously grown in a low phosphate (0.1 mM Na3HPO4) medium is connected with phosphate regulation. Mutation in the porin phoE, in the regulatory gene phoR, and in genes related to the high-affinity phosphate-specific transport system pstA, phoS and phoT, decreased survival 10–1500-fold after 6 days in filtered sterilized seawater [100]. Furthermore, E. coli CLG 302, totally deprived of alkaline phosphatase activity due to a phoA mutation, was found to have increased seawater sensitivity when previously grown in a low phosphate (0.1 mM K3HPO4) medium [75].

A few other genes were also implicated in the ability of E. coli to form colonies on solid media, but only in conjunction with additional stress factors or following specific growth conditions. Numerous genes involved in protection against reactive oxygen species (rpmS, katE katG, dps, sodA sodB and oxyR) proved to be important when the cells were exposed to visible light [39,41] (see Section 4.1). The absence of two genes encoding for glycine betaine transport, prop and proU (E. coli EF047), was found to increase E. coli MC4100 seawater sensitivity only when previously exposed for 1 h to high osmolarity and glycine betaine (0.1 mM) [47].

Similarly, mutations in two potassium transport systems, kdpABC (osmotically inducible high-affinity transport, E. coli TK2240) and trkA (constitutive low-affinity transport, E. coli TK2205) were found to have a deleterious effect on E. coli (MC4100) survival in seawater only when previously grown in a minimal medium at high osmolarity (0.5 M NaCl) [47].

The absence of one gene, induced by high osmolarity, but with an unknown function – osmB (strain E. coli JJ2)
was found to have a surprisingly protective effect on seawater survival compared to its parental strain [47]. No explanation for this phenomenon was provided. The same effect was observed for *E. coli* strain SASX41B, mutated in *hemA*, when incubated in seawater exposed to visible light. In this case, a decrease in intracellular photosensitizer concentrations was suggested [39].

Table 1 summarizes previously published data concerning the effect of specific gene mutations on colony formation ability of *E. coli*. It also includes results of further gene screening in our laboratory, in which additional gene mutations were tested for their effect; the survival of none of these was significantly different from that of its respective wild-type.

Another potential approach to discovering genes the products of which may be important is not only to expose the cells to seawater, but also to force them to attempt to grow under these conditions. This can be carried out by amending the seawater with sufficient nutrients. Table 1 also includes the results of such growth experiments carried out under nutrient-rich conditions for all the genes listed, each mutant compared to its own wild-type parental strain. An effect was noted when the deleterious influence of the mutation on growth in seawater-based Luria–Bertani broth (LB) was significantly higher than its effect on growth in regular LB. Out of 34 mutations screened, there were several that did not affect survival in unamended seawater but had a marked effect either on the growth rate or the duration of the lag phase that preceded it. Among those were strains with mutations in genes involved in osmoregulatory processes (*kdpABC* *trkA*, *kdpABC* *trkE*, *ompF* *ompC*), defenses against oxidative stress (*sodA* *sodB*), the σ factor *rpoE*, the gene encoding the histone-like protein H-NS (*hns*), and the sodium proton antiporter (*nhaA*). The effect of the latter mutation was the most dramatic: no growth at all was observed in the mutant. The mutation in *rpoS* had the expected effect in both incubation conditions.

6.2. Genes induced upon seawater exposure

Another possible strategy in the search for genes playing a role in seawater survival is to look for those activated in response to seawater exposure. Such a study was conducted by Rozen et al. [139] who screened a 687-member *E. coli* promoter::luxCDABE fusion library [135]. Twenty-two promoters were found to be activated in seawater amended by sufficient nutrients to allow bioluminescent expression but not growth. The luminescence driven by these promoters (*osmY*, *ldcC*, *treA*, *otsA*, *poxB*, *yciG*, *ybhP*, *yohF*, *yohC*, *ybdK*, *yeaG*, *yhdW*, *prpR*, *ytrF*, *ykgC*, *phrB*, *yjhT*, *ykgD*, *cysL*, *pstS*, *yglL*, *yjhG*) increased at least 2-fold; only nine of the corresponding genes have previously been assigned a function. The most prominent characteristic of the induced genes was that most of them (17 out of 22) were under *rpoS* positive control (*osmY*, *ldcC*, *treA*, *otsA*, *poxB*, *yciG*, *ybhP*, *yohF*, *yohC*, *ybdK*, *yeaG*, *yhdW*, *prpR*, *ytrF*, *ykgC*, *phrB*, *yjhT*, *ykjD*, *cysL*, *pstS*, *yglL*, *yjhG*) increased at least 2-fold; only nine of the corresponding genes have previously been assigned a function. The most prominent characteristic of the induced genes was that most of them (17 out of 22) were under *rpoS* positive control (*osmY*, *ldcC*, *treA*, *otsA*, *poxB*, *yciG*, *ybhP*, *yohF*, *yohC*, *ybdK*, *yeaG*, *yhdW*, *prpR*, *ytrF*, *ykgC*, *phrB*, *yjhT*, *ykjD*, *cysL*, *pstS*, *yglL*, *yjhG*) increased at least 2-fold; only nine of the corresponding genes have previously been assigned a function. The most prominent characteristic of the induced genes was that most of them (17 out of 22) were under *rpoS* positive control (*osmY*, *ldcC*, *treA*, *otsA*, *poxB*, *yciG*, *ybhP*, *yohF*, *yohC*, *ybdK*, *yeaG*, *yhdW*, *prpR*, *ytrF*, *ykgs*, *phrB*, *yjhT*) and one was under *rpoS* negative control (*yjbG*). The most significant environmental factors responsible for inducing the majority of these promoters appeared to be nutrient limitation. Salinity or osmolarity were instrumental in only four cases (*osmY*, *yciG*, *ybhP* and *yohG*), and in three promoters (*cysL*, *yglL* and *yjhG*) increased pH also appeared to play a role [139].

To our knowledge, no data were published regarding de novo synthesis of proteins in response to seawater incubation, though there are clear evidences that low-level protein synthesis occurs under such conditions for at least 3 weeks (Rozen and Belkin, unpublished).

7. Concluding remarks

A review of the scientific literature on the survival of enteric bacteria in the marine environment reveals a set of attempts, many of them highly empirical in nature, to describe the actual fate of wastewater or enteric bacteria upon exposure to seawater. Only in recent years have these been expanded to include also some molecular aspects of the studied phenomena. In this review we attempted to clarify the extent of our knowledge in the field, and derive from it a definition of desirable future research directions.

While it is generally accepted that one of the earlier phenomena observed in enteric bacteria exposed to seawater is the loss of the ability to form colonies on solid media, there is a controversy in regard to the physiological state of the nonculturable cells. Bogosian et al. [16,17] claim that VBNC cells are either dead or of no significance as they cannot be resuscitated if experimental procedures are carefully carried out. In contrast, other evidence suggests that they are not only viable but that the pathogenic among them may still be infective [6]. Such an observation has a major significance in view of the worldwide practice of releasing non-disinfected wastewaters into the sea and its potential public health consequences. The seawater-related VBNC controversy is a part of the broader issue [136–138] of the true meaning of different viability criteria and of the molecular and biochemical mechanisms controlling the shift from colony forming to VBNC and vice versa. These regulatory systems are only partially understood, and probably vary according to the stimuli imposed on the cells.

When enteric bacteria are exposed to seawater they are simultaneously challenged by a combination of stress factors, including pH, temperature, salinity, nutrient availability, light radiation and its associated oxidative stress. It is claimed [30–33] that the latter two may probably be the most significant components of this hostile combination, though relevant only for shallow waters of coastal areas. Salinity in itself appears to be less significant; in-
rpoS possibly the most significant among such adaptive systems; necessary defense circuits. Can be handled better if the cells can first mobilize at least some of the risks inherent in seawater exposure. Clearly indicates the existence of an adaptation potential: previous growth history also has a major influence on subsequent survival in the hostile marine environment. This clearly indicates the existence of an adaptation potential: at least some of the risks inherent in seawater exposure can be handled better if the cells can first mobilize the necessary defense circuits.

The molecular data available to date clearly point at possibly the most significant among such adaptive systems: the rpoS regulon. At least 50 different genes were previously shown to be under rpoS control; they are induced upon a shift to a stationary growth phase, as well as by diverse stresses including salinity and starvation. Not all rpoS-controlled genes were assigned a clear function, but among those that were, many were involved in combating the effects of such stresses. The dominance of this regulatory circuit was observed both in the significant negative effect that rpoS mutations had on E. coli survival [42,43] and in the observation that rpoS-dominated genes accounted for 18 out of 22 shown to be induced by seawater exposure [139].

The impetus for many of the studies quoted in this review was the desire to protect near-shore areas from fecal pollution and its ultimate effects on human health. In the design of marine sewage effluent outlets, engineers often make use of bacterial (mostly E. coli) ‘die off’ constants. Such values may be based on both laboratory and field experiments, and it is of importance to recognize the limitation of both approaches. The significance of many of the field experiments is often site-specific, and they tend to ignore previous growth history of the monitored strains. Thus, mathematical models based on such results cannot replace the need for routine bacterial pollution monitoring. Conversely, laboratory experiments can be accurately designed to test the effects of specific parameters, but cannot simulate or imitate the complexity of the real marine environment. Nevertheless, a true understanding of responses of enteric bacteria to the imposed conditions and their molecular controls can only be obtained in the laboratory.

Another front in which basic molecular studies will be able to markedly affect wastewater pollution monitoring practices are the detection methodologies of either pathogenic or indicator microorganisms. Present approaches are based upon a few simple though highly informative indicator assays. In recent years, several molecular approaches have been proposed that allow fast and sensitive detection of bacteria, viruses and other organisms. Such methods may lead to a more accurate and rapid wastewater pollution detection, including bacteria in the VBNC state. These methods will provide a broader understanding of the survival of these organisms, thus meeting the challenge of future health management of marine waters.

In order to continue to unravel the dominant mechanisms in seawater survival of enteric bacteria, future research will have to combine traditional field and laboratory viability experiments with the molecular insight allowed by genetic approaches. In addition to enhancing our understanding of the basic mechanisms involved in the defenses against multiple stress conditions, such studies will surely also allow a more knowledgeable forecast of bacterial decay rates in the sea.

One of the directions these studies should take in order to be effective will be towards a broadening of the spectrum of the studied responses. This should include a screening of all E. coli genes, as is now possible with modern DNA array technologies, and be expanded to comprehensive expression assays, specific mutation effects and protein identification. The specific roles played by individual genes and proteins identified in this manner should be studied in greater detail. In parallel, additional enteric bacteria should be studied as well, with a greater focus on infectivity bioassays in addition to the various viability parameters. In this context, the significance of the ‘viable but nonculturable’ issue in seawater calls for a clearer resolution than it has been awarded to date.

Acknowledgements

The use of many E. coli strains from the collection of T.K. Van Dyk and R.A. LaRossa (Dupont, Wilmington, DE, USA), S. Dukan (CNRS/INSU UMR, Marseille, France) and M. Berlyn at CGSC (E. coli Genetic Stock Center, Yale University, New Haven, CT, USA) is gratefully acknowledged. M. Gouremelon is warmly thanked for providing her PhD thesis. This study was supported by the Israel Science Foundation Grant No. 113-00-1 and by the Israeli Ministry of Science and the Arts. Y.R. is a Rieger-JNF fellow.

References

[2] Grimes, D.J., Atwell, R.W., Brayton, P.R., Palmer, L.M., Rollins,


Barcina, I., Gonzalez, J.M., Irberri, J. and Egea, L. (1992) Role of


[129] Lederberg, J., The Rockefeller University, Suite 400 (Founders Hall), 1230 York Avenue, New York, NY 0021-6399, USA.


[131] Garen, A., Molecular Biology and Biophysics, Yale, New Haven, CT, USA.


