Influence of Osmoregulation Processes on Starvation Survival of *Escherichia coli* in Seawater

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The adaptation of enteric bacteria in seawater has previously been described in terms of nutrient starvation. In the present paper, we bring experimental arguments suggesting that survival of these microorganisms could also depend on their ability to overcome the effects of osmotic stress. We analyzed the influence of osmoregulatory mechanisms (potassium transport, transport and accumulation of organic osmolytes) on the survival of *Escherichia coli* in seawater microcosms by using mutants lacking components of the osmotic stress response. Long-term protection was afforded to cells by growth in a medium whose osmotic pressure was increased by either NaCl, LiCl, or saccharose. Achievement of the protection state depended at least partly on osmoregulatory mechanisms, but differed when these were activated or induced during prior growth or in resting cells suspended in phosphate buffer or in seawater. When achieved during growth, K⁺ transport, glycine-betaine (GBT) synthesis or transport, and trehalose synthesis helped increase the ability to survive in seawater. Protection by GBT was also obtained with resting cells in a phosphate buffer at high osmotic pressure. However, when added only to the seawater, GBT did not change the survival ability of cells no matter what their osmoregulation potential. These results showed that the survival of *E. coli* cells in seawater depends, at least partly, on whether they possess certain genes which enable them to regulate osmotic pressure and whether they can be stimulated to express those genes before or after their release into the environment. This expression requires nutrients as the substrates from which the corresponding gene products are made.

Coliform bacteria, like other enteric bacteria, rapidly decline in seawater both "in situ" (2, 16, 39, 54) and in laboratory experiments (1, 10, 27, 64) when counted by conventional methods. Over the past decade, the concept of die-off has been challenged due to reports indicating the existence of sublethal stress in coliforms exposed to unfavorable conditions, such as those found in marine environments (1, 4, 16, 31, 60).

The apparent decay of these bacteria in natural waters may result from enumeration techniques. Injured cells are unable to grow on selective medium (5) and exhibit a higher sensitivity to temperature (1, 31) and salinity (1). In addition, coliforms and enteric pathogens can undergo morphological and physiological changes when starved in seawater and rapidly evolve toward a viable but nonculturable state; they remain alive for some time and, at least in some cases, capable of producing pathological processes when introduced into test animals (55, 62, 64).

More recently, however, experimental observations have emphasized the possible adaptation of enteric bacteria to stress in seawater. They have shown an increase in their survival in nutrient-free seawater after previous growth on a salt medium (22, 48). It was then assumed that the survival capacity of these bacteria in seawater depended on osmolyte-regulatory processes induced by salts, which possibly involve the intracellular accumulation of organic or inorganic components (47). The ability of enteric bacteria to adapt to fluctuations in the ambient osmolarity is of fundamental importance for their survival. In order to grow, bacterial cells must maintain a positive turgor, i.e., an outwardly directed pressure that is derived from maintaining cytoplasmic osmotic pressure at a point higher than that of the environment. In *Escherichia coli*, this is achieved through osmoregulatory processes that produce a cytoplasm which both has an optimal osmotic pressure and is conducive to enzyme function (6). In response to osmotic stress and to a decrease in cell turgor pressure, *E. coli* cells transiently accumulate potassium ions (17, 20, 32, 45) and activate systems for the transport and synthesis of several organic osmolytes compatible with metabolism (34, 35, 37, 59, 61), which prevent dehydration of cells and stabilize enzyme activity in solutions of high ionic strength (50). Both low and high levels of osmotic tolerance can be produced through glutamate or trehalose synthesis (59) and uptake or synthesis of betaines and other amines (34, 36, 38, 49).

The present work was carried out to analyze the influence of these osmoregulation mechanisms on the survival of *E. coli* strains exposed to seawater, developed either during a previous growth period or in resting cells suspended in a buffer or in seawater. The contribution of particular gene products involved in osmoregulation to the survival mechanism(s) was investigated by using mutants lacking components of the osmotic stress response.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains used are listed in Table 1. *E. coli* 12 (LT⁺) is a wild nontypable strain that produces labile enterotoxin (LT⁺) and was isolated from human stool in Bangladesh. It was provided by J. M. Scheftel (Faculté de Pharmacie, Université de Strasbourg, France).

MC4100, a well-known strain derived from *E. coli* K-12 (11), is able to transport glycine betaine (GBT) via the ProP constitutive low-affinity system and the ProU osmotically inducible high-affinity system (42). The EF strains were all derivatives of MC4100, and their construction has been described previously (7, 9, 42). All lacked the major proline

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permease I (encoded by putP). EF038 and EF046 lacked the ProP and the ProU system, respectively. EF047 strain lacked both of them. MC4100, EF038, EF046, and EF047 strains were provided by E. Bremer (University of Konstanz, Federal Republic of Germany) and M. Villarejo (University of California, Davis).

The CSH7 strain is a wild-type E. coli able to oxidize choline (CHO) to GTB. Mutant FF48 carries a mutation in the betA gene, which appears to be the structural gene for choline dehydrogenase. It is therefore unable to synthesize GBT from CHO. The betA mutation was generated in E. coli K10 and transduced into CSH7 (24, 34, 61). Mutant FF4169 carries an otsA::Tn10 mutation in an MC4100 background. The otsA mutation blocks the synthesis of trehalose-phosphate synthase, making the mutant incapable of accumulating trehalose under osmotic stress. Strains CSH7, FF48, and FF4169 were provided by A. Strom (University of Tromsø, Norway).

The FRAG-1 strain is wild type for potassium transport. It is able to accumulate K⁺ ions by means of both a TrkA constitutive low-affinity transport system and a Kdp osmotically inducible high-affinity system (18, 19). The FRAG-5 and TK2240 mutants lack Kdp and TrkK activity, respectively, and the TK2205 strain has neither Kdp nor Trk activity. Strains FRAG-1, FRAG-5, TK2240, and TK2205 were provided by W. Epstein (University of Chicago).

The MPh2 strain produces an osmotically induced outer membrane lipoprotein (28) encoded by the osmotically stimulated omxB locus. The J2J strain is a mutant lacking the omxB product. Both were provided by M. Villarejo.

**Growth conditions.** Stock cultures were preserved in liquid nitrogen. Bench cultures of the LT⁺ strain were maintained on nutrient agar (NA; Difco Laboratories, Detroit, Mich.) or in nutrient broth (NB; Difco). Strains MC4100, EF038, EF046, and EF047 were grown in mineral medium A (MMA) (42) containing (in millimoles per liter) K₂HPO₄, 60; KH₂PO₄, 32; (NH₄)₂SO₄, 7.5; sodium citrate - 2H₂O, 1.5; MgSO₄·7H₂O, 0.4; d-glucose, 10. CSH7, FF48, and FF4169 were grown in mineral medium 63 (M63) (46) containing (in millimoles per liter) KH₂PO₄, 100; KOH, 90; (NH₄)₂SO₄, 15; MgSO₄·7H₂O, 0.003; d-glucose, 20. Cultures of FRAG-1, FRAG-5, TK2205, TK2240, Mph2', and JJ2 strains were made in M9 medium mineral (M9K⁺) (41) containing (in millimoles per liter) Na₂HPO₄, 44; KH₂PO₄, 22; NH₄Cl, 18; MgSO₄, 0.4; CaCl₂, 1; d-glucose, 10. In some tests, M9 medium was prepared without potassium salts (M9K⁻), using NaH₂PO₄ (44 mmol/liter) instead of KH₂PO₄; the residual K⁺ concentration of this medium, measured spectrophotometrically (Philips PU 9200 atomic absorption spectrophotometer), was 25 ± 2 μM. Some tests were also made on LT⁺ cells grown in M9K⁺ and with MC4100, EF038, EF046, and EF047 resting cells in phosphate buffer (0.02 M, pH 7.5). MMA, M63, and M9 were supplemented with thiamine (1 mmol/liter) except for cultivation of E. coli LT⁺. The osmotic strength of these media was increased when necessary by the addition of NaCl (500 mmol/liter), LiCl (400 mmol/liter), or saccharose (800 mmol/liter). For tests with LT⁺ strains, GBT, CHO, and proline were added to NB to obtain a final concentration of 10 mmol/liter. In mineral media MMA, M63, and M9, GBT and CHO were used at lower concentrations (10 and 100 μmol of GBT per liter and 5 mmol of CHO per liter). Cells were incubated aerobically at 37°C in a shaking water bath. Growth was measured spectrophotometrically at 650 nm with a Uvikon 720LC spectrophotometer (Kontron, Paris, France). Mineral chemicals were from Carlo Erba (Farmitalia, Paris), and organic chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

**Survival tests.** Survival tests were performed in microcosms, which were made up of 250-ml Erlenmeyer flasks containing 100 ml of filtered natural seawater. Coastal seawater was collected along a rocky shore (Cape of Nice) and filtered through membranes (pore size, 0.2 μm; Millipore Corp., Bedford, Mass.). Microcosms were inoculated with cells previously grown in NB, MMA, M63, or M9, supplemented or unsupplemented with NaCl and osmoprotectants. Cells were harvested during the early logarithmic phase of growth (A₆5₀ of 0.2) and then washed three times by centrifugation (10,000 × g, 20 min, 4°C) in artificial seawater (40). The pellets were suspended in 5 ml of artificial seawater, and microcosms were finally inoculated with these cell suspensions to a density of 10⁸ to 10¹⁰ CFU/ml. They were then gently stirred in the dark at room temperature (20 to 24°C).

**Culturable-cell counts.** All microcosms were sampled immediately after inoculation (T₀) and then periodically during the following 8 to 10 days. Bacterial counts were made in triplicate by the membrane filtration technique (Millipore filters; pore size, 0.45 μm) on NB. The number of CFU was counted after 24 h of incubation at 37°C.

**Reproducibility of results.** All tests were made in duplicate and some in triplicate, and data reported are replicate means.

**RESULTS**

The enhancement of the survival ability of E. coli LT⁺ cells in seawater after growth at high osmolarity was first described for complex media prepared with full-strength seawater (22). It now appears that this protection is highly effective, since it allowed the maintenance of culturability of about 5% of starved cells after 5 months in seawater (unpublished data). Such an intriguing effect, which at first analysis could be considered to prevent cellular processes leading to the nonculturable stage, was also induced by sodium chloride, lithium chloride, or saccharose (Fig. 1 and 2). It was therefore not specific to some ions but due to the overall osmolarity of the medium. A threshold was previously observed in the efficiency with which NaCl strengthened cell
resistance in nutrient-free seawater; a protective effect was obtained only for NaCl concentrations greater than 300 mM, which could correspond to the threshold for induction of protective mechanisms. In addition, this effect appeared to be partly persistent, since an intermediate level of protection was maintained in LT⁺ cells grown first in a salted medium and then subcultured for 24 h in a freshwater medium, which then disappeared after a second subculture in a medium of low osmotic pressure (Fig. 3).

The addition of organic osmolytes to the medium used for the growth of LT⁺ cells also influenced their behavior in seawater. Proline and choline, although involved in osmoregulation in E. coli (15, 43), were not able to protect cells and maintain their survival in seawater as did NaCl (or saccharose) added to the medium alone or together with osmolytes (Fig. 4). On the other hand, GBT was able to protect cells from decay in seawater. It is worth noting that this effect was observed with cells grown in a medium of low osmolarity, even though GBT was shown to be accumulated only under conditions of osmotic stress (8, 49).

Experiments carried out with LT⁺ strains, however, remained rather imprecise, since the modalities of osmoregulation in this bacterium were not known. The specific influence of the most important mechanisms involved in the osmotic stress response of enteric bacteria on their survival in seawater was then analyzed by using mutant E. coli strains lacking these mechanisms, which were systematically compared with their corresponding wild-type parent strains.

The protective effect of GBT was confirmed with mutants lacking GBT uptake or synthesis systems. When provided to cells during prior growth, this osmolyte enhanced their survival in seawater and/or maintained their ability to grow on bacteriological complex medium, when accumulated via either the ProP or ProU transport system (Fig. 5) or synthesized from choline (Fig. 6). In both cases, however, the effect of GBT did not account for that of NaCl; GBT protected cells even when added to a medium of low osmolarity, whereas NaCl increased the overall resistance of cells unable to transport or synthesize GBT. This could be due to the induction of additional osmoregulation mechanisms, such as intracellular production and accumulation of osmolytes different from GBT, or potassium ion uptake.
The protective activity of GBT was also analyzed when this osmolyte was provided to cells after growth in a medium of low osmolarity, either in resting cells suspended for 1 h in a phosphate buffer or directly in microcosm seawater. In the former case, GBT protected cells containing both ProP and ProU transport systems only in the buffer of high osmolarity (Fig. 7). Partial protection, however, was observed in cells maintained in the saline buffer without GBT. Cells lacking ProP and ProU systems were also partially protected by NaCl, with or without GBT. When added directly to seawater, GBT was not able to protect E. coli LT'. MC4100, EF038, and EF046 cells previously grown at low osmolarity (Fig. 8 and 9). It was even toxic to LT+ cells at high concentrations (>100 mM) (Fig. 9).

Experiments performed with mutants lacking trehalose synthetase showed that the induced synthesis of trehalose in E. coli cells during prior growth in a salt medium could also account for a part of their protection; FF4169 cells, more sensitive to seawater than MC4100 parental cells, were less protected than the latter when previously grown at high osmolarity (Fig. 10). Nevertheless, the slight but significant increase in resistance of mutant cells grown in the salt medium compared with that of cells grown at low osmolarity could not be explained solely by the accumulation of trehalose.

The protective influence of potassium accumulation was tested with mutants lacking Trk, Kdp, or both of these K+ transport systems (Fig. 11). Cells grown at high osmolarity in M9K medium were protected during 3 days in seawater regardless of their ability to accumulate potassium. Later on, cells with both Trk and Kdp transport systems showed a higher resistance until the end of the experiments, while those lacking these systems were not protected and adapted in the same way as reference cells grown at low osmolarity. The Kdp system made cells partially resistant to seawater; its protective influence, however, remained imprecise, as kdp gene expression was probably repressed during growth in the M9K+ medium due to its high K+ content (20 mM) (53). The role of K+ ions in the protection of E. coli was further investigated by using FRAG-1 cells (Trk+ Kdp+) grown in M9 medium with high (20 mM K+, M9K+) or low (25 μM K+, M9K−) K+ concentration, supplemented or unsupplemented with NaCl (500 mM) (Fig. 12). The high level of protection conferred by growth at high osmolarity...
was achieved in the presence of high K⁺ concentration as well as when the K⁺ content of the medium was very low (Fig. 12). This result showed that the Kdp system was also able to afford protection to cells, since it was actually functioning at a K⁺ concentration as low as 25 μM (17, 20, 21).

At the least, the osmB gene product seemed to disadvantage cells grown at high osmolarity, as it reduced their viability in nutrient-free seawater (Fig. 13).

FIG. 9. Survival of E. coli LT⁺ cells grown in medium M9 of low osmolarity, in seawater supplemented with 0.1 (□), 1 (×), 10 (●), 100 (■), or 600 (△) mmol of GBT per liter. ○, Control cells grown in M9 medium supplemented with NaCl (500 mmol/liter).

FIG. 11. Survival of E. coli FRAG-1 (Trk⁺ Kdp⁺) (●, ○), FRAG-5 (Trk⁺ Kdp⁻) (■, □), TK2240 (Trk⁻ Kdp⁺) (▲, △), and TK2205 (Trk⁻ Kdp⁻) (+, ×) cells grown in M9 medium supplemented (●, ■, ▲, ×) or not (○, □, △, +) with NaCl (500 mmol/liter). Bars represent 1 standard deviation from two experiments.

FIG. 10. Influence of trehalose synthesis on the survival of E. coli MC4100 (●, ○) and mutant FF4169 (▲, △) cells in seawater when previously grown in MMA medium supplemented (○, △) or not (●, ▲) with NaCl (500 mmol/liter). Bars represent 1 standard deviation from two experiments.

FIG. 12. Survival of E. coli FRAG-1 cells (Trk⁻ Kdp⁺) grown in M9K⁺ (20 mM K⁺) (●, ▲) and M9K⁻ (25 μM K⁺) (○, △) media at low (●, ○) or high (▲, △) osmolarity (NaCl, 500 mmol/liter). Bars represent 1 standard deviation from two experiments.

DISCUSSION

When they enter the sea, enteric bacteria are subjected to simultaneous stresses whose impact on cell metabolism will lead to either their maintenance or their decay in marine environments. Up till now, the long-term adaptation of these bacteria in seawater has mainly been analyzed and discussed in terms of resistance to nutritional starvation (12, 14, 30, 47, 51, 52, 65), since the earliest research on the survival of enteric bacteria in marine water emphasized the protective influence of organic matter (10, 23, 25, 33, 63). Whatever the processes are which allow adaptation to nutrient scarcity, survival also depends on the maintenance of osmotic integrity, which is essential to preserve cell homeostasis. In other words, adaptation of enteric bacteria in seawater should also depend on how they achieve an effective level of osmoregulation prior to adaptation to nutrient starvation. The increased survival of E. coli cells when preadapted to high osmolarity in salt medium (22) strongly supports this hypothesis.

The present results suggest that the major osmoregulatory processes, e.g., K⁺ uptake and accumulation of compatible solutes, influence the medium- or long-term maintenance of E. coli viability in nutrient-free seawater and its ability to undergo further growth on bacteriological complex medium. In this way, however, the protective effect of osmoregulation mechanisms was different when they were induced or activated during prior growth, in resting cells, or during starvation survival in seawater.

When developed during growth, all of the osmoregulation mechanisms tested during this study helped increase the ability of E. coli cells to survive in seawater. This is in accordance with the present conception of osmoregulation in enteric bacteria, which is considered a homeostatic system
involving the integrated functioning of several regulated transport and metabolic mechanisms (6). At high osmolarity, both Trk and Kdp K⁺ transport systems can contribute to the achievement of a resistance state in E. coli cells. Uptake and accumulation of osmoles also increase the survival of E. coli in seawater. In this way, trehalose synthesis seems of little importance, while GBT gives cells a level of protection as high as that induced by prior growth at high osmolarity. As observed with strain MC4100, the influence of GBT is constant whatever the means of accumulation, i.e., uptake via ProP and ProU systems or synthesis from intracellular precursors. Nevertheless, the protection of cells by GBT after growth in a medium of low osmolarity suggests that a small amount of GBT could be sufficient to induce the survival-competent state, as this osmolyte is also transported, but at a very low level, under such conditions (5 to 13 times less than in a medium of high osmotic pressure) (49). When GBT is provided to nongrowing cells suspended in a phosphate buffer, protection is achieved rapidly (1 h) only at high osmolarity, via the ProP and ProU systems. In nutrient-free seawater, GBT cannot confer an adaptive advantage to E. coli cells, even when they possess the necessary transport systems and GBT is provided at concentrations compatible with induction of ProU (10 μmol/liter) or activation of ProP (100 μmol/liter) (7). This could be explained by (i) the lack of induction or activation of these systems, (ii) the lack of GBT transport due to energy deficiency (lack of ATP, depletion of proton motive force) (9, 26) or to inhibition of transport under starvation conditions, as described for carbohydrates (56, 58), (iii) the inability of GBT, even if transported and accumulated, to generate further hypothetical structural or metabolic modifications favoring survival in seawater. Some of these explanations are currently under evaluation. In contrast, high concentrations of GBT are toxic to E. coli cells in seawater, although they offer protection when present during growth; this effect remains unexplained. The role of the osmB gene product, an outer membrane lipoprotein (3, 28), is also unknown. However, it is worth noting that the osmB mutant carrying an osmB::TnphoA insertion mutation is both more resistant to inhibition of metabolism by high osmolarity (28) and less sensitive to starvation in seawater than the parent strain. This suggests that both properties could proceed from a common mechanism. Whatever the reason, the OsmB protein appeared to be a disad-

vantage to cells and impaired their survival in seawater. In more general terms, the present results show that survival is improved when cells possess certain genes and are stimulated to express those genes and that the expression of such genes requires nutrients as the substrates from which corresponding gene products are made. Genes encoding osmoregulation mechanisms, expected to contribute to survival of enteric bacteria in seawater, demonstrably did so.

The resistance of cells preadapted to high osmotic pressure probably results from the prevention of drastic cellular modifications which could allow cells to adapt readily to seawater by maintaining expression of essential metabolic functions and energy production. Such preadaptation can also help cells to overcome osmotic downshift when they are placed on the culture media of low osmolarity used for isolation or enumeration or reintroduced into the human intestinal tract. In this way, we must emphasize the influence of GBT (and possibly other betaines), since it is known to protect enteric bacteria from various inhibitory effects of osmotic stress (4, 44, 50, 57, 58) and has been shown to restore colony-forming activity in osmotically stressed E. coli cells (57). Under natural conditions, the protective effect of this universal osmolyte could be achieved during the growth of cells either in the urinary tract, where GBT has been detected (13), or in marine sediments, which contains GBT and many quaternary amines (29). The study of the behavior of enteric bacteria in marine sediments could thus be of high interest from both fundamental and sanitation points of view, since it could provide interesting answers to the question raised concerning the transit of enteric pathogens in marine deposits, which for a long time have been considered favorable to their maintenance in the marine environment.

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LITERATURE CITED


FIG. 13. Influence of osmB gene product on survival of E. coli cells in seawater when grown in M9 medium of low (□, △) or high (○, △) osmotic strength (NaCl, 500 mmol/liter). Circles, strain MPh2 (osmB⁺); triangles, strain JJ2 (osmB). Bars represent 1 standard deviation from two experiments.

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