CURRENT MICROBIOLOGY Vol. 15 (1987), pp. 5–10

Current Microbiology

Influence of Salts and Sodium Chloride on the Recovery of *Escherichia coli* from Seawater

Michel J. Gauthier, Patrick M. Munro, and Soussan Mohajer

National Institute of Health and Medical Research, Unité 303, Nice, France

Abstract. The objective of this study was to evaluate the influence of seawater salts, and more specially sodium chloride, on the recovery of *Escherichia coli* cells after exposure to natural seawater in laboratory microcosms, and the possible adaptation in this bacterium to high salinity. The recovery efficiency of a complex organic medium supplemented with sodium chloride largely depended on the strain and varied with starvation time and salinity. Moreover, cells previously grown on salted medium appeared more able to survive after exposure to seawater. It is assumed that, within *E. coli* populations, some cells are able to adapt to seawater in the presence of both salts and organic matter.

Coliform bacteria exhibit a rapid decline in seawater when enumerated through standard conventional methods. Such die-off has been described both in situ [2, 9, 12, 25, 27, 33, 40] and in laboratory experiments [1, 5, 15, 17, 27, 35, 41].

Recently, investigators have challenged such die-off, with reference to reports indicating the existence of a sublethal stress in coliforms exposed to unfavorable conditions such as those found in natural aquatic environments and especially seawater [1, 3, 9, 23, 37].

It has been noted that the enumeration technique itself may be responsible, at least partly, for the apparent decay of these bacteria in natural waters [3, 23, 32]. Injured cells are unable to grow on selective media [3] and exhibit a higher sensitivity to temperature [1, 23] and salinity [1]. Coliforms starved in seawater can also undergo morphological and physiological changes and rapidly evolve toward a "viable but nonculturable" state, as demonstrated for *Vibrio cholerae* [39] and *Salmonella enteritidis* [35] in freshwater environments.

With the recognition of sublethal stress, studies were made to improve enumeration methods and recover injured cells. Processes were proposed, grounded on the progressive transition from environmental to cultural conditions: increase in temperature and inhibitory activity of the medium [36, 37]. In other respects, even when several investigators have attempted to evaluate the influence of salinity on *E. coli* die-off in marine or estuarine environments [6, 12, 40], only Dawe and Penrose [9] have evidenced, a contrario, the repairing role of salts in culture media used for the enumeration of injured coliforms from seawater.

The present work was developed to analyze the influence of seawater salts and specially sodium chloride on the recovery of *E. coli* cells after exposure to seawater using the membrane filter technique at high temperature (44.5°C), and the possible adaptation of this bacterium to high salinity.

Materials and Methods

A nontypable *E. coli* 12 producing labile enterotoxin (strain LT+), isolated from human stools in Bengladesh, was mainly used in this study. It was kindly provided by Dr. Scheftel (Faculté de Médecine, Strasbourg, France). Seven other strains were used for complementary tests: *E. coli* B (Institut Pasteur Collection), *E. coli* K12 (Institut Pasteur Collection no. 2518), *E. coli* CFAI (H 10407, kindly provided by Dr. Le Minor, Institut Pasteur, Paris), and four wild *E. coli* strains isolated one week before test, from wastewater (EE1 and EE2) and polluted seawater (M1 and M2).

The LT+ strain was maintained and stocked in Evans broth (EB) [11] or on Evans agar (EA) (EB + 15 g agar/liter).

The survival of this strain in seawater was analyzed in laboratory microcosms formed by use of 1-liter Erlenmeyer flasks containing 300 ml of natural seawater (NSW) untreated or filter-sterilized (Millipore filters, pores 0.22 μm), collected along the shore near Nice harbor.

Laboratory microcosms were inoculated with a cell suspension prepared by inoculation of 5 ml of EB with a loopful of a

Address reprint requests to: Dr. Michel J. Gauthier, Institut National de la Santé et de la Recherche Médicale, Unité 303, 1 avenue Jean Lorrain, F-06300 Nice, France.

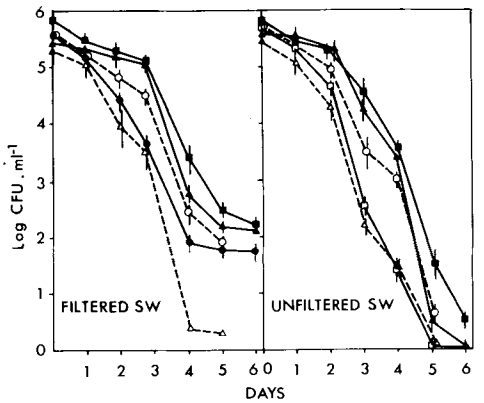

Fig. 1. Survival of *Escherichia coli* (strain LT+) in filtered and unfiltered seawater, evaluated by counting CFU on nutrient agar medium prepared with distilled water (●), distilled water supplemented with sodium chloride (23 g/liter) (■), or artificial seawater (▲), and on mFC agar prepared with distilled water (○) or artificial seawater (△). Bars represent standard deviations from three experiments.

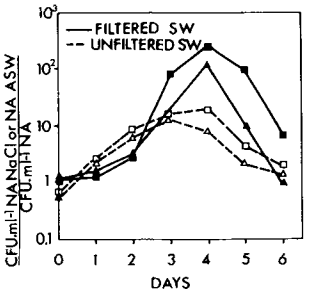

Fig. 2. Variation with time of the efficiency of nutrient agar medium supplemented with sodium chloride (23 g/liter) (■, □) or prepared with artificial seawater (▲, △), for the recovery of *Escherichia coli* strain LT+ from filtered and unfiltered natural seawater (same experiment as in Fig. 1).

previous culture (24 h, 37°C) of the strain in the same medium, incubation for 6 h at 37°C (end of the logarithmic phase of growth), and washing cells by centrifugation in artificial seawater (ASW). The pellet was finally suspended in 10 ml of ASW. The total contact time of ASW with the cell suspension, including washing and diluting, was approximately 2 h. Each microcosm was then inoculated with 0.2 ml of the final suspension and gently stirred in the dark at room temperature (22° ± 2°C).

All microcosms were sampled immediately after inoculation and again at 24 h intervals. *Escherichia coli* counts were made in triplicate, using the membrane filter technique (Millipore filters, porosity 0.45 μm) on different solid media: nutrient agar (NA) (Difco), NA supplemented with NaCl (23 g/liter) or prepared with ASW, and m-FC medium (Difco) prepared with distilled water or with ASW. Colony-forming units (CFU) were counted after 24 h incubation at 44.5°C (±0.1°C). The influence of salts or NaCl on the recovery of *E. coli* was evaluated by calculating the ratio between the number of CFU on salted media and the number of CFU on the corresponding unsalted media. This *recovery factor* was also evaluated on NA in the presence of increasing concentrations of NaCl: 1, 5, 10, 15, and 30 g/liter.

Comparative survival tests were made with a clone of the strain LT+, subsequently isolated and maintained on a salted medium (Marine Agar, Difco). This salt-adapted strain (SALT+) was subcultured for a week on MA before the test. A similar experiment was then performed with the strain SALT+ readapted to freshwater conditions by culture on normal NA for one week (strain SDALT+).

A shortened study of the survival on filtered seawater of the seven additional *E. coli* strains maintained on NA or on MA was later carried out in the same conditions as described for strain LT+; the number of recoverable cells on NA and NA supplemented with NaCl was evaluated, however, only at the beginning of the experiment and after 3 days at 25°C.

All survival tests were made in duplicate or triplicate, except for the experiments with strain SDALT+ and with various *E. coli* strains (one test). In the latter case, an analysis of variance was performed to define the significance of differences between strains with respect to their sensitivity to seawater after culture in freshwater or saltwater medium, and the efficacy of the salted medium for their enumeration.

Results

Comparative survival studies in filtered or unfiltered seawater (Fig. 1) indicated that the decay of *E. coli* 12 was almost the same in both conditions during the first 2 days, further die-off being therefore more pronounced in the presence of the natural microflora of seawater. This observation agreed with previously published data showing the antagonistic influence of indigenous marine flora on enteric bacteria in the sea [18, 29, 30].

On the other hand, the enumeration efficiency of injured cells was largely dependent on the salt content of the enumeration medium. Highest recovery rates were observed with the nonselective medium (NA) supplemented with sodium chloride. On the contrary, both selective and nonselective media prepared with seawater were less effective than the unsalted corresponding media. Besides, the recovery efficiency of NA supplemented with sodium chloride varied with starvation time (Fig. 2); the recovery factor increased after 1–2 days of contact with NSW and reached a maximal value (about ×250) after 4 days. The period of increasing efficiency (2–4 days) corresponded to the faster die-off of cells.

The survival of the "salt-adapted" strain SALT+ in sterile NSW was much higher than that of the original LT+ strain (Fig. 3), and the recovery efficiency of injured cells on Na-salted medium only increased for strain LT+ and corresponded again to the faster die-off of cells (1–2 days) (Fig. 4). The

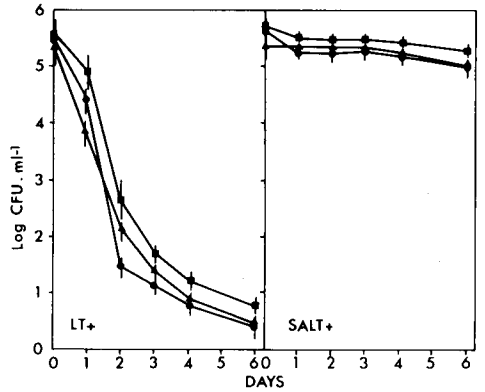

Fig. 3. Comparative survival in natural seawater of *Escherichia coli* previously cultivated on nutrient agar medium (LT+) or marine agar medium (SALT+). CFU were counted on nutrient agar medium prepared with distilled water (●), artificial seawater (▲), or distilled water supplemented with sodium chloride (23 g/liter) (■). Bars represent standard deviations from three experiments.

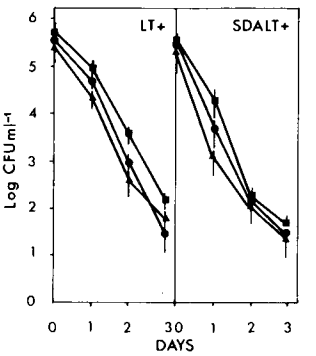

Fig. 5. Comparative survival of *Escherichia coli* strains LT+ and SDALT+ (SALT+ readapted to freshwater medium) in the same natural seawater. CFU were counted on nutrient agar medium prepared with distilled water (●), with distilled water added with sodium chloride (23 g/liter) (■), or with seawater (▲). Bars represent standard deviations evaluated from three numerations of CFU (one experiment).

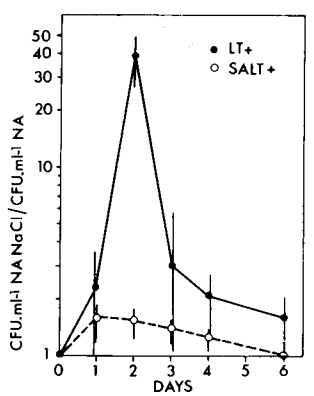

Fig. 4. Variation with time of the efficiency of nutrient agar medium supplemented with sodium chloride (23 g/liter) for the recovery of *Escherichia coli* strains LT+ (maintained on freshwater medium) and SALT+ (maintained on seawater medium) from filtered or unfiltered seawater (same experiment as in Fig. 3). Bars represent standard deviations from three experiments.

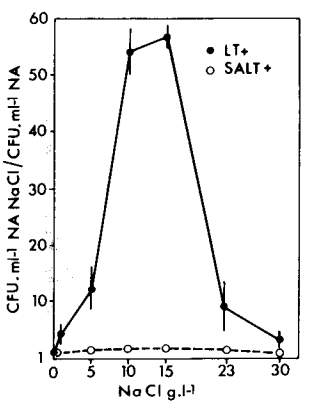

Fig. 6. Efficiency of the recovery of *Escherichia coli* adapted to freshwater (LT+) or seawater (SALT+), after 3 days in filtered seawater (same experiment as in Fig. 1), evaluated by counting CFU on nutrient agar medium supplemented with NaCl at several concentrations. Bars represent standard deviations from three experiments.

readaptation of strain SALT+ to freshwater conditions restored its sensitivity to the injuring activity of seawater (Fig. 5). In other respects, the recovery efficiency of the nonselective medium varied with its sodium chloride concentration (Fig. 6) and was maximal for intermediate values (10–15 g NaCl/liter) between freshwater (0 g/liter) and seawater (23 g/liter).

All of the seven *E. coli* strains used for complementary tests showed a more or less developed sensitivity to saline conditions after three days in filtered natural seawater (Fig. 7). Cells previously grown on freshwater medium appeared less able to survive in seawater (significant to the 0.1% level), wild strains recently isolated from natural waters being more resistant. Furthermore, the recovery rate of cells on salted nutrient agar was highly dependent on the strain regardless of previous culture history or exposure to seawater: except for strains CFAI and B, no statistically significant difference was found between CFU counts with respect to salt content of the enumeration medium. For strains B and CFAI, the recovery rate was yet very high: ×8600 and ×47 for strain CFAI cultivated on fresh-

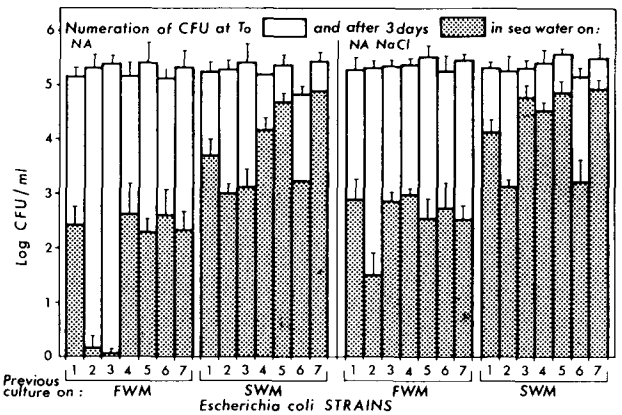

Fig. 7. Influence of the previous adaptation of *Escherichia coli* strains to salinity on their survival in natural seawater and on the recovery efficiency of salted NA medium for counting CFU after 0 and 3 days in seawater: NA, nutrient agar medium prepared with distilled water; and NA NaCl, the same added with 23 g NaCl/liter. Each strain was previously cultivated on freshwater medium (FWM) or seawater medium (SWM). *Escherichia coli* strains: 1, K12; 2, B; 3, CFAI; 4, EE1; 5, EE2; 6, M1; and 7, M2. Bars represent standard deviations for three numerations of CFU (one experiment).

water or seawater media, respectively, and ×33 for strain B maintained on freshwater medium.

Discussion

The addition of sodium chloride to an organic complex medium like nutrient agar made it possible to recover an important fraction of *E. coli* cells from seawater microcosms. Nevertheless, the recovery factor largely differed with strains (from ×1.2 to ×8600) and varied with starvation time in seawater. This could lead to a misinterpretation of results presented in Fig. 7: for some *E. coli* strains, the higher recovery rate of cells on salted medium could have been reached after a shorter or a longer exposure to seawater. On the other hand, none of the eight tested strains was completely recovered on salted medium, as previously reported by Dawe and Penrose [9] in the case of fecal coliforms maintained for 6 days in the marine environment and counted on NA prepared with natural seawater. On the other hand, the recovery rate was significantly different in different experiments carried out with the same strain (×60 to ×250 for LT+). This could, at least partly, be due to the seasonal variation of the antibacterial activity of natural seawater, which has been previously reported [19, 31]; our experiments were effectively performed with natural seawater collected from November to March. In other respects, these discrepancies could not be due to variations in temperature, which can influence the survival of enteric bacteria in seawater [1, 7, 12, 14, 23, 31]: all the tests were performed at a nearly constant temperature (22°–23°C), whatever the temperature of seawater in situ. Besides, the unfavorable influence of ASW observed in these experiments could be a consequence of the antagonistic effect of its metallic components, as already stated by Jones [20, 21].

Three successive phases were observed in survival experiments with strain LT+, regarding the recovery efficiency of the salted medium (Fig. 1). During the first day, the number of culturable cells decreased to about 50% of the initial population and their ability to grow on enumeration media was not significantly modified by salts or sodium chloride. During the next 1–3 days, most of the remaining cells disappeared, or possibly evolved to a non-culturable state, recoverable cells exhibiting a higher need for sodium chloride or a higher accommodation to salt. The corresponding increase of the ratio between the number of CFU on salted medium and the number of CFU on unsalted medium was then probably due to an increase in the numerator (cells repaired by, or adapted to salts) rather than to a decrease in the denominator (cells able to readapt to an unsalted medium). The strain LT+, previously grown on saline organic medium, was equally culturable on salted or unsalted nutrient agar after up to 6 days in the same seawater microcosm (Fig. 3). Later, the remainder of the culturable cells, representing less than one-thousandth of the initial population, evolved more slowly; bacterial growth was then less and less influenced by salt, a property that seems to characterize salt-adapted cells.

In other respects, these results emphasize the influence of organic matter in enhancing survival of fecal coliforms in seawater, often previously described [7, 14, 22]. Furthermore, they suggest a reversible adaptation of cells to seawater in the presence of both salts and nutrients. Such physiological accommodation could result from the development of an osmoregulatory mechanism induced by salts and probably sodium chloride, and from the intracellular accumulation of organic components such as amino acids [28, 37, 38], polyhydroxy alcohols [4], or carbohydrates [34] from available organic nutrients. It could also be a consequence of some structural modifications in the outer membrane of salt-medium grown cells, as previously described [8, 26].

On this assumption, the survival capability of

E. coli and possibly other related enteric bacteria in marine environments could be greatly increased every time they are simultaneously brought in the presence of salts and organic matter: this is particularly the case for marine sediments, where coliforms can actually survive far longer than in water [10, 13, 16, 24].

ACKNOWLEDGMENTS

We thank B. Chabanne and H. Olagnero for their technical assistance.

Literature Cited

1. Anderson IC, Rhodes MW, Kator HI (1979) Sublethal stress in *Escherichia coli*: a funcion of salinity. Appl Environ Microbiol 38:1147–1152
2. Anderson IC, Rhodes MW, Kator HI (1983) Seasonal variation in survival of *Escherichia coli* exposed in situ in membrane diffusion chambers containing filtered and nonfiltered estaurine waters. Appl Environ Microbiol 45:1877–1883.
3. Bissonnette GK, Jezeski JJ, McFeters GA, Stuart DG (1975) Influence of environmental stress on enumeration of indicator bacteria from natural water. Appl Microbiol 29:186–194
4. Brown AD (1974) Microbial water relations: features of the intracellular composition of sugar-tolerant yeasts. J Bacteriol 118:769–777
5. Burdyl P, Post FJ (1979) Survival of *Escherichia coli* in Great Salt Lake water. Water Air Soil Pollut 12:237–246
6. Carlucci AF, Pramer D (1959) Factors influencing the survival of bacteria in seawater. Appl Microbiol 7:388–391
7. Carlucci AF, Pramer D (1960) An evaluation of factors affecting the survival of *Escherichia coli* in seawater. II. Salinity, pH and nutrients. Appl Microbiol 8:247–250
8. Chai TJ (1983) Characteristics of *Escherichia coli* grown in bay water as a compared with rich medium. Appl Environ Microbiol 45:1316–1323
9. Dawe LL, Penrose WR (1978) "Bactericidal" property of seawater: death or debilitation? Appl Environ Microbiol 35:829–833
10. Erkenbrecher CW Jr (1981) Sediment bacterial indicators in an urban shellfishing subestuary of the lower Chesapeake Bay. Appl Environ Microbiol 42:484–492
11. Evans DJ, Evans DG, Gorbach SL (1973) Production of vascular permeability factor by enterotoxigenic *E. coli* isolated from man. Infect Immun 8:725–730
12. Faust MA, Aotaky AE, Hargadon ML (1975) Effect of physical parameters on the in situ survival of *Escherichia coli* MC-6 in an estuarine environment. Appl Microbiol 30:800–806
13. Gerba CP, McLeod JS (1976) Effect of sediments on the survival of *Escherichia coli* in marine waters. Appl Environ Microbiol 32:114–120
14. Graham JJ, Sieburth JMcN (1973) Survival of *Salmonella typhimurium* in artificial and coastal seawater. Rev Int Oceanogr Med 29:5–29
15. Guthrie RK, Scovill MA (1984) Recovery of *Escherichia coli* and *Vibrio cholerae* from aquatic microcosms. Water Res 8:1055–1057
16. Hendricks CW (1971) Increased recovery rate of *Salmonella* from stream bottom sediments versus surface waters. Appl Microbiol 21:379–380
17. Hood MA, Ness GE (1982) Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. Appl Environ Microbiol 43:578–584
18. Jannasch HW (1968) Competitive elimination of enterobacteriaceae from seawater. Appl Microbiol 16:1616–1618
19. Jensen A (1976) Chemical conditioning of seawater by algal growth and development. In: Faulkner DJ, Fenical WH (eds) Marine natural products chemistry. New York: Plenum, pp 332–335
20. Jones GE (1964) Effect of chelating agents on the growth of *Escherichia coli* in seawater. J Bacteriol 87:483–499
21. Jones GE (1967) Growth of *Escherichia coli* in heat and copper treated synthetic seawater. Limnol Oceanogr 12:167–172
22. Jones GE (1971) The fate of freshwater bacteria in the sea. Dev Indust Bact 12:141–151
23. Klein DA, Wu S (1974) Stress: a factor to be considered in heterotrophic micro-organism enumeration from aquatic environments. Appl Environ Microbiol 27:429–431
24. La Liberté P, Grimes DJ (1982) Survival of *Escherichia coli* in lake bottom sediment. Appl Environ Microbiol 43:623–628
25. Lessard EJ, Sieburth JMcN (1983) Survival of natural sewage population of enteric bacteria in diffusion chambers in the marine environment. Appl Environ Microbiol 45:950–959
26. Lugtenberg B, Peters R, Bernheimer H, Berendsen W (1976) Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol Gen Genet 147:251–262
27. McFeters GA, Cameron SA, Le Chevallier MW (1982) Influence of diluents, media and membrane filters on detection of injured water-borne coliform bacteria. Appl Environ Microbiol 43:97–103
28. Measures JC (1975) Role of amino acids in osmoregulation of nonhalophylic bacteria. Nature 257:398– 400
29. Mitchell R (1974) The effects of water movement on lysis of non-marine micro-organisms by marine bacteria. Sartia 34:263–266
30. Mitchell R, Nevo Z (1964) Decomposition of structural polysaccharides of bacteria by marine micro-organisms. Nature 205:1007–1008
31. Moebius K (1972) Seasonal changes in antibacterial activity of North Seawater. Marine Biol 13:1–3
32. Olson BH (1976) Enhanced accuracy of coliform testing in seawater by a modification of the most-probable number method. Appl Environ Microbiol 36:438–444
33. Rhodes MW, Anderson IC, Kator HI (1983) In situ development of sublethal stress in *Escherichia coli*: effects on enumeration. Appl Environ Microbiol 45:1870–1876
34. Roller SD, Anagnostopoulos GD (1982) Accumulation of carbohydrates by *Escherichia coli* B/r/1 during growth at low water activity. J Appl Bacteriol 52:425–434
35. Roszak DB, Grimes DJ, Colwell RR (1984) Viable but unrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can J Microbiol 30:334–338
36. Speck ML, Ray B, Read RB Jr (1975) Repair and enumeration of injured coliforms by plating procedure. Appl Microbiol 29:549–550
37. Stuart DG, McFeters, GA, Schillinger JE (1977) Membrane filter technique for the quantification of stressed fecal coliforms in the aquatic environment. Appl Environ Microbiol 34:42–46

38. Sugiura M, Kisumi M (1985) Osmoregulation in a proline-producing strain of *Serratia marcescens*. J Gen Microbiol 134:2515–2520
39. Tempest DW, Meers JL, Brown CM (1970) Influence of the environment on the content and composition of microbial free amino-acid pools. J Gen Microbiol 64:171–185
40. Vasconselos GJ, Swartz RG (1976) Survival of bacteria in seawater using a diffusion chamber apparatus in situ. Appl Environ Microbiol 31:913–920
41. Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8:313–323