

Restoration of Colony-Forming Activity in Osmotically Stressed *Escherichia coli* by Betaine

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Exposure of *Escherichia coli* to 0.8 M NaCl caused a rapid and large decrease in colony-forming activity. When such osmotically upshocked cells were exposed to betaine, colony-forming activity was restored. Betaine was able to restore colony-forming activity even when chloramphenicol inhibited protein synthesis. Thus, restoration was not the result of cell turnover. The cells were not killed by exposure to 0.8 M NaCl, because during exposure they accumulated ATP intracellularly. Betaine treatment caused this cellular ATP to decrease to a lower level. This work may provide the foundation for a simple plating procedure to quantitatively detect nonculturable *E. coli* in ocean beach recreational waters.

In connection with our studies on the effect of hyperosmolarity on carbohydrate transport in *Escherichia coli* (18, 19), we obtained preliminary results that colony-forming activity decreased over time in our test system. Our system is a normoosmotic minimal growth medium to which 0.8 M NaCl is added. We found that within 6 h after NaCl addition, about 90% of these osmotically stressed cells lost colony-forming activity. We undertook the present study to try to establish whether this large percentage of osmotically stressed cells was truly dead or merely nonculturable, that is, alive but unable to form colonies on a solid medium (16).

The work presented here shows that this loss of colony-forming activity after osmotic upshock is not the result of cell death, because these upshocked cells (i) accumulated high levels of ATP intracellularly, (ii) did not release this ATP into the extracellular environment, and (iii) after treatment with betaine (even in the presence of chloramphenicol), regained their ability to form colonies upon plating to a solid medium.

Plating methods are often used to access microbial contamination in hyperosmolar samples such as salted foods and seawater. The difficulty in accurately enumerating living bacteria in seawater samples, and the public-health threat that this difficulty poses, have recently been reviewed (16). In seawater samples the vast majority of bacteria are nonculturable, and the lack of a simple plating method for the accurate estimation of the number of truly viable *E. coli* is of particular concern.

The observations presented here, that incubation with the nonmetabolizable (13) compound betaine restores colony-forming activity and that this restoration occurs in the presence of chloramphenicol, which prevents any increase in cell number during the incubation, may provide the basis of a simple plating procedure that will give an accurate enumeration of the viable cells in hyperosmolar samples.

MATERIALS AND METHODS

Cell culture. *E. coli* CA8000 (*thi*) was grown aerobically with shaking at 32°C in a minimal medium (supplemented with glucose, NH₄Cl, and thiamine [18]), and the optical density of the culture was measured at 450 nm, all as previously described (6).

Upshock procedure. At an optical density of 0.4, equivalent to 50 mg of cellular protein per liter, a portion of the culture was upshocked by rapid mixing with an equal volume of prewarmed, supplemented minimal medium containing 1.6 M NaCl. In certain experiments, as noted in the text, chloramphenicol (25 mg/liter, final concentration) was added when the culture achieved the optical density of 0.4; 1 h later this culture was upshocked as described above, except that the upshock medium also contained chloramphenicol (25 mg/liter). Betaine, as the monohydrate, was obtained from Sigma Chemical Co., St. Louis, Mo. When betaine was added, its final concentration was 2 mM.

Colony and cell counting. Samples for colony counting were removed from the cultures, and 2 portions of each sample were diluted with 0.9% NaCl to achieve about 60 cells per ml. (This >10⁶-fold dilution was large enough to restore normoosmolarity and to reduce chloramphenicol, when present, to a noninhibitory concentration.) Five separate 1-ml portions of each dilution were collected by filtration onto separate Bacteriological Monitors (Millipore Corp., Bedford, Mass.) and incubated with added tryptic soy broth (Difco Laboratories, Detroit, Mich.) as the growth medium. Essentially the same values for CFU per milliliter were obtained in preliminary experiments in which dilutions were also plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Samples for total cell counting were diluted with 0.9% NaCl that contained formaldehyde sufficient for a final concentration of 0.75%. Total cell number was determined by phase microscopy, using a hemacytometer; approximately 80 cells were counted in each of four portions of each sample.

Leucine incorporation. Cultures received L-[U-¹⁴C]leucine (Dupont, NEN Research Products, Boston, Mass.) with a specific activity and final concentration of either 1.3 μCi/μmol and 76 μM (for exponentially growing cultures) or 90 μCi/μmol and 2.4 μM (for upshocked or chloramphenicol-treated cultures). Samples (1 volume) of the cultures were periodically added to 0.1 volume of 100% trichloroacetic acid. After mixing and centrifugation, 1 volume of supernatant was removed, and the precipitate was washed four times by successively adding 1 volume of 10% trichloroacetic acid, mixing, centrifuging, and then removing 1 volume of supernatant. One volume of NaOH, to give a concentration of 1 N, was added to the precipitate in the remaining 0.1 volume

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of acid, and the samples were heated at 100°C for 45 min to solubilize the precipitate. Portions of these solubilized samples (0.6 ml in 10 ml of Picofluor-30 scintillation fluid [Packard Instrument Co., Inc., Rockville, Md.]) were counted for radioactivity; counting efficiency (about 0.9 cpm/dpm) was determined by recounting after addition of a known amount of [¹⁴C]toluene. Protein in the solubilized samples was determined by the method of Lowry et al. (11), using crystalline bovine serum albumin as the standard; values were corrected for the consistent 20% loss of measurable protein that we have observed in samples that are boiled in strong alkali in this manner, a phenomenon also observed by Lowry et al. (11).

Rates of leucine incorporation. Rates of [¹⁴C]leucine incorporation into cellular protein are expressed as micromoles per gram of protein per hour. For nongrowing cultures (treated with chloramphenicol or upshocked or both), in which the cellular level of protein in the culture was constant, the actual rate of incorporation was obtained from the slope of a linear regression analysis of micromoles per gram of protein versus hours. (The values of micromoles per gram of protein in these stationary cultures were obtained by dividing the micromoles incorporated at a given time by the constant cellular level of protein in the culture.)

For exponential growth, the actual rate was calculated by multiplying the slope, obtained by linear regression analysis, of the linear differential plot (micromoles of leucine incorporated versus grams of protein) by the growth constant, $(\ln 2)/1.2$ h (5). Alternatively, the values of the micromoles incorporated at a given time were divided by the amount of protein in the culture at the time of [¹⁴C]leucine addition, and these values for micromoles per gram of protein were plotted versus time. The slope of the linear portion of this plot (about the first 20 min under the experimental conditions used here) is a very close approximation of the actual rate. This plot allows a direct visual comparison of the rate of incorporation during growth with the rates in the various stationary cultures when the lines for micromoles per gram of protein versus time for each of the cultures are placed on the same graph. Correlation coefficients (r) of at least 0.99 were obtained with the linear regression analyses used in the calculations of the various actual rates and the approximate rate.

ATP determination. For total ATP, samples of the cultures were collected into chilled perchloric acid. After centrifugation, the clear supernatant was neutralized with KHCO₃. Samples for extracellular ATP were first filtered (0.22- μ m-pore-size Millex-GV membrane filters; Millipore). ATP in the neutralized extracts was determined luminometrically by using firefly luciferase (ATP bioluminescence CLS; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Intracellular ATP was obtained by subtracting extracellular ATP from total ATP. In the experiments presented here, no extracellular ATP was detected. An enzymatic spectrophotometric method was used for ATP standardization (10). ATP is expressed as micromoles per gram of protein.

RESULTS

Exposure of *E. coli* to 0.8 M NaCl caused no decrease in the total number of cells, observed by microscopic examination, but caused a rapid and large decrease in colony-forming activity (Fig. 1). When such osmotically upshocked cells were exposed to betaine before being diluted to normoosmolarity and cultured, colony-forming activity was restored (Fig. 1). Within 2 h after exposure to betaine, the

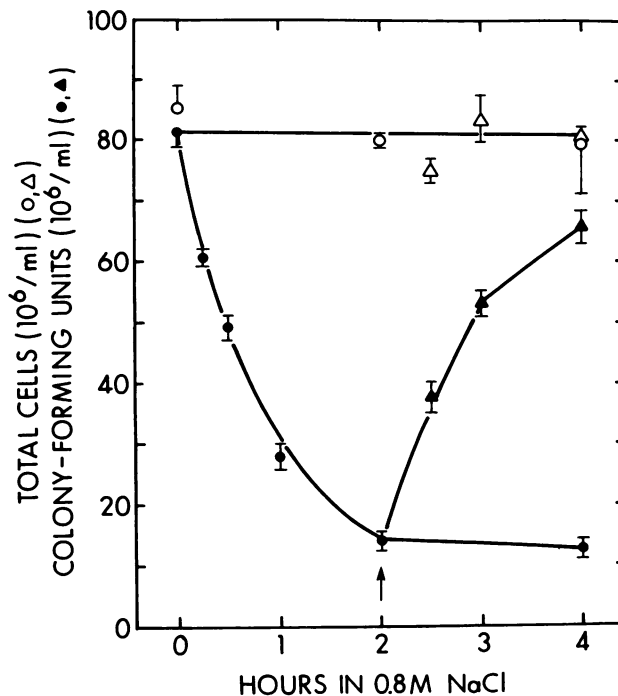


FIG. 1. Effect of osmotic upshock with 0.8 M NaCl on the total cell number and on the number of CFU of *E. coli* without (○, ●) and with (△, ▲) betaine. The arrow shows the time of betaine addition (2 mM, final concentration). Each value is the mean \pm 1 standard error. An exponentially growing culture was upshocked at zero time as described in Materials and Methods.

number of colony-forming units returned nearly to the control level, and the total number of cells was still unchanged. (Incubation with betaine during upshock was necessary to restore colony-forming activity; betaine had no effect when added later, that is, when betaine was present either during the dilution back to normoosmolarity or in the growth medium.) Even after 24 h of exposure to 0.8 M NaCl, there was no decrease in the total number of cells, and treatment with betaine was still effective; betaine treatment for 2 h restored colony-forming activity to about 80% of the cells present. Betaine restored colony-forming activity after 24 h of upshock even when chloramphenicol was present (Fig. 2). This restoration in the presence of chloramphenicol rules out cell turnover (that is, cryptic growth [14]) as the cause of the recovery of colony-forming activity, because, as shown below, chloramphenicol did actually block protein synthesis by the upshocked cells.

The following observations demonstrate that chloramphenicol blocks protein synthesis in osmotically upshocked cells. No net protein synthesis occurred when *E. coli* CA8000 was upshocked with 0.8 M NaCl, but approximately 2 h after the addition of betaine to the upshocked culture, net protein synthesis was restored (Fig. 3A), in agreement with our previous observations (19). However, in upshocked cultures pretreated with chloramphenicol, there was no subsequent restoration of net protein synthesis after betaine addition (Fig. 3B). Furthermore, pretreatment with chloramphenicol before upshock produced a nearly 10-fold-lower rate of [¹⁴C]leucine incorporation than did upshock alone (Fig. 4). During the first hour after upshock alone, the rate gradually increased to a constant rate of 2.40 μ mol/g of protein per h. A rate of only 0.28 μ mol/g of protein per h was

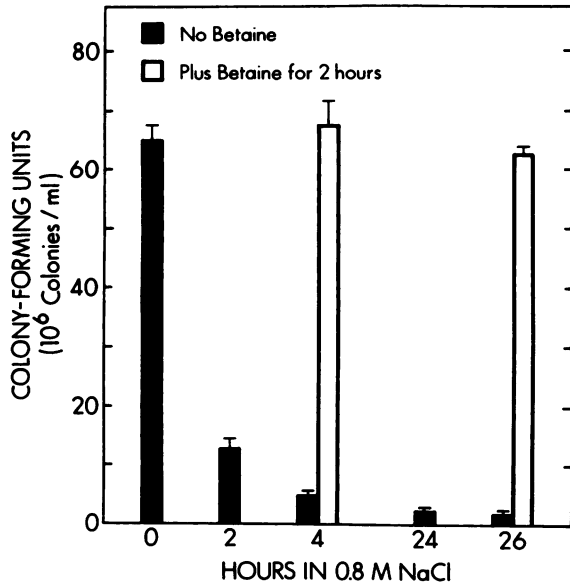


FIG. 2. Loss of colony-forming activity and restoration by betaine in a 0.8 M NaCl-upshocked culture of *E. coli* treated with chloramphenicol for 1 h before upshock. Each value is the mean \pm 1 standard error.

observed with chloramphenicol-treated, upshocked cells. This result unequivocally demonstrates that chloramphenicol virtually eliminated protein synthesis in the upshocked cells.

The intracellular steady-state level of ATP in exponentially growing *E. coli* CA8000 was approximately 9.5 μ mol/g

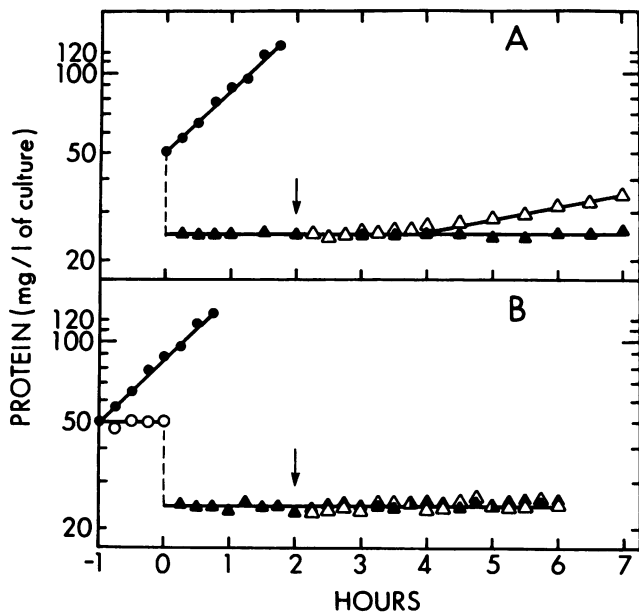


FIG. 3. Net protein synthesis in exponentially growing *E. coli* in the presence of 0.8 M NaCl without or with added betaine and without (A) or with (B) a 1-h pretreatment with chloramphenicol. Symbols: ●, control (no additions); ○, chloramphenicol; ▲, 0.8 M NaCl; △, 0.8 M NaCl plus betaine. Note that a 1:2 dilution of the culture resulted from the upshock procedure. Betaine was added 2 h after upshock (arrows).

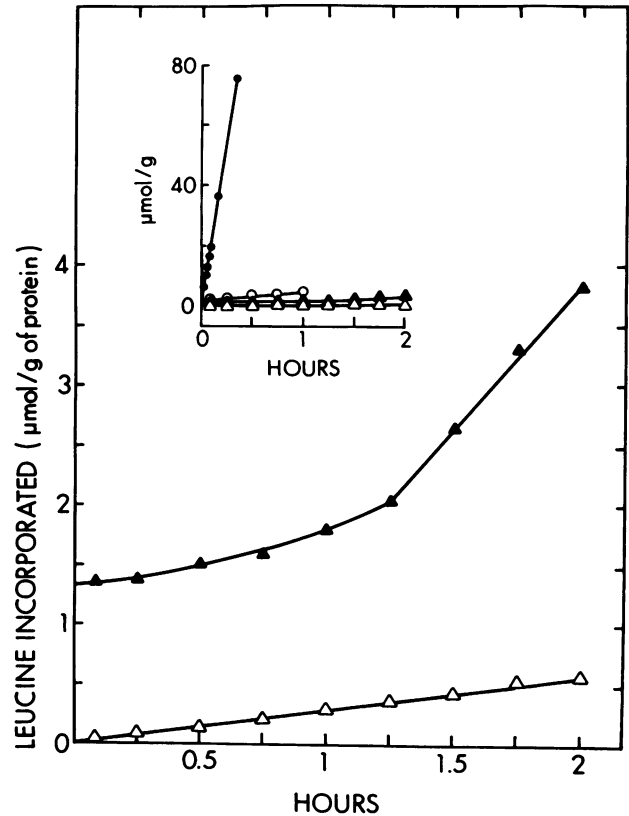


FIG. 4. Rates of [¹⁴C]leucine incorporation into cellular protein in exponentially growing *E. coli* cultures that were upshocked with 0.8 M NaCl (▲) or upshocked after a 1-h pretreatment with chloramphenicol (△). For comparison, the rates during exponential growth without (216 μ mol/g of protein per h) (●) or with (3.53 μ mol/g of protein per h) chloramphenicol (○) are shown in the inset. All cultures except the exponentially growing control were prelabeled with [¹⁴C]leucine for 15 s before upshock or (in the culture that was never upshocked) before chloramphenicol addition.

of protein (Fig. 5). After upshock with 0.8 M NaCl the intracellular level of ATP decreased transiently and then rose, and by 4 h after upshock ATP approached a level that was approximately 1.5-fold greater than the level in growing cells (Fig. 5). (No ATP was leaked into the medium.) Thus, the cells were not killed by exposure to 0.8 M NaCl, but somehow during the same period that these cells were generating a high intracellular level of ATP, most of these upshocked cells lost their ability to demonstrate colony-forming activity (Fig. 1).

The addition of betaine 2 h after upshock caused an abrupt transient rise in intracellular ATP, followed by a decrease, until by 2 h after betaine addition an ATP level was approached that was about two-thirds of the steady-state level in growing cells (Fig. 5). (No ATP was leaked into the medium.) These betaine-treated cells maintained a significant cytoplasmic concentration of ATP (at least 1.6 mM, on the basis of 4 ml of cytoplasmic water per g of protein [21]). In addition, betaine-treated cells were almost all capable of showing colony-forming activity by 2 h after betaine treatment (Fig. 1 and 2). Thus, by either criterion, i.e., cellular level of ATP or colony-forming activity, the betaine-treated cells were not dead.

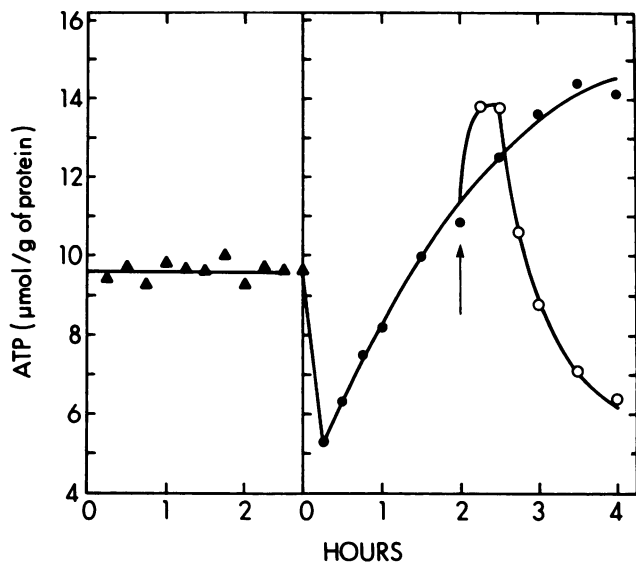


FIG. 5. Cellular ATP during exponential growth in control (▲) and 0.8 M NaCl-upshocked *E. coli* without (●) and with (○) added betaine. The arrow shows the time of betaine addition.

DISCUSSION

We show here that *E. coli* cells upshocked with 0.8 M NaCl are, in fact, alive despite a decrease in colony-forming activity. First, the upshocked cells accumulated high levels of ATP intracellularly (Fig. 5) and did not release ATP to the extracellular environment. Second, the ability to form colonies was restored to upshocked cells (by betaine) in the presence of chloramphenicol (Fig. 2), which inhibited protein synthesis (Fig. 3 and 4) and thus prevented the formation of any new cells. Thus, the cells whose colony-forming activity was restored by betaine were the same cells that had originally been upshocked. Therefore, in order for these cells to have been revived by betaine, they would have had to be alive at the time betaine was added. In fact, these cells were alive even after 24 h of upshock, because betaine still restored their colony-forming activity (Fig. 2).

Betaine treatment increases glucose transport by 0.8 M NaCl-upshocked cells (19). However, despite this increase of the energy supply to the upshocked cells, cellular ATP decreased by half (Fig. 5). Thus, betaine must not only stimulate ATP production via an increase in glucose transport but must also stimulate, and to an even greater extent, an ATP-consuming process. This decrease in cellular ATP was paralleled by restoration of colony-forming activity (Fig. 1).

Apparently, there is a general correlation between lowering cellular ATP and preventing or restoring the stress-induced loss of colony-forming activity. Anaerobiosis prevents this loss for thymine-starved *thy* mutants (that is, prevents thymineless death) (20), 2,4-dinitrophenol prevents this loss in substrate-accelerated death (15), and we show here that betaine treatment lowers ATP and restores colony-forming activity in osmotic stress.

The restoration of colony-forming activity in osmotically stressed *E. coli* that we show here may be useful in solving a major public-health problem. *E. coli* is an indicator organism in environmental samples because it is the only coliform biotype consistently and exclusively associated with the fecal wastes of warm-blooded animals (2). Therefore, its

presence demonstrates contamination from such wastes and indicates the existence of a potential health hazard from microbial or viral enteric pathogens. Plating on a solid medium is routinely used for detection of *E. coli* in ocean beach recreational waters. However, the vast majority of bacteria in seawater samples are nonculturable (16), that is, alive (show metabolic activity [16]) but unable to form colonies on a solid medium. Consequently, the number of bacteria able to form colonies on solid media are several orders of magnitude lower than the number that are actually alive and demonstrated to be capable of expressing their pathogenicity in a susceptible host (3, 4, 17, 22). Therefore, in order to accurately assess the public-health risk, there is clearly a need for a simple plating procedure that would detect both the culturable and nonculturable *E. coli* in seawater samples.

On the basis of the work presented here, we propose that before plating, *E. coli* in seawater samples be resuscitated by incubation after the addition of glucose (the preferred carbon source of *E. coli* [7]), NH_4^+ (the simplest nitrogen source), betaine, and chloramphenicol.

Glucose and NH_4^+ are added at the resuscitation step because seawater lacks adequate sources of carbon and nitrogen (1, 12). Chloramphenicol is added at the resuscitation step to prevent proliferation during resuscitation and thus prevent obtaining a falsely high count upon plating. This addition is necessary because we have shown previously that, in the absence of chloramphenicol, osmotically stressed *E. coli* cultures eventually begin to proliferate after treatment with betaine (19). On the other hand, we show here that although chloramphenicol blocks this unwanted proliferation, it does not block the required resuscitation by betaine. Our proposed resuscitation procedure remains to be tested.

The work presented here, which shows that the loss of colony-forming activity after osmotic upshock is not the result of cell death, is relevant to basic science studies of osmotic stress. If cell death could not have been ruled out, then the responses to osmotic stress observed by us (18, 19) and by others (for example, see reviews in references 8 and 9) could have been interpreted to be occurring only in the cells capable of forming colonies, that is, only in a small fraction of the total cell population. This interpretation would suggest that a particular observed response was not characteristic of an organism but was either atypical or the result of mutation. Our present work appears to rule out that suggestion.

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LITERATURE CITED

1. Amy, P. S., C. Pauling, and R. Y. Morita. 1983. Starvation-survival processes of a marine vibrio. *Appl. Environ. Microbiol.* 45:1041-1048.
2. Cabelli, V. J. 1977. Indicators of recreational water quality, p. 222-238. In A. W. Hoadley and B. J. Dutka (ed.), *Bacterial indicators/health hazards associated with water*. American Society for Testing and Materials, Philadelphia.
3. Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin. 1982. Swimming-associated gastroenteritis and water quality. *Am. J. Epidemiol.* 115:606-616.
4. Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer. 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment. *Bio/Tech-*

- nology 3:817-820.
5. Dietzler, D. N., M. P. Leckie, P. E. Bergstein, and M. J. Sughrue. 1975. Evidence for the coordinate control of glycogen synthesis, glucose utilization, and glycolysis in *Escherichia coli*. I. Quantitative covariance of the rates of glucose utilization and the cellular level of fructose 1,6-diphosphate during exponential growth and nutrient limitation. *J. Biol. Chem.* **250**:7188-7193.
 6. Dietzler, D. N., M. P. Leckie, and C. J. Lais. 1973. Rates of glycogen synthesis and the cellular levels of ATP and FDP during exponential growth and the nitrogen-limited stationary phase of *Escherichia coli* W4597(K). *Arch. Biochem. Biophys.* **156**:684-693.
 7. Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr. 1980. Carbohydrate transport in bacteria. *Microbiol. Rev.* **44**:385-418.
 8. Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine. 1984. Molecular biology of osmoregulation. *Science* **224**:1064-1068.
 9. Le Rudulier, D., and R. C. Valentine. 1982. Genetic engineering in agriculture: osmoregulation. *Trends Biochem. Sci.* **7**:431-433.
 10. Lowry, O. H., and J. V. Passonneau. 1972. A flexible system of enzymatic analysis, p. 151-152. Academic Press, Inc., New York.
 11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
 12. Novitsky, J. A., and R. Y. Morita. 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. *Appl. Environ. Microbiol.* **33**:635-641.
 13. Perroud, B., and D. Le Rudulier. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J. Bacteriol.* **161**:393-401.
 14. Postgate, J. R., and J. R. Hunter. 1962. The survival of starved bacteria. *J. Gen. Microbiol.* **29**:233-263.
 15. Postgate, J. R., and J. R. Hunter. 1964. Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. *J. Gen. Microbiol.* **34**:459-473.
 16. Rosak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51**:365-379.
 17. Roszak, D. B., D. J. Grimes, and R. R. Colwell. 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* **30**:334-338.
 18. Roth, W. G., M. P. Leckie, and D. N. Dietzler. 1985. Osmotic stress drastically inhibits active transport of carbohydrates of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **126**:434-441.
 19. Roth, W. G., S. E. Porter, M. P. Leckie, B. E. Porter, and D. N. Dietzler. 1985. Restoration of cell volume and the reversal of carbohydrate transport and growth inhibition of osmotically upshocked *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **126**:442-449.
 20. Stacey, K. A. 1976. The consequences of thymine starvation, p. 365-382. In T. R. G. Gray and J. R. Postgate (ed.), *The survival of vegetative microbes*. Twenty-sixth Symposium of the Society for General Microbiology. Cambridge University Press, Cambridge.
 21. Stock, J. B., B. Rauch, and S. Roseman. 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. *J. Biol. Chem.* **252**:7850-7861.
 22. Xu, H.-S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb. Ecol.* **8**:313-323.