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Influence of the RpoS (KatF) Sigma Factor on Maintenance of Viability and Culturability of *Escherichia coli* and *Salmonella typhimurium* in Seawater

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The sigma factor RpoS is essential for stationary-phase-specific, multiple-stress resistance. We compared the viabilities (direct viable counts) and culturabilities (colony counts) in seawater of *Escherichia coli* and *Salmonella typhimurium* strains and those in which *rpoS* was deleted or which were deficient in guanosine 3′,5′-bispyrophosphate (ppGpp) synthesis (*relA spoT*). RpoS, possibly via ppGpp regulation, positively influenced the culturability of these bacteria in oligotrophic seawater. This influence closely depended, however, upon the growth state of the cells and the conditions under which they were grown prior to their transfer to seawater. The protective effect of RpoS was observed only in stationary-phase cells grown at low osmolarity. A previous exposure of cells to high osmolarity (0.5 M NaCl) also had a strong influence on the effect of RpoS on cell culturability in seawater. Both *E. coli* and *S. typhimurium* RpoS mutants lost the ability to acquire a high resistance to seawater, as observed in both logarithmic-phase and stationary-phase RpoS$^+$ cells grown at high osmolarity. A previous growth of *S. typhimurium* cells under anoxic conditions also modulated the incidence of RpoS on their culturability. When grown anaerobically at high osmolarity, logarithmic-phase *S. typhimurium* RpoS$^+$ cells partly lost their resistance to seawater through preadaptation to high osmolarity. When grown anaerobically at high osmolarity until stationary phase, both RpoS$^+$ and RpoS$^-$ cells retained very high levels of both viability and culturability and then did not enter the viable but nonculturable state for over 8 days in seawater because of an RpoS-independent, unknown mechanism.

For enteric bacteria, seawater is an extreme environment in which they are submitted to multiple physicochemical stresses: high osmolarity, low temperature, nutrient starvation (34), and oxidation through solar light irradiation (1, 4). In addition to biological interactions, these have previously been considered responsible for the decay of enteric bacteria in natural marine waters (2, 17). When maintained in seawater under nutrient starvation, *Escherichia coli* and *Salmonella typhimurium* cells respond to this situation of multiple environmental stresses by entering a dormant, viable but nonculturable (VNC) state (41, 43). At present, very little is known about the VNC state of members of the *Enterobacteriaceae* family in natural waters, and the mechanisms allowing some of them to retain culturability under such conditions remain unknown.

Compared with enteric conditions, those in most marine waters are relatively oligotrophic, so enteric bacterial cells exist in seawater in a state that resembles, to some extent, the stationary phase of growth as defined for laboratory cultures (43). The involvement of stationary-phase resistance processes in the VNC response might then be suspected. The stationary-phase response involves the synthesis of a set of stress proteins which confer on *E. coli* cells a marked resistance to several stress conditions, including heat shock, oxidation, hyperosmolarity, acidity, and nutrient scarcity (18, 31). One of the stationary-phase-induced genes, *rpoS* (*katF*), encodes a regulator of central importance for gene expression and multiple stress resistance during the stationary phase. This regulator is designated σ^s in *E. coli* (19, 26, 31) as well as in *S. typhimurium* (11). A positive influence of RpoS on the survival of *E. coli* in seawater has been recently reported (36), and although it has been suspected (41), the possible implication of RpoS in the VNC response has not been directly investigated.

The aim of this study was to test this relationship by comparing the viabilities and culturabilities in seawater of isogenic sets of *E. coli* and *S. typhimurium* strains bearing a functional or a nonfunctional *rpoS* gene. Since RpoS synthesis typically depends on growth phase, experiments were performed with cells taken from the logarithmic and stationary phases of growth. In addition, as RpoS synthesis is positively regulated by the cytoplasmic level of guanosine 3′,5′-bispyrophosphate (ppGpp) (16), the influence of ppGpp in the maintenance of viability and culturability was analyzed with *E. coli* mutant strains with a partial or complete ppGpp deficiency. As enteric bacteria grow in the intestine (and urinary tract) under anoxic conditions at high osmolarity (24, 28, 46), the influence of ppGpp and RpoS was also studied with cells grown either aerobically or anaerobically at low or high osmolarity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. *E. coli* RH90 (26) and *S. typhimurium* SF1005 (11) are mutant strains in which *rpoS* (RpoS$^-$ phenotype) has been deleted. *E. coli* CF1652 is a *relA* null mutant of CF1648 with residual ppGpp synthetic activity (RelA$^-$ SpoT$^+$ RpoS low phenotype), and CF1693 is a double *relA spoT* null mutant in which this residual synthesis is eliminated (RelA$^-$ SpoT$^-$ RpoS$^-$ phenotype) (47).

All strains were maintained at −80°C in Luria-Bertani (LB) broth (33) supplemented with glycerol (15%, vol/vol). For the experiments, the cells were grown aerobically or anaerobically at 37°C in LB broth (97 mosM) or in LB broth supplemented with 0.5 M NaCl (1,027 mosM). Growth was monitored by measuring A_{600}. Anaerobic growth was obtained in an H_2-plus-CO_2 atmosphere (GasPack System; BioMérieux, Marcy l'Etoile, France). To prevent the accumulation of suppressor mutations and ensure consistent basal levels of RpoS in ppGpp-deficient (ppGpp$^-$) cells (CF1652 and CF1693), the cells were prepared as suggested by Gentry et al. (16).

Survival assays. Survival tests were run in 100 ml of natural seawater (Cape of

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
E. coli		
MC4100	F′ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 ptsF25 deoC1 rbsR*	44
RH90	MC4100 *rpoS359*::Tn*10*	26
CF1648	λ⁻ F⁻ (prototroph)	M. Cashel
CF1652	CF1648 Δ*relA251*::*kan* (prototroph)	46
CF1693	CF1648 Δ*relA251*::*kan* Δ*spoT207*::*cat*	46
NM522	*recA supE* Δ(*lac-proAB*) *hsd-5* (F′ *proAB lacI*^{q}*15*)	33
S. typhimurium		
14028s	Prototroph	ATCC[a]
SF1005	14028s *rpoS*	11
Plasmids		
pRSkatF5	*katF*::*lacZ* (transcriptional fusion)	35
pFP2	*katF*::*lacZ* (translational fusion)	29

[a] American Type Culture Collection, Rockville, Md.

Nice, France; 980 mosM) filtered through membranes (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) and autoclaved (115°C for 15 min) in 250-ml Erlenmeyer flasks. Microcosms were inoculated with cells grown aerobically or anaerobically and harvested either during early logarithmic phase of growth (A_{600} = 0.25) or during stationary phase (overnight). The cells were washed by centrifugation (10,000 × *g* for 10 min at 20°C) with three 25-ml portions of autoclaved seawater and then suspended in 2 ml of autoclaved seawater. The microcosms were inoculated with these suspensions at a density of approximately 1×10^6 to 3×10^6 CFU/ml. The microcosms were then incubated in the dark at room temperature (22 to 24°C).

Enumeration of bacteria. All microcosms were sampled immediately after inoculation and after 2, 5, and 8 days of incubation. The colony-forming ability of the cells was determined in triplicate by the membrane filtration technique (Millipore filters; pore size, 0.45 μm) on nutrient agar (Difco Laboratories, Detroit, Mich.). The colonies on the agar plates were counted after a 24-h incubation at 37°C, and the results are expressed as culturable cells per milliliter. The total number of viable cells was estimated with a microscope by a direct viable count method derived from that described by Kogure et al. (23) in which cephalexin is used instead of nalidixic acid (27). This method is based on the propensity of living cells to elongate when incubated with organic substrates in the presence of an antibiotic inhibiting cell division. Elongated cells were stained with acridine orange and counted by fluorescence microscopy. Results were expressed as viable cells per milliliter.

β-Galactosidase assay. Quantitative β-galactosidase assays were performed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate, and activities were expressed in Miller units (33).

Reproducibility of results. All experiments were performed at least in duplicate. An analysis of variance was performed to test the level of significance of some observed differences.

RESULTS

Expression of *rpoS* in seawater. RpoS synthesis is regulated at both transcriptional (35) and posttranscriptional (29, 32) levels. The expression of *rpoS* in seawater was therefore analyzed with *E. coli* NM522 (33) carrying a transcriptional *lacZ* fusion (pRSkatF5 plasmid) (35) or a translational *lacZ* fusion (pFP2 plasmid) (29). The transcriptional fusion (Fig. 1A) was not expressed in logarithmic-phase cells grown either at low or high osmolarity. In seawater microcosms, the β-galactosidase activity of stationary-phase cells, whether grown at low or high salinity, was very high at the outset of the experiments (2,300 to 2,500 Miller units) and progressively decreased (30 to 45%) until the end of the experiments. Expression of the translational fusion was very low (10 to 60 Miller units) in logarithmic-phase cells grown at low osmolarity (Fig. 1B). As previously reported by Loewen et al. (29), the β-galactosidase activity of stationary-phase cells was about five times lower than that of the cells harboring the transcriptional fusion. This activity increased during the first day of incubation in logarithmic-phase cells adapted to high salinity. The fusion was expressed at a high level in stationary-phase cells grown at either low or high osmolarity. In these cells, the high β-galactosidase activity initially acquired in the LB medium (15 times that of the logarithmic-phase cells) remained the same during the incubation period (4 days) for cells grown at high osmolarity, while it decreased with time in cells grown at low osmolarity. The expression of *rpoS* was not analyzed for more than 4 days, since β-galactosidase activity significantly decreases in *E. coli* cells after a longer incubation in seawater (38).

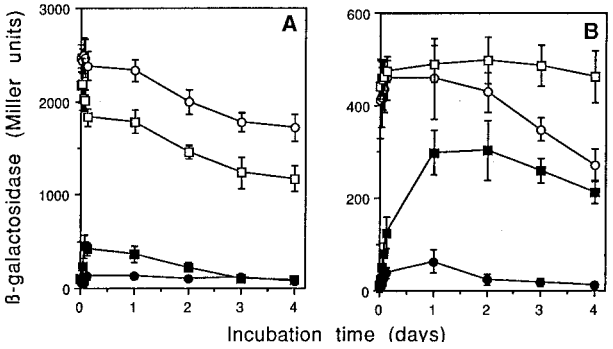

FIG. 1. Expression in seawater of transcriptional (A) and translational (B) *rpoS-lacZ* fusions in *E. coli* NM522 cells grown in LB broth with low (circles) or high (squares) osmolarity and taken from logarithmic phase (solid symbols) or stationary phase (open symbols). Bars represent standard deviations as calculated from two separate experiments.

Influence of RpoS on viability and culturability of *E. coli*. In cells grown aerobically at low osmolarity, the loss of RpoS resulted in a marked decrease in the culturability of cells from a stationary-phase culture, whereas both RpoS$^+$ and RpoS$^-$ cells exhibited a similar rapid decline when taken from early logarithmic phase (Fig. 2A). RpoS$^+$ and RpoS$^-$ populations retained, however, high levels of viability for over 8 days, although viability was slightly but significantly ($P < 0.05$) less pronounced in RpoS$^-$ cells (Fig. 2B).

When grown aerobically at an osmolarity similar to that of seawater, *E. coli* RpoS$^+$ cells exhibited a resistance to seawater (15, 37, 39) characterized by a high level of culturability whether they were collected and tested during logarithmic phase or stationary phase (Fig. 2C). The log-phase RpoS$^-$ mutant did not show this resistance, but stationary-phase cells did show some resistance. Nearly 100% of the RpoS$^+$ populations remained viable throughout the experiments regardless of their initial growth state (Fig. 2D). The RpoS$^-$ stationary-phase cells also retained viability, whereas only about 1% of the RpoS$^-$ logarithmic-phase cells proved substrate responsive after 8 days of incubation.

Influence of ppGpp synthesis in *E. coli*. *E. coli* CF1648 (RelA$^+$ SpoT$^+$), CF1652 (RelA$^-$ SpoT$^+$), and CF1693 (RelA$^-$ SpoT$^-$) exhibited a rapid decline in culturability in seawater when grown aerobically at low osmolarity and harvested during the logarithmic phase (Fig. 3A). When taken from the stationary phase, the wild-type CF1648 cells proved much less sensitive to seawater, as was observed with *E. coli* MC4100, whereas the RelA$^-$ SpoT$^-$ cells lost culturability nearly as rapidly as they did in logarithmic phase. The RelA$^-$ SpoT$^+$ cells exhibited a loss in culturability significantly ($P > 0.01$) lower than that of the wild-type cells. The level of viability of CF1648 and CF1652 cells (Fig. 3B) remained very high for over 8 days (near 50% viable cells), whereas that of CF1693 cells decreased significantly (0.2% viable cells at 10 days).

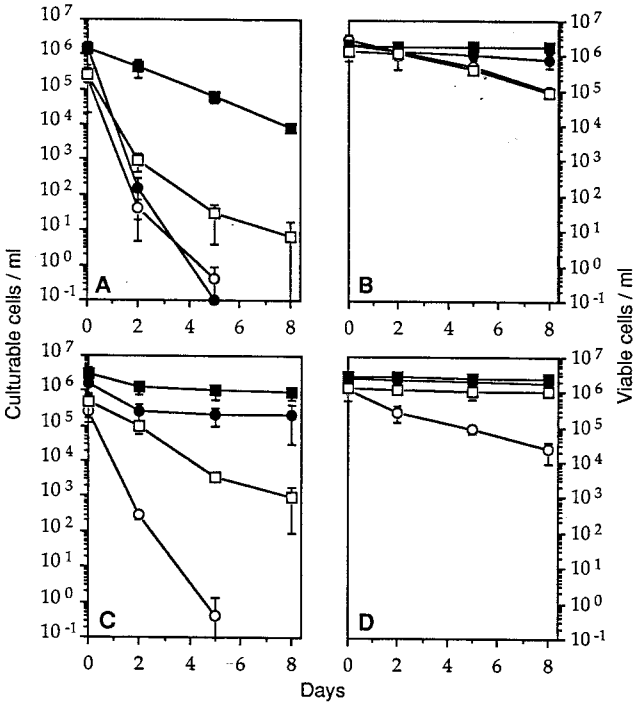

FIG. 2. Time course changes in the numbers of culturable cells (A and C) and viable cells (B and D) in seawater of *E. coli* MC4100 ($RpoS^+$) (solid symbols) and RH90 ($RpoS^-$) (open symbols) grown aerobically in LB broth with low (A and B) or high (C and D) osmolarity and taken from logarithmic phase (circles) or stationary phase (squares). Bars represent standard deviations as calculated from three separate experiments.

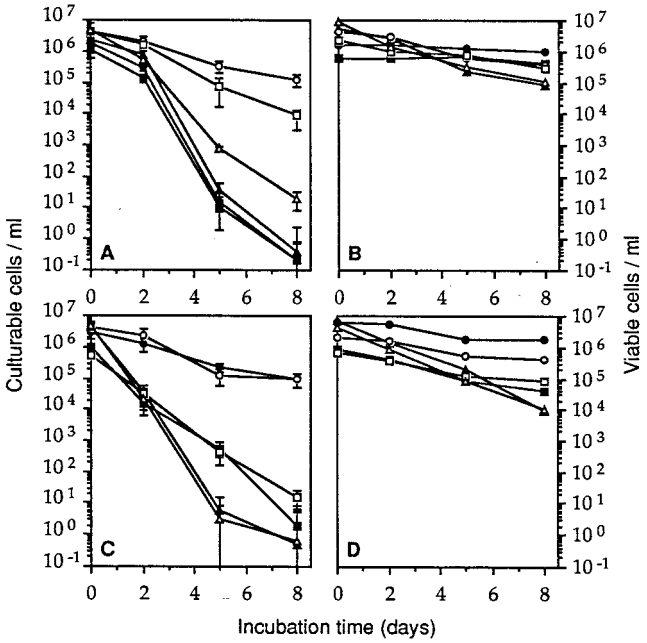

FIG. 3. Time course changes in the numbers of culturable cells (A and C) and viable cells (B and D) in seawater of *E. coli* CF1648 ($RelA^+$ $SpoT^+$) (circles), CF1652 ($RelA^-$ $SpoT^+$) (squares), and CF1693 ($RelA^-$ $SpoT^-$) (triangles) grown aerobically in LB broth with low (A and B) or high (C and D) osmolarity and taken from logarithmic phase (solid symbols) or stationary phase (open symbols). Bars represent standard deviations as calculated from three separate experiments.

When grown at an elevated osmolarity, only the wild-type CF1648 cells retained a high level of culturability (3%) throughout the experiments (Fig. 3C). The $RelA^-$ $SpoT^-$ cells lost culturability at a rate comparable to that observed in cells grown at low osmolarity. The culturability loss of the $RelA^-$ $SpoT^+$ population was not significantly different from that of the $RelA^-$ $SpoT^-$ population after 8 days of incubation, although the decline in culturability of the $RelA^-$ $SpoT^+$ cells was less rapid during the first 5 days. The decrease in viability of the $RelA^-$ $SpoT^-$ cells grown at high salinity was higher than that observed with these cells grown at low salinity (Fig. 3D). The remainder of substrate-responsive cells at the end of the experiments, expressed as the percentage of viable cells at day 8 relative to that of the initial concentration, was estimated at 42, 5, and 0.2% for CF1648, CF1652, and CF1693 cells, respectively. It is noteworthy that unlike cells grown at low osmolarity which proved more sensitive when taken from the exponential phase, no significant difference in both viability and culturability between logarithmic-phase cells and stationary-phase cells was observed.

Influence of RpoS on viability and culturability of *S. typhimurium*. When grown aerobically in low-salt LB medium (Fig. 4A and B), *S. typhimurium* $RpoS^+$ and $RpoS^-$ cells exhibited a behavior similar to that of *E. coli* $RpoS^+$ and $RpoS^-$ cells (Fig. 2A and B). The effect of a previous exposure to high osmolarity was, however, more marked with $RpoS^+$ than with $RpoS^-$ logarithmic-phase cells (Fig. 4C), and the loss of RpoS had a less dramatic effect for *S. typhimurium* than for *E. coli* (Fig. 2C). The level of viability of $RpoS^+$ and $RpoS^-$ cells (Fig. 4D) was comparable to that of *E. coli* cells.

Anaerobically grown cells. Strains RH90 and CF1693 showed a very faint and inconsistent growth under an H_2-plus-CO_2 atmosphere; therefore, it was not possible to test the effect of the loss of RpoS and ppGpp on survival and viability in seawater of *E. coli* grown anaerobically. In *S. typhimurium* grown at low osmolarity (Fig. 5A), *rpoS* mutant cells, both log and stationary phase, exhibited low culturability. This lower culturability was, however, less pronounced than that observed with cells grown aerobically (Fig. 4A) and significantly ($P < 0.05$) higher in logarithmic-phase cells after 2 days of incubation in seawater. The level of viability was as high as that of aerobically grown cells, and the loss of RpoS decreased the viability of logarithmic-phase cells only ($P < 0.05$) (Fig. 5B). When grown at a high osmolarity, $RpoS^+$ cells retained a very high level of culturability regardless of the initial growth state, and the loss of RpoS was followed by a slight decrease in CFU numbers throughout the experiments only in logarithmic-phase cells (Fig. 5C). The level of viability of $RpoS^+$ and $RpoS^-$ populations remained very high (nearly 100% of the cells were substrate responsive) for over 8 days whether they were taken from logarithmic or stationary phase (Fig. 5D).

Influence of RpoS and ppGpp on the nonculturable response. The effect of RpoS and ppGpp production on the entry of *E. coli* and *S. typhimurium* into the nonculturable state in seawater can be estimated more easily by examining the ratios of culturable cells to viable cells in the microcosms at day 8 (Table 2).

The σ^s factor was responsible for highly significant increases of the culturability/viability ratios observed for stationary-phase cells grown at low osmolarity as well as for log-phase and stationary-phase cells grown at high osmolarity, provided they were grown aerobically. This effect was not observed when cells were grown anaerobically.

The ability of *E. coli* cells to synthesize ppGpp also modified their nonculturable response in seawater. The Δ*relA* and Δ*relA*

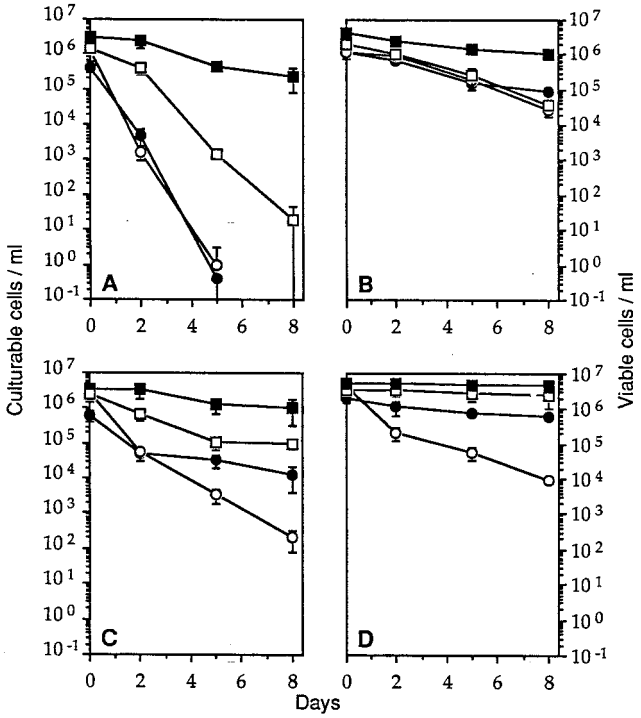

FIG. 4. Time course changes in the numbers of culturable cells (A and C) and viable cells (B and D) in seawater of *S. typhimurium* 14028s (RpoS$^+$) (solid symbols) and SF1005 (RpoS$^-$) (open symbols) grown aerobically in LB broth with low (A and B) or high (C and D) osmolarity and taken from logarithmic phase (circles) or stationary phase (squares). Bars represent standard deviations as calculated from three separate experiments.

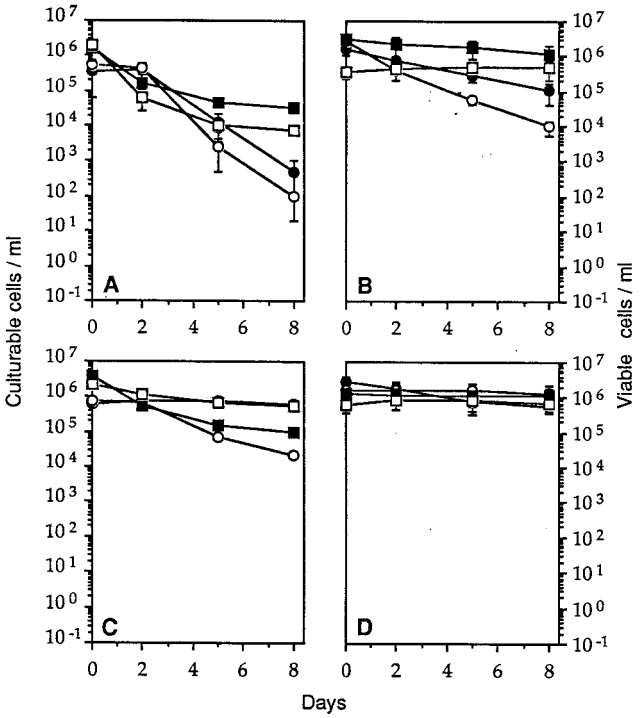

FIG. 5. Time course changes in the numbers of culturable cells (A and C) and viable cells (B and D) in seawater of *S. typhimurium* 14028s (RpoS$^+$) (solid symbols) and SF1005 (RpoS$^-$) (open symbols) grown anaerobically in LB broth with low (A and B) or high (C and D) osmolarity and taken from logarithmic phase (circles) or stationary phase (squares). Bars represent standard deviations as calculated from three separate experiments.

Δ*spoT* deletions resulted in significant increases in the ratios of nonculturable cells, with the increases being more marked in the double mutant. This effect was similar to that of RpoS in *E. coli* MC4100: it affected only stationary-phase cells grown at low osmolarity and both logarithmic-phase and stationary-phase cells grown at high osmolarity.

DISCUSSION

The sigma factor RpoS is essential for the expression of a variety of stationary-phase-induced genes and for stationary-phase-specific, multiple-stress resistance. Our results show that its influence on the maintenance of culturability of *E. coli* and *S. typhimurium* in seawater closely depends upon conditions under which cells are grown prior to their transfer to seawater. The growth state of the cells is obviously critical since the protective effect of RpoS was observed only in stationary-phase cells when they were grown at low osmolarity. This is in agreement with what we know about specific induction of *rpoS* expression in late exponential and stationary phases of growth of *E. coli* and *S. typhimurium* as a consequence of nutrient limitation (18, 19, 26, 31, 45).

A previous exposure of cells to high osmolarity also modified the effect of RpoS on cell viability in seawater (Table 2). *E. coli* and *S. typhimurium* cells deficient in RpoS did not acquire the marked resistance to seawater observed in logarithmic-phase and stationary-phase RpoS$^+$ cells grown aerobically on media of high osmolarity, as previously reported for various enteric bacteria (39). The mechanism responsible for resistance is RpoS dependent and very likely involves osmoregulatory processes.

This observation supports the hypothesis of a contribution of RpoS to osmotic regulation (3, 19–21). The expression of *rpoS* itself is osmotically induced at the posttranscriptional level (19a). Our data showed that RpoS favored the maintenance of cell culturability and that *rpoS* was expressed posttranscriptionally in seawater. It can be hypothesized that the VNC response of aerobically grown *E. coli* cells in oligotrophic seawater may be regulated at the translational level. Studies of the roles of ribosomes and protein synthesis on the survival of enteric bacteria in oligotrophic environments (10, 30, 42) support this hypothesis. In addition, recent data suggest that the enhanced survival of marine vibrios (22) and *E. coli* (25) subjected to nutrient starvation does not depend on the number of ribosomes but is rather related to the efficiency of ribosomal translation kinetics. This efficiency might be, at least partly, regulated by RpoS in natural waters.

The highly similar responses exhibited by *E. coli* cells deficient in either RpoS or ppGpp (Fig. 1; Table 2) suggest an indirect effect of ppGpp via RpoS synthesis. Indeed, ppGpp functions as a signal of perturbations in steady-state growth as well as the entrance into stationary phase and induces *rpoS* expression (16). The *relA* null mutant CF1652, deficient in ppGpp synthetase I, exhibited the same survival behavior in seawater as the double *relA spoT* mutant CF1693 when cells were grown at high osmolarity. This probably means that the residual ppGpp content of *relA* cells (47) was not high enough to increase the RpoS concentration to a sufficient level.

Another important feature that modulates the influence of RpoS on the maintenance of culturability of enteric bacterial cells in seawater is the previous growth of the cells under anoxic conditions (Table 2). Both RpoS$^+$ and RpoS$^-$ logarith-

TABLE 2. Ratio of culturable cells to viable cells in seawater microcosms of *E. coli* and *S. typhimurium* cells after 8 days of incubation at room temperature[a]

Strain	Relevant phenotype	Culturable cell/viable cell ratio							
		Low osmolarity				High osmolarity			
		Aerobiosis		Anaerobiosis		Aerobiosis		Anaerobiosis	
		LoPh[b]	StPh[c]	LoPh	StPh	LoPh	StPh	LoPh	StPh
E. coli									
MC4100	$RpoS^+$	1.3×10^{-8}	4.8×10^{-2}	1.2×10^{-3}	5.0×10^{-2}	0.12	0.27	2.1×10^{-2}	0.86
RH90	$RpoS^-$	5.9×10^{-8}	8.2×10^{-5}[d]	ND[e]	ND	4.0×10^{-7}[d]	8.0×10^{-4}[d]	ND	ND
CF1648	$RelA^+$ $SpoT^+$	2.0×10^{-7}	0.33	ND	ND	5.3×10^{-2}	0.2	ND	ND
CF1652	$RelA^-$ $SpoT^+$	5.0×10^{-7}	3.0×10^{-2}[d]	ND	ND	4.8×10^{-5}[d]	1.7×10^{-4}[d]	ND	ND
CF1693	$RelA^-$ $SpoT^-$	4.7×10^{-6}	2.0×10^{-4}[d]	ND	ND	5.6×10^{-5}[d]	5.9×10^{-4}[d]	ND	ND
S. typhimurium									
14028s	$RpoS^+$	1.1×10^{-2}	0.22	4.8×10^{-3}	3.0×10^{-2}	0.14	0.23	8.5×10^{-2}	1.05
SF1005	$RpoS^-$	3.6×10^{-2}	5.0×10^{-4}[d]	9.7×10^{-3}	2.5×10^{-2}	2.8×10^{-2}[d]	3.7×10^{-2}[d]	1.6×10^{-2}	0.85

[a] Cells were previously grown at low or high osmolarity and taken from exponential or stationary phase.
[b] LoPh, logarithmic phase.
[c] StPh, stationary phase.
[d] Ratio significantly different ($P < 0.01$) from that obtained with the corresponding wild-type strain.
[e] ND, not determined.

mic-phase cells of *S. typhimurium* grown anaerobically at low osmolarity exhibited a lower culturability when compared with cells grown aerobically after 8 days of incubation in seawater. Furthermore, the similar sensitivities to seawater exhibited by $RpoS^+$ and $RpoS^-$ cells taken from stationary phase in anaerobic cultures disclosed a drastic decrease in the protective role of RpoS. When grown anaerobically at high osmolarity, logarithmic-phase $RpoS^+$ cells of *S. typhimurium* showed lower resistance to seawater, despite previous exposure to high osmolarity. The most important effect of anaerobic growth on the further VNC response of cells in seawater appeared, however, when they were grown anaerobically at high osmolarity until stationary phase. Both $RpoS^+$ and $RpoS^-$ cells retained very high levels of both viability and culturability and did not enter the VNC state for over 8 days in seawater because of an unknown RpoS-independent process.

The development of such a hyperresistant state in enteric bacteria grown in media of high osmolarity and low oxygen content might have interesting ecological and sanitary implications. It is now well documented that both the intestinal content and the urine are media with high osmotic strengths (24, 28, 46) in which enteric bacteria can grow and exhibit structural and physiological adaptations to hyperosmolarity (5–7, 24), such as OmpC porins (40) that lower the VNC response of *E. coli* in seawater (12). Moreover, the very high resistance to seawater of freshly voided fecal coliforms from human origin has been reported recently (13). In view of the results shown in Table 2, it is clear that the origin of inocula from logarithmic phase or stationary phase is crucial in terms of survival in seawater. Outside of pathological episodes during which cells of the pathogen can multiply more actively, the growth of enterobacteria in the enteric environment is probably slow (generation time of coliforms in the gut = 6 to 24 h) (8). Both conditions are sufficient to induce *rpoS* expression (16, 35). Since these bacteria are, under natural conditions, from an anoxic environment with high osmolarity, it can be assumed that their behavior and VNC response in seawater are independent of the sigma factor RpoS. This is only true, however, for cells directly released into seawater. Additional studies are needed to analyze changes in the growth state of the cells and RpoS expression during transit in wastewater. As far as we know, a short period of incubation in wastewater (<100 mosM) increases the resistance of *E. coli* to seawater (9) in spite of inducing a loss of cellular compounds involved in osmoprotection (14).

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REFERENCES

1. **Arana, I., A. Muela, J. Iriberri, L. Egea, and I. Barcina.** 1992. Role of hydrogen peroxide in loss of culturability mediated by visible light in *Escherichia coli* in a freshwater ecosystem. Appl. Environ. Microbiol. **58:**3903–3907.
2. **Aubert, M., J. Aubert, M. J. Gauthier, and P. Bernard.** 1981. Les systèmes d'information des microorganismes marins. Rev. Int. Océanogr. Méd. **60–61:**37–91.
3. **Boos, W., U. Ehmann, H. Forkl, W. Klein, M. Rimmele, and P. Postma.** 1990. Trehalose transport and metabolism in *Escherichia coli*. J. Bacteriol. **172:** 3450–3461.
4. **Chamberlin, C. E., and R. Mitchell.** 1978. A decay model for enteric bacteria in natural waters, p. 325–348. *In* R. Mitchell (ed.), Water pollution microbiology, vol. 2. John Wiley, New York.
5. **Chambers, S. T., and C. M. Kunin.** 1985. The osmoprotective properties of urine for bacteria: the protective effect of betaine and human urine against low pH and high concentrations of electrolytes, sugars and urea. J. Infect. Dis. **152:**1308–1315.
6. **Chambers, S. T., and C. M. Kunin.** 1987. Isolation of glycine betaine and proline betaine from human urine. Assessment of their role as osmoprotective agents for bacteria and the kidney. J. Clin. Invest. **79:**731–737.
7. **Chambers, S. T., and C. M. Kunin.** 1987. Osmoprotective activity for *Escherichia coli* in mammalian renal inner medulla and urine: correlation of glycine and proline betaines and sorbitol with response to osmotic stress. J. Clin. Invest. **80:**1255–1260.
8. **Chapoy, P.** 1989. Bacterial overgrowth in children with severe gastrointestinal disorders, p. 73–86. *In* E. Bergogne-Berezin (ed.), Microbial ecology and intestinal infections. Springer-Verlag, Paris.
9. **Combarro, M.-P., M. J. Gauthier, G. N. Flatau, and R. L. Clément.** 1992. Effect of a transient incubation in wastewater on the sensitivity to seawater of *Escherichia coli* cells grown under intestinal-like conditions. Biomed. Lett. **47:**185–190.
10. **Davis, B. D., S. M. Luger, and P. C. Tai.** 1986. Role of ribosome degradation in the death of starved *Escherichia coli* cells. J. Bacteriol. **166:**439–445.
11. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J.**

Harwood, and D. G. Guiney. 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA **89:**11978–11982.
12. **Gauthier, M. J., S. A. Benson, G. N. Flatau, R. L. Clément, V. A. Breittmayer, and P. M. Munro.** 1992. OmpC and OmpF porins influence viability and culturability of *Escherichia coli* cells incubated in seawater. Microb. Releases **1:**47–50.
13. **Gauthier, M. J., and R. L. Clément.** 1994. Effect of a short period of starvation in oligotrophic waters on the resistance of enteric bacterial pathogens to gastric pH conditions. FEMS Microbiol. Ecol. **14:**275–284.
14. **Gauthier, M. J., G. N. Flatau, D. LeRudulier, R. L. Clément, and M.-P. Combarro-Combarro.** 1991. Intracellular accumulation of potassium and glutamate specifically enhances survival of *Escherichia coli* in seawater. Appl. Environ. Microbiol. **57:**272–276.
15. **Gauthier, M. J., P. M. Munro, and S. Mohadjer.** 1987. Influence of salt and sodium chloride on the recovery of *Escherichia coli* from seawater. Curr. Microbiol. **15:**5–10.
16. **Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel.** 1993. Synthesis of the stationary-phase sigma factor σ^s is positively regulated by ppGpp. J. Bacteriol. **175:**7982–7989.
17. **Grimes, D. J., R. W. Atwell, P. R. Brayton, L. M. Palmer, D. M. Rollins, D. B. Roszak, F. L. Singleton, M. L. Tamplin, and R. R. Colwell.** 1986. The fate of enteric pathogenic bacteria in estuarine and marine environments. Microbiol. Sci. **3:**324–329.
18. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *Escherichia coli*. Cell **72:**165–168.
19. **Hengge-Aronis, R.** 1993. The role of *rpoS* in early stationary phase gene regulation in *Escherichia coli* K-12, p. 171–200. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
19a. **Hengge-Aronis, R.** Personal communication.
20. **Hengge-Aronis, R., W. Kein, R. Lange, M. Rimmele, and W. Boos.** 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. J. Bacteriol. **173:**7918–7924.
21. **Hengge-Aronis, R., R. Lange, N. Henneberg, and D. Fischer.** 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. **175:**259–265.
22. **Kjelleberg, S., N. Albertson, K. Flärdh, L. Holmquist, A. Jouper-Jaan, R. Marouga, J. Ostling, B. Svenblad, and D. Weichart.** 1993. How do non-differentiating bacteria adapt to starvation. Antonie Leeuwenhoek **63:**333–341.
23. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
24. **Kunin, C. M., T. H. Hua, L. van Arsdale, and M. Villarejo.** 1992. Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine. J. Infect. Dis. **166:**1311–1315.
25. **Kurland, C. G., and R. Mikkola.** 1993. The impact of nutritional state on the microevolution of ribosomes, p. 225–237. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
26. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:**49–59.
27. **Lebaron, P., V. Roux, M. C. Lett, and B. Baleux.** 1993. Effect of pili rigidity and energy availability on conjugative plasmid transfer in aquatic environments. Microb. Releases **2:**127–133.
28. **Leclerc, H., and D. A. A. Mossel.** 1989. Le tube digestif, p. 141–162. *In* Microbiologie: le tube digestif, l'eau et les aliments. Doin, Paris.
29. **Loewen, P. C., I. von Ossowski, J. Switala, and M. R. Mulvey.** 1993. KatF (σ^s) synthesis in *Escherichia coli* is subject to posttranscriptional regulation. J. Bacteriol. **175:**2150–2153.
30. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Annu. Rev. Microbiol. **43:**293–316.
31. **McCann, M. P., J. P. Kidwell, and A. Matin.** 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. **173:**4188–4194.
32. **McCann, M. P., J. P. Kidwell, and A. Matin.** 1993. The putative σ factor KatF is regulated posttranscriptionally during carbon starvation. J. Bacteriol. **175:**2143–2149.
33. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Moriarty, D. J. W., and R. T. Bell.** 1993. Bacterial growth and starvation in aquatic environments, p. 25–54. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
35. **Mulvey, M. R., J. Switala, A. Borys, and P. C. Loewen.** 1990. Regulation of transcription of *katE* and *katF* in *Escherichia coli*. J. Bacteriol. **172:**6713–6720.
36. **Munro, P. M., R. L. Clément, G. N. Flatau, and M. J. Gauthier.** 1994. Effect of thermal, oxidative, acidic, osmotic or nutritional stresses on subsequent culturability of *Escherichia coli* in seawater. Microb. Ecol. **27:**57–63.
37. **Munro, P. M., M. J. Gauthier, V. A. Breittmayer, and J. Bongiovanni.** 1989. Influence of osmoregulation processes on starvation survival of *Escherichia coli* in seawater. Appl. Environ. Microbiol. **55:**2017–2024.
38. **Munro, P. M., M. J. Gauthier, and F. M. Laumond.** 1987. Changes in *Escherichia coli* cells starved in seawater or grown in seawater-wastewater mixtures. Appl. Environ. Microbiol. **53:**1476–1481.
39. **Munro, P. M., F. M. Laumond, and M. J. Gauthier.** 1987. A previous growth of enteric bacteria on a salted medium increases their survival in seawater. Lett. Appl. Microbiol. **4:**121–124.
40. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
41. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
42. **Reeve, C. A., P. S. Amy, and A. Matin.** 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. J. Bacteriol. **160:**1041–1046.
43. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
44. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Spector, M. P., and J. W. Foster.** 1993. Starvation-stress (SSR) of *Salmonella typhimurium*: gene expression and survival during nutrient starvation, p. 201–224. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
46. **Wadolkovski, E. A., D. C. Laux, and P. S. Cohen.** 1988. Colonization of the streptomycin-treated mouse large intestine by a human fecal *Escherichia coli* strain: role of growth in mucus. Infect. Immun. **56:**1030–1035.
47. **Xiao, X., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutation. J. Biol. Chem. **266:**5980–5990.