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Trehalose Synthesis Genes Are Controlled by the Putative Sigma Factor Encoded by *rpoS* and Are Involved in Stationary-Phase Thermotolerance in *Escherichia coli*

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The *rpoS* (*katF*) gene of *Escherichia coli* encodes a putative sigma factor (σ^S) required for the expression of a variety of stationary phase-induced genes, for the development of stationary-phase stress resistance, and for long-term starvation survival (R. Lange and R. Hengge-Aronis, Mol. Microbiol. 5:49–59, 1991). Here we show that the genes *otsA*, *otsB*, *treA*, and *osmB*, previously known to be osmotically regulated, are also induced during transition into stationary phase in a σ^S-dependent manner. *otsA* and *otsB*, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, are involved in σ^S-dependent stationary-phase thermotolerance. Neither σ^S nor trehalose, however, is required for the development of adaptive thermotolerance in growing cells, which might be controlled by σ^E.

Escherichia coli is able to use trehalose [*O*-α-D-glucosyl-(1→1)-α-D-glucoside] as a carbon source but can also synthesize and accumulate trehalose as an osmoprotectant when grown in high-osmolarity medium. Trehalose uptake is mediated by a phosphotransferase system ($EII^{Tre}/EIII^{Glc}$), and the resulting trehalose-6-phosphate is hydrolyzed to trehalose. Degradation of trehalose is catalyzed by amylotrehalase and results in one glucose residue, with the simultaneous transfer of the other to a polysaccharide acceptor (4). EII^{Tre} and amylotrehalase, which are encoded by *treB* and *treC*, respectively, are induced only at low osmolarity by the presence of trehalose (4), with trehalose-6-phosphate being the physiological internal inducer (18). However, under conditions of high medium osmolarity, *E. coli* can still grow on trehalose as a carbon source, using an osmotically inducible periplasmic trehalase, the gene product of the *treA* gene. Trehalase releases glucose, which is transported into the cytoplasm by the glucose phosphotransferase system (3). Moreover, the cells synthesize and accumulate trehalose as an osmoprotectant. Trehalose synthesis is dependent on *otsA* and *otsB*, which are induced at high osmolarity and encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase (9).

A series of osmotically inducible genes (*osmA* to *osmK*) was identified by isolating gene fusions to *phoA*, the structural gene for the periplasmic alkaline phosphatase (12). *osmA* and *treA* were found to be the same gene (11). Expression of several *osm* genes not only responds to changes in medium osmolarity but also is growth phase dependent. Such a dual control has been shown for *osmB*, a gene encoding a lipoprotein (17). Also, we observed higher trehalase levels in stationary-phase cultures, which could be due to a similarly complex regulation for the *treA* gene (our unpublished results).

During recent years, it has become apparent that the physiology of stationary-phase cells differs greatly from that of rapidly growing cells (26). Stationary phase much better resembles the conditions that *E. coli* encounters in its natural environments. It is therefore not surprising that interest in growth phase-dependent gene regulation has risen considerably. Using a *lacZ* gene fusion approach, we were able to identify a central regulator for the processes occurring during the transition into stationary phase (20). The transcription of this regulatory gene (*rpoS*) is induced during entry into stationary phase in rich medium, and its expression is inversely correlated with growth rate. *rpoS* is required for glycogen synthesis, thermotolerance, H_2O_2 resistance, long-term starvation survival, and induction of at least 15 to 20 proteins, as determined on two-dimensional O'Farrell gels (20). *rpoS* is also involved in the determination of cell shape and cell size in stationary phase, and it controls the morphogene *bolA* (19). *rpoS* is identical to *katF* (20), which was identified as a positive regulator for catalase HPII (*katE*) (25, 30) and exonuclease III (*xthA*) (33). In addition, *rpoS* is allelic with *appR*, which positively regulates a periplasmic acidic phosphatase (*appA*) (20, 37). The *katF* sequence (29) exhibits extensive homology to *rpoD*, which encodes σ^{70}, the housekeeping sigma subunit of RNA polymerase in *E. coli*. This finding suggested that its gene product acted as an alternate sigma factor, hence the novel designations *rpoS* and σ^S for the gene and its product, respectively. Also, a putative consensus sequence for σ^S recognition has been found in the promoter regions of *katE*, *xthA* (42), and *bolA* (19).

Although *rpoS* mutants have a very pleiotropic phenotype, only the few *rpoS*-regulated genes mentioned above have been identified so far. Here we report that expression of the genes involved in trehalose synthesis (*otsA* and *otsB*) is both osmotically and growth phase regulated in a σ^S-dependent manner and that the *ots* genes are involved in σ^S-dependent stationary-phase thermotolerance. We also show that two other osmotically regulated genes (*treA* and *osmB*) exhibit growth phase-dependent regulation that involves σ^S.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are described in Table 1. P1 transductions

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Reference or source
MC4100	F⁻ Δ(*arg-lac*)*U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*	34
RH90	MC4100 *rpoS359*::Tn*10*[a]	20
FF4169	MC4100 *otsA1*::Tn*10*	9
FF2032	MC4100 Φ(*otsA-lacZ*)*7* (λp*lac*Mu55)[b]	9
FF1112	MC4100 Φ(*otsB-lacZ*)*8* (λp*lac*Mu55)[b]	9
WK170	FF2032 *rpoS359*::Tn*10*	This study
WK171	FF1112 *rpoS359*::Tn*10*	This study
RO22	MC4100 *otsA1*::Tn*10* Φ(*otsB-lacZ*)*8* (λp*lac*Mu55)	This study
MPh2	MC4100 Δ(*brnQ-phoA-proC*)	12
CLG2	MPh2 *osmB411*::Tn*phoA*	12
CLG11	MPh2 *treA259*::Tn*phoA*	11
RO23	CLG2 *rpoS359*::Tn*10*	This study
RIM170	CLG11 *rpoS359*::Tn*10*	This study

[a] *rpoS359*::Tn*10* is identical to *csi2*::Tn*10* previously described (20).

[b] For the sake of readability, these fusions are indicated in the text as *otsA*::*lacZ* and *otsB*::*lacZ*, respectively.

performed according to the method of Miller (28) were used for strain constructions. *rpoS359*::Tn*10* is located between the *Dra*I and *Hin*cII restriction sites (20) within *rpoS* (12a).

Cultures were grown under aeration in Luria broth (LB), in minimal medium A (MMA), or in minimal medium M9 (28) with 0.4% glycerol as a carbon source. The osmolarity of the medium was increased by adding NaCl in the concentrations indicated. Growth was monitored by measuring the optical density at 578 nm (OD_{578}). When stationary-phase induction of *lacZ* or *phoA* fusions was tested, the cultures were grown sufficiently long in the exponential phase either before or during the experiments shown in the respective figures that a basal steady-state level of exponential-phase specific activities could be determined.

Enzyme assays. β-Galactosidase and alkaline phosphatase activities were visualized on plates containing the indicator substrates 5′-bromo-4′-chloro-3′-indolyl-β-D-galactopyranoside and 5′-bromo-4′-chloro-3′-indolyl phosphate, respectively. Quantitative β-galactosidase assays were performed by using *o*-nitrophenylgalactopyranoside (ONPG) as a substrate, and activities are given in micromoles per minute per milligram of protein (28). Alkaline phosphatase assays were performed in 250 mM Tris-HCl (pH 8.0) with *p*-nitrophenyl phosphate as a substrate. The assay procedure was as described by Gutierrez et al. (12), and activities are given in micromoles per minute per milligram of protein.

Thermotolerance assays. Resistance against high-temperature shocks was tested by plating samples from a heat-shocked cell suspension on LB plates and counting colonies after growth overnight.

For assaying stationary-phase thermotolerance, cells were grown overnight at 37°C in MMA or M9 with 0.4% glycerol as a carbon source. Immediately before the temperature shift, the cultures were diluted in 0.9% NaCl to approximate cell densities of 5×10^3 (MC4100), 1×10^4 (RO22), and 2×10^4 (RH90) cells per ml. One-milliliter samples were then shifted to 55°C, and 100-μl aliquots were withdrawn at the times indicated and plated on LB plates. Initial 100% survival was determined immediately before heat shock by plating 100-μl samples of these suspensions which were not further diluted (MC4100) or were diluted threefold (RO22) or fivefold (RH90).

For testing adaptive thermotolerance of exponentially growing cells, overnight cultures grown in MMA and 0.4% glycerol at 30°C were diluted in the same medium and grown at 30°C for 4 h to an approximate OD_{578} of 0.2. The cultures were divided into two aliquots, one of which was shifted to 42°C, and incubation was continued. Thermotolerance assays were performed 30 and 90 min after splitting for the 30 and 42°C cultures, respectively. Cells were diluted in 0.9% NaCl to an approximate cell density of 6×10^3 cells per ml, heat shocked at 51.5°C, plated, and counted as described above.

RESULTS

σ^S controls genes involved in trehalose synthesis. A high internal concentration of trehalose is essential for growth in high-osmolarity medium in the absence of other osmoprotective substances such as glycine betaine (9). The genes *otsA* and *otsB*, which are required for trehalose synthesis, as well as *treA* belong to a family of osmotically inducible genes. *osmB*, which is similarly regulated, exhibits increased expression also during entry into stationary phase (17). Using *lacZ* and *phoA* fusions in *otsA*, *otsB*, and *treA*, we therefore tested whether these genes were also growth phase regulated. As shown in Fig. 1, this was indeed the case. Moreover, a Tn*10* insertion in the *rpoS* gene, which encodes a putative stationary-phase sigma factor (σ^S), abolished stationary-phase induction of *otsA*::*lacZ* and *otsB*::*lacZ* and reduced *treA*::Tn*phoA* expression. The same experiment was also performed with an *osmB*::Tn*phoA* fusion, and stationary-phase induction of this fusion proved to be similarly dependent on σ^S (Fig. 1).

Neither sequences nor any promoter studies have been reported for the *ots* genes so far. Osmotic and growth phase regulation of *otsA* and *otsB* could be mediated by separate promoters which might be recognized by different sigma factors, or a single promoter responsive to various regulatory signals might be used. We therefore wanted to determine whether *rpoS* was involved not only in growth phase regulation but also in osmotic induction of the *ots* genes. These experiments were done in minimal medium, because LB medium contains glycine betaine and proline and the presence of these osmoprotective substances in the medium generally reduces the osmotically inducible expression of genes involved in coping with osmotic stress. Upon addition of 0.3 M NaCl during exponential growth, *rpoS*⁺ cells immediately reacted with increased expression of *otsA*::*lacZ* and *otsB*::*lacZ* (Fig. 2). In *rpoS*-deficient cells, however, the *otsA* and *otsB* fusions remained uninduced. To be sure that *rpoS*-deficient cells do not simply need a more drastic osmotic signal for induction of the *ots* genes, an NaCl concentration range between 0 and 800 mM was tested. Since osmotically induced expression levels of *otsA*::*lacZ* and *otsB*::*lacZ* remained stable in stationary phase (our unpublished results), β-galactosidase activities were determined in overnight cultures grown in the presence of NaCl as indicated (Fig. 3). Expression of the *ots* fusions was maximal when the cultures were grown in the presence of 200 to 400 mM NaCl. At higher concentrations, both the growth rate and the growth yield, as well as the expression of the *ots* fusions, were reduced. This is unlike the actual trehalose concentrations in a corresponding wild-type strain and shows that at very high osmolarities, stimulation of the activity of the trehalose-synthesizing enzymes is more important than their increased expression (9, 36). The *ots* genes

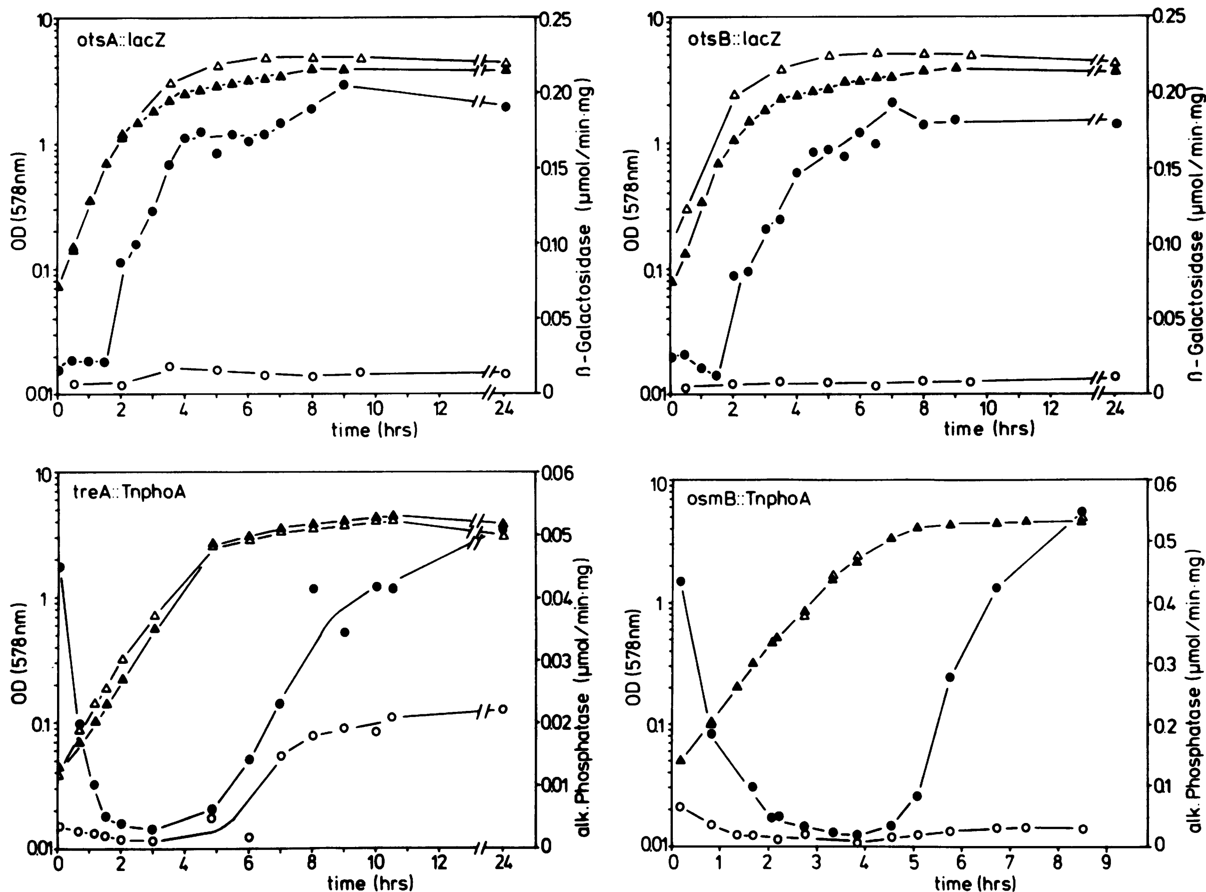

FIG. 1. Involvement of *rpoS* in stationary-phase induction of *otsA*, *otsB*, *treA*, and *osmB*. Expression of *lacZ* or Tn*phoA* gene fusions in *otsA*, *otsB*, *treA*, and *osmB* was determined in isogenic *rpoS*$^+$ (closed symbols) and *rpoS*::Tn*10* (open symbols) strains. Cells were grown in LB. Growth curves measured as OD_{578} (triangles) and specific β-galactosidase or alkaline phosphatase activities (circles) are shown.

remained uninduced in the *rpoS* mutants grown in the presence of up to 800 mM NaCl.

Trehalose is involved in stationary-phase thermotolerance. Stationary-phase *E. coli* cells are resistant to a variety of stresses (26). We previously reported that thermotolerance and H_2O_2 resistance are dependent on *rpoS* (20). For *Saccharomyces cerevisiae*, trehalose has been implicated in thermotolerance, because cellular trehalose contents correlate with the ability to survive heat shock (14). Since the trehalose synthesis genes of *E. coli* proved to be σ^S controlled, we speculated that trehalose was at least part of the σ^S-dependent thermotolerance system. Therefore, we tested the stationary-phase thermotolerance of a strain unable to synthesize trehalose as the result of an *otsA otsB* double mutation in comparison with the wild-type and the *rpoS* mutant strains (Fig. 4). The trehalose-free strain was indeed much more sensitive than the wild-type strain to a 55°C heat shock but still more resistant than the *rpoS* mutant. This finding indicated that trehalose acts as a thermoprotectant also in *E. coli*, but that it is probably not the only factor in σ^S-dependent thermotolerance. Trehalose was especially important for thermoprotection when the growth medium contained less Mg^{2+} than is present in regular M9 medium (1 mM; Fig. 4B). This was shown with MMA (0.4 mM; Fig. 4A) or with M9 supplemented with 0.2 mM Mg^{2+} only (Fig. 4D). The presence or absence of Ca^{2+} did not play a role (compare Fig. 4B and C).

Adaptive thermotolerance in growing cells is independent of both σ^S and trehalose. Before thermotolerance was demonstrated in starved (15) or stationary-phase (20) cells, it was reported that also growing cells acquire resistance against lethal temperatures when grown at an elevated temperature for at least 30 min (45). In general, most treatments that lead to a heat shock response also result in increased thermotolerance (see references 10 and 31 for reviews), although inducing the heat shock proteins alone proved insufficient for the induction of thermotolerance (40).

To determine whether *rpoS* and trehalose are involved in adaptive thermotolerance as well, the experiment shown in Fig. 5 was performed. The wild-type, the *rpoS* mutant, and the trehalose-free strains were grown at 30°C. The cultures were divided during exponential growth, one aliquot was shifted to 42°C, and incubation was continued for 90 min. The cells were heat shocked at 51.5°C, because a temperature of 55°C usually applied to stationary-phase cells (as in Fig. 4) killed exponential nonadapted cells so rapidly that die-off kinetics could hardly be monitored. Surprisingly, σ^S-deficient as well as trehalose-free strains exhibited an adaptive thermotolerance indistinguishable from that of the wild-type strain. The same result was obtained when the challenge temperature (between 50 and 53°C) or the incubation time at 42°C (30 or 90 min) was varied (data not shown). We therefore conclude that while being essential for stationary-phase thermotolerance, σ^S is dispensable for adaptive

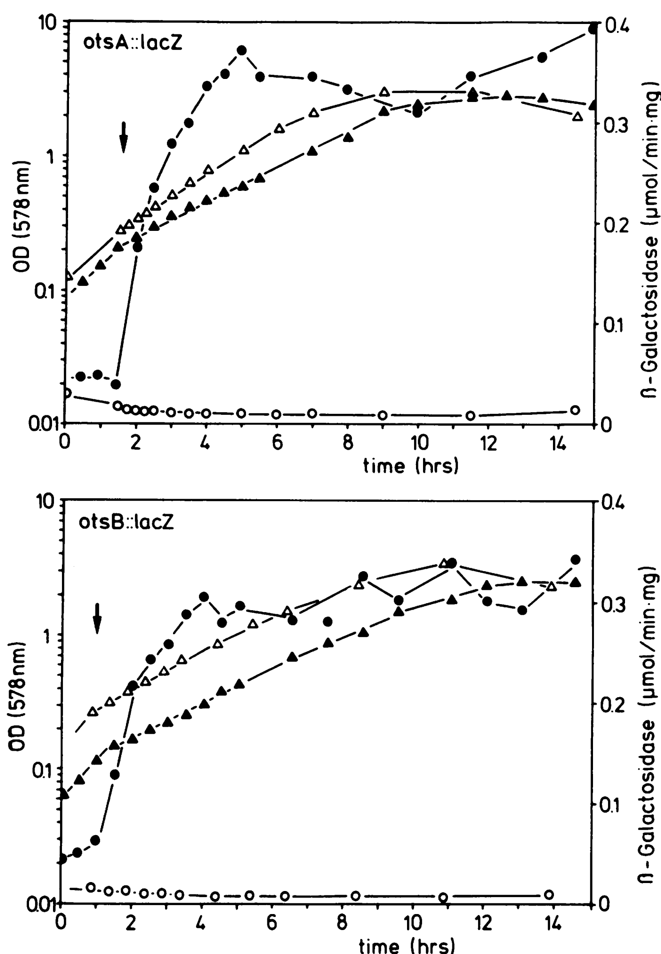

FIG. 2. Dependence on *rpoS* of osmotic induction of the *ots* genes. Strains FF2032 (*otsA*::*lacZ*) and FF1112 (*otsB*::*lacZ*) (closed symbols) as well as their isogenic *rpoS*::Tn*10* derivatives (open symbols) were grown in MMA with 0.4% glycerol as a carbon source. At the time indicated by the arrows, NaCl was added to a final concentration of 0.3 M. OD_{578} was monitored (triangles), and specific β-galactosidase activities (circles) were determined.

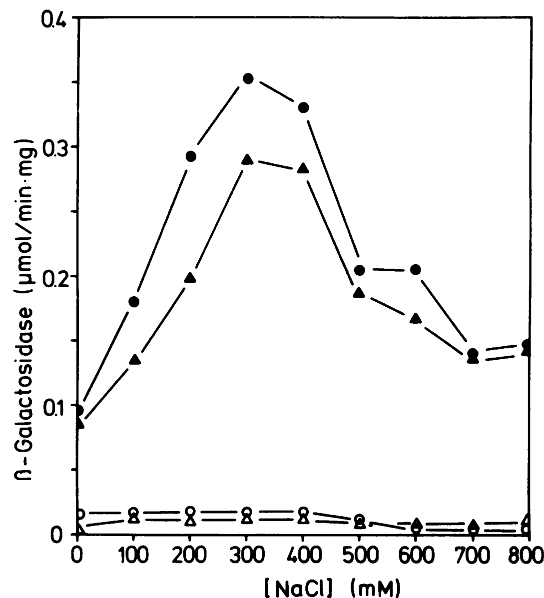

FIG. 3. *rpoS*-dependent expression of the *ots* genes as a function of medium osmolarity. Strains FF2032 (*otsA*::*lacZ*) (closed circles) and FF1112 (*otsB*::*lacZ*) (closed triangles) as well as their isogenic *rpoS*::Tn*10* derivatives WK170 (open circles) and WK171 (open triangles), respectively, were grown overnight in MMA containing 0.4% glycerol and NaCl as indicated. Specific β-galactosidase activities determined in the overnight cultures are shown as a function of NaCl concentration in the growth medium.

thermotolerance in growing cells and also that trehalose is not involved in this adaptive response.

DISCUSSION

The genes involved in trehalose biosynthesis in *E. coli* (*otsA* and *otsB*) exhibit increased expression at elevated medium osmolarity (9). We found that they were induced to a similar extent also during the transition into stationary phase. This growth phase regulation was dependent on *rpoS*, which encodes the putative alternate sigma factor σ^S. Osmotic induction of the *ots* genes in exponentially growing cells also required σ^S, indicating that a certain cellular level of active σ^S was present in cells growing in the minimal glycerol medium used for our osmotic induction experiments. The expression of a transcriptional *rpoS*::*lacZ* fusion (20) under these conditions was indeed almost half the maximum level observed in LB in stationary phase and further increased after the addition of 0.3 M NaCl (data not shown). This finding reflects the inverse growth rate correlation of *rpoS*::*lacZ* expression, with the doubling times being approximately 60 min in minimal glycerol medium and 130 min after osmotic upshift. In the case of the *ots* genes, however, osmotic induction cannot be a consequence of the reduced growth rate alone, since use of potassium acetate as a carbon source to slow down growth (to a similar doubling time of 135 min) produced only a twofold increase in the activity of the *otsA*::*lacZ* fusion (data not shown), whereas osmotic induction is five- to eightfold. This finding indicated a specific osmoregulatory process. The *ots* genes might belong to a subclass of *rpoS*-dependent genes which require, in addition to σ^S, a specific activator for transcription at high medium osmolarity. Nevertheless, the osmotic induction of the *ots* genes is still rather weak compared with that of other osmotically regulated genes or operons such as *proU* (5), which encodes a transport system for the osmoprotectant glycine betaine and exhibits a more than 100-fold σ^{70}-dependent (16) osmotic induction. Our results also demonstrate that σ^S not only is involved in stationary phase gene regulation but also plays a role in osmotic stress adaptation in minimal medium which does not contain glycine betaine and under conditions in which the ability to synthesize trehalose is essential for growth at elevated osmolarity (9).

The promoters of the genes under study here might be directly or indirectly controlled by σ^S. For the *otsBA* operon, the promoter region has not yet been sequenced. Two other osmotically regulated genes, *treA* and *osmB*, also exhibited σ^S-dependent growth phase regulation. Stationary-phase expression of *treA* was partially reduced in an *rpoS* mutant. Only the osmotically induced *treA* promoter has been mapped, and σ^{70} appears to recognize it (32). Stationary-phase induction of *osmB* was entirely dependent on σ^S. The *osmB* P2 promoter is responsible for both osmotic and growth phase regulation. P2 exhibits reasonable homology to the σ^{70} consensus but has a somewhat short spacing (15 bp between the putative −35 and −10 regions) and a region of

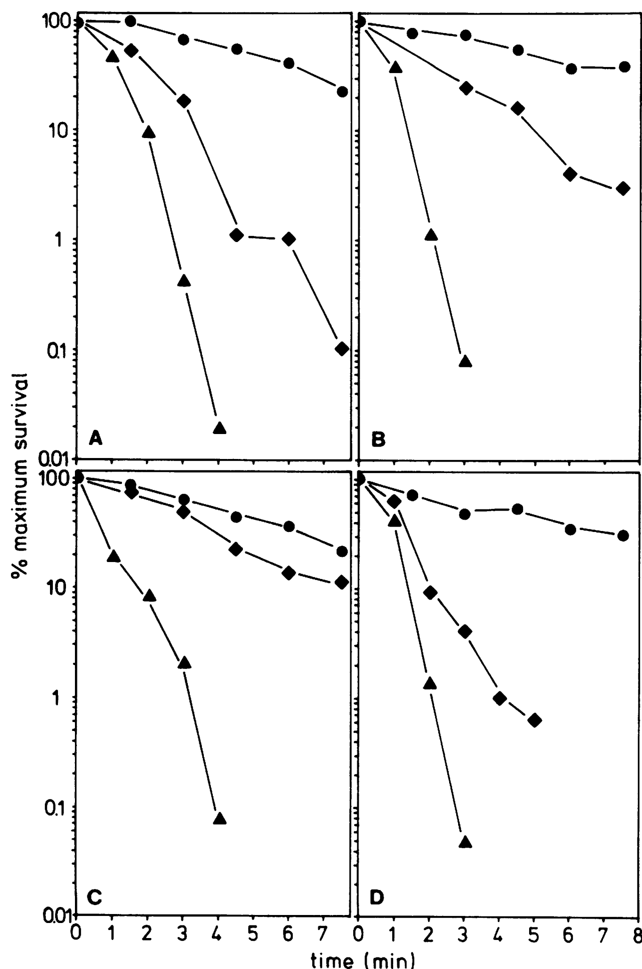

FIG. 4. Stationary-phase thermotolerance. Strains MC4100 (*rpoS*$^+$ *ots*$^+$) (circles), RO22 (*otsA*::Tn*10* *otsB*::*lacZ*) (diamonds), and RH90 (*rpoS*::Tn*10*) (triangles) were grown overnight in the following media: (A) regular MMA (contains 0.4 mM Mg^{2+} and no Ca^{2+}; (B) regular M9 (1 mM Mg^{2+}, 0.1 mM Ca^{2+}); (C) modified M9 (1 mM Mg^{2+}, no Ca^{2+}); (D) modified M9 (0.2 mM Mg^{2+}, no Ca^{2+}). The carbon source in all cases was 0.4% glycerol. After appropriate dilution (see Materials and Methods for details), the cells were transferred to prewarmed (55°C) tubes, and viable cell numbers were determined by plating aliquots onto LB plates. One hundred percent viability corresponds to the viable cell number determined immediately before heat shock.

dyad symmetry with the two inverted repeats flanking the −35 region (17). At positions located appropriately with respect to the start of transcription, no homology to the potential σ^S consensus was found (−35, GTTAAGC; −10, CGTCC [19]). It might be that σ^S indirectly regulates the genes under study here by controlling the expression of unknown secondary regulators which could even act together with σ^{70} to stimulate transcription.

What is the function of trehalose in nongrowing *E. coli* cells? The concentration of trehalose was much lower in stationary-phase cells than in osmotically stressed cells (data not shown). This finding suggests that in nongrowing cells, trehalose acts in a more specific way than in osmotically stressed cells, in which it plays the role of a counteracting osmolyte that has to be present in high concentrations to be effective. This finding also indicated that the massive trehalose accumulation observed under high-osmolarity conditions is due mainly to direct osmotic activation of the trehalose-synthesizing enzymes. Whereas trehalose was described only as an osmoprotectant in *E. coli*, in such diverse organisms as *Streptomyces griseus* (27), *S. cerevisiae* (13, 14), *Dictyostelium discoideum* (7), and even nematodes (43), the intracellular trehalose concentration correlates well with the ability to survive various stresses such as hyperosmotic shock, dehydration, and high-temperature shocks. This suggests that trehalose is a stress protectant rather than a storage compound (41). We therefore investigated whether trehalose acted as a thermoprotectant also in *E. coli*. As we show here, the ability to synthesize trehalose was essential for stationary-phase thermotolerance, especially if the Mg^{2+} concentration of the growth medium was below 1 mM.

The bacterial membranes are a critical target for heat treatment (38, 39). Trehalose binds to the polar head groups of phospholipids, where it replaces water, and acts to preserve the properties of a hydrated membrane (6, 21). In vitro experiments have shown that trehalose can inhibit membrane fusion (44). Besides its membrane protective effect, trehalose may also increase the thermal stability of proteins as do other sugars and polyols (1). Thus, trehalose is clearly suited to play the role of a thermoprotectant. However, it is clear that trehalose is not the only factor in σ^S-dependent stationary-phase thermotolerance, since *rpoS*-deficient cells are considerably more thermosensitive than the *otsA otsB* double mutant in all media tested.

Growing cells also develop thermotolerance when adapted to a growth temperature of 42°C before they are challenged with a lethal temperature (45). Although the heat shock system has been implicated in this adaptive response, induction of the heat shock proteins alone in a strain carrying a plasmid with *rpoH* under the control of the IPTG-inducible P_{tac} promoter is clearly not sufficient to produce this response (40), and the underlying mechanisms have remained obscure. Interestingly, also in yeast cells the development of thermotolerance is not strictly correlated with the induction of heat shock proteins (2). Here we demonstrate that σ^S, while being essential for stationary-phase thermotolerance (20), is not required for the development of adaptive thermotolerance, and also the ability to synthesize trehalose is dispensable. It has been suggested that the alternate sigma factor σ^E is involved in thermotolerance (8). σ^E stimulates transcription from the *rpoH* P3 promoter at very high temperature and thus is involved in the σ^{32}-dependent induction of heat shock proteins (8). σ^E also recognizes the promoter of *htrA*, a gene required for growth at elevated temperatures (22–24, 35), and might regulate other heat-inducible but σ^{32}-independent genes (8). An attractive hypothesis suggested by our results is that σ^S is required for stationary-phase thermotolerance whereas σ^E might be crucial for the development of adaptive thermotolerance.

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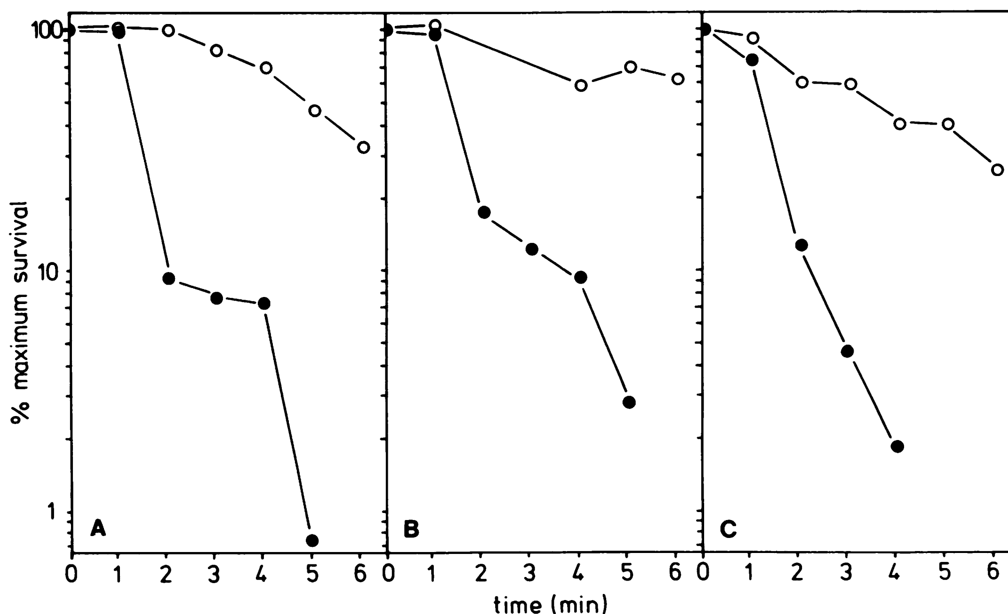

FIG. 5. Adaptive thermotolerance in exponentially growing cells. Cultures of MC4100 (*rpoS*$^+$ *ots*$^+$) (A), RO22 (*otsA*::Tn*10* *otsB*::*lacZ*) (B), and RH90 (*rpoS*::Tn*10*) (C) were divided during exponential growth at 30°C in MMA–0.4% glycerol, and incubation of the aliquots was continued at 42 and 30°C. Survival of heat shock at 51.5°C was tested (see Materials and Methods for details) for the 42°C heat-adapted (open circles) and the nonadapted (closed circles) cultures.

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