

The stationary-phase morphogene *bolA* from *Escherichia coli* is induced by stress during early stages of growth

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Summary

The *Escherichia coli* morphogene *bolA* causes round morphology when overexpressed. The expression of *bolA* is mainly regulated by a σ^S -dependent *gearbox* promoter *bolA1p*. Such regulation results in increased relative levels of expression at slow growth rates, as seen with those attained at the onset of stationary phase. We demonstrate that *bolA1p* is also induced during early logarithmic growth in response to several forms of stress, and that this induction can be partially σ^S independent. Sudden carbon starvation results in a 17-fold increase in mRNA levels derived from *bolA1p* 1 h after stress imposition. Increased osmolarity results in a more than 20-fold increase after the same period. Considerable increases in *bolA1p* mRNA levels were also detected as a result of heat shock, acidic stress and oxidative stress, which has been shown to inhibit σ^S translation. The orders of magnitude of *bolA1p* induction in log phase due to sudden starvation, osmotic shock and oxidative stress surpass the levels reached in stationary phase. Under sudden carbon starvation and osmotic shock, the cells changed their morphology, resembling those cells in which *bolA* is overexpressed in stationary phase. Increased expression and morphological changes due to sudden carbon starvation and osmotic shock still occur when σ^S is not present in a *rpoS*⁻ background. The results show that expression of *bolA* is not confined to stationary phase, but it can also play an important role in general stress response. We propose that *bolA1p* stress induction overrides the normal regulation imposed by growth rate, which is strictly the result of σ^S -directed transcription.

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Introduction

Escherichia coli cells become thinner and shorter after a period of starvation or stationary-phase conditions. Altered morphology is induced by a large group of adaptive responses available to *E. coli* when facing a general form of stress. It is assumed that this capacity of fast adaptation allows bacteria to cope with rapid environmental changes. The gene *bolA* is an *E. coli* morphogene whose product causes round morphology when overexpressed (Aldea *et al.*, 1988). This gene seems to be involved in the switching between cell elongation and septation systems during the cell division cycle (Aldea *et al.*, 1988; 1989). Normally, the expression of *bolA* is growth-rate regulated, being induced during the transition into stationary phase (Aldea *et al.*, 1989; 1990; Lange and Hengge-Aronis, 1991a,b). This type of regulation has been linked to the presence of *gearbox* promoters from which RNA is transcribed at levels inversely proportional to growth rate (Aldea *et al.*, 1990; 1993; Vicente *et al.*, 1991). Expression of *bolA* is governed by two promoters. P2 is located further upstream from the structural gene, is under the control of σ^D and transcribes *bolA* constitutively. In turn, the main promoter P1, proximal to the structural gene, is a *gearbox* promoter under the control of σ^S from which *bolA* has been shown to be transcribed in an inverse growth rate-dependent fashion (Aldea *et al.*, 1989; Lange and Hengge-Aronis, 1991a).

The alternate sigma factor σ^S is encoded by the gene *rpoS* and has been described as a central regulator for the induction of a set of specific genes involved in adaptation to stationary phase (Loewen and Hengge-Aronis, 1994). It has, nevertheless, been shown that σ^S function is not confined to stationary phase. Significant increases in σ^S cellular levels were seen during exponential growth in response to forms of stress and to genes under its control code for important adaptive regulators for general stress conditions (Hengge-Aronis, 1993; Lee *et al.*, 1995; Muffler *et al.*, 1996; Bearson *et al.*, 1997). Because *bolA1p* is under direct σ^S control (Bohannon *et al.*, 1991; Lange and Hengge-Aronis, 1994) and because it has been shown to be triggered by σ^S in response to osmotic shock (Hengge-Aronis, 1993; 1996), we investigated whether it was also induced in response to other forms of stress during exponential growth. We demonstrate that *bolA1p*, the main promoter regulating the expression of the *E. coli*

morphogene *bolA*, is triggered in response to all forms of stress imposed in early exponential phase of growth and that this induction can be independent from σ^S .

Results

Growth phase-dependent expression of bolA1p in minimal medium

To circumvent the problems associated with the very low constitutive levels of *bolA* mRNA during exponential growth, cells were transformed with the plasmid pMAK580 from which the *bolA2p* and *bolA1p* mRNAs can be detected (Aldea *et al.*, 1988; 1989; Santos *et al.*, 1997). Cells were grown in M9 minimal medium supplemented with 0.4% glucose as the sole carbon source. This choice of medium was dictated by the fact that it has been the type of medium most frequently used to study the role of σ^S during the stress response (Hengge-Aronis, 1993; McCann *et al.*, 1993; Muffler *et al.*, 1996; Notley and Ferenci, 1996). The results shown in the right inset of Fig. 1 clearly show that *bolA1p* remains constant during exponential growth but is induced at the entrance to stationary phase, reaching a fivefold increase at an OD_{620} of 1.8. This fivefold increase in *bolA1p* mRNA levels obtained in stationary phase is in agreement with the recently reported (Zgurskaya *et al.*, 1997) four- to sixfold net increase in σ^S levels

seen in the same stage of growth. As expected, *bolA2p* was constitutively expressed during exponential growth and gradually silenced as the culture reached stationary phase (Fig. 1). The left inset in Fig. 1 shows a full-length blot corresponding to a RNA sample extracted at an OD_{620} of 1. In all other experiments, only the bands corresponding to *bolA2p* and *bolA1p* will be shown.

Induction of bolA1p in response to sudden carbon starvation and osmotic shock during early exponential growth

In all stress experiments, cells were grown in M9 minimal medium, maintaining growth conditions that were rigorously the same (see *Experimental procedures*). Different stresses were imposed when the cultures reached an OD_{620} of 0.3 (a in Fig. 1). Sudden carbon starvation was evoked by resuspension of washed cells in glucose-free M9 minimal medium. In contrast to the entrance into stationary phase, in which the exhaustion of energy source is gradual and accompanied by an increase in metabolic by-products, this procedure causes an abrupt depletion of the energy source with no further stress imposed by accumulation of metabolites. It is well established that metabolic by-products can induce transcription of the *rpoS* gene (McCann *et al.*, 1993). A 17-fold increase (representing more than a threefold

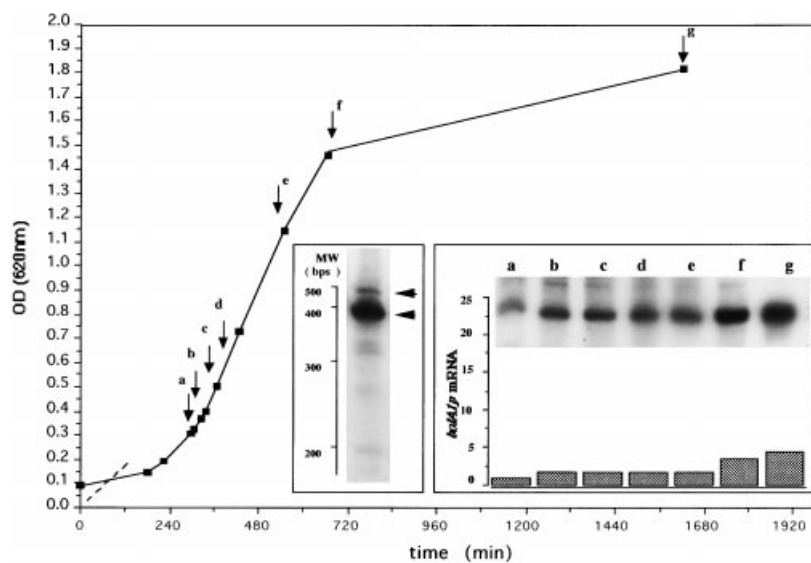


Fig. 1. Growth phase-dependent expression of *bolA* in minimal medium. Optical densities were measured at 620 nm (OD_{620}). CMA10 cells growing exponentially in M9 minimal medium supplemented with 0.4% glucose were diluted to an OD_{620} of 0.08. Dark squares represent the time points when optical density was monitored. Points marked with an arrow and a letter (a–g) correspond to times when total RNA was extracted for Northern blot analysis. Point a represents an OD_{620} of 0.3 and corresponds to the time when the different stresses were imposed in the following experiments (time 0 min). Points b–g represent the times 8', 15', 60', 240', 6 h and 22 h, respectively, relative to a. The inset on the right shows the Northern blot using total RNAs extracted at points a–g that were hybridized with a *bolA* probe spanning the whole gene. To visualise a full-length blot, we show a lane corresponding to a total RNA sample extracted at an OD_{620} of 1.0 in a different experiment (Fig. 1, inset on the left). Arrows on top show the positions of the bands corresponding to *bolA2p* (470 nts) and *bolA1p* (397 nts). Densitometric quantifications of the bands obtained by Northern blot are represented in the graph. Column values are an average of the performed experiments and represent relative *bolA1p* levels, using the quantification at time 0 min (a) as the reference unit for each experiment.

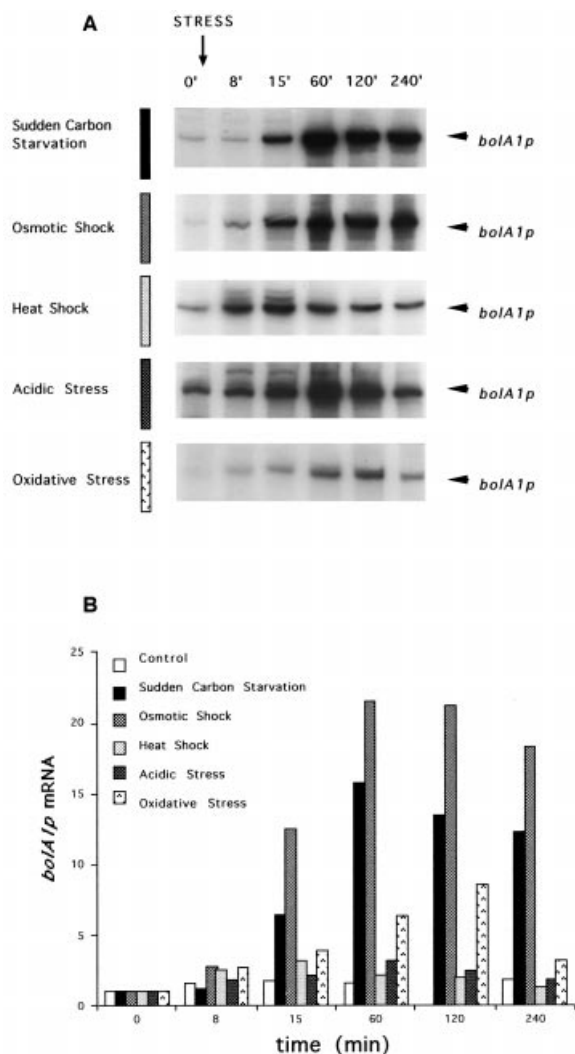


Fig. 2. Expression of *bolA* under stress conditions. A. Examples of Northern blots obtained from total RNA extracted at the time points indicated at the top from CMA10 cells to which stresses were imposed 20 s after cultures reached an OD_{620} of 0.3 (time 0 min). Experiments were carried out at least twice for each stress, rigorously maintaining constant growth parameters. In each case, the black arrow indicates the position of the band corresponding to *bolA1p* (397 nts). B. Densitometric quantifications of the bands obtained by Northern blot. Column values are an average of the performed experiments and represent relative *bolA1p* mRNA levels, always using the quantification at time 0 min (a in Fig. 1) as the reference unit for each experiment. For each case, *bolA1p* response was monitored for a period of 4 h (240 min). The control values, in white, arose from experiments depicted in Fig. 1.

increment relative to stationary phase) in the *bolA1p* mRNA levels was observed in starved cells 60 min after sudden glucose starvation (Fig. 2A and B). These results were somewhat surprising and showed that the induction of *bolA1p* could also occur in early exponential growth as a consequence of the stress imposed by sudden carbon starvation. As expected, cells stopped growing and dividing

after stress was imposed (Fig. 3A). Cell viabilities accrued an abrupt drop immediately after the onset of starvation, but started recovering 120 min after stress imposition (Fig. 3B).

An even higher increase in the levels of *bolA1p* mRNA was observed in response to osmotic shock. Sixty minutes after addition of NaCl to a final concentration of 0.35 M, *bolA1p* mRNA levels had increased approximately 22-fold (Fig. 2A and B). These values represent more than four times what had been seen in stationary phase. The time at which *bolA1p* induction reached its maximum (60 min) coincided with the time that cells resumed growth and had increased viabilities after a lag period of adaptation to the new medium osmolarity (Fig. 3A and B).

Heat shock, acidic stress and oxidative stress

Heat shock, acidic stress and oxidative stress also caused induction of *bolA1p* mRNA levels. A threefold increase was observed in response to both heat shock and acidic stress (Fig. 2A and B). The rapid change from 30°C to 42°C caused an almost immediate induction of *bolA1p*, reaching a maximum level after 15 min and progressing back to lower levels as seen at the following time points (Fig. 2A and B). The fall in *bolA1p* mRNA levels detected was accompanied by a dramatic increase in optical densities and by a moderate recovery in viabilities (Fig. 3A and B). One interpretation for this fact would be that permanence at 42°C rendered the cells less apt to divide whereas mass increase was not inhibited. In fact, a lower rate of cell division compared with the rate of cell growth was further substantiated by the gradual increase in cell length and filamentation along the time interval spanned by the experiments (results to be shown elsewhere). Induction of *bolA1p* expression was more gradual when pH decreased from 7.2 to 4.4 (Fig. 2A and B). Although the optical density of acid-shocked cultures started to increase very slowly 15 min after stress, they never resumed their original growth rate (Fig. 3A). However, cells rapidly regained their ability to form colonies on LB plates (Fig. 3B).

In contrast, oxidative stress caused by the addition of 15 mM H_2O_2 besides inhibiting growth also caused a permanent loss of viabilities (Fig. 3A and B). That this viability loss was not due to a failure to induce the stress response was shown by the observation that *bolA1p* mRNA levels increased gradually, reaching up to eight times the initial value 2 h after the onset of oxidative stress (Fig. 2A and B). These results were surprising because it has been established that OxyS regulatory RNA, which integrates the adaptive response to hydrogen peroxide, represses *rpoS* translation (Zhang *et al.*, 1998). Growth inhibition as a result of oxidative stress was nevertheless reversible because 14 h after the addition of H_2O_2 the OD_{620} had increased up to 0.5 (results not shown).

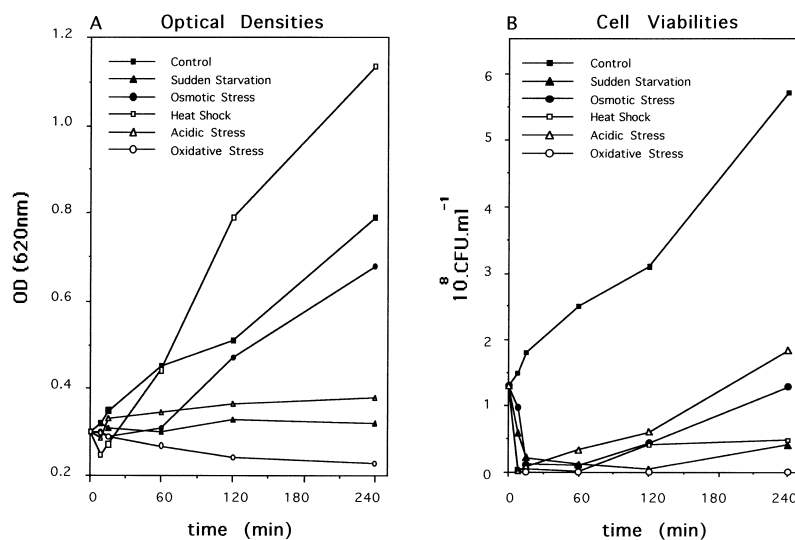


Fig. 3. Effect of the imposed stress conditions on cell growth.

A. CMA10 cells growing exponentially in M9 minimal medium supplemented with 0.4% glucose were diluted to an OD_{620} of 0.08. Stresses were imposed 20 s after these cultures reached an OD_{620} of approximately 0.3 (time 0 min), and their effect on optical densities was monitored thereafter at the same time points as when total RNA was extracted for Northern analysis. In the graph, values were treated such that OD_{620} at time 0 min matched 0.30 for all stresses. Real average values for time 0 min were $OD_{620} = 0.30$ for the control, 0.28 for sudden carbon starvation, 0.35 for osmotic shock, 0.31 for heat shock, 0.32 for acidic stress and 0.29 for oxidative stress.

B. Effect of the imposed stress conditions on cell viabilities. Cell viabilities were measured as the number of colonies obtained in Luria agar plates after 48 h at 30°C, and is shown as $cfu\ ml^{-1}$. CMA10 cells growing exponentially in M9 minimal medium supplemented with 0.4% glucose were diluted to an OD_{620} of 0.08. Stresses were imposed 20 s after these cultures reached an OD_{620} of 0.3 (time 0 min), and their effect on cell viabilities was monitored thereafter at the same time points as when total RNA was extracted for Northern analysis. In the graph, values were treated such that the number of $cfu\ ml^{-1}$ at time 0 min matched 1.3×10^{10} for all stresses. Real average values for time 0 min were 1.30 for the control, 1.02 for sudden carbon starvation, 2.15 for osmotic shock, 2.06 for heat shock, 2.41 for acidic stress and 2.05 for oxidative stress.

Stressed *E. coli* cells reveal morphological alterations

Sudden starvation and osmotic shock were the two conditions eliciting higher responses in terms of *bolA1p* induction and their effect on cell morphology was investigated. Cells in early exponential growth (at an early OD_{620} of 0.3) are shown in Fig. 4A, whereas cells in stationary phase (at an OD_{620} of 1.8) are shown in Fig. 4B. All cells in both populations were rod shaped; however, early exponential rods were twice the length of the rods in stationary phase. Cultures that had undergone a prolonged carbon starvation stress (up to 4 h, Fig. 4C) contained a mixed population of short rods (70%) and round-shaped cells (30%). This latter phenotype resembles the morphology of cells in which *bolA* was overexpressed from the runaway plasmid pMAK552 (Aldea *et al.*, 1988). These cells, as seen in Fig. 3A, stopped dividing during carbon depletion. Cell morphology was also greatly altered in response to increased osmosis. Addition of NaCl caused a general reduction of cell size and produced a mixed population of short rods (60%) and cells with an ovoid morphology (40%) (Fig. 4D). In this case, cells resumed division 15 min after addition of salt (Fig. 3A), at the same time *bolA* started to be induced to high levels (Fig. 2A and B). The morphology of osmotically stressed cells

resembled that which had been seen in *ftsQ* and *ftsA* cell division mutants in which *bolA* was overexpressed by the same runaway plasmid (Aldea *et al.*, 1988). These results show that the changes in cell morphology seen during stress conditions, stationary phase or when *bolA* was overexpressed could be correlated. This strongly implicates this gene in the observed phenotypes.

Sigma S response to sudden starvation and osmotic shock

To quantitatively correlate *bolA1p* induction with actual σ^S levels at late stages when induction was considerable, a Western blot using antibodies against σ^S was performed (Fig. 5). Quantification of the bands corresponding to σ^S in the Western blot showed that the sigma factor also responded to the imposed stress conditions. However, relative levels of σ^S reached upon sudden carbon starvation did not account for the observed *bolA1p* mRNA inductions, and the σ^S response to osmotic shock showed different kinetics from the corresponding *bolA1p* mRNA synthesis. Two hours after sudden carbon starvation, the σ^S levels attained (an approximately 10-fold increase) could not account for more than two-thirds of the observed *bolA1p* full-transcript induction (Figs 5 and 2B). Four hours after

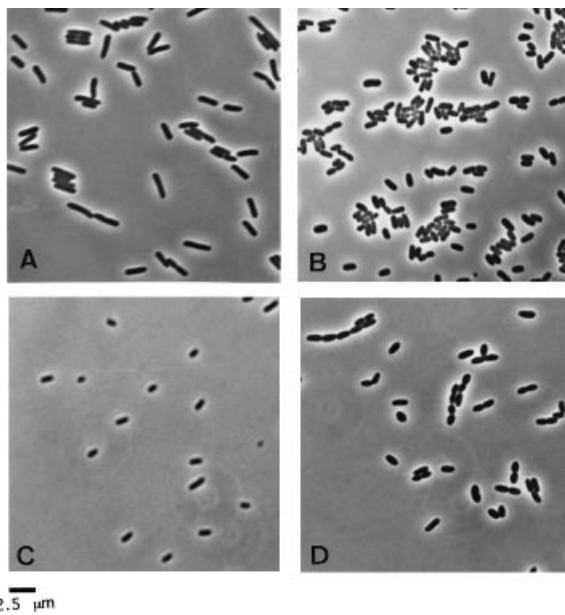


Fig. 4. CMA10 cell shapes produced by stationary phase, sudden carbon starvation and osmotic shock. A. CMA10 cells were grown in M9 minimal medium and harvested when growing exponentially at an OD_{620} of 0.3. B. Stationary-phase cells harvested at an OD_{620} of 1.8. C. Cells harvested 4 h after imposition of sudden carbon starvation at an OD_{620} of 0.3. D. Cells harvested 4 h after imposition of osmotic shock (0.3 M NaCl) at an OD_{620} of 0.30. Bar, 2.5 μm .

starvation, both mRNA and σ^S levels decreased. Still, the amount of protein detected (an approximately sixfold increase) did not account for the observed *bolA1p* full transcript, being, at this time, about half the mRNA induction. The increase in σ^S levels 2 h after the addition of NaCl was also half of that which would be necessary to explain

the 20-fold induction of *bolA1p* mRNA. However, 4 h after osmotic shock, the σ^S levels matched what had been observed for *bolA1p* induction (Figs 5 and 2B). These results suggested a differential dependence on σ^S for induction of *bolA1p* mRNA levels under these two stress conditions. Although the σ^S levels attained 4 h after sudden glucose starvation did not account for the observed *bolA1p* induction, the same was not true for the osmotic shock response. In this case, the stress response could be totally accounted for by the levels of σ^S protein measured 4 h after salt addition. To test for this possibility, an isogenic *rpoS* mutant strain was constructed (CMA11) and subjected to the same stress conditions. The absence of RpoS activity in CMA11 was tested indirectly by measuring catalase (HPH) activity (Jishage *et al.*, 1996), and the absence of RpoS was confirmed by Western blot (results not shown).

Stress induction of *bolA1p* in the absence of σ^S

The results in Fig. 6 show that basal (time 0 min) *bolA1p* mRNA levels could still be detected in the σ^S mutant strain. Moreover, as in the case of the wild type, sudden carbon depletion in the σ^S mutant caused a four- to fivefold increase in *bolA1p* levels in the first 15 min after the onset of stress (Fig. 6A and B). This expression of *bolA1p* mRNA lasted for at least 60 min and then reverted down to basal levels, which shows that the σ^S -dependent character is more accentuated during the early stages of stress response and that the lower levels of σ^S , relative to *bolA1p* mRNA inductions, were enough to substantiate the detected transcription levels at the late stages of stress response (Figs 5 and 2B). Although in the wild-type strain higher *bolA1p* mRNA levels were attained by 60 min, the same drop was observed at points 120 min and 240 min (Fig. 6A and B).

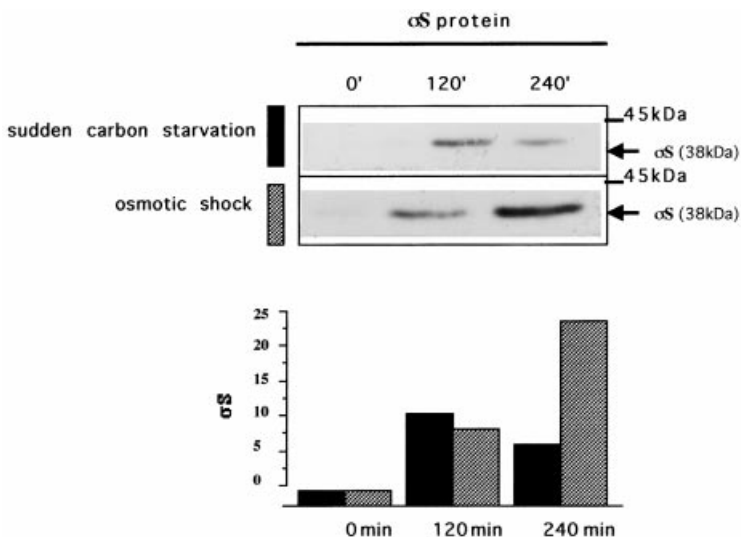


Fig. 5. Expression of RpoS upon sudden carbon starvation and osmotic shock. Western blot of total protein extracted at the indicated time points after imposition of sudden carbon depletion and osmotic shock to non-transformed wild-type MG1693 cells. The black arrow indicates the position of the band corresponding to σ^S . Bands corresponding to σ^S were quantified by densitometry and quantifications were plotted graphically using the quantification at time 0 min (a in Fig. 1) as the reference unit for each experiment.

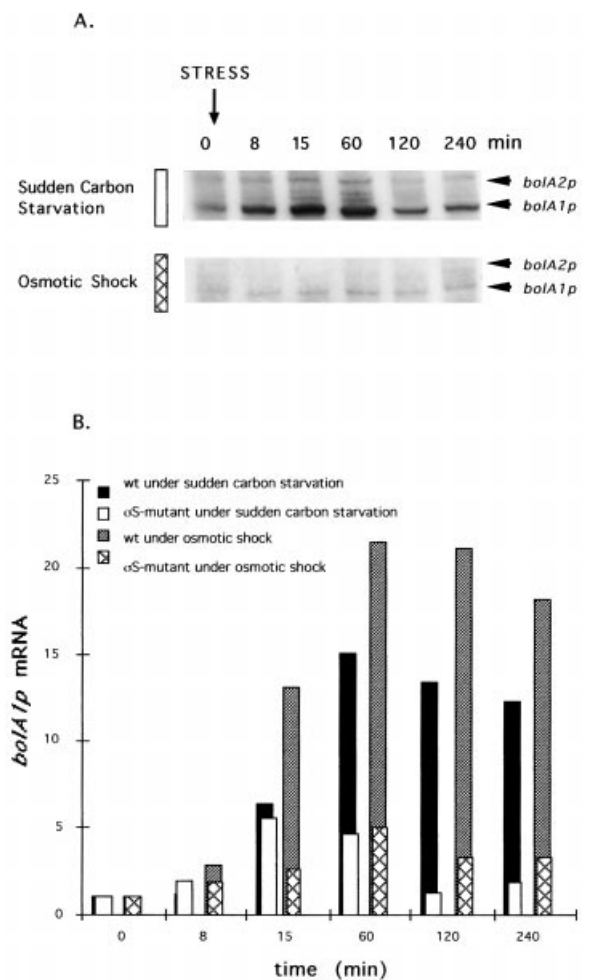


Fig. 6. Expression of *bolA* under sudden carbon starvation and osmotic shock in the absence of RpoS. **A.** Examples of Northern blots obtained from total RNA extracted at the time points indicated at the top from CMA12 cells to which sudden carbon starvation and osmotic shock were imposed 20 s after cultures reached an OD_{620} of 0.3 (time 0 min). Experiments were carried out at least twice for each stress, rigorously maintaining constant growth parameters. In each case, the black arrows indicate the position of the bands corresponding to *bolA2p* (470 nts) and *bolA1p* (397 nts). **B.** Densitometric quantifications of the bands obtained by Northern blot. Column values are an average of the performed experiments and represent relative *bolA1p* mRNA levels, always using the quantification at time 0 min (a in Fig. 1) as the reference unit for each experiment. Values for the wild-type strain CMA10 are also plotted to allow for better comparison. For each case, the *bolA1p* response was monitored for a period of 4 h (240 min). Wild-type values arose from experiments depicted in Fig. 2.

In both the wild type and σ^S mutant, a fall in *bolA1p* levels of a magnitude of about four- to fivefold was observed 60 min after induction. These results suggested the existence of an early mechanism for *bolA1p* induction that was σ^S independent. In contrast, and according to what had been previously reported (Hengge-Aronis *et al.*, 1993), osmoregulation of *bolA1p* was shown to be highly

dependent on σ^S . The response to osmotic shock was considerably reduced in the *rpoS::Tn10* background throughout the full time interval spanned by the experiments. Unlike sudden carbon starvation, the difference between the wild type and the σ^S mutant was already significant in the first 15 min after induction by the addition of salt (Fig. 6). However, a fivefold increase in *bolA1p* mRNA levels was still observed 60 min after osmotic shock (Fig. 6A and B). Overall, the data demonstrated the existence of a basal stress response (upon starvation or shift to high osmolarity) that operated in the absence of σ^S . This condition-dependent mechanism was boosted and prolonged by σ^S in the wild type but, in the case of sudden carbon starvation, it seemed capable of promoting *bolA1p* transcription alone.

The early σ^S -independent character of *bolA1p* induction seen in the first 15 min after sudden carbon starvation was further substantiated with a Western blot, again using antibodies against σ^S (Fig. 7). To obtain better kinetics of σ^S induction in response to sudden carbon starvation that could be related to the full time interval spanning the *bolA1p* induction seen in Fig. 2, we added the early time points after stress imposition to the experiment. This way we could also determine whether a maximum level of RpoS induction would be achieved earlier because in the experiment depicted in Fig. 5 induction proved to be reversible during the time period spanned by the experiments. As seen in Fig. 7, σ^S was not detected in the early stages after starvation, meaning it was not induced immediately by sudden carbon starvation. Furthermore, a maximum level of induction was reached at 60 min, matching the pattern of *bolA1p* mRNA induction seen in previous experiments (Fig. 2). We, thus, conclude that in response to carbon starvation there must exist an early mechanism(s) for *bolA1p* induction that is(are) independent of σ^S . This alternate transcription enhancement is both capable of maintaining relatively high basal steady-state levels of *bolA1p* mRNA in the exponential phase of cell culture growth (Fig. 6A, time 0 min) and is responsible for the *bolA1p* induction observed in the first 15 min after sudden carbon starvation (Fig. 7).

Morphology of *E. coli rpoS* mutant cells under stress conditions

To further investigate the implication of *bolA* in cell morphology changes due to stress, we have monitored the cell shapes of the *rpoS::Tn10* mutant CMA12 under sudden carbon starvation and osmotic shock. Cells that were growing exponentially at an OD_{620} of 0.3 are shown in Fig. 8A. Cells were also rod shaped and apparently resembled the wild type (Fig. 4A). Four hours after sudden glucose starvation, mutant cells still appeared to be rod shaped, however the total average length was reduced to about half

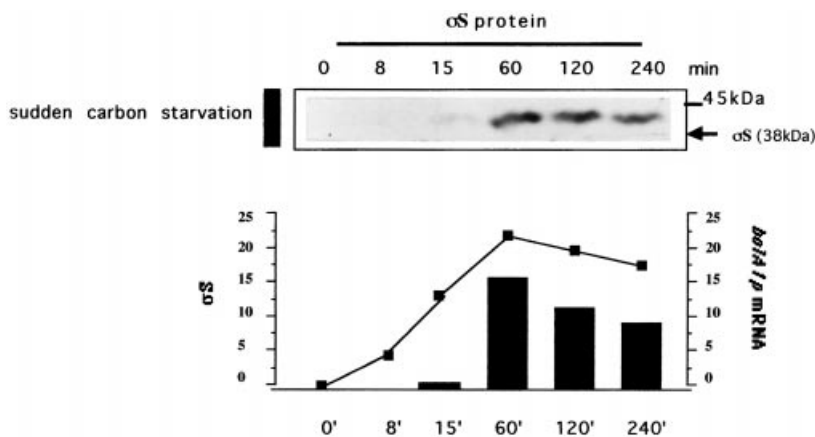


Fig. 7. Expression of RpoS upon sudden carbon starvation. Western blot of total protein extracted at the indicated time points after imposition of sudden carbon starvation to wild-type MG1693 cells. The black arrow indicates the position of the band corresponding to σ^S . Bands corresponding to σ^S were quantified by densitometry and quantifications were plotted graphically using the quantification at time 0 min (a in Fig. 1) as the reference unit for each experiment (dark columns). Relative levels of *bolA1p* mRNA are also plotted according to the same quantifications performed in the experiments depicted in Fig. 2B (dark line).

(Fig. 8B). This is consistent with the *bolA1p* induction still seen without σ^S (Figs 6 and 7). In turn, the effect of osmotic shock induction of the round-shaped morphology of the *rpoS* mutant cells was significantly reduced compared with the effect seen in the wild type (Fig. 8C). Four hours after salt addition, the culture consisted of a mixed population of mainly unaltered rod-shaped cells (87%) with still a few that were ovoid (13%). These observations agreed with what would be expected from Northern analyses. The induction of *bolA1p* as a result of osmotic shock was more dependent on σ^S than induction due to sudden starvation. Accordingly, the observed effects on morphology seen in *rpoS::Tn10* mutant cells were consistently more general and pronounced under sudden carbon depletion than under increased osmosis.

Discussion

General stress response genes are induced whenever the cell needs to adapt and survive under adverse growth conditions. Among them are genes that encode for transcription regulation factors exerting pleiotropic effects in the

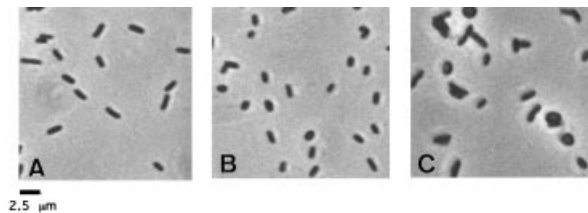


Fig. 8. CMA12 cell shapes produced by sudden carbon starvation and osmotic shock.

A. CMA12 cells were grown in M9 minimal medium and harvested when growing exponentially at an OD_{620} of 0.3.
B. CMA12 cells harvested 4 h after imposition of sudden carbon starvation at an OD_{620} of 0.3.
C. CMA12 cells harvested 4 h after imposition of osmotic shock (0.3 M NaCl) at an OD_{620} of 0.3. Bar, 2.5 μ m.

cell, such as RpoS. In this study, we show that the function of *bolA* is not confined to stationary phase, and that its sensitivity to several forms of stress during early growth gives this gene the potential of playing a role in the general stress response.

The mechanism by which an overexpression of *bolA* induces altered cell morphology is not yet known. However, an helix–turn–helix motif found in the protein sequence, which could correspond to a DNA-binding domain, and data obtained by two-dimensional protein electrophoresis led to the suggestion that BoIA can induce, at the transcription level, the synthesis of a penicillin-binding protein (PBP6) (Aldea *et al.*, 1989). PBP6, along with PBP5, is one of the low-molecular-weight carboxypeptidases involved in murein synthesis in *E. coli* (Markiewicz *et al.*, 1982).

The instantaneous behaviour of the *bolA1p* activity after the cessation of growth imposed by the stress conditions agrees with it being a *gearbox* promoter (Vicente *et al.*, 1991). However, stress-dependent induction of *bolA1p* does not always inversely correlate with changes in growth rate. For example, the drop in *bolA1p* mRNA levels found 120 min after sudden starvation does not follow the expected *gearbox* pattern because growth rate did not increase. Although cell death is likely to be the trivial explanation for the drop in promoter activity observed 240 min after oxidative stress, the response of *bolA1p* to acidic stress did not agree with the *gearbox* pattern of induction (Vicente *et al.*, 1991). The promoter *bolA1p* was induced despite the simultaneous increase in growth rate (Fig. 3A and B). The transient stress induction of *bolA1p* could form part of a general stress response directed towards survival that would be initiated independently of growth rate and therefore independent of *gearbox* regulation. In agreement with this is the fact that *mcbAp*, a promoter containing –35 and –10 sequences highly homologous to *bolA1p*, does not transcribe the *mcbA* gene upon imposition of all conditions used in this study with the exception of heat shock (M. Cruz and J. Schottel, personal communication). These

observations together indicate that stress-dependent induction of *bolA* is specific for this gene and that other mechanisms aimed at correcting cell distress may overtake unconditioned regulation, with the consequence of the *gearbox* mode of expression (Vicente *et al.*, 1991) being masked.

Sigma S-independent transcription of *bolA1p* seen with the *rpoS::Tn10* mutant could be driven by the RNA polymerase holoenzyme containing RpoD ($E\sigma^D$). The potential of the vegetative $E\sigma^D$ core enzyme to transcribe *bolA1p* has been shown *in vitro* (Nguyen *et al.*, 1993). Closer interaction of $E\sigma^D$ with the *bolA* promoter region agrees with the enhanced expression of the housekeeping *bolA2p* promoter seen in both stress conditions tested with the σ^S mutant strain (Fig. 6). These observations *in vivo*, together with the previously reported *in vitro* results (Nguyen *et al.*, 1993; Ballesteros *et al.*, 1998), suggest a much higher affinity of the $E\sigma^S$ holoenzyme for *bolA1p* than $E\sigma^D$. Increased *bolA2p* expression in the σ^S mutant strain could be due to an increase in overall $E\sigma^D$ holoenzyme concentration in the cell, as a result of the absence of cores combined with σ^S . This hypothesis is supported by the recently reported evidence for specific competition between σ^D and σ^S for the core polymerase (Farewell *et al.*, 1998). However, σ^D is a constitutive sigma subunit that is not induced by stress. Thus, enhanced $E\sigma^D$ -driven transcription due to lack of competitor could explain higher steady-state levels of *bolA* mRNA at time 0 min, but it does not explain the sequential induction of *bolA1p* and *bolA2p* promoters seen in the σ^S mutant. These results suggest the existence of another transcriptional factor (or factors) specific for the stress-mediated induction of the gene *bolA*. Such variety would also help to interpret the occasional concomitant induction of the constitutive *bolA2p* promoter when facing specific challenges such as heat shock or acidic stress (Fig. 2A). Furthermore, our results show that both σ^S -mediated and σ^S -independent pathways for *bolA1p* induction have different kinetics under sudden carbon starvation and under osmotic shock. The alternative response seems to be relatively more important for survival in the early stages of sudden glucose starvation than when cells were subjected to increased medium osmolarity. However, there is still a minor osmotic induction of *bolA* in the *rpoS* mutant at all times, in contrast to the much more drastic reaction in the wild type. This is similar to the osmotic inducibility of *osmY*, another σ^S -dependent gene seen in stationary phase and in rich medium (Lange *et al.*, 1993). In the first 15 min after depletion of glucose, *bolA1p* was induced equally in both the wild type and the σ^S mutant strain, clearly showing a σ^S -independent character of induction. In contrast, osmotic shock caused a 12-fold induction of *bolA1p* after the same period of time, contrasting to the threefold increase seen in the mutant. Different patterns of induction such as these leave open the hypothesis of

existing redundant regulatory mechanisms that control the expression of *bolA* under distinct forms of stress.

We propose that the smaller morphology caused by stress-induced overexpression of *bolA* renders to the cell both an important reduction in the surface area exposed to the damaging agent and, at the same time, a decrease in surface to volume ratio. We think that both parameters are important, and that they may act alone or in a concerted way to increase cell resistance. The stress-dependent activation of *bolA1p* confers to the *bolA* gene a general role in protection against stress, and uncovers additional mechanisms that can override any growth rate or growth phase-dependent regulation.

Experimental procedures

Bacterial strains, plasmids and genetic manipulations

Strains used were *Escherichia coli* MG1693 (*thyA715*) (Bachmann and Low, 1980), RH-90 (MC4100 *rpoS359::Tn10*) (Lange and Hengge-Aronis, 1991b), CMA10 (MG1693 + pMAK580), CMA11 (MG1693 *rpoS359::Tn10*) and CMA12 (CMA11 + pMAK580). CMA11 was constructed by transferring the *rpoS359::Tn10* allele from RH-90 into MG1693 using standard P1 transduction techniques (Miller, 1972). Transductants were tested qualitatively by measuring catalase (HPII) activity by applying small drops of H₂O₂ (30%) onto colonies or patches grown overnight on LB plates (Lange and Hengge-Aronis, 1991b). Positive control colonies showed strong bubbling upon contact with 30% H₂O₂, whereas transductants showed no reaction. When necessary, strains were transformed with plasmid pMAK580 (Aldea *et al.*, 1988) containing *bolA* under the regulation of its own promoters. Transformations were carried out as described by Sambrook *et al.* (1989).

Media, growth conditions and stress inductions

Luria broth, Luria agar and M9 minimal medium compositions were carried out according to Miller (1972). When required, the media were supplemented with thymine (Sigma Chemical) to a final concentration of 50 $\mu\text{g ml}^{-1}$, and also 20 $\mu\text{g ml}^{-1}$ chloramphenicol (Sigma Chemical), and 0.4% (w/v) glucose (Merck). During stress experiments, batch cultures were launched from overnight growths that were diluted to an optical density of 0.08 measured at 620 nm (OD₆₂₀). Cultures were grown aerobically in M9 minimal medium containing 0.4% (w/v) glucose at 30°C and 120 r.p.m. Stress was imposed when the cultures reached 0.3 at OD₆₂₀. Sudden depletion of glucose was performed as follows: cells were harvested by centrifugation for 10 min at 7520 $\times g$ at 4°C and washed twice with two volumes of sterile ice-cold M9 medium with no carbon source. The cells were resuspended in the same volume of minimal medium devoid of glucose and were incubated as before. Culture samples were then extracted at different time points for mRNA analysis. As a control for the effects of centrifugation, washing and resuspension, a culture was subjected to the same treatment but resuspended in medium supplemented with the original concentration of glucose. Analysis of RNA extracted at time points after this procedure

showed no induction when probed for *bolA* (data not shown). For hyperosmotic shock, NaCl was added to a final concentration of 0.35 M (Muffler *et al.*, 1996). To rule out possible artefactual results due to increasing plasmid copy number, we have repeated the sudden starvation and osmotic shock procedures using the non-transformed wild-type strain MG1693 (results not shown). Levels of *bolA1p* induction obtained using the non-transformed MG1693 strain were, with little variations, very similar to that which had been obtained using the plasmid-containing strain CMA10. Thus, 4 h after sudden starvation, *bolA1p* underwent a 12-fold induction, whereas the same time period after osmotic shock caused a 24-fold increase. Because of the proximity of these values to that which had been observed with the plasmid-containing strain, we continued to use transformed cells for better detection. For oxidative stress, H₂O₂ was added to the culture at a final concentration of 15 mM (Imlay and Linn, 1986). Heat shock was applied by shifting the culture from 30°C to 42°C (Muffler *et al.*, 1997), and acid challenge was attained by adding HCl (30%) to bring the pH down from 7.2 to 4.4 (Bearson *et al.*, 1997). To evaluate cell viabilities, exactly the same growth conditions and stress procedures were carried out as described above. The number of viable cells was determined by harvesting 100 µl of culture at the desired times. Cells were then plated on Luria agar and incubated for 48 h at 30°C. The number of viable cells was extrapolated directly from the number of colonies obtained and is expressed as cfu ml⁻¹.

RNA preparation, probe preparation and Northern blot hybridization

Culture samples were taken at different time points, starting 20 s before stress imposition (time 0 min) and total RNA was extracted as described previously (Santos *et al.*, 1997). The RNA was treated with RNase-free DNase I (Boehringer Mannheim Biochemicals) before use. In all experiments, RNA was quantified by spectrophotometry and verified by examining rRNA bands in ethidium bromide-stained agarose gels. Samples containing 20 µg of total RNA were dissolved in 90% formamide, 0.01 M EDTA, pH 7.0, 1 mg ml⁻¹ xylene cyanol, 1 mg ml⁻¹ bromophenol blue buffer (Sambrook *et al.*, 1989) and heated for 10 min at 85°C for denaturation. RNA samples were then electrophoresed on a 6% denaturing polyacrylamide gel and transferred to a nylon membrane (Biodyne A; Pall) according to the procedure described previously by Fitzwater *et al.* (1987). The RNA was then fixed to the membrane by UV light and hybridized with the probe that had been radio-labelled with [α -³²P]-dCTP, using the Multiprime DNA-labelling system from Amersham. The probe spanned the entire *bolA* transcriptional unit and was prepared by either digesting the plasmid pMAK580 with the restriction enzymes *Clal* and *HincII* (Boehringer Mannheim Biochemicals) or, alternatively, this fragment was obtained by PCR using *EcoTaq* from Ecogen and primers A (5'-GCAGTGTAATCGTCGGGG-3') and B (5'-CGCCCCATTCTCAACTGG-3'). Hybridization was carried out as described previously by Thomas (1983), at 42°C in 50% formamide. Filters were autoradiographed at -70°C using either Amersham hyperfilm MP or Biomax MR from Kodak. Bands were quantified by densitometry with a Molecular Dynamics IMAGEQUANT densitometer using Molecular Dynamics software.

Total protein SDS-PAGE and Western blot analysis

Samples containing 15 µg of total protein were separated by 10% SDS-PAGE as described previously (Sambrook *et al.*, 1989) and were transferred to nitrocellulose by electroblotting (Schleicher and Schuell). Western blot was carried out as described by Zilhão *et al.* (1996). The primary antibody used was against σ^S (Jishage and Ishihama, 1995), incubated at a dilution of 1:2500. The blots were exposed to radiographic film (Amersham), and the signal in the autoradiograms was quantified by scanning densitometry.

Microscope preparations

CMA10 and CMA12 cells were harvested from cultures growing under sudden carbon starvation and osmotic shock to allow us to observe the effect of these stresses on morphology. Growth conditions were the same as those used during the stress experiments to enable us to compare cell shape before and after stress imposition. Preparations were carried out according to Donachie *et al.* (1976). Cells were harvested and fixed onto slides coated with a thin layer of agar (1%) containing 0.9% NaCl and 0.1% sodium azide to prevent further cell growth. Cells were either photographed with a Zeiss Ultra-phot microscope (CMA10) or a Leica DMRB microscope (CMA12) under phase-contrast optics.

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