Molecular Microbiology (1994) 11(3), 537–544

Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest

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Summary

The synthesis of the small, cytoplasmic protein UspA (universal stress protein A) of *Escherichia coli* is induced as soon as the cell growth rate falls below the maximal growth rate supported by the medium, regardless of the condition inhibiting growth. The increase in UspA synthesis appears to be the result of induction of the monocistronic *uspA* gene. Induction of this gene during a heat-shock treatment is demonstrated to be the result of transcriptional activation of a σ^{70}-dependent promoter which has previously been shown to be activated also during carbon starvation-induced growth arrest. Mutant cells lacking UspA grow at rates indistinguisible from the isogenic parent at different temperatures and in the presence of different growth inhibitors but are impaired in their ability to survive prolonged periods of complete growth inhibition caused by a variety of diverse stresses, including $CdCl_2$, H_2O_2, DNP, CCCP exposure, and osmotic shock. Moreover, the *uspA* mutation results in an increased sensitivity of cells to carbon-source starvation (i.e. glucose, glycerol or succinate depletion). Also, the mutation causes a marked alteration in the timing of starvation protein expression but protein expression during steady-state growth appears to be normal. The results presented have prompted us to postulate that UspA may have a general protective function related to the growth arrest state.

Introduction

Transition of *Escherichia coli* cells into the stationary phase significantly diminishes the cells' sensitivity to a variety of potentially harmful stresses, such as heat shock, H_2O_2 exposure (Jenkins *et al.*, 1988; Lange and Hengge-Aronis, 1991), osmotic challenge (Jenkins *et al.*, 1990), near-u.v. irradiation (Sammartano *et al.*,1986) and treatments with antibiotics (Godell and Tomasz, 1980; Toumanen *et al.*, 1988). A few genes required for the development of 'stationary-phase' stress resistance have been identified (reviewed in Hengge-Aronis, 1993), and the induction of several of these genes has been demonstrated to require an intact *rpoS* gene (Hengge-Aronis *et al.*, 1991; Lange and Hengge-Aronis, 1991; Sak *et al.*, 1989), encoding the stationary phase sigma factor, σ^s (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991; Mulvey and Loewen, 1989; Tanaka *et al.*, 1993). Mutations in the *rpoS* gene render the cells sensitive to different stresses and also diminish the cells' ability to survive starvation in general (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991). In addition, genes of the heat-shock response (Spence *et al.*, 1990), the *relB* gene of the stringent-starvation regulon (Mosteller, 1978; Mosteller and Kwan, 1976), the *pcm* gene encoding L-isoaspartyl methyltransferase (Li and Clark, 1992) and a few genes referred to as *sur* (survival; Tormo *et al.*, 1990) have been reported to be important in the maintenance of viability during starvation-induced growth arrest.

We have previously demonstrated that cells of *E. coli* greatly increase the synthesis of a small cytoplasmic protein as soon as cell growth ceases, regardless of the condition inhibiting growth (Nyström and Neidhardt, 1992). This protein, UspA (universal stress protein A), is thus a general, non-specific responder to growth arrest (this paper; Nyström and Neidhardt, 1992), and its induction is independent of RpoS as well as of all other global regulators so far tested, including PhoB, RelA, SpoT, AppY, OmpR, H-ns, Lrp, and RpoH (Nyström and Neidhardt, 1992). The effect of mutations in *cya* or *crp* on UspA expression has not been tested, but the universal property of *uspA* of responding to diverse stresses rules out catabolite repression as the sole mechanism for *uspA* regulation. In this paper we demonstrate that mutants devoid of UspA synthesis are highly susceptible to killing when subjected to complete and prolonged growth inhibition by the stresses studied, including osmotic shock, H_2O_2, $CdCl_2$, carbonylcyanide-3-chlorophenylhydrazone (CCCP) and dinitrophenol (DNP) exposure and long-term carbon-source starvation. Also, the *uspA* mutation markedly affected the timing of expression

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of glucose-starvation-inducible proteins. It is also demonstrated that induction of UspA during a shift in growth temperature from the Arrhenius range to 42°C results primarily from transcriptional activation of a σ^{70}-dependent 'housekeeping' promoter which has previously been demonstrated to be activated during carbon-starvation-induced growth inhibition (Nyström and Neidhardt, 1992).

Results and Discussion

Protein UspA is induced immediately as a result of growth inhibition

The kinetics of UspA synthesis during inhibition of growth by a variety of stress agents were determined by quantitative two-dimensional gel electrophoresis as described in the *Experimental procedures*. Levels of UspA expression were low but detectable throughout the exponential phase of growth but were induced immediately as a result of pertubations of growth, regardless of the stress condition employed (Fig. 1). All treatments, except a shift from 28 to 42°C, led to an immediate, complete and prolonged growth inhibition (Fig. 1). The induction of UspA peaked within 50 min after a pertubation in the growth rate was observed, after which the rate of UspA production reached a new 'steady-state' level (Fig. 1). Depletion of different macronutrients has been demonstrated previously to cause a similar pattern of UspA induction (Nyström and Neidhardt, 1992). Temperature shifts within the Arrhenius range (15 to 37°C) did not cause an increased expression of UspA (data not shown), but a shift from 28 to 42°C resulted in a threefold induction (Fig. 1). At a shift from 28 to 50°C, the protein was made at a rate indistinguishable from the preshift rate (Fig. 1).

Induction of uspA *is a result of transcriptional activation of a single σ^{70}-dependent promoter*

We have previously demonstrated that the increased level of protein UspA during carbon (glucose) starvation is a result of the activation of a promoter with −10 and −35 regions characteristic of a σ^{70}-dependent promoter (Nyström and Neidhardt, 1992). We conducted primer extension analysis of *E. coli* RNA isolated from cells growing in steady state at 28°C and at intervals after a shift to 42°C to address the question of whether the same promoter is activated during heat stress and carbon starvation. The transcriptional start site, depicted in Fig. 2, from which transcription is initiated during heat stress was found to be identical to that activated in response to carbon starvation (Nyström and Neidhardt, 1992). The increased level of the *uspA* transcript, as measured by densitometry of the autoradiogram depicted in Fig. 2, can fully account for the threefold increase in UspA synthesis during the shift from 28 to 42°C shown in Fig. 1. Further, the half life of the *uspA* transcript and the UspA protein was not altered in response to heat stress (data not shown). Therefore, we conclude that the increased level of UspA during heat stress and carbon starvation is primarily the result of transcriptional activation of the same promoter.

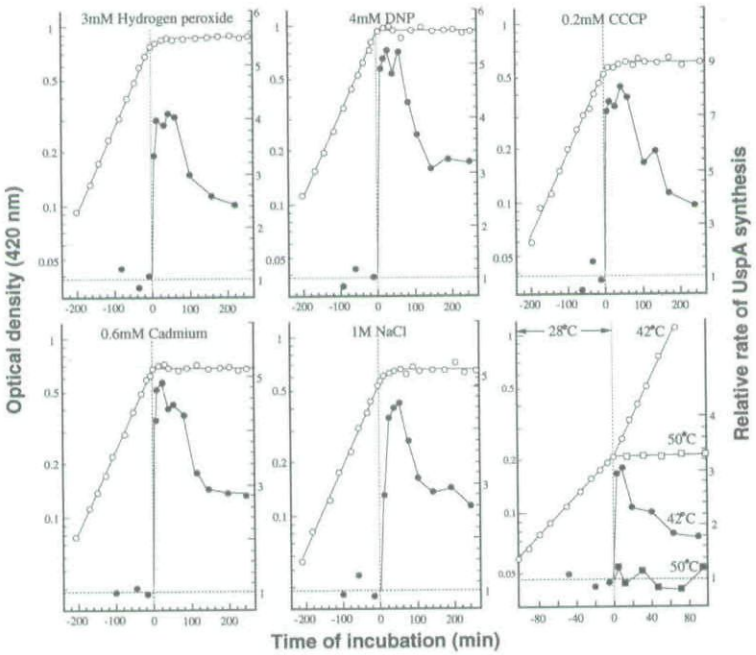

Fig. 1. Effect of different stress conditions on growth (○, □) and UspA induction (●, ■). Strain W3110 was grown aerobically in glucose minimal MOPS at 37°C except where noted in the graph. The time at which temperature shifts were initiated and stress agents were added to the growing culture was designated time zero. A portion of the culture was labelled for 5 min at time intervals during steady-state growth and at intervals after the imposition of stress on the cells. The differential rates of UspA synthesis were determined as described in the *Experimental procedures*. All rates are plotted relative to the mean differential rate obtained during steady-state growth, which was defined as 1.0.

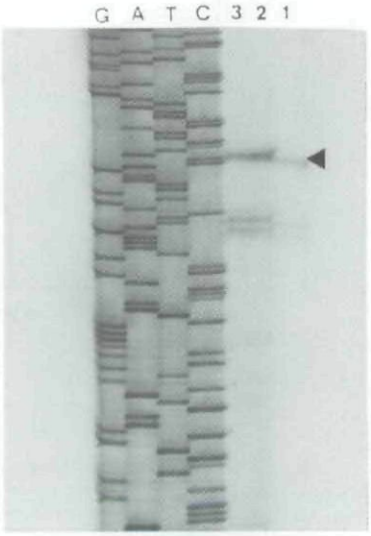

Fig. 2. Primer extension analysis of *uspA* transcripts. RNA was isolated from *E. coli* W3110 growing at steady state at 28°C in glucose minimal MOPS (1), and at 5 min (2) and 10 min (3) after a shift to 42°C. A radiolabelled oligonucleotide was incubated with the RNA, and reverse transcriptase was added to produce a DNA copy of the transcript, as described in the *Experimental procedures*. The same oligonucleotide was used to prime dideoxy sequencing products from a DNA template (pTN6091) that contained the *uspA* gene. The letters on top of each lane indicate the dideoxynucleotide used to terminate each reaction. The arrow indicates the transcriptional start site.

A uspA *mutant does not display increased sensitivity to stresses causing only partial growth inhibition*

A *uspA* mutant was isolated after insertional mutagenesis of the *uspA* gene using the kanamycin-resistance geneblock of Pharmacia Biochemicals (Nyström and Neidhardt, 1993). The mutation was crossed into the chromosome of strain JC7623 (*arg ara his leu pro recB21 recC22 sbcB15 thr*) and subsequently introduced into strain JM105 and W3110 by standard P1 transduction procedures.

As a preliminary investigation of the nature of the physiological alterations in the *uspA* mutant, its growth characteristics were compared to that of the isogenic parent during the imposition of different stress conditions. Overnight cultures of the *uspA*::*kan* mutant and its isogenic parent were inoculated in minimal MOPS media (see the *Experimental procedures*) with glucose as the single source of carbon and the growth of the cells was followed by measurement of culture turbidity. Temperature shifts and the addition of stress agents were initiated when the cultures growing in steady state had been diluted once in fresh, prewarmed medium and allowed to grow for at least four more generations. A shift from 28 to 50°C led to a complete inhibition of growth in both the *uspA* mutant and the wild-type parent while shifts from 28 to 37 or 42°C led to an immediate and identical increase in the growth rate of both strains (data not shown). Similarly, addition of DNP, CCCP, H_2O_2, NaCl, HCl and $CdCl_2$ at different concentrations causing partial reductions in the growth rate affected growth of the *uspA* mutant and the wild-type parent to the same extent (data not shown). In other words, the *uspA* mutant did not display an increased sensitivity to stresses that caused only partial growth inhibition.

The uspA *mutant strain is more sensitive to stresses causing complete growth arrest*

The effect of the addition of stress agents at concentrations causing complete and prolonged growth arrest was tested on culture viability. The addition of stress agents and temperature shifts was initiated when cultures of wild-type and *uspA* mutant strains reached an optical density at 420 nm of 0.5–0.8. Cells were grown in glucose minimal MOPS medium at 37°C, except where noted. As depicted in Fig. 3, the sensitivity of the *uspA* mutant TN3151 and the isogenic parent W3110 during a shift in temperature from 28 to 50°C was found to be indistinguishable. However, wild-type cultures preadapted to a non-lethal temperature

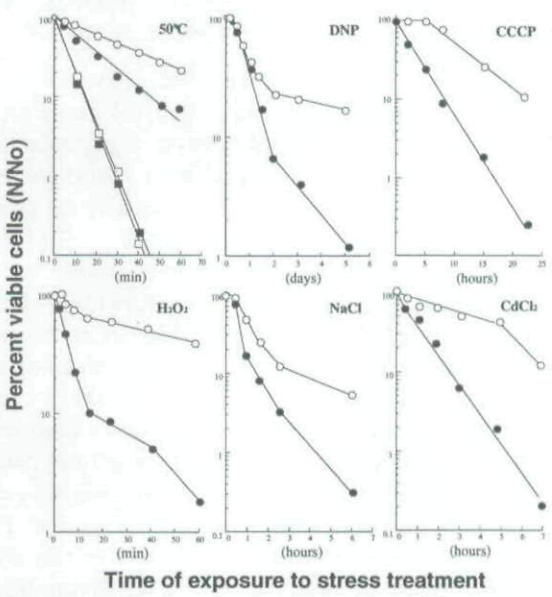

Fig. 3. Comparison of the stress sensitivity of exponentially growing wild-type W3110 with that of the *uspA*::*kan* mutant strain TN3151. Cells were grown in glucose minimal MOPS medium and allowed to reach an OD_{420} of 0.5–0.8 before the imposition of growth stress was initiated (4 mM DNP, 200 μM CCCP, 600 μM $CdCl_2$, 1 M NaCl and 3 mM H_2O_2). Open symbols denote wild-type W3110 and closed symbols the *uspA*::*kan* mutant. Squares in the top, left graph denote the viability of cells shifted from 28 to 50°C, while circles in the same graph signify the viability of cells preadapted for 15 min at 42°C and then shifted to 50°C. Viability is expressed as the number of cfu at time t (N) divided by the number of cfu before the imposition of stress (No).

of 42°C for 15 min were found to be somewhat more resistant than preadapted *uspA* cultures (Fig. 3). Moreover, the *uspA* mutant was found to be highly sensitive to different stresses causing complete and prolonged growth inhibition. The conditions tested that caused complete inhibition of growth included, 4 mM DNP, 200 μM CCCP, 600 μM $CdCl_2$, 1 M NaCl, and 3 mM H_2O_2. The kinetics of die-off differed between stress conditions and all stress conditions resulted in a loss of viability in both wild-type and *uspA* mutant cultures. However, the *uspA* mutant strain was found to be markedly more susceptible to killing by all the stresses tested (Fig. 3). This stress sensitivity could be complemented with plasmid pTN6094 (Nyström and Neidhardt, 1992), carrying the entire structural *uspA* gene (not shown). The introduction of the *uspA* insertion mutation in strain JM105 resulted in a similar sensitivity to the stresses tested. The striking sensitivity of the *uspA* mutant to diverse stresses causing growth inhibition may indicate that protein UspA has a general protective function related to the growth arrest state rather than being implicated in different specific repair mechanisms needed during the imposition of the different damaging agents.

The uspA *mutant strain is impaired in its ability to survive prolonged periods of carbon-source starvation*

To determine the effect of different starvation conditions on the viability of the *uspA* mutant strain, cells were grown in minimal MOPS medium with 5% of the normal concentration of the appropriate nutrient (Nyström and Neidhardt, 1992). This procedure resulted in growth arrest of cells at an OD_{420} of 0.5–0.65 (1.5×10^8 to 2.5×10^8 cells ml^{-1}). Viable counts, total counts and cell size distributions were followed throughout starvation periods of 24 d. Figure 4 shows the viability of the *uspA* mutant strain TN3151 and the isogenic parent during prolonged glucose, glycerol, phosphate and nitrogen starvation. As depicted in this figure, the *uspA* mutant cells were markedly impaired in their ability to survive carbon source depletions. No difference in culture viability could be detected prior to 5–8 d of glucose or glycerol starvation, after which the *uspA* mutant cells lost viability at an increased and exponential rate (Fig. 4). Similar results were obtained for succinate-starved cells (data not shown), indicating that the *uspA* mutant is impaired in its ability to survive carbon-source starvations in general. The total cell number remained constant after the first few hours of starvation and no lysis could be detected during the rapid die-off of the *uspA* mutant strain. In addition, the median cell volume of the *uspA* mutant culture and the isogenic parent was identical throughout the starvation period studied and no significant filamentation or sphaeroplast formation could be detected in either culture.

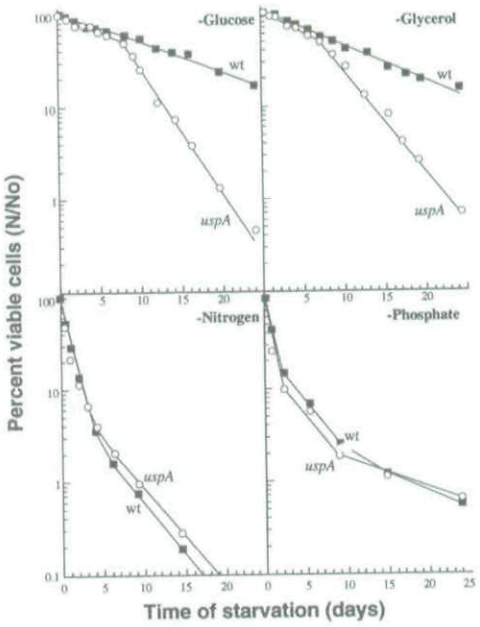

Fig. 4. Starvation survival of strain TN3151 carrying *uspA*::*kan*. Wild-type strain W3110 (closed squares) and the *uspA* mutant strain TN3151 (open circles) were grown in glucose minimal MOPS medium with 5% the normal concentration of the appropriate nutrient (see the *Experimental procedures* for details). After growth arrest commenced, incubation was continued for 24 d under the same conditions. Viable cells were counted as colonies plated on LB plates after appropriate dilutions. One-hundred per cent viability corresponds to the number of viable cells counted 1 h after growth was arrested owing to the depletion of the limiting nutrient.

The survival of the *uspA* mutant and the wild-type strains were found to be indistinguishible during depletion of either phosphate or nitrogen (Fig. 4). Notably, the ability of the wild-type *E. coli* strains studied to survive starvation for nitrogen or phosphate in the presence of high concentrations of glucose is markedly less impressive than the survival of cells subjected to starvation for carbon (Fig. 4). Similar results, with regard to the poor ability of cells to withstand nitrogen or phosphate starvation in the presence of a good carbon source, have been demonstrated for other heterotrophic prokaryotes as well (Nyström *et al.*, 1992).

The phenotype of the *uspA* mutant regarding its sensitivity to different stress conditions and long-term starvation is reminiscent of the phenotype displayed by *rpoS* mutants (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991; Mulvey *et al.*, 1990). However, induction of *uspA* is independent of RpoS (Nyström and Neidhardt, 1992) and thus constitutes an additional response of *E. coli* involved in enhancing the cells' capacity to survive potentially harmful stress and starvation conditions.

The uspA *mutation causes a marked alteration in the timing of starvation protein expression*

To approach the question of whether UspA constitutes a global regulator reminiscent of RpoS, we performed two-dimensional gel electrophoresis of the *uspA* mutant and wild-type cells labelled with radioactive methionine during steady-state growth in glucose minimal medium and at times during prolonged periods of glucose depletion. The pattern of proteins synthesized during steady-state growth in both *uspA*::*kan* and wild-type strains was virtually identical with the exception of one high molecular weight protein which was absent in the wild-type strain (Fig. 5, A, B, E and F). Similarly, no apparent differences in the protein profiles could be detected after starvation for 30 min (Fig. 5, B and F) to 24 h (data not shown), while dramatic differences between the strains were noticeable after 3 d of glucose starvation. Specifically, the *uspA* mutant strain failed to express several proteins that were induced in the wild-type strain as well as displaying the synthesis of a number of proteins that were lacking in the parent (Fig. 5, C and G). These differences were even more pronounced after 5 d of starvation (Fig. 5, D and H). When glucose was added back to the cell suspensions starved for 5 d, both strains recovered with equal efficiency and regained the pattern of protein expression depicted in Fig. 5, A and E. Therefore, the altered pattern of protein expression displayed by the *uspA* mutant strain did not seem to be the result of the occurrence of stable, secondary mutations.

The results presented in Fig. 5 may indicate that UspA functions as a global regulator of gene expression during prolonged starvation. However, when the patterns of protein expression and the differential rates of synthesis of some selected proteins were analysed at more frequent intervals during starvation, it became apparent that the difference in protein expression was the result of an altered timing of starvation-protein expression in the mutant. More specifically, several glucose starvation-inducible proteins, e.g. Gsi1-5, induced early during starvation in both strains were repressed much earlier in the *uspA* mutant (Fig. 6, top panels). In addition, several Gsi proteins (e.g. Gsi11, 12, 14, 15 and 17), normally induced after 3–5 d of starvation were induced already after 1–3 d in the *uspA* mutant (Fig. 6, bottom panels). Although it is possible that the induction and repression of the Gsi proteins, depicted in Figs 5 and 6, are an integral part of an ordered and progressive programme of starvation-induced gene expression serving to adapt the cells to the growth-arrested state, the induction of Gsi proteins after several days of starvation may constitute a pathological

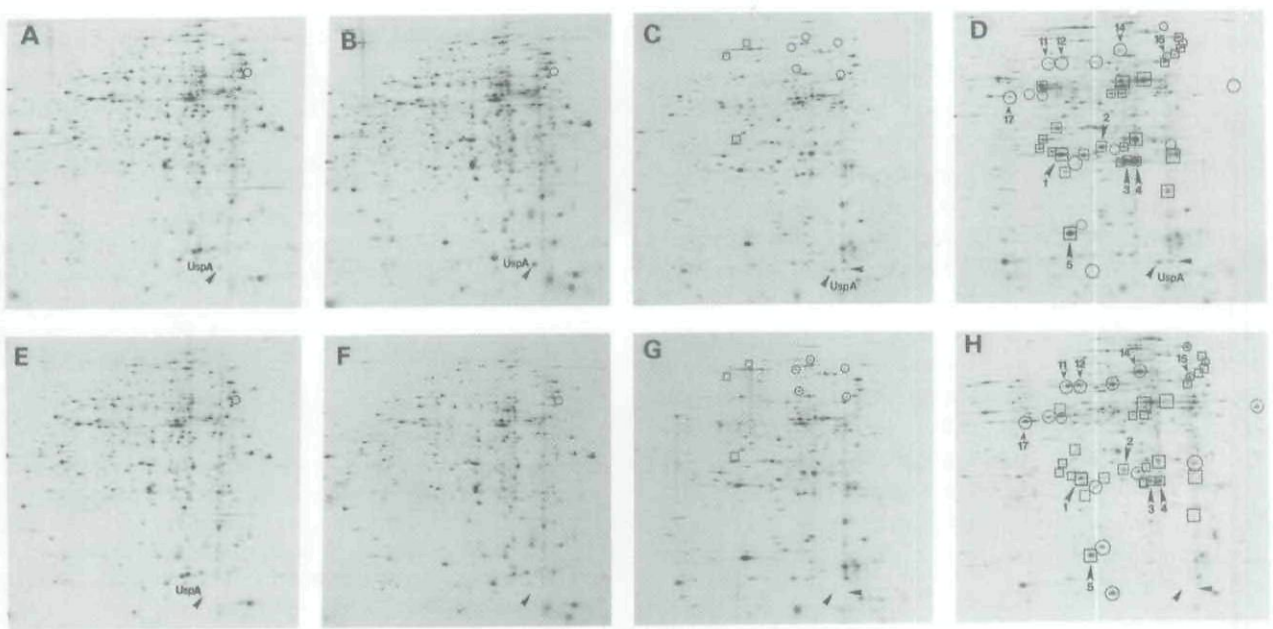

Fig. 5. Autoradiograms of two-dimensional polyacrylamide gels of extracts of *E. coli* W3110 (A–D) and the *uspA*::*kan* mutant strain TN3151 (E–H) grown aerobically in glucose minimal MOPS at 37°C (A and E), and glucose starved for 30 min (B and F), 3 d (C and G), and 5 d (D and H). Cells were labelled with [^{35}S]-methionine for 5 min during exponential growth, for 15 min after 30 min of starvation, and for 4 h after 3 and 5 d of starvation. Circled spots denote proteins that exhibit a markedly higher rate of expression in strain TN3151 as compared to W3110, while boxed spots represent proteins that have a higher rate of expression in W3110. Gsi proteins (Gsi1–5, 11, 12, 14, 15 and 16) for which kinetics of expression were analysed and accounted for in Fig. 6 are marked with their numbers in D and H. The location of UspA is marked with arrows. For pI and molecular weight designations of protein spots in the autoradiograms consult the reference gels of the 'Gene–protein database of *Escherichia coli*' (VanBogelen *et al.*, 1990).

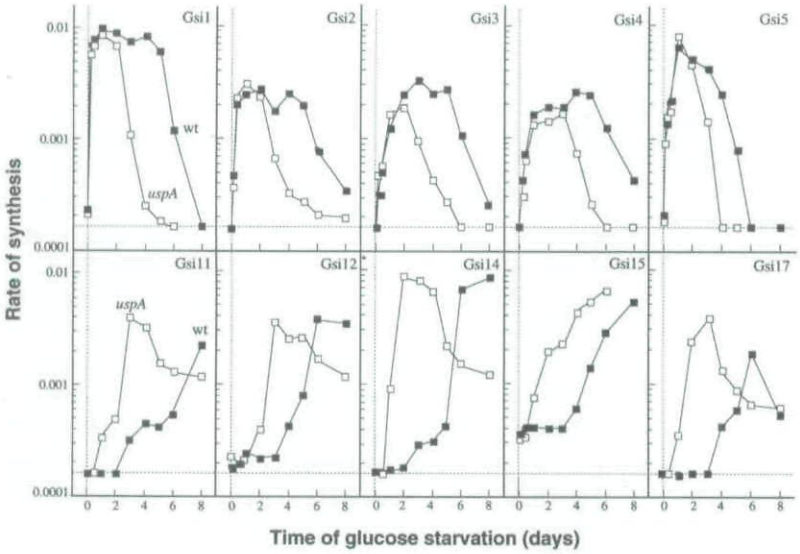

Fig. 6. Time course of expression of some Gsi proteins in wild-type strain W3110 (closed symbols) and *uspA::kan* mutant strain TN3151 (open symbols). Cells were grown and labelled, and differential rates of synthesis were determined as described in the *Experimental procedures*. The rates are expressed as the rate of synthesis of the particular Gsi protein per total rate of protein synthesis. The horizontal, dotted line in the graphs denote the detection limit. Symbols on this dotted line signify undetectable rates of synthesis.

response as a result of starvation-induced cellular damage. It should be noted that the viability of both strains is decreasing during the time studied (Fig. 4, top left panel). Thus, the 'accelerated' repression and induction of Gsi proteins in the *uspA* mutant may reflect simply that this strain encounters harmful cellular damage earlier during starvation. Nevertheless, analysis of the identity and function of these 'late' starvation-inducible proteins may prove useful in elucidating the cause of cell death during starvation-induced cell stasis.

In conclusion, mutant cells lacking UspA display a pleiotrophic phenotype with respect to carbon utilization (Nyström and Neidhardt, 1993), stress and starvation resistance (this paper) and patterns of protein expression during prolonged periods of carbon starvation (this paper). However, there is no supporting evidence indicating that UspA is a global regulator of gene expression. On the other hand, overproduction of UspA appears to affect the pI of several other proteins, indicating a possible role of UspA in post-translational modification (T. Nyström and F. C. Neidhardt, unpublished). Studies are under way in our laboratories to explore this possibility, to determine the nature of such post-translational modification, to identify the targets for UspA-mediated modifications and to elucidate whether such modification of several proteins forms the basis for the pleiotrophic effects on the cell of a *uspA* mutation.

Experimental procedures

Bacterial strains and plasmids

The K-12 strains of *E. coli* W3110 (Smith and Neidhardt, 1983) and JM105 (F′ *traD36 lacI*^{q} D[*lacZ*]M15 *proAB/thi rpsL endA sbcB15 hsdR4* D[*lac–proAB*]) were used as noted. Strain JC7623 (*arg ara his leu pro recB21 recC22 sbcB15 thr*) was used to recombine the *uspA::kan* mutation into the chromosome. The mutation was transduced into *E. coli* W3110 and JM105 by standard P1*vir* transduction procedures (Miller, 1972). Strain DH5αF′ (*supE44* Δ*lacU169* (f80*lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for transformations.

Media and growth conditions

Cultures were grown in liquid MOPS (morpholine–propanesulphonic acid) medium (Neidhardt *et al.*, 1974), supplemented with glucose (0.4%) and thiamine (10 mM) for minimal medium or glucose (0.4%), 20 amino acids, five nucleotides, and five vitamins (Wanner *et al.*, 1977) for rich medium, in Erlenmeyer flasks placed in a rotary shaker at indicated temperatures. Luria broth/agar for plates was prepared as described by Sambrook *et al.*, (1989). When appropriate, the Luria broth medium was supplemented with kanamycin (50 μg ml^{-1}), carbenicillin (50 μg ml^{-1}), and/or Xgal (0.8 mg per plate). For analysis of proteins induced by starvation for different levels, cells were grown aerobically in glucose minimal MOPS medium with 5% of the normal concentration of the appropriate nutrient (Nyström and Neidhardt, 1992). This procedure resulted in growth arrest of cells at an OD_{420} of ~0.5 (1.5×10^8 cells ml^{-1}). Temperature shifts, treatments with $CdCl_2$, H_2O_2, (VanBogelen *et al.*, 1987) and osmotic challenge (Jenkins *et al.*, 1990) were performed as previously described. DNP and CCCP were added at varied concentrations as indicated.

Bacterial cell counts and cell volume

Samples (2 ml) from cell suspensions were fixed using 250 μl of 2.5% (v/v) formaldehyde. Total counts and size distributions were obtained using a microcomputerized (Coulter Channelyzer 256) Coulter Counter ZM.

Resolution of proteins on two-dimensional polyacrylamide gels

Culture samples were processed to produce extracts for resolution on two-dimensional polyacrylamide gels by the methods of O'Farrell (1975) with modifications (VanBogelen and Neidhardt, 1990).

Measurement of rates of synthesis of individual proteins

At indicated times, a portion (1 ml) of a culture was removed and placed in a flask containing [^{3}H]-leucine (5 mCi mmol^{-1}, 100 μCi ml^{-1}). Incorporation was allowed to proceed for different lengths of time as indicated, after which non-radioactive leucine (2.4 mM) was added for a chase. To this sample was added a portion of a culture of the same strain grown in [^{35}S]-methionine labelling medium (glucose minimal MOPS supplemented with thiamine). The mixed sample was then prepared as previously described (VanBogelen and Neidhardt, 1990) to produce extracts for resolution of the cellular proteins on two-dimensional gels. An autoradiogram was prepared to permit visualization of labelled proteins. Protein spots chosen for quantitative assay were sampled from the dried gel with a syringe needle and treated as described (Pedersen *et al.*, 1976) to permit measurement of their ^{3}H and ^{35}S content by scintillation counting. The differential rate of synthesis of a sampled protein was defined as the ^{3}H/^{35}S ratio of the sampled spot divided by the same isotope ratio of unfractionated mixed extracts.

Promoter mapping

Oligonucleotide primers were end labelled using 50 μCi of [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) and T4 polynucleotide kinase as described (Sambrook *et al.*, 1989). Labelled primer (0.02–0.05 pmol) was added to *E. coli* RNA in 10 μl reaction volumes containing 0.05 M Tris-HCl (pH 8.2) and 0.1 M KCl. Reaction mixtures were incubated at 90°C for 1 min, 60°C for 2 min, and then on ice for 15 min to allow annealing of primer to template. The annealing reaction mixes were added as 6 μl samples to tubes containing 1 μl of 2.5 mM dATP, dCTP, dGTP and dTTP mix; 2 μl of reverse transcriptase buffer (0.25 M Tris-HCl (pH 8.2), 0.2 M KCl, 0.036 M Mg acetate and 0.01 M dithiothreitol) and 1 μl (8 U) of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). Reaction mixtures were incubated at 48°C for 45 min, and then 4 μl of Sequenase stop buffer (USB) was added to terminate reactions. The primer extension reaction mixtures (2.5–6.0 μl) were subjected to electrophoresis next to a sequencing ladder obtained using the same primer in 5% polyacrylamide gels containing 8 M urea and followed by autoradiography. The primer used in the extension analysis shown in Fig. 2 is the same as that used in Nyström and Neidhardt (1992; Fig. 11).

Acknowledgements

This work was supported by Public Health Service grant GM-17892 from the National Institutes of Health to F.C.N., and by the Swedish Natural Science Research Council to T.N. We are grateful to Nan H. Albertson for valuable comments on the manuscript.

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