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Starvation-Induced Cross Protection against Heat or H_2O_2 Challenge in *Escherichia coli*

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Glucose- or nitrogen-starved cultures of *Escherichia coli* exhibited enhanced resistance to heat (57°C) or H_2O_2 (15 mM) challenge, compared with their exponentially growing counterparts. The degree of resistance increased with the time for which the cells were starved prior to the challenge, with 4 h of starvation providing the maximal protection. Protein synthesis during starvation was essential for these cross protections, since chloramphenicol addition at the onset of starvation prevented the development of thermal or oxidative resistance. Starved cultures also demonstrated stronger thermal and oxidative resistance than did growing cultures adapted to heat, H_2O_2, or ethanol prior to the heat or H_2O_2 challenge. Two-dimensional gel electrophoresis of ^{35}S-pulse-labeled proteins showed that subsets of the 30 glucose starvation proteins were also synthesized during heat or H_2O_2 adaptation; three proteins were common to all three stresses. Most of the common proteins were among the previously identified Pex proteins (J. E. Schultz, G. I. Latter, and A. Matin, J. Bacteriol. 170:3903–3909, 1988), which are independent of cyclic AMP positive control for their induction during starvation. Induction of starvation proteins dependent on cyclic AMP was not important in these cross protections, since a Δ*cya* strain of *E. coli* K-12 exhibited the same degree of resistance to heat or H_2O_2 as the wild-type parent did during both growth and starvation.

Procaryotic cells respond to environmental or chemical stresses by inducing specific sets of proteins characteristic to each stress (5). The proteins within each set and the genes that encode them constitute a stimulon (23); well-characterized procaryotic examples include heat shock (14), the SOS response (24), and oxidative stress (1). For some stimulons, induction of the stress proteins by exposure to nonlethal levels (an adaptive dose) of a stress agent has been shown to confer protection against subsequent exposure to lethal levels (a challenge dose) of the same stress agent (1, 2, 25).

In some cases, proteins associated with one stimulon can be induced during other stresses. For instance, various heat shock proteins in *Escherichia coli* are also synthesized when the cells are exposed to hydrogen peroxide (23), ethanol (21, 23), UV light (9), puromycin (4), or amino acid deprivation (8). Similarly, *Salmonella typhimurium* synthesizes some heat shock proteins when exposed to H_2O_2 (1, 11), ethanol (11), or nutrient deprivation (20). For a limited number of stimulons that share common proteins, a cross-protective effect has also been demonstrated. Thus, *E. coli* exposed to an adaptive dose of H_2O_2 or ethanol develops increased resistance to a heat challenge (22); likewise, *S. typhimurium* adapted to H_2O_2 stress demonstrates enhanced thermal resistance (1).

We have previously shown that *E. coli* K-12 responds to glucose starvation by inducing about 30 proteins (6, 7, 17). These proteins confer enhanced starvation resistance on the bacterium (15, 16, 18). Several of the 30 starvation proteins were heat shock proteins (6, 17), prompting us to investigate whether starvation cross protects *E. coli* K-12 against a challenge by heat. We report here that starvation cross protects *E. coli* not only against a heat challenge but also against an oxidative challenge. Furthermore, we have found additional starvation proteins that overlap with heat adaptation, as well as some that are common to H_2O_2 adaptation conditions.

MATERIALS AND METHODS

Strains and starvation protocol. Wild-type K-12 used in this study was a Stanford strain (λ^-, F^-) (17). Strain AMS2 is a Δ*cya-854* derivative of this strain (17). Cells were grown in M9 minimal medium or morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with glucose as previously described (17). The starvation protocols were the same as described in the accompanying paper (17), except that the cells were grown at 29°C where specified.

Adaptation and challenge protocols. An *E. coli* culture was grown at 29°C in M9 medium containing 0.3% glucose. It was divided into four aliquots on reaching a density of ca. 3×10^8 cells per ml; each aliquot received one of the following treatments: (i) 42°C for 30 min (heat adaptation/heat shock), (ii) 60 μM H_2O_2 for 60 min (H_2O_2 adaptation), (iii) 10% ethanol for 15 min (ethanol adaptation), or (iv) no treatment (growth). A separate culture was grown at 29°C in M9 medium containing 0.025% glucose and allowed to starve for 4 h. Control experiments with different concentrations of H_2O_2 or various temperatures and exposure periods established that these adaptation doses conferred maximal resistance to challenge by their respective stresses.

For heat challenge, a culture aliquot was diluted to ca. 3×10^3 cells per ml in M9 medium without glucose, and 1 ml was pipetted into a microcentrifuge tube prewarmed in a 57°C heat block. Samples were removed every 2 min and plated onto LB plates (10). Oxidative challenge involved adding 15 mM H_2O_2 to a culture aliquot (ca. 3×10^8 cells per ml); samples were then serially diluted at various times in M9 medium without glucose and were plated onto LB plates to determine the percent viability.

Pulse-labeling of proteins and two-dimensional gel electrophoresis. Cells were grown to mid-log phase (ca. 3×10^8 cells per ml) in M9 medium plus 0.3% glucose either at 29°C with

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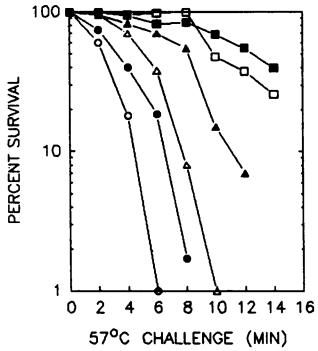

FIG. 1. Induction of thermal resistance in *E. coli* during glucose starvation. A wild-type K-12 culture at 37°C was challenged at 57°C during exponential growth (○) or at 1 h (△), 2 h (▲), 4 h (□), or 24 h (■) after glucose depletion from the medium. A control experiment was also performed, in which chloramphenicol (100 μg/ml) was added 20 min after the onset of starvation and the culture was allowed to starve for 4 h before heat challenge (●). On the *y* axis, 100% is equivalent to ca. 3×10^3 cells per ml.

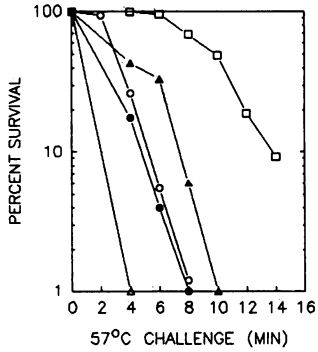

FIG. 2. Comparison of the thermal resistance of a glucose-starved *E. coli* K-12 culture with that of a growing culture adapted to heat, H_2O_2, or ethanol. Aliquots of an exponential culture (29°C) were either untreated (○), treated with 60 μM H_2O_2 for 60 min (△), treated with 10% ethanol for 15 min (●), shifted to 42°C for 30 min (▲), or glucose starved for 4 h (□). Each was then challenged at 57°C for up to 14 min and tested for viability. On the *y* axis, 100% is equivalent to ca. 3×10^3 cells per ml.

a shift to 42°C (for labeling during heat adaptation) or at 37°C with exposure to 60 μM H_2O_2 (for H_2O_2 adaptation). Culture aliquots were pulse-labeled at selected intervals with 10^{-8} M L-[^{35}S]methionine (12 μCi/ml; 1,072 to 1,134 Ci/mmol), chased with 10^{-5} M unlabeled methionine, and then precipitated with 10% (wt/vol) trichloroacetic acid at 4°C as described previously (7). A 2-min pulse followed by a 2-min chase was used during heat adaptation, and a 3-min pulse followed by a 1-min chase was used during H_2O_2 adaptation.

Two-dimensional gel electrophoresis was performed as previously described (7), except LKB ampholytes were used in the first isoelectric focusing dimension. The second dimension was a sodium dodecyl sulfate–10.5%-polyacrylamide gel. Equivalent amounts of radioactivity were loaded for each sample (approximately 750,000 cpm). Labeled proteins were visualized by autoradiography on XAR-5 film (Eastman Kodak Co.).

RESULTS

Effect of glucose starvation on heat resistance. Aliquots from an *E. coli* culture during logarithmic growth and at various times after glucose exhaustion were challenged by a rapid upshift in temperature from 37 to 57°C, and cell viability was determined. Starved cells survived heat challenge markedly better than exponentially growing cells did, and the degree of resistance increased with the time for which they were starved prior to the heat challenge (Fig. 1). For example, after a 6-min challenge at 57°C, a culture in logarithmic growth was essentially nonviable, whereas aliquots of the same culture starved for 1 or 4 h were 38 and 95% viable, respectively. Starvation for 4 h provided the maximal thermal protection, since cells starved for 24 h exhibited no further increase in heat resistance (Fig. 1).

To determine whether protein synthesis during starvation was required for the development of enhanced thermal resistance, a culture starved in the presence of chloramphenicol (100 μg/ml) for 4 h was similarly challenged by heat. Little thermal resistance developed (Fig. 1); thus, protein synthesis was essential for the observed cross protection.

To determine whether stresses other than starvation could confer cross protection against heat and, if so, their relative effectiveness in affording such protection, an *E. coli* culture was grown at 29°C and aliquots were adapted to heat, ethanol, or H_2O_2 before being subjected to challenge by heat. Both heat and starvation adaptations conferred enhanced heat resistance, but the resistance exhibited by starved cells was markedly greater than that exhibited by heat-adapted cells (Fig. 2). Adaptation by H_2O_2 and by ethanol conferred no enhanced resistance to heat; in fact, cells adapted to H_2O_2 were more sensitive to the thermal challenge (Fig. 2). Other workers have reported that H_2O_2 adaptation protects bacteria against heat challenge (1, 22); however, our heat challenge involved a higher temperature and longer duration.

Effect of glucose starvation on H_2O_2 resistance. Glucose-starved cells of *E. coli* were also strikingly more resistant to a challenge of 15 mM H_2O_2 than were their unstarved, logarithmically growing counterparts. As with heat resistance, H_2O_2 resistance increased as the cells were starved for longer periods, and starvation for 4 h provided the maximal protection (Fig. 3). The enhanced resistance to H_2O_2 was associated with starvation protein synthesis, since addition of chloramphenicol 20 min after the onset of starvation prevented the full development of oxidative resistance (Fig. 3).

Adaptive treatments with heat, H_2O_2, or ethanol all protected against the H_2O_2 challenge (Fig. 4); however, glucose-starved cells exhibited the strongest H_2O_2 resistance, showing up to twice the protection afforded to even H_2O_2-adapted cells.

The increased resistance conferred by starvation to both heat and oxidative challenges was not confined to glucose starvation. Nitrogen-starved K-12 cultures exhibited a similar degree of cross protection to heat or H_2O_2 (data not shown).

Effect of glucose starvation on heat and H_2O_2 resistance in an *E. coli* Δ*cya* strain. We have previously shown that about two-thirds of the starvation proteins are not synthesized in strain AMS2 (17), an adenylate cyclase deletion mutant otherwise isogenic to *E. coli* K-12. We have also shown that these cyclic AMP (cAMP)-dependent starvation proteins play no role in conferring resistance to starvation (17). It was therefore of interest to determine whether the synthesis of these proteins was involved in the starvation-induced cross protection against heat or H_2O_2 challenge. When the experiments whose results are shown in Fig. 1 and 3 were repeated with strain AMS2 and compared with those for K-12, essen-

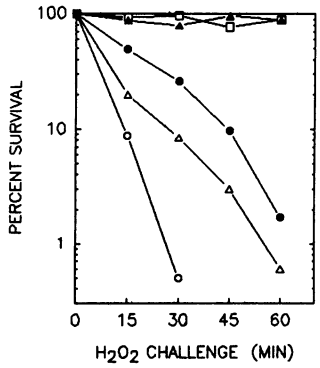

FIG. 3. Induction of H_2O_2 resistance in *E. coli* during glucose starvation. A wild-type K-12 culture was challenged with 15 mM H_2O_2 during exponential growth (○) or at the following times after glucose depletion: 15 min (△), 4 h (▲), or 26 h (□). A control experiment was also performed, in which chloramphenicol (100 μg/ml) was added 20 min after the onset of starvation and the culture was allowed to starve for 4 h before H_2O_2 challenge (●). All cultures were kept at 37°C throughout the experiment. On the *y* axis, 100% is equivalent to ca. 3×10^8 cells per ml.

tially identical results were obtained in that comparable thermal and oxidative resistances were found during growth as well as during starvation.

Two-dimensional gel analysis of proteins induced by glucose starvation, heat, or H_2O_2 adaptation. The chloramphenicol experiments described above implicate at least some of the starvation proteins in the cross protection that starvation provided against heat or H_2O_2. To identify which proteins were involved, we compared the two-dimensional gel patterns of *E. coli* cells subjected to glucose starvation, heat adaptation, or H_2O_2 adaptation. Protein synthesis patterns for all of these stresses have been previously published, but they were produced by investigators working in different laboratories and using slightly different methods (1, 6, 13, 25); they are therefore not directly comparable.

Some 27 proteins were induced by heat adaptation, and 20 were induced by H_2O_2 adaptation. Eleven of the heat shock proteins and six of the oxidation stress proteins were common to starvation proteins; only three proteins were common to all three stresses (Fig. 5). Eight proteins common to starvation and heat adaptation (polypeptides 4, 6, 17, 22, 36, 37, 38, and 39) and five proteins common to starvation and H_2O_2 stress (polypeptides 17, 19, 23, 34, and 36) have been previously found to be independent of positive cAMP regulation during glucose or nitrogen starvation; i.e., they are Pex proteins (17).

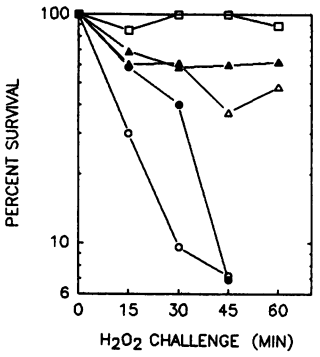

FIG. 4. Comparison of the H_2O_2 resistance of glucose-starved K-12 cultures to growing cultures adapted by heat, H_2O_2, or ethanol. Culture conditions and treatments were the same as described in the legend to Fig. 2, except that cultures were challenged with 15 mM H_2O_2. All cultures were kept at 29°C, unless otherwise noted. On the *y* axis, 100% is equivalent to ca. 3×10^8 cells per ml. Symbols: ○, untreated; ●, ethanol adapted; △, heat adapted; ▲, H_2O_2 adapted; □, glucose starved.

DISCUSSION

Previous studies from this laboratory have shown that induction of new proteins in the first several hours after the onset of starvation alters cells in such a way that they become more resistant to starvation stress (15, 16). The experiments described in this paper demonstrate that starvation also protects the cells against heat and oxidation challenges. Because starvation in the presence of chloramphenicol fails to confer either type of cross protection, induction of starvation proteins is necessary for enhancing the resistance of the cells to these stresses. Thus, the molecular changes that *E. coli* cells undergo during the first few hours of starvation appear to make them more hardy with respect to several different stresses. Previous studies (3, 19) have suggested that stationary-phase cells are in general more resistant; however, our studies are the first to correlate this enhanced resistance to the synthesis of starvation proteins.

Although starvation, heat shock, and H_2O_2 stress each produced its own individual pattern of protein induction, several starvation proteins were common to either heat or oxidation stress, and a few were common to both. This is consistent with the idea that starvation protein synthesis is required for the cross protection against heat or H_2O_2. Only starvation proteins induced independently of cAMP regulation appear relevant, however, because a glucose-starved Δ*cya* mutant survived heat or H_2O_2 challenge as well as the wild-type parent strain did. Furthermore, the majority of the proteins common to starvation and either of these two stresses were Pex proteins (i.e., independent of positive cAMP regulation [17]). Thus, of the 11 proteins common to starvation and heat shock, the 8 that are Pex proteins are the most likely to be involved in conferring thermal resistance. The same reasoning suggests that of the six starvation proteins induced during H_2O_2 adaptation, the five Pex proteins would appear to be the most critical for oxidative protection. The three proteins common to all three stresses cannot be sufficient for heat or H_2O_2 resistance, because H_2O_2-adapted cells, despite synthesizing these three proteins, were not heat resistant. These findings support our hypothesis that it is primarily the Pex proteins that are involved in stabilizing stressed, growth-impaired bacterial cells (17).

Although the induction of starvation proteins is coincident with the development of cross protection to heat or H_2O_2, this does not necessarily establish a cause-and-effect relationship. Such a point has been recently emphasized by the results of studies on the role of heat shock proteins in thermal resistance. Because heat shock proteins regulated by *rpoH* (*htpR* or *hin*) are characteristically induced during adaptive temperature upshifts, they have been thought to be involved in conferring thermotolerance. VanBogelen et al. (22), however, have shown that the synthesis of these proteins without a coincident increase in temperature is insufficient for the development of thermotolerance. This finding does not rule out the possibility that non-*rpoH*-

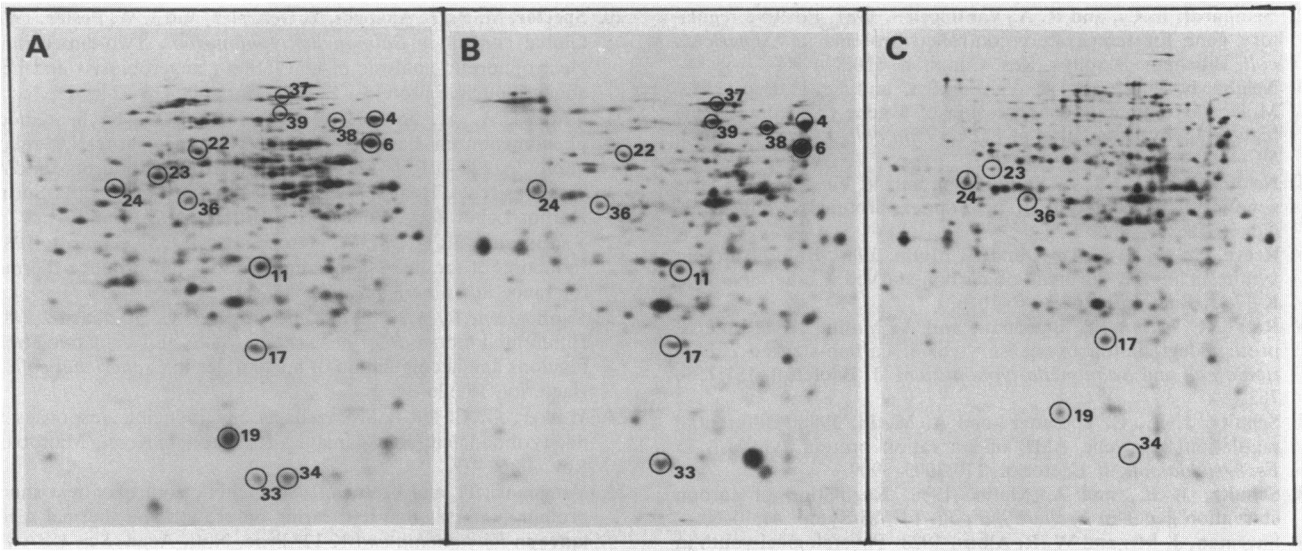

FIG. 5. Two-dimensional autoradiograms of polypeptides commonly synthesized in *E. coli* K-12 during (A) glucose starvation (at 30 min), (B) heat adaptation (at 5 min), or (C) H_2O_2 adaptation (at 60 min). Of the 30 proteins induced during starvation, only the 14 polypeptides commonly induced by either heat or H_2O_2 adaptation are circled and numbered in panel A; the numbering system used previously has been retained to facilitate comparison with previous work from this laboratory (7, 17). *E. coli* K-12 cultures were pulse-labeled with [^{35}S]methionine after glucose depletion from the medium (carbon starvation), a temperature shift from 29 to 42°C (heat adaptation), or addition of 60 μM H_2O_2 (H_2O_2 adaptation). Heat- or H_2O_2 adaptation-specific polypeptides were identified by comparing the gel patterns shown with those of corresponding unstressed control cultures. Slight variations in the isoelectric points of polypeptides among panels A, B, and C occurred owing to differences in the pH gradient established from different ampholyte sources; however, specific polypeptide identity could be ascertained by their relative position to other spots for the full set of gels prepared for each stress. Because the maximal synthesis rate differed for individual polypeptides during the adaptation periods, the representative gels do not show the maximal induction for all proteins.

regulated heat shock proteins play a role in thermal resistance.

We do not know whether any of the starvation proteins induced by heat or H_2O_2 adaptation are controlled by the known regulator genes, *rpoH* or *oxyR*, respectively (1, 12). If any of the starvation proteins are *rpoH* or *oxyR* controlled, it is possible that their regulation involves multiple promoters or individual promoters responding to different stress stimuli. An example of the latter case has been found in the stringent response, in which some heat shock promoters exhibit different requirements for induction during the stringent response from those shown during heat shock (8).

It is striking that starved cultures were so much more resistant to heat or H_2O_2 challenge than were cultures specifically adapted to these stresses, even though optimal adaptive conditions were used. The fact that cells required 4 h of starvation before developing maximal resistance to heat or H_2O_2 suggests that proteins belonging to the late temporal class of starvation proteins (7) play the more crucial role or that the pertinent proteins must accumulate to a critical level before eliciting maximal cross protections. If the latter case is true, the relatively less effective protections developed during heat or H_2O_2 adaptations could be attributed to insufficient protein levels caused either by continued cell division or by a shorter period of relevant protein synthesis during these adaptive responses.

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