Starvation-Induced Cross Protection against Osmotic Challenge in *Escherichia coli*

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Stationary-phase *Escherichia coli* cultures showed enhanced osmotic resistance as compared with cultures in mid-logarithmic growth or preadapted to osmotic stress. The osmotolerance that developed during starvation or osmotic adaptation required de novo protein synthesis. Of the 22 polypeptides induced during osmotic shock, five were also starvation proteins.

Stationary-phase bacterial cultures exhibit several physiological properties not present during exponential growth. These include increased proteolysis (5, 10, 12), induction of about 40 starvation-related proteins (6, 7, 10), and enhanced resistance to heat, oxidative stress (9), and antibiotics (3, 10, 14).

Our laboratory previously demonstrated that starvation survival depends on de novo protein synthesis (12); correlative evidence suggests that a subset of starvation proteins, the Pex proteins, play a significant role (11, 13). Pex proteins are synthesized when cultures are deprived of carbon, nitrogen, or phosphorus and are further distinguished by a lack of dependence on the positive effector cyclic AMP for induction during starvation. Additionally, several Pex proteins show induction during heat or oxidation stress and have been correlated with the enhanced thermal and oxidative resistance that develops during starvation (9). The fact that starvation conferred a strong cross protection against very different stresses prompted the present investigation in which the cross-protective effect of starvation against osmotic stress was examined.

We assessed the osmotic resistance of glucose-starved cells by comparing the viability of *Escherichia coli* K-12 cultures osmotically challenged during mid-logarithmic growth or 4 h after entering the postexponential phase. Cultures were grown at 37°C in M9 medium (8 mM NaCl) supplemented with either 0.025% glucose for starvation at an approximate optical density at 660 nm of 0.35 (4×10^8 cells per ml) or 0.3% glucose for sampling during logarithmic growth at a similar optical density at 660 nm. Cultures were osmotically challenged by adding granular NaCl to the medium to reach a final concentration of 2.2 to 2.5 M. Percent viability was determined by colony counts on LB plates, with 100% viability representing the colony count of the cultures just prior to osmotic challenge. The starving cultures were allowed to remain in the stationary phase for 4 h before osmotic challenge, since the induction of starvation proteins is essentially complete (7) and maximal resistance to heat and oxidation challenges occurs (9) by this time.

Starved cultures consistently showed higher osmotic resistance than did similar cultures challenged during logarithmic growth (Fig. 1). The cross protection that starvation conferred against osmotic challenge depended on de novo protein synthesis during starvation, since the addition of chloramphenicol, a protein synthesis inhibitor, at the onset

of starvation prevented the development of osmotic protection (Fig. 1). An adenylate cyclase mutant of *E. coli* K-12, AMS2 ($\Delta cya-854$), which is unable to synthesize cyclic AMP (13), exhibited approximately the same degree of osmotic resistance during starvation as did the wild-type parent (Fig. 1). This finding suggests that the starvation proteins important in osmotic resistance are cyclic AMP independent for their induction during starvation.

Logarithmically growing cultures adapted to a low, non-growth-inhibiting osmotic shock (0.30 to 0.35 M NaCl) before osmotic challenge (2.5 or 2.2 M NaCl [Fig. 2]) also showed enhanced osmotic resistance, although it was less marked than that induced by starvation. A 1- to 2-h adaptation period was sufficient for maximal osmotic resistance; cultures osmotically adapted for 4 or 6 h showed the same degree of osmotic resistance (Fig. 2, inset). A lack of

FIG. 1. Osmotic resistance to 2.5 M NaCl of an *E. coli* K-12 culture growing exponentially ($\square$), after 4 h of glucose starvation ($\blacksquare$), after 4 h of glucose starvation with chloramphenicol added at the onset of starvation ($\bullet$) and of a $\Delta cya-854$ derivative of K-12 (AMS2 [13]) growing exponentially ($\triangle$) and after 4 h of starvation ($\blacktriangle$). The data for growing K-12 cultures and K-12 cultures starved for 4 h reflect the means of three independent experiments. Standard deviations were ± 4.6 , ± 1.6 , and $\pm 1.0\%$ for growing K-12 cultures at 6, 10, and 20 h, respectively, and ± 4.6 , ± 3.6 , ± 6.2 , and $\pm 9.0\%$ for starved K-12 cultures at 10, 20, 30, and 40 h, respectively.

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FIG. 2. Osmotic resistance to 2.5 M NaCl of a logarithmically growing *E. coli* K-12 culture after no osmotic adaptation ($\square$) or after adaptation to 0.35 M NaCl for 1 h ($\blacktriangle$), 2 h ($\blacksquare$), or 2 h in the presence of chloramphenicol ($\bullet$). The inset shows the osmotic resistance to 2.2 M NaCl of a logarithmically growing culture after adaptation to 0.30 M NaCl for 2 h ($\blacktriangle$), 4 h ($\blacksquare$), or 6 h ($\bullet$).

resistance following the addition of chloramphenicol at the onset of osmotic adaptation demonstrated that de novo protein synthesis was also important in this protective response (Fig. 2). These results document an osmotic adaptation response corresponding to the established adaptation responses to heat and oxidation (1, 15).

The fact that 4 h of starvation provided stronger osmotic resistance than did osmotic adaptation is consistent with our previous observation that starvation conferred greater resistance to heat or oxidation challenge than did the respective maximal adaptive treatments (9). This cross protection against three disparate stresses suggests that a strong, general resistance develops in starving bacterial cultures.

Since glucose starvation and osmotic adaptation both led to enhanced osmotic resistance and protein synthesis was required for protection in either case, we investigated whether common proteins were induced during these treatments. Two-dimensional gel patterns of proteins synthesized by *E. coli* K-12 cultures during osmotic adaptation were compared with those determined previously for glucose-starved cultures (6, 7). A K-12 culture growing in M9 medium (8 mM NaCl) was upshifted to 0.35 M NaCl by the addition of granular NaCl. Samples were taken at 0, 30, 60, and 120 min after osmotic shock, pulse-labeled for 3 min with L-[35 S]methionine, and chased for 1 min with unlabeled methionine. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10.5% acrylamide was performed as previously described (7).

By 30 min of osmotic adaptation, nearly all 22 proteins induced during osmotic shock (Fig. 3) had attained the maximal level of synthesis; five of these corresponded to previously identified starvation proteins. All five were Pex starvation proteins (9, 13). Two heat shock proteins (numbers 17 and 38; Fig. 3) were among the starvation proteins

FIG. 3. Two-dimensional autoradiograms of logarithmically growing *E. coli* K-12 showing polypeptides synthesized during nonstress (a) and osmotic adaptation (b) conditions. Polypeptides induced at 30 min of 0.35 M NaCl osmotic adaptation are circled; the five starvation proteins common to osmotic adaptation are indicated by previously published numbers (see references 7, 9, and 13).

induced (9). That widespread induction of heat shock proteins does not occur during osmotic stress has also been reported in other bacteria (1, 8).

Induction per se may not be the only criterion upon which to judge the significance of a protein in osmotic resistance. Pertinent proteins might reach functional levels by maintaining a constant rate of synthesis coupled with increased protein stability. Protein accumulation may indeed have a role in the development of osmotic tolerance, since starvation proteins induced during osmotic adaptation reached maximal induction rates as early as 30 min of osmotic shock, but maximal osmotic resistance did not develop until 1 to 2 h of osmotic adaptation.

Osmotic stress conditions mimic other physiological features of starvation (10) in addition to inducing similar proteins. For example, increased osmolarity in bacterial cells is associated with the inhibition of DNA replication, cell growth, and nutrient uptake (4). Whether common survival mechanisms exist during these two stresses and what role common proteins play in these processes are questions remaining to be explored.

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LITERATURE CITED

- Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* 41:753-762.
- Clark, D., and J. Parker. 1984. Proteins induced by high osmotic pressure in *Escherichia coli*. *FEMS Microbiol. Lett.* 25:81-83.
- Cozens, R. M., E. Tuomanen, W. Tosch, O. Zak, J. Suter, and A. Tomasz. 1986. Evaluation of the bactericidal activity of β -lactam antibiotics on slowly growing bacteria cultured in the chemostat. *Antimicrob. Agents Chemother.* 29:797-802.
- Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* 53:121-147.
- Goldberg, A. L., and A. C. St. John. 1976. Intracellular protein degradation in mammalian and bacterial cells: part 2. *Annu. Rev. Biochem.* 45:747-803.
- Groat, R. G., and A. Matin. 1986. Synthesis of unique polypeptides at the onset of starvation in *Escherichia coli*. *J. Ind. Microbiol.* 1:69-73.
- Groat, R. G., J. E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin. 1986. Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. *J. Bacteriol.* 168:486-493.
- Hecker, M., C. Heim, U. Völker, and Wölfel. 1988. Induction of stress proteins by sodium chloride treatment in *Bacillus subtilis*. *Arch. Microbiol.* 150:564-566.
- Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H₂O₂ challenge in *Escherichia coli*. *J. Bacteriol.* 170:3910-3914.
- Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* 43:293-316.
- Reeve, C. A., P. S. Amy, and A. Matin. 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *J. Bacteriol.* 160:1041-1046.
- Reeve, C. A., A. T. Bockman, and A. Matin. 1984. Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* 157:758-763.
- Schultz, J. E., G. I. Latter, and A. Matin. 1988. Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. *J. Bacteriol.* 170:3903-3909.
- Tuomanen, E., D. T. Durack, and A. Tomasz. 1986. Antibiotic tolerance among clinical isolates of bacteria. *Antimicrob. Agents Chemother.* 30:521-527.
- Yamamori, T., and T. Yura. 1982. Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* 79:860-864.