Requirement of the *Escherichia coli* *dnaK* gene for thermotolerance and protection against H₂O₂

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Thermotolerance in *Escherichia coli* is induced by exposing cells to a brief heat shock (42 °C for 15 min). This results in resistance to the lethal effect of exposure to a higher temperature (50 °C). Mutants defective in the *recA*, *uvrA* and *xthA* genes are more sensitive to heat than the wild-type. However, after development of thermotolerance these mutants are like the wild-type in their heat sensitivity. This suggests that thermotolerance is an inducible response capable of protecting cells from the lethal effects of heat, independently of *recA*, *uvrA* and *xthA*. Thermotolerance does not develop in a *dnaK* mutant. In addition, the *dnaK* mutant is sensitive to heat and H₂O₂, but is resistant to UV irradiation. This implies that the *E. coli* heat-shock response includes a mechanism that protects cells from heat and H₂O₂, but not from UV.

Introduction

The heat-shock response is a highly conserved genetic response to environmental stress. This response is ubiquitous in nature, having been found in every organism studied for its presence, from bacteria to man (Lindquist, 1986; Neidhardt *et al.*, 1984; Schlesinger *et al.*, 1982). Induction of the heat-shock response in *Escherichia coli* involves the expression of at least 17 genes, and is caused by stimuli such as H₂O₂ treatment, ethanol, UV irradiation and viral infection (Neidhardt *et al.*, 1984), in addition to heat. Whilst the heat-shock response has been studied extensively, its function is essentially unknown.

When *E. coli* is exposed to a brief, sub-lethal dose of heat it becomes more resistant to subsequent, potentially lethal heat exposure. This phenomenon is termed thermotolerance and has been observed in many organisms (Lindquist, 1986). Interestingly, thermotolerance in *E. coli* does not develop simply through induction of the heat-shock regulon (VanBogelen *et al.*, 1987); however, there are many data which imply that the development of thermotolerance depends upon the heat-shock response. For example, *E. coli* *htpR* mutants, in which heat-shock gene expression is blocked, fail to exhibit thermotolerance (Yamamori & Yura, 1982). Agents other than heat, such as ethanol, which induce the heat-shock

response also induce thermotolerance (Lindquist, 1986). In mammalian cells, the accumulation of heat-shock proteins is directly related to the degree to which thermotolerance is expressed (Subject & Sciandra, 1982). Of all heat-shock proteins, the concentration of the 70 kDa heat-shock protein, Hsp70, best correlates with the development of thermotolerance (Lindquist, 1986).

Many inducers of the heat-shock response in *E. coli*, particularly heat, H₂O₂ and UV, are DNA-damaging agents (Ananthaswamy & Eisenstark, 1977; Bridges *et al.*, 1969a; Massie *et al.*, 1972; Neidhardt *et al.*, 1984; Woodcock & Grigg, 1972). This finding suggests that a function of the heat-shock response, and/or thermotolerance, may be to protect against the lethal effects of certain environmental agents known to cause DNA damage. To address this idea, the inactivating effect of heat, H₂O₂ and UV on repair-deficient mutants and a *dnaK* mutant of *E. coli* have been studied. The results indicate that the *E. coli* *dnaK* mutant is sensitive to heat and H₂O₂ but not to UV, and suggest that the *dnaK* gene may be needed to protect *E. coli* from the lethal effects caused by heat and H₂O₂.

Methods

Bacterial strains. All mutant *E. coli* strains were derived from AB1157 (Howard-Flanders & Theriot, 1966), which is designated wild-type. Strains AB2463 (Howard-Flanders & Theriot, 1966), AB2480 (Howard-Flanders *et al.*, 1969), BW9101 (Demple *et al.*, 1983) and

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GW4813 (Paek & Walker, 1987) will henceforth be referred to as *recA*, *recA uvrA*, *xthA* and *dnaK*, respectively. The *recA* and *recA uvrA* mutants have missense mutations, whereas the *xthA* and *dnaK* mutants both have deletion mutations.

Heat treatment. Fresh cultures of bacteria were grown overnight at 30 °C in Hershey's broth (Steinberg & Edgar, 1962) prior to each experiment. These cultures were diluted into fresh Hershey's broth and incubated with shaking at 30 °C to a concentration of about 1×10^8 cells ml⁻¹. Culture (1 ml) was then pipetted into each of seven Eppendorf tubes which were placed in a 50 °C waterbath for the desired time. Time points represent the total time spent at 50 °C, and do not include the time needed for the culture to come up to temperature. This 'come-up' time was measured to be no more than 2 min in each experiment. At each time of treatment, a tube was withdrawn from the waterbath, and the bacterial suspensions were immediately serially diluted in M-9 salts solution and plated on Hershey's agar plates using the agar overlay method (Adams, 1959). The bacterial suspensions at all time points were diluted in duplicate, except those at the zero dose time point which were diluted in triplicate. Plates were incubated for 36–48 h at 30 °C. As a precaution against possible light-induced DNA repair, photoreactivation was avoided by maintaining darkened conditions during these experiments and during plate incubation. Survival at any given time of heat treatment was determined as the ratio of colony-forming units (c.f.u.) after treatment to the number of c.f.u. at the zero time point.

Thermotolerance. Thermotolerance experiments were done as described above for heat treatment except that prior to the 50 °C exposure, the bacterial suspensions (1.0 ml in plastic Eppendorf tubes) were placed at 42 °C for 15 min (not including 'come-up' time). Following this 42 °C heat treatment, the bacteria were immediately transferred to 50 °C. Survival was determined as above.

H₂O₂ treatment. Bacteria were grown and suspensions were pipetted into Eppendorf tubes as described above. CuSO₄ was added to each tube to a final concentration of 0.1 mM to increase the rate of hydroxyl radical formation (Brandi *et al.*, 1987; McCord & Day, 1978). H₂O₂ (Mallinckrodt 30%) was then added to a final concentration of either 4.4 mM or 8.8 mM. All tubes were placed on ice for the desired reaction time (reaction temperature was approximately 1.0 °C). After a known period of time, one tube was withdrawn from the ice and 2000 units of catalase (Sigma) was added. The bacterial suspension was then serially diluted, immediately plated and incubated as above. Survival was determined as indicated above.

UV treatment. Bacterial cultures grown to about 1×10^8 cells ml⁻¹ were centrifuged at 1000 *g* for 20 min, and the cell pellet was resuspended in an equal volume of M-9 salts solution. The suspension was centrifuged, and the cell pellet resuspended again in an equal volume of M-9 salts solution. After standing at room temperature for at least 20 min 1 ml samples of the suspension were placed into each of seven small dishes (35 × 10 mm), one dish for each time point being studied. The dishes were placed individually under a UV lamp (General Electric) emitting a dose of 1.0 J m⁻² s⁻¹, for a specific length of time. After exposure, the dish was removed from the UV light, and the bacteria were serially diluted, plated and incubated as described above. Photoreactivation was avoided, and survival was determined as above.

Results

Sensitivity of *E. coli* mutants to heat

Repair-defective mutants, *recA* and *recA uvrA*, were more sensitive to heat than their wild-type parent (Fig.

1a, b). These results are in agreement with previous reports (Bridges *et al.*, 1969a, b), and support the conclusion that heat causes DNA damage. Furthermore, the *recA uvrA* double mutant was more sensitive to heat than the *recA* strain. This indicates that both excision and recombinational pathways are employed in the repair of heat-induced DNA damages.

In *E. coli*, the *xthA* gene encodes the DNA repair enzyme exonuclease III. *xthA* mutants are sensitive to H₂O₂, a known DNA-damaging agent (Ames *et al.*, 1985; Demple *et al.*, 1983, 1986). An *xthA* deletion mutant was also more sensitive to heat than the wild-type (Fig. 1c). This result implies that exonuclease III, the product of the *xthA* gene, may be used to repair heat-induced DNA damage.

The DnaK protein is a major heat-shock protein in *E. coli*. As shown in Fig. 1(d), the *dnaK* deletion mutant was very sensitive to heat. This result was previously obtained by Paek & Walker (1987). The inactivation curves of the wild-type and the *dnaK* mutant were replotted with best-fit straight lines obtained through linear regression analysis (data not shown). Comparison of the slopes of these curves reveals that the slope of the *dnaK* inactivation curve is 3.6 times greater than the slope of the wild-type inactivation curve. These results indicate that the *dnaK* gene is important in protecting *E. coli* from the lethal effect of heat.

Thermotolerance protects against heat-induced damage

The three mutants *recA*, *recA uvrA* and *xthA* with defects in known repair pathways, and wild-type *E. coli* were strongly thermotolerant (Fig. 2). In other words, after heat-shock at 42 °C for 15 min there was almost no killing by subsequent treatment at 50 °C, even though they are sensitive to a direct treatment of 50 °C (compare Fig. 2a–c to Fig. 1a–c). This suggests that, after establishment of thermotolerance, there is little or no need to repair heat-induced DNA damage by means of repair pathways employing the *recA*, *uvrA* or *xthA* gene products.

In contrast, when the *dnaK* deletion mutant was also heat-shocked at 42 °C for 15 min and then heat-treated at 50 °C, it was killed very rapidly (Fig. 2d). Comparison of the killing curves of wild-type and the *dnaK* mutant showed that the slope of the *dnaK* inactivation curve was 19.6 times greater than the slope of the wild-type inactivation curve. The rate of inactivation of the *dnaK* mutant at 50 °C was essentially the same whether pretreated at 42 °C (Fig. 2d) or not (Fig. 1d).

The *dnaK* deletion mutant is sensitive to H₂O₂

Since the *dnaK* gene is induced by H₂O₂ treatment (VanBogelen *et al.*, 1987), the effect of H₂O₂ on the

Fig. 1. Sensitivity of *E. coli* strains to a temperature of 50 °C. Each point is a mean value based on determinations from at least three experiments. Error bars indicate standard error of the mean. WT, Wild-type.

Fig. 2. Sensitivity of *E. coli* strains to a temperature of 50 °C following exposure at 42 °C for 15 min. Each point is a mean value based on determinations from at least three experiments. Error bars indicate standard error of the mean. WT, Wild-type.

Fig. 3. Sensitivity of *E. coli* strains to exposure to (a) 4.4 mM- H_2O_2 and (b) 8.8 mM- H_2O_2 . Each point is a mean value based on determinations from at least three experiments. Error bars indicate standard error of the mean. WT, Wild-type.

survival of the *dnaK* mutant was studied. An *xthA* mutant, previously reported to be sensitive to H_2O_2 (Dempsey *et al.*, 1983) was also tested for comparison (Fig. 3). Both the *dnaK* and *xthA* mutants were more sensitive to 4.4 mM- H_2O_2 than the wild-type (Fig. 3a), which was relatively insensitive to H_2O_2 up to 60 min of exposure. Both the *dnaK* and *xthA* mutants were inactivated by H_2O_2 at approximately the same rate. At the higher H_2O_2 concentration (8.8 mM), wild-type cells were killed more rapidly (Fig. 3b). Here, wild-type cells were relatively resistant to H_2O_2 until 30 min of exposure, when survival declined rapidly. The *dnaK* and *xthA* strains were initially much more sensitive to this concentration of H_2O_2 than to the lower concentration. Killing was most rapid in the *dnaK* mutant during the early treatment times, such that only 20% of the cells survived after 5 min of exposure (Fig. 3b). For the *xthA* mutant, killing was also most rapid during the first 5 min of treatment. These results indicate that the *dnaK* gene, like *xthA*, is needed to protect *E. coli* from H_2O_2 .

UV irradiation turns on the heat-shock response and induces the *dnaK* gene (Krueger & Walker, 1984). To determine if the *dnaK* gene protects a cell generally or if it

is limited to specific types of environmental stress, the effect of UV on the *dnaK* deletion mutant was studied. Wild-type and *dnaK* mutant cells were irradiated with up to 180 J of UV light, a dose which has been shown to induce the *E. coli* heat-shock response (Krueger & Walker, 1984). The results of these experiments indicated that the *dnaK* mutant is not more sensitive to UV than the wild-type (data not shown). This implies that the *dnaK* gene is not involved in protecting cells from UV-induced DNA damage.

Discussion

Treatment of *E. coli* with a sub-lethal dose of heat results in resistance to subsequent heat exposure, i.e. thermotolerance. This induced heat resistance occurs independently of *recA*-, *uvrA*- or *xthA*-mediated repair systems, but is absent in a *dnaK* mutant. These results imply that the development of thermotolerance, an inducible protective mechanism, abrogates the need for the *recA*, *uvrA* and *xthA* DNA repair pathways. Therefore, another heat-inducible system must exist which eliminates the DNA damaging potential of heat.

Deletion of the *dnaK* gene from *E. coli* results in cellular defects at a wide range of temperatures (Bukau & Walker, 1989). This suggests that the DnaK protein has important cellular functions at both normal and high growth temperatures, and, in fact, the DnaK protein is essential for growth at high temperatures (Bukau & Walker, 1989; Itikawa & Ryu, 1979; Paek & Walker, 1987). In addition, DnaK is necessary for initiation of DNA replication in *E. coli* at high temperatures (Sakakibara, 1988) and in bacteriophage λ at all temperatures (Dodson *et al.*, 1986). I have shown that the *dnaK* mutant is very sensitive to H_2O_2 . Therefore, the DnaK protein may also function to protect a cell from oxidative stress. Since heat and H_2O_2 at the concentration used may cause nucleic acid, protein and membrane damage, any of these may be the targets of DnaK protection.

The *dnaK* deletion mutant is very sensitive to heat even under conditions in which thermotolerance would otherwise be induced (Fig. 2*d*). This suggests that the DnaK protein provides some function which is essential for the development of thermotolerance. The precise nature of this function is not known, but many possibilities exist. *E. coli dnaK* mutants are deficient in both DNA and RNA synthesis and cell division is blocked at non-permissive temperatures (Bukau & Walker, 1989; Itikawa & Ryu, 1979; Massie *et al.*, 1972; Saito & Uchida, 1977). Therefore, DNA and/or RNA synthesis may be required for the development of thermotolerance in a *dnaK* deletion mutant. Since the DnaK protein inhibits the heat-shock response in *E. coli* (Tilly *et al.*, 1983), another possibility is that DnaK provides some type of regulatory function for the development of thermotolerance which is missing in the *dnaK* deletion mutant. Alternatively, the DnaK protein may be required for replication or repair of DNA at high temperatures.

Spontaneous DNA damage due to heat or oxidative processes appears to be a serious problem with which organisms must cope (Ames *et al.*, 1985; Setlow, 1987). One function of the *E. coli* heat-shock response could be to specifically repair the type of DNA damage caused by heat and H_2O_2 . The heat-shock response may be well suited to repair the DNA damage induced by both heat and oxidative reactions because these damages are similar. Both heat and H_2O_2 cause base alterations which result in apurinic and apyrimidinic (AP) sites (Hagensee & Moses, 1986; Massie *et al.*, 1972; Ripley, 1988). Maintenance of the replicative integrity of DNA at high temperatures would allow a cell to survive heat stress more efficiently than a cell without such a process, and might result in the high degree of conservation seen in the heat-shock response.

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