Identification of a gene, closely linked to *dnaK*, which is required for high-temperature growth of *Escherichia coli*

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We have constructed four deletion derivatives of the cloned *dnaK* gene. Plasmid pDD1, in which the last 10 amino acids of the DnaK protein have been replaced by three different amino acids derived from the pBR322 vector, was as effective as plasmid pKP31, from which it was derived, in restoring the ability of a *dnaK* null mutant, *Escherichia coli* BB1553, to plate λ phage and to grow at high temperatures. The other three mutations, involving much larger deletions of the *dnaK* gene, did not restore the ability to plate λ phage or the ability to grow at high temperatures. Plasmid pKUC2, which contains the whole *dnaK* gene and its promoters, was capable of restoring the ability of *E. coli* BB1553 to plate λ phage but, surprisingly, it did not restore the ability to grow at high temperatures, even though it was shown that the DnaK protein was efficiently expressed in these cultures. By transposon mutagenesis and sub-cloning, we have shown the presence of a second gene in plasmid pKP31 which is required for high-temperature growth of *E. coli* BB1553. This gene, which we call *htgA*, is presumably also defective in the *dnaK* null mutant *E. coli* BB1553. We have also demonstrated that the inability of *E. coli* K756 to grow above 43.5 °C is complemented by sub-clones which contain the *htgA* gene, but not by plasmid pKUC2.

Introduction

Exposure of cells to high temperatures induces the synthesis of a specific group of proteins, known collectively as the heat-shock proteins (for a review see Lindquist & Craig, 1988). Heat-shock proteins are conveniently subdivided into families according to their molecular mass. One of the most studied of these is the hsp70 family, which includes the *dnaK*-encoded heat-shock protein of *Escherichia coli*. The *dnaK* gene has been cloned and sequenced (Bardwell & Craig, 1984); it encodes a protein of 69 kDa. The 70 kDa heat-shock proteins appear to be amongst the most highly conserved proteins in nature. The hsp70 proteins of eukaryotes are extremely difficult to study, since individual cells encode families of similar proteins, which are regulated differently and may have different functions in the cell (Wadsworth, 1982; Ingolia *et al.*, 1982). In contrast, *E. coli* only produces one hsp70-type heat-shock protein, the product of the *dnaK* gene (Bardwell & Craig, 1984). Therefore the DnaK protein may perhaps be expected to be a multifunctional protein.

Synthesis of the DnaK protein is inducible, along with 19 other heat-shock proteins in *E. coli*, such that it constitutes 4.3% of total cell protein at 46 °C (Herendeen

et al., 1979). The induction of heat-shock proteins is dependent upon the product of the *rpoH* gene, σ^{32} , which is required for transcription (Neidhardt & Van Bogelen, 1981; Yamamori & Yura, 1982) and regulation (Grossman *et al.*, 1987) of heat-shock genes. The DnaK protein is essential for the growth of λ phage in *E. coli*, being required for the initiation of λ DNA replication. It appears to be necessary for the dissociation of the DnaB protein from the λ P protein, thereby allowing the action of DNA helicase (Liberek *et al.*, 1988; Zylicz *et al.*, 1989).

The functions of the DnaK protein in *E. coli* cells are not fully understood. It physically interacts with the GrpE heat-shock protein, both *in vitro* (Zylicz *et al.*, 1987; Johnson *et al.*, 1989) and *in vivo* (Johnson *et al.*, 1989). The DnaK protein is reported to be essential for growth of the cells at high and at low temperatures (Paek & Walker, 1987; Bukau & Walker, 1989a). The fact that the DnaK protein is also synthesized at 37 °C, where it constitutes 1.4% of total cell protein (Herendeen *et al.*, 1979), suggests a role for this protein in growth at normal temperatures. *dnaK* mutants of *E. coli* filament readily at non-permissive temperatures, although such mutants rapidly acquire secondary mutations which suppress filamentation (Bukau & Walker, 1989a). The filamenta-

tion of *dnaK* mutants is also suppressed by plasmids that carry the *ftsZ* gene, which is necessary for normal cell division (Paek & Walker, 1987). This is perhaps suggestive of a role of the DnaK protein in normal cell division. Recently the DnaK protein was also shown to have a role in mini-F plasmid replication (Ezaki *et al.*, 1989; Kawasaki *et al.*, 1990), in P1 plasmid replication (Bukau & Walker, 1989b; Tilly & Yarmolinsky, 1989; Wickner, 1990), and in chromosome segregation (Bukau & Walker 1989b).

Our initial objective in this work was to investigate the possible functional domains of the DnaK protein. We have constructed deletion mutants of the cloned *dnaK* gene. The 'mutant' *dnaK* genes were then used to complement previously characterized *dnaK* mutations, in order to determine whether they were able to restore one, or more, of the phenotypic properties of the wild-type *dnaK* gene.

Methods

Bacterial strains and media. The strains used in this work are listed in Table 1. All cultures were routinely grown in LB broth, or on plates of LB agar (Miller, 1972), supplemented where required with ampicillin (100 µg ml⁻¹), chloramphenicol (15 µg ml⁻¹), streptomycin (100 µg ml⁻¹), or tetracycline (15 µg ml⁻¹).

Plasmids. Plasmid pKP31, which contains the complete *dnaK* gene and its promoters, within a 5.3 kb *Hind*III fragment cloned into the *Hind*III site of pBR322, was kindly provided by Graham Walker (Department of Biology, Massachusetts Institute of Technology, USA). The cloning vectors pUC19 (Vieira & Messing, 1982) and pACYC184 (Chang & Cohen, 1978) have been previously described. Plasmids constructed in this work are described in the text or in Fig. 1.

Enzymes and reagents. Restriction enzymes and T4 DNA ligase were purchased from Pharmacia/LKB, or NBL.

Plasmid restriction, ligation and electrophoresis. Digestion of DNA with restriction endonucleases, electrophoresis of restriction fragments, ligation of DNA fragments and transformation into *E. coli* JM83 were carried out as described by Maniatis *et al.* (1982).

Transposon mutagenesis. Transposon mutagenesis of plasmid pKP31 with Tn1000 was performed as previously described (Chak & James 1984).

Plasmid transformation into *dnaK* mutants. Transformation into *E. coli* BB1553 or *E. coli* K756 was done by the freeze-thaw method as described by Merrick *et al.* (1987).

SDS-PAGE and Western blotting. *E. coli* cultures were grown to an OD₅₅₀ of between 0.9 and 1.1 at 30 °C, or were grown to an OD₅₅₀ of 0.6 at 30 °C before being transferred to 42 °C until the OD₅₅₀ reached 0.9 to 1.1. Two-millilitre volumes of these cultures were then centrifuged at 12000 g for 60 s and the supernatant completely removed. The cell pellets were resuspended in 100 µl loading buffer (40 mM-Tris/HCl pH 6.8 containing 50 mM-DTT, 1%, w/v, SDS, 7.5%, w/v, glycerol and 0.00015%, w/v, bromophenol blue), boiled for 5 min and centrifuged again briefly. Twenty-microlitre volumes were then loaded on a 10% (w/v) SDS-PAGE gel and electrophoresis was carried out in an ATTO electrophoresis system following the protocol recommended by the manufacturer. The SDS-PAGE gels were then either stained for

Table 1. *E. coli* strains used in this work

Strain	Genotype/phenotype
K756	<i>dnaK756</i> derivative of <i>E. coli</i> C600
MC4100	Ara ⁻ Δ(<i>argF-lac</i>) <i>relA1 deoC1 ptsF25 rpsA</i>
BB1553	Δ <i>dnaK52::Cm'</i> derivative of MC4100 with a <i>sidB1</i> secondary mutation
JM83	Lac ⁻ Pro ⁻ Thi ⁻ <i>rpsL</i> ϕ80d <i>lacZ</i> M15

protein with Coomassie blue, or electroblotted for 1 h at 80 mA onto nitrocellulose using the Sartoblot II system. The nitrocellulose filters were washed in TBS (10 mM-Tris/HCl pH 7.4, 140 mM-NaCl) to remove excess SDS and then blocked with 2% (w/v) BSA in TBS. Subsequently the filters were washed for 1 h, with agitation, in 3% (w/v) BSA in TBS containing a 1 in 10000 dilution of a polyclonal DnaK antibody (kindly provided by Barbara Dalie, Roche Institute of Molecular Biology, Nutley, NJ, USA), and then in 3% (w/v) BSA in TBS, containing a 1 in 3000 dilution of goat anti-rabbit antibody labelled with horseradish peroxidase (Bio-Rad). The excess antibody in each case was removed by washing for 6 × 5 min with TBS. The immunoactive bands were visualized by staining with 4-chloronaphthol.

λ phage plating efficiency. Stocks of bacteriophage λ*dnaK*⁺ and λ*clear* were prepared as previously described (Maniatis *et al.*, 1982). *E. coli* cultures containing the plasmid being tested were grown up with shaking at 30 °C overnight in LB broth containing maltose (0.2%, w/v) and MgSO₄ (8 mM). Aliquots (100 µl) of each test culture were then mixed with 100 µl of a suitable dilution of the λ stock suspensions and incubated at 30 °C for 30 min, to allow phage adsorption, before plating in a soft-agar overlay on LB agar plates containing ampicillin, maltose and MgSO₄. After overnight incubation at 30 °C the plaques were counted and the number of plaques was averaged for the three plates used for each culture. The number of plaques was then expressed as a percentage of the number of plaques found with λ*dnaK*⁺ plated on the *E. coli* host containing plasmid pKUC2.

High-temperature growth. Cultures of *E. coli* K756, or BB1553, carrying recombinant plasmids were grown up overnight at 30 °C with shaking, and were then streaked onto plates of LB agar containing the required antibiotics. After overnight incubation at 30 °C, 42 °C or 45 °C, the plates were inspected for growth of the streaks. The results were double-checked by restreaking from the 30 °C plate onto plates which were incubated overnight at 30 °C, 42 °C or 45 °C.

Results

Construction of deletion mutants of *dnaK*

In order to facilitate the construction of in-frame deletion mutants of the *dnaK* gene, we determined a more detailed restriction map of plasmid pKP31 (Fig. 1). The published nucleotide sequence of the *dnaK* gene (Bardwell & Craig, 1984) was also analysed for restriction sites. Plasmid pDD1 was generated from pKP31 by deletion of the 901 bp *Sal*I fragment from the 3' end of the *dnaK* gene to the *Sal*I site in the vector pBR322 (Fig. 1). The resulting plasmid encoded a mutant protein which consisted of the first 628 of the 638 amino acids of the DnaK protein. The last 10 amino acids of DnaK

Fig. 1. Subcloning of plasmid pKP31. The restriction sites present in the 5.3 kb *Hind*III fragment of pKP31 are indicated in the top line of the figure. The restriction fragments present in the plasmids listed are indicated.

(AEFEEVKDKK) were replaced by three different amino acids (RCP) derived from the vector. The resulting mutant protein therefore consists of 631 amino acids.

Inspection of the restriction map of pKP31 revealed the presence of an *Ava*I site at approximately 2.3 kb. The 3 kb *Ava*I–*Hind*III fragment which includes the whole of the *dnaK* gene was sub-cloned in pUC19. The resulting plasmid was designated pKUC2. Two further constructs were derived from pKUC2 by deletion sub-cloning, using either *Eco*RV, or *Hpa*I. The resulting plasmids, pDD2 and pDD3, contained in-frame, internal deletions of the *dnaK* gene. In pDD2 the deletion removed 144 amino acids, from 333 to 527 (22.6% of the DnaK protein), whilst in pDD3 the deletion removed 84 amino acids, from 281 to 365 (13.2% of the protein). The final deletion mutant of *dnaK*, pDD4, was isolated by chance during *Tn*1000-mediated transposon mutagenesis of pKUC2. Plasmid pDD4 contains a *Tn*1000 insert in the *dnaK* gene 3' to the *Eco*RV site at 1431 bp (Fig. 1). It presumably results in a fusion between the *dnaK* gene and *Tn*1000-derived sequences until an in-frame stop codon is reached. The resulting plasmid has lost approximately 25% of the 3' end of the *dnaK* gene.

Expression of the mutant DnaK proteins

In order to demonstrate expression of the DnaK protein in these constructs we used the *dnaK* null mutant *E. coli*

BB1553 as the host strain. This strain, which carries the Δ *dnaK*52::Cm^r deletion described by Paek & Walker (1987), also carries a secondary mutation, *sidB*1, which allows growth at 37 °C (Bukau & Walker, 1990). Total cell protein extracts were prepared and run on SDS-PAGE gels, which were either stained for protein, or Western blotted using the polyclonal anti-DnaK antibody (Fig. 2). The results clearly show that the size of the truncated DnaK proteins encoded by the deletion mutants were as predicted from the extent of the in-frame deletions in the *dnaK* gene. The relative amounts of the truncated DnaK proteins encoded by pDD2, pDD3 and pDD4 appeared to be significantly less than with the full-size DnaK protein. This may well be due to some proteolytic degradation. Indeed, the Western blot of pDD2 reproducibly yielded two fragments, presumably as a result of degradation of the upper full-size fragment.

Phenotypes of the mutant DnaK proteins

λ phage plating efficiency. *E. coli* K756 (Georgopoulos & Herskowitz, 1971; Saito & Uchida, 1977), or *dnaK* null mutants like *E. coli* BB1553 (Paek & Walker, 1987), will not support the growth of λ phage, due to a defect in DNA initiation. Phage λ *dnaK*⁺ will however plate on both of these *E. coli dnaK* mutant strains. The wild-type *dnaK*⁺ gene in either plasmid pKP31 or pKUC2 complemented the *dnaK* null mutation of *E. coli* BB1553

Fig. 2. SDS-PAGE and Western blot of total cell extracts of the *dnaK* constructs. Total cell extracts of *E. coli* BB1553 containing pDD4 (lane 2), pDD3 (lane 3), pDD2 (lane 4), pDD1 (lane 5), pKP31 (lane 6), pKUC2 (lane 7) or pBR322 (lane 8), were run on SDS-PAGE gels and then stained for protein (a), or Western blotted with DnaK antibody (b). Lanes 1 and 9 of both gels contained molecular mass markers.

Table 2. Restoration of λ plating by cloned *dnaK*

The phage titres were obtained by plating λ clear or λ *dnaK*⁺ on either *E. coli* BB1553 or *E. coli* K756 containing the plasmid indicated. This experiment was repeated twice with similar results each time.

Phage Plasmid	<i>E. coli</i> BB1553		<i>E. coli</i> K756	
	λ clear	λ <i>dnaK</i> ⁺	λ clear	λ <i>dnaK</i> ⁺
pBR322	<0.0008	4.5 $\pm$ 2.5	0.001 $\pm$ 0.0001	7.4 $\pm$ 0.8
pKP31	60.3 $\pm$ 0.49	69.1 $\pm$ 4.8	54.0 $\pm$ 1.5	69.2 $\pm$ 2.1
pDD1	68.6 $\pm$ 0.25	81.9 $\pm$ 1.6	68.8 $\pm$ 7.3	74.6 $\pm$ 0.8
pUC19	<0.0008	2.7 $\pm$ 0.2	0.0009 $\pm$ 0.0001	8.1 $\pm$ 0.7
pKUC2	82.6 $\pm$ 6.5	100 $\pm$ 3.3	90.6 $\pm$ 10.9	100 $\pm$ 3.6
pDD2	<0.0008	28.1 $\pm$ 0.7	<0.0008	29.6 $\pm$ 3.5
pDD3	<0.0008	37.9 $\pm$ 1.7	<0.0008	38.0 $\pm$ 4.4
pDD4	<0.0008	7.1 $\pm$ 0.2	<0.0008	11.6 $\pm$ 0.6

and allowed plating of λ phage. It was interesting that the presence of the wild-type gene on these multi-copy plasmids further increased the plating efficiency of λ *dnaK*⁺ on *E. coli* BB1553. This suggests that there is a gene dosage effect of the DnaK protein on phage plating. Plasmid pDD1 restored the ability of *E. coli* BB1553 to plate λ phage to the level seen with plasmid pKP31, from which it is derived (Table 2). This suggests that the last 10 amino acids of the DnaK protein, which are

conserved in both *E. coli* and *Bacillus*, are not required for initiation of λ DNA replication. None of the other mutant *dnaK* genes restored the ability of *E. coli* BB1553 to plate λ phage (Table 2), although both plasmids pDD2 and pDD3 had some effect in increasing the plating efficiency of λ *dnaK*⁺. This perhaps suggests that the DnaK proteins encoded by these constructs retain some activity in the reactions required for λ plating on *E. coli*. Similar results were obtained using *E. coli* K756 as the host strain, except that the actual number of plaques was some 20 fold higher than with *E. coli* BB1553 (Table 2).

High-temperature growth. Deletions of the *dnaK* gene in *E. coli* prevent growth at above 30 °C. *E. coli* BB1553 contains a secondary mutation, *sidB1*, which allows growth at 37 °C. Plasmid pKP31 restored the ability of *E. coli* BB1553 to grow at either 42 °C or 45 °C (Table 3). A similar result was observed with plasmid pDD1. Surprisingly, plasmid pKUC2, which contains the whole *dnaK* heat-shock gene and its promoters, and which efficiently expresses the DnaK protein (Fig. 2), did not allow growth of *E. coli* BB1553 at 42 °C. This suggests that the inability of *E. coli* BB1553 to grow at high temperature is not solely the result of the deletion of the *dnaK* gene. One possible hypothesis is that there is a mutation, or deletion, in another gene closely linked to *dnaK* which was introduced during the construction of this *dnaK* deletion. This possibility is perhaps strengthened by

Table 3. High-temperature growth of *E. coli* BB1553 and *E. coli* K756

E. coli BB1553, or *E. coli* K756, transformed with the plasmid shown was tested for its ability to grow at the three temperatures shown. +, Growth; −, no growth; ±, poor growth. This experiment was repeated three times.

Plasmid	<i>E. coli</i> BB1553			<i>E. coli</i> K756		
	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C
None	+	−	−	+	+	−
pBR322	+	−	−	+	+	−
pKP31	+	+	+	+	+	+
pDD1	+	+	+	+	+	+
pUC19	+	−	−	+	±	−
pKUC2	+	−	−	+	±	−
pDD2	+	−	−	+	±	−
pDD3	+	−	−	+	±	−
pDD4	+	−	−	+	±	−
pRJ325	+	−	−	+	+	+
pRJ327	+	−	−	+	±	−
pRJ328	+	−	−	+	±	−
pDD11	+	−	−	+	+	+
pDD12	+	−	−	+	+	+

consideration of the technical details of the derivation of this *dnaK* null mutant. This involved the replacement of 933 bp at the 5' end of the *dnaK* gene, together with some 800 bp of the sequence upstream of the *dnaK* gene, with a chloramphenicol resistance cassette (Paek & Walker, 1987). Our results show that the mutation in this hypothetical second gene is complemented by plasmid pKP31, but not by plasmid pKUC2. In order to confirm this hypothesis and to localize this second gene we performed transposon Tn1000 mutagenesis of plasmid pKP31.

Transposon mutagenesis of pKP31

Transconjugant colonies of *E. coli* BB1553, containing pKP31 with random Tn1000 inserts, were screened for their inability to grow at 42 °C. Six independent Tn1000 inserts were obtained which inactivated the ability of pKP31 to restore high-temperature growth to *E. coli* BB1553; none of them affected λ plating. Restriction mapping of the Tn1000 inserts further confirmed that they are not located in the *dnaK* gene. The insertions were located between 2.0 and 2.4 kb from the left-hand HindIII site of pKP31, which is at least 700 bp upstream of the 5' end of the *dnaK* gene. Western blots of these cultures revealed that they contain similar amounts of the DnaK protein as cultures containing pKP31 (data not shown). We propose to call the gene inactivated by these Tn1000 inserts *htgA*, for high temperature growth.

Table 4. High-temperature growth of *E. coli* BB1553 with a binary vector system

E. coli BB1553 containing the plasmid shown in the left-hand column was transformed with either pDD1841 or pACYC184, and representative transformants were then tested for their ability to grow at the three temperatures shown. This experiment was repeated twice with identical results each time.

Plasmid	pDD1841			pACYC184		
	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C
None	+	−	−	+	−	−
pRJ325	+	+	+	+	−	−
pD11	+	+	+	+	−	−
pDD12	+	+	+	+	−	−

The *htgA* gene alone does not restore high-temperature growth

In order to investigate whether the *htgA* gene alone, or in combination with *dnaK*, is required for high-temperature growth of *E. coli* BB1553, we made further recombinant constructs. Plasmid pRJ327 is the 2.1 kb HindIII–BglII fragment of pKP31 cloned into pUC19, whilst pDD11 is the same fragment cloned into pBR322. Plasmid pRJ328 is the 3.6 kb HindIII–EcoRI fragment of pKP31 cloned into pUC19, whilst pDD12 is the same fragment cloned into pBR322. None of these four plasmids restored the ability of *E. coli* BB1553 to grow at either 42 °C or 45 °C (Table 3). This result suggests that both the *htgA* and the *dnaK* genes are required for high-temperature growth of *E. coli*. We demonstrated the requirement for both genes by using a binary vector system, using two compatible cloning vectors. Plasmid pDD1841 consists of the 3.0 kb *AvaI*–HindIII fragment from pKUC2, which contains the *dnaK* gene, cloned into pACYC184. This plasmid restored the ability of *E. coli* BB1553 containing plasmid pRJ325, pDD11, or pDD12, to grow at high temperatures (Table 4). The result of this experiment also clearly shows that the *dnaK* gene present in pKUC2 is not defective in any way.

The DnaK756 heat-shock protein

E. coli K756 is capable of growth up to 43.5 °C (Georgopoulos, 1977), but not at 45 °C. This phenotype has been assumed to be the result of the production of an altered DnaK protein, which has a different pI. Plasmids pRJ325, pDD11 and pDD12 were all capable of restoring growth of this *dnaK* mutant at 45 °C (Table 3). This suggests that the inability to grow at high temperatures is the result of a second mutation in *E. coli* K756, possibly in the *htgA* gene, rather than due to the altered DnaK756 protein. The DnaK756 mutant pro-

tein, although not capable of the functions necessary to allow the plating of λ phage, must be able to substitute for the wild-type DnaK protein in its role in high-temperature growth of *E. coli*. An alternative explanation is that the alterations in the DnaK756 protein require a compensating increase in the gene dosage of the *htgA* gene. We are attempting to clone the *htgA* gene from *E. coli* K756 to distinguish these possibilities.

A comparison of the results in this experiment with plasmids pDD11 and pDD12, which are both recombinant constructs using pBR322 as the vector, and the equivalent constructs in pUC vectors, pRJ327 and pRJ328, reveals an interesting effect (Table 3). The higher-copy-number pUC-based recombinant plasmids, unlike the equivalent pBR322-based constructs, do not restore growth of *E. coli* K756 at 45 °C. This perhaps suggests that when the *htgA* gene is present in a very high copy number (> 200 in the case of pUC vectors), its gene product is present in too great an excess compared with the chromosomally encoded DnaK756 protein, which results in the inability to grow at high temperatures. An alternative explanation of these results is that the burden imposed upon the host cells, caused by the DNA replication, transcription and translation associated with high-copy-number plasmids, may be just too much for the cell's machinery to cope with. Preliminary evidence to support the latter comes from our observation that *E. coli* K756 containing pUC19, or recombinant plasmids based on this vector, do not grow well even at 42 °C. In contrast *E. coli* K756 containing pBR322, or recombinant plasmids based on this vector, grows well at 42 °C (Table 3).

Conclusions

We have identified a new gene, *htgA*, which is required for high-temperature growth of *E. coli*. The *E. coli* *dnaK* null mutant BB1553 must have a mutation/deletion in this gene as well as in *dnaK*. Previous workers have probably missed the presence of the *htgA* gene because of the way in which they tested for genetic complementation of *dnaK* mutants using P1 transduction with the *thr* marker located upstream of both the *dnaK* and the *htgA* genes. Our results with *E. coli* K756 suggest that this strain may also have a defective *htgA* gene. We are currently attempting to confirm that the *htgA* gene is defective in both *E. coli* BB1553 and K756 and sequencing the wild-type *htgA* gene. It will be interesting to observe whether this gene has a characteristic heat-shock promoter and whether it has any sequence homology to any other heat-shock genes.

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