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Escherichia coli dnaK Null Mutants Are Inviable at High Temperature

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DnaK, a major *Escherichia coli* heat shock protein, is homologous to major heat shock proteins (Hsp70s) of *Drosophila melanogaster* and humans. Null mutations of the *dnaK* gene, both insertions and a deletion, were constructed in vitro and substituted for *dnaK*$^+$ in the *E. coli* genome by homologous recombination in a *recB recC sbcB* strain. Cells carrying these *dnaK* null mutations grew slowly at low temperatures (30 and 37°C) and could not form colonies at a high temperature (42°C); furthermore, they also formed long filaments at 42°C. The shift of the mutants to a high temperature evidently resulted in a loss of cell viability rather than simply an inhibition of growth since cells that had been incubated at 42°C for 2 h were no longer capable of forming colonies at 30°C. The introduction of a plasmid carrying the *dnaK*$^+$ gene into these mutants restored normal cell growth and cell division at 42°C. These null mutants showed a high basal level of synthesis of heat shock proteins except for DnaK, which was completely absent. In addition, the synthesis of heat shock proteins after induction in these *dnaK* null mutants was prolonged compared with that in a *dnaK*$^+$ strain. The well-characterized *dnaK756* mutation causes similar phenotypes, suggesting that they are caused by a loss rather than an alteration of DnaK function. The filamentation observed when *dnaK* mutations were incubated at a high temperature was not suppressed by *sulA* or *sulB* mutations, which suppress SOS-induced filamentation. Our results indicate that DnaK function is normally essential only at elevated temperatures. Nevertheless, cells did not have an absolute requirement for DnaK function for growth at 42°C since we were able to isolate spontaneously arising suppressors of *dnaK* deletion mutations that permitted growth at that temperature.

The heat shock response is an example of a biological response to an environmental change that has been extremely strongly conserved during evolution (5, 25, 31). A characteristic feature of the heat shock responses of organisms as diverse as *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and humans is the induction of a ca. 70-kilodalton (kDa) protein. Furthermore, many eucaryotic organisms encode families of related ca. 70-kDa proteins (Hsp70s), some members of which are not induced by heat. In contrast, *E. coli* encodes only one Hsp70 protein, the product of the *dnaK* gene, and its synthesis is induced by heat and other forms of stress (14, 19, 34). DNA sequencing studies have shown that the amino acid sequence of DnaK is about 50% homologous with the heat-induced Hsp70s of *D. melanogaster* and humans (1, 17).

The strong conservation of Hsp70 structure throughout evolution suggests that the Hsp70 proteins induced by heat may carry out common or related roles in various organisms and, moreover, that there may be common elements of function between various members of the Hsp70 family within a given organism. To date, the *E. coli* DnaK protein has been the heat-induced Hsp70 protein most extensively characterized at a biochemical level. It has been purified and shown to have several biochemical activities: (i) it is essential for both lambda and M13 in vitro DNA replication systems that are dependent on the lambda *O* and *P* proteins (21, 38, 39); (ii) it autophosphorylates at a threonine residue (39); and (iii) it has a weak DNA-dependent ATPase activity (38, 39). Although the heat-induced Hsp70s from other organisms have been less well characterized biochemically, it is interesting that they bind strongly to ATP-agarose (4, 36), suggesting that they may have ATPase activities, and that an Hsp70 of *Dictyostelium discoideum* is phosphorylated at a threonine residue (22). A particularly intriguing development in this area has been the discovery that a constitutively synthesized member of the Hsp70 family of proteins in bovine brain cells is the uncoating ATPase that releases clathrin triskelions from coated vesicles in an ATP-dependent manner (4).

Studies of the *E. coli dnaK* gene have focused on the *dnaK756* allele, derived by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, that was originally identified as a mutation blocking bacteriophage lambda replication (12, 13). Cells carrying the *dnaK756* mutation make a DnaK protein of the wild-type size but with a more-acidic isoelectric point (14). The *dnaK756* mutation makes *E. coli* temperature sensitive for growth and prevents both DNA and RNA synthesis at restrictive temperatures (18, 30). Furthermore, bacteria carrying the *dnaK756* mutation show a high basal level of synthesis of heat shock proteins at 30°C and fail to turn off the heat shock response at 42°C, suggesting that DnaK functions as a negative regulatory modulator in the heat shock response (33). DiDomenico et al. (9) have suggested that the heat-induced Hsp70 plays a role in the turn-off of the heat shock response in *D. melanogaster*.

The fact that *dnaK756* mutants are temperature sensitive for growth had suggested that *dnaK* is an essential gene. However, we report here the construction and analysis of null mutations, both insertions and a deletion, in the *dnaK*

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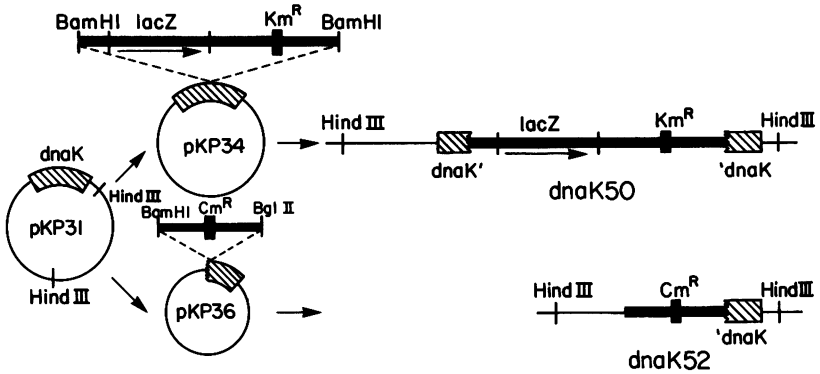

FIG. 1. Construction of *dnaK50* insertion and *dnaK52* deletion mutations. Mutations and plasmids were constructed as described in the text. The insertion and deletion mutations were crossed onto the chromosome by transforming a *recB recC sbcB* strain with linearized plasmid DNA.

gene of *E. coli*. The resulting *dnaK* null mutants were temperature sensitive for growth. We furthermore report that it was possible to isolate suppressors of the *dnaK* null mutations that permitted growth at 43°C.

MATERIALS AND METHODS

Media, bacterial strains, and plasmid construction. The media used in this study were described previously (27). Selection for antibiotic resistance was done on LB agar plates (24) containing 25 μg of kanamycin or chloramphenicol per ml or 50 μg of ampicillin per ml. Strains B178, B178 *dnaK756* (33), and JC7623 (37) were described before. Strain GC4540 (7) carrying *sulA*::Tn*5* was used in P1 transduction experiments (24) to move a *sulA* allele into various recipient strains. The *sulB103* allele was transduced from strain GC694 (provided by J. Lutkenhaus) into the *dnaK52* mutants by selecting for Leu$^+$.

The starting material for pKP31 and pKP33 was λ *dnaK*$^+$ (12). The 5.3-kilobase (kb) *Hin*dIII fragment from λ *dnaK*$^+$ was subcloned into the *Hin*dIII site of pBR322 and pSE101 (11), respectively. pKP34 carrying the *dnaK'-lacZ*$^+$ operon fusion was constructed in the following way. pKP31, carrying a *Hin*dIII fragment of *E. coli* chromosomal DNA that includes the *dnaK*$^+$ gene, was linearized at the *Bgl*II site at 933 base pairs (bp) on the *dnaK* coding region, by *Bgl*II partial digestion. The 7.8-kb *Bam*HI fragment of pGS100 (G. DeVos, personal communication), containing a *lacZ* operon fusion fragment and a Km^r gene next to it, was cloned into the above *Bgl*II site. Restriction enzyme digestion analysis was used to confirm that the desired construct had been obtained.

In vivo labeling of proteins and gel electrophoresis. For [^{35}S]methionine labeling, early log cultures grown in M9 glucose medium at 30°C were labeled with [^{35}S]methionine (final concentrations for one-dimensional gels, 5 μCi/ml; for two-dimensional gels, 30 μCi/ml) during growth at 30°C (control) or after a shift to 42°C (heat shock) (19). The cells were allowed to incorporate radiolabel for 5 min and then were rapidly chilled on ice and spun down. The pellets were prepared for electrophoresis as described previously (20), and about 100,000 cpm of each sample was subjected to electrophoresis in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels as originally described by Laemmli (20). Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (26). Extracts were prepared as for one-dimensional gels and were then diluted 1:2 with sample dilution buffer. One-dimensional isoelectric focusing gels were subjected to 6,400 V/h. The resolving portion of the two-dimensional gel was 10% SDS-polyacrylamide gel. Autoradiography was done by exposing the dried polyacrylamide gels to XAR-5 X-ray film (Eastman Kodak Co.) at −70°C.

Southern blot analysis. Either 5 μg of chromosomal DNA or 0.1 μg of plasmid DNA was digested with appropriate restriction endonucleases (*Hin*dIII or *Eco*RI) and electrophoresed in 0.8% agarose gels. The DNA was transferred to nitrocellulose paper (Schleicher & Schuell, Inc.) by the method of Southern (32). The immobilized DNA was hybridized to a probe labeled with [^{32}P]dATP (Amersham Corp.) by nick translation (28) The *dnaK*$^+$-containing probe used in the analysis was a 5.3-kb *Hin*dIII fragment isolated from the plasmid pKP31. The DNA fragment was purified by the electroelution method (23). The hybridization was performed at 37°C in 50% formamide–0.75 M NaCl–0.75 M sodium citrate–0.2% SDS–1× Denhardt solution (8).

Western blotting. Proteins electrophoresed in a 10% SDS-polyacrylamide gel were electroeluted onto nitrocellulose (2) and developed (35) by published methods. The nitrocellulose filters were exposed to 1:1,000 dilutions of a rabbit antisera against DnaK and then a 1:1,000 dilution of anti-rabbit donkey immunoglobulin G antibodies coupled to horseradish peroxidase (Amersham Corp.) and developed with diaminobenzidine and hydrogen peroxide. The rabbit antibody raised against purified DnaK was a gift from C. Georgopoulos.

Phase-contrast microscopy. Cells from exponentially growing cultures at 30°C or after a shift to 42°C were prepared on glass microscope slides. A Zeiss photomicroscope with a ×40 phase objective lens and Kodak Tri-X pan film were used in the photography.

RESULTS

Construction of *dnaK* insertion and deletion mutants. Our initial strategy for obtaining *dnaK* null mutations was to construct insertion mutations of *dnaK* in a recombinant plasmid and then to cross these mutations into the *E. coli* chromosome. We generated these mutations by inserting, into the cloned *dnaK*$^+$ gene, DNA cassettes carrying (i) a drug resistance marker and (ii) a DNA sequence capable of creating transcriptional fusions, as shown in Fig. 1; our hope was that these mutations would eventually prove useful in analyses of *dnaK* regulation as well as the *dnaK* function. By these procedures, insertions creating transcriptional fusions to *lacZ* were generated at nucleotide 933 of the *dnaK* coding

sequence (1,917 bp total length [1]) and at a *Sau*3A1 site located near nucleotide 600 of the *dnaK* coding sequence (*dnaK50* and *dnaK51*, respectively). Since each of these insertions carried a selectable antibiotic resistance marker, we were able to cross these mutations into the *E. coli* chromosome by linearizing the cloned DNA and transforming it into a *recB recC sbcB* strain (Fig. 1). The *recB recC* mutations inactivate exonuclease, preventing it from degrading the linear DNA, while the *sbcB* mutation restores recombination proficiency (37). This method is functionally equivalent to the one-step gene replacement method of Rothstein (29) in *S. cerevisiae*. Although the existence of temperature-sensitive lethal mutations of *dnaK* suggested that *dnaK* might be essential, we were able to cross these *dnaK* insertion mutations into a haploid *E. coli* genome. Km^r colonies were selected and screened for failure to support phage lambda growth. The resulting strains were temperature sensitive for cell survival at 42°C as judged by colony-forming ability.

The temperature sensitivity of the strains carrying *dnaK* insertion mutations suggested two possibilities to us. Either the *dnaK* gene of *E. coli* is not essential for cell survival at low temperatures or truncated products of DnaK produced in the insertion mutants might retain some activity necessary for survival at low temperatures. To examine these two possibilities, we constructed a *dnaK* deletion mutant.

The strategy used for constructing the *dnaK52* deletion mutation (Fig. 1) was similar to that used to construct the insertion mutations. First, we deleted a 1.7-kb *Bgl*II fragment from the $dnaK^+$ plasmid pKP31. This fragment contained the first 933 bp of the *dnaK* coding sequence and approximately 800 bp of the sequence upstream of the *dnaK* gene. Next, we replaced this fragment with a 3.1-kb *Bgl*II-*Bam*HI fragment from pSE150 that encodes chloramphenicol resistance (11). Then we linearized this plasmid (pKP36) with *Hin*dIII, transformed it into a *recB recC sbcB* strain, and selected for Cm^r colonies at 30°C. As with the *dnaK* insertion mutants, we found that colonies which could not support phage lambda growth were also temperature sensitive for cell survival at 42°C.

These results strongly suggested that null mutations of the *dnaK* gene make *E. coli* cells temperature sensitive for growth at 42°C. By transducing the *dnaK52* deletion mutation and the *dnaK50* insertion mutation into a $recB^+$ $recC^+$ $sbcB^+$ strain, we were able to show that these phenotypes were not unique to a *recB recC sbcB* background. In all cases, the transductants were unable to propagate phage lambda or form colonies at 42°C. We experienced some technical difficulties in carrying out these transductions because of the poor growth of phage P1 on the *recB recC sbcB dnaK* strains, but we were able to improve phage growth sufficiently to carry out these experiments by introducing a $recB^+$ $recC^+$ plasmid into the strains.

Confirmation of *dnaK* null mutants by several experiments. We carried out three kinds of experiments to confirm that our manipulations had indeed resulted in the construction of the anticipated *dnaK* null mutants.

(i) Southern hybridization analyses of chromosomal DNA extracted from the wild type and *dnaK* null mutants. To confirm that the wild-type *dnaK* gene was physically disrupted at the desired sites in the deletion and insertion mutants, we carried out Southern blot analyses (32). Chromosomal DNA was extracted from the isogenic wild-type strain (JC7623), the *dnaK50* insertion mutant, and the *dnaK52* deletion mutant. A hybridization probe was prepared from the 5.3-kb *Hin*dIII fragment that contained the $dnaK^+$ region from pKP31. As expected, this probe hybridized to the single 5.3-kb fragment of *Hin*dIII-digested JC7623 DNA (Fig. 2, lane a). As anticipated, *Hin*dIII-digested DNA from the *dnaK50* insertion mutant showed two hybridizing fragments of 9.3 and 3.1 kb but no 5.3-kb fragment (lane b). For the *dnaK52* deletion mutant, we deleted a 1.7-kb fragment and replaced it with a 3.1-kb Cm^r fragment; there was no *Hin*dIII site in the inserted fragment. Consistent with our expectations, our probe hybridized to a single 6.7-kb fragment of *Hin*dIII-digested DNA from the *dnaK* deletion mutant (lane c). Similarly, hybridization with the same probe to *Eco*RI-digested DNA from these same three strains also showed the patterns we expected. These Southern hybridization analyses clearly confirmed that the insertion and deletion mutations we constructed actually disrupted the restriction fragments expected to lie within the *dnaK* gene and also ruled out any undesired occurrences of rearrangements or deletions of the *dnaK* region of the chromosome in these mutants.

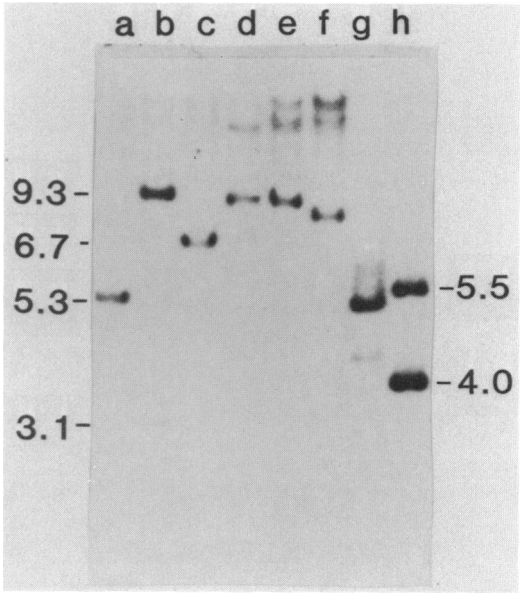

FIG. 2. Southern hybridization analyses of chromosomal DNA extracted from the wild type and *dnaK* null mutants. The *lacZ* Km^r fragment contains both *Hin*dIII and *Eco*RI sites. The Cm^r insertion used in the construction of the deletion does not contain *Hin*dIII or *Eco*RI sites. Lanes: a, JC7623 cut with *Hin*dIII; b, *dnaK50*::*lacZ* Km^r insertion cut with *Hin*dIII (although the 3.1-kb fragment was less intense than expected in this experiment, in other experiments it had the expected intensity); c, *dnaK* deletion cut with *Hin*dIII; d, JC7623 cut with *Eco*RI; e, *dnaK50*::*lacZ* Km^r insertion cut with *Eco*RI; f, *dnaK* deletion cut with *Eco*RI; g, pKP31 cut with *Hin*dIII; h, pKP31 cut with *Eco*RI.

(ii) Two-dimensional protein gel analyses of *dnaK* null mutants. The 70-kDa DnaK protein was not present on two-dimensional protein gels of *dnaK* null mutants. The [^{35}S]methionine-labeled proteins synthesized by $dnaK^+$ and *dnaK* strains after a 42°C heat shock were separated by two-dimensional gel electrophoresis (26). The positions of the GroEL, DnaK, and other heat shock proteins, after a similar treatment, have been described (26, 27). The DnaK protein was completely missing in both *dnaK50* insertion and *dnaK52* deletion mutants (Fig. 3B and C, respectively), whereas DnaK was one of the most prominent proteins in the wild type (Fig. 3A). The relative positions of other

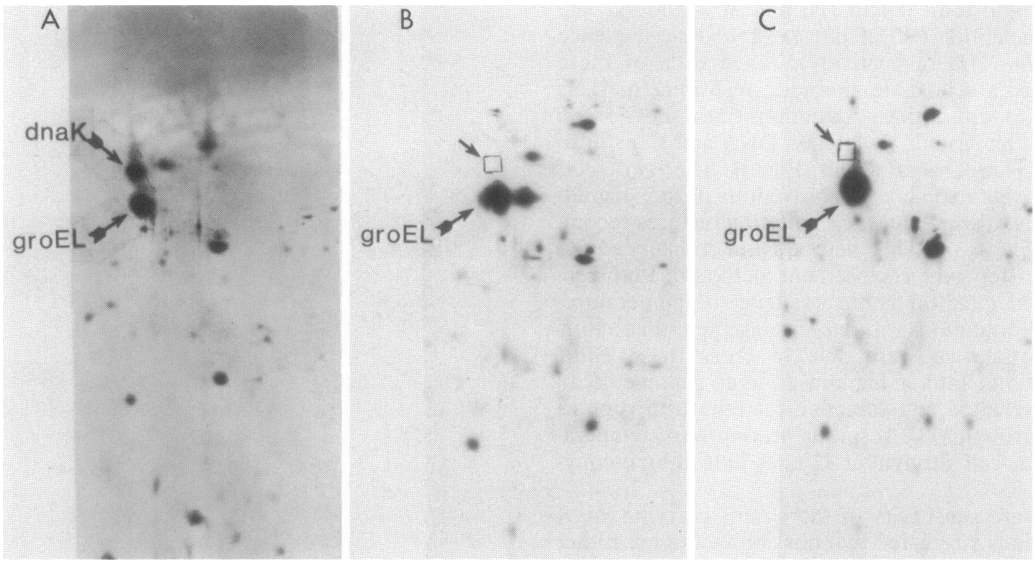

FIG. 3. Two-dimensional polyacrylamide gel electrophoretic separation of [^{35}S]methionine-labeled proteins of *dnaK*$^+$ and *dnaK* null mutants after a temperature shift from 30 to 42°C. Cultures were labeled for 5 min. Equal counts of acid-precipitable samples were applied to each gel. Isoelectric-focusing analysis was in the horizontal dimension (acidic side is on the left), and SDS-PAGE was in the vertical dimension (largest proteins are at the top). Arrows indicate the positions of the *groEL* (*mopA*) gene product (M_r 62,000) and the *dnaK* gene product (M_r 70,000). (A) Strain JC7623; (B) *dnaK50*::*lacZ* Km^r insertion mutant; (C) *dnaK* deletion mutant.

proteins were the same in these mutants and in the wild type. Even though we expected a truncated derivative (about 35 kDa) of DnaK in the *dnaK50* insertion mutant, we did not detect such a product. Instead, we observed an extra spot next to GroEL which had a molecular weight of 62,000 (Fig. 3B). We assume that this product was the result of a fusion of the *dnaK* reading frame to a reading frame upstream of the *lacZ* coding sequence in the operon fusion fragment. This explanation is consistent with the Western blot analysis described below.

(iii) Immunoblot analysis for DnaK protein with anti-DnaK antibody. Even though the DnaK spot was completely missing from the *dnaK50* insertion and *dnaK52* deletion mutants on the autoradiogram of our two-dimensional gels, we wanted additional confirmation that cells carrying these mutations truly lacked the native DnaK proteins. Therefore, we carried out an immunoblot analysis with an antibody raised to the purified DnaK protein (a generous gift from C. Georgopoulos) (Fig. 4). Anti-DnaK antibodies cross-reacted strongly with the 70 kDa protein of the JC7623 strain, the isogenic parent of the *dnaK50* deletion and *dnaK52* insertion mutants (Fig. 4, lane e), the *dnaK756* mutant (lane c), and its isogenic parent, strain B178 (lane d). For the *dnaK52* deletion and *dnaK50* insertion mutants (lanes a and b), there was no 70-kDa protein which strongly cross-reacted with DnaK antibody, whereas all the background was the same as for other strains. The expected mobility of the putative *dnaK* fusion protein based on results shown in Fig. 3 was almost the same as that of one of the other bands that cross-reacted with the DnaK antiserum (Fig. 4, lane b). Upon close inspection of the original gel, we could discern a closely spaced doublet near the 62-kDa region instead of a singlet as in the other lanes. We interpret this to mean that the top band of this doublet corresponded to the extra spot near GroEL on a two-dimensional gel electrophoresis autoradiogram (Fig. 3B). These results confirmed the disruption of the *dnaK* gene in the insertion and deletion mutants, revealed the presence of a hybrid protein containing the DnaK sequence in the insertion mutant, and established the absence of any cross-reacting carboxy-terminal fragments of DnaK that might have arisen from restarts in the deletion mutant.

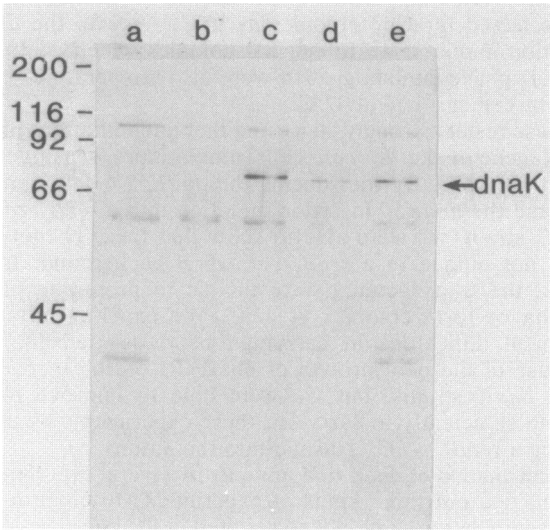

FIG. 4. Western blot analysis of extracts of cultures separated on an SDS-polyacrylamide gel by using anti-DnaK antibody. Proteins electrophoresed in a 10% SDS-polyacrylamide gel were electroeluted onto the nitrocellulose. The nitrocellulose was exposed to 1:1,000 dilutions of a rabbit antisera against DnaK and then a 1:1,000 dilution of anti-rabbit donkey immunoglobulin G antibodies coupled to horseradish peroxidase and developed with diaminobenzidine and hydrogen peroxide. Cultures were labeled for 5 min at 5 min after a temperature shift from 30 to 42°C. Equal counts were loaded in all lanes. Lanes: a, JC7623 *dnaK* deletion; b, JC7623 *dnaK50*::*lacZ* Km^r insertion; c, B178 *dnaK756*; d, B178; e, JC7623.

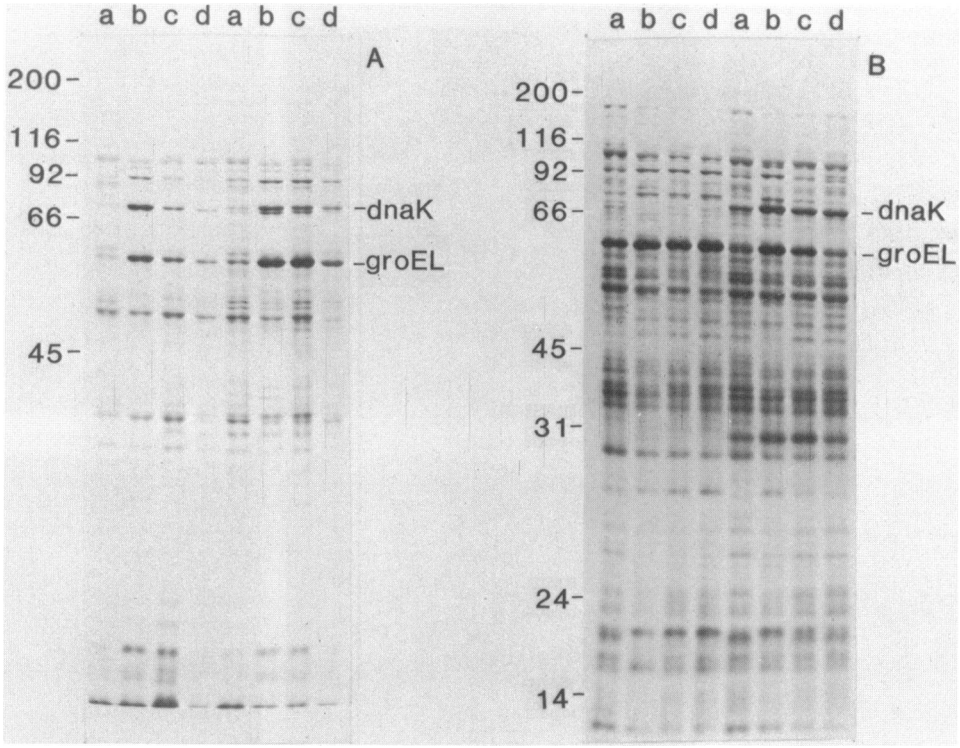

FIG. 5. Comparison of heat shock protein synthesis pattern in strain JC7623, a *dnaK756* mutant, a *dnaK* deletion mutant, and a *dnaK* deletion mutant with pKP33. Cultures were labeled for 5 min. Equal counts were loaded in all lanes. Panels A and B were separate gels that were exposed independently. Molecular weights (10^{-3}) of standard proteins are indicated on the left. (A) four left lanes, strain JC7623; four right lanes, B178 *dnaK756*; (B) four left lanes, JC7623 *dnaK* deletion; four right lanes, JC7623 *dnaK* deletion (pKP33). Lanes and times of labeling after indicated temperature shifts were as follows: a, control, 30°C; b, 42°C, 5 min; c, 42°C, 30 min; d, 42°C, 1 h.

Expression of heat shock proteins in *dnaK* null mutants. Cells carrying the *dnaK756* allele have been previously shown to exhibit a high basal level of synthesis and a prolonged heat shock response after a shift to 42°C. Both our *dnaK* insertion and deletion mutants exhibited the same phenotype. A *dnaK756* mutant exhibited a high basal level of synthesis of heat shock proteins and a prolonged synthesis of these proteins after a shift to 42°C (Fig. 5A, left lanes a through d). The pattern of synthesis of heat shock proteins in a $dnaK^+$ strain is also shown in Fig. 5A. The pattern of synthesis of heat shock proteins observed with the *dnaK52* deletion mutant is shown in Fig. 5B, left lanes a through d. This mutant exhibited a high basal level of synthesis of heat shock proteins such as GroES, GroEL, F84.0, and Lon (DnaK was of course missing) and a prolonged heat shock response after a shift to 42°C, as did the *dnaK756* mutant. This phenotype appeared to be caused solely by the absence of DnaK, since introduction of a low-copy-number plasmid carrying $dnaK^+$ (pKP33) into the *dnaK* deletion mutant lowered the basal level of synthesis of heat shock proteins and led to a rapid decrease in the synthesis of heat shock proteins seen after a shift to 42°C (Fig. 5B). These results suggest that the effects of the *dnaK756* mutation on the basal synthesis and induction of heat shock proteins are caused by a loss rather than an alteration of the DnaK function.

***dnaK* mutants grow more slowly than the wild type at low temperatures and produce filaments after a shift to 42°C.** The *dnaK* deletion and insertion mutants grew more slowly than wild-type cells at low temperatures. The growth rate of *dnaK* mutants was 67% that of their $dnaK^+$ parent at 30°C and 75% of the parental rate at 37°C. These observations indicate that, although the $dnaK^+$ gene was not absolutely required for cell growth at 30 and 37°C, its product did play a role in normal growth at these temperatures.

When the *dnaK* insertion and deletion mutants, growing logarithmically at 30°C, were shifted to 42°C, no immediate lag in growth was observed. However, upon continued incubation at 42°C, a reduced rate of increase in optical density was observed, and no further increase was observed after 2 h. The shift of the *dnaK* mutants to 42°C evidently resulted in a loss of cell viability rather than simply an inhibition of growth since cells that had been incubated at 42°C for 2 h were no longer capable of forming colonies at 30°C. Thus, it appears that the DnaK protein was required both for cell growth and viability at 42°C.

As reported previously for the *dnaK756* mutant (18), a temperature upshift of our *dnaK* insertion and deletion mutants resulted in an inhibition of both DNA and RNA synthesis but only a slight inhibition of protein synthesis (data not shown).

To further investigate the basis of cell death at 42°C, we examined cells by phase-contrast microscopy before and after a temperature shift from 30 to 42°C. Wild-type cells showed uniform cell length before and after temperature shift (Fig. 6A and B). On the other hand, our *dnaK* mutants produced filaments vigorously after shifts to the high temperature. (Fig. 6C through F). Similar filamentation of a *dnaK756* mutant has been briefly mentioned before (10). The time point at which maximum filamentation occurred after the temperature shift coincided with the complete cessation of cell growth (about 2 h after the shift). The filamentation was clearly caused by the *dnaK* mutation since the presence

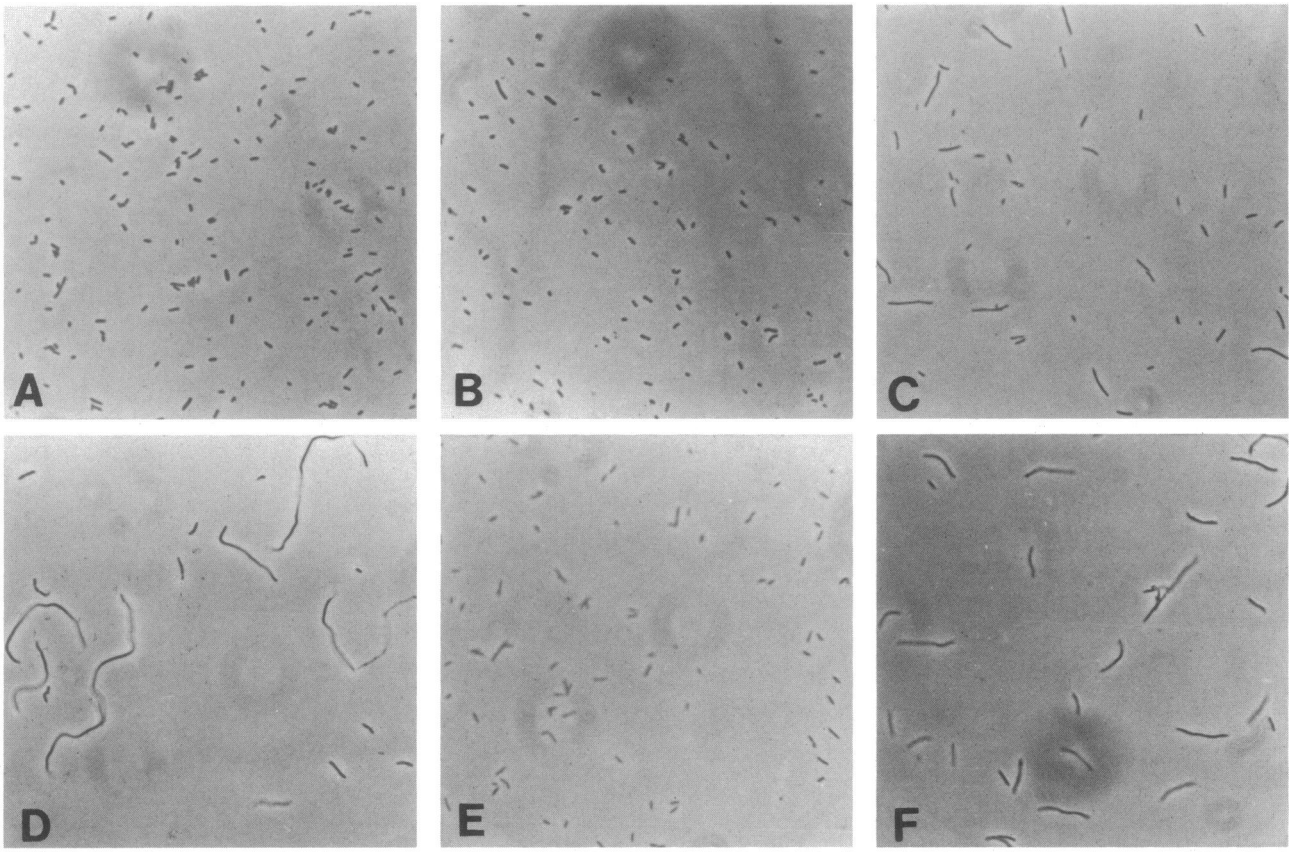

FIG. 6. Phase-contrast microscopic observation of cellular shape of *dnaK*$^+$ and *dnaK* mutants. Cells from exponentially growing cultures at 30°C or 3 h after a shift to 42°C were prepared on the glass objective and Kodak Tri-X pan film was used in the photography. (A) JC7623, 30°C; (B) JC7623, 42°C; (C) JC7623 *dnaK* deletion, 30°C; (D) JC7623 *dnaK* deletion, 42°C; (E) JC7623 *dnaK50*::*lacZ* Km^r insertion, 30°C; (F) JC7623 *dnaK50*::*lacZ* Km^r insertion, 42°C.

of a *dnaK*$^+$ plasmid (pKP33) in the *dnaK* deletion mutant restored normal growth and prevented filamentation at 42°C (data not shown). Since the filamentation was irreversible, it is possible that it accounts, totally or partially, for the cell death observed upon the shift of a *dnaK* mutant to 42°C.

We have investigated whether the filamentation of *dnaK* mutants shifted to 42°C is related to the filamentation observed during the SOS response. In the SOS response, *sulA* mutations can suppress the lethality that is associated with constitutive expression of the SOS system. The *sulA* gene product is an SOS-regulated inhibitor of cell division that appears to act by inhibiting septum formation (15, 16); *sulA* mutations suppress the excessive filamentation caused by UV irradiation of a *lon* mutant or the shift of a *lon recA441* (*tif-1*) mutant to 42°C (3). We found that a *sulA11 dnaK50* double mutant still filamented at the high temperature and was temperature sensitive like *dnaK* mutants. Thus, the filamentation induced by a temperature upshift in *dnaK* mutants is not *sulA* dependent. Neither was it inhibited by *sulB103*, an allele of *ftsZ*, that also prevents SOS-induced filamentation. More generally, it appears that this filamentation is not mediated by the SOS response. There is no SOS-inducing signal generated in *dnaK* mutants by a temperature upshift. This was determined by introducing a plasmid carrying a *lacZ* operon fusion to the SOS-regulated *umuD* gene (11) into our *dnaK*$^+$ and *dnaK* strains and monitoring the beta-galactosidase level. Even though there was a good induction of beta-galactosidase by UV irradiation, an SOS-inducing agent in these strains, there was no change in the beta-galactosidase level after a temperature upshift of our *dnaK* strains (data not shown).

It appears that cells did not have an absolute requirement for the DnaK function for growth at 42°C, since we were able to isolate suppressors of *dnaK* null mutants that could grow at that temperature. Specifically, by plating cultures of *dnaK52* deletion mutants on LB plates containing chloramphenicol at 42°C, we were able to isolate derivatives at a frequency of approximately 10^{-7} that would grow at that temperature; several independent derivatives were isolated in both *recB recC sbcB* and *recB*$^+$ *recC*$^+$ *sbcB*$^+$ backgrounds. Southern analyses of these strains, with a *dnaK* probe, gave patterns identical to those shown in Fig. 1, indicating that there had been no observable rearrangment of the chromosome in these mutants. Examination by phase-contrast microscopy of cultures of these derivatives grown at 42°C revealed that they exhibited less extensive filamentation than the original *dnaK* null mutants, with some members of the population having the size of wild-type cells. These derivatives did not plate lambda, indicating that the suppression of the defect in *dnaK* null mutants for growth at 42°C did not result in a simultaneous suppression of all other phenotypes of *dnaK* mutants.

DISCUSSION

In the present study we describe the construction and characterization of insertion and deletion mutants of *dnaK*,

the gene coding for the heat-inducible Hsp70 protein of *E. coli*. These precisely defined null mutations were first constructed in plasmids and then crossed into the chromosomal copy of the *dnaK* gene by homologous recombination. We have used several methods to demonstrate that the chromosomal copy of *dnaK* is indeed disrupted or deleted in these mutants and that these strains fail to synthesize the DnaK protein.

Our *dnaK* null mutants, both the deletion and insertion mutants, had altered growth properties. At low temperatures (30 and 37°C), the mutants grew more slowly than did the wild type. At a higher temperature (42°C), the *dnaK* mutants were unable to form colonies. Furthermore, our *dnaK* mutants were killed by incubation at 42°C, since they could not grow when subsequently plated out at 30°C. This temperature sensitivity of *dnaK* null mutants was caused totally by the absence of *dnaK* function, since the introduction of a $dnaK^+$ plasmid into our *dnaK* mutants completely restored cell growth and survival. These results indicate that null mutations in the *dnaK* gene confer a temperature sensitivity for cell survival in *E. coli*. In *S. cerevisiae*, it has been reported that a double mutant containing mutations in both the *YG100* and *YG102* genes, two of the Hsp70-related genes, is temperature sensitive for growth (6). At low temperatures this double mutant grew more slowly than did the wild type. At higher growth temperatures the mutant would not form colonies. The *dnaK* gene is the only Hsp70-related gene in the *E. coli* chromosome, while *S. cerevisiae* contains at least six more Hsp70-related genes in addition to *YG100* and *YG102*. It is intriguing that a yeast *YG100 YG102* double mutant has the same phenotype as *E. coli dnaK* mutants with respect to cell growth.

Several facts suggest that the cell death of *dnaK* mutants at 42°C is closely connected with the observed filamentation. (i) Cultures of *dnaK* mutants at 42°C stopped increasing in optical density at about the same time (2 h after the temperature upshift) that they exhibited their maximum filamentation. (ii) *dnaK* cells were killed by incubation at 42°C, and filamentation was irreversible after a shift back to the low temperature. (iii) The presence of a $dnaK^+$ plasmid restored normal cell division and cell survival at 42°C to *dnaK* mutants. (iv) Suppressors that allowed *dnaK* null mutants to grow at 42°C reduced the amount of filamentation. Nevertheless, our results were insufficient to determine whether filamentation is a direct or indirect consequence of the loss of the DnaK function. However, we have been able to show that filamentation is not mediated via SulA as it is in the SOS response. Despite the failure of the *sulB103* allele of *ftsZ* to block filamentation in *dnaK* mutants, it is possible that the key cell division protein FtsZ does play a role in this filamentation.

Although our results indicate that cells normally require DnaK function for growth at 42°C, this was not an absolute requirement since we were able to isolate suppressors, at a frequency of approximately 10^{-7}, that permitted growth at elevated temperatures. Since *dnaK* null mutants became blocked for DNA synthesis, RNA synthesis, and cell division after a shift to 42°C, the presence of these suppressors must have resulted in sufficient restoration of these major cellular processes to permit growth. One explanation of these observations is that these three processes are coupled and the suppressor is able to act at a single step to circumvent the normal requirement for DnaK function. Another explanation is that the suppressor bypasses the normal requirement for the DnaK function by affecting each process individually. Whatever the nature of the suppression, it did not reverse all of the phenotypes of *dnaK* mutants since the suppressed strains still failed to plate phage lambda. *E. coli dnaK756* mutants showed a high basal level of synthesis of heat shock proteins and failed to turn off the heat shock response at 42°C, continuing to synthesize the altered *dnaK756* gene product as well as other heat shock proteins (33). Reversion and transduction experiments have shown that the *dnaK756* mutation is responsible for the high basal level of heat shock protein synthesis and the prolonged synthesis of heat shock proteins (33). Our *dnaK* null mutants, both the deletion and the insertion mutants, exhibited the same phenotype as a *dnaK756* mutant. That is, they showed a high basal level of heat shock protein synthesis and a prolonged heat shock protein synthesis except for the DnaK protein, which was completely absent. Our results strongly suggest that the same phenotype as that of the *dnaK756* mutant was not caused by an alteration of DnaK function but rather by a lack of DnaK function, and these results confirm the suggestion of Tilly et al. (33) that DnaK functions as a negative regulator of the heat shock response in *E. coli*.

It is tempting to suggest that there may be a close connection between temperature-sensitive bacterial growth, accompanied by filament formation, and abnormal heat shock protein synthesis in *dnaK* mutants. It is also intriguing that *dnaK* mutants are defective in aspects of proteolysis (C. Dykstra and G. C. Walker, manuscript in preparation). Certain types of proteolytic protein cleavages may be important during some stages of the cell cycle, and these changes may be blocked in *dnaK* bacteria which do not divide at high temperatures.

Finally, our results provide an interesting example of a situation in which (i) the ability to isolate temperature-sensitive lethal alleles of a gene does not necessarily imply that the function of the gene is required at the nonrestrictive temperature and (ii) an inability of an organism carrying a disrupted gene to grow under a particular condition does not necessarily imply that the organism has an absolute requirement for the product of that gene.

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