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DnaK Mutants Defective in ATPase Activity Are Defective in Negative Regulation of the Heat Shock Response: Expression of Mutant DnaK Proteins Results in Filamentation

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Site-directed mutagenesis has previously been used to construct *Escherichia coli dnaK* mutants encoding proteins that are altered at the site of in vitro phosphorylation (J. S. McCarty and G. C. Walker, Proc. Natl. Acad. Sci. USA 88:9513–9517, 1991). These mutants are unable to autophosphorylate and are severely defective in ATP hydrolysis. These mutant *dnaK* genes were placed under the control of the *lac* promoter and were found not to complement the deficiencies of a Δ*dnaK* mutant in negative regulation of the heat shock response. A decrease in the expression of DnaK and DnaJ below their normal levels at 30°C was found to result in increased expression of GroEL. The implications of these results for DnaK's role in the negative regulation of the heat shock response are discussed. Evidence is also presented indicating the existence of a 70-kDa protein present in a Δ*dnaK52* mutant that cross-reacts with antibodies raised against DnaK. Derivatives of the *dnaK*$^+$ *E. coli* strain MC4100 expressing the mutant DnaK proteins filamented severely at temperatures equal to or greater than 34°C. In the *dnaK*$^+$ *E. coli* strain W3110, expression of these mutant proteins caused extreme filamentation even at 30°C. Together with other observations, these results suggest that DnaK may play a direct role in the septation pathway, perhaps via an interaction with FtsZ. Although Δ*dnaK52* derivatives of strain MC4100 filament extensively, a level of underexpression of DnaK and DnaJ that results in increased expression of the other heat shock proteins did not result in filamentation. The Δ*dnaK52* allele could be transduced successfully, at temperatures of up to 45°C, into strains carrying a plasmid expressing *dnaK*$^+$ *dnaJ*$^+$, although the yield of transductants decreased above 37°C. In contrast, with a strain that did not carry a plasmid expressing *dnaK*$^+$ *dnaJ*$^+$, the yield of Δ*dnaK52* transductants decreased extremely sharply between 39 and 40°C, suggesting that DnaK and DnaJ play one or more roles critical for growth at temperatures of 40°C or greater.

Escherichia coli, in common with many organisms, responds to sudden shifts in temperature by transiently inducing members of the heat shock regulon, which consists of genes and operons that are under the transcriptional regulation of the heat shock sigma factor σ^{32} (42). By genetic analysis, this regulon has been shown to be negatively regulated by three of these heat shock proteins, DnaK, DnaJ, and GrpE. *dnaK*, *dnaJ*, and *grpE* mutants have the phenotype of overproducing the proteins of the heat shock regulon (50). The primary mechanisms for this negative regulation appear to be effects on the level of synthesis, stability, and activity of σ^{32} (19, 49–51, 53). Recently, the physical interaction of DnaK, DnaJ, and GrpE with σ^{32} has been demonstrated (17, 28).

Models that incorporate roles for DnaK, DnaJ, and GrpE have been proposed to account for the cellular mechanism that detects and responds to changes in environmental temperature. The homeostatic model proposed by Craig and Gross invokes a titration of DnaK, DnaJ, and GrpE away from σ^{32} by denatured or malfolded proteins that arise as a consequence of cellular stress (13). This is hypothesized to lead to an increased level of unbound σ^{32} and the subsequent increase in heat shock protein expression. In a variation on this theme, Gamer et al. have recently proposed a separate model invoking titration of chaperones and suggest that DnaJ may also have a central role in this coupling of chaperone substrates with the regulation of σ^{32} (17). As a distinct mechanism, we have proposed that DnaK may be capable of responding directly to increases in temperature as part of its regulation of heat shock protein expression (36). The highly temperature-responsive nature of DnaK may be integral to the mechanisms behind the rapid increase in heat shock protein expression in response to temperature shifts (36). Evidence supporting each model has been published (13, 17, 36, 44), and as these modes of regulation are not mutually exclusive (7, 36), each may play a role in integrating cellular responses to changes in environmental conditions.

Genetic analyses have identified other physiological processes in addition to negative regulation of the heat shock response in which DnaK plays a role under nonstress conditions. For some of these processes, experiments with purified DnaK have identified activities that may be relevant to these in vivo functions. For example, DnaK is required for the replication of λ DNA in vivo and has been shown in vitro both to bind to the λ P protein (30) and to play a role in dissociation of the λ P protein from the λ initiation complex, leading to activation of the DnaB helicase (1, 29, 37). Also, DnaK facilitates the maintenance of P1 miniplasmids in vivo and is known to be required in vitro for converting P1-encoded RepA dimers to monomers (55, 56). The monomeric form of RepA has been shown to be active in binding the origin of replication of these miniplasmids in vitro (56) and is essential for P1 replication (54). These in vitro processes which require DnaK have also been shown to require the hydrolysis of ATP. DnaK that has been highly purified has been shown to possess a very weak ATPase activity (6, 36) that is activated under certain condi-

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tions, suggesting that ATP hydrolysis by DnaK is essential for its activities in vivo and in vitro.

Recently, we reported the use of site-directed mutagenesis to construct *dnaK* mutants encoding proteins that are altered at the site of in vitro autophosphorylation, threonine 199 (36). These mutant DnaK proteins, which were unable to autophosphorylate, were also found to be severely defective in the hydrolysis of ATP. All three mutant DnaK proteins (DnaK T199D, DnaK T199A, and DnaK T199V) were found to purify to homogeneity in a manner indistinguishable from the wild type and were capable of binding ATP, as judged by retention on ATP-agarose (36). Biophysical characterizations of one of these mutant proteins, DnaK T199A, showed that its physical properties were essentially identical to those of wild-type DnaK protein (44). These included thermal stability as monitored by far-UV circular dichroism, the ability to refold upon lowering of temperature, and ATP-induced conformation change as monitored by tryptophan fluorescence. Together, these studies indicate that these three mutant proteins are very similar to the wild-type DnaK protein except that they are severely defective in ATPase activity and are unable to autophosphorylate.

During purification, both the wild-type and mutant DnaK proteins appear in multiple forms during purification (35). Data from two different gel filtration columns, an anion exchange column, and glutaraldahyde cross-linking experiments indicated that both the mutant and wild-type DnaK proteins purified in at least two forms as well as in a fraction that contained DnaJ (35). Other workers have observed higher-molecular-weight forms of DnaK during purification (18), including a form that appeared to be dimeric DnaK (44). The existence of these multiple forms raises the possibility that oligomerization of DnaK may be important for at least some of its activities and also that *dnaK* mutations that alter the residue at position 199 might exhibit negative dominance (21) over *dnaK*$^+$. Recently, certain other alleles of *dnaK* were reported to be dominantly negative over *dnaK*$^+$ (6, 57).

We have been interested in using *dnaK* mutants encoding proteins altered at residue 199 to investigate the requirement for DnaK ATPase activity in vivo. The physiological effects of these mutant proteins are of particular interest, since these DnaK derivatives are altered at a residue localized in the interior and are defective in ATPase activity but appear to be normal by a number of biophysical criteria. In this article, we describe experiments in which we examined the effect of expressing these mutant DnaK proteins in both Δ*dnaK52* and *dnaK*$^+$ backgrounds on the regulation of the heat shock response of *E. coli*. In addition, we provide evidence to suggest that DnaK is directly involved in septation and also describe the requirements for DnaK in cellular growth as a function of incubation temperature.

MATERIALS AND METHODS

Media and growth conditions. Strains containing plasmids carrying mutant and wild-type *dnaK* alleles were maintained on plates and in liquid LB medium that contained 0.2% glucose and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.6). Plasmids were maintained by addition of kanamycin and ampicillin to the medium at the concentrations described before (32). Isopropylthiogalactopyranoside (IPTG) was made up as a 100 mM stock solution and sterile filtered. Cell labeling experiments were performed in M9 glucose minimal salt medium containing vitamin B_1 (0.0005%), arginine (40 μg/ml), and threonine (40 μg/ml) (38). Incubator temperatures were closely monitored, particularly around 39 and 40°C. Two separate thermometers were used, one exposed to air and another in a flask of water.

Microscopic techniques. For light microscopic examination, cells from fresh colonies (unless otherwise indicated) grown on LB agar plates were prepared on glass slides with a drop of liquid LB medium and observed in a microscope (Axioplan Universal; Zeiss) with a Plan 100 objective and Nomarski differential interference contrast optics. Photographs were taken of representative cells from multiple colonies with Tri-X-Pan 400 films (Eastman Kodak Co., Rochester, N.Y.) at 1600 ASA.

Strains and strain construction. Plasmids were transformed by protocols described before (32) or with an electroporation apparatus (Bio-Rad) by the manufacturer's instructions. Δ*dnaK52*::Cm^r was transduced as described before (38) by using P1 *vir* lysates of strain GW8306 grown in 0.5 mM IPTG at 30°C. Δ*dnaK52* strains and Δ*dnaK52* strains expressing mutant DnaK proteins were taken directly from the agar plates used to select transductants of the Δ*dnaK52* allele so as to minimize the chance of enriching for strains containing suppressors that arise spontaneously (8). These strains were grown to the late log stage in M9 minimal medium containing 0.5 mM IPTG and the drugs required to maintain plasmids and to maintain selection for the Δ*dnaK52* allele prior to being subcultured for protein labeling experiments. GW8319 was constructed by transducing the Δ*dnaK52*::Cm^r allele into GW8305 at 30°C.

Efficiency of P1 transduction of the Δ*dnaK52* allele. The ability of a strain to be transduced with the Δ*dnaK52* allele was determined as a function of the incubation temperature after transduction. *dnaK*$^+$ strains containing the appropriate plasmids were grown to late log stage in LB containing 5 mM $CaCl_2$. Cells were immediately mixed with 1/10 volume of the P1 lysate described above. After 10 min at room temperature, the cells were spun at 5,000 rpm in a Sorvall centrifuge for 5 min at 4°C. The pellet was resuspended in an equivalent volume of the original culture in LB containing 10 mM sodium citrate at 4°C. The cells were pelleted again at 4°C and resuspended in 1/10 volume of the original culture in LB (10 mM sodium citrate) at 4°C. Then 50 μl was aliquoted from the same transduction mixture for each strain and spread on plates containing chloramphenicol (30 μg/ml), ampicillin (100 μg/ml), and kanamycin (50 μg/ml). These plates were immediately placed in a loosely closed box within incubators at the various temperatures. The relative efficiency of transduction at a given temperature was calculated by dividing the number of colonies arising by the maximum number of colonies obtained at any incubation temperature for that strain.

Plasmid constructions. Enzymes were used as specified by the manufacturers, and all DNA manipulations were done as described before (32). Site-directed mutagenesis was performed with the Muta-Gene kit (Bio-Rad) to introduce a *Bam*HI site ca. 230 bp upstream of the start codon and upstream of the operator sequence for the lactose operon promoter (P_{lac}) in the sequence of the Bluescript vector SK+ (Stratagene). This *Bam*HI site was introduced independently into the vector portion of plasmids pJM2, pJM3, pJM4, and pJM5 (encoding wild-type DnaK, DnaK T199D, DnaK T199A, and DnaK T199V, respectively [36]), resulting in plasmids pJM11, pJM12, pJM13, and pJM14, respectively. The *Bam*HI fragment from these vectors containing the *dnaKJ* operon and the entire P_{lac} sequence contained on the Bluescript SK+ vector was subcloned into the pBR322 *Bam*HI site. This procedure yielded plasmids pJM41, pJM42, pJM43, and pJM44, encoding wild-type DnaK, DnaK T199D, DnaK T199A, and DnaK T199V, respectively. Clones of each plasmid

that had the *dnaKJ* operon oriented so that the direction of transcription was opposite that of the *bla* gene were selected. Plasmid pJM100 is a derivative of pACYC177 that encodes Km^r but not Ap^r and carries the gene encoding $LacI^q$. It was constructed by first disrupting the coding sequence for Amp^r by digestion with *Aha*II, which cuts twice within the *bla* gene, and religation. The gene encoding $LacI^q$ was subcloned from pMJR1560 (Amersham) by digestion with *Eco*RI and *Hin*dIII and blunt-ended with the Klenow fragment of DNA polymerase I. This fragment was blunt-end ligated into the *Bam*HI site of the Amp^s derivative of pACYC177.

Production of antibodies directed against DnaK. DnaK was overproduced from plasmid pBB1 (36) and purified as described (11). The purified DnaK was then cut out from a 10% polyacrylamide gel, and the gel piece was macerated and injected directly into rabbits. In order to eliminate cross-reactive antibodies, the resulting serum was affinity purified. A 50% ammonium sulfate cut of the cleared rabbit serum was passed over a column containing purified DnaK (36) cross-linked to CNBr-activated Sepharose (Pharmacia) by the manufacturer's directions. The column was thoroughly washed with a buffer containing 200 mM Tris (pH 7.5) and 200 mM NaCl until the optical density at 280 nm (OD_{280}) was <0.05. Antibodies were eluted with glycine (pH 2.3) and collected in tubes containing 0.5 volume of 1 M Tris buffer (pH 7.5). The fractions of significant optical density were pooled and dialyzed against buffer containing 200 mM Tris (pH 7.5), 200 mM NaCl, and 0.02% sodium azide. The resulting antibodies were extremely specific for DnaK. Optimal conditions included antibody dilutions of 1:1,000 on proteins blotted from cells (1 ml at an OD_{600} of 0.5) separated on 10% polyacrylamide gels (14 by 18 cm) and the Western (immunoblot) hybridization conditions described previously (43). Under these conditions, the purified antibodies gave strong signals to DnaK from wild-type strains but no reaction to any other proteins from Δ*dnaK52* strains. In contrast, at a high concentration of antibodies, with a 10-fold-higher concentration of proteins, and during immunoprecipitations with excess antibodies, some cross-reacting proteins were observed.

Western blot analyses of DnaK. Cellular concentrations of DnaK were determined by quantitative Western blot analysis. For these determinations of DnaK expression, plasmids carrying the wild-type and mutant *dnaK* alleles were transformed into a Δ*dnaK52 sidB1* strain (BB1553). This strain does not contain any protein that cross-reacts with the affinity-purified antibodies described above unless the antibodies or the proteins being probed are in a concentrated form. In addition, these strains carry a suppressor, *sidB1*, that allows a normal cell growth rate and nonfilamentous growth of Δ*dnaK52* strains (8). Strains were grown in LB liquid or M9 glucose minimal medium to the mid-log stage under inducing conditions, subcultured in identical medium, and grown to an OD_{600} of ca. 0.3. An aliquot was added to an equal volume of 2× loading buffer (27), boiled for 5 min, and spun in an Eppendorf centrifuge for 10 min. Cell extracts were loaded on 10% polyacrylamide gels, varying the volumes to correct for slight differences in optical density of the cultures. Proteins were then separated by polyacrylamide gel electrophoresis (PAGE) (27) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore) by the manufacturer's directions (Bio-Rad). DnaK was visualized by probing with antibodies specific to DnaK, and Western blots were developed with a chemiluminescence system (Tropix) with the reagents and secondary antibodies supplied by the manufacturer. Quantification of DnaK was performed by comparing the intensity of bands on X-ray film as a function of exposure time and by scanning bands with a densitometer (LKB 2202 Ultroscan Laser Densitometer).

Immunoprecipitation. Immunoprecipitations were done as described before (61) except that 1% Nonidet P-40 was used instead of 1% Brij. Antibodies and bound proteins were precipitated by the addition of Formalin-fixed *Staphylococcus aureus* cells (Boehringer Mannheim) followed by centrifugation. The pellet was washed in a buffer composed of the separate buffers used in the cell lysis in the same ratio described (61). The pellet was resuspended in 1× loading buffer, boiled for 5 min, and centrifuged for 10 min.

Temperature-induced protein synthesis. The procedures used to examine temperature-induced protein synthesis were adapted from a previously published procedure (58). Cell cultures were grown to the late log stage in M9 minimal medium under inducing conditions and subcultured at least 1/100 into the same medium. Temperature shifts were performed when the OD_{600} was between 0.2 and 0.35. An equal volume of identical, warm medium was added, and the flask was transferred to an incubator at the higher temperature. Medium at 54°C was added in experiments to examine the response to a shift from 30 to 42°C, and 70°C medium was added to cultures in experiments to examine the response to a shift from 30 to 50°C. At the indicated times after addition of warm medium, 1-ml samples were placed in warmed tubes containing 10 μCi of [^{35}S]methionine at 42°C (or 50°C) with agitation. After incubation for 1 min, 50 μl of warm methionine (10 mg/ml) was added, and the tube was incubated for 1 min with agitation before transfer to ice. Steady-state protein expression at 30 and 37°C was monitored by the same 1-min labeling protocol.

For the experiments examining heat shock protein expression, steady-state expression at 30°C was determined ca. 5 min prior to the temperature shift by aliquoting 0.5 ml of culture into 0.5 ml of warm medium containing 10 μCi of [^{35}S]methionine. Protein was precipitated by addition of 1/10 volume of 100% trichloroacetic acid followed by incubation on ice for at least 30 min. Protein was pelleted by spinning for at least 30 min in an Eppendorf centrifuge and washed with acetone at −20°C. Pellets were dried and resuspended in 100 ml of 1× loading buffer, boiled for 5 min, vortexed, and spun in an Eppendorf centrifuge for 10 min. The specific activity of the lysate was determined by counting an aliquot in Hydrofluor (National Diagnostics) with a scintillation counter (Beckman). Proteins were analyzed by separation by 10% PAGE (27). For each sample, 500,000 cpm was loaded per lane, and bands were visualized by autoradiography with Kodak X-Omat films.

RESULTS

Construction of strains that express mutant and wild-type DnaK proteins under control of the *lac* promoter. The level of expression of DnaK in *E. coli* is altered as a function of the ambient temperature (20). Previously characterized *dnaK* mutants have been shown to express increased levels of heat shock proteins under non-heat-stress conditions (52), and DnaK has been demonstrated to be involved in negative regulation of the heat shock response (50). Thus, it was possible, a priori, that expression of mutant DnaK proteins altered at residue 199 might result in defects in the regulation of heat shock protein synthesis and consequently in DnaK expression as well. Such an effect would have made a careful characterization of the defects resulting from the expression of the mutant DnaK proteins in *E. coli* difficult, because phenotypes resulting from changes in the level of DnaK expression could not then be

unambiguously distinguished from phenotypes resulting more directly from defects in DnaK activity.

Therefore, we chose to carry out our physiological studies of the behavior of these mutant DnaK proteins in regulation of the heat shock response with a system in which expression of the mutant DnaK proteins was controlled by a heterologous promoter. The system we used consisted of pBR322-derived plasmids in which the wild-type or mutant *dnaK* coding sequences (as well as the wild-type *dnaJ* coding sequence) were directly fused to the start codon of the *lacZ* gene and were thus under the transcriptional control of the P_{lac} promoter. The $dnaJ^+$ gene was included in these constructions because the level of DnaJ in Δ*dnaK52* strains is significantly lowered than in wild-type strains (35). The LacI repressor was encoded by a second compatible plasmid (pJM100, containing $lacI^q$), so that expression of the DnaK and DnaJ proteins could be controlled by the concentration of IPTG added to the medium. As described below, this combination of vector copy number and regulated promoter allowed us to find conditions under which the expression of DnaK protein approximated that of DnaK expressed from its own chromosomal promoter at 30°C. This system allowed us to carry out experiments in which both the wild-type and mutant DnaK proteins were expressed at the same level irrespective of any effects of the *dnaK* mutations on the regulation of the heat shock response. In addition, this system enabled us to examine the physiological consequences of expressing DnaK at lower than normal levels.

Restriction fragments encoding wild-type and mutant *dnaK* sequences under P_{lac} control were subcloned from the Bluescript SK+ vector (Stratagene) (36) to the lower-copy-number pBR322 vector. The Bluescript vectors are derived from the pUC vectors, which are high copy number and, furthermore, increase in copy number with increased temperature (41), an undesirable property in these studies. The fragments from the Bluescript plasmids encoding wild-type DnaK and the mutant proteins DnaK T199D, DnaK T199A, and DnaK T199V under P_{lac} control were subcloned into pBR322 to yield plasmids pJM41, pJM42, pJM43, and pJM44, respectively. In addition, these plasmids express wild-type DnaJ, since the entire coding sequence of the *dnaKJ* operon was subcloned. In all cases, clones in which the *dnaKJ* operon was in the opposite orientation from the *bla* gene were identified by restriction analysis. The resulting plasmids were transformed into a $dnaK^+$ strain (MC4100) carrying a compatible plasmid (pJM100) that expresses $LacI^q$ (Table 1).

Expression of mutant and wild-type DnaK from pBR322-derived plasmids. We established the concentration of IPTG that resulted in a level of expression of the plasmid-encoded DnaK that was equal to the level of expression of DnaK in a wild-type strain growing logarithmically at 30°C. We chose this temperature because it is the incubation temperature for many experiments examining the in vivo characteristics of *dnaK* mutants. In the experiments reported here, 30°C was the initial temperature used when examining the transient induction of heat shock protein synthesis in response to rapid increases in temperature. In addition, P1-mediated transduction of the Δ*dnaK52* allele is efficient at this temperature. The expression of wild-type DnaK from the P_{lac}-$dnaK^+$ plasmid pJM41 was determined in a Δ*dnaK52* strain (GW8306) as a function of the IPTG concentration and compared with that of the corresponding $dnaK^+$ strain MC4100. This Δ*dnaK52* strain also contains the *sidB1* suppressor, an allele of *rpoH* that allows approximately normal growth at 30°C (8). The level of DnaK produced in these strains was determined by a chemiluminescence Western hybridization system (Tropix).

We determined that 0.5 mM IPTG in liquid LB medium (Fig. 1) or liquid M9 glucose minimal medium (Fig. 2) resulted in levels of DnaK expression approximately equal to those resulting from the expression of DnaK under its wild-type promoter in strain MC4100 at 30°C. Further increases in the concentration of IPTG added to the medium resulted in only minor increases in expression of DnaK from the plasmid. Therefore, the addition of amounts of IPTG greater than 0.5 mM did not result in a level of expression of DnaK from the plasmid carrying P_{lac}-$dnaK^+$ that was equivalent to that seen in a wild-type strain at 42°C.

In addition, we determined that, at 0.5 mM IPTG, each of the mutant DnaK proteins was expressed to the same level as the wild-type DnaK protein expressed from the corresponding plasmid, indicating that these DnaK mutant proteins were not substantially altered in stability (Fig. 2). The similarity of the stabilities of the mutant and wild-type DnaK proteins is consistent with the similar biophysical properties of these proteins in vitro (44). Because *dnaK* is in an operon with *dnaJ* and the Δ*dnaK52* allele also results in significantly lowered expression of DnaJ (35), expression of DnaJ from the plasmid with 0.5 mM IPTG should also result in DnaJ levels near those found in a wild-type strain at 30°C.

Heat shock regulation by wild-type DnaK under the transcriptional control of P_{lac} from a plasmid. We examined the regulation of GroEL in a Δ*dnaK52* strain expressing wild-type DnaK from plasmid pJM41 with 0.5 mM IPTG added to M9 glucose minimal medium. Two aspects of the heat shock response were examined: (i) steady-state expression at 30°C and (ii) steady-state expression at 42°C (determined after 30 min at 42°C). We found that the steady-state expression of GroEL at 30°C under these conditions (Fig. 3C) was indistinguishable from that seen in a wild-type strain (Fig. 3A). This supports the conclusion that the expression of DnaK at 30°C from these plasmids in response to 0.5 mM IPTG (Fig. 3C) approximates the expression of DnaK in a wild-type strain (Fig. 3A) at this temperature. In addition, after shift in temperature from 30 to 42°C, normal induction of GroEL was

TABLE 1. Bacterial strains

Strain	Genetic markers	Source or reference
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*	10
BB1553	MC4100 Δ*dnaK52 sidB1*	8
W3110	F⁻ λ⁻ *IN*(*rrnD-rrnE*)*1*	B. Bachman
GW8301	MC4100(pJM100)	This study
GW8302	BB1553(pJM100)	This study
GW8303	GW8301(pBR322)	This study
GW8304	GW8302(pBR322)	This study
GW8305	GW8301(pJM41)	This study
GW8306	GW8302(pJM41)	This study
GW8307	GW8301(pJM42)	This study
GW8308	GW8302(pJM42)	This study
GW8309	GW8301(pJM43)	This study
GW8310	GW8302(pJM43)	This study
GW8311	GW8301(pJM44)	This study
GW8312	GW8302(pJM44)	This study
GW8313	W3110(pJM100)	This study
GW8314	GW8313(pBR322)	This study
GW8315	GW8313(pJM41)	This study
GW8316	GW8313(pJM42)	This study
GW8317	GW8313(pJM43)	This study
GW8318	GW8313(pJM44)	This study
GW8319	GW8305 Δ*dnaK52*	This study
BB1130	MC4100 Δ*dnaK52 sidA1*	8

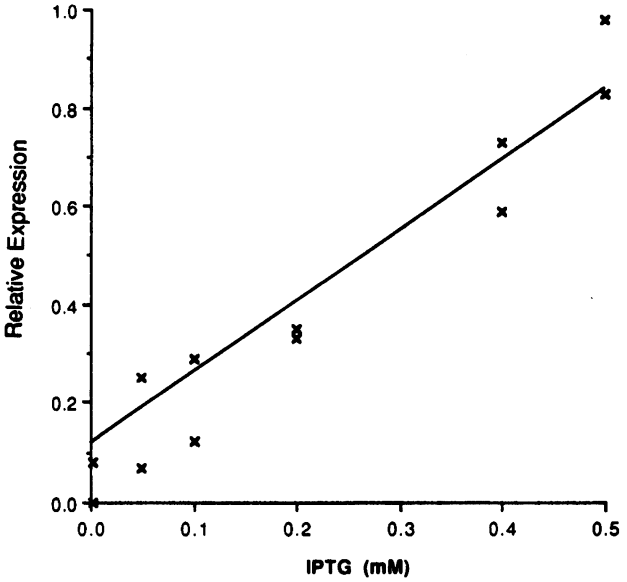

FIG. 1. Expression of wild-type DnaK proteins in a Δ*dnaK52* strain (GW8306), which carries P_{lac}-*dnaK*$^+$ on pBR322, as a function of IPTG concentration. Cells were grown in LB medium at 30°C with appropriate drugs and the indicated levels of IPTG to the late log stage and then diluted approximately 1:1,000 into the same medium. An aliquot was taken when the cells reached an OD_{600} of ca. 0.4, mixed with an equal volume of 2× loading buffer, and prepared as described in Materials and Methods. The amount of cellular material loaded per lane was adjusted for the optical density of the culture. Western hybridization blots were developed with a chemiluminescence system (Tropix Western Light), and exposed films were quantitated with a densitometer. The relative amounts of DnaK protein are expressed as a fraction of that observed for wild-type protein in an MC4100 strain at 30°C.

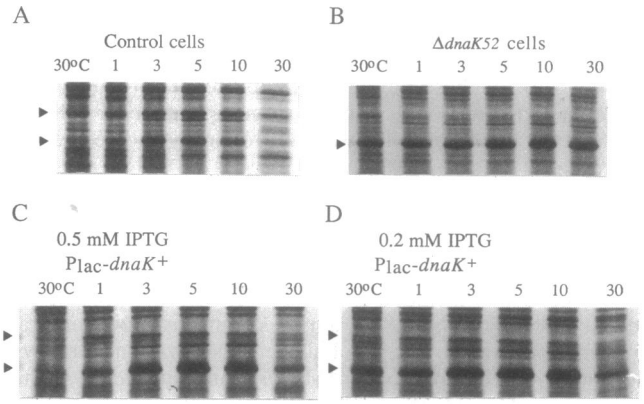

FIG. 3. One-minute [^{35}S]methionine labeling of cells before and after a 30 to 42°C heat shock. Cells were grown in M9 glucose minimal medium containing appropriate drugs and the indicated concentrations of IPTG to an OD_{600} of approximately 0.2 to 0.35. Aliquots were taken before heat shock (first lane) or 1, 3, 5, 10, and 30 min after the addition of an equal volume of identical medium at 54°C. After 1 min of incubation with agitation in the presence of [^{35}S]methionine, 50 μl of methionine (10 mg/ml) was added, and the agitation was continued for 1 min before the culture was placed on ice. Proteins were analyzed as described in Materials and Methods. (A) Wild-type *dnaK*$^+$ strain GW8303; (B) a fresh Δ*dnaK52* transductant of GW8303; (C and D) Δ*dnaK52* strain carrying P_{lac}-*dnaK*$^+$ (GW8319) induced with 0.5 mM (C) or 0.2 mM (D) IPTG. The upper arrow indicates DnaK, and the lower arrow indicates GroEL.

observed, with maximum induction at ca. 5 min. However, the steady-state expression of GroEL at 42°C was higher than in a wild-type strain. This was the expected result, given that DnaK's role in the negative regulation of the heat shock response is probably dependent on the concentration of DnaK in the cell. At 42°C, the expression of DnaK from the plasmid encoding P_{lac}-*dnaK*$^+$ is less than that observed in wild-type *E. coli*.

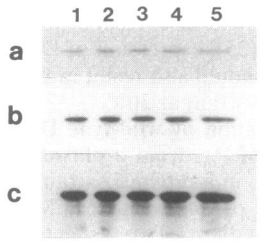

FIG. 2. Expression of wild-type DnaK in MC4100 and expression of wild-type and mutant DnaK in Δ*dnaK52 sidB1* MC4100 strains that carry pBR322 encoding P_{lac}-*dnaK* derivatives induced with 0.5 mM IPTG in M9 glucose minimal medium at 30°C. Panels a, b, and c are exposures of (approximately) 1 s, 5 s, and 20 s, respectively, of a chemiluminescence Western hybridization analysis (Tropix Western Light system) with affinity-purified anti-DnaK antibodies. Each lane had an equivalent cell density from the following strains: lane 1, MC4100; lane 2, GW8306 (expressing wild-type DnaK); lane 3, GW8308 (expressing DnaK T199D); lane 4, GW8310 (expressing DnaK T199A); lane 5, GW8312 (expressing DnaK T199V).

DnaK expression from P_{lac}-*dnaK*$^+$ appears to be regulated in response to heat shock. Interestingly, the expression of DnaK from a plasmid under the transcriptional control of P_{lac} appeared to increase transiently during a heat shock shift from 30 to 42°C, although not to the extent found in a wild-type strain after a similar heat shock. This result was surprising, given that the transcription of DnaK in this strain is under the control of P_{lac}.

There are several possible explanations for this apparent increase in expression. First, the transcription of P_{lac}-*dnaK*$^+$ may increase transiently, perhaps a result of fluctuations in the copy number or topology of the pJM41 plasmid due to the temperature shift. Another possibility is that the expression of DnaK may be controlled posttranscriptionally, perhaps at the level of translation, as is the case for σ^{32} (24, 40).

An additional possibility is that there may be another protein that has a molecular mass identical to that of DnaK (70 kDa) that is also increased in this strain. We observed a protein that is expressed weakly (Fig. 4A, lane 3) during a 30 to 50°C heat shock treatment in a Δ*dnaK52* strain that migrated similarly to DnaK (Fig. 4A, lane 1) on 10% PAGE. Under these conditions, the heat shock proteins are strongly induced but very few other proteins are expressed (42). Immunoprecipitation analysis with affinity-purified DnaK antibodies indicates that there is a protein of the same molecular weight as DnaK present in a Δ*dnaK52 sidB1* strain grown at 30°C (Fig. 4B, lane 4) that cross-reacts with these antibodies. A protein of this molecular weight is also observed when these affinity-purified DnaK antibodies were used in Western hybridization analyses of membranes prepared from gels that had been heavily loaded with a lysate of a Δ*dnaK52* strain, BB1130, grown at 37°C (data not shown). These results suggest that the protein induced during the 30 to 50°C heat shock may also be

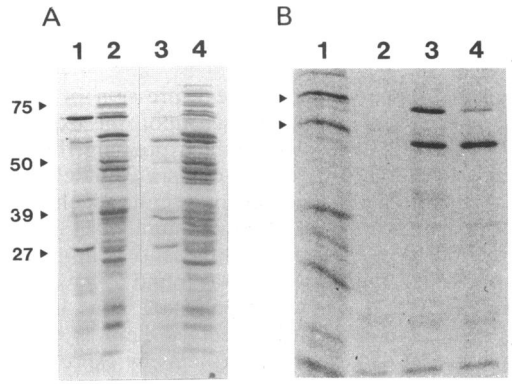

FIG. 4. Expression of proteins after a 30 to 50°C heat shock and analysis of proteins precipitating with purified anti-DnaK antibodies. (A) Autoradiogram of proteins labeled for 1 min with [^{35}S]methionine that are expressed at 30°C (lanes 2 and 4) or 5 min after a shift in temperature from 30 to 50°C (lanes 1 and 3) by the addition of an equal volume of identical medium at 70°C. Lanes 1 and 2 are extracts of a *dnaK*$^+$ strain (MC4100), and lanes 3 and 4 are extracts of a Δ*dnaK52* strain (BB1130). DnaK is the most prevalent band in lane 1 and is about 70 kDa. The approximate molecular weights are indicated on the left (in thousands) and were from Bio-Rad low-molecular-weight standards. (B) Autoradiogram of proteins labeled for 1 min with [^{35}S]methionine. Immunoprecipitated proteins labeled in the *dnaK*$^+$ strain MC4100 (lane 3) grown at 30°C to mid-log stage and a Δ*dnaK52* strain (BB1553, lane 4) grown at 30°C to mid-log stage. Immunoprecipitations were carried out as described in Materials and Methods with affinity-purified anti-DnaK antibodies. Lane 1 shows the total proteins labeled after a 30 to 50°C heat shock of MC4100 as in lane 1 of panel A. Lane 2 is an immunoprecipitation reaction performed on MC4100 with no added antibodies. The upper arrow indicates DnaK, and the lower arrow indicates GroEL.

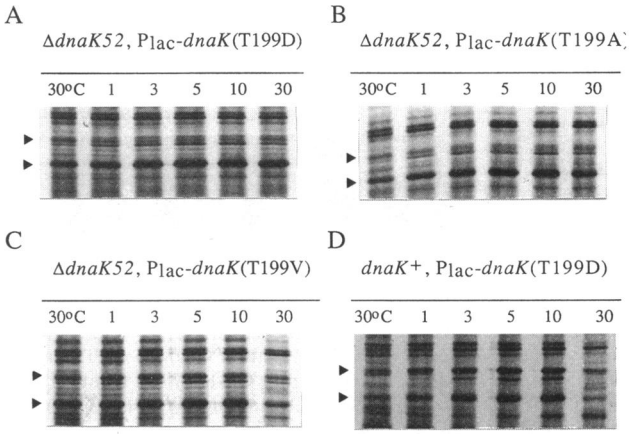

FIG. 5. One-minute [^{35}S]methionine labeling of strains expressing mutant DnaK proteins before and after a 30 to 42°C heat shock. Cells were grown in M9 glucose minimal medium containing appropriate drugs and 0.5 mM IPTG to an OD_{600} of approximately 0.2 to 0.35. Aliquots were taken before heat shock (first lane) or 1, 3, 5, 10, and 30 min after the addition of an equal volume of identical medium at 54°C. After 1 min of incubation with agitation in the presence of [^{35}S]methionine, 50 ml of methionine (10 mg/ml) was added, and the agitation was continued for 1 min before the culture was placed on ice. Proteins were analyzed as described in Materials and Methods. Shown are fresh Δ*dnaK52* transductants of (A) GW8307 (expressing DnaK T199D), (B) GW8309 (expressing DnaK T199A), (C) GW8311 (expressing DnaK T199V), and (D) GW8307, a derivative of a *dnaK*$^+$ strain expressing DnaK T199D. The upper arrow indicates DnaK, and the lower arrow indicates GroEL.

recognized by antibodies made and affinity purified against DnaK.

Thus, it appears that a minor 70-kDa protein normally masked by DnaK is expressed both at 30°C and after a shift from 30 to 50°C. However, unless the expression of this protein is higher in strains containing DnaK than in Δ*dnaK52* cells, the expression of this minor protein as observed in a Δ*dnaK52* strain would not be sufficient to result in the apparent increase in DnaK expressed from P_{lac} after a 30 to 42°C heat shock. Therefore, the increase in expression observed at the region of the gel containing DnaK may also result from an increase in translation of the *dnaK* message or a transient alteration in transcription of *dnaK* from P_{lac} as a function of temperature.

Underexpression of DnaK leads to defects in regulation of the heat shock response. We took advantage of the availability of the Δ*dnaK52* strain carrying the P_{lac}-*dnaK*$^+$*J*$^+$ construction to investigate heat shock protein expression under conditions in which DnaK and DnaJ were present at lower-than-normal levels. We found that there were significant defects in the regulation of GroEL expression when DnaK was induced with only 0.2 mM IPTG added to the medium (Fig. 3D). We had previously determined that, during induction of DnaK$^+$ from P_{lac}-*dnaK*$^+$ with 0.2 mM IPTG, only about one-third the normal expression of DnaK observed at 30°C is obtained (Fig. 1). The level of GroEL was increased under steady-state conditions at both 30 and 42°C (Fig. 3D). No major defects were noted in the time and extent of maximum GroEL induction in response to a 30 to 42°C heat shock. Within the limits of detection and with the time points shown, induction was maximum at ca. 5 min.

Alleles of *dnaK* encoding DnaK derivatives altered at residue 199 do not complement Δ*dnaK52* in negative regulation of the heat shock response. The derivatives of DnaK altered at the site of autophosphorylation (DnaK T199D, DnaK T199A, and DnaK T199V) are defective in ATPase activity but appear by a number of biophysical criteria to be similar to wild-type DnaK protein (44). We found that in Δ*dnaK52* strains expressing these mutant DnaK proteins from plasmids, the steady-state level of expression of GroEL at both 30 and 42°C was greatly increased (Fig. 5) compared with that in a similar strain expressing wild-type DnaK from a plasmid. The elevated levels of GroEL expression observed in this experiment were similar to those seen at 30°C and at 42°C in a Δ*dnaK52* strain containing only pBR322 vector sequence and the vector encoding LacI^q (pJM100).

A Δ*dnaK52* strain does not appear to exhibit a transient increase in GroEL in response to a temperature shift, as seen in a wild-type strain, indicating that there is no regulation in response to a heat shock (Fig. 3B). Expression of GroEL, however, may be greater at 42 than at 30°C, although this appears to be manifested as a gradual increase in GroEL expression after the shift in temperature. The Δ*dnaK52* strains expressing the mutant DnaK proteins, however, exhibit a slight increase in expression of GroEL at ca. 10 min after a heat shock. This may indicate that these mutant DnaK proteins retain a limited capacity to negatively regulate heat shock protein synthesis in response to a shift in temperature. However, these mutant DnaK proteins do not function normally in the regulation of GroEL expression under any conditions tested.

Expression of mutant DnaK in a wild-type strain leads to defects in steady-state regulation of heat shock protein synthesis. The observation that DnaK may exist as oligomers (18,

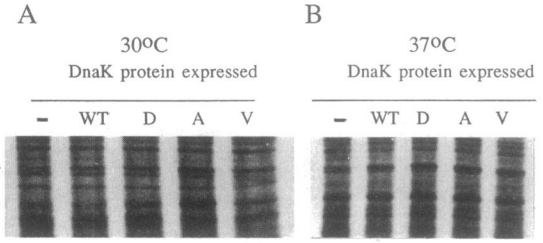

FIG. 6. One-minute [^{35}S]methionine labeling of a *dnaK*$^+$ strain and *dnaK*$^+$ strains expressing wild-type and mutant DnaK proteins from plasmids during steady-state growth at (A) 30°C and (B) 37°C. Proteins were labeled and analyzed as described in Materials and Methods. Lanes: —, no plasmid encoding DnaK, strain GW8303; WT, DnaK$^+$, strain GW8305; D, DnaK T199D, strain GW8307; V, DnaK T199V, strain GW8309; and A, DnaK T199A, strain GW8311. The upper arrow indicates DnaK, and the lower arrow indicates GroEL.

35, 44) and the observation of dominance in other alleles of *dnaK* (6, 57) raised the possibility that the *dnaK* mutations encoding DnaK proteins altered at residue 199 might exhibit negative dominance (21) over *dnaK*$^+$. Therefore, we examined expression of GroEL in *dnaK*$^+$ strains expressing the mutant DnaK proteins DnaK T199D, DnaK T199A, and DnaK T199V from plasmids. We found that the mutant DnaK proteins altered at the site of phosphorylation and defective in ATPase activity appear to be partially dominant over the wild type with respect to DnaK's activity in the negative regulation of the heat shock response. At 30°C, expression of these proteins from plasmids with 0.5 mM IPTG in *dnaK*$^+$ strains results in increased expression of GroEL as well as an increased intensity in the band on the gel in the region of DnaK (Fig. 6A). Scanning densitometry measurements indicated that this represented two- to threefold overproduction of DnaK and GroEL relative to the levels in the wild type, depending on the mutant DnaK expressed (data not shown). This apparent increase in the expression of DnaK probably results from increased expression of wild-type DnaK located on the chromosome, which contains a wild-type promoter subject to σ^{32}-directed transcription (60). This conclusion is supported by the observation that a similar increase in this band is not observed after expression of the mutant DnaK proteins in the Δ*dnaK52* strains (Fig. 5). Therefore, this dominant effect of the mutant DnaK proteins is occurring under conditions in which the level of wild-type DnaK is apparently greater than the level of the mutant DnaK protein.

There also appears to be a slight increase in the expression of GroEL during growth at 37°C for strains expressing mutant DnaK from plasmids induced with 0.5 mM IPTG (Fig. 6B). This less obvious defect in the regulation of GroEL at 37°C than at 30°C could result from lower stoichiometry of mutant protein to wild-type DnaK at 37°C. The temperature dependence of wild-type DnaK expression from the chromosome (20) predicts that the level of wild-type DnaK will increase at this temperature. In addition, the dominance effect itself may be dependent on the temperature.

In contrast, the expression in a *dnaK*$^+$ strain of wild-type DnaK from plasmids in response to 0.5 mM IPTG in the medium leads to a slight decrease in the expression of GroEL at 30°C. This is consistent with the demonstrated role of DnaK in the negative regulation of the heat shock response (50, 52). In addition, it supports the conclusion that the observed increase in expression of GroEL in wild-type strains expressing the mutant DnaK proteins results from defects in DnaK activity, as opposed to simply an increased level of DnaK. This also demonstrates that the expression of heat shock proteins is closely regulated as a function of the concentration of DnaK in the cell.

The most notable defect in the expression of GroEL at steady-state conditions was observed upon the expression of DnaK T199A. In a wild-type strain expressing this protein, GroEL appeared to be overexpressed approximately threefold at 30°C. As mentioned above, this protein has been characterized in vitro and found to have a number of biophysical properties indistinguishable from those of wild-type DnaK (44). In particular, this derivative was found to have a thermal stability identical to that of wild-type DnaK. Thus, it is unlikely that the induction of GroEL observed here is due to the general induction of heat shock proteins by expression of an unstable protein (45).

Derivatives of the *dnaK*$^+$ MC4100 strain expressing wild-type or mutant DnaK from plasmids have similar growth rates. We examined the growth rate and viability of *dnaK*$^+$ strains (MC4100) containing plasmids that carried either wild-type *dnaK*$^+$ or one of the three mutant alleles under the control of the P_{lac} promoter. These strains, induced with 0.5 mM IPTG in M9 glucose liquid medium, showed no dramatic differences in growth rate, as measured by an increase in the OD_{460} (5) at 30, 37, or 42°C (data not shown). Cultures of these four strains grown at 30°C had identical numbers of colony-forming units per OD_{460} when assayed on LB plates containing 0.5 mM IPTG. In addition, the same number of colony-forming units was observed when samples of these cultures grown at 30°C were plated and then incubated at any temperature in the range from 25 to 45°C. Furthermore, at any given temperature, the colonies of all four strains were also of equal size. Incubations at 16 and 20°C resulted in lower numbers of colony-forming units for all four strains as well as for control strains (*dnaK*$^+$ strains carrying the vector pBR322 and the plasmid expressing LacI, pJM100; data not shown).

A slightly different result was obtained for cells grown in liquid medium at 37 and 42°C. Strains expressing the mutant DnaK proteins in a wild-type MC4100 background had fewer colony-forming units than the control strain (*dnaK*$^+$ strain containing only the pBR322 vector and the *lacI*^{q}-carrying plasmid pJM100; data not shown) when grown at the higher temperature. Light microscopic examination revealed that cells of *dnaK*$^+$ strains expressing the mutant DnaK proteins were filamented when grown in liquid medium at 37°C and higher. Thus, while the acquisition of cellular mass, as determined by the OD_{460} (5), was unaffected by expression of the mutant DnaK proteins, there appeared to be defects in septation at higher temperature in strains expressing mutant DnaK. This phenotype is similar to that observed for Δ*dnaK52* strains that do not carry suppressors (9) (see Fig. 11). Therefore, we systematically examined the morphology of derivatives of *dnaK*$^+$ strains expressing wild-type or mutant DnaK proteins from plasmids to further characterize the nature of the defect.

Derivatives of *dnaK*$^+$ MC4100 strains expressing mutant DnaK proteins show temperature-dependent filamentation. We used differential interference contrast optics (Nomarski) light microscopy to examine the morphology of *dnaK*$^+$ MC4100 derivatives expressing mutant DnaK proteins which are altered at position 199 when the strains were grown on LB plates containing IPTG. As discussed in the previous section, over the range of incubation temperatures from 16 to 45°C, *dnaK*$^+$ strains expressing any of the mutant DnaK proteins from a plasmid were found to produce colonies equal in size to those of the control strain (*dnaK*$^+$ strain carrying the vector pBR322 and the plasmid expressing LacI^q, pJM100). Multiple representative colonies were examined for each strain at each

temperature (Fig. 7). Duplicate strains were also constructed and examined in parallel to avoid the possibility that strains containing suppressors had been inadvertently selected.

We observed that the *dnaK*$^+$ MC4100 strains expressing wild-type DnaK from the plasmid contained almost no cells of abnormal length at low and intermediate temperatures. At these temperatures, a substantial increase in the concentration of wild-type DnaK in the cells compared with that in control cells would be expected because of expression of DnaK$^+$ from the plasmid. Furthermore, increases in the temperature at which the plates were incubated did not substantially alter the average cell length but did result in an increased frequency of cells of abnormal length. However, even at 45°C, only a subpopulation (<1%) of cells were typically several times longer than the majority of cells. We observed that cells of control strains (*dnaK*$^+$ strains carrying the vector pBR322 and the plasmid expressing LacI^a, pJM100) also demonstrated this minor defect in septation in a small fraction of cells at the higher temperature as well. Thus, strains that expressed wild-type DnaK from both the chromosome and the plasmid in response to 0.5 mM IPTG were indistinguishable from strains expressing DnaK from the chromosome only. This observation indicates that the range of levels of wild-type DnaK used in these experiments does not affect septation in MC4100 cells.

In contrast, *dnaK*$^+$ MC4100 strains containing plasmids encoding mutant DnaK proteins altered at residue 199 exhibited temperature-dependent filamentation when grown in medium containing 0.5 mM IPTG. We observed that at 30°C and lower, cells from these strains were very similar in morphology to cells of the control strain that expressed wild-type DnaK from the plasmid. A slight propensity, however, towards the formation of filaments of intermediate length was detected in strains expressing DnaK T199A at these intermediate and low temperatures (Fig. 7). These filamenting cells represented only a small percentage of the total cells present, although their proportion varied somewhat between independent colonies. However, at temperatures as moderate as 34°C, striking defects in septation were observed concomitant with expression of the various mutant DnaK proteins. The severity of this cell division defect increased in response to increases in incubation temperature. At 40°C, most of the cells expressing mutant DnaK proteins were long filaments, with some cells extending for over 100 mm.

At temperatures greater than 37°C, the strains expressing the mutant DnaK proteins in a *dnaK*$^+$ background exhibited an alteration in the morphology of colonies as well as defects in cellular morphology. We noted that defects in cellular morphology correlated with this alteration in colony morphology. Colonies incubated at higher temperatures (40°C and higher) appeared similar to colonies of Δ*dnaK52* derivatives of strain MC4100 (9). These colonies are also composed of extensively filamented cells and are flat and transparent in appearance. Upon longer incubation (for example, >24 h), *dnaK*$^+$ strains expressing mutant DnaK from plasmids formed colonies that appeared flat and transparent at the higher incubation temperatures and then began to acquire regions that appeared more white and opaque. In addition, microscopic examination of these older colonies revealed that the cells exhibited a range of lengths. This variation in cell length was observed in some younger colonies as well (Fig. 7). In general, however, the proportion of cells that were short filaments or were of normal length was found to increase substantially with increased time of incubation. Thus, the presence of either slightly filamented or normal cells in a colony from *dnaK*$^+$ strains expressing the mutant DnaK at high temperatures correlated directly with the appearance of opaque regions in the colonies.

The opaque regions of the colonies could arise either from acquisition of suppressors or from loss of the plasmid expressing the mutant DnaK protein. Since the filamentous phenotype is a direct consequence of expression of mutant DnaK from a plasmid, the loss of the plasmid from a given cell would be expected to give rise to daughter cells which no longer had the tendency to filament. Loss of plasmid and acquisition of the associated regions in the colonies would be expected as a result of extracellular local depletion of ampicillin as a result of β-lactamase activity. The selective pressure for plasmid maintenance would then be lost within the colony, and cells containing no plasmid could proliferate. Consistent with this explanation, colonies of the control strain (*dnaK*$^+$ strain carrying the vector pBR322 and the plasmid expressing LacI^q, pJM100) and strains expressing mutant DnaK proteins contained few colony-forming units when grown on plates containing ampicillin at 30°C (data not shown). Occasionally, colonies were observed that contained significant numbers of cells that were intermediate in length between the control cells and long filaments. It seems more likely that these cells contain suppressors that allow nonfilamentous growth than that they have lost the plasmid encoding the mutant DnaK.

Plasmid-encoded wild-type DnaK under control of P_{lac} allows chromosomal deletion of DnaK in MC4100 at high temperatures. The Δ*dnaK52* allele (43) contains an insertion encoding chloramphenicol resistance (Cm^r), so that P1 transductants which have received the Δ*dnaK52* allele can be selected directly by use of plates containing chloramphenicol. Strains carrying this allele are not viable at high temperatures (9, 43). However, we were able to transduce this Δ*dnaK52* allele efficiently into *dnaK*$^+$ MC4100 strains containing a plasmid expressing wild-type DnaK under the control of the *lac* promoter (pJM41). We found that the relative number of Cm^r colonies arising from P1 transductions was dependent on the incubation temperature of the transduced cells (Fig. 8). In these experiments, the expression of DnaK from the plasmid was not adjusted at any temperature by varying the concentration of IPTG; only 0.5 mM IPTG was used. In the range from 25 to 39°C, there was no significant difference in the number of colonies arising upon incubation at a given temperature. However, the efficiency of transduction, as measured by the relative number of colonies arising, decreased above 39°C. Nevertheless, even at the highest temperature tested, 45°C, we were still able to transduce the Δ*dnaK52* allele with reasonable efficiency. This is in contrast to a recent report (14) that a strain carrying the Δ*dnaK52* allele could not be complemented by a plasmid carrying wild-type DnaK when strains were grown at 42 or 45°C. This difference is probably due to the presence of temperature-sensitive suppressors which map to *rpoH* (8) in the strains used in the previous study.

We observed that growth of the strains carrying the plasmid with P_{lac}-*dnaK*$^+$ and transduced with the Δ*dnaK52* allele at temperatures above 40°C was dependent on the presence of 0.5 mM IPTG in the medium (data not shown). We also determined that the colonies obtained during incubation at high temperatures resulted from true deletion of the chromosomal *dnaK*$^+$. Five of five Cm^r colonies tested that were isolated at 45°C demonstrated an IPTG-dependent λ sensitivity (Fig. 9). This indicated that chromosomal *dnaK*$^+$, with the wild-type promoter, was replaced by the Δ*dnaK52* allele in these isolates and that expression of DnaK was now under control of the *lac* promoter.

The lower frequency of transduction of the Δ*dnaK52* allele at higher temperatures may be a result of significantly lower

A
B
C
D
E
DnaK T199V
DnaK T199A
DnaK T199D
DnaK+
Control

FIG. 7. Nomarski differential interference contrast optics microscopy of cells from a control strain and *dnaK*$^+$ MC4100 strains expressing the following proteins from plasmids carrying P_{lac}-*dnaK*: wild-type DnaK (strain GW8305) and mutant proteins DnaK T199D (strain GW8307), DnaK T199A (strain GW8309), and DnaK T199D (strain GW8311). Cells were taken from LB plates containing 0.5 mM IPTG, kanamycin, and ampicillin that had been incubated at 30 (A), 34 (B), 37 (C), 40 (D), or 45°C (E). Multiple colonies were examined for each strain at each temperature, and representative cells were photographed. Each strain was made in duplicate, and cells were observed from multiple colonies for each strain. The control strain was GW8303, a *dnaK*$^+$ MC4100 strain carrying plasmids pJM100 and pBR322. Cells were examined from colonies that were about 2 to 3 mm in diameter.

expression of DnaK from the plasmid than from the wild-type promoter on the chromosome at these temperatures (20, 42). Thus, the lower transduction frequency may simply reflect a higher susceptibility to heat stress due to lower levels of DnaK in the cell. As demonstrated above and in other reports, *dnaK* null and point mutants are temperature sensitive for growth (8, 22, 25, 43), and DnaK has been implicated in protection from and amelioration of heat stress (47).

We also observed that Δ*dnaK52* derivatives of the P_{lac}-*dnaK*$^+$ strain that had been constructed at the lower temperature of 30°C displayed a similar decrease in viability when grown at higher temperatures in the presence of IPTG. Strains that were cultured at 30°C and then counted on plates containing 0.5 mM IPTG had about 10-fold more colonies when incubated at 30°C than at 45°C (data not shown). This temperature dependence in colony formation is consistent with the decreased frequency of P1 transduction of the Δ*dnaK52* allele into *dnaK*$^+$ strains at high temperatures, as discussed above. The cellular morphology of Δ*dnaK52* strains complemented by the plasmid carrying P_{lac}-*dnaK*$^+$ and induced with 0.5 mM IPTG in the medium was not substantially different from wild-type cellular morphology at any of the temperatures tested. The only exception was when incubation was done at the extreme temperature of 45°C, when a small percentage of the cells were severalfold longer than normal (Fig. 10). Consistent with these observations, colonies of these strains were similar in morphology to colonies of *dnaK*$^+$ strains at all temperatures.

In addition, cells grown at 30°C with lower levels of IPTG also had few defects in septation (Fig. 10). It was particularly interesting that cells grown at 30°C with 0.2 mM IPTG appeared normal, even though heat shock protein expression is

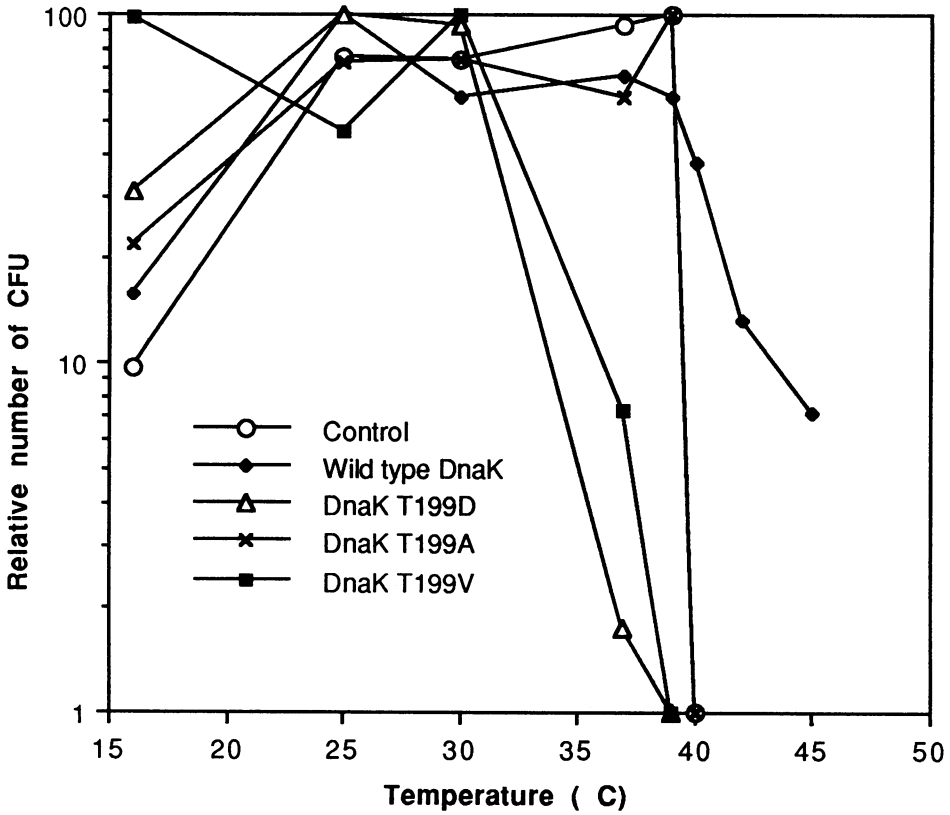

FIG. 8. Relative efficiency of transduction of Δ*dnaK52* via generalized P1 transduction as a function of incubation temperature. The control strain (GW8303, a derivative of MC4100) and MC4100 derivative strains expressing wild-type DnaK (GW8305), DnaK T199D (GW8307), DnaK T199A (GW8309), and DnaK T199V (GW8311) from plasmids carrying P_{lac}-*dnaK* and induced with 0.5 mM IPTG. Recipient cells were grown to the late log stage at 30°C, and phage absorption was done for 10 min at room temperature. After the cells were washed at 4°C, aliquots were spread on plates containing chloramphenicol, ampicillin, kanamycin, and 0.5 mM IPTG and placed in incubators at the indicated temperatures. Temperatures were monitored with thermometers in a flask containing water as well as thermometers exposed to the air. Efficiency of transduction was calculated by dividing the number of colonies arising after transduction of Δ*dnaK52* at a given temperature by the maximum number of colonies arising for that strain at any temperature.

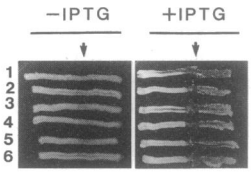

FIG. 9. Ability of Δ*dnaK52* derivatives of MC4100 carrying pBR322 P_{lac}-*dnaK*$^+$ (strain GW8305) to grow λ phage with and without induction by 0.5 mM IPTG. The arrow indicates the location of λ phage (ca. 10^8 PFU) in a streak perpendicular to the bacterial streaks. Bacteria were subsequently streaked across the region containing phage from left to right. The strains in streaks numbered 1 through 5 were generated from P1 transductions of Δ*dnaK52* and isolated at 45°C, while the strain in streak number 6 was isolated at 30°C.

altered under these conditions of reduced DnaK and DnaJ expression. A small percentage of cells were slightly elongated during incubation at 30°C with 0.05 mM IPTG. Substantial defects in septation, however, were evident when IPTG was completely absent from the plates. Cells of a Δ*dnaK52* strain carrying a plasmid with the P_{lac}-*dnaK*$^+$ plasmid pJM41 and grown on medium containing no added IPTG were extensively filamentous (Fig. 10), as observed for a Δ*dnaK52* strain (Fig. 7). These results indicate that wild-type levels of DnaK as well as the normal stoichiometries of DnaK to other heat shock proteins are not crucial for proper septation at a variety of temperatures but that it is critical that some DnaK be present.

Efficiency of P1 transduction of Δ*dnaK52* decreases sharply at approximately 40°C. We have examined in more detail the dependence of cell growth on the presence of DnaK as a function of temperature. This was done by measuring the efficiency with which we could transduce the Δ*dnaK52* allele at various temperatures into cells that contained the vector only with no complementing *dnaK*$^+$. The P1 phage lysate was grown on a Δ*dnaK52* strain carrying P_{lac}-*dnaK*$^+$ on a plasmid and grown at 30°C with 0.5 mM IPTG. When recipient cells were incubated at 30°C, we were able to obtain nearly 10^4 Cm^r colony-forming transductants per ml of P1 lysate grown on a Δ*dnaK52* strain. The same number of Δ*dnaK52* transductants were obtained at 30°C with a strain complemented with wild-type DnaK expressed from the P_{lac}-*dnaK*$^+$ plasmid pJM41. The efficiency of transduction in these experiments is near the maximum efficiency of P1 transduction noted by other researchers (33). By these criteria, no effect on cell viability is associated with deletion of *dnaK* at 30°C.

This high efficiency of transduction was obtained for incubation temperatures between 25 and 39°C (Fig. 8). However, the frequency of obtaining transductants carrying the Δ*dnaK52* allele decreased sharply over a very narrow temperature range above 39°C. We were unable to move the Δ*dnaK52* allele into a control strain (*dnaK*$^+$ strain carrying the vector pBR322 and the plasmid expressing LacI^q, pJM100) when the plates were incubated at 40°C. This experiment was repeated with similar results. Thus, there appears to be a transitional temperature above which cells require DnaK for growth. We found this transition point to be between 39 and 40°C. This transition temperature is very similar to that discussed above, when a lower frequency of transduction of Δ*dnaK52* was observed in a

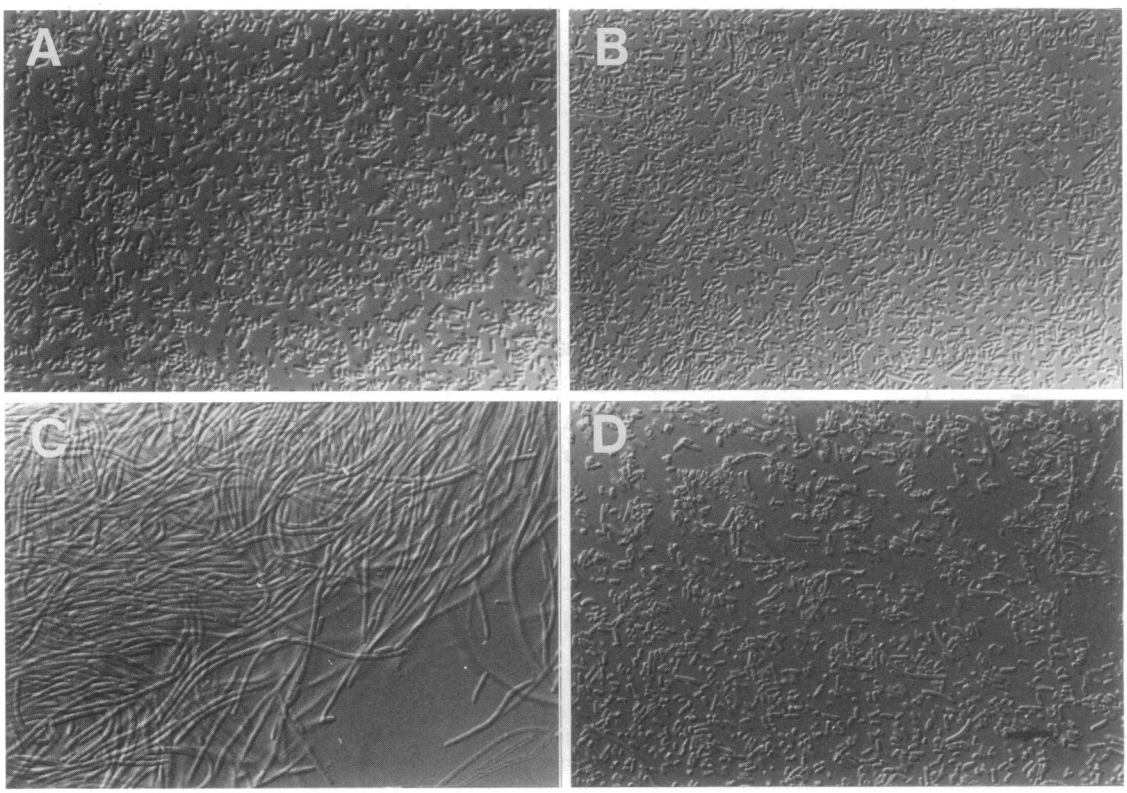

FIG. 10. Light microscopy of a Δ*dnaK52* derivative of an MC4100 strain carrying pBR322 P_{lac}-*dnaK*$^+$ (strain GW8319) induced with the indicated level of IPTG at the indicated temperature: (A) 0.2 mM IPTG, 30°C; (B) 0.05 mM IPTG, 30°C; (C) 0 mM IPTG (20 mM HEPES [pH 7.6], 0.2% glucose), 30°C; (D) 0.5 mM IPTG, 45°C. The LB plates also contained ampicillin, chloramphenicol, and kanamycin. Multiple colonies were examined, and representative cells were photographed. Bar, 10 mm.

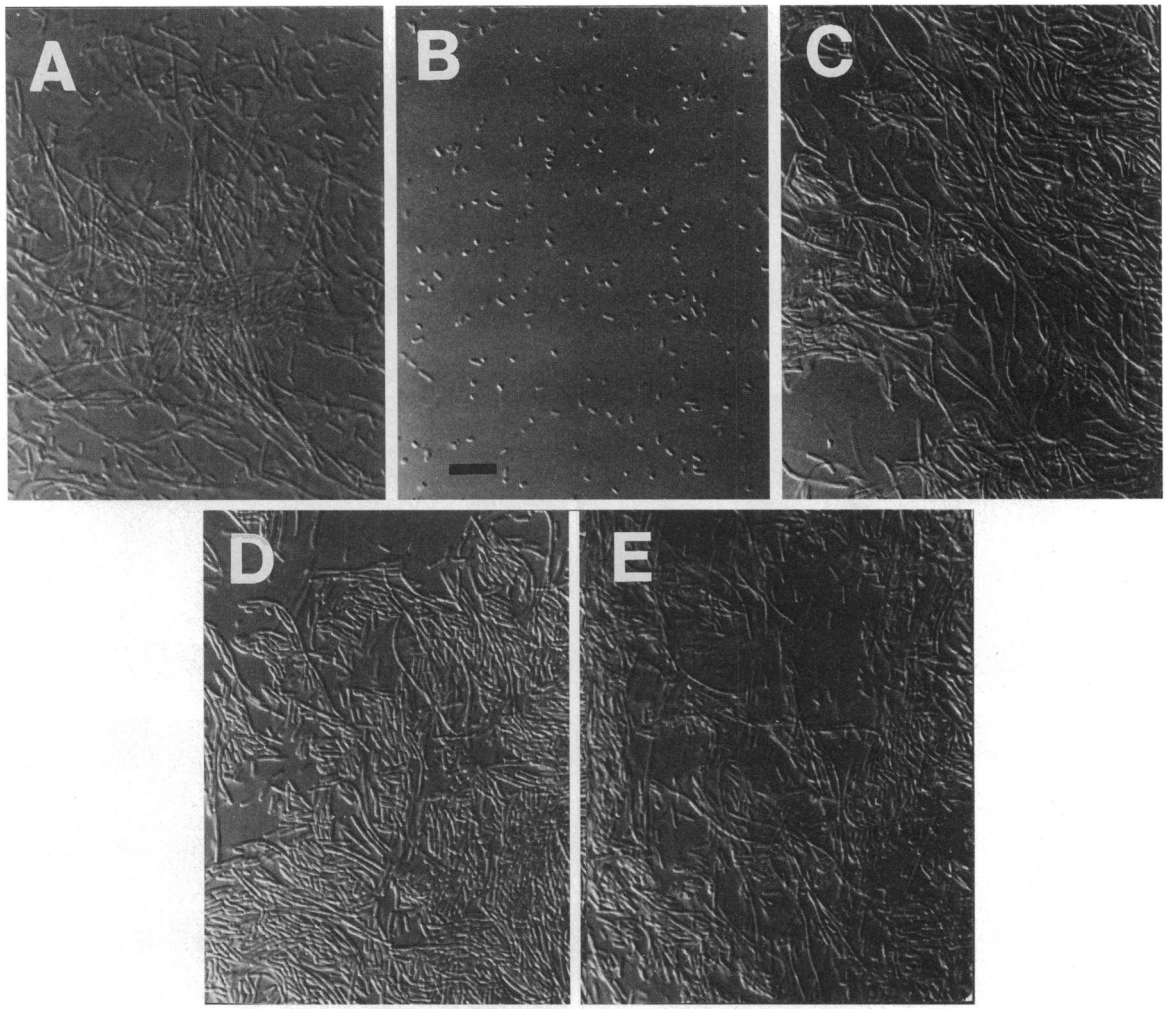

FIG. 11. Light microscopy of cells of Δ*dnaK52* derivatives of (A) MC4100 control strain GW8303 and MC4100 strains expressing (B) wild-type DnaK (GW8305), (C) DnaK T199D (GW8307), (D) DnaK T199A (GW8309), and (E) DnaK T199V (GW8311) expressed from plasmids carrying P_{lac}-*dnaK*. Multiple colonies were examined, and representative cells were photographed. Cells were taken directly from colonies arising after selection for transduction of Δ*dnaK52* into the above strains on plates containing chloramphenicol, ampicillin, kanamycin, and 0.5 mM IPTG and incubated at 30°C.

strain that was complemented by P_{lac}-*dnaK*$^+$ but whose expression of DnaK was not increased to compensate for the higher temperature. These results imply not only that DnaK is required at high temperatures, but that the levels of DnaK are important as well. Any Cm^r colony tested that arose from transduction of the Δ*dnaK52* allele at 40°C and higher was found to be λ sensitive. We have interpreted this to mean that these strains were derived from the subpopulation of cells that contained a duplication of the *dnaK* region (9) and thus still expressed DnaK.

Expression of the mutant DnaK proteins from plasmids is detrimental to growth of Δ*dnaK52* strains in a temperature-dependent manner. Strains that expressed DnaK T199D or DnaK T199V from plasmids had a strikingly decreased ability to be transduced with the Δ*dnaK52* allele by P1 at temperatures above 30°C (Fig. 8). In contrast, expression of DnaK T199A from a plasmid did not substantially alter the range of temperatures allowing growth of cells transduced with the Δ*dnaK52* allele but may have slowed growth during incubations at 39°C, as an extra day was required to achieve colonies equal in size to those of a control Δ*dnaK52* strain. The results for each strain were verified by multiple P1 transduction experiments with two separate constructions of each strain.

At 30°C, expression of any of the three mutant DnaK proteins did not substantially alter the cellular morphology of Δ*dnaK52* strains (Fig. 11) or the colony morphology of strains newly transduced to Δ*dnaK52*. All Δ*dnaK52*-transduced strains that were not complemented with wild-type DnaK from a plasmid developed colonies that were flat and transparent in appearance, while the colonies of Δ*dnaK52* transductant strains complemented by P_{lac}-*dnaK*$^+$ on pJM41 and induced with IPTG were indistinguishable from the colonies of a control *dnaK*$^+$ strain.

Expression of mutant DnaK proteins in *dnaK*$^+$ W3110 and Δ*dnaK52* W3110 strains results in alterations in septation. We found that introduction of the Δ*dnaK52* allele into *E. coli* W3110 results in only minor increases in cell length (Fig. 12). This is in sharp contrast to the major effects of the Δ*dnaK52* allele on cell length observed in *E. coli* MC4100 (Fig. 11). Therefore, we examined the effects caused by expression of the three mutant DnaK proteins in the W3110 background. We found that the parental W3110 strain (Fig. 12) was more

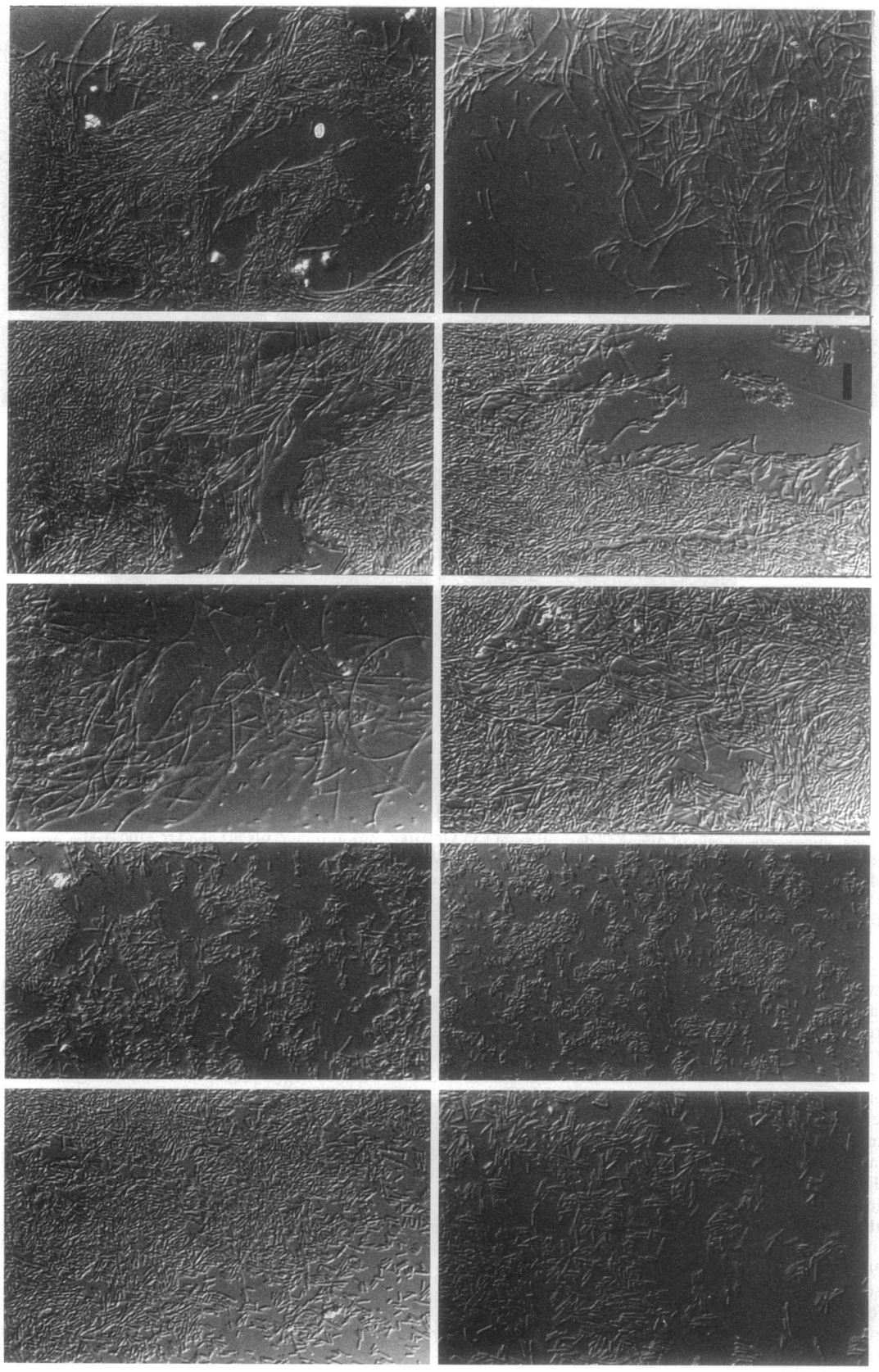

FIG. 12. Light microscopy of *dnaK*$^+$ and Δ*dnaK52* derivatives of strain W3110 and *dnaK*$^+$ and Δ*dnaK52* derivatives of W3110 strains expressing mutant DnaK proteins from plasmids carrying P_{lac}-*dnaK* and induced with 0.5 mM IPTG. The control W3110 strain carries the *lacI*^{q}-containing plasmid pJM100 and plasmid pBR322. The strains were GW8315 (wild-type DnaK), GW8316 (DnaK T199D), GW8317 (DnaK T199A), and GW8318 (DnaK T199V). All cells were taken from plates incubated at 30°C. Multiple colonies were examined, and representative cells were photographed. The cells of Δ*dnaK52* derivatives were photographed from colonies that arose directly from the P1 transduction.

heterogeneous in cell length than MC4100 (Fig. 7). We also observed that there were defects in septation associated with expression of any of the three mutant DnaK protein in *dnaK*$^+$ W3110 at high temperatures (data not shown) that were similar to the defects observed for the *dnaK*$^+$ MC4100 strains discussed above. However, in this case, septation defects were manifested at 30°C as well (Fig. 12). Thus, the defects in septation associated with expression of the mutant DnaK in *dnaK*$^+$ strains was not dependent on the higher temperatures in a W3110 background.

The expression of mutant DnaK proteins in Δ*dnaK52* W3110 strains also leads to an alteration in cell length. Normally, Δ*dnaK52* W3110 cells do not form extensive filaments; instead, only slightly longer cells are observed. Expression of the mutant DnaK proteins, however, resulted in filamentation of Δ*dnaK52* W3110 cells that was similar to the filamentation observed in Δ*dnaK52* MC4100 strains. These long filamented cells were also similar to the long filaments that resulted from expression of the mutant DnaK protein in *dnaK*$^+$ MC4100 strains at high temperatures. The colony morphology of Δ*dnaK52* W3110 strains expressing mutant DnaK was also altered (data not shown), consistent with the long filamented cells.

DISCUSSION

In this work, we have demonstrated that DnaK is required for cell growth at temperatures above 39°C. In the range from 25 to 39°C, there is no alteration in the ability of a Δ*dnaK52* strain to grow, as judged from the number of colonies arising after P1 transduction of the Δ*dnaK52* allele. In contrast, we determined that incubation at temperatures above 40°C did not allow Δ*dnaK52* cells to grow. Previously, our laboratory has reported that Δ*dnaK52* mutants will not grow at 42°C and higher (43), and it has been independently reported that a mutant carrying another null allele of *dnaK* is restricted for growth at 40.5°C (25). However, we have demonstrated in these experiments that this alteration in the ability of cells deleted in *dnaK* to grow appears to occur over a narrow temperature range. The transition temperature of 39.5°C for the DnaK requirement is intriguing, as maximum *E. coli* growth occurs at 39°C (20).

Our observation that DnaK can be deleted at temperatures below 40°C with no apparent loss of viability complements an earlier study which demonstrated that there is a requirement for DnaK for cell growth only at high temperature (26). Δ*rpoH* (formerly referred to as *htpR*) strains, which are not viable at temperatures of >20°C, have decreased expression of GroES/EL and probably contain little or no DnaK (4, 26, 60). Expression of DnaK from the tryptophan promoter alone did not substantially increase the upper growth temperature of these Δ*rpoH* cells. In contrast, spontaneous suppressors that mapped to the promoter of *groES/EL* increased expression of GroES/EL and allowed growth of the Δ*rpoH* cells at up to 40°C. However, further increases in growth temperature, up to at least 42°C (26), were possible if these strains also expressed DnaK. These experiments led to the similar conclusion that DnaK is required for growth only at temperatures above 40°C (26, 50, 59).

In addition, we found that three *dnaK* mutants with alterations in DnaK at the site of autophosphorylation are defective in negative regulation of the heat shock response. Expression of these proteins from plasmids at wild-type levels at 30°C in Δ*dnaK52* strains resulted in severe defects in the steady-state regulation of heat shock protein synthesis, similar to those observed for a strain completely deficient in DnaK. Thus, these *dnaK* mutations failed to complement a null mutation for heat shock protein regulation, just as they did in the cases of λ growth (34, 35), efficient lytic growth of P1 phage (34), and cell viability at high temperature.

The failure of these particular *dnaK* mutants, which encode proteins defective in ATPase activity and autophosphorylation, to complement a *dnaK* null mutant with respect to regulation of the heat shock response suggests a correlation between this activity of DnaK in vivo and the ability of DnaK to efficiently hydrolyze ATP or to autophosphorylate. The deficiencies in ATPase activity and autophosphorylation are the only two biochemical characteristics in which these three mutant proteins have been found to differ from wild-type DnaK. In the case of λ replication, the correlation between DnaK's ATPase activity and its activity in λ DNA replication has been investigated in vitro (34). We found that DnaK's ATPase activity correlated very well with DnaK's activity in λ replication. Specifically, DnaK T199D, the only mutant protein of the three to have a measurable ATPase activity (1/15 of wild-type DnaK ATPase activity at 37°C with 70 μM ATP [36]), was the only mutant protein to exhibit a measurable activity in λ replication in vitro at high concentrations of mutant DnaK protein (34). Together, these results suggest that the lack of negative regulation of heat shock protein synthesis observed for cells expressing these mutant DnaK proteins may be a direct result of the ATP hydrolysis defects of the mutant proteins. Formally, it must also be considered that the defects in autophosphorylation activity of the DnaK mutants examined here may be directly responsible for the defects in heat shock protein regulation rather than the defects in ATPase activity. The role of DnaK autophosphorylation is not clear, and unfortunately, the fact that the mutant DnaK proteins that cannot be autophosphorylated are also defective in ATPase activity does not allow an unambiguous determination of the relative physiological importance of these two activities (36).

Interestingly, this result is perhaps contrary to what might have been predicted on the basis of previous reports. Genetic experiments have shown that part of the mechanism for DnaK's negative regulation of the heat shock response probably involves an effect of DnaK on σ^{32} stability (13, 50). DnaK is hypothesized to lead to a decrease in the stability of σ^{32} that is transiently relieved in response to stress (13). One possible mechanism is that DnaK plays a role in directing σ^{32} to some sort of proteolytic fate (13), resulting in the observed half-life for σ^{32} of less than 1 min under nonstress conditions (50). In addition, interactions with DnaK may prevent σ^{32} from efficiently complexing with RNA polymerase and directing the transcription of heat shock promoters. It has been suggested that the cellular consequences of stress (13) or perhaps temperature itself, by increasing the intrinsic activities of DnaK such as the ATPase rate (36), may result in an increase in free σ^{32} in the cell, which can then effect transcription of the heat shock regulon. Strains carrying one particularly well-studied allele of *dnaK*, *dnaK756*, exhibit about twice the normal level of heat shock proteins at 30°C (50), indicating an increase in σ^{32} activity. Mutant DnaK protein from *dnaK756* strains has been purified and reported to have an ATPase rate of up to 50-fold that of wild-type DnaK (31). In addition, it has recently been demonstrated that ATP results in the dissociation of DnaK isolated in a complex with σ^{32} (17). These results suggest a simplistic model in which DnaK that is more active in ATP hydrolysis may be less effective in the sequestration of σ^{32}. By simple extension, *dnaK* mutants whose gene products are defective in ATPase activity would then be expected to exhibit lower heat shock protein expression, the result opposite of that reported here.

The difference between our observations with the alleles of *dnaK* encoding an altered residue at position 199 and what might have been predicted from previous investigations can be rationalized in two ways. One possibility is not only that a defect in the ATPase activity of DnaK may affect the ability of DnaK to make σ^{32} available for interaction with RNA polymerase, but the defect in DnaK ATPase activity may also lead to defects in the ability of DnaK to direct σ^{32} to a proteolytic fate. Thus, the net effect may be an actual increase in the levels of active σ^{32} in the cell. A second possibility involves the observed interaction of DnaK with a variety of proteins. There is direct or indirect evidence for DnaK's association with RNA polymerase (48), λ P protein (30), denatured bovine pancreatic trypsin inhibitor (31), mutant p53 protein (12), a small peptide (36), as well as DnaJ, GrpE, and σ^{32} (17, 23, 28, 62). In addition, it has been hypothesized that the closely related HSP70 proteins in eukaryotes may interact with a variety of peptides (15, 16), nascent proteins (2), and proteins imported into the mitochondria (46). In many of these cases, the interaction of DnaK with these substrates has been demonstrated to be disrupted by addition of ATP (12, 62) or even by the hydrolysis of ATP (15, 31). Thus, the interaction of these proteins with DnaK may be similar to that observed for DnaK with σ^{32} (17). Therefore, it would not be surprising if mutant DnaK proteins that were not able to efficiently hydrolyze ATP in vitro, such as those studied here, become associated with a variety of proteins in the cell in a prolonged or irreversible fashion, even under nonstressed conditions, and are thus not available for interaction with σ^{32}. Essentially, these mutant DnaK proteins may become titrated in a manner similar to that hypothesized in the homeostatic model (13) used to explain the transient increase in heat shock protein expression.

We have also demonstrated that the level of DnaK and perhaps DnaJ is critical for the negative regulation of the heat shock response both at 30°C and after 30 min at 42°C. This is consistent with the homeostatic model for heat shock protein regulation (13), in which the level of free DnaK (as well as DnaJ and GrpE) is critical for control of heat shock protein expression. Expression of wild-type DnaK (and DnaJ) from a plasmid in Δ*dnaK52* strains at levels lower than that in a wild-type strain (ca. one-third normal) resulted in overexpression of GroEL at 30°C. In addition, expression of wild-type DnaK from a plasmid in wild-type strains, which should result in approximately double the level of DnaK, resulted in decreased expression of GroEL.

In addition to the function of DnaK at intermediate temperatures in the regulation of heat shock protein synthesis (8, 50), we present evidence that DnaK may be involved in the normal septation pathway during cell division at temperatures above 30°C. We observed a temperature-dependent filamentation associated with expression of mutant DnaK proteins altered at residue 199 in strains carrying *dnaK*$^+$ on their chromosomes. This suggests that *dnaK* alleles encoding DnaK proteins that are defective in ATPase activity are dominant negatives (21) with respect to the requirement for *dnaK*$^+$ for normal septation.

Previous reports have indicated that defects in septation are associated with the introduction of the Δ*dnaK52* mutation into strain MC4100 (9, 43). These septation defects of an MC4100 Δ*dnaK52* derivative were suppressible, however, by mutations arising spontaneously at 30°C in *rpoH* (8). These particular alleles of *rpoH* resulted in lowered activity of σ^{32} and in decreased levels of heat shock proteins in both *dnaK*$^+$ and Δ*dnaK52* strains (8). Thus, in the absence of the heat shock protein DnaK, suppression of septation defects at 30°C can be achieved by a reduction in the abnormally high levels of the other heat shock proteins. This result was interpreted as indicating that the septation defects in Δ*dnaK52* MC4100 at 30°C were a result of altered heat shock protein levels or abnormal stoichiometries of heat shock proteins associated with the absence of DnaK and overexpression of the remaining heat shock proteins (8). Our observations here suggest that the situation may be more complicated. A normal stoichiometry between DnaK and other heat shock proteins (other than perhaps DnaJ) does not appear to be essential for normal septation. At 30°C, underexpression of DnaK and DnaJ from P_{lac}-*dnaK*$^+$*J*$^+$ in a Δ*dnaK52* MC4100 strain with 0.2 mM IPTG resulted in defects in heat shock protein expression but not in defects in septation. Substantial defects in septation were only observed when expression of DnaK (and DnaJ) was not induced by the addition of IPTG. In addition, relative underexpression of DnaK (and DnaJ) in this strain during growth at 45°C with 0.5 mM IPTG also did not result in substantial defects in septation.

An alternative explanation is that DnaK interacts directly with a protein in the septation pathway. At least one such putative target protein for DnaK is FtsZ, which is essential in the septation pathway and has been shown to form a ring structure at the site of septum formation (3). Our lab has previously demonstrated that overexpression of FtsZ from a plasmid is capable of suppressing the severe filamentation defect but not the temperature sensitivity and other phenotypes associated with the Δ*dnaK52* allele (9). Interestingly, the FtsZ protein has recently been purified to homogeneity with the exception of copurification with DnaK (39). Interestingly, the defect in septation associated with expression of the mutant DnaK proteins appears to be particularly well revealed in the W3110 background. This strain has a less consistent cell length than MC4100 and was not observed to filament extensively upon introduction of the Δ*dnaK52* allele, a characteristic that distinguishes it from two other commonly used derivatives of *E. coli* K-12, MC4100 and AB1157 (9, 43). However, expression of the mutant DnaK proteins resulted in filamentation of both Δ*dnaK52* and *dnaK*$^+$ derivatives of W3110 at 30°C. This result is consistent with mutant DnaK proteins interacting irreversibly with a protein essential for normal septation.

The apparent dominance over wild-type DnaK of these mutant DnaK proteins defective in ATPase and autophosphorylation for some but not all activities in vivo suggests that the mutant proteins can act as "antimorphs" (21) with respect to these activities. In contrast to the heat shock regulation and filamentation, however, these mutant proteins had no detectable dominance over wild-type DnaK with respect to supporting efficient λ or P1 phage growth (34). In addition, no dominance was detected in vitro with respect to wild-type DnaK activity in λ DNA replication or the activation of P1 RepA protein by any of these mutant proteins (34). It is also possible that the differential dominance is a reflection of the sensitivity of the particular function of DnaK in vivo to the concentration of free DnaK.

We observed that there appeared to be a protein in Δ*dnaK52* strains whose expression increases in response to heat shock and which migrates to a position in 10% polyacrylamide gels very close to that of DnaK. It is likely that the same protein is increased in expression in response to the 30 to 42°C and 30 to 50°C heat shock treatments and may also cross-react to antibodies directed against DnaK. These results suggest that *E. coli* may have two heat shock proteins of ca. 70 kDa that may have common epitopes.

Finally, we have demonstrated that the temperature-sensitive growth phenotype associated with the Δ*dnaK52* allele can

be suppressed at temperatures of up to 45°C, provided that DnaK and DnaJ are expressed from plasmid pJM41. In addition, induced expression of DnaK (and DnaJ) from pJM41 allows efficient formation of colonies at 45°C from Δ*dnaK52* strains constructed and maintained at 30°C. Since the pJM41 plasmid contains no *dnaK* sequences upstream of the starting codon of *dnaK*$^+$, we conclude that the Δ*dnaK52* allele does not alter any upstream genes required for growth at temperatures of up to 45°C. This result is in contrast to a previous report (14) suggesting the existence of "*htpA*," hypothesized to be upstream of *dnaK*, that is required at high temperatures and mutated in a Δ*dnaK52* strain. The discrepancy between this result and ours, however, may be explained by the fact that the strains used in the previous study (14) contained an additional mutation (8) in the gene encoding σ^{32}, which has been demonstrated to be required at high temperature (26).

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