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Mutations altering heat shock specific subunit of RNA polymerase suppress major cellular defects of *E.coli* mutants lacking the DnaK chaperone

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An *Escherichia coli* mutant lacking HSP70 function, Δ*dnaK52*, is unable to grow at both high and low temperatures and, at intermediate temperature (30°C), displays defects in major cellular processes such as cell division, chromosome segregation and regulation of heat shock gene expression that lead to poor growth and genetic instability of the cells. In an effort to understand the roles of molecular chaperones such as DnaK in cellular metabolism, we analyzed secondary mutations (*sid*) that suppress the growth defects of Δ*dnaK52* mutants at 30°C and also permit growth at low temperature. Of the five suppressors we analyzed, four were of the *sidB* class and mapped within *rpoH*, which encodes the heat shock specific sigma subunit (σ^{32}) of RNA polymerase. The *sidB* mutations affected four different regions of the σ^{32} protein and, in one case, resulted in a several fold reduction in the cellular concentration of σ^{32}. Presence of any of the *sidB* mutations in Δ*dnaK52* mutants as well as in *dnaK*$^+$ cells caused down-regulation of heat shock gene expression at 30°C and decreased induction of the heat shock response after shift to 43.5°C. These findings suggest that the physiologically most significant function of DnaK in the metabolism of unstressed cells is its function in heat shock gene regulation.

Key words: DnaK/heat shock proteins/HSP70/*rpoH*/sigma 32

Introduction

Hsp70 proteins are proposed to be molecular chaperones or polypeptide chain binding proteins that catalyze protein folding as well as assembly and disassembly of oligomeric protein complexes (for reviews see Pelham, 1986; Ellis, 1987; Lindquist and Craig, 1988; Rothman, 1989). The chaperone function of Hsp70 proteins appears to be biologically extremely important since these proteins have been found in all organisms and are closely related in sequence (for review see Lindquist and Craig, 1988). Biochemical data indicate functions for members of the Hsp70 protein family in initiation of replication of bacteriophage λ DNA (for review see Echols, 1986; Liberek *et al.*, 1988), protein secretion (Chirico *et al.*, 1988; Deshaies *et al.*, 1988), clathrin uncoating (for review see Rothman and Schmid, 1986), and lysosomal protein degradation (Chiang *et al.*, 1989). Genetic evidence indicates that the chaperone function of Hsp70 proteins is important for normal cell growth of *Saccharomyces cerevisiae* and *Escherichia coli* not only under stress conditions such as heat shock, but also under normal metabolic conditions in unstressed cells (Craig *et al.*, 1987; Bukau and Walker, 1989a,b). Systematic analyses of the cellular functions of Hsp70 proteins in eukaryotes have not yet been performed mainly because of the high complexity of the genetics and pattern of expression of these proteins. For example, *S.cerevisiae* encodes at least nine different Hsp70 proteins that are closely related but differentially regulated and that partially complement each other (for review see Lindquist and Craig, 1988). In contrast, *E.coli* contains a single Hsp70 member, encoded by the *dnaK* heat shock gene (Bardwell and Craig, 1984). *E.coli* therefore is an attractive candidate for analysis of the cellular function of Hsp70 proteins.

The *E.coli* DnaK protein has ATPase activity (Zylicz *et al.*, 1983), is capable of autophosphorylation (Zylicz *et al.*, 1983), and physically interacts with the DnaJ and GrpE heat shock proteins (Sell, 1987; Johnson *et al.*, 1989). The *dnaK* gene forms an operon with the promoter distal *dnaJ* gene. DnaK is expressed at a very high level at 37°C and is further induced upon heat shock of the cells, along with 16–19 other heat shock proteins (Lemaux *et al.*, 1978; Herendeen *et al.*, 1979; for review see Neidhardt and Van-Bogelen, 1987). Expression of DnaK at normal growth temperatures as well as the heat shock induction of DnaK is regulated at the transcriptional level by the *rpoH* gene product, σ^{32}, which confers to RNA polymerase core the specificity to transcribe heat shock genes (Grossman *et al.*, 1984; Cowing *et al.*, 1985; Bloom *et al.*, 1986). DnaK is essential for initiation of replication of λ DNA (for review see Echols, 1986; Liberek *et al.*, 1988; Zylicz *et al.*, 1989). In *E.coli* cells DnaK is essential for growth at 42°C (Paek and Walker, 1987) and genetic evidence indicates functions for DnaK after heat shock in synthesis of RNA and DNA, and in cell division (Itikawa and Ryu, 1979; Paek and Walker, 1987; Sakakibara, 1988). The functions for DnaK in cellular metabolism at temperatures below 42°C are poorly understood as well. *dnaK756* missense mutants were shown to be defective at 30°C in degradation of abnormal proteins (Straus *et al.*, 1988) and of the normally highly unstable σ^{32} protein (Grossman *et al.*, 1987; Straus *et al.*, 1987; Tilly *et al.*, 1989; D.B.Straus and C.Gross, personal communication). Partial stabilization of σ^{32} in *dnaK* mutants contributes to increased expression of heat shock genes at 30°C (Paek and Walker, 1987) and to defects in recovery from the heat shock response (Tilly *et al.*, 1983).

We previously reported a systematic analysis of cellular defects at a variety of growth temperatures of Δ*dnaK52* mutants which completely lack the DnaK heat shock protein (Paek and Walker, 1987; Bukau and Walker, 1989a,b; Bukau *et al.*, 1989). Δ*dnaK52* mutants are cold sensitive as well as heat sensitive and thus possess a very narrow temperature range for growth. At permissive (30°C)

temperature, Δ*dnaK52* mutants have multiple cellular defects that lead to slow growth, poor viability and genetic instability of the cells. We identified severe defects of Δ*dnaK52* mutants in cell division, chromosome segregation and replication of low copy number plasmids. The roles for DnaK in these processes as well as in processes that lead to cold sensitivity and temperature sensitivity of growth remain unclear. It is possible that DnaJ plays a role in these cellular processes as well. Recent work (Sell *et al.*, 1990) revealed that *dnaJ* null mutations cause cell division defects and growth defects at 30°C as well as temperature sensitivity of growth, although these defects are less severe than in Δ*dnaK52* mutants. The fact that Δ*dnaK52* mutants contain very little DnaJ (Sell *et al.*, 1990) might therefore contribute to the observed cellular defects of these mutants. However, lysogenization of Δ*dnaK52* mutants with λ phages carrying *dnaK*$^+$ but not *dnaJ*$^+$ complemented major cellular defects which indicates that it is lack of DnaK rather than reduction of DnaJ that is primarily responsible for the observed defects of Δ*dnaK52* mutants (Bukau and Walker, 1989a).

To gain more insights into the cellular functions of DnaK in normal metabolism we isolated and characterized a set of suppressor mutations that efficiently suppress cold sensitivity of growth as well as growth defects at intermediate (30°C) temperatures of Δ*dnaK52* mutants. The major class of suppressor mutations described here map within the *rpoH* gene and cause downregulation of expression of heat shock genes. The biological implications of these findings are discussed.

Results

Spontaneous secondary mutations suppress major cellular defects at 30°C of ΔdnaK52 mutants

Prolonged incubation of Δ*dnaK52* transductant colonies isolated at 30°C led to frequent appearance of papillae (Bukau and Walker, 1989a). Upon incubation of the colonies at 30°C for ~7 days, only cells from papillae retained their viability and could be restreaked well. They were thus suppressed in whatever cellular defect of Δ*dnaK52* mutants it is which causes their low viability at 30°C. Furthermore, the shape of colonies formed by cells taken from papillae was indistinguishable from that of wild-type cells and was clearly distinct from the flat and translucent colonies of fresh Δ*dnaK52* transductants (Bukau and Walker, 1989a). Microscopic analysis of cells contained within colonies of five independent isolates of papillae that were chosen for further analysis revealed that cell division defects at 30°C were greatly suppressed in all five isolates as compared with newly isolated Δ*dnaK52* transductants. Figure 1 shows cells of one isolate, BB1553. Moreover, cells contained within papillae grew considerably faster than newly isolated Δ*dnaK52* transductants. Four of the five isolates (BB1553, BB1554, BB1556 and BB1557) had generation times that were similar to that of wild-type cells, while one isolate (BB1130) had a generation time that was between that of wild-type cells and Δ*dnaK52* transductants (Table I). Here we focused on the four isolates BB1553, BB1554, BB1556 and BB1557 that showed the greatest improvement of growth as judged by their generation times.

We used two different approaches to establish that the cells of isolates BB1553, BB1554, BB1556 and BB1557 carried secondary mutations, unlinked to the *dnaKJ* operon,

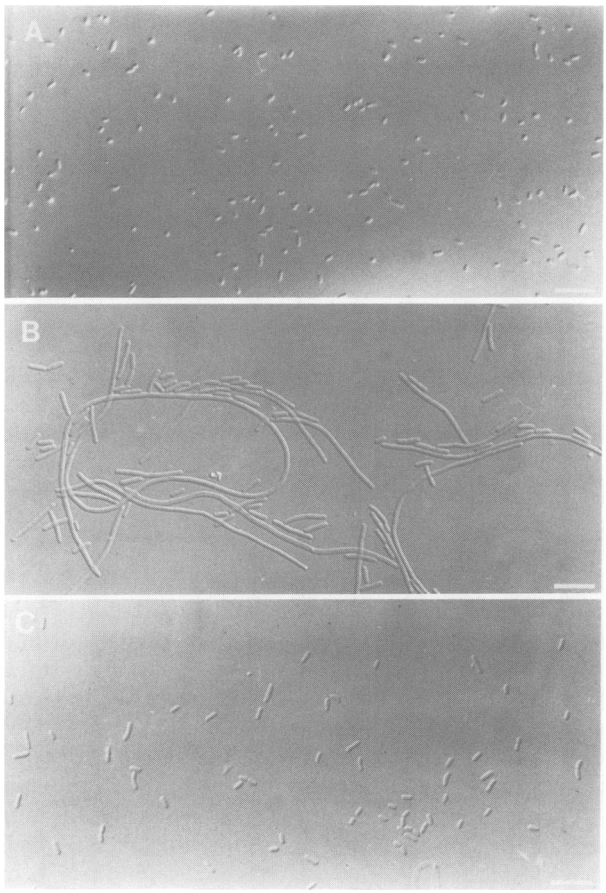

Fig. 1. *sidB1* mutation suppresses cell division defects of Δ*dnaK52* mutants at 30°C. Wild-type cells (MC4100) (**A**), fresh Δ*dnaK52* transductants (**B**) and Δ*dnaK52 sidB1* mutants (BB1553) (**C**) were subjected to light microscopic analysis after growth at 30°C on LB/Cm or LB agar plates. Bars, 10 μm.

Table I. Presence of suppressor mutations increases growth rates at 30°C of Δ*dnaK52* mutants

Strain	Relevant marker	Generation time[a] (min)
MC4100	wild-type	43
Δ*dnaK52* transductant	Δ*dnaK52*	138
BB1130	Δ*dnaK52 sidA1*	58
BB1553	Δ*dnaK52 sidB1*	44
BB1554	Δ*dnaK52 sidB2*	42
BB1556	Δ*dnaK52 sidB3*	46
BB1557	Δ*dnaK52 sidB4*	42

[a]Cells were grown at 30°C in LB with or without (MC4100) Cm and the increase in optical density during growth was measured and the generation times determined.

which suppress cell division defects, slow growth and low viability of the cells at 30°C. In one experiment we transduced the Δ*dnaK52* alleles of all four isolates into wild type cells at 30°C and found that none of the transductants exhibited any of the phenotypes of the suppressed strains. In another experiment we transduced the four strains first to *dnaK*$^+$ and then reintroduced the Δ*dnaK52* allele and found that the strains still exhibited the suppressed phenotypes. We will refer to this class of suppressor

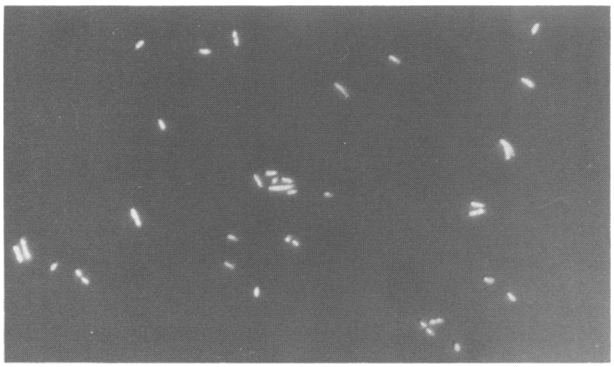

Fig. 2. *sidB1* mutation suppresses chromosome segregation defects of Δ*dnaK52* mutants at 30°C. *sidB1* Δ*dnaK52* mutant cells (BB1553) were grown in LB to the logarithmic phase and subjected to fluorescence microscopic analysis.

mutation isolated at 30°C as *sid* (suppressor at intermediate temperature of Δ*dnaK52*), in contrast to mutations which were isolated by selection for suppression of temperature sensitivity of Δ*dnaK52* mutants (*std*) (unpublished results). Based on their similar growth behavior at 30°C and their map position (see below) we name the *sid* mutations present in BB1553, BB1554, BB1556 and BB1557 *sidB1*, *sidB2*, *sidB3* and *sidB4*, respectively (as opposed to the *sidA1* mutation present in strain BB1130 that is not described here).

Effects of the sidB1 mutation on chromosome segregation and P1 plasmid stability at 30°C

For one *sidB* Δ*dnaK52* isolate, BB1553, we determined whether the *sidB1* mutation present in this strain not only suppresses cell division defects but also suppresses defects at 30°C of Δ*dnaK52* mutants in chromosome segregation (Bukau and Walker, 1989b) and stability of low copy number plasmids (Bukau and Walker, 1989b; Tilly *et al.*, 1989). To detect chromosome segregation, we stained cells of strain BB1553 grown at 30°C with a fluorescent dye that binds to DNA and subjected the cells to fluorescence microscopic analysis. Figure 2 shows that virtually all cells contained nucleoids, demonstrating that the chromosome segregation defects of Δ*dnaK52* mutants were efficiently suppressed. However, we cannot completely exclude the possibility that a small fraction of the cell population might be anucleated. Plasmid stability was determined in cells of strain BB1553 which were lysogenized with a derivative of bacteriophage P1, λ*kan cI*857-P1:R5-3 (λminiP1) (Sternberg and Austin, 1983; Funnell, 1988). λminiP1 replicates as a plasmid with a copy number similar to that of the host chromosome. This plasmid is very stably maintained in wild-type cells (MC4100) (0.25% plasmid loss/generation) but becomes highly unstable in Δ*dnaK52* mutants (4.57% plasmid loss/generation) (Bukau and Walker, 1989b). Plasmid stability tests revealed that λminiP1 is lost in BB1553 λminiP1 lysogens at a rate of 2.61% plasmid loss/generation. Although this rate of loss of λminiP1 is slightly lower than in *sidB*$^+$ Δ*dnaK52* mutants, it is still ~10-fold higher than in wild-type cells. Thus, presence of the *sidB1* mutation efficiently suppresses major cellular defects of Δ*dnaK52* mutants at 30°C including defects in cell division and chromosome segregation, but fails to suppress efficiently instability of low copy number plasmids derived from P1.

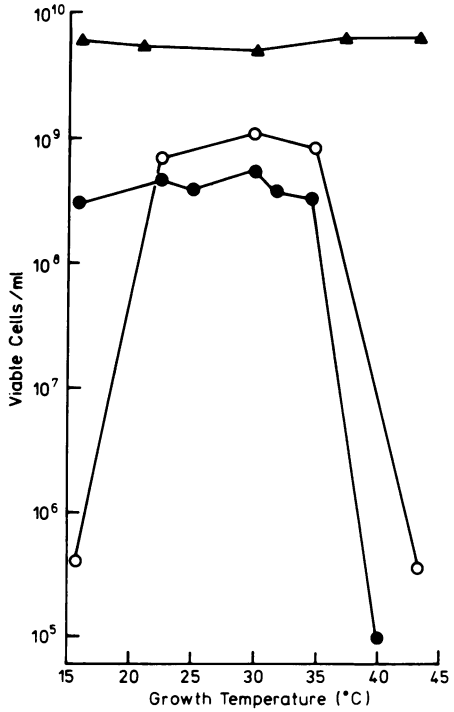

Fig. 3. Temperature range for growth of *sidB1* Δ*dnaK52* mutants. Wild-type cells (MC4100) (▲), fresh Δ*dnaK52* transductants (○), and *sidB1* Δ*dnaK52* mutants (BB1553) (●) were grown in LB or LB/Cm at 30°C, and dilutions plated on LB agar plates that were incubated at the indicated temperatures. Differences in the absolute numbers of viable cells at 30°C result from differences in cell densities of the liquid cultures used. The number of colonies was determined as a measure for viable cells.

sidB mutations suppress cold sensitivity but not temperature sensitivity of ΔdnaK52 mutants

We tested the ability of *sidB1*, *sidB2*, *sidB3* and *sidB4* mutations to suppress cold sensitivity of growth of Δ*dnaK52* cells. Cells of all four *sidB* Δ*dnaK52* isolates (BB1553, BB1554, BB1556, BB1557) were able to grow at low (16°C) temperature, while they remained temperature sensitive (at 42°C) for growth. Figure 3 shows the growth temperature range for *sidB1* Δ*dnaK52* cells. These observations suggest that the cellular requirements for DnaK at high temperature are not identical to the cellular requirements for DnaK at low temperature.

Further examination of strain BB1553 revealed that growth of the cells at 16°C led to formation of pale and translucent colonies similar to those formed at 30°C by Δ*dnaK52* mutants lacking any suppressor. Cells within these colonies were present as filaments which were, however, shorter and contained more invaginations at presumptive sites of septum formation than cell filaments of Δ*dnaK52* mutants at 30°C (data not shown). Thus, while the *sidB1* mutation is able to suppress cold sensitivity of growth of Δ*dnaK52* mutants, defects in cell division increase at low temperatures. Furthermore, prolonged incubation at 16°C led to the appearance of papillae indicating that secondary mutations had occurred which further increase the growth rate of *sidB1* Δ*dnaK52* mutants at low temperatures. Also, we observed that a temperature shift to 42°C of cells of strain BB1553 led to formation of cell filaments (data not shown). These findings suggest that the requirements for DnaK protein in

major cellular processes such as cell division change with growth temperature.

Genetic mapping of sidB mutations

We determined the locations of *sidB* mutations on the chromosome of the four isolates BB1553, BB1554, BB1556 and BB1557. The strategy for mapping was to replace specifically parts of the chromosome using Hfr matings and P1 transductions and to screen for loss of *sidB* mutations on the basis of loss of their suppressive effects on filamentation of Δ*dnaK52* cells at 30°C. The *sidB1*, *sidB2*, *sidB3* and *sidB4* mutations were all located at ~75–76 min on the genetic map, linked to two Tn*10* insertions (*zhf-50*::Tn*10*; *malT*::Tn*10*) (Singer *et al.*, 1989) located in that region. Using the *zhf-50*::Tn*10* as selection, we transduced the *sidB* mutations into wild-type cells and verified that suppression at 30°C of defects in cell division leading to extensive filamentation of Δ*dnaK52* mutants were cotransducible.

Genetic complementation and marker rescue of sidB mutations by rpoH$^+$ plasmids

The *rpoH* gene, which encodes the activator of heat shock gene expression σ^{32}, has similar linkage to Tn*10* insertions in the 75–76 min region of the chromosome to the *sidB* mutations. This proximity of the map positions of *rpoH* and *sidB*, as well as additional physiological evidence (see below) led us to consider that *sidB* mutations are mutated alleles of *rpoH*. To test this hypothesis we introduced a multicopy plasmid (pKP11) that encodes *rpoH*$^+$ into *sidB* Δ*dnaK52* mutant cells of strains BB1553, BB1554, BB1556 and BB1557 and determined whether the presence of this plasmid would complement the *sidB* mutations. Transformation of cells of all four *sidB* Δ*dnaK52* mutant isolates with pKP11 resulted in slow growth of the transformants at 30°C and rapid loss of viability during further purification of the colonies. Cells contained within transformant colonies formed long unseptated filaments similar to those formed by Δ*dnaK52* mutants without any suppressor. Thus, multiple copies of *rpoH*$^+$ complement major *sidB* suppressor phenotypes at 30°C, suggesting that *sidB* mutations are recessive mutant alleles of *rpoH*.

To test further the hypothesis that *sidB* mutations map within *rpoH* we performed marker rescue experiments in which we determined whether it is possible to cross *sidB* mutations by homologous recombination with plasmids encoding functionally inactive forms of the *rpoH* gene product. This rare recombination event would occur only if the *sidB* mutations are located within the part of *rpoH* that is also encoded for by the plasmid, and it would result in cellular defects as found in Δ*dnaK52* mutants lacking any suppressor mutation. For these experiments we chose the *sidB1* Δ*dnaK52* isolate, BB1553, and transformed it with plasmid pFN92 which contains all but the 15 amino acid codons of the 3′-end of *rpoH* (Landick *et al.*, 1984). While the presence of pFN92 in BB1553 cells did not alter the morphology of most colonies at 30°C, ~4×10^{-4} of the colonies were flat and translucent and contained strongly filamenting cells. Prolonged incubation of these colonies at 30°C led to frequent formation of papillae, indicating genetic instability. The presence in BB1553 cells of the vector lacking the *rpoH* insert did not lead to *sidB1* marker rescue. These results indicated that the plasmid pFN92 carries genetic information which allows the conversion of *sidB1* to *sidB*$^+$ and thus provided further genetic evidence that at least *sidB1* maps within *rpoH*.

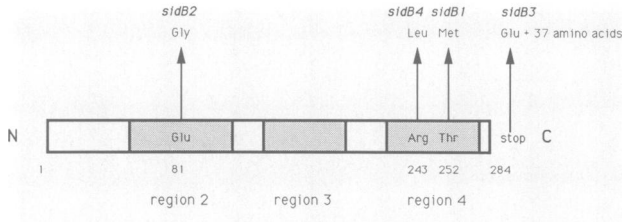

Fig. 4. Localization of *sidB* mutations within σ^{32}. A schematic representation of σ^{32} protein is shown. Amino acid residues that are affected by *sidB1*, *sidB2*, *sidB3* and *sidB4* mutations are indicated. Regions of homology of σ^{32} to other σ factors (regions 2, 3 and 4) (Helmann and Chamberlin, 1988) are represented as darkened boxes.

sidB mutations are alleles of rpoH

To confirm our hypothesis that *sidB* mutations are alleles of *rpoH* we sequenced the *rpoH* genes of *sidB* Δ*dnaK52* mutant strains BB1553, BB1554, BB1556 and BB1557. Our strategy was to amplify the *rpoH* genes of these mutants by the polymerase chain reaction, using primers complementary to DNA sequence upstream of the regulatory region (within the 3′-end of the *ftsX* gene) and downstream of the stop codon of *rpoH*, and to determine the DNA sequences of the amplified genes. Sequencing revealed that the *sidB1* mutation is a C to T transition at position 755 of the *rpoH* coding sequence leading to a Thr252 to Met change; *sidB2* is an A to G transition at position 242 leading to a Glu81 to Gly change; *sidB3* is a T to G transversion in the TAA stop codon (position 853) of *rpoH* leading to addition of a Glu residue at this position and to further addition of 37 amino acids to the C terminus of σ^{32}; *sidB4* is a G to T transversion at position 728 leading to an Arg243 to Leu change (Figure 4). Thus, all four *sidB* mutations analyzed were located within the coding region of the *rpoH* gene.

sidB mutations decrease heat shock gene expression at 30°C of ΔdnaK52 mutants and of dnaK$^+$ cells

Given that *sidB* mutations map within *rpoH* it was reasonable to assume that the presence of these mutations affect heat shock gene expression at 30°C in Δ*dnaK52* cells. To test this hypothesis we pulse-labeled *sidB* Δ*dnaK52* cells at 30°C with [^{35}S]methionine and determined the rate of synthesis of heat shock proteins. In each of several independent experiments all four *sidB* Δ*dnaK52* mutants were clearly reduced in synthesis of GroEL and other major heat shock proteins as compared with Δ*dnaK52* mutants lacking any suppressor (Figure 5). Scanning of the autoradiographs revealed ~2- to 4-fold reduction of GroEL synthesis in the *sidB* Δ*dnaK52* mutants, as compared with *sidB*$^+$ Δ*dnaK52* mutants. However, in none of the four *sidB* Δ*dnaK52* mutants tested did heat shock gene expression reach levels as low as in *dnaK*$^+$ cells (6-fold less than in Δ*dnaK52* cells). For the *sidB1* mutation, we tested whether it affects heat shock gene expression at the transcriptional level. We transduced the Δ*dnaK52* allele into *sidB1* and *sidB*$^+$ cells carrying a *groEL–lacZ* transcriptional fusion and monitored the level of *groEL* expression in the cells by determining β-galactosidase activity. We found that β-galactosidase activity in *sidB1* Δ*dnaK52* transductants was ~2-fold lower

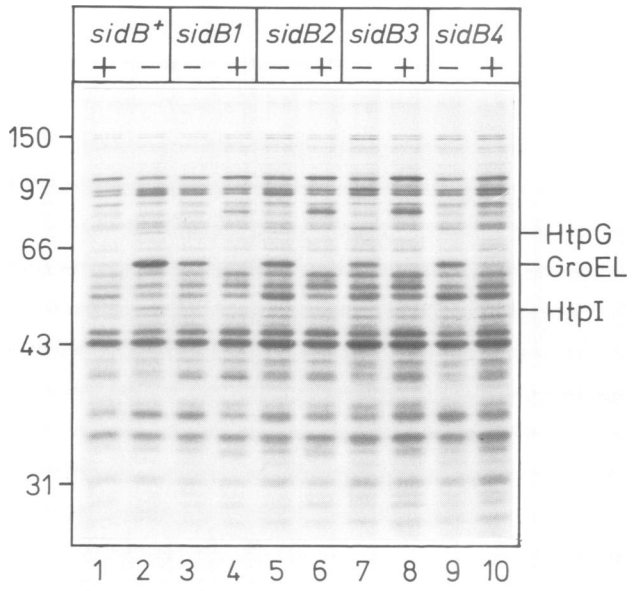

Fig. 5. *sidB* mutations decrease heat shock gene expression at 30°C. Cells of *sidB* mutants carrying *dnaK*$^+$ (+) or Δ*dnaK52* (−) alleles were grown at 30°C, pulse-labeled with [^{35}S]methionine, and the proteins separated on SDS−PAGE and visualized by autoradiography. Migration positions of some major heat shock proteins (right side) and of mol. wt standards (left side) are indicated. Strains used are MC4100 (lane 1), fresh Δ*dnaK52* transductants lacking any suppressor mutation (lane 2), BB1553 (lane 3), BB1699 (lane 4), BB1554 (lane 5), BB1700 (lane 6), BB1556 (lane 7), BB1701 (lane 8), BB1557 (lane 9) and BB1702 (lane 10).

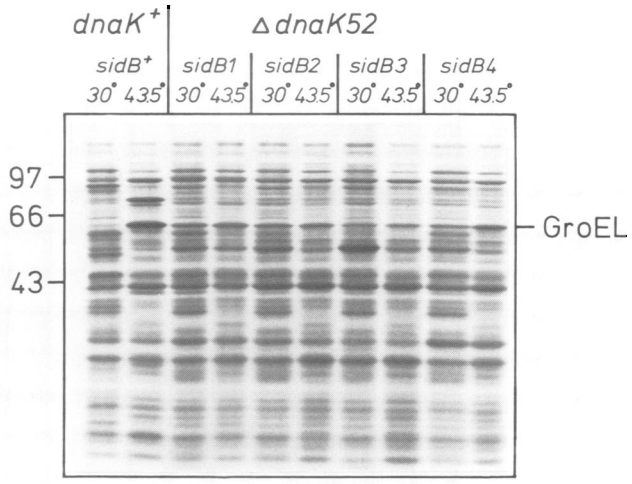

Fig. 6. *sidB* mutations affect heat shock response of Δ*dnaK52* mutants. Cells of *sidB1* Δ*dnaK52* mutants (BB1553), *sidB2* Δ*dnaK52* mutants (BB1554), *sidB3* Δ*dnaK52* mutants (BB1556), *sidB4* Δ*dnaK52* mutants (BB1557) and *sidB*$^+$ *dnaK*$^+$ cells (MC4100) were grown at 30°C and pulse-labeled with [^{35}S]methionine at 30°C or at 5 min after shift to 43.5°C. The proteins were separated on SDS−PAGE and autoradiographed. The migration positions of the GroEL protein (right side) and of mol. wt standards (left side) are indicated.

as compared with *sidB*$^+$ Δ*dnaK52* transductants, indicating that *sidB1* affects *groEL* expression at the transcriptional level. This finding is consistent with the fact that *sidB1* maps within the gene that encodes the transcriptional activator of the heat shock response. From our results we conclude that it is down-regulation of heat shock gene expression which leads to suppression of cellular defects of Δ*dnaK52* mutants at intermediate and low temperatures.

sidB mutations might also affect heat shock gene expression at 30°C of *dnaK*$^+$ cells. To address this question we first transduced the *sidB* Δ*dnaK52* mutants to *dnaK*$^+$ and then pulse-labeled the transductant cells at 30°C with [^{35}S]methionine and determined the rate of synthesis of major heat shock proteins such as GroEL. Figure 5 shows that the rate of GroEL synthesis is reduced in *sidB2 dnaK*$^+$ isolates as compared with wild-type cells; also, although difficult to detect in the printed Figure 5, we repeatedly found *dnaK*$^+$ cells carrying *sidB1, sidB3* or *sidB4* mutations to be reduced in GroEL synthesis as well. Thus, the mutated σ^{32} proteins of *sidB1, sidB2, sidB3* and *sidB4* mutants seem to have decreased activities at 30°C in both Δ*dnaK52* and *dnaK*$^+$ cells. We also observed alterations in *sidB dnaK*$^+$ cells in synthesis at 30°C of proteins other than heat shock proteins (e.g. appearance of a protein of ~80 kd in *dnaK*$^+$ cells carrying *sidB1, sidB2* or *sidB3* mutations), the nature and significance of which are unknown.

sidB mutations cause defects in heat shock response and cell growth at 43.5°C

We were interested in establishing whether *sidB* mutations also affect induction of the heat shock response of Δ*dnaK52* and *dnaK*$^+$ cells. For Δ*dnaK52 sidB* mutants, we performed pulse−chase experiments where the cells were shifted from 30°C to 43.5°C and labeled with [^{35}S]methionine after incubation at 43.5°C for 5 min, followed by SDS−PAGE of the cellular proteins. Δ*dnaK52* cells carrying any of the *sidB1, sidB2, sidB3* or *sidB4* mutations were reduced in their heat shock response as compared with wild-type cells (Figure 6) and with Δ*dnaK52 sidB*$^+$ mutants (not shown). *sidB1* Δ*dnaK52* mutants and *sidB4* Δ*dnaK52* mutants exhibited a reduced but still detectable heat shock induction whereas *sidB2* Δ*dnaK52* mutants and *sidB3* Δ*dnaK52* mutants failed to exhibit detectable induction of major heat shock proteins upon shift to 43.5°C (Figure 6). For *dnaK*$^+$ *sidB* mutants, we performed similar pulse−chase experiments except that we also labeled the cells at later stages after heat shock in order to gain information on the capability of the cells to recover from heat shock response. All four *sidB dnaK*$^+$ mutants tested exhibited reduced heat shock induction of the major heat shock proteins as compared with wild-type cells. Figure 7 shows *sidB3 dnaK*$^+$ and *sidB4 dnaK*$^+$ mutants as examples. In addition, *sidB4 dnaK*$^+$ mutants cells were defective in the recovery from heat shock and continued to synthesize heat shock proteins at induced levels >60 min. The failure to down-regulate heat shock gene expression might be explained by assuming defects in regulation of cellular concentration of the mutant σ^{32} proteins of *sidB4* cells.

We wondered if the defects of *sidB dnaK*$^+$ mutants in induction of the heat shock response might be severe enough to cause impairment of cell growth at that temperature. We tested this possibility by streaking the cells on agar plates that were incubated at 43.5°C and found that two of the four strains, *sidB2 dnaK*$^+$ and *sidB3 dnaK*$^+$ mutants, were unable to grow at that temperature. Taken together, all four *sidB* mutations tested confer defects to Δ*dnaK52* and *dnaK*$^+$ cells in expression of heat shock proteins at intermediate temperatures as well as in induction of the heat shock response at high temperature. These results indicate general functional defects of the mutated σ^{32} proteins.

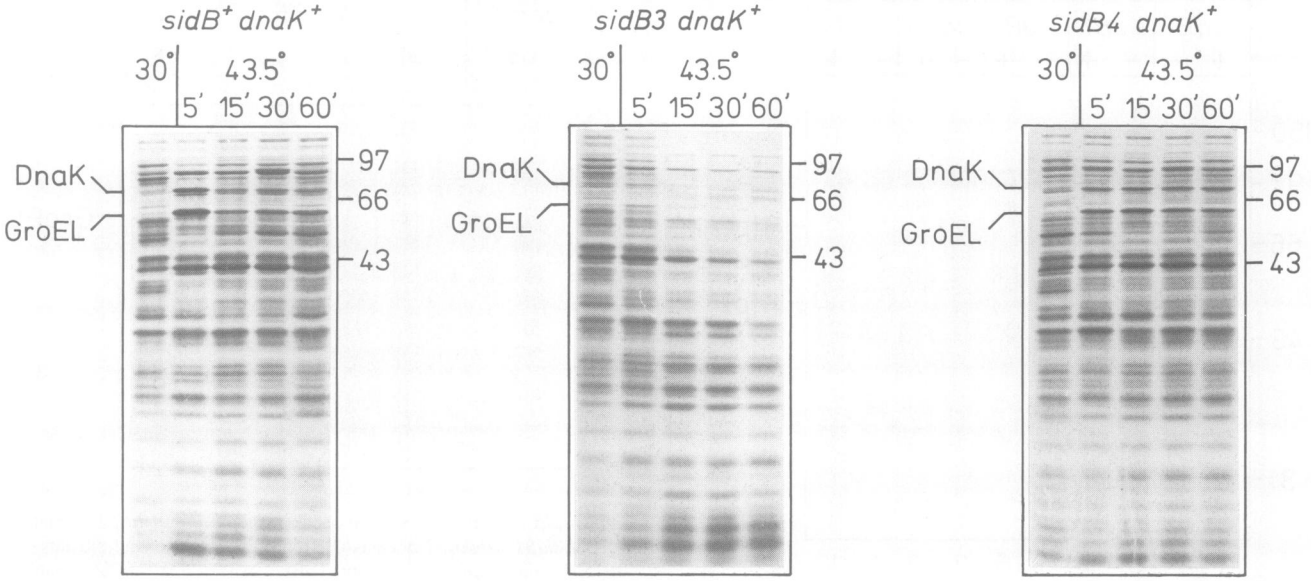

Fig. 7. *sidB* mutations affect heat shock response of *dnaK*$^+$ cells. Cell culture of *sidB3 dnaK*$^+$ mutants (BB1701), *sidB4 dnaK*$^+$ mutants (BB1702) and of *sidB*$^+$ *dnaK*$^+$ cells (MC4100) were pulsed-labeled with [^{35}S]methionine at 30°C or at 5, 15, 30 and 60 min after shift to 43.5°C. The proteins were separated on SDS–PAGE and autoradiographed. The migration positions of the DnaK and GroEL proteins (left side) and of mol. wt standards (right side) are indicated.

Cellular concentration at 30°C and apparent mol. wt of σ^{32} proteins of sidB mutants

The cellular concentration of the normally very unstable σ^{32} protein is the rate limiting factor for expression of heat shock genes (Grossman *et al.*, 1987; Straus *et al.*, 1987). It is therefore possible that defects in heat shock gene expression at 30°C of *sidB* mutants is due to a decrease in cellular concentration of the mutated σ^{32} proteins, e.g. by their further destabilization. To test this hypothesis, we performed quantitative Western hybridization experiments using anti-σ^{32} antiserum and determined the cellular concentration at 30°C of σ^{32} in Δ*dnaK52* and *dnaK*$^+$ cells carrying *sidB1*, *sidB2*, *sidB3* and *sidB4* mutations (Figure 8). In Δ*dnaK52* mutants lacking any suppressor the cellular concentration of σ^{32} is several fold higher than in wild-type cells, as expected from the known function of DnaK in degradation of σ^{32} (Grossman *et al.*, 1987; Straus *et al.*, 1987; Tilly *et al.*, 1989; D.B.Straus and C.Gross, personal communication). The presence of *sidB1* or *sidB2* mutations in Δ*dnaK52* and *dnaK*$^+$ cells did not significantly alter the cellular concentration of σ^{32} as compared with the respective *sidB*$^+$ cells. However, very slight alterations (<2-fold) in σ^{32} concentration would not be detectable due to limits of resolution of the Western technique. Thus, our data indicate that *sidB1* and *sidB2* mutations affect the activity rather than the cellular concentration of σ^{32} although we cannot exclude the possibility that small alterations in the cellular concentration of σ^{32} might contribute to the observed defects in heat shock gene expression. In contrast, the *sidB3* mutation caused a several fold decrease in cellular concentration of σ^{32} in both Δ*dnaK52* and *dnaK*$^+$ cells, as compared with the respective *sidB*$^+$ cells. These changes in concentration of σ^{32} might be sufficient to account for the observed defects of *sidB3* mutants in heat shock gene expression although additional defects in activity of the mutated σ^{32} proteins cannot be excluded. The presence of the *sidB4* mutation in Δ*dnaK52* and *dnaK*$^+$ cells did not

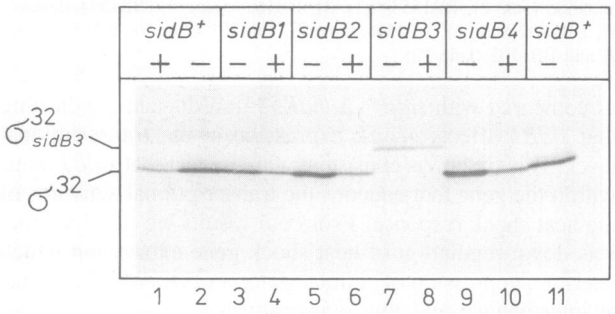

Fig. 8. Immunoblot analysis of σ^{32} proteins of *sidB* mutants. Total cell lysates of Δ*dnaK52* mutants (−) and *dnaK*$^+$ cells (+) with and without *sidB* suppressor mutations were electrophoresed and subjected to immunoblot analysis using anti-σ^{32} antiserum. Strains used are MC4100 (lane 1), fresh Δ*dnaK52* transductants lacking any suppressor mutation (lanes 2 and 11), BB1553 (lane 3), BB1699 (lane 4), BB1554 (lane 5), BB1700 (lane 6), BB1556 (lane 7), BB1701 (lane 8), BB1557 (lane 9) and BB1702 (lane 10).

reduce the cellular concentration of σ^{32} but there appears to be even a slight (2- to 3-fold) increase in the cellular concentration of σ^{32} as compared with the respective *sidB*$^+$ cells. Thus, as for *sidB1* and *sidB2* mutants, activity defects rather than concentration changes of σ^{32} account for the defects of *sidB4* mutants in the regulation of heat shock gene expression. It is not yet clear whether the slightly higher level of σ^{32} in the *sidB4* mutant is related to the defect of this strain in the down-regulation of heat shock gene expression.

The Western analysis shown in Figure 8 also revealed that the σ^{32} proteins of *sidB3* and *sidB4* mutants have altered mobilities. The apparent mol. wt of σ^{32} of *sidB3* mutants was considerably increased as compared with wild-type σ^{32}, which is consistent with our sequencing data that predict the addition of 38 extra amino acids to the C terminus of the *sidB3* mutant protein. The *sidB4* mutation caused a slight increase in mobility of σ^{32} which is presumably related to a small change in the ability of the protein to bind SDS.

Table II. Bacterial strains

Strains	Genetic markers	Reference or source
MC4100	F⁻ *araD139* Δ(*argF–lac*) *U169 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*	lab collection
BB1042	MC4100, *thr*::Tn*10* Δ*dnaK52*::Cm^r	Bukau and Walker (1989a)
BB1031	MC4100, *thr*::Tn*10*	this study
BB1130	MC4100, Δ*dnaK52*::Cm^r *sidA1*	this study
BB1553	MC4100, Δ*dnaK52*::Cm^r *sidB1*	this study
BB1699	BB1553, *dnaK*$^+$ *thr*::Tn*10*	this study
BB1554	MC4100, Δ*dnaK52*::Cm^r *sidB2*	this study
BB1700	BB1554, *dnaK*$^+$ *thr*::Tn*10*	this study
BB1556	MC4100, Δ*dnaK52*::Cm^r *sidB3*	this study
BB1701	BB1556, *dnaK*$^+$ *thr*::Tn*10*	this study
BB1557	MC4100, Δ*dnaK52*::Cm^r *sidB4*	this study
BB1702	BB1557, *dnaK*$^+$ *thr*::Tn*10*	this study

Discussion

We isolated and analyzed secondary mutations that suppress major cellular defects of Δ*dnaK52* mutants at intermediate (30°C) as well as at low (16°C) temperatures. A number of general conclusions concerning the role for DnaK in cellular metabolism can be drawn from this suppressor analysis. First, while *dnaK* function is required for normal growth at intermediate and low temperatures, this requirement can be circumvented by mutational alteration and thus is not absolute. Second, although we could isolate suppressors that permitted growth of Δ*dnaK52* mutants at intermediate and low temperatures, we did not isolate single suppressors that allowed growth of the Δ*dnaK52* cells over the entire temperature range for growth of *E.coli* wild-type cells. Thus, by selection for suppression of growth defects at an intermediate temperature (30°C), we did not obtain mutations (out of 100 isolates tested) which concomitantly allowed Δ*dnaK52* mutants to grow at 42°C. However, by selection for growth of Δ*dnaK52 sidB1* mutants at 42°C it is possible to obtain suppressors, named *std*, that allow growth of the cells at high temperatures (unpublished data). Thus it seems that suppression of growth defects of Δ*dnaK52* mutants at all growth temperatures requires multiple mutational events. While our experiments do not exclude the possibility that single secondary mutations exist which suppress growth defects of Δ*dnaK52* mutants within the entire growth temperature range of *E.coli*, they indicate that at least some of the cellular requirements for DnaK at high temperatures are different from those at intermediate and low temperatures. It should be emphasized that Δ*dnaK52* mutants have low cellular concentrations of DnaJ (Sell *et al.*, 1990) which might also be a cause of the cellular defects described above. However, lysogenization of Δ*dnaK52* mutants with λ phages carrying *dnaK*$^+$ but not *dnaJ*$^+$ complement cellular defects of Δ*dnaK52* mutants (Bukau and Walker, 1989a). Thus, it appears that it is lack of DnaK and not reduction of DnaJ which is primarily responsible for these defects.

A surprising finding of our suppressor analysis was that four out of five *sid* mutations that we analyzed mapped within the coding region of *rpoH*, the gene encoding σ^{32}. These four *sidB* mutations reduced the basal level of expression of GroEL and the other major heat shock proteins at 30°C both in Δ*dnaK52* and *dnaK*$^+$ strains by reducing transcription. They also reduced or eliminated the ability of both Δ*dnaK52* and *dnaK*$^+$ strains to induce heat shock proteins after a shift to 43.5°C. Furthermore, these four *sidB* mutations affected four different regions of the σ^{32} protein and, in one case, resulted in a several fold reduction in the cellular concentration of σ^{32} due to addition of 38 extra amino acids to its C terminus. On the basis of these observations it seems most likely that the *sidB* mutations are suppressing the major cellular defects of Δ*dnaK52* mutants at 30°C by reducing the elevated basal level of expression of heat shock proteins at 30°C seen in *dnaK* mutants rather than by causing differential changes in the levels of expression of particular heat shock proteins. This conclusion implies that regulation of heat shock gene expression is the single most important cellular function of DnaK that is required for normal growth and genetic stability of the cells at intermediate temperatures as well as for growth at low temperatures. Our results do not distinguish between the defects of Δ*dnaK52* mutants in cell division and chromosome segregation at 30°C (Bukau and Walker, 1989a,b) resulting from inappropriately elevated levels of expression of (i) the entire set of heat shock genes, (ii) the set of heat shock genes specifically minus *dnaK*, or (iii) one or more particular heat shock genes. However, the observation of Bahl *et al.* (1987) that the overexpression of the entire set of heat shock genes caused by the λ cIII protein is deleterious suggests that inappropriately elevated levels of heat shock protein expression is sufficient to cause deleterious effects even if the *dnaK*$^+$ gene is present. Furthermore, the deleterious effects do not appear to result from overexpression of the *lon* gene (Goff and Goldberg, 1987; Neidhardt and VanBogelen, 1987) since introduction of the Δ*dnaK52* allele into Δ*lon* mutants caused cellular defects at 30°C that were similar to those of *lon*$^+$ Δ*dnaK52* mutants (unpublished results). Nor do they appear to be due to overexpression of the *groES groEL* operon since overexpression of these genes on multicopy plasmids does not confer cellular defects to wild-type cells at 30°C (unpublished results).

Even if the most important function of DnaK at intermediate and low temperatures is regulation of the expression of heat shock genes, it is possible that DnaK may play additional, more direct roles in cell division and chromosome segregation. Our results do not exclude the possibility that DnaK may directly participate in such processes in *dnaK*$^+$ cells but that other molecular chaperones or polypeptide binding proteins can carry out its functions if DnaK is absent. However, it is clear that there are certain roles of DnaK that cannot be suppressed by *rpoH* mutations. For example, the instability of P1 miniplasmids was not suppressed in Δ*dnaK52 sidB1* mutants nor was the inability of Δ*dnaK52* mutants to plate λ suppressed by *sid* mutations. It is interesting to note that a direct role of DnaK has been demonstrated both in *in vitro* replication of λ DNA (for review see Echols, 1986; Liberek *et al.*, 1988; Zylicz *et al.*, 1989) and in *in vitro* replication of P1 plasmids (Wickner, 1990). In these cases, it does not appear that another molecular chaperone or polypeptide binding protein is able to replace the specific requirement for DnaK.

At low temperature, an essential role for DnaK in heat shock gene regulation would explain the initially puzzling finding that in striking contrast to Δ*dnaK52* mutants (Bukau and Walker, 1989a) Δ*rpoH* mutants were able to grow only at temperatures below 20°C (Kusukawa and Yura, 1988; Zhou *et al.*, 1988). While at 16°C Δ*rpoH* mutants express heat shock genes at low levels which correspond to the reduced cellular requirement at that temperature for heat shock proteins, Δ*dnaK52* mutants express heat shock genes at elevated levels that appear to be lethal at lower temperatures.

Taken together, our data indicate that cellular concentrations of heat shock proteins have to be tightly regulated with temperature, and that it is DnaK that plays a key function in this regulation. Even moderate alteration of the cellular concentration of heat shock proteins appears to result in severe growth defects and cell death. A requirement for precise, DnaK mediated adjustment to temperature of heat shock gene expression may explain our inability to isolate single secondary mutations that suppress cellular defects of Δ*dnaK52* mutants over the entire growth temperature range of *E.coli* cells; it may also explain why efficient suppression of cell division defects and genetic instability at 30°C of Δ*dnaK52 sidB* cells became less efficient at 16°C (although cold sensitivity of growth was suppressed at 16°C). Suppressors of Δ*dnaK52* mutations might adjust the cellular concentration of heat shock proteins to the cellular requirements at the particular selection temperature, but not to cellular requirements at a variety of temperatures.

Our data indicate that in *sidB1, sidB2* and *sidB4* mutants the defects in heat shock gene expression are primarily due to defects in activity of the mutated σ^{32} proteins. Until now, only very few mutations in *rpoH* have been described so that our analysis of *sidB* mutations might help in understanding the relevance of the particular protein domains for σ^{32} activity. All three *sidB1, sidB2* and *sidB4* mutations alter amino acids located within regions of σ^{32} that are conserved among sigma factors (Landick *et al.*, 1984; Doi and Wang, 1986; Helmann and Chamberlin, 1988) (Figure 4). The *sidB2* mutation is in the center of subregion 2.2. of conserved region 2 and changes the conserved Glu81 residue to Gly. From studies on various bacterial sigma factors it is thought that subregion 2.2. forms the heart of the RNA polymerase core binding region (Helmann and Chamberlin, 1988). The *sidB1* mutation is in subregion 4.2. of conserved region 4 and changes the highly conserved Thr252 to Met. Thr252 is immediately upstream of a predicted helix-turn-helix motif that, in the case of σ^{70} protein, appears to directly contact the −35 region of promoter sequences (Helmann and Chamberlin, 1988; Siegele *et al.*, 1989). The *sidB4* mutation is in subregion 4.1. of region 4 and changes the conserved Arg243 to Leu. Subregion 4.1. has unknown functions in sigma factor activity although C.Gross and co-workers recently showed that altered amino acids in this region decrease the activity of σ^{70} in transcription initiation (Siegele *et al.*, 1989). In summary, the *sidB1, sidB2* and *sidB4* mutations provide genetic evidence for the functional importance of conserved subregions 2.2., 4.1. and 4.2. of σ^{32}.

The *sidB3* mutation is of particular interest since it leads to synthesis of a mutant σ^{32} protein that differs from wild-type σ^{32} only in the presence of 38 additional amino acids at the C terminus. These extra amino acids considerably decrease the cellular concentration of σ^{32} which might be the major reason for reduction in heat shock gene expression in *sidB3* mutants. It is likely that the decrease in cellular concentration is due to increased proteolytic degradation of the mutant σ^{32} proteins. It has been reported recently that addition of extra amino acids to the C terminus of the N-terminal domain of λ repressor results in increased proteolytic degradation of this protein in *E.coli* cells (Parsell *et al.*, 1990). It is tempting to speculate that it is the C terminus of regulatory DNA binding proteins such as σ^{32} that determines their stability *in vivo*.

Materials and methods

Bacterial strains, phages and plasmids

Bacterial strains are listed in Table II. In addition, we used a collection of Hfr srains and a Tn*10* mapping strain kit constructed by M.Singer and C.Gross (Singer *et al.*, 1989). Plasmids pFN92, pKP11 and λ *kan cI*857-P1: R5-3 were described earlier (Landick *et al.*, 1984; Paek and Walker, 1986; Funnell, 1988). Bluescript SK$^+$ vector was purchased from Stratagene.

Media and growth conditions

Bacteria were grown aerobically at 30°C or at temperatures indicated in Luria Broth (LB) or M9 minimal media (Miller, 1972) supplemented with glucose (0.4%), thiamine (0.001%) and appropriate amino acids (50 μg/ml). Antibiotics were used in the following concentrations: chloramphenicol (Cm), 25 μg/ml; tetracycline (Tc), 10 μg/ml; kanamycin (Km), 60 μg/ml; ampicillin (Ap), 100 μg/ml. β-Galactosidase assays were done according to Miller (1972).

Genetic techniques and DNA manipulations

P1*vir* transductions, Hfr crosses, transformations and λ lysogenizations were done using standard techniques (Miller, 1972; Maniatis *et al.*, 1982; Silhavy *et al.*, 1984). Preparations of chromosomal and plasmid DNA and cloning of PCR amplified fragments were done as described (Maniatis *et al.*, 1982).

Strain constructions and mapping of sidB mutations

dnaK$^+$ derivatives of Δ*dnaK52* mutants (BB1699, BB1700, BB1701, BB1702) were constructed by first transducing the cells to *thr*::Tn*10* using P1*vir* lysates grown on a *dnaK*$^+$ *thr*::Tn*10* strain and by then screening for Cm^s, λ sensitive transductants.

sidB mutations were mapped by interrupted matings with Hfr strains and by P1*vir* transductions using P1*vir* lysates grown on a collection of strains carrying Tn*10* insertions at different locations on the *E.coli* chromosome (Singer *et al.*, 1988). Loss of *sidB* mutations in Δ*dnaK52* backgrounds during Hfr matings and P1*vir* transductions was monitored by determining the appearance of flat translucent colonies containing filamenting cells. Hfr mapping of the *sidB1, sidB2, sidB3* and *sidB4* mutations of strains BB1553, BB1554, BB1556 and BB1557, respectively, revealed that the *sidB* mutations were all located between 65 min and 84 min on the *E.coli* map. The *sidB* mutations in all four strains could be efficiently transduced out by co-transduction with two Tn*10* insertions, located at 75.25 min (*malT*::Tn*10*) and 75.5 min (*zhf-50*::Tn*10*).

Plasmid stability measurements

Plasmid stability tests were done as described before (Bukau and Walker, 1989b).

Marker rescue experiments

Logarithmically growing cells of strain BB1553 carrying the plasmid pFN92 were irradiated with 50 J/m^2 UV light in order to stimulate recombinational events. Then ~ 10^4 cells were plated on LB/Ap plates and incubated at 30°C. After 48 h the number of colonies that appeared pale and translucent were determined and the cells contained within these colonies were tested for the presence of cell division defects.

PCR amplification and cloning of rpoH genes

Chromosomal DNA (~ 0.5 μg) of strains BB1553, BB1554, BB1556 and BB1557 were used to amplify the coding region and the 5′ regulatory region of *rpoH* with PCR according to the instructions of the supplier of Taq polymerase (Perkin Elmer Cetus) with the following specifications. The incubation buffer contained 1.5 mM $MgCl_2$ and 0.5 μM primers. As

primers, we used 28mers that had 18 nucleotides at their 3'-end homologous to *rpoH* sequences and *Bam*HI linkers at their 5'-end. PCR amplification consisted of 30 cycles of incubation of the reaction mixture at 93°C for 1 min, 45°C for 0.5 min and 72°C for 4 min. Under these conditions very specific DNA fragments of the expected size of 1.2 kb were obtained. PCR amplification mixtures were subjected to four cycles of centrifugation with Centricon 30 filters (Amicon Corp.), digested with *Bam*HI restriction enzyme and cloned into the *Bam*HI site of Bluescript (SK^+) vector.

DNA sequencing

Two approaches were used to sequence PCR amplified *rpoH* genes of *sidB* mutants. First, amplified 1.2 kb fragments were gel purified and directly subjected to double strand sequencing according to the dideoxy method (Sanger *et al.*, 1977) using Sequenase (USB). However, this approach was not always useful since the quality of PCR DNA was not consistently good enough for sequencing. Second, PCR amplified *rpoH* fragments of *sidB* mutants were cloned into Bluescript plasmids and then subjected to double strand dideoxy sequencing. In this case the *rpoH* genes of at least three independent clones were sequenced to ensure that the sequence changes observed were not due to PCR mediated errors.

Pulse–chase experiments

Cell cultures were grown at 30°C to an OD_{600} of 0.4 in M9 minimal medium and, in the case of temperature shift experiments, subsequently incubated at 43.5°C. At the indicated times 1 ml aliquots were labeled with 10 μCi L-[^{35}S]methionine (sp. act. >800 Ci/mmol; Amersham Corp.) for 5 min followed by a chase for 5 min with unlabeled methionine (1 mM final concentration). The labeled cells were transferred on ice, washed in 0.9% NaCl, lysed by boiling in cracking buffer (20 mM EDTA, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 50 mM Tris–HCl, pH 6.8), and lysates were electrophoresed on 12.5% polyacrylamide–SDS gels (SDS–PAGE) (Laemmli, 1970). Gels were autoradiographed using Kodak X-Omat films. Band intensities in autoradiograms were measured using a Hirschmann elscript 400 (Biosys GmbH) laser densitometer.

Immunoblot analysis

For immunoblots, cells were grown at 30°C to early logarithmic phase in LB media and continuously labeled with 0.5 μCi [^{35}S]methionine/10 ml of culture for at least two generations prior to harvesting the cells. Cell lysates were prepared as described for the pulse–chase experiments and the amount of [^{35}S]methionine incorporated into cell protein was determined in a liquid scintillation counter. The volume of each sample loaded on polyacrylamide–SDS gels (12.5%) was adjusted to give a constant number of counts per lane. After electrophoresis, Western blots were performed according to the manufacturer's instructions using a polyclonal anti-σ^{32} antiserum that was preabsorbed to Δ*rpoH* cells (Kusukawa and Yura, 1988) according to D.Straus *et al.* (1987). As a secondary antibody horseradish peroxidase conjugated anti-rabbit IgG was used.

Microscopic techniques

For light microscopic analysis, cells from exponentially grown LB cultures were prepared on glass microscopic slides. For fluorescence microscopy, cells from exponentially growing LB cultures were OsO_4 fixed and stained with Fluorochrome 33342 (Hoechst–Roussel Pharmaceuticals, Inc.) as described before (Bukau and Walker, 1989b). Microscopic analysis was performed with a Zeiss Axioplan Universal microscope using a Plan 100 objective and Nomarsky differential interference contrast optics. Photographs were taken using Kodak Tri-X-Pan 400 films (Eastman Kodak Co.).

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