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Construction and Analysis of Hybrid *Escherichia coli-Bacillus subtilis dnaK* Genes

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The highly conserved DnaK chaperones consist of an N-terminal ATPase domain, a central substrate-binding domain, and a C-terminal domain whose function is not known. Since *Bacillus subtilis dnaK* was not able to complement an *Escherichia coli dnaK* null mutant, we performed domain element swap experiments to identify the regions responsible for this finding. It turned out that the *B. subtilis* DnaK protein needed approximately normal amounts of the cochaperone DnaJ to be functional in *E. coli*. The ATPase domain and the substrate-binding domain form a species-specific functional unit, while the C-terminal domains, although less conserved, are exchangeable. Deletion of the C-terminal domain in *E. coli* DnaK affected neither complementation of growth at high temperatures nor propagation of phage λ but abolished degradation of σ^{32}.

Hsp70 chaperones are a highly conserved group of proteins which participate in various cellular processes, including the folding of nascent polypeptides, assembly and disassembly of multimeric protein structures, membrane translocation of secreted proteins, and protein degradation (3, 11, 12). The DnaK protein, the prokaryotic Hsp70 homologue, has been found in all of the eubacterial species examined so far. Its chaperone activity relies on the transient association of DnaK with substrates in a process controlled by ATP and the cochaperones DnaJ and GrpE (12, 18). In the ATP conformation, DnaK binds and releases substrates very rapidly (18, 21, 25, 27). Upon ATP hydrolysis, DnaK is switched into the ADP conformation, which exchanges substrates slowly. The ATPase and substrate-binding activities of DnaK are divided into two separable functional units: the N-terminal ATPase domain (amino acid [aa] 1 to 385; ~44 kDa) and the central substrate-binding domain (aa 386 to 540; ~17 kDa). These two domains are followed by a C-terminal domain (aa 543 to 637; ~10 kDa) whose function is unknown. During our work on *Bacillus subtilis dnaK*, we found that the *Escherichia coli* Δ*dnaK52* mutant could not be complemented by the *dnaK* gene of *B. subtilis* for growth defects at high temperatures and for propagation of phage λ. Therefore, this study was performed to identify the region(s) within the *B. subtilis* DnaK protein which is responsible for this failure to complement the Δ*dnaK52* mutant of *E. coli* for growth at high temperatures and for propagation of phage λ. For this purpose, a series of hybrid genes have been constructed based upon the domain model of the Hsp70 proteins.

Construction of hybrid *dnaK* genes. To allow reciprocal exchange of the different domains, a restriction site was first introduced into the interdomain region separating the ATPase and the peptide-binding domains (*Afl*II site) and that separating the peptide-binding domain and the C-terminal domain (*Spe*I site). The amino acid exchanges caused by the introduction of a restriction site in pHK05, pHK06, and pHK07 did not change the complementation profiles in comparison to the corresponding wild-type counterparts (data not shown). The different hybrid genes shown in Fig. 1 were then constructed. All *dnaK* genes, the two wild types and the mutant alleles, were ligated into plasmid pUHE21-2fdΔ12 (1) downstream of an isopropyl-β-D-thiogalactopyranoside (IPTG)-controllable promoter and transformed into MC4100 (24) and isogenic *dnaK* derivatives containing plasmid pDMI,1 (15) coding for the LacI repressor. Upon addition of 1 mM IPTG, all *dnaK* genes from plasmids pHK01 through pHK14 were stably expressed to equal levels, as visualized by examining cell lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue, and reached levels about fivefold higher than that of chromosomally expressed wild-type DnaK (data not shown).

Complementation experiments with Δ*dnaK52* and *dnaK756 E. coli* mutants. First, we tested the hybrid *dnaK* genes for complementation of the Δ*dnaK52* null mutant for growth defects at two different temperatures. In this mutant, an about 1-kb internal fragment of *dnaK* has been replaced with a *cat* cassette (23), which causes cold and heat sensitivity, and thus the mutant possesses a very narrow temperature range for growth (5, 6). The different pHK plasmids were transformed into strain BB2414, a Δ*dnaK52* derivative of MC4100 (6), and plated on Luria-Bertani agar plates containing IPTG at a final concentration of 250 μM to induce the *dnaK* alleles; these plates were incubated at either 30 or 40°C, and growth was recorded. In the presence of either the empty vector pUHE21-2fdΔ12 (data not shown) or the different recombinant plasmids, strain BB2414 formed colonies at 30°C (Fig. 2A). When plated at 40°C, it was unable to form colonies in the absence of IPTG with either plasmid (data not shown). Growth occurred only in the presence of the inducer with a plasmid expressing either wild-type *E. coli* DnaK (pHK01), hybrid DnaK with the ATPase and the peptide-binding domain from *E. coli* and the C-terminal domain from *B. subtilis* (pHK11), or a truncated version of *E. coli* DnaK lacking the C-terminal domain (pHK13; Fig. 2A). We conclude from these results that *B. subtilis* DnaK cannot substitute for the *E. coli* homologue in the Δ*dnaK52* strain and that the C-terminal domain is completely dispensable for growth at least up to 42°C.

As already observed for the Δ*dnaK52* allele, *E. coli* BB2362, an MC4100 derivative carrying the *dnaK756* allele (6), was able to form colonies at 30°C in the presence of the vector plasmid

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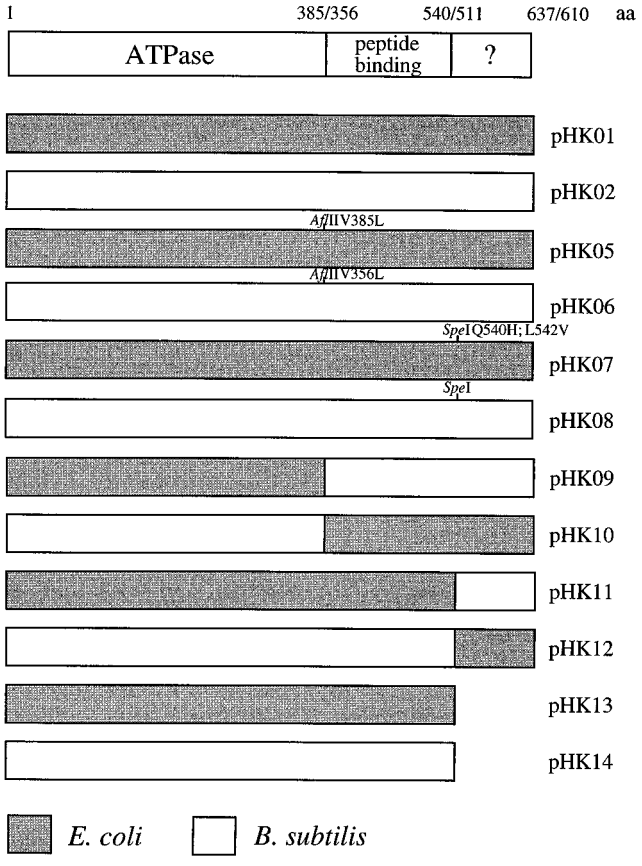

FIG. 1. Construction of hybrid *dnaK* genes. The domain structures of the DnaK proteins and the positions of the domain boundaries (*E. coli*/*B. subtilis*) are given. Amino acid exchanges caused by the introduction of restriction sites are indicated. By using sequence-specific mutagenesis (14), two different restriction sites were introduced into the two *dnaK* genes. An *Afl*II site was introduced in the region separating the ATPase and peptide-binding domains, leading to V385L and V356L exchanges in the DnaK proteins of *E. coli* and *B. subtilis*, respectively. An *Spe*I site was created within the interdomain region between the peptide-binding and C-terminal domains of both genes, leading to Q540H and L542V exchanges in the *E. coli* protein only. The hybrid genes shown here were constructed by using these modified prototype genes.

pUHE21-2fdΔ12 (data not shown) or any of the hybrid plasmids (Fig. 2A). When these different strains were incubated at 46°C, they were able to form colonies only in the presence of 50 μM IPTG. Here, both the *E. coli* and *B. subtilis* wild-type alleles were able to complement the *dnaK756* mutant for growth (Fig. 2A, pHK01 and pKH02). Proteins consisting of the ATPase domain from one species and the two other domains from the second species turned out also to be active (pHK09 and pKH10). As already observed for the Δ*dnaK52* allele, the small C-terminal domain was exchangeable without significantly influencing the complementing activity of the DnaK proteins (pHK11 and pHK12) and was even dispensable (pHK13 and pHK14).

These results seem to be in contrast to those obtained with the Δ*dnaK52* mutant strain and raise the question of why there are allele-specific differences in complementation. Two different possibilities can be envisaged: (i) the amount of DnaJ protein in the *dnaK* null mutant is reduced due to a polar effect of the Cm^r marker on the downstream *dnaJ* gene compared to the wild type or the *dnaK756* allele (27), or (ii) the mutant DnaK756 protein retains some residual activity which can be increased in the presence of the wild-type protein. To distinguish between these two possibilities, we constructed plasmid p*dnaJ*, which carries the *dnaJ* gene under the control of an arabinose- and IPTG-inducible promoter. This plasmid was transformed into all BB2414 derivate strains containing wild-type and hybrid *dnaK* genes. The amounts of DnaJ in the different *E. coli* strains used here was determined by immunoblotting. They were below the level of detection in the Δ*dnaK52* mutant and in the same strain carrying the p*dnaJ* plasmid in the absence of the inducer and restored to wild-type or *dnaK756* levels when the latter strain was grown in the presence of 0.5% arabinose and 1 mM IPTG (data not shown). Under *dnaJ*-inducing conditions, wild-type *B. subtilis* DnaK (pHK02) and the hybrid derivates containing the ATPase and substrate-binding domains of *B. subtilis* were now able to complement *E. coli* Δ*dnaK52* for growth at high temperature (Fig. 2A). Therefore, the *B. subtilis* DnaK protein is able to complement Δ*dnaK52* for growth at high temperature, provided that sufficient amounts of DnaJ are present. Quite recently, the exact ratios of DnaK and DnaJ have been determined to be 30:1 in *E. coli* (28) and 3:1 in *B. subtilis* (21). The fact that these ratios differ by a factor of 10 might indicate that the *B. subtilis*

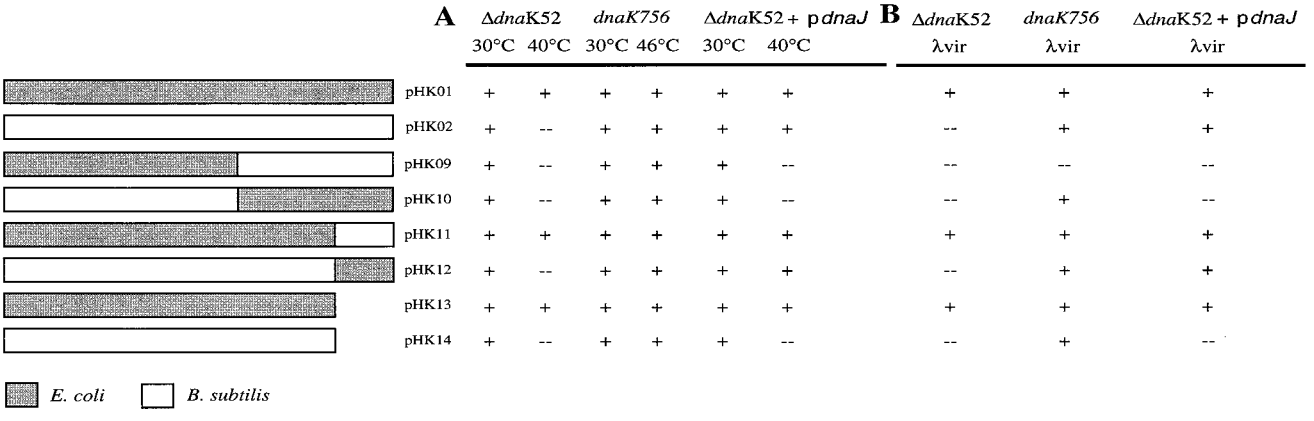

FIG. 2. Complementation of temperature-sensitive phenotypes and phage λ propagation by plasmid-encoded hybrid *dnaK* genes. Δ*dnaK52*, *dnaK756* and Δ*dnaK52* mutant strains carrying the *dnaJ* gene under arabinose and IPTG control and containing the different *dnaK* hybrids were tested for growth at 30 and 40 or 46°C (A) and for propagation of bacteriophage λ at 30°C (B). Growth was assayed by determining the ability of the cells to form colonies or plaques on Luria Bertani agar plates. +, wild-type number and size of colonies or plaques; - -, no colonies or plaques. All strains were grown in the presence of 250 μM IPTG to induce the expression of the *dnaK* genes, and those carrying p*dnaJ* also received 0.5% arabinose. Plates were scored for colonies and plaques after 20 h of incubation.

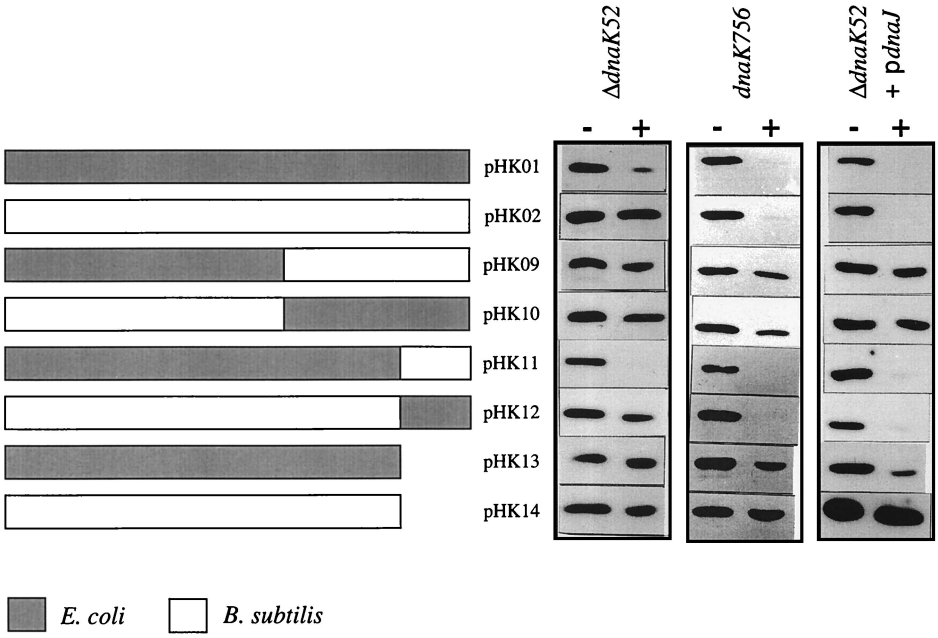

FIG. 3. Amounts of σ^{32} in the presence of different hybrid proteins. Δ*dnaK52* or *dnaK756* mutant strains carrying hybrid *dnaK* genes were grown to mid-exponential phase at 30°C and induced with 250 μM IPTG. Whole-cell fractions corresponding to identical amounts of cell culture were collected before (−) or 2 h after (+) the addition of IPTG, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The membranes were probed with anti-σ^{32} antibodies (1:10,000 dilution) and developed by a colorimetric assay as previously described (13).

DnaK protein needs this large amount of DnaJ to stimulate its ATPase activity.

Plating efficiency of bacteriophage λ in the presence of the different hybrid DnaK proteins. The *dnaK* gene has been discovered to be a conditional-lethal mutation which does not allow replication of phage λ DNA (10), and DnaK is involved in the dissociation of the complex consisting of the λ P and *E. coli* DnaB proteins (8). Therefore, we asked which hybrid DnaK proteins would allow the propagation of phage λ in *dnaK* mutant strains. All three of the strains described above (*dnaK756*, Δ*dnaK52*, and Δ*dnaK52* plus p*dnaJ*) were infected with λ *vir*, and growth was recorded in the presence of 250 μM IPTG at 30°C. In principle, we observed a growth pattern of phage λ comparable to that observed for complementation of growth defects at high temperature (Fig. 2B). Deletion of the C-terminal domain of *E. coli* DnaK did not abolish λ propagation but caused inactivation of *B. subtilis* DnaK in the *dnaK* null mutant, even in the presence of wild-type levels of DnaJ. These results confirm those for complementation for growth at high temperature and highlight that *B. subtilis* DnaK needs increased amounts of DnaJ to be active and, in addition, that DnaK756 must possess some residual activity.

Degradation of σ^{32}. In *E. coli*, the genes encoding cytosolic heat shock proteins form a regulon that is positively controlled by the *rpoH* gene product, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (2, 20, 29). A key aspect of this regulation, the sensing of stress and transmission of this information to σ^{32}, involves the DnaK chaperone machine (9). The DnaK chaperone system was shown to physically interact with σ^{32} (7, 16, 17), and two sites with high affinity for DnaK have been located within σ^{32} (19). Therefore, we asked whether hybrid DnaK proteins would be able to interact with σ^{32}, causing destabilization and thereby a reduction in its total amount within the cells. The amount of σ^{32} was determined in the three *E. coli* indicator strains containing the different DnaK-expressing plasmids in the absence or presence of 250 μM IPTG to induce the *dnaK* genes from the plasmids. While induction of the *E. coli* wild-type *dnaK* gene clearly reduced the amount of σ^{32} in all three indicator strains, the *B. subtilis* wild-type homologue needed larger amounts of the DnaJ protein to lower the intracellular level of σ^{32} (Fig. 3). Hybrid DnaK proteins containing the ATPase domain of one species and the peptide-binding and C-terminal domains of the other species exhibited only slight activity in the Δ*dnaK52* mutant strain expressing DnaJ (pHK09 and pHK10; Fig. 3). The C-terminal domains were again exchangeable (pHK11 and pHK12) without changing the complementation profile in comparison to that of the wild-type counterpart. Deletion of this domain in *E. coli* DnaK (pHK13) rendered it inactive in the *dnaK* null mutant and caused a reduction of its activity in *dnaK756*. The truncated *B. subtilis* DnaK protein (pHK14) was no longer active, even in the presence of sufficient DnaJ levels. In summary, exchange of the ATPase domain resulted in nearly inactive proteins, as already described for complementation for growth defects at high temperature and λ propagation. The deletion of the C-terminal domain of *E. coli* or *B. subtilis* DnaK resulted in strongly reduced complementation in both the *dnaK756* and the Δ*dnaK52* mutants independent of the amount of DnaJ. The major conclusion from these results is that the C-terminal domain is essential for degradation of σ^{32} in vivo. It might be involved in the binding of σ^{32}, its unfolding, or both. Since this domain is not involved in binding of the λ P protein and of thermosensitive cytoplasmic proteins, σ^{32} might represent a specific substrate.

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