Structure-Function Analysis of HscC, the *Escherichia coli* Member of a Novel Subfamily of Specialized Hsp70 Chaperones*

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Hsp70 chaperones assist protein folding processes through nucleotide-controlled cycles of substrate binding and release. In our effort to understand the structure-function relationship within the Hsp70 family of proteins, we characterized the *Escherichia coli* member of a novel Hsp70 subfamily, HscC, and identified considerable differences to the well studied *E. coli* homologue, DnaK, which together suggest that HscC is a specialized chaperone. The basal ATPase cycle of HscC had $k_{cat}$ and $K_m$ values that were 8- and 10,000-fold higher than for DnaK. The HscC ATPase was not affected by the nucleotide exchange factor of DnaK GrpE and stimulated 8-fold by DjIC, a DnaJ protein with a putative transmembrane domain, but not by other DnaJ proteins tested. Substrate binding dynamics and substrate specificity differed significantly between HscC and DnaK. These differences are explicable by distinct structural variations. HscC does not have general chaperone activity because it did not assist refolding of a denatured model substrate. In vivo, HscC failed to complement temperature sensitivity of *ΔdnaK* cells. Deletion of *hscC* caused a slow growth phenotype that was suppressed after several generations. Triple knock-outs of all *E. coli* genes encoding Hsp70 proteins (*ΔdnaK ΔhsA ΔhscC*) were viable, indicating that Hsp70 proteins are not strictly essential for viability. An extensive search for ΔhscC phenotypes revealed a hypersensitivity to Cd$^{2+}$ ions and UV irradiation, suggesting roles of HscC in the cellular response to these stress treatments. Together our data show that the Hsp70 structure exhibits an astonishing degree of adaptive variations to accommodate requirements of a specialized function.

The 70-kDa heat shock proteins (Hsp70s) constitute a large family of highly conserved chaperones that assist a multitude of protein folding processes including de novo folding of proteins, prevention of aggregation and refolding of stress denatured proteins, disaggregation of protein aggregates, control of activity and stability of regulatory proteins, and degradation of unfolded proteins (1–3). Such a large and diverse array of functions has been attributed to no other class of chaperones.

The chaperone activity of the Hsp70 proteins is based on several properties: (i) The C-terminal substrate-binding domain of Hsp70s interacts with substrates by binding to short peptide stretches of −5 amino acids in length (4). (ii) This interaction is controlled by the nucleotide status of the adjacent N-terminal ATPase domain (5). (iii) The nucleotide status is regulated by co-chaperones of the DnaJ protein family and a nucleotide exchange factor (GrpE in bacteria, Bag in eukaryotic cytosol) (3, 6). This mode of action of Hsp70 chaperones and their co-chaperones has been elucidated mainly using *Escherichia coli* DnaK with DnaJ and GrpE and mammalian Hsc70 with Hdj-1, the human DnaJ homologue, and Bag-1 M as model systems. However, more recently it was found that there are significant differences between the Hsp70 systems. In the yeast cytosol Ssa1 and Ssb1 and in *E. coli* DnaK and HscA (Hsc66) differ greatly with respect to their ATPase cycle. HscA and Ssb1 have much lower affinities for ATP than DnaK and Ssa1 (7–9). These differences go along with differences in the cellular functions of these proteins, although it is not clear whether a causal correlation exists. Although Ssa1 is involved in a large variety of protein folding processes, Ssb1 seems to be mainly engaged in the translation process and the de novo folding of proteins (10, 11). Similarly, whereas DnaK is involved in many different folding processes in *E. coli*, HscA seems to be specialized for the assembly of iron-sulfur cluster proteins (12).

Sequencing of the *E. coli* genome identified a third Hsp70 homologue, termed HscC (Hsc62). HscC shares approximately 30% amino acid identity with DnaK and HscA, which is much lower than the sequence identity between DnaK and human Hsc70 of approximately 50% (13). HscC thus belongs to the most distant Hsp70 relatives of DnaK and is therefore anticipated to exhibit strong differences to DnaK in functional and mechanistic features. In an effort to dissect the molecular basis for the functional variability among Hsp70 chaperones and to understand the roles for this newly identified third Hsp70 homologue of *E. coli*, we characterized the HscC protein in *vitro* and searched for its function *in vivo*.

**EXPERIMENTAL PROCEDURES**

*Bacteria and Phages*— Routinely, the bacterial strains listed in Table 1 were cultured at 30 °C in NZY medium (10 g yeast extract, 5 g peptone, 1 g NaCl). The antibiotics ampicillin, chloramphenicol, kanamycin, spectinomycin, and tetracycline (Sigma) were used at final concentrations of

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The abbreviations used are: Hsp70, 70-kDa heat shock protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IAANS, 2-(4-amido-anilino)napthalene-6-sulfonic acid.
100, 15, 25, 50, and 10 μg ml⁻¹, respectively. λ plaque formation was tested using λmut and, as control, a dnaK and dnaJ transducing λ phage (a generous gift from C. Georgopoulos).

Protein Production and Purification—The open reading frame encoding for HscC was PCR-amplified from MC4100 genomic DNA and cloned into the vector pUHE21-26ΔΔ12 using BamHI and HindIII restriction sites. The vector contains a strong IPTG-regulatable promoter. HscC was produced in ΔdnaK2 mutant cells (BB1553) and purified as follows. The hscC expressing strain was grown in 2× YT at 30 °C to mid-log phase and induced by the addition of IPTG to a final concentration of 1 mm for 5 h. The cells were disrupted using a French Press, and cell debris was removed by centrifugation. The cleared lysate was subjected to ammonium sulfate precipitation at 35% saturation. The pellet was resuspended in a minimal volume of buffer A (25 mM Hepes-KOH, pH 7.6, 50 mM KC1, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 1 mM EDTA, 5% glycerol, and 10 mM N-octylglucopyranosid) and chromatographed on a Superdex 200 gel filtration column (Amersham Biosciences) pre-equilibrated in buffer A. HscC-containing fractions were pooled and applied to a DMAE-Fractogel anion exchange column (Merck) equilibrated in buffer A. HscC was eluted using a linear gradient of 50–100 mM KC1. The pooled HscC-containing fractions were divided into aliquots and stored at −80 °C. To produce DicJCATM as a C-terminus fusion with intein and chitin-binding protein, the DicJCATM encoding DNA sequence was amplified by PCR and cloned into vector pYTB2 (New England Biolabs). DicJCATM-intein-chitin-binding protein was produced at 15 °C in BL21(DE3) cells grown in 2× YT medium and purified in the presence of 0.2% Triton X-100 on a chitin column according to the instructions of the manufacturer (New England Biolabs). Cleavage of the intein portion was performed in 50 mM dithiothreitol for 36 h at 4 °C. DnaK, DnaJ, CbpA, HscB, and GrpE were expressed in E. coli and purified according to published protocols (14–18).

Substrate Interaction, ATPase, and Chaperone Assays—Peptide binding to HscC was determined using a IAANS-labeled peptide (see Fig. 4B) as published (19). Peptide library scanning was performed essentially as described (20). Briefly, DnaK (0.2 μM) and HscC (0.8 μM) were incubated with cellulose membranes onto which 13-mer peptides were spotted according to the following equation.

\[
\text{f}_i(m) = \frac{1}{m} \sum_{n=1}^{m} \frac{N_n(i)}{n} 
\]

(Eq. 1)

where \(N_n(i)\) is the total number of the amino acid X in peptide \(i\), \(n\) is the total number of peptides in the library (557), and \(m\) is a number between 1 and \(n\), e.g. \(f_1(3)\) is the number of alanines in peptide 1 divided by the average number of A in the library; \(f_5(2)\) is the average number of alanines in peptides 1 and 2 divided by the average number of alanines in the library; \(f_{557}(557)\) is the average occurrence of alanines in peptides 1–557 divided by the average number of alanines in the library, which is equal to 1. These cumulative frequencies \(f_i(m)\) were plotted versus \(m\) (see Fig. 4B), and the \(m\) values between 100 and 400 were analyzed by linear regression analysis. The resulting intercepts represent the relative occurrence of each amino acid in a theoretical peptide with the characteristics of the library that has the highest affinity to DNAK and HscC, respectively.

RESULTS

Unique Sequence Characteristics of HscC—To guide our biochemical studies of HscC we first analyzed its amino acid sequence using ClustalW and Swiss Model (25–27). To search for elements that had been shown for other Hsp70 homologues to be important for the ATPase cycle and the interactions with co-chaperones and substrates, the HscC sequence was aligned with the sequences of E. coli DnaK, E. coli HscA, and human Hsc70 as representatives of the three recently identified subfamilies of the Hsp70 chaperones (9) (Fig. 1, A–C).

The ATPase domain of HscC is overall well conserved, including the residues, which coordinate the phosphate groups of ATP and are important for catalysis (e.g. Lys⁷⁰, Glu¹⁷¹, Thr¹⁹⁹), numbering according to DnaK throughout the paper unless otherwise stated; for review see Ref. 28). However, it was surprising to discern a deletion of 19 amino acids corresponding to residues 93–111 of DnaK that otherwise exclusively occurs in Hsp70s of Gram-positive bacteria and archaees (29), the functional consequences of which are unknown so far (not shown). In addition, the structural elements that were identified in DnaK to be important for the tight binding of nucleotides and interaction with GrpE, including two salt bridges and an exposed loop (9), are absent in HscC. HscC is therefore expected to have high nucleotide dissociation rates and to be incapable of interacting with GrpE (Fig. 1B and data not shown). The residues in DnaK that are important for the interaction with DnaJ (Tyr⁴¹⁴, Asn¹⁴⁷, Asp¹⁴⁸, Arg¹⁶⁷, Glu¹⁷¹, and Val¹⁷⁵) (30, 31) are all conserved in HscC except Asn¹⁴⁷, which is conservatively
exchanged by serine. It therefore seems likely that this Hsp70 also interacts with a DnaJ protein.

The substrate-binding domain (residues Val 354–Pro556) is less well conserved. A homology model of residues Val 354–Arg486 of HscC using Swiss Model and all available structures of the substrate-binding domain of Hsp70 proteins as template is shown in Fig. 1D. The helical lid domain (residues Gln 538–Ala607 in DnaK) also seems to be present, although helices A and/or B are probably shorter by a total of four turns because of a deletion in this region (corresponding to residues 514–526 in DnaK). The substrate-interacting residues are reasonably well conserved except amino acids that form an arch over the substrate backbone in the DnaK crystal structure (Fig. 1C) (4, 32).

A striking difference to DnaK and Hsc70 is an insertion of four residues in the loop L1,2 and a small deletion of two amino acids in loop L5,6 leading to a displacement of the opening of the substrate-binding cavity in direction of the outer loops (Fig. 1, C and D). Together with helix B, which has probably been shortened, this could have profound influences on substrate binding by HscC compared with DnaK and most other Hsp70s, in particular because we recently showed that residues in L1,2 and L3,4 modulate substrate specificity (33).

Using these characteristics in the substrate-binding domain of HscC, we performed a Blast search for homologues. Homologues with an insertion of 4 amino acids in loop L1,2 were found in the closely related enterohemeoragic E. coli, Salmonella typhimurium, and Salmonella enteritis. These Hsp70 proteins have a high degree of identity over the whole sequence and are therefore HscC orthologues. In addition, we found Hsp70 homologues with an even larger insertion of 8 or 9 residues in L1,2 in Burkholderia fungorum, Pseudomonas fluorescens, Novosphingobium aromaticivorans, Bacillus anthracis, and Ralstonia solanacearum (Fig. 1, A–C). Whether these Hsp70 homologues belong to the HscC type will be discussed below.

Basal ATPase Cycle of HscC—To analyze the ATPase cycle of HscC, we first determined $K_m$ and $k_{cat}$ of the basal steady-state ATPase activity and found that $K_m$ and $k_{cat}$ of HscC were $112 \pm 6 \mu M$ and $0.0046 \pm 0.0002 s^{-1}$ (Fig. 2A). Although the $k_{cat}$ is almost 8- and 3-fold higher than the values for DnaK and HscA,
Characterization of E. coli HscC

Fig. 2. ATPase activity of HscC. A, Michaelis-Menten kinetic of HscC ATPase activity. The reaction mixtures contained 0.3 μM HscC and 3–1000 μM ATP in HKM buffer at 30 °C. The initial rates were determined from least square linear regression analysis of the data and are plotted as the rate observed (Vₜ) versus ATP concentration. The curve shown represents a least square fit of the Michaelis-Menten equation to the data resulting in a Kₘ = 112 ± 6 μM and kₚ = 0.0046 ± 0.0002 s⁻¹. The inset shows the Lineweaver-Burk plot. B, stimulation of the steady-state ATPase activity of HscC by DnaJ proteins normalized to HscC basal rate.

The catalytic efficiency kₚ/Kₘ is much lower for HscC (41 M⁻¹s⁻¹) than for DnaK (30,000–70,000 M⁻¹s⁻¹) or even HscA (109 M⁻¹s⁻¹). It is surprising that Hsp70 proteins vary that much in their basal ATPase activity. The Kₘ for ATP is 10,000-fold higher for HscC than for DnaK and still 10-fold higher than for HscA, indicating that the affinity of HscC for nucleotide is relatively low, consistent with a high nucleotide dissociation rate as predicted above. To confirm this result, we tried to determine the dissociation rate for ADP directly using the fluorescently labeled nucleotide analogue (N8-(4′-thraniloylaminobutyl)-8 aminoadenosine 5′-phosphate) and indirectly using intrinsic tryptophane fluorescence (22, 23, 34). However, the measured fluorescence differences between the free and nucleotide-bound forms were too small to give reliable results (not shown). Nevertheless, circumstantial evidence suggests that the dissociation rates for nucleotides are relatively high. First, HscC (like HscA) does not bind to ATP-agarose; second, a complex of ATP with HscC cannot be isolated by rapid gel filtration. Both properties are in contrast to the behavior of DnaK and mammalian Hsp70s.

Stimulation of the ATPase Cycle of HscC by Co-chaperones—The ATPase rate of HscC was not stimulated significantly by DnaJ, GrpE, or a combination of both (Fig. 2B and data not shown), arguing that neither DnaJ nor GrpE functionally interacts with HscC. In the view of our sequence analysis, this was not surprising for GrpE (Fig. 1B). In the case of DnaJ, however, the result was unexpected, and we conclude that DnaJ is not the appropriate co-chaperone for HscC.

Aside from DnaJ there are five additional DnaJ proteins in E. coli: CbpA, DjIA, HscB, YbeS, and YbeV. The unique common feature of all the DnaJ homologues is a short sequence of about 75 amino acid residues called the J-domain, which is essential for the interaction with an Hsp70 chaperone partner and for the stimulation of its ATPase activity (Fig. 1E) (6, 35). It has been shown genetically and/or biochemically that CbpA and DjIA interact with DnaK and HscB with HscA (18, 36–38). They are therefore not likely to interact functionally with HscC. The remaining DnaJ proteins, YbeS and YbeV, which exhibit 50% amino acid identity, are encoded by two open reading frames that are in close proximity to the HscC encoding gene hscC (ybeW) on the E. coli chromosome (13). Because we show here (see below) that YbeV is a bona fide DnaJ protein, we propose to change the sequencing project names of YbeS and YbeV into DjlB and DjlC (DnaJ-like proteins B and C) in analogy to DjIA. The coding sequences of both potential co-chaperones were cloned and expressed from a regulatable promoter. Overproduction of DjIB was toxic. DjlC was very unstable in vivo, and after overproduction it was found in the insoluble membrane fraction. Both proteins are predicted to have a transmembrane domain at their C terminus. Therefore, we produced DjlC without the potential transmembrane domain (DjICΔTM) fused C-terminally to intein and chitin-binding protein and purified it by affinity chromatography over a chitin column and dithiothreitol-induced cleavage from the intein. DjlCΔTM stimulated the ATPase activity of HscC about 8-fold and therefore seems to be the appropriate DnaJ co-chaperone for HscC (Fig. 2B). However, it did not stimulate the ATPase activity of DnaK, nor was the J-domain of DjlC when grafted onto the C-terminal domains of DnaA able to replace DnaJ in vivo (data not shown). Therefore, DjlC is not cooperating with DnaK and is most likely specific for an interaction with HscC. Addition of GrpE did not increase the DjlC-stimulated ATPase activity of HscC (not shown).

HscC-Substrate Interactions—Sequence analysis and modeling have suggested that the interaction of HscC with substrates might be significantly different from the DnaK-substrate interaction. Because peptides are good model substrates for Hsp70 chaperones (39, 40), we used a fluorescently labeled peptide to analyze the HscC-substrate interaction. The peptide α32-132-Q132-144-CIAANS (19) was found to bind with good affinity to HscC, and the binding resulted in an increase in fluorescence at 420 nm when excited at 335 nm. Using this peptide we determined the dissociation equilibrium constant (Kₜ) in the absence and presence of ATP. Both values were very similar, approximately 1.5 μM (Fig. 3A and Table I). This contrasts with the affinity of this peptide to DnaK, which is about 20 times higher in the absence (Kₜ = 0.08 μM) than in the presence of ATP (Kₜ = 1.8 μM) (41). Because the Kₜ for the peptide-HscC complex in all nucleotide states is comparable with the Kₜ of the peptide-DnaK complex in the ATP state, we determined for α32-132-Q132-144-CIAANS whether the substrate-binding domain of HscC is in a constitutively open conformation with high association and dissociation rate constants (kₜₐₐ and kₜₐₜ) (Fig. 3B). In the absence of ATP the kₜₐₐ of the peptide-HscC complex (1.4 × 10⁻³ s⁻¹) was similar to the kₜₐₜ measured for the peptide-DnaK complex (0.9 × 10⁻³ s⁻¹) (41), indicating that HscC is not in an open conformation but that kₜₐₜ is very low. ATP increased kₜₐₜ by a factor of 19 as compared with the factor of 440–2500 determined for DnaK (39, 41). Therefore, ATP does influence the conformation of the substrate-binding domain of HscC, but it increases the kₜₐₜ and kₜₐₐ to a similar extent, thereby not influencing the equilibrium constant. We conclude that the kinetics of the HscC-substrate interaction is significantly different from the DnaK-substrate interaction.

Substrate Specificity of HscC—Because our sequence alignment showed that residues contributing to substrate specificity in DnaK are replaced in HscC by different residues (Fig. 1C; Met494 → Asn-Arg-Gln-Gly-Val; Ala429 → Met), we investigated the substrate specificity of HscC using the peptide library approach employed earlier for DnaK (20) and compared HscC with DnaK. For this approach we choose peptides scanning the sequences of three E. coli proteins, EF-Tu, MetE, and
Characterization of E. coli HscC

**Fig. 3. Peptide binding by HscC.** A, equilibrium titration of the complex formation of HscC with the fluorescently labeled peptide α29-Q132-Q144-C-IAANS. Increasing concentrations of HscC (0.1–4 μM) were incubated with 0.1 μM α29-Q132-Q144-C-IAANS for at least 1 h, and the emission spectra (350–500 nm) were recorded at an excitation wavelength of 335 nm. The maxima of fluorescence were plotted against the HscC concentration, and the quadratic solution of the binding equation was fitted to the data using nonlinear regression analysis (Grafal, Erithacus). The plot shows representative data of one of five independent experiments. B, dissociation kinetic of the HscC-peptide complex in the absence (upper panel) and presence (lower panel) of ATP. HscC (0.5 μM) and α29-Q132-Q144-C-IAANS (0.5 μM) were preincubated at 30 °C for 1 h and mixed with a 100-fold excess of unlabeled peptide. Single or double exponential decay functions were fitted to the data.

### Table II
HscC interaction with a substrate peptide

| Without nucleotide | With ATP | Fold increase |
|--------------------|----------|---------------|
| K_{d} (μM) | 1.5 ± 0.6 | 1.4 ± 1.5 | 0.93 |
| k_{on} (s^{-1}) | 0.0014 ± 0.00002 | 0.0269 ± 0.0073 | 19.2 |
| k_{off} (s^{-1}) | 0.0013 ± 0.0003 | 933 | 20.6 |
| k_{on} (M^{-1} · s^{-1}) | 0.0014 ± 0.00002 | 0.0269 ± 0.0073 | 19.2 |

**Table III**

| Relative contribution of different amino acids | HscC | DnaK | Ratio |
|-----------------------------------------------|------|------|-------|
| Glycine                                       | 52%  | 48%  | 1.08  |
| Serine                                        | 54%  | 46%  | 1.18  |
| Threonine                                     | 56%  | 44%  | 1.27  |
| Alanine                                       | 60%  | 50%  | 1.20  |
| Valine                                        | 62%  | 58%  | 1.07  |

GlnRS, that are thermolabile proteins and substrates of DnaK (42). The chosen proteins are large enough to give a representative number of different peptide sequences (shown for MetE in Fig. 4A). HscC showed a clear pattern of binding and nonbinding peptides. A comparison with the pattern of DnaK revealed that a number of peptides, which are good binders for DnaK, were not recognized by HscC (37% of all DnaK binders), and vice versa, several peptides that were bound by HscC with high affinity were not bound by DnaK (38% of all HscC binders; Fig. 4A). A statistical analysis for all three peptide scans with a total number of 557 peptides is given in Table III. These data indicate that the substrate specificity of HscC is clearly different from DnaK.

Although the sample size is not large enough to determine the exact binding motif of HscC, using an algorithm detailed under “Experimental Procedures” we were able to determine the amino acid preferences of HscC and of DnaK as a control (Fig. 4, A and B). Our data indicate that for both chaperones positively charged amino acids contribute to binding, whereas negatively charged residues were strongly disfavored for binding. Aromatic amino acids also contribute to binding to HscC and DnaK. Leucine, which is important for binding to DnaK as published earlier (20), was not enriched in HscC-binding peptides (Fig. 4, A and B). Interestingly, proline was enriched in HscC-binding peptides but not in DnaK-binding peptides. In contrast to earlier publications, isoleucine disfavored binding to DnaK as well as to HscC. However, a closer analysis revealed that this result is due to the small sample size and the non-statistical distribution of isoleucine in our library. Although 46% of our peptides were negatively charged, 53% of all isoleucines were found in negatively charged peptides. These peptides are likely disfavored for binding to DnaK and also HscC because of their negative charge. Similarly, 58% of all methionines were found in negatively charged peptides. Glycine, on the other hand, was strongly enriched in positively charged peptides. All of the other amino acids were statistically distributed to neutral and positively and negatively charged peptides. Therefore, with the exception of isoleucine, methionine, and glycine, our data (Fig. 4C) give a good representation of the relative contribution of the different amino acids to binding to DnaK and HscC.

**Chaperone Activity of HscC and DjlC—**To investigate the potential chaperone activity of HscC and DjlC, we first tested whether they prevent the aggregation of chemically denatured luciferase. HscC was able to reduce the aggregation of denatured luciferase in a concentration-dependent manner as demonstrated by the reduction of light scattering at 550 nm (Fig. 5). At a 10-fold excess of HscC over luciferase, no increase in light scattering was observed, indicating that the formation of large aggregates was suppressed by HscC. This prevention of aggregation was independent of ATP consistent with the effect of ATP on substrate binding. Similarly, DjlC(TM) was able to prevent the aggregation of denatured luciferase in a concentration-dependent manner. Combination of DjlC(TM) and HscC had only additive effects. Therefore, both HscC and DjlC(TM) are able to act according to the minimal definition of a chaperone in the sense that they suppress aggregation of an unfolded test substrate. However, no combination of HscC with a DnaJ co-chaperone (DnaJ, HscB, and DjlC(TM)) and with or without GrpE assisted the refolding of chemically denatured luciferase (not shown). We therefore conclude that although HscC can prevent aggregation of denatured substrates, it is not a general folder chaperone such as DnaK.

**HscC Is a Nonessential Cytosolic Protein That Is Functionally Distinct from DnaK—**To gain insights into the in vivo roles of HscC, we first analyzed the levels of HscC in different E. coli strains and at various temperatures. As shown in Fig. 6A, we detected HscC by a specific antibody in C600 and MC4100 but not in W3110, B178, DH5α, and MG1655 (the last not shown). Quantification by immunoblotting of cell extracts using purified HscC as standard revealed that approximately 780 molecules of HscC exist per cell in MC4100. This number compares to 2,500 and 12,000 molecules of HscA and DnaK, respectively (43, 44). Because the detection limits of our antibody are between 200 and 500 molecules/cell, we cannot exclude the possibility that HscC is expressed in other E. coli strains (e.g. MG1655, W3110) at very low levels. We also identified HscC by mass spectrometry as an individual spot on two-dimensional gels of MC4100 total cell extracts (not shown). HscC was produced to similar levels in MC4100 cells grown at 15, 30, and 42 °C in logarithmic and stationary phase (not shown).

Because the DnaJ co-chaperone of HscC, DjlC, contains a putative transmembrane domain, we then asked whether HscC is membrane-associated. We separated membrane and cytosolic fractions of MC4100 cells grown at 30 °C and analyzed the fractions by SDS-PAGE and immunoblotting. HscC was com-
Fig. 4. HscC and DnaK have different substrate specificities. A, DnaK (upper panel) and HscC (lower panel) binding to cellulose-bound 13-mer peptides scanning the amino acid sequence of MetE with an overlap of 10 residues. Some striking differences between DnaK and HscC are indicated by circles. B, cumulative frequency for the amino acids lysine (squares), aspartic acid (circles), isoleucine (triangles), and leucine (inverted triangles) plotted against the peptides, which are sorted from left to right according to decreasing affinity to DnaK (closed symbols) or HscC (open symbols). For clarity only every 20th value is shown.

C, relative occurrence of each amino acid in high affinity binding peptides for HscC (dark gray bars) and DnaK (light gray bars) determined as described under “Experimental Procedures.”

Table III
Comparison of the substrate specificity of HscC and DnaK

|  | Number of peptides bound |
|---|--------------------------|
|  | Nonbound | HscC and DnaK | HscC and not DnaK | DnaK and not HscC | Total |
| EF-Tu | 100 (78%) | 15 (12%) | 8 (6%) | 5 (4%) | 128 |
| MetE | 158 (64%) | 41 (17%) | 25 (10%) | 24 (10%) | 248 |
| GlnRS | 126 (70%) | 28 (15%) | 17 (9%) | 10 (6%) | 181 |
| Total | 384 (69%) | 84 (15%) | 50 (9%) | 39 (7%) | 557 |

As a third approach to elucidate the in vivo function of HscC, we investigated whether HscC is able to functionally replace DnaK. Deletion of dnaK causes temperature-sensitive growth and resistance to plaque formation by bacteriophage \( \lambda \). We expressed dnaK or hscC from plasmids in an IPTG-regulated manner in a \( \lambda \) dnaK52 strain (BB153) (45) and determined the number of colony forming units at 30 or 40 °C. Even high overexpression of hscC was not able to support growth at 40 °C (Table IV). Similarly, \( \lambda \) only plated on the dnaK expressing strain, and hscC expression could not replace it (Table IV). Moreover, co-overexpression of djlC with hscC did not complement the temperature sensitivity phenotype of the Delta dnaK52 strain (not shown). HscC thus cannot functionally replace DnaK in E. coli.

As a fourth approach we replaced the hscC open reading frame (codons 2–551) by an interposon carrying a spectinomycin \( \beta \) or a kanamycin resistance gene \( \beta \). These interposons contain transcriptional terminators in both directions and on both ends stop codons in all three reading frames (46). It is therefore unlikely that the insertion of the resistance cassette disturbed any adjacent open reading frames.
Characterization of E. coli HscC

Hsc is not able to complement the temperature sensitivity and resistance to bacteriophage λ plaque formation of a ∆dnaK strain

| Temperature sensitivity at 40 °C | λ plaque formation |
|---------------------------------|-------------------|
| 0 µM IPTG | 50 µM IPTG | 100 µM IPTG | 250 µM IPTG | 0 µM IPTG | 100 µM IPTG | 250 µM IPTG |
| dnaK | - | - | - | + | - | - |
| hscC | - | - | + | + | - | - |

Reading frame. Furthermore, because there is no downstream open reading frame in the same direction of hscC, a polar effect was not to be expected. The deletion was made in the presence of an hscC expressing plasmid to circumvent the possible problem of an essential gene. The deletion was verified by PCR (not shown).

To test whether hscC is essential under regular growth conditions, we performed co-transduction experiments. Using P1 transduction we introduced into the ∆dnaK strain BB1553 (45) harboring the lacI expressing plasmid pDMI1 that was transformed with plasmids encoding for dnaK or hscC under an IPTG-inducible promoter. Serial dilutions of an overnight culture were spotted onto LB plates containing the indicated IPTG concentration and grown at 30 or 40 °C. Plating efficiency compared to plating at 30 °C: +++, +, −1; +, 0.1 to 1, small colonies; −, <10−6. Plaque formation was tested by spotting serial dilutions of a λ<sub>wp</sub> phage onto a lawn of a ∆dnaK strain expressing the indicated gene.

| Strain | Tc | Km | Cotransduction |
|--------|----|----|---------------|
| MC4100 | 126 | 14 | 11 | 15 | 100 | 15 |
| MG1655 | 227 | 32 | 14 | 2 | 23 | 9 |
| C600  | 432 | 56 | 13 | 5 | 20 | 25 |

To analyze the mutual relationship of the three Hsp70 homologues in E. coli, we constructed double and triple knock-out strains and analyzed their growth at 30 and 37 °C (upper growth temperature limit for the ∆dnaK52 strain) (Fig. 8). The combined deletion of hscC, dnaK, and hscA did not aggravate the growth phenotype under the conditions tested relative to the single mutant strains. This result shows that E. coli is viable in the complete absence of Hsp70 chaperones.

DISCUSSION

This study represents the first characterization of a novel member of the Hsp70 family in E. coli, HscC. In summary, we demonstrate that HscC is a specialized Hsp70 chaperone that shows significant differences to DnaK in key features, including the ATPase cycle and its regulation by co-chaperones, the substrate binding properties, and the in vivo function.

The HscC Subfamily of Hsp70 Chaperones—HscC seems to be a representative of a new subfamily of Hsp70 proteins. The most striking characteristics of HscC proteins are an insertion of 4–9 residues in L1,2 and a deletion of 13 residues in the helical part of the substrate-binding domain. In addition, there are more than 40 residues spread over the entire sequence that are conserved within this subfamily but differ from corresponding residues in DnaK. The residues found in DnaK in these positions are often highly conserved within the DnaK and Hsc70 homologues. Striking is also the 19-residue deletion in the ATPase domain that is otherwise only found in the DnaK homologues of Gram-positive bacteria and archaea. Because this deletion has been taken as evidence that the Hsp70 homologues of archaea have arisen from horizontal gene transfer from Gram-positive bacteria, it is also possible that HscC was acquired by Gram-negative bacteria through a similar mechanism. Consistent with this hypothesis is the occurrence of HscC homologues in Gram-positive bacteria like B. anthracis.

The ATPase Cycle—Although the basic ATPase cycle of E. coli HscC is similar to that of DnaK and Hsc70 with respect to its low overall rate, it has a significantly higher nucleotide dissociation rate, as determined indirectly by measuring the K<sub>m</sub> for ATP, binding to ATP-agarose, and size exclusion chromatography of HscC-ATP complexes. This difference is corre-
were spotted onto LB plates containing 600 

\[
\text{CdcCl}_2 (\mu\text{M})
\]

cannot rescue /H9004 open square type; hscC cultures of the different MC4100 wild type and /H9004 DnaJ protein DjlC, which is consistent with the presence of the other Hsp70s nucleotide release is fast and independent of exchange of some Hsp70 chaperones is regulated, whereas in HscA (9, 12). It is still an unsolved question why nucleotide interaction with HscC. This is in agreement with both the absence of HscA, or r, hscC, hypersensitive to CdCl₂. Serial dilutions of overnight cultures of different DnaJ proteins and the C-terminal domains of DnaJ /H9004, the hscC strain is hypersensitive to UV irradiation. Serial dilutions of overnight cultures of the different MC4100 wild type and mutant strains were spotted onto LB plates, irradiated at 312 °C overnight. 

\[
\text{CdcCl}_2 + 600 \mu\text{M CdcCl}_2
\]

time (min)

\[
\text{wt phscC, wild type cells harboring an hsc expressing plasmid; ΔhscC, hscC deleted MC4100 strain; ΔhscC phscC, hscC deleted MC4100 strain harboring an hscC expressing plasmid. B, growth curves of the MC4100 wild type and mutant strains in LB liquid medium in the absence and presence of 600 μM CdcCl₂. Filled circle, wild type; open square, ΔDjlB ΔdjlC double knock-out strain; filled triangle, ΔhscC phscC; open triangle, ΔhscC, overexpression of dnaK and hscA cannot rescue ΔhscC sensitivity to CdcCl₂. Serial dilutions of overnight cultures of the different MC4100 wild type and ΔhscC strains expressing hscC, dnaK, or hscA under control of an IPTG-regulated promoter were spottedonto LB plates containing 600 μM CdcCl₂ and 100 μM IPTG. D, the ΔhscC strain is hypersensitive to UV irradiation. Serial dilutions of the indicated strains were spotted onto LB plates, irradiated at 312 nm for the indicated time, and incubated at 30 °C overnight.

The deletion of hscC does not decrease viability of ΔdnaK, ΔhscA, and ΔdnaK ΔhscA strains at 30 and 37 °C on rich medium. Serial dilutions of the indicated strains were spotted onto LB plates and incubated overnight at the indicated temperatures.

![Figure 7](image)

**Figure 7.** In vivo phenotype of ΔhscC strains. A, the ΔhscC strain is hypersensitive to Cd²⁺ ions. 10-fold dilutions of overnight cultures of different MC4100 wild type and mutant strains were spotted onto LB plates containing 600 

\[
\text{CdcCl}_2 (\mu\text{M})
\]

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![Figure 8](image)

**Figure 8.** The deletion of hscC does not decrease viability of ΔdnaK, ΔhscA, and ΔdnaK ΔhscA strains at 30 and 37 °C on rich medium. Serial dilutions of the indicated strains were spotted onto LB plates and incubated overnight at the indicated temperatures.

Altered with and probably caused by the absence of two structural elements found in DnaK, two salt bridges that span the nucleotide binding cleft and an exposed loop in subdomain IIB. Alterations of both structural elements in DnaK increase synergistically the rates of nucleotide dissociation from DnaK (9).

The nucleotide exchange factor GrpE did not functionally interact with HscC. This is in agreement with both the absence in HscC of the GrpE signature motif, which in DnaK is essential for GrpE binding (9), and the intrinsically high nucleotide dissociation rate of HscC. HscC shares these two features with HscA (9, 12). It is still an unsolved question why nucleotide exchange of some Hsp70 chaperones is regulated, whereas in other Hsp70s nucleotide release is fast and independent of exchange factors.

The rate of ATP hydrolysis by HscC was stimulated by the DnaJ protein DjlC, which is consistent with the presence of the conserved channel in the ATPase domain identified earlier to be important for interaction of DnaK with DnaJ (30, 31). However, although the residues, which had been identified to be essential for interaction of DnaK with DnaJ, are present in HscC, HscC did not interact with DnaJ, and DnaK did not interact with DjlC. This indicates that additional residues in the conserved channel or elsewhere in HscC and DnaK are responsible for the DnaJ protein specificity. Furthermore, a hybrid protein consisting of the J-domain of DjlC and the other domains of DnaJ was not functional in vivo. This is in contrast to a number of other hybrid proteins made up of the J-domain of different DnaJ proteins and the C-terminal domains of DnaJ (47, 48). Therefore, the J-domain of DjlC bears a high degree of specificity for HscC. In support of this conclusion, we found that every sequenced organism that encodes an HscC homologue also encodes a DjlC-like protein. In E. coli and some of its close relatives, DjlB is yet another candidate for an HscC-specific DnaJ protein.

**Interaction with Substrates**—HscC exhibits within its substrate-binding domain the greatest divergence among Hsp70 proteins (with the exception of the distantly related Hsp110 and Hsp170 proteins). HscC is therefore a natural model system to study the evolutionary plasticity of Hsp70-substrate interactions. The degree of identity between HscC and DnaK within the substrate-binding domain was nevertheless sufficient to model a large part of the structure of this domain onto the structures of the substrate-binding domain of E. coli DnaK and bovine Hsc70. This model revealed a similar structure with a characteristic insertion into one of the substrate enclosing loops. Because this part of the Hsp70 substrate-binding domain is generally conserved to a very high degree, we propose that this insertion defines the new subfamily of HscC proteins. This insertion in L₁₂ is accompanied by a deletion of one residue corresponding to position 461 in DnaK and of 13 residues corresponding to 514–526 in DnaK in all nine HscC sequences present in public databases. The first deletion shortens the outer loop L₂₅₆ and the second most likely shortens helices A and/or B by a total of four full turns. These alterations will probably relocalize the entrance to the substrate-binding cavity toward the outer loops. Thereby the entrance of the substrate-binding cavity may not be covered by the shortened lid as illustrated in the tentative model of the substrate-binding domain of HscC shown in Fig. 9A.
Characterization of E. coli HscC

Our data support such a model of the substrate-binding domain of HscC. First, the overall affinity to peptide substrates was lower as compared with DnaK. Even the peptide ε52-Q132-Q144, which shows high affinity binding to HscC in peptide library screenings, had a comparatively high $K_d$ of 1.5 μM. This is 20-fold higher than the $K_d$ of this peptide for DnaK (0.08 μM (40, 41)). However, the dissociation rate ($k_{diss}$) of the HscC-peptide complex was similar to the $k_{diss}$ of the DnaK-peptide complex. This is consistent with the earlier finding that the major contribution to the restriction of peptide dissociation comes from the β-sheet domain (41). The consequence of the high $K_d$ and the low $k_{diss}$ is a very low association rate ($k_{ass}$) of approximately 900 M$^{-1}$s$^{-1}$ as compared with 10$^4$ M$^{-1}$s$^{-1}$ for DnaK. This could be interpreted in the way that in comparison with DnaK, the substrate-binding cavity of HscC is less well accessible or needs more rearrangement to accommodate a substrate in an induced fit-like mechanism. This interpretation would be consistent with a more flexible loop L1,2 caused by the inserted four amino acids.

Second, the $k_{diss}$ value was increased ~19-fold by addition of ATP contrasting the 440–2500-fold increase in the case of DnaK (39, 41, 49, 50). This observation would also be consistent with a more flexible loop L1,2 that could hinder dissociation of the substrate even when an ATP-induced rearrangement of the β-sheet domain opens up the substrate-binding cavity, although alternative explanations may also exist.

Finally, the observed differences in substrate specificity of HscC as compared with DnaK (one-third of all screened peptides to which HscC bound was not recognized by DnaK and vice versa) most likely result from the insertion in L1,2 because most other substrate-interacting residues are identical or conservatively exchanged as compared with DnaK-type Hsp70 chaperones. Our data are consistent with an earlier publication that demonstrated an influence of the arch forming amino acids of L1,2 and L3,4 on substrate specificity (33). However, other parts of the substrate-binding domain also seem to contribute to the different substrate specificity. This idea is supported by the surprising observation that leucine, which was considered to be the ideal amino acid for DnaK, seems less important for substrate binding by HscC. However, leucine is a very frequent residue and appears on average once in every 13-mer peptide. Therefore, an enrichment of leucine would only be detected when the absence of leucine would be strongly disfavorable and/or when more than one leucine would contribute synergistically to binding affinity. This is the case for DnaK, which is much more biased toward peptides with two and more leucines than toward peptides containing a single leucine (data not shown). The reason for this is probably that one leucine binds to the hydrophobic pocket, and adjacent leucines interact with the hydrophobic arch spanning over the peptide backbone. The situation may be different for HscC because the arch forming loop L1,2 also contains hydrophilic amino acids (NRQ). The enrichment of proline in HscC-binding peptides but not in DnaK-binding peptides is especially interesting but not well understood. Positively charged amino acids contributed most to substrate affinity. This specificity can be explained by a very high density of negatively charged surface residues of the β-sheet domain of HscC (Fig. 9B).

Functional Analysis of HscC—HscC prevented aggregation of chemically denatured luciferase completely at 2–4-fold stoichiometric excess. DjlCΔTM alone was also able to prevent aggregation in a concentration-dependent manner, an ability that it shares with other DnaJ proteins. However, HscC in combination with DjlCΔTM was not able to refold chemically denatured luciferase. We interpret this behavior as an indication that HscC and DjlC do not act in the general refolding of unfolded or misfolded proteins. Consistently, the cellular levels of HscC do not increase upon heat treatment of the cells, and HscC cannot replace DnaK in vitro in protein refolding and in vivo in heat-treated ΔdnaK52 mutants. We instead postulate a more specialized activity for HscC and DjlC, which, in view of the existence of a predicted transmembrane helix at the C termini of both DjlC and DjlB, may involve cellular processes related to the inner membrane of E. coli.

The first indications of the potential cellular functions of HscC come from our analysis of ΔhscC mutants. The sensitivity of ΔhscC mutant cells to exposure to UV light and Cd$^{2+}$ provides a first indication that HscC may be involved in the repair of damage induced by radiation and Cd$^{2+}$. The detected UV sensitivity of ΔhscC cells is consistent with the recent finding of a DNA array study (51) that the level of the HscC encoding mRNA increased about 8-fold after a 20-min exposure of wild type cells to UV.

The Cd$^{2+}$ sensitivity seems to point in a different direction. The toxicity of Cd$^{2+}$ is due to two different effects. Firstly, unspecific interactions with sulfhydryl groups of proteins cause protein misfolding and loss of protein function. Similar damage is caused by Hg$^+$ and Zn$^{2+}$ ions. Secondly, Cd$^{2+}$ causes oxidative damage on proteins and DNA similar to Cu$^+$ ions. Because the ΔhscC strain only showed an increased sensitivity to Cd$^{2+}$
but not to Cu++, Hg++, Zn++, or H2O2, it is not likely that HscC is involved in repair or degradation of proteins damaged by heavy metal ions or oxidation induced damage. We rather speculate that HscC affects detoxification and somehow aids the specific transport systems that pump Cd2+ out of the cytosol of E. coli. Such a hypothesis is supported by the fact that the specific DnaJ cofactors of HscC, DjlB and DjlC, are membrane-associated, suggesting an action of HscC on membrane proteins. In addition, the hscD and djlC homologues in R. solanacearum are found on a megaplasmid, which also encodes specific transport systems for Cd2+ ions (52).

However, our findings show that whatever the in vivo role of HscC is, it is not essential for viability at laboratory conditions. Even more, we show here that mutants with deletions in all three genes encoding the E. coli members of the Hsp70 family (hscC, hscA, and dnaK) are viable within the growth temperature range of ΔdnaK mutant cells. This shows that E. coli can survive in the complete absence of Hsp70 activities.

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REFERENCES

1. Georgopoulos, C., Liberek, K., Zylcic, M., and Ang, D. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperones (Morrison, R. L., Tissières, A., and Georgopoulos, C., eds) pp. 209–220, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
3. Mayer, M. P., Brehmer, D., Gassler, C. S., and Bukau, B. (2001) in Advances in Protein Chemistry: Protein Folding in the Cell (Horwich, A. L., ed) Vol. 59, pp. 1–44, Academic Press, San Diego
4. Zhu, X., Zhao, Z., Gappert, S., Opatra, C. M., Gottesman, M., and Hendrickson, W. A. (1996) EMBO J. 15, 2725–2734
5. Bukau, B., and Horwich, A. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15229–15234
6. Schmid, D., Baci, A., Gehring, H., and Christen, P. (1994) Science 263, 971–973
7. Mayer, J. S., Brehmer, A., Reid, M. P., and Bukau, B. (1999) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York
8. Genevaux, P., Wawrzaszyn, A., Zylcic, M., Georgopoulos, C., and Kelley, W. L. (2001) J. Biol. Chem. 276, 7906–7912
9. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
10. Brehmer, D., Rüdiger, S., Gassler, C. S., Klotzleimer, M., Packesch, L., Heinrich, J., Mayer, M. P., and Bukau, B. (2001) Nat. Struct. Biol. 8, 427–432
11. Fink, A. L., and Goto, Y. (eds) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York
12. Mayer, M. P., Brehmer, A., Reid, M. P., and Bukau, B. (1999) EMBO J. 18, 6934–6949
13. Hesterkamp, T., and Bukau, B. (1999) EMBO J. 17, 4818–4826
14. Fukai, T., Ogura, T., Tatsuta, T., and Bukau, B. (1999) Mol. Microbiol. 33, 567–581
15. Kelley, W. L., and Georgopoulos, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3679–3684
16. Apolinario, E., Fink, A. L., and Goto, Y. (eds) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York
17. Apolinario, E., Fink, A. L., and Goto, Y. (eds) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York
18. Kelley, W. L., and Georgopoulos, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3679–3684
19. Kelley, W. L., and Georgopoulos, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3679–3684
20. Gómez, B., Fink, A. L., and Goto, Y. (eds) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York
21. Gómez, B., Fink, A. L., and Goto, Y. (eds) Molecular Chaperones in Proteins: Structure, function, and Mode of Action (Fink, A. L., and Goto, Y., eds) pp. 241–374, Marcel Dekker, New York