First publ. in: Nature 400 (1999), pp. 693-696

Trigger factor and DnaK cooperate in folding of newly synthesized proteins

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The role of molecular chaperones in assisting the folding of newly synthesized proteins in the cytosol is poorly understood. In *Escherichia coli*, GroEL assists folding of only a minority of proteins[1] and the Hsp70 homologue DnaK is not essential for protein folding or cell viability at intermediate growth temperatures[2]. The major protein associated with nascent polypeptides is ribosome-bound trigger factor[3,4], which displays chaperone and prolyl isomerase activities *in vitro*[3,5,6]. Here we show that Δ*tig::kan* mutants lacking trigger factor have no defects in growth or protein folding. However, combined Δ*tig::kan* and Δ*dnaK* mutations cause synthetic lethality. Depletion of DnaK in the Δ*tig::kan* mutant results in massive aggregation of cytosolic proteins. In Δ*tig::kan* cells, an increased amount of newly synthesized proteins associated transiently with DnaK. These findings show *in vivo* activity for a ribosome-associated chaperone, trigger factor, in general protein folding, and functional cooperation of this protein with a cytosolic Hsp70. Trigger factor and DnaK cooperate to promote proper folding of a variety of *E. coli* proteins, but neither is essential for folding and viability at intermediate growth temperatures.

Because trigger factor can associate with ribosomes and nascent polypeptide chains, it is a prime candidate for a chaperone that is dedicated to assist folding of newly synthesized proteins. To elucidate its *in vivo* role genetically, we replaced the complete coding sequence of the *tig* gene (encoding trigger factor) with a kanamycin cassette (Fig. 1a). The Δ*tig::kan* mutant lacking trigger factor was viable and showed no growth defects between 15 and 42 °C in rich (Fig. 1b) and minimal media (not shown). Furthermore, Δ*tig::kan* mutants showed no defects in protein folding at 30 and 37 °C, as judged by unaltered specific activities of a reporter enzyme (firefly luciferase) and solubility of cellular proteins. Trigger factor is thus not essential for viability and protein folding in *E. coli*.

We investigated whether other chaperones compensate for the missing activity of trigger factor in Δ*tig::kan* cells by determining the phenotypes resulting from combining the Δ*tig::kan* allele with additional chaperone-gene mutations. The Δ*tig::kan* allele could be transduced with normal efficiency into the chromosomes of Δ*htpG::lacZ* (ref. 7), *secB::Tn5* (ref. 8), Δ*ibpAB::kan* (unpublished results) and temperature-sensitive *groEL* mutants (L140, L673 and L44)[9] without apparent alteration of the existing growth phenotypes. However, it could not be introduced into Δ*dnaK52* mutant cells[10], which lack DnaK and have low levels of the DnaJ co-chaperone. This finding was further substantiated by co-transduction experiments in which a Tn*10* selective marker (*zba-3054::Tn10*) placed close to the Δ*tig::kan* allele was transduced into Δ*dnaK52* and *dnaK*$^+$ cells. The co-transduction frequency of the Δ*tig::kan* allele was 83% in *dnaK*$^+$ cells and 0% in Δ*dnaK52* mutant cells. Combination of the Δ*tig::kan* and Δ*dnaK52* mutations thus causes synthetic lethality.

To investigate the cause of the synthetic lethality, we constructed Δ*tig::kan* and *tig*$^+$ strains in which expression of the chromosomal *dnaK dnaJ* operon is under the control of the IPTG-regulatable promoter $P_{A1/lacO-1}$ (P_{IPTG}*dnaKJ*, Fig. 1a)[11]. On plates containing 1 mM IPTG, the P_{IPTG}*dnaKJ* Δ*tig::kan* and P_{IPTG}*dnaKJ tig*$^+$ strains formed colonies at all temperatures tested (15, 30, 37 and 42 °C). On plates lacking IPTG, the P_{IPTG}*dnaKJ tig*$^+$ cells did not form colonies at 15 and 42 °C (Fig. 1b), consistent with the cold-sensitive and heat-sensitive phenotype of Δ*dnaK52* mutants[12]. In the absence of IPTG, P_{IPTG}*dnaKJ* Δ*tig::kan* cells did not form colonies at any temperature tested (Fig. 1b); growth of these mutants at 30 and 37 °C was restored by expression of the *tig* gene from plasmids (not shown). The onset of synthetic lethality could also be monitored in liquid medium (Fig. 1c). After overnight growth in medium with IPTG, P_{IPTG}*dnaKJ* Δ*tig::kan* and P_{IPTG}*dnaKJ tig*$^+$ cells were diluted into medium lacking IPTG. DnaK and DnaJ levels decreased from about twice wild-type levels to undetectable amounts after 8 h growth without IPTG (time point 3, Fig. 1c). P_{IPTG}*dnaKJ* Δ*tig::kan* cells depleted for DnaK and DnaJ started to show slower growth after 8 h (time point 3). At 8 and 10 h (time points 3 and 4), viability (determined as plating efficiency) decreased by 3 and 5 orders of magnitude, respectively, below the P_{IPTG}*dnaKJ* Δ*tig::kan* control grown with IPTG.

To account for synthetic lethality, we first ruled out that it resulted from DnaK's regulatory role as a negative modulator of σ^{32}, the transcriptional activator of chaperone and protease genes of the heat-shock regulon[13]. Accordingly, overproduction of chaperones and proteases resulting from DnaK and DnaJ depletion may be poisonous for Δ*tig::kan* cells. We artificially induced the heat-shock response in Δ*tig::kan* cells using a plasmid expressing the *rpoH* gene (encoding σ^{32}) under the control of an IPTG-inducible promoter[14]. IPTG-induced chaperone overproduction was at least as high as chaperone overproduction caused by DnaK and DnaJ depletion (monitored, for example, for GroEL, Fig. 1d) but did not affect the viability of Δ*tig::kan* cells at 30 and 37 °C (Fig. 1d, lower panel).

To investigate whether synthetic lethality is caused by protein-

folding defects, we first tested the *in vivo* folding efficiency of firefly luciferase. At 5 h of growth without IPTG (time point 2, Fig. 1c) at 30 °C, DnaK and DnaJ levels were about 6-times lower than wild-type levels. Growth, viability and protein synthesis were not yet affected (data not shown). At this time point, synthesis of plasmid-encoded luciferase was induced for 30 min. Luciferase activity was reduced by 60–70% in P_{IPTG}*dnaKJ Δtig::kan* cells as compared to P_{IPTG}*dnaKJ tig*$^+$ cells, although the amounts of luciferase present in both strains were similar (Fig. 2a). This decrease in specific activity was accompanied by increased luciferase aggregation (Fig. 2b).

We then tested the folding efficiency of *E. coli* bulk proteins. After 5 h of growth without IPTG (time point 2), cells were pulse-labelled with ^{35}S-methionine for 15 s and analysed for protein aggregation. Aggregated proteins and some membrane proteins were recovered by centrifugation and separated by two-dimensional-gel electrophoresis, followed by staining of pre-existing proteins and autoradiographic detection of newly synthesized proteins (Fig. 3). There was a large increase in the number (~40 proteins) and total amount (2.5-fold) of pelleted pre-existing proteins in P_{IPTG}*dnaKJ Δtig::kan* cells as compared to P_{IPTG}*dnaKJ tig*$^+$ cells (Fig. 3a) and cells of both strains grown in IPTG (not shown). Most spots that increased in intensity correspond to cytosolic proteins and thus represent aggregated proteins (for example, MetE and RpoB showed 4- and 25-fold increased aggregation, respectively; Fig. 3a, right panel). The amounts of membrane proteins present in the pellet fraction remained similar in the two strains tested (for example, inner and outer membrane proteins AtpD and OmpA; Fig. 3a), indicating that protein-folding defects affected predominantly non-membrane proteins. The autoradiogram revealed quantitatively (2.5-fold overall increase) and qualitatively similar changes in the solubility of newly synthesized proteins (Fig. 3b). Exposure of the gel was extended to allow detection of minor spots, but this also increased background staining, including that by nascent polypeptide chains.

The extent to which proteins misfold in DnaK- and DnaJ-depleted *Δtig::kan* cells may be larger than detected, because proteases may degrade misfolded proteins and other chaperones may partially prevent aggregation. Furthermore, folding problems probably become more severe at later time points after IPTG withdrawal, conditions that were difficult to investigate because of general defects in, for example, protein synthesis.

To determine the functional relationship between DnaK and

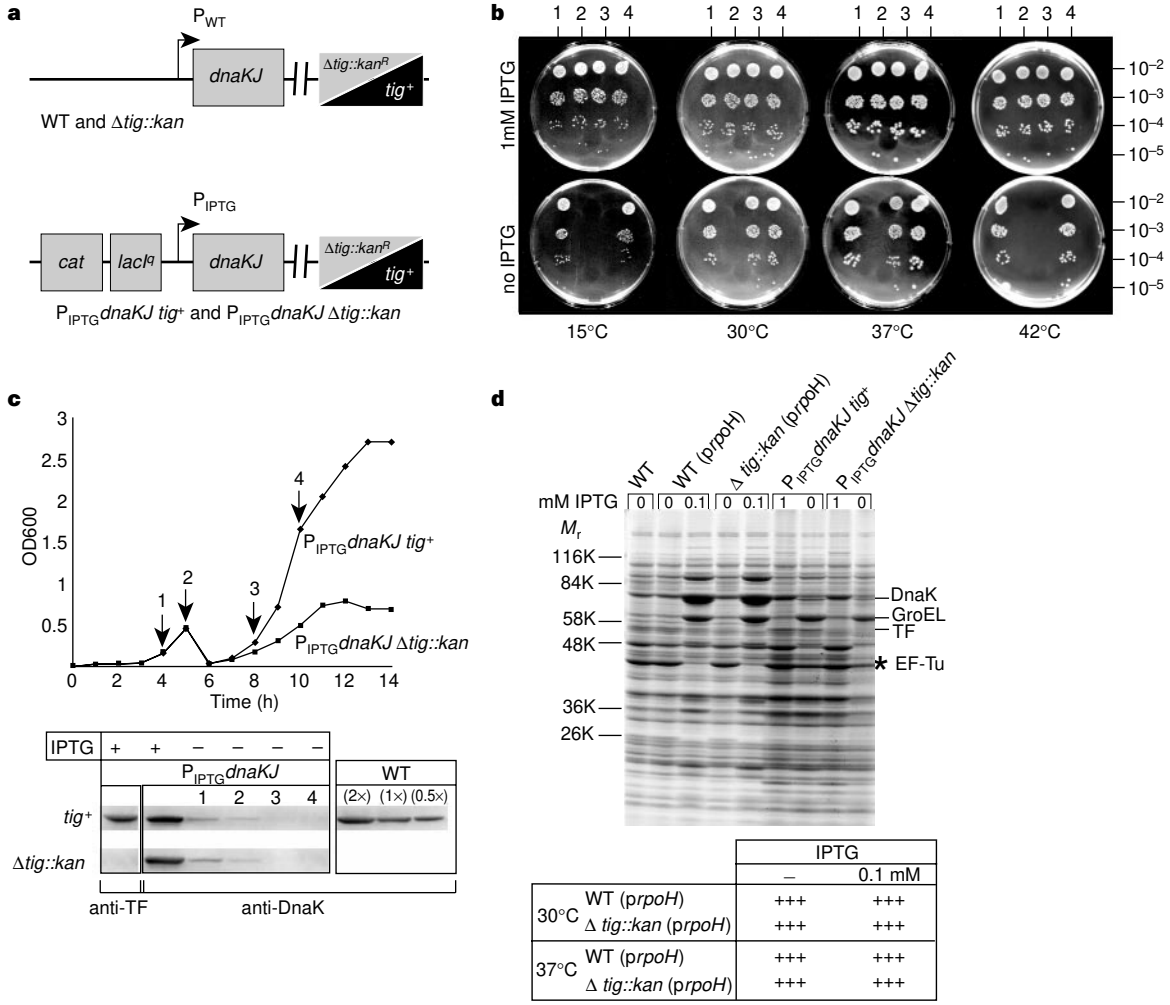

Figure 1 Synthetic lethality. **a**, Relevant genotypes of used strains. WT, wild type. **b**, Growth of wild-type (1), P_{IPTG}*dnaKJ Δtig::kan* (2), P_{IPTG}*dnaKJ tig*$^+$ (3) and *Δtig::kan* (4) cells on LB plates with or without IPTG at indicated temperatures for 24 or 96 h (15 °C). 10 μl of diluted cultures were spotted. **c**, Growth of P_{IPTG}*dnaKJ tig*$^+$ and P_{IPTG}*dnaKJ Δtig::kan* cells in LB liquid medium without IPTG at 37 °C. Top: at an OD_{600} of 0.6, cells were diluted to maintain logarithmic growth. Arrows indicate time points of sample withdrawals. Similar growth behaviour was found for cells grown in M9 medium at 37 °C. Bottom: to detect DnaK and trigger factor levels during depletion, identical OD_{600} equivalents were harvested at time points 1 to 4 (upper panel) and immunoblot analysis was performed using specific antisera. As controls, different OD_{600} equivalents of wild-type cells were applied. **d**, Protein patterns of σ^{32} and *dnaK*, *dnaJ*-regulatable *tig*$^+$ and *Δtig::kan* cells. Top: Coomassie-stained SDS–PAGE of total lysates (identical OD_{600} equivalents) of cultures grown in LB at 37 °C with IPTG or depleted from IPTG for 5 h. EF-Tu, for unknown reasons, is reduced in σ^{32}-overproducing cells. Bottom: growth of wild-type and *Δtig::kan* cells with and without IPTG-induced σ^{32} overproduction on LB agar.

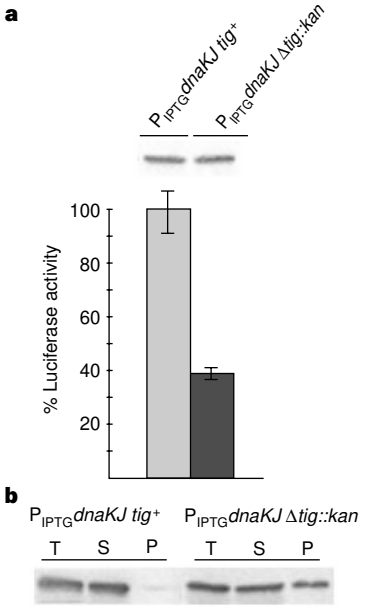

Figure 2 Specific activity and aggregation of firefly luciferase. **a**, *In vivo* activity and cellular levels of firefly luciferase in P_{IPTG}*dnaKJ tig*$^+$ (light-grey bar) and P_{IPTG}*dnaKJ Δtig::kan* cells (dark-grey bar). DnaK- and DnaJ-depleted cells were induced for 30 min to produce luciferase and assayed for luciferase activity[2] (set 100% for P_{IPTG}*dnaKJ tig*$^+$ cells). Upper panel: identical OD_{600} culture equivalents of the cells were subjected to immunoblot analysis with luciferase-specific antiserum. **b**, Solubility of luciferase. Lysates of cells treated as for **a** were prepared, divided into soluble and insoluble fractions by centrifugation and analysed by immunoblotting with luciferase-specific antiserum. Total lysate (T), supernatant (S) and aggregated material recovered in the pellet (P; double amount loaded).

trigger factor, we tested whether, in the absence of trigger factor, DnaK shows altered association with newly synthesized proteins *tig*$^+$ and *Δtig::kan* cells were labelled for 15 s with ^{35}S-methionine and DnaK-bound substrates were rapidly recovered by co-immunoprecipitation with DnaK-specific antisera (Fig. 4a). About 2–4% of total labelled protein co-immunoprecipitated with DnaK from *tig*$^+$ cells, whereas 2–3-fold more labelled material was initially co-immunoprecipitated from *Δtig::kan* cells (Fig. 4a, lower panel). The labelled material consisted of various proteins appearing as a smear (Fig. 4a), probably because nascent polypeptides were among these substrates. Chasing with non-radioactive methionine for 1–10 min before co-immunoprecipitation converted the smear to distinct bands, some of which associated with DnaK to similar extents in *tig*$^+$ and *Δtig* cells (Fig. 4a). Some nascent chains showed a rapid flux through DnaK (<1 min half-life), the extent, or perhaps the kinetics, of their association with DnaK being affected by trigger factor. Other proteins showed prolonged interaction with DnaK (>10 min half life), to an extent that is apparently not affected by trigger factor. The mechanistic basis for these protein classes remains unclear. Taking into account the efficiency of co-immunoprecipitation and the amount of recovered labelled protein, we estimate that 9–18% and 26–39% of newly synthesized proteins interact transiently with DnaK in wild-type and *Δtig::kan* cells, respectively.

We also found that, in *Δtig*::kan cells, a slightly increased amount (1.3-fold) of newly synthesized protein associates with GroEL (Fig. 4b). The most notable difference between the DnaK and GroEL substrates is an upper size limitation for GroEL substrates (relative molecular mass (M_r) of ~65K). This size exclusion by GroEL of large proteins, which are particularly prone to aggregation in *Δtig::kan* mutants depleted for DnaK and DnaJ, explains the importance of the DnaK chaperone system for protein folding in *Δtig::kan* cells.

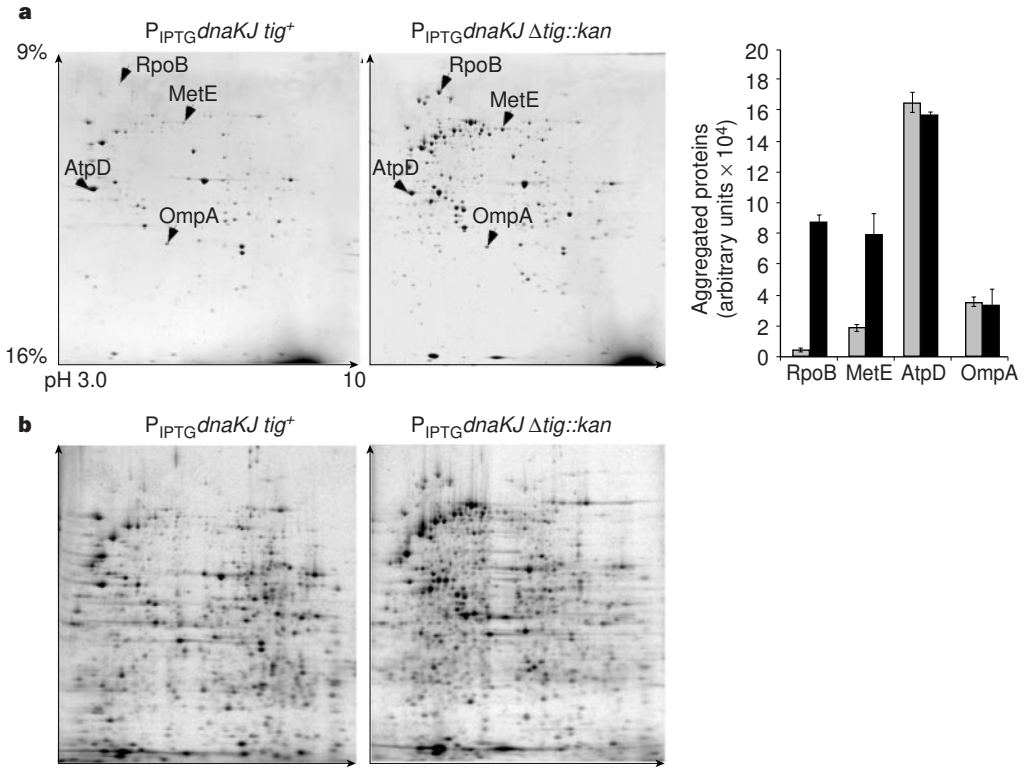

Figure 3 Aggregation of pre-existing and newly synthesized *E. coli* proteins. **a**, Coomassie-blue-stained gels of the pellet fractions of P_{IPTG}*dnaKJ tig*$^+$ and P_{IPTG}*dnaKJ Δtig::kan* cells grown for 5 h in the absence of IPTG. Arrows indicate cytosolic (MetE, RpoB) and membrane (AtpD, OmpA) proteins identified by mass spectrometry. Right: spot quantification of the Coomassie-stained gels. P_{IPTG}-*dnaKJ tig*$^+$ cells, light-grey bars; P_{IPTG}*dnaKJ tig::kan* cells, dark-grey bars. Quantification was performed using ImageMaster 2D program (Amersham/Pharmacia). **b**, Autoradiography of the Coomassie-stained gels shown in **a**.

Our findings indicate cooperative functions for trigger factor and DnaK in folding of newly synthesized proteins, although neither function is essential for folding. Trigger factor probably acts co-translationally, as it associates with ribosomes and nascent polypeptide chains[3,15]. DnaK associates co- and/or post-translationally with a subset of newly synthesized proteins even in the presence of trigger factor, indicating that it regularly participates in protein folding even in wild-type cells. The nature of the functional cooperation between trigger factor and DnaK remains unclear. □

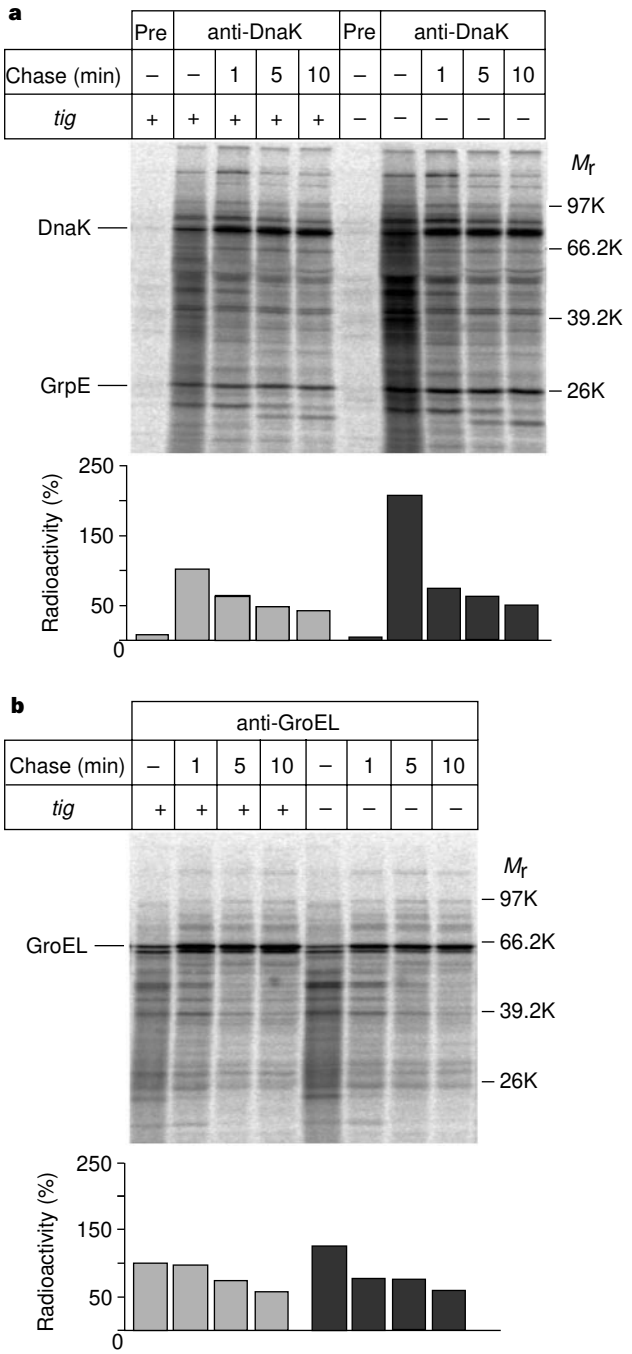

Figure 4 Association of DnaK with nascent polypeptide chains. **a**, Co-immunoprecipitation with DnaK-specific antisera. The lower panel shows quantification of radioactivity of the immunoprecipitated material above; *tig*$^+$, light-grey bars; *Δtig::kan*, dark-grey bars. Radioactivity immunoprecipitated in the *tig*$^+$ lysate after 15 s pulse is set 100%. **b**, Co-immunoprecipitation with GroEL-specific antibodies. The lower panel shows quantification of radioactivity of the immunoprecipitated material. Radioactivity immunoprecipitated in the *tig*$^+$ lysate after 15 s pulse is set 100%.

Methods

Growth of P_{IPTG}*dnaKJ* cells. The strains used for depletion experiments are in the C600 background. Cells were grown overnight with 1 mM IPTG (isopropyl-β-D-thiogalactoside), then washed three times in saline to remove IPTG, inoculated in fresh medium to an absorbance at 600 nm (OD_{600}) of 0.015–0.03 and grown for analyses without IPTG at 37 °C for ~5 h to OD_{600} = 0.6–0.8.

Luciferase activity. Cultures of P_{IPTG}*dnaKJ tig*$^+$ and *Δtig::kan* cells containing plasmid pBB516 (ref. 11) were grown overnight at 30 °C in LB medium with ampicillin (100 μg ml^{-1}) containing 1 mM IPTG, washed three times in saline (0.8% NaCl) and inoculated in LB medium lacking IPTG to an OD_{600} of 0.030. At mid-log phase, luciferase production was induced by the addition of 0.4% arabinose and *in vivo* activity was determined as described[2].

Isolation of insoluble proteins. Cultures were grown at 37 °C in M9 medium containing 0.2% glucose and all L-amino acids except methionine to an OD_{600} of 0.6. Cells were pulsed with ^{35}S-methionine (15 μCi ml^{-1} final concentration) for 15 s, rapidly chilled on ice, lysed as described[2] and the protein content was determined (Bradford assay, Biorad). We subjected 2.4 mg protein of each lysate to a two-step centrifugation protocol where the first low-speed centrifugation removes most membrane proteins[2]. 2D-gel electrophoresis of pellet fractions was performed according to the distributor (Amersham/Pharmacia). Experiments were reproduced at least three times.

Co-immunoprecipitation. Ten-millilitre aliquots of cells grown in M9 medium at 37 °C to mid-log phase were pulsed for 15 s with ^{35}S-methionine (15 μCi ml^{-1}; Amersham) and chased by addition of unlabelled methionine (160 μg ml^{-1}) when indicated. Cells were immediately transferred to 10 ml of frozen crushed solution (0.8% NaCl, 10 mM EDTA; pH 8.0), incubated for 5 min and then pelleted by low-speed centrifugation. Lysates were prepared as described[2], except that lysis buffer contained 100 mM EDTA to prevent ATP-dependent dissociation of DnaK-bound substrates, and that lysates were subjected to additional centrifugation for 5 min at 13,000*g* to remove insoluble material before immunoprecipitation. Eighty microlitres of lysate (2 mg ml^{-1}) was incubated with 80 μl protein A–Sepharose beads in PBS, 4 μl of appropriate antibodies and 1 ml of RIPA-buffer (50 mM Tris/HCl, 10 mM EDTA, 150 mM NaCl, 0.5% NP-40, pH 7.5) for 45 min at 8 °C. Beads were washed 3 times with RIPA-buffer, once with PBS/5 mM EDTA and finally boiled in 80 μl SDS-sample buffer. Incorporation of ^{35}S-methionine in TCA-precipitated lysates was determined to correct for differences in the labelling of the lysates.

Received 6 May; accepted 29 June 1999.

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Acknowledgements. We thank members of the Bukau lab, T. Hesterkamp, M. Müller and H.-G. Koch for comments on the manuscript and discussions. This work was supported by grants of the DFG and the Fonds der Chemie to B.B.

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