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Trigger Factor and DnaK possess overlapping substrate pools and binding specificities

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Summary

Ribosome-associated Trigger Factor (TF) and the DnaK chaperone system assist the folding of newly synthesized proteins in *Escherichia coli*. Here, we show that DnaK and TF share a common substrate pool *in vivo*. In TF-deficient cells, Δ*tig*, depleted for DnaK and DnaJ the amount of aggregated proteins increases with increasing temperature, amounting to 10% of total soluble protein (approximately 340 protein species) at 37°C. A similar population of proteins aggregated in DnaK depleted *tig*$^+$ cells, albeit to a much lower extent. Ninety-four aggregated proteins isolated from DnaK- and DnaJ-depleted Δ*tig* cells were identified by mass spectrometry and found to include essential cytosolic proteins.

Four potential *in vivo* substrates were screened for chaperone binding sites using peptide libraries. Although TF and DnaK recognize different binding motifs, 77% of TF binding peptides also associated with DnaK. In the case of the nascent polypeptides TF and DnaK competed for binding, however, with competitive advantage for TF. *In vivo*, the loss of TF is compensated by the induction of the heat shock response and thus enhanced levels of DnaK. In summary, our results demonstrate that the co-operation of the two mechanistically distinct chaperones in protein folding is based on their overlap in substrate specificities.

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Introduction

In the *E. coli* cytosol, a fraction of the newly synthesized proteins requires the activity of molecular chaperones for folding to the native state. The major chaperones implicated in this folding process are the ribosome-associated Trigger Factor (TF), and the DnaK and GroEL chaperones with their respective co-chaperones (Horwich *et al*., 1993; Ellis and Hartl, 1999; Bukau *et al*., 2000; Hartl and Hayer-Hartl, 2002).

Trigger Factor is an ATP-independent chaperone and displays chaperone and peptidyl-prolyl-*cis*-*trans*-isomerase (PPIase) activities *in vitro* (Stoller *et al*., 1995; Hesterkamp *et al*., 1996; Scholz *et al*., 1997). It is composed of at least three domains, an N-terminal domain which mediates association with the large ribosomal subunit, a central substrate binding and PPIase domain with homology to FKBP proteins, and a C-terminal domain of unknown function (Hesterkamp and Bukau, 1996; Stoller *et al*., 1996; Hesterkamp *et al*., 1997). The positioning of TF at the peptide exit channel, together with its ability to interact with nascent chains as short as 57 residues (Valent *et al*., 1997; Lill *et al*., 1988; Bukau *et al*., 2000), renders TF a prime candidate for being the first chaperone that binds to the nascent polypeptide chains. DnaK requires ATP and its co-chaperones DnaJ and GrpE to refold a large variety of misfolded proteins through repeated cycles of substrate binding and release (Bukau and Horwich, 1998). At 30°C DnaK associates with approximately 9–18% of newly synthesized proteins including nascent polypeptides (Deuerling *et al*., 1999; Teter *et al*., 1999). This suggests that DnaK acts co- and post-translationally in the *de novo* folding of a subset of newly synthesized proteins. GroEL, which constitutes an oligomeric ATP-dependent chaperone system and acts together with its GroES co-chaperone, was shown to associate post-translationally with at least 10–15% of newly synthesized polypeptides (Horwich *et al*., 1993; Ewalt *et al*., 1997; Houry *et al*., 1999).

The functional relationship between these chaperone systems is complicated and only partially understood. The *groEL* gene is essential for growth at all temperatures, but it is unclear whether this severe phenotype is due to the role of the GroEL system in folding of newly synthesized proteins. The *dnaK* gene is not essential for growth and protein folding at 30°C, but is required at temperatures

above 37° and below 15°C (Bukau and Walker, 1989; Fayet *et al*., 1989; Deuerling *et al*., 1999; Teter *et al*., 1999). At least 150 protein species aggregate as a result of the missing repair function of DnaK at 42°C (Mogk *et al*., 1999). In contrast to DnaK and GroEL, TF is not a heat shock induced chaperone. Deletion of the *tig* gene encoding TF does not impair growth of *E. coli* cells at any temperature and does not lead to detectable protein folding defects (Deuerling *et al*., 1999; Teter *et al*., 1999).

The importance of the combined activity of TF and the DnaK system for the folding of newly synthesized proteins has been indicated by the recent finding that deletion of the *tig* gene in DnaK and DnaJ depleted cells or *ΔdnaK52* mutant cells causes synthetic lethality at 37°C (Deuerling *et al*., 1999; Teter *et al*., 1999). In *Δtig* cells, depleted for the DnaK system, more than 40 species of newly synthesized proteins show increased aggregation (Deuerling *et al*., 1999). Interestingly, the amount of newly synthesized polypeptides that associate with DnaK was two to threefold higher in *Δtig* cells as compared to *tig*$^+$ cells, consistent with a co-operative mode of action of both chaperones.

In this study, we investigated how the two mechanistically distinct chaperones TF and DnaK can co-operate with each other in protein folding. For that purpose we: (i) set out to identify *in vivo* substrates of these chaperones; (ii) compared their binding specificites by mapping binding sites in natural substrates; (iii) analysed whether binding of TF and DnaK to nascent polypeptide substrates is independent, co-operative or competitive; and (iv) investigated how the cells compensate for the loss of TF in *Δtig* cells.

Results

Protein aggregation is temperature dependent in DnaK and DnaJ depleted Δtig::kan *cells*

It was reported previously that the depletion of DnaK and DnaJ in *Δtig::kan* cells causes aggregation of more than 40 cytosolic species of newly synthesized proteins (Deuerling *et al*., 1999). In *tig*$^+$ and *Δtig::kan* cells carrying the *dnaK dnaJ* operon under transcriptional control of an IPTG-inducible promoter (P_{IPTG}*dnaKJ*) synthesis of DnaK and its DnaJ co-chaperone is shut off by omission of IPTG in the growth medium (Deuerling *et al*., 1999). To obtain more precise information on the aggregated protein species we now used an improved method which includes repetitive washing of the pellet with 2% NP40 (Tomoyasu *et al*., 2001). This allowed separation of membrane proteins from aggregated proteins and detection and quantification of even minor aggregates.

In contrast to the previous study (Deuerling *et al*., 1999), we now found that, even in *tig*$^+$ cells depleted for DnaK and DnaJ, aggregates can be detected after growth at 30°C and 37°C, which amounted to 0.4% and 1.4% of total soluble cellular protein, respectively (Fig. 1A, lanes 2 + 5). In *Δtig::kan* cells depleted for DnaK and DnaJ, protein aggregation was strongly increased, amounting to 1.2% and 10% of total soluble protein at 30°C and 37°C respectively (Fig. 1A). It is important to note that the depleted cells were harvested at conditions at which DnaK and DnaJ were only partially depleted (DnaK levels approximately 10% of wild type, Deuerling *et al*., 1999) and the cells were not impaired in growth and protein biosynthesis. The observed protein aggregation may therefore represent an underestimation of the full extent of aggregation occurring when the DnaK system and TF are missing completely. Together these findings demonstrate the importance of TF and/or DnaK for the assistance of protein folding *in vivo*.

Identification of in vivo *substrates of DnaK and Trigger Factor*

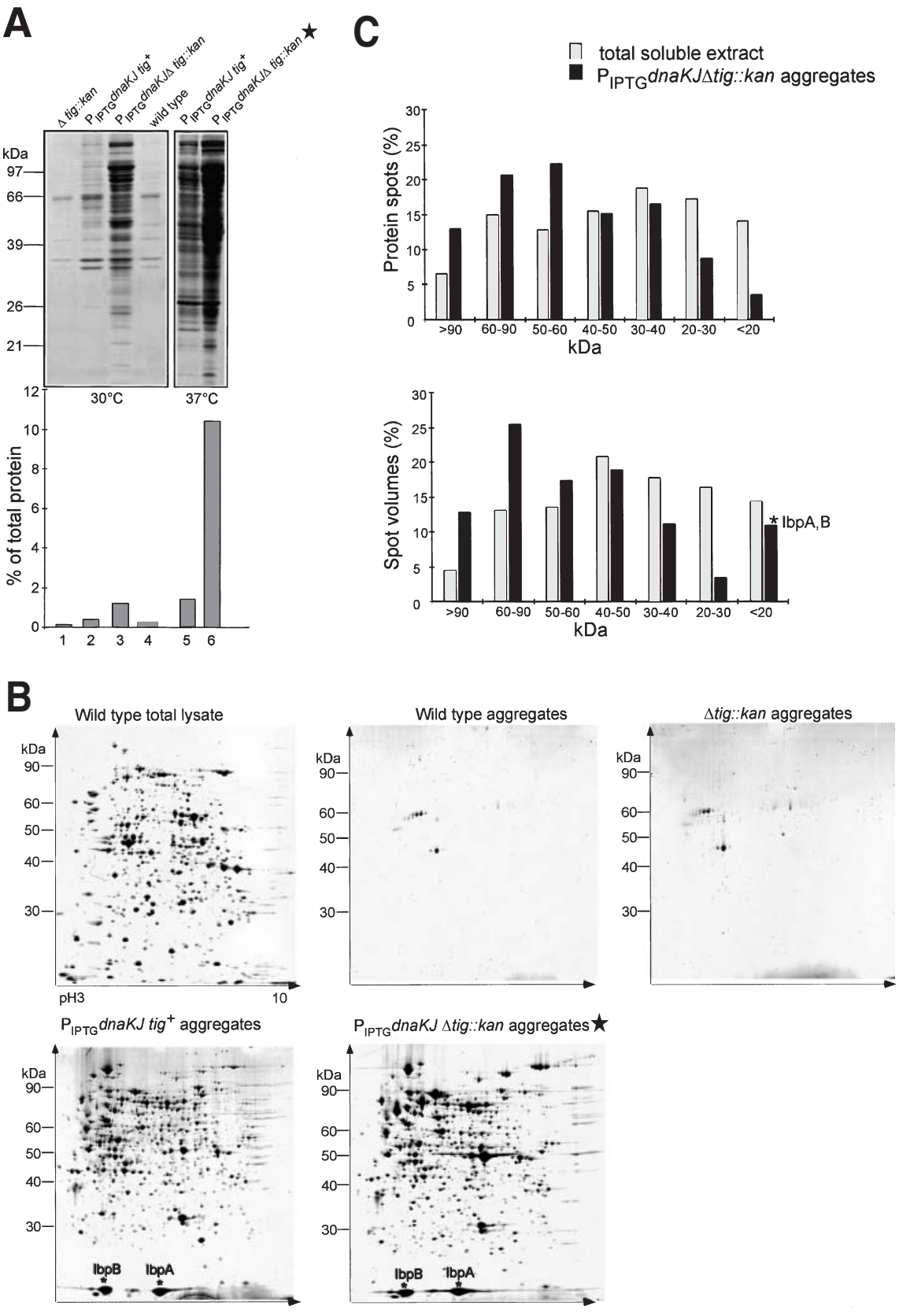
Two dimensional gel analysis of the aggregated protein fraction revealed approximately 340 spots of aggregation prone proteins in DnaK and DnaJ depleted *Δtig::kan* cells at 37°C, and a similar number of spots in DnaK and DnaJ depleted *tig*$^+$ cells (Fig. 1B). Spot matching revealed that all major aggregated protein species were shared between the two strains. Although the detectable protein spots ranged in their molecular weights from ~16–167 kDa, large proteins ≥ 60 kDa were enriched (41% of total aggregated proteins versus 20% of total soluble proteins). The majority of protein spots ≥ 60 kDa detected in total soluble cytosolic extract of wild-type cells were aggregation prone in DnaK and DnaJ depleted *tig*$^+$ and

Fig. 1. Aggregation of cytosolic proteins in DnaK and DnaJ depleted *tig*$^+$ and *Δtig* cells. Cells (wild-type C600, *Δtig::kan* and DnaK/DnaJ-regulatable P_{IPTG}*dnaKJ tig*$^+$ and *Δtig::kan* cells) were grown in LB with 1 mM IPTG overnight and diluted into LB without IPTG to a final OD_{600} of 0.03. At logarithmic phase, cells were lysed and the insoluble pellet fractions were isolated. Identical equivalents of OD_{600} were used for analyses except when indicated by star (only one third was loaded).

A. Temperature-dependent aggregation of proteins. Aggregated proteins separated by 12% SDS-PAGE (upper panels) and quantified by Bradford (lower panels). Please notice that aggregation in wild-type C600 and *Δtig::kan* cells was similar at 30 and 37°C and therefore is only shown at 30°C in A (lane 1 + 4).

B. Aggregated proteins (isolated from cells grown at 37°C in LB) were separated by 2D gel electrophoresis and Coomassie stained. Total lysate is shown for comparison. Asterisks indicate small heat shock proteins (Ibp) in this fraction.

C. Comparison of the number of protein spots and spot volumes from 2D gels. Please note that the spot volumes detected in the aggregated material <20 kDa of DnaK/DnaJ depleted *Δtig::kan* cells are mainly a result of the high abundance of the IbpA and IbpB chaperones known to associate with aggregates (see asterisks in Fig. 1B and C).


A
Δ tig::kan
P IPTG dnaKJ tig+
P IPTG dnaKJΔ tig::kan
wild type
P IPTG dnaKJ tig+
P IPTG dnaKJΔ tig::kan ★
kDa
97
66
39
26
21
12
30°C
37°C
% of total protein
10
8
6
4
2
0
1 2 3 4 5 6
C
total soluble extract
P IPTG dnaKJΔtig::kan aggregates
Protein spots (%)
30
25
20
15
10
5
0
>90 60-90 50-60 40-50 30-40 20-30 <20
kDa
Spot volumes (%)
* IbpA, B
B
Wild type total lysate
Wild type aggregates
Δtig::kan aggregates
kDa
90
60
50
40
30
pH3
10
P IPTG dnaKJ tig+ aggregates
P IPTG dnaKJ Δtig::kan aggregates ★
IbpB
IbpA

Δtig::kan cells (Fig. 1B). Large proteins, composed of multiple domains, are thus highly vulnerable to misfolding and aggregation during *de novo* folding (Fig. 1C) and require TF and/or DnaK for folding assistance. In addition, several protein spots visible in the aggregated fractions of DnaK and DnaJ depleted *tig*$^+$ and *Δtig::kan* cells were not detected in the total cytosolic protein extract (Fig. 1B), indicating that some low abundant proteins are aggregation prone.

Using mass spectrometry we identified 94 major spots of aggregated proteins isolated from DnaK and DnaJ depleted *Δtig::kan* cells, all of which were also detected in DnaK and DnaJ depleted *tig*$^+$ cells albeit in reduced amounts (Table 1). These potential substrates are all cytosolic proteins, involved in a variety of cellular processes including transcription, translation and metabolism and include several essential proteins (for example EF-Tu, RpoB). The identified species do not possess common features regarding their pI or content of α-helices or β-strands. Interestingly, although TF is a PPIase, the substrates are not enriched in prolyl residues compared to *E. coli* proteins in general. Remarkably, 72% of these proteins were also identified as thermolabile proteins which are prone to aggregation in *ΔdnaK52* cells after heat treatment (Table 1) (Mogk *et al*., 1999). This finding suggests that the majority of the proteins which depend on the assistance of DnaK and TF during *de novo* folding at regular growth temperature are thermolabile and tend to unfold at heat shock temperatures.

Physical association of Trigger Factor and DnaK with identified in vivo *substrates*

To obtain further direct evidence on whether the identified aggregated proteins represent natural substrates of TF and DnaK we attempted to detect physical interactions between the chaperones and their substrates by co-immunoprecipitation. DnaK associated substrates could be co-immunoprecipitated with DnaK specific antiserum under ATP depleted conditions from extracts of ^{35}S-methionine labelled *Δtig::kan* cells grown at 37°C (Deuerling *et al*., 1999; Teter *et al*., 1999). For analysing the specificity of substrate interactions, 10 mM ATP was added during co-immunoprecipitation, which resulted in a substantial release of substrates from DnaK (data not shown). We performed two-dimensional gel electrophoresis of the co-precipitated proteins and subsequent spot matching of the autoradiography with reference gels of aggregates isolated from DnaK and DnaJ depleted *Δtig::kan* cells and total cell lysate. From about one hundred spots visible in the audioradiography, 39 could be mapped unambigously with reference gels. Twenty-nine out of the 39 identified proteins co-immunoprecipitating with DnaK correspond to proteins identified as aggregates in DnaK and DnaJ depleted *Δtig::kan* cells. Identified proteins that co-immunoprecipitate with DnaK but were not found in the aggregated fraction are shown in Table 2. Taken together, about one-third of the proteins identified as aggregation prone also showed a direct physical interaction with DnaK by co-immunoprecipitation (summarized in Table 1). Considering the technical difficulties in catching unstable chaperone-substrate complexes by this method, the observed overlap provides evidence that many of the aggregation prone proteins are substrates for DnaK.

In contrast, no TF-associated substrates were co-immunoprecipitated with TF specific polyclonal serum (data not shown). The stability of TF-substrate complexes was probably too low to allow their detection by this method, consistent with high dissociation rates determined for protein substrates *in vitro* (Maier *et al*., 2001). As an alternative approach, we generated arrested nascent polypeptide chains of two of the identified aggregation-prone proteins [isocitrate dehydrogenase (IcdH) and pyruvate kinase (PykF)] in an *E. coli* based *in vitro* transcription/translation system and tested the ability of TF to associate with these chains. Translation was carried out in the presence of ^{35}S-methionine to label the nascent polypeptides, and at a physiological 1 : 3 molar ratio of ribosomes to TF. Addition of the chemical cross-linker DSS led to the appearance of cross-linking products of about 80 kDa and 90 kDa for nascent IcdH and of 70 kDa for nascent PykF (Fig. 2A). By co-immunoprecipitation TF was identified as cross-linking partner of both nascent polypeptides (Fig. 2A). This demonstrates that TF can interact directly with the nascent polypeptide chains of two proteins identified as aggregation-prone in DnaK and DnaJ depleted *Δtig* cells.

Because IcdH and PykF were also detected in the aggregated fraction of DnaK and DnaJ depleted *tig*$^+$ cells, we investigated whether nascent IcdH and PykF are substrates of DnaK. DnaK cross-linked to nascent polypeptide chains of IcdH and PykF generated in an *in vitro* transcription/translation system in the presence of physiological ratios of the DnaK system, ribosomes and TF (Fig. 2B). However, DnaK was only cross-linked by EDC, whereas TF could be cross-linked exclusively by DSS (Fig. 2). The difficulties to cross-link both chaperones to substrates using the same cross-linker was already reported earlier (Schaffitzel *et al*., 2001).

Taken together, we demonstrated by independent approaches that at least a subset of the aggregation prone proteins identified in this study physically interact with DnaK and TF.

Lack of Trigger Factor induces the heat shock response

Our finding that no significant protein aggregation occurred in *Δtig::kan* cells carrying the authentic *dnaK dnaJ* operon (Fig. 1A, lane 1) is consistent with two inter-

Table 1. DnaK and Trigger Factor substrates.

#	name	size (kDa)	function	ColP	thermo-labile 42°C	#	name	size (kDa)	function	ColP	thermo-labile 42°C
1	GltB	167	glutamate-synthase			48	LeuA	58	leucine-biosynthesis		
2	RpoC	156	RNA polymerase			49	AspA	55	Asp-Ammonia-lyase	+	
3	RpoB	151	RNA polymerase	+	+	50	PepA	55	aminopeptidase		
4	PutA	145	proline DH		+	51	YdgA	55	unknown		
5	PurL	142	purine biosynthesis	+	+	52	TnaA	54	tryptophanase	+	
6	MetH	137	methionine biosynthesis		+	53	AsnS	53	Asn-tRNA-synthetase		+
7	NarG	141	nitrate reductase		+	54	GlnA	52	Gln-synthetase	+	+
8	TrcF	130	transcription repair coupling factor		+	55	Gnd	52	6-P-gluconate dehydrogenase	+	
9	CarB	119	carbamoylphosphate-synthetase	+	+	56	ImdH	52	IMP dehydrogenase		+
10	HepA	110	RNA polymerase associated helicase			57	PykF	51	pyruvate kinase		
11	ValS	109	Val-tRNA-synthetase			58	LpdA	51	E3 of 2-oxoglutarate dehydrogenase	+	
12	SucA	106	E1 of 2-oxoglutarate dehydrogenase		+	59	NuoF	50	NADH dehydrogenase I		
13	AceE	100	E1 of pyruvate dehydrogenase	+	+	● 60	HslU	50	chaperone		+
14	PepN	99	Aminopeptidase N			61	SerS	49	Ser-tRNA-synthetase		+
15	LeuS	98	Leu-tRNA-synthetase			62	PurA	48	AMP-biosynthesis	+	
16	GyrA	97	DNA Gyrase			● 63	ClpX	47	chaperone		+
17	AdhE	97	alcohole dehydrogenase		+	64	DeoA	47	thymidine-Phosphoryl.		+
18	AlaS	96	Ala-tRNA synthetase			65	Rho	47	transcriptional terminator		+
19	AcnB	94	aconitase	+	+	66	IcdH	46	isocitrat dehydrogenase		
20	PheT	88	Phe-tRNA-synthetase	+		67	MurA	45	peptidoglycan biosynthesis	+	+
● 21	Lon	87	ATP-dependent protease		+	68	DeoB	45	Phosphopentomutase	+	
22	NrdA	86	ribonuclease reductase1, α-subunit			69	SerA	44	3-phosphogycerate dehydrogenase	+	+
23	PflB	86	Formate-acetyl-transferase 1			70	TufBA	43	elongation factor EF-Tu	+	
24	MetE	85	methionine biosynthesis	+	+	71	Glf	43	LPS biosynthesis		+
25	B2463	83	unknown			72	FabF	43	fatty acid biosynthesis		
26	CadA	82	Lys-decarboxylase			73	CarA	42	carbamoylphosphat synthetase		+
27	DeoD	80	purine-nucleoside-phosphorylase		+	74	SucC	42	Suc-CoA-synthetase		
28	KatG	80	catalase			75	Pgk	41	phosphoglycerate-kinase		+
29	FusA	78	elongation factor EF-G	+	+	76	FtsZ	40	cell division	+	+
30	Pta	77	phosphotransacetylase	+		77	Fba	39	Fructose-biphosphate-aldolase II		
31	MetG	77	Met-tRNA-synthetase	+		78	RecA	38	DNA recombination		
32	ThrS	75	Thr-tRNA-synthetase			79	FliM	38	flagellar motor switch prot.		
33	TktA	72	transketolase 1	+		80	TrpS	38	Trp-tRNA synthetase		
34	RpoD	70	Sigma70			81	Fbp	37	fructose-bisphophatase		
35	AceF	66	E2 comp. of pyruvate-dehydrogenase	+	+	82	RpoA	37	RNA-polymerase		+
36	TypA	66	elongation factor EF-G homologue	+	+	83	AsnA	37	Asn-synthetase A		+
37	CysJ	66	cystein-biosynthesis			84	GapA	36	GAP-dehydrogenase		+
38	SfcA	65	malate-dehydrogenase (malic enzyme)	+	+	85	CysB	36	transcriptional activator		
39	TreC	64	trehalose-phosphate hydrolase			86	TalB	35	transaldolase B		
40	ProS	64	Pro-tRNA-synthetase	+		87	FdoH	34	formate dehydrogenase (aerobic)		
41	GlnRS	64	Gln-tRNA-synthetase	+		88	MinD	30	cell division	+	+
42	YjjK	63	putative ABC-transporter			89	GpmA	29	phosphoglycerate-mutase 1		+
43	PyrG	61	CTP synthase			90	Udp	27	uridin phophorylase		
44	FumA	61	Fumarase		+	91	NuoB	25	NADH-dehydrogenase I		
45	GuaA	59	GMP synthase		+	92	Efp	21	translation		
46	PrfC	59	Release factor 3			● 93	IbpA	16	chaperone		+
47	LysU	58	Lys-tRNA-synthetase			● 94	IbpB	16	chaperone		+

94 proteins from the aggregated fraction (37°C) of DnaK/DnaJ-depleted *tig*$^+$ and Δ*tig::kan* cells were identified by mass spectrometry. This list comprises proteins identified in LB and in M9 minimal media on the basis of at least three independent experiments. Proteins are listed according their size (kDa) with names, sizes and functions indicated. Identified chaperones and proteases that most probably co-aggregated with substrates are marked with closed circles. Proteins which were also identified by co-immunoprecipitation with DnaK specific antibodies (this study) or by aggregation at 42°C in Δ*dnaK52* cells are indicated by "+".

Table 2. DnaK bound proteins.

No.	Name	Size (kDa)	Function
1	PepD	53	Aminoacyl-histidine dipeptidase
2	Eno	46	Enolase
3	SerC	40	Phosphoserine aminotransferase
4	Asd	40	Aspartate-semialdehyde dehydrogenase
5	PurM	37	Phosphoribosylformylglycinamidine cyclo-ligase
6	TrxB	34	Thioredoxin reductase
7	XthA	31	Exonuclease III
8	EF-Ts	30	Elongation factor Ts
9	GrpE	23	Hsp70 cofactor
10	Crr	18	Phosphotransferase enzyme II

Proteins that could be co-immuniprecipated with DnaK-specific antibodies but were not found in the aggregated protein fractions of DnaK/DnaJ-depleted Δ*tig*::*kan* cells. Proteins are listed according to their size (kDa) with names, sizes and functions indicated.

pretations: Either TF does not play an important role in protein folding, except that it backs up DnaK in its absence, or TF is important for protein folding but the DnaK system is highly efficient in backing up TF in its absence. The second interpretation predicts that misfolded proteins would accumulate in *Δtig::kan* cells which then become substrates for DnaK. This accumulation of DnaK substrates should induce the heat shock response since the level of misfolded proteins is tightly sensed by DnaK and transduced directly to the heat shock transcription factor, σ^{32} (Tomoyasu *et al*., 1998). In consequence, the levels of the σ^{32} regulated chaperones and proteases, and hence the cell's capacity to cope with misfolded proteins, would increase. To test this hypothesis we analysed the levels of the DnaK and GroEL heat shock proteins at 37°C. We detected that *Δtig::kan* cells have two to threefold enhanced steady state levels of DnaK and GroEL compared with wild-type cells (Fig. 3), whereas the level of a control chaperone (HscB), which is not regulated as part of the heat shock response, is similar in both strains (data not shown). This observed increase in the steady state chaperone levels is similar to the increase occurring after a shift of *E. coli* cells from 30°C to 42°C (Bukau, 1993), and hence corresponds to a heat shock-like situation. This finding supports the hypothesis that cells lacking TF generate misfolded proteins that induce the heat shock response. As we did not find a single cytosolic protein species that aggregated in *Δtig::kan* cells in the presence of the increased levels of DnaK (Figs 1A,B) this further substantiates our finding that TF and DnaK have highly similar substrate populations *in vivo*.

The disaggregation activity of ClpB is not crucial for cells lacking Trigger Factor.

The finding described above suggests that enhanced DnaK levels in *Δtig::kan* cells can fully compensate the loss of TF and thus no aggregates are formed in these cells. It is known that DnaK is the most efficient chaperone in preventing the aggregation of insoluble proteins. In addition, DnaK acts together with the ClpB chaperone to solubilize aggregated proteins (Goloubinoff *et al*., 1999; Mogk *et al*., 1999). To analysis whether the disaggregation of proteins is crucial in *Δtig::kan* cells, we constructed a *ΔtigΔclpB* strain. Growth anaylsis revealed that the double knockout mutant grew similarly well compared to the single knockout and wild-type cells at all temperatures tested (30–42°C, Fig. 4A). Next, we investigated the capacity of these strains to reverse aggregation after heat

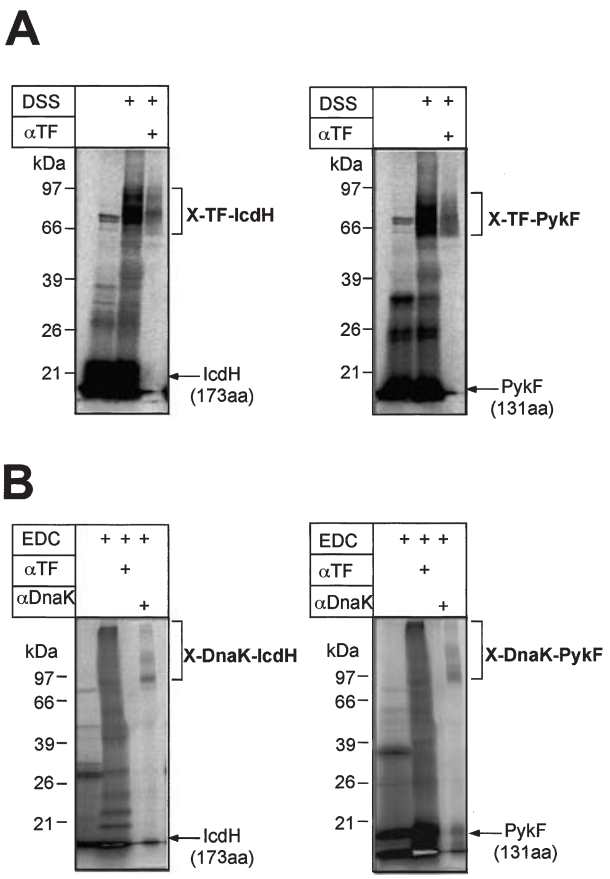


Fig. 2. Trigger Factor and DnaK associate with IcdH and PykF nascent polypeptides. Using an *in vitro* cell-free transcription/translation system with physiological ratios of the TF, DnaK and ribosomes (3 : 2 : 1) we produced arrested ^{35}S-labelled nascent chains. Association of nascent chains with TF was investigated by chemical cross-linking with DSS, interaction of nascent chains with DnaK was monitored by using the cross-linker EDC. Subsequent co-immunoprecipitation of cross-linking products with specific antibodies were performed to identify cross-linked chaperones. The fuzziness of the cross-linking products is probably due to the chemical cross-linker, which may cross-link the proteins at different positions thereby generating variations in the apparent molecular weight of the cross-linking products.
A. Cross-linking of nascent IcdH and PykF with TF.
B. Cross-linking of nascent IcdH and PykF with DnaK.

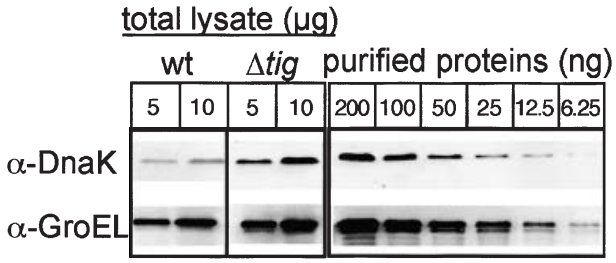


Fig. 3. Loss of Trigger Factor induces the heat shock response. Immunoblots against DnaK and GroEL using specific antisera were performed with different amounts of total lysates prepared from wild-type (wt) and *Δtig::kan* cells grown at 37°C. The titration of purified proteins is shown for control.

shock. As reported earlier (Mogk *et al*., 1999), aggregates generated in *ΔclpB* cells by a 30 min heat treatment at 45°C could not be solubilized during the recovery period at 30°C, whereas aggregated proteins found in heat treated wild-type cells were completely solubilized after 30 min recovery at 30°C (Fig. 4B). *ΔtigΔclpB* cells behaved similar to *ΔclpB* cells, whereas *Δtig* cells behaved like wild-type cells indicating that the loss of TF neither enhances protein aggregation in *ΔclpB* cells nor does it influence the protein disaggregation capacity (Fig. 4B). Moreover, it appears that aggregation is less pronounced after the heat pulse in *Δtig* cells compared to wild-type cells. This result can be explained by our finding that *Δtig* cells have higher levels of DnaK, which may result in a more efficient prevention of protein aggregation. Taken together, the disaggregation activity of ClpB is not crucial for cell survial in a *Δtig* background.

Overlap of Trigger Factor and DnaK binding sites in protein sequences

To directly compare the substrate binding characteristics of TF and DnaK, we determined the binding pattern of both chaperones to peptide libraries scanning the sequences of *in vivo* substrates identified in this study (EF-Tu, MetE, IcdH, GlnRS; see Fig. 5A for examples). Seventy-seven per cent of the TF binding peptides also displayed affinity for DnaK. This substantial overlap in substrate binding may be a prerequisite for DnaK and TF to assist the folding of the same set of protein substrates *in vivo*. Trigger Factor recognizes a motif of eight consecutive residues in which aromatic and basic residues are favoured and acidic residues are disfavoured, whereby the positions of these residues within the motif are not important (Patzelt *et al*., 2001) (Fig. 5B). The binding motif of DnaK consists of a hydrophobic core of five residues, among which leucine is particularly enriched, and flanking regions enriched in basic residues (Rüdiger *et al*., 1997) (Fig. 5B). The common feature of both motifs is the recognition of hydrophobic and basic amino acid residues providing the basis for overlapping binding specificities.

Trigger Factor and DnaK compete for association with nascent polypeptides

Because TF and DnaK share the majority of potential binding sites in protein sequences, we investigated by cross-linking whether they can also bind to the same nascent polypeptide, and analysed whether they compete for binding. We used a cell-free transcription/translation system derived from *E. coli* with adjusted physiological molar ratios of 1 : 3 : 2 of ribosomes to TF to DnaK (and adjusted levels of DnaJ and GrpE). For technical reasons we could not use IcdH and PykF nascent polypeptides as model substrates because different cross-linking agents are required to cross-linking both chaperones (see above). However, we could use nascent polypeptide chains of proOmpA, which can be cross-linked specifically to TF and DnaK by EDC (Schaffitzel *et al*., 2001).

^{35}S-labelled arrested nascent proOmpA chains of 125 residues length were generated. From peptide scan analysis of proOmpA (data not shown) it was known that this polypeptide has overlapping binding sites for TF and DnaK (residues 28–40). Chemical cross-linking with EDC and subsequent co-immunoprecipitation with specific antisera showed that TF is the most prominent cross-linking partner of nascent proOmpA (Fig. 6, lanes 2, 3). We also detected a faint cross-linking adduct with DnaK (Fig. 6, lanes 2, 4). Addition of a 10-fold molar excess of DnaK with its co-chaperones as compared to TF resulted in more efficient cross-linking of nascent proOmpA with DnaK, concomitant with a decreased cross-linking with TF (Fig. 6, lanes 6–8). Co-addition of a 10-fold overshoot of the DnaK system as well as TF resulted in a cross-linking pattern similar to the physiological conditions (Fig. 6, lanes 10–12). Furthermore, when extracts prepared from *Δtig::kan* cells were used for the *in vitro* transcription/translation assays, the cross-linking with DnaK was more pronounced (data not shown). This is in agreement with earlier data showing that two to three times more newly synthesized proteins associate with DnaK in the absence of TF (Deuerling *et al*., 1999; Teter *et al*., 1999). In summary, we show here that both chaperones can associate with the same peptide stretch within a nascent polypeptide, however, with a competitive advantage for binding of TF.

Discussion

This study: (i) identified *in vivo* substrates of TF and DnaK at regular growth temperature of *E. coli;* (ii) showed that TF and DnaK overlap in their binding specificities; (iii) revealed that both chaperones compete for binding to

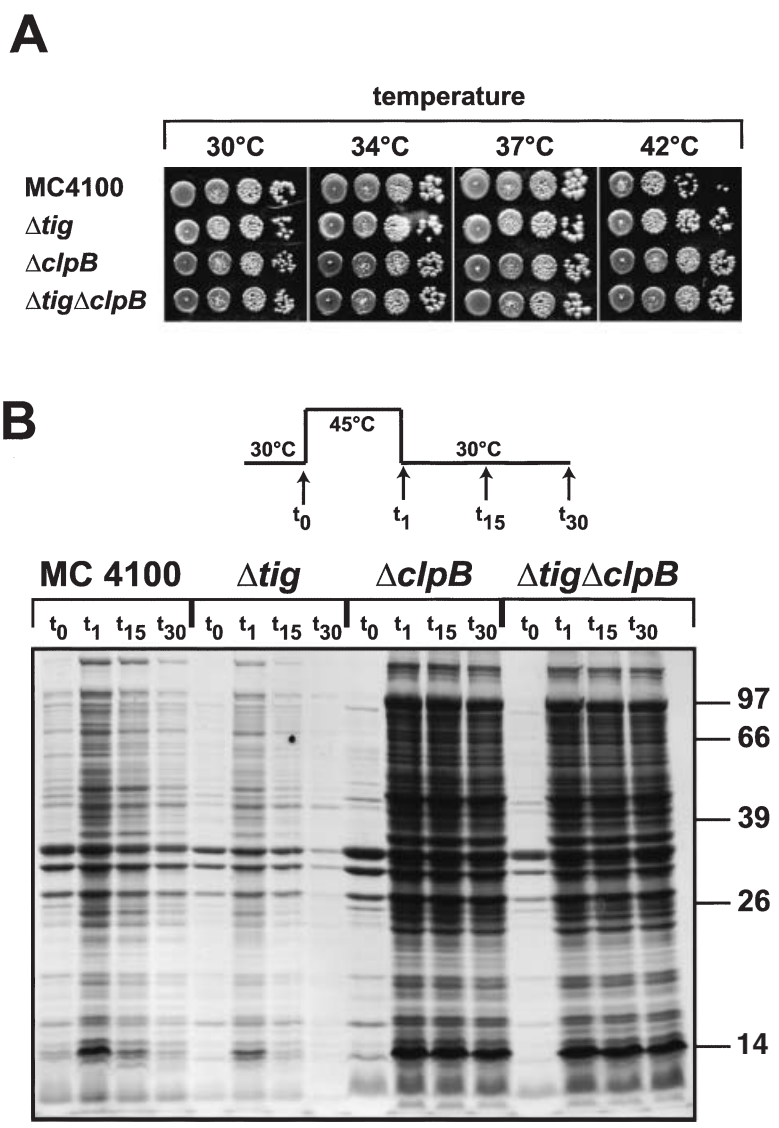


Fig. 4. Analysis of cells lacking Trigger Factor and ClpB.
A. Growth analysis. Cells were spotted in serial dilutions on LB-plates and incubated for 24 h at indicated temperatures.
B. Coomassie Blue stained SDS-PAGE of isolated aggregates.
Cells were grown in LB medium at 30°C to logarithmic phase, then shifted to 45°C for 30 min followed by an incubation at 30°C for 30 min. At indicated time points before (t_0) and 1, 15, and 30 min after the heat treatment (t_1, t_{15}, t_{30}) samples were withdrawn and insoluble fractions isolated (Mogk *et al.*, 1999).

nascent polypeptides; and (iv) demonstrated that *Δtig* cells compensate the loss of TF by increased cellular levels of heat shock proteins.

Analysis of proteins that are prone to aggregation in DnaK and DnaJ depleted *Δtig* mutant cells led to the identification of a subset of *in vivo* substrates for TF and DnaK. The number of these substrates (≥340 protein species) is by far higher than the number estimated from an earlier study (≥40 proteins) in which a less sensitive method for isolation of protein aggregates was used (Deuerling *et al.*, 1999). The number of *in vivo* substrates for both chaperones is probably even higher, as not all substrates are prone to aggregation and/or are degraded rapidly by cytosolic proteases. A comparison of the aggregation prone proteins identified here with the previously identified 52 proteins which interact with GroEL (Houry *et al.*, 1999) showed that only eight proteins (GyrA, Pta, ThrS, ClpX, TufBA, RpoA, GapA, MinD) are identical. However, this finding might be caused by the different methodological approaches used.

Interestingly, temperature is a major factor that determines the chaperone requirement for the *de novo* folding of proteins since protein aggregation in DnaK and TF deficient cells increased approximately 10-fold from 30° to 37°C. Moreover, the majority of these aggregated proteins (72%) is identical with the population of thermolabile proteins which aggregate in *ΔdnaK52* cells at 42°C (Mogk *et al.*, 1999). Multidomain proteins are enriched in the aggregated protein fraction of DnaK and DnaJ depleted *Δtig::kan* cells grown at 37°C and in *ΔdnaK52* cells subjected to a 42°C heat shock treatment. It is unclear which features of large sized proteins render them vulnerable to misfolding and aggregation during *de novo* folding as well as during thermal stress. Unfolded or misfolded conform-

ers of large proteins expose statistically more hydrophobic surface patches than small proteins, and in consequence might have a higher chance of undergoing intra- and intermolecular unspecific aggregation. Furthermore, hydrophobic interdomain contacts may be particularly endangered to become exposed and thus may nucleate aggregation during co- and post-translational folding and thermal unfolding. Finally, the rates of *de novo* folding and refolding after heat denaturation may be slower for large proteins than for small proteins, with the consequence that aggregation could occur more likely.

The *E. coli* heat shock response is triggered by misfolded proteins, which accumulate upon heat exposure (Goff and Goldberg, 1985; Connolly *et al.*, 1999). As a consequence, DnaK binds to the misfolded species thereby releasing bound heat shock transcription factor σ^{32}. Subsequently, σ^{32}-dependent heat shock genes are induced leading to enhanced levels of heat shock proteins, proteases and chaperones like DnaK. The treatment of *E. coli* cells with puromycin can as well induce the heat shock response. In this case misfolded proteins were generated by the premature release of truncated nascent polypeptides.

A similar situation obviously arises in cells lacking the ribosome-associated chaperone TF at permissive temperature. In *Δtig* cells we detected a heat shock like situation as monitored by permanently increased levels of heat shock proteins DnaK and GroEL. Two explanations are possible: (i) misfolded proteins are generated by the loss of TF function at 30°C and 37°C; or (ii) DnaK substitutes TF as a nascent chain binding chaperone. In both cases DnaK is titrated out from σ^{32} and heat shock is induced. Since no aggregates were detectable in *Δtig* cells, we hypothesize that all proteins which require the assistance of TF during *de novo* folding can be rescued by the heat shock induced proteins including chaperones like DnaK, GroEL and several proteases. Interestingly, cells lacking TF do not require ClpB function as a disaggregating chaperone since *ΔtigΔclpB* cells are neither impaired in growth at permissive (30–37°C) and heat shock temperature (42°C) nor in the solubilization of aggregates generated by a heat treatment.

Using peptide libraries we show that TF and DnaK share the majority of potential binding sites in protein substrates like EF-Tu or GlnRS. Moreover, we found that TF and DnaK compete for cross-linking to a shared bind-

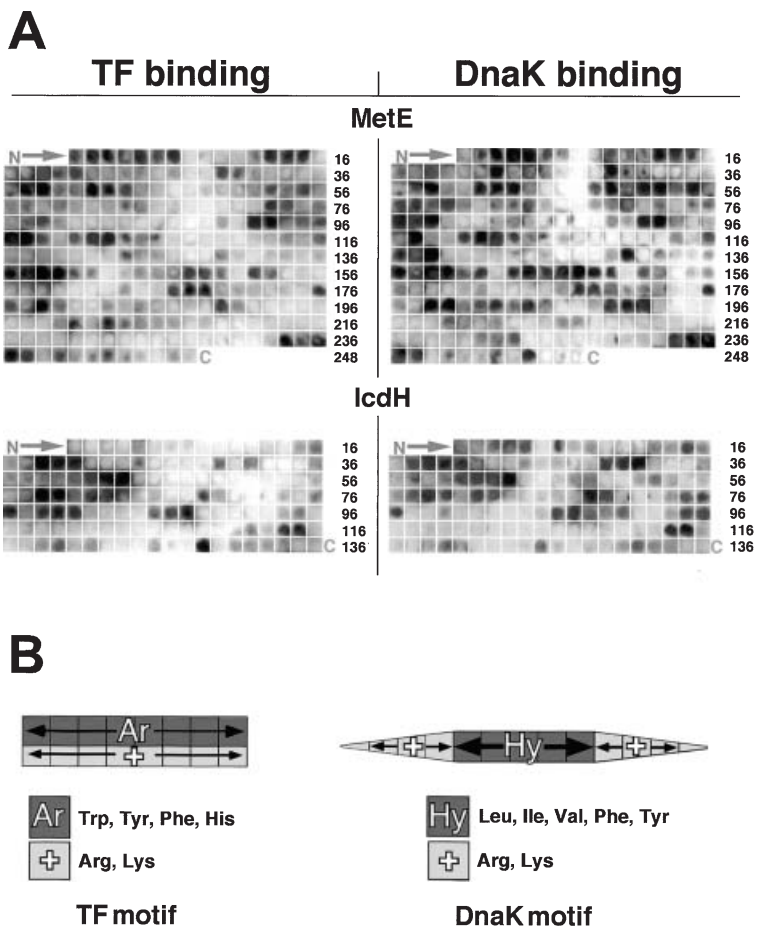


Fig. 5. Trigger Factor and DnaK overlap in their binding specificities.
A. Comparison of TF and DnaK binding to peptide libraries.
B. The recognition motifs of both chaperones are shown schematically. TF and DnaK data were taken from (Patzelt *et al.*, 2001) and (Rüdiger *et al.*, 1997) respectively.

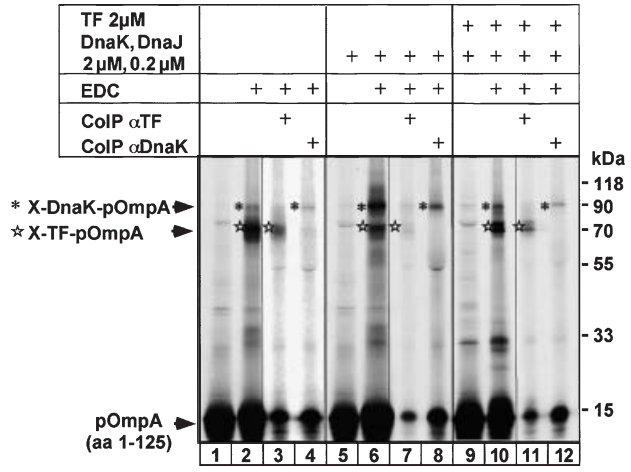


Fig. 6. Trigger Factor and DnaK compete for binding to nascent polypeptide chains. Using an *in vitro* cell free transcription/translation system with physiological ratios of the DnaK-system, TF and ribosomes, arrested ^{35}S-labelled nascent chains of proOmpA were created bearing overlapping binding sites for both chaperones. Association of nascent chains with chaperones was investigated by chemical cross-linking with EDC. Open stars indicate cross-links immunoprecipitated with TF specific antibodies, asterisks represent cross-links co-immunoprecipitated with DnaK antibodies. Where indicated a 10-fold excess of DnaK/DnaJ (2 μM/0.2 μM) or TF (2 μM) was added to the extract.

ing site in a short nascent polypeptide chain of proOmpA. DnaK, however, is not cross-linked efficiently to this chain in the presence of physiological molar concentrations of TF, indicating a hierarchical order of chaperone association with newly synthesized proteins. The positioning of TF next to the polypeptide exit tunnel on the large ribosomal subunit might be crucial for this hierarchy (Kramer *et al*., 2002). DnaK acts downstream of TF in the folding of newly synthesized proteins, perhaps by serving as a rescue system which assists the refolding of misfolded protein species which accumulate in the absence of TF in *Δtig* mutants.

Experimental procedures

Strains, culture conditions and preparation of aggregates

Escherichia coli strains were derivatives of MC4100. *Escherichia coli ΔtigΔclpB* strain was constructed by P1 transductions using *ΔclpB* cells (Mogk *et al*., 1999) and a P1 lysate prepared from *E. coli Δtig::kan zba-3054::Tn10* (Deuerling *et al*., 1999). P_{IPTG}*dnaKJ* strains used for depletion experiments were treated as described (Deuerling *et al*., 1999). For quantitative isolation of aggregates, 100 ml of cultures grown to log phase in LB liquid media were divided into aliquots of 10 ml, harvested and lysed (Deuerling *et al*., 1999). Aggregated material was isolated as reported (Tomoyasu *et al*., 2001). Appropriate amounts were withdrawn for protein determination by Bradford, the remainder was centrifuged (30 min, 10.000 *g*, 4°C), resolved in urea buffer (Mogk *et al*., 1999) and subjected to 2D gel electrophoresis.

Two dimensional gel electrophoresis and identification of proteins by mass spectrometry

Two dimensional gel electrophoresis and mass spectrometry were performed as described (Mogk *et al*., 1999). For protein identification, spots were analysed by mass spectrometry and/or protein spot matching with reference gels using ImageMaster software (Pharmacia) (Mogk *et al*., 1999).

Screening of cellulose membrane-bound peptides

Screening was performed according to published procedures (Rüdiger *et al*., 1997; Knoblauch *et al*., 1999; Patzelt *et al*., 2001). The 13mer peptides were derived from the sequences of EF-Tu, MetE, IcdH (isocitrate dehydrogenase) and GlnRS (glutamine-tRNA-synthetase). Peptide libraries were incubated with 500 nM TF or 100 nM DnaK (Buchberger *et al*., 1994; Hesterkamp *et al*., 1997) and detected by fluoroimaging using TF and DnaK specific antisera (Rüdiger *et al*., 1997; Patzelt *et al*., 2001).

In vitro *transcription/translation and chemical cross-linking*

Preparation of extracts and generation of arrested nascent chains were performed as described (Behrmann *et al*., 1998; Beck *et al*., 2000; Schaffitzel *et al*., 2001). Extracts were analysed for chaperone contents by quantitative immunoblotting with specific antiserum, showing that DnaK, DnaJ were present in reduced amounts (Schaffitzel *et al*., 2001). To obtain concentrations close to physiological molar ratios, DnaK (200 nM, final concentration), DnaJ (20 nM, final concentration), 100 nM ribosomes and 300 nM TF were added to the translation extracts. For generation of arrested nascent chains from PykF and IcdH, both genes were amplified from chromosomal DNA of *E. coli* strain C600 by PCR using the primers P5′(ggccaatcatatgaaaaagaccaaaattgtttgc) and P3′(cgggatccttacaggacgtgaacagatgc) for PykF and I5′(acgtc catggaaagtaaagtagttgttccg) and I3′(cgggatccttacatgttttcgat gatcgcg) for IcdH. Polymerase chain reaction products were digested with *Nco*I and *Bam*HI in case of IcdH and with *Nde*I and *Bam*HI in case of PykF and cloned into pET3d and pET3a respectively. Transcription was started with 0.4 ng μl^{-1} of pET-PykF or 2 ng μl^{-1} of pET-IcdH. Arrested nascent chains were produced by the addition of 40 ng μl^{-1} of antisense-oligonucleotide (ccttcaatggcggtaacttcc for PykF and cccccatctcttcacgcagg for IcdH). In case of OmpA, transcription was started by adding 0.4 ng μl^{-1} of p717OmpA plasmid (Beck *et al*., 2000; Schaffitzel *et al*., 2001). Translation extracts were additionally supplemented with 0.3 U μl^{-1} of T7 polymerase and 0.3 μCi μl^{-1} ^{35}S-methionine. After 30 min, cross-linker DSS (disuccinimidyl-suberate, for PykF and IcdH, 25 mM final concentration) or EDC (*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbidiimide, 80 mM final concentration) was added for 30 min at RT for DSS cross-linking or 30°C for EDC cross-linking. The reaction was quenched with 50 mM Tris/HCl pH 7.5 or 100 mM glycin, 10 mM $NaHCO_3$, respectively, for 15 min on ice (EDC) or at room temperature (DSS) and ribosomal complexes were purified (Hesterkamp *et al*., 1997). The pellet was resolubilized in PBS buffer and

co-immunoprecipitation was performed with DnaK or TF specific antisera (Deuerling *et al*., 1999).

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3370/mmi3370sm.htm

Fig. S1. Association of DnaK with substrates.

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