

# The *Escherichia coli* small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured *in vivo* during extreme heat shock

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**The roles of the *Escherichia coli* IbpA and IbpB chaperones in protection of heat-denatured proteins against irreversible aggregation *in vivo* were investigated. Overproduction of IbpA and IbpB resulted in stabilization of the denatured and reversibly aggregated proteins (the S fraction), which could be isolated from *E. coli* cells by sucrose gradient centrifugation. This finding is in agreement with the present model of the small heat-shock proteins' function, based mainly on *in vitro* studies. Deletion of the *ibpAB* operon resulted in almost twofold increase in protein aggregation and in inactivation of an enzyme (fructose-1,6-biphosphate aldolase) in cells incubated at 50 °C for 4 h, decreased efficiency of the removal of protein aggregates formed during prolonged incubation at 50 °C and affected cell viability at this temperature. IbpA/B proteins were not needed for removal of protein aggregates or for the enzyme protection/renaturation in cells heat shocked at 50 °C for 15 min. These results show that the IbpA/B proteins are required upon an extreme, long-term heat shock. Overproduction of IbpA but not IbpB caused an increase of the level of  $\beta$ -lactamase precursor, which was localized in the S fraction, together with the IbpA protein, which suggests that the unfolded precursor binds to IbpA but not to IbpB. Although in the wild-type cells both *E. coli* small heat-shock proteins are known to localize in the S fraction, only 2% of total IbpB co-localized with the aggregated proteins in the absence of IbpA, while in the absence of IbpB, the majority of IbpA was present in the aggregates fraction.**

Keywords: protein aggregation,  $\beta$ -lactamase precursor, DnaK

## INTRODUCTION

Small heat-shock proteins (sHsps) are one of the families of molecular chaperones preventing the irreversible aggregation and assisting in the refolding of denatured proteins. The sHsps have been found in all organisms tested so far and are characterized by their low molecular mass (12–30 kDa), oligomeric structure and the presence of a conserved 'α-crystallin' domain (Jacob & Buchner, 1994). The sequence of this domain is highly similar to the vertebrate protein α-crystallin which prevents the formation of cataracts associated with protein aggregation in the eye lens (MacRea, 2000).

**Abbreviations:** CP, cytoplasmic and periplasmic; IM, inner membrane; OM, outer membrane; sHsps, small heat-shock proteins.

According to the present model, ATP-independent sHsps bind and stabilize denatured proteins in a folding competent state, preventing their irreversible aggregation. The stabilized proteins are subsequently delivered to the Hsp70–Hsp40 chaperone system for ATP-dependent refolding (Ehrnsperger *et al.*, 1997; Veinger *et al.*, 1998; Lee & Vierling, 2000). *Escherichia coli* has two members of the sHsps family, IbpA and IbpB proteins, sharing 50% amino acid homology and identified for the first time as proteins bound to heterologous proteins forming inclusion bodies in *E. coli* cells (Allen *et al.*, 1992). We have previously found that IbpA and IbpB are major components of the S fraction which contains endogenous, heat-denatured *E. coli* proteins, separated from the

membranes and soluble proteins by sucrose gradient ultracentrifugation (Kucharczyk *et al.*, 1991; Laskowska *et al.*, 1996a). The S fraction appears in *E. coli* wild-type (WT) cells 15 min after the temperature change from 30 °C to 45 °C. During subsequent growth at 37 °C, the S fraction disappears due to refolding and degradation of the aggregates by heat-shock proteins. Removal of the S fraction is inhibited or retarded by mutations affecting the heat-shock proteases and chaperones Lon, HtrA, ClpP, ClpX, ClpA, ClpB, DnaK, DnaJ and GroEL/ES (Laskowska *et al.*, 1996b; Kędzierska *et al.*, 1999). The S fraction is also stable in *rpoH* cells, which lack active  $\sigma^{32}$ , the transcriptional activator of heat-shock genes (Kucharczyk *et al.*, 1991).

To date, IbpB protein is the only *E. coli* sHsp examined for its chaperone activity *in vitro*, whereas IbpA has not been tested, as it tends to aggregate during all stages of purification (Shearstone & Baneyx, 1998). It was found that IbpB reduced thermal aggregation of model substrates: citrate synthase and alcohol dehydrogenase (Shearstone & Baneyx, 1998) and formed a stable and soluble complex with MDH which was refolded subsequently by the DnaK/DnaJ and GroEL/GroES systems (Veinger *et al.*, 1998). Compared to the considerable amount of data derived from studies on the *in vitro* systems, there is not much information available on the *in vivo* function of the IbpA/B proteins. Kitagawa *et al.* (2000) found that *E. coli* strains overproducing IbpA or IbpB acquired higher levels of resistance to heat and oxidative stresses and accumulated lower amounts of aggregated proteins after exposure to 50 °C than the WT strain. Deletion of the *ibpA/B* operon affected neither the cell viability nor the intracellular protein aggregation at high temperature. The only phenotypic change observed in a  $\Delta ibpA/B$  mutant was a slightly slower growth at 46 °C (Thomas & Baneyx, 1998).

It was demonstrated that IbpB assembles *in vitro* into oligomeric structures of heterogeneous size. The basic 600 kDa oligomers are roughly spherical and interact to form larger complexes (Shearstone & Baneyx, 1998). There is no evidence that IbpA and IbpB are assembled into mixed complexes in *E. coli* cells, although in organisms containing more than one sHsp, different sHsp species form hetero-oligomers, for example: vertebrate  $\alpha$ A- $\alpha$ B-crystallin,  $\alpha$ B-crystallin-Hsp27 and sHsps from *Bradyrhizobium japonicum* (HspH/HspB and HspC/HspF) (MacRea, 2000; Liu & Welsh, 1999; Studer & Narberhaus, 2000). IbpB was also found to form mixed oligomers with HspB and HspH of *B. japonicum* (Studer & Narberhaus, 2000). It is not clear either whether IbpA and IbpB are functionally equivalent. The fact that overproduced IbpA in  $\Delta ibpA/B$  cells was partitioned between soluble and insoluble cellular fractions whereas overproduced IbpB was located mostly as a soluble protein (Shearstone & Baneyx, 1998) suggests that *E. coli* sHsp may have a different affinity for unfolded proteins. The studies on sHsps of *B. japonicum* revealed that the chaperone activity of purified hetero-oligomers is indistinguishable from the activity of homo-oligomers. On the other hand,

it was reported that vertebrate  $\alpha$ -crystallin homo-oligomeric complex containing  $\alpha$ A- or  $\alpha$ B-subunits differs in lens plasma-membrane-binding properties from heteromeric  $\alpha$ -crystallin (Cobb & Petrash, 2000). Furthermore,  $\alpha$ B-crystallin is a more efficient chaperone in preventing aggregation of the heat-inactivated citrate synthase than  $\alpha$ A-crystallin (Rajaraman *et al.*, 2001).

The purpose of this work was to investigate further the role of IbpA and IbpB in the protection of heat-denatured proteins against irreversible aggregation *in vivo*. It was found previously that excess of DnaK/DnaJ or GroEL/GroES chaperones prevented the formation of the S fraction during heat shock (Kędzierska *et al.*, 1999). In this work we examined whether overproduction of IbpA and/or IbpB proteins resulted in the opposite effect, stabilization of the S fraction, as should be expected from the current model of the sHsps function. Finally, we have characterized the effect of deletion of the *ibp* operon on the protein aggregation in *E. coli* cells upon extreme heat shock at 50 °C.

## METHODS

**Bacterial strains, plasmids and culture conditions.** MC4100 [*araD139*  $\Delta$ (*lacI*POZYA *argF*)U169 *fla* *relA* *rpsL*] was used as a WT strain. In MC4100  $\Delta ibpA/B$ , the majority of the *ibpAB* operon (892 bp *Bst*XI region) is replaced by the *Bsa*AI–*Bcl*II fragment containing the *cm* gene from pACYC184. pCA, pCB and pCAB plasmids are pUC derivatives bearing *ibpA*, *ibpB* and *ibpAB* genes, respectively, under control of the *pTac* promoter. The XL-1 Blue strain with pKEN8 plasmid overproducing the *E. coli* fructose-1,6-bisphosphate aldolase was purchased from the American Type Culture Collection (ATCC 77472). For the S fraction isolation, cultures were grown to exponential phase at 30 °C or 37 °C in 100 ml LB medium supplemented with 100  $\mu$ g ampicillin ml<sup>-1</sup>, if required, and shifted to 45 °C or 50 °C.

**Cell fractionation and isolation of the S fraction.** This was done essentially as described by Kucharczyk *et al.* (1991). Briefly, cells were harvested, converted into spheroplasts, lysed by sonication and fractionated by ultracentrifugation in a two-step SG0 sucrose density gradient to separate insoluble material [aggregated proteins (S fraction), and outer (OM) and inner (IM) membranes] from soluble, cytoplasmic and periplasmic proteins. Then the insoluble fraction was loaded on a six-step SG1 gradient to separate the S fraction containing aggregated proteins from the OM and IM. Thirty subfractions were collected from the bottom of a tube and analysed for protein concentration according to the method of Bradford (1976).

**Analytical methods.** Proteins were resolved by SDS-PAGE according to Laemmli (1970). To estimate the contribution of overproduced IbpA and IbpB to the S fraction, the aggregated proteins were resolved by SDS-PAGE and Coomassie blue-stained gels were analysed by scanning densitometry (Sigma Gel). Two-dimensional PAGE was performed as described by Laskowska *et al.* (1996b), using 1.6% pH 6–8 and 0.4% pH 3.5–10 carrier ampholite mixture (Pharmacia). Polyclonal rabbit antisera against IbpA/B, DnaK and Fda, anti-rabbit peroxidase conjugate (Sigma) and substrates 4-chloro-1-naphthol and H<sub>2</sub>O<sub>2</sub> (Serva) were used for protein immunodetection. Membranes were scanned and analysed with the Sigma Gel program. For microsequencing, proteins were

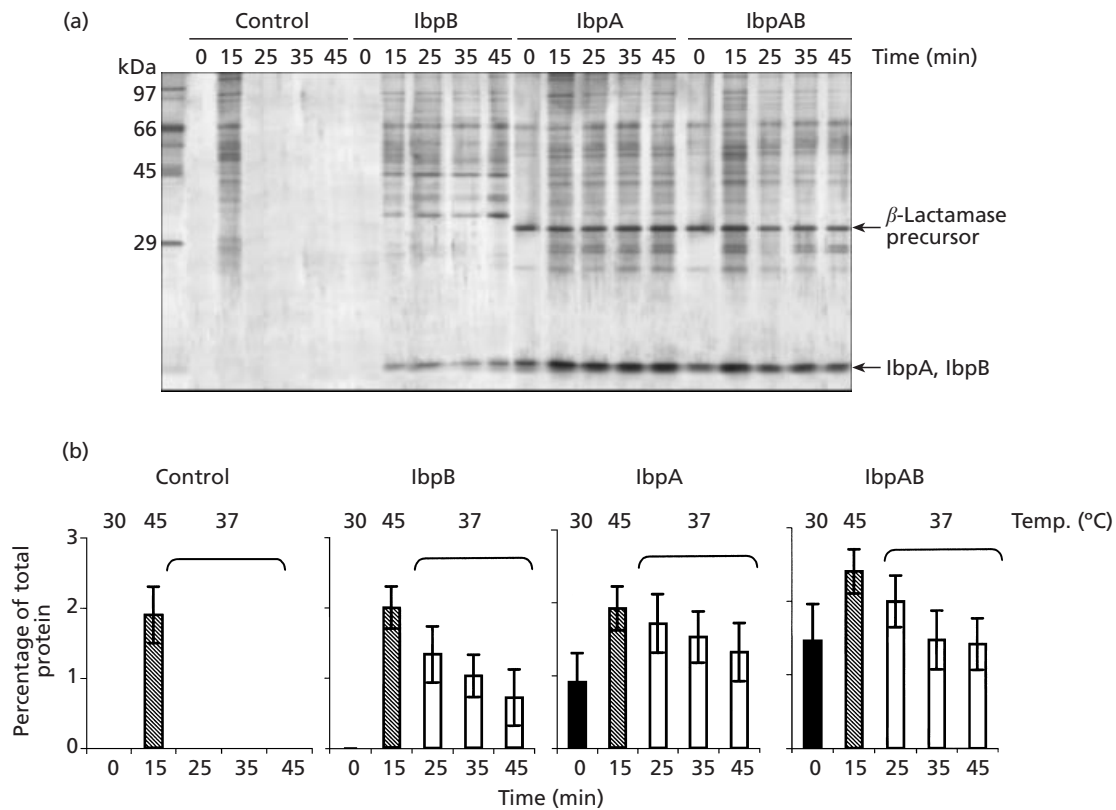
subjected to 0.1% SDS-12% PAGE and electrotransferred to PVDF (Serva) according to the method of Wilson & Yuan (1989). The N-terminal amino acid sequence analysis was performed on a gas-phase sequencer (model 491, Perkin Elmer Applied Biosystems) at Biocenter (Jagiellonian University, Krakow, Poland). For the Fda activity determination in cell extracts, a coupled-enzyme assay for the fructose 1,6-bisphosphate was carried out according to the Sigma protocol as described by Kędzierska *et al.* (2001), except that the cultures were grown in LB medium. The activity of  $\beta$ -lactamase was determined using penicillin G as a substrate (Meerman & Georgiou, 1994) in cell lysates prepared for the S fraction isolation.

**RESULTS**

**Overproduction of IbpA and IbpB stabilizes aggregates of denatured proteins *in vivo***

To distinguish the role of IbpA and IbpB in the S fraction formation and removal, we used the MC4100  $\Delta$ *ibpA/B* mutant strain transformed with pUC18 derivatives: pCA, pCB, or pCAB plasmids bearing *ibpA*, *ibpB* and *ibpAB* genes, respectively. Since MC4100 is  $\Delta$ *lac*, IbpA

and/or IbpB proteins were produced without the addition of IPTG at a constant level, approximately eightfold higher than that in the WT cells heat shocked at 45 °C for 15 min. For the S fraction isolation, culture samples were taken at time 0, i.e. just before the temperature shift to 45 °C, after 15 min at 45 °C, and during growth at 37 °C at the 25th min, 35th min and 45th min of the experiment. SDS-PAGE analysis of the aggregated proteins showed that IbpA and IbpB were the most abundant proteins in the S fraction (Fig. 1a); however, the amount of IbpB was lower than that of IbpA. Overproduction of both IbpA and IbpB and their localization in the S fraction of the strain transformed with pCAB was confirmed by two-dimensional PAGE (not shown). To compare the levels of aggregation in the control and the IbpA/B overproducing strains (Fig. 1b), the amount of aggregated proteins was calculated in relation to the total protein (100%) after subtraction of the IbpA/B level (see Methods). In the control strain MC4100  $\Delta$ *ibpA/B*(pUC18), at the 15th min after heat shock, 2% of total cellular proteins were aggregated and formed the S fraction, which disappeared in the next

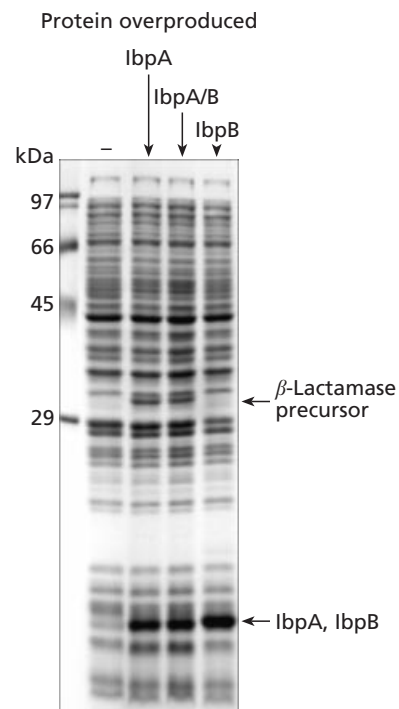


**Fig. 1.** The rise and removal of the S fraction in *E. coli* cells overproducing IbpA and IbpB proteins. *E. coli* MC4100- $\Delta$ *ibpA/B*(pUC18) control strain, MC4100 $\Delta$ *ibpA/B*(pCAB), MC4100 $\Delta$ *ibpA*(pCA) and MC4100 $\Delta$ *ibpA/B*(pCB) were grown at 30 °C in LB medium. At an OD<sub>595</sub> of 0.3 the bacteria were submitted to heat shock (15 min, 45 °C) and then transferred to 37 °C. The S fraction was isolated from samples taken at time 0 (before the temperature shift), after 15 min at 45 °C and at the 25th, 35th and 45th min during incubation at 37 °C, as described in Methods. (a) SDS-PAGE analysis of the S fraction isolated from MC4100 $\Delta$ *ibpA/B* overproducing IbpA and/or IbpB. Samples (20  $\mu$ l) of the S fraction were subjected to 0.1% SDS-15% PAGE followed by silver staining. The positions of IbpA/B and pre- $\beta$ -lactamase are shown by arrows. (b) The amount of aggregated proteins (the S fraction) was calculated in relation to the total protein (100%) after subtraction of the IbpA/B level. Error bars represent the standard deviation of four values.

10 min of incubation at 37 °C (Fig. 1b). The same result was observed in the WT strain transformed with pUC18 (results not shown). In the mutant strain overproducing IbpB, the amount of aggregated proteins was similar to that in the control (2% of total protein); however, removal of the S fraction was delayed: at the 45th min of the experiment, almost one-third of the aggregated proteins remained in cells. The overproduction of IbpA in the mutant strain had a more pronounced effect: the aggregates were formed even at 30 °C (0.9% of total proteins). After the temperature shift (15th min), the S fraction reached 1.9% and after transfer to 37 °C decreased to 1.3% of total proteins at the 45th min. When both *E. coli* sHsps were overproduced, larger amounts of proteins were aggregated: 2.4% and 1.4% of total cellular proteins were insoluble at the 15th and 45th min, respectively, suggesting that IbpA and IbpB may act synergistically in the stabilization of the S fraction. Interestingly, the growth of IbpA/B-overproducing strains was not affected by the stabilized aggregates (results not shown).

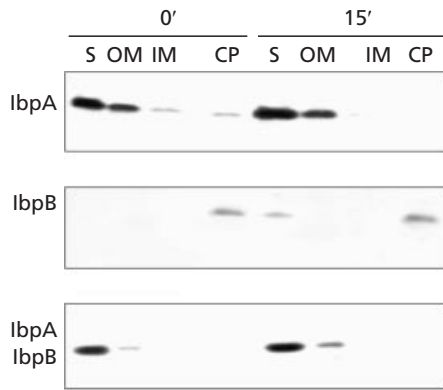
### The $\beta$ -lactamase precursor aggregates with IbpA but not IbpB

We analysed the protein content of the S fraction of the  $\Delta ibpA/B$  cells overproducing IbpA and/or IbpB proteins (Fig. 1a). The main difference in the protein content of the S fraction aggregates was the presence of a 32 kDa protein visible on the gel as a dominant component of the S fraction isolated from cells overproducing either IbpA or both IbpA and IbpB. Aggregates containing IbpB (Fig. 1a) were not abundant in the 32 kDa protein and were poorly separated from the OM fraction in the SG1 gradient, since the OM proteins OmpA and OmpF could be detected in the S fraction (identified by Western blotting; not shown). The 32 kDa protein, which aggregates in the presence of IbpA at 30 °C as well as at 45 °C, was identified by N-terminal sequencing and appeared to be a precursor of periplasmic  $\beta$ -lactamase coded by the *bla* gene carried by the IbpA/B-overproducing plasmids. We found that cells overexpressing IbpA or IbpA/IbpB also overproduced pre- $\beta$ -lactamase, whilst the cells carrying only the pUC vector or overexpressing IbpB did not (Fig. 2). However, the activity of  $\beta$ -lactamase was comparable in all strains (results not shown). The observed overproduction of the  $\beta$ -lactamase precursor could be explained as follows. We assume that the unfolded precursor may be stably bound by IbpA but not by IbpB and, in consequence, in cells with high levels of IbpA (or IbpA/B) large amounts of the precursor would be trapped and not processed. This situation, in the presence of antibiotic (100  $\mu\text{g ml}^{-1}$ ) would lead to increased production of the precursor which, after saturating available IbpA molecules, could be transported to the periplasm for processing. In conclusion, our results suggest that unfolded proteins (represented here by the  $\beta$ -lactamase precursor) may be bound stably by IbpA but not IbpB. This conclusion is supported by the results of experiments showing cellular localization of the IbpA/B proteins.

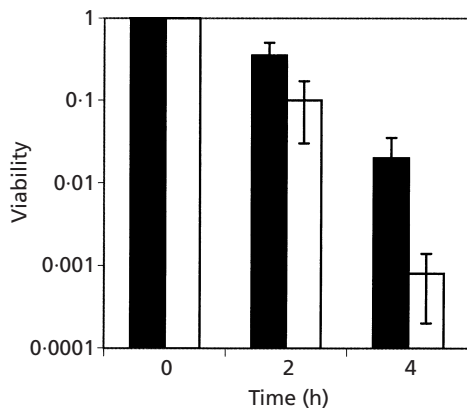


**Fig. 2.** The overproduction of IbpA results in increased levels of pre- $\beta$ -lactamase. MC4100 $\Delta ibpA/B$  bacteria transformed with plasmids pUC, pCA, pCB or pCAB were grown to exponential phase at 30 °C in LB medium. Samples of cell extracts corresponding to the same amount of bacteria were prepared and analysed by 0.1% SDS-15% PAGE. The gel was stained with Coomassie brilliant blue. The  $\beta$ -lactamase precursor is indicated by an arrow.

The immunodetection of the Ibp proteins in cellular fractions isolated from the  $\Delta ibpA/B$  mutant overproducing IbpA or IbpB revealed that most of the IbpA co-localized with the insoluble, aggregated proteins of the S fraction, both at 30 °C and after heat shock, whereas the vast majority of IbpB was found in a soluble cytoplasmic and periplasmic (CP) protein fraction (Fig. 3). Approximately only 2% of total IbpB was present in the S fraction after heat shock. IbpA protein was also detected in the OM and IM fractions. We suppose that some IbpA molecules bind to the membranes after cell lysis, which would be consistent with the fact that IbpA, a cytoplasmic protein, is found mainly in the OM, not in the IM. When IbpA and IbpB were overproduced simultaneously, either at 30 or at 45 °C, both were present predominantly in the S fraction and IbpB disappeared from the CP fraction (some IbpA/B was found in the OM fraction, probably as an artefact). In the last experiment, the presence of both Ibp proteins in the S fraction was confirmed by two-dimensional electrophoresis and immunoblotting (results not shown). These results support an idea that IbpA by itself, but not IbpB, may bind to unfolded (and aggregated) proteins and also show that IbpB localization changes in the presence of IbpA. The latter could be explained by the formation of IbpA-IbpB complexes,

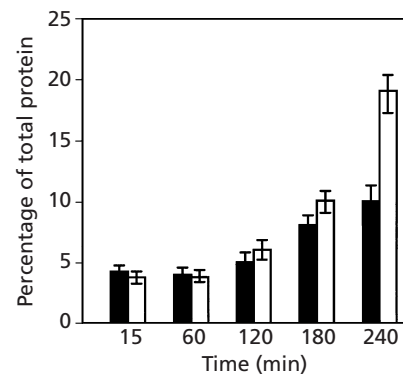


**Fig. 3.** Localization of IbpA and IbpB in cellular fractions of *E. coli*. The fraction of aggregated proteins (S fraction), outer membrane (OM), inner membrane (IM) and soluble, cytoplasmic and periplasmic proteins (CP) were isolated by sucrose density gradient ultracentrifugation from MC4100  $\Delta$ *ibpA/B* transformed with pCA (*ibpA*), pCB (*ibpB*) or pCAB plasmid (*ibpA/B*) grown at 30 °C (0') and heat shocked at 45 °C for 15 min (15'). Aliquots of the S, OM and IM fractions, each containing 6  $\mu$ g protein and 24  $\mu$ g soluble (CP) proteins were subjected to 0.1% SDS-15% PAGE and Western blotting with anti-IbpA/B serum. For determination of the IbpA/B level in each fraction (see text) it was taken into account that the insoluble proteins were loaded in 10-fold excess compared to the soluble CP fraction. Positions of IbpA/B proteins are indicated by arrows. Soluble proteins constitute 87% of total cellular proteins, whilst the S fraction contains 2% of the total protein.



**Fig. 4.** Effect of the  $\Delta$ *ibpA/B* mutation on viability of *E. coli* submitted to extreme heat shock at 50 °C. MC4100 (black bars) and MC4100 $\Delta$ *ibpA/B* bacteria (white bars) were grown in LB medium to a mid-exponential phase at 37 °C and transferred to 50 °C. Serial dilution of cells were plated onto LB agar plates. The plates were incubated at 37 °C for 18 h and colonies were counted. Error bars represent the standard deviation of three values.

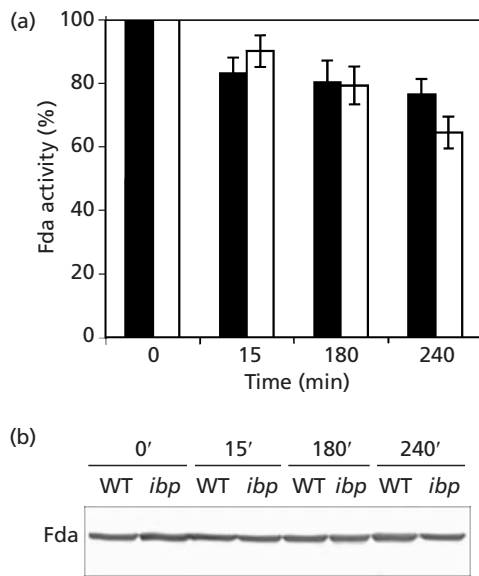
localizing in the aggregate fraction. Similar results concerning localization of the IbpA/B proteins were obtained when the cells were heat shocked at 50 °C (not shown).



**Fig. 5.** Protein aggregation in MC4100 and MC4100  $\Delta$ *ibpA/B* cells (black and white bars, respectively) submitted to extreme heat shock (50 °C). The cells were grown at 37 °C in LB medium to mid-exponential phase and transferred to 50 °C. The S fraction was isolated from samples taken at the 15th min and the first, second, third and fourth hour after the temperature shift as described earlier (Fig. 1). The amount of aggregated proteins was calculated in relation to the total protein (100%).

### IbpA/B participate in the removal of the S fraction upon extreme heat shock

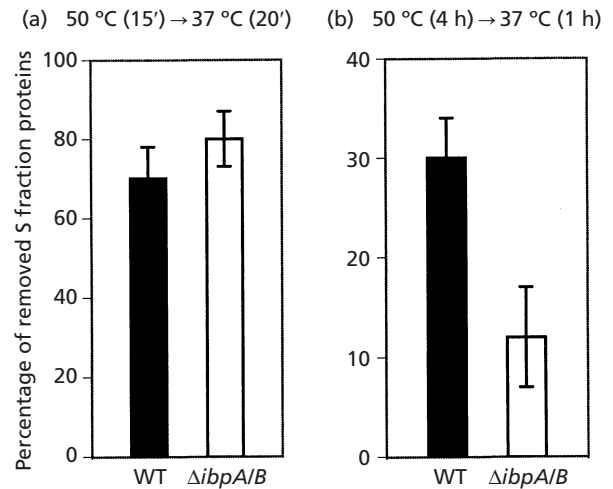
We found that IbpA/B were dispensable for removal of the S fraction proteins in cells exposed to heat stress at 45 °C for 15 min. The size of the S fraction formed at the 15th min after the temperature shift to 45 °C was about 10% smaller in the mutant than in the WT bacteria, but we did not observe any difference in the rate of S fraction removal between the  $\Delta$ *ibpA/B* mutant and WT cells (results not shown). Therefore, we exposed the  $\Delta$ *ibpA/B* mutant to a more severe heat stress. We investigated the S-fraction formation and removal after the transfer of the WT and  $\Delta$ *ibpA/B* cultures from 37 °C to 50 °C, because we had found that at this temperature the  $\Delta$ *ibpA/B* deletion substantially decreased viability of *E. coli* cells (Fig. 4). After 15 min and after 1 h following the temperature shift, about 4% of total protein was present in the S fraction of both strains (Fig. 5). We observed again that slightly less protein was aggregated in the mutant cells than in the WT, similar to the case of cells submitted to heat shock at 45 °C. Prolonged exposure of the cultures to 50 °C revealed more noticeable differences. We observed that starting from the second hour after the temperature change, the level of aggregated proteins in the  $\Delta$ *ibpA/B* strain increased faster and reached 18% of total protein at the fourth hour, whereas the S fraction in the WT contained only 10% of the total protein. These data indicate that IbpA/B proteins participate in preventing aggregation of proteins after prolonged exposure to an extreme temperature. This result was consistent with the activity assay of fructose-1,6-bisphosphate aldolase (Fda) under extreme heat stress. Fda had previously been identified as one of the S-fraction proteins (Kędzierska *et al.*, 2001). For this experiment *E. coli*(pKEN8) bacteria, overproducing Fda, were used, since the level of Fda in non-transformed cells was too low for reliable measure-



**Fig. 6.** Effect of  $\Delta ibpA/B$  mutation on inactivation of Fda in *E. coli* cells submitted to heat shock. MC4100 (black bars) and MC4100 $\Delta ibpA/B$  (white bars) transformed with pKEN8 were grown at 30 °C in LB medium. At an OD<sub>595</sub> of 0.3, IPTG was added to 1 mM. After 1 h growth, the bacteria were submitted to heat shock at 50 °C. (a) Fda activity was determined in cell lysates from samples collected at time 0 (before the temperature shift), after 15 min, and 3 and 4 h at 50 °C. Error bars represent the standard deviation of three values. (b) The level of Fda was compared in cell extracts (10 µg) submitted to 0.1% SDS-12% PAGE and Western blotting with anti-Fda serum.

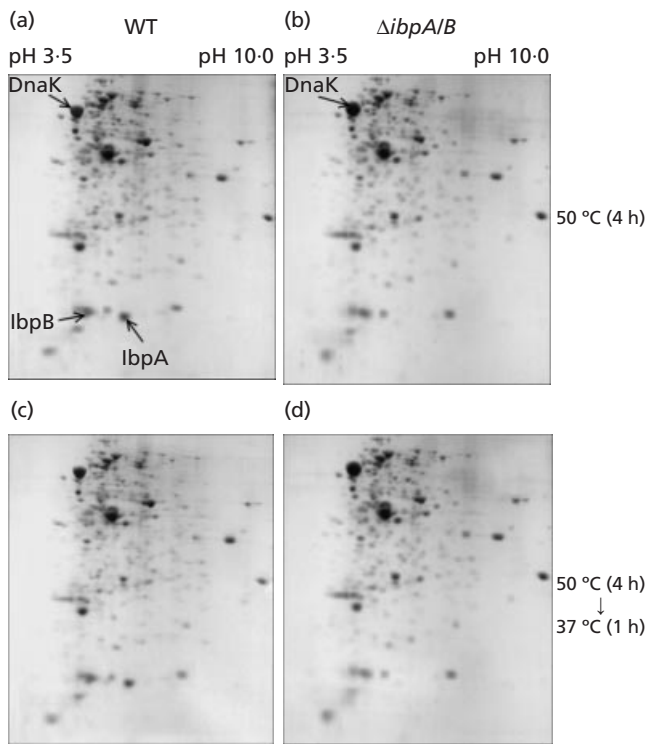
ments. It should be noted that the  $\Delta ibpA/B$  mutation did not influence the level of the Fda in cells (Fig. 6b). Fifteen minutes after heat shock in the WT cells, the Fda activity decreased to 83% of its initial value, whereas in the  $\Delta ibpA/B$  cells, 90% of the enzyme remained active (Fig. 6a). However, after a prolonged incubation (4 h) at 50 °C, the activity of Fda decreased to 76% and to 64% in the WT and  $\Delta ibpA/B$  cells, respectively. These data indicate that IbpA/B protect Fda from inactivation upon extreme heat stress.

We also tested the capability of both strains to remove the S fraction during the recovery phase at 37 °C. When the cultures were heat shocked at 50 °C for 15 min and then transferred to 37 °C for 20 min, 80% and 70% of aggregates were removed from  $\Delta ibpA/B$  and WT cells, respectively (Fig. 7a). These results are in agreement with our previous notion that the elimination of S fraction at 45 °C is slightly more efficient in the absence of the IbpA/B proteins. However, this was not the case when the cultures were incubated at 50 °C for a longer period. Only 13% of the S fraction formed within 4 h of incubation at 50 °C was removed from  $\Delta ibpA/B$  cells during 1 h at 37 °C (Fig. 7b). Under the same conditions, WT cells coped more efficiently with the aggregates and 30% of the S-fraction proteins were removed. These data indicate that IbpA and IbpB are required for elimination of aggregates formed during long (4 h) exposure to 50 °C.

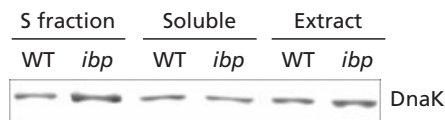


**Fig. 7.** Removal of the S fraction from *E. coli* MC4100 (black bars) and MC4100 $\Delta ibpA/B$  (white bars) submitted to extreme heat shock. (a) The cells were grown at 37 °C in LB medium to mid-exponential phase and transferred to 50 °C for 15 min. Thereafter the cultures were incubated at 37 °C. The S fraction was isolated from samples taken 15 min after the temperature shift to 50 °C and 20 min after subsequent growth at 37 °C (at the 35th min of experiment). (b) The S fraction was isolated from cultures shocked at 50 °C for 4 h and thereafter incubated at 37 °C for 1 h. Size of the S fraction isolated at 50 °C was set to 100%. Graphs present percentage of removed S fraction after recovery from heat shock. Error bars represent the standard deviation of three values.

To elucidate whether IbpA/B inhibit protein aggregation in general or protect some particular S-fraction proteins from aggregation upon extreme heat shock, we compared by two-dimensional electrophoresis the composition of the S fractions isolated from the WT and  $\Delta ibpA/B$  cells exposed to 50 °C for 4 h and after 1 h recovery time at 37 °C (Fig. 8). The overall pattern of the S-fraction proteins seemed to be similar in all cases (compare Fig. 8a and b; c and d). One evident difference was a twofold higher level of DnaK in the S fraction of  $\Delta ibpA/B$  cells when compared to the WT aggregates (Fig. 8a, b). Taking into account that the same amount of protein was loaded on the two-dimensional gels and that the S fraction of the  $\Delta ibpA/B$  strain was almost twice as large as that of the WT strain (see Fig. 5, time point 240 min), we estimate that after 4 h of heat shock the total amount of aggregated DnaK was approximately fourfold higher in the mutant than in the WT cells. It is interesting to note that under these conditions the levels of soluble DnaK in the mutant and WT cells were similar, though the total amount of DnaK was higher in mutant than in WT (Fig. 9). Two-dimensional PAGE analysis of the S fraction isolated from cells stressed at 50 °C and subsequently incubated at 37 °C revealed that all S fraction proteins were removed to a similar extent in both strains (Fig. 8c, d). Therefore, we assume that IbpA/B are responsible for inhibition of protein aggregation in general and participate in the removal of a whole spectrum of the S-fraction components rather than of particular proteins.



**Fig. 8.** Two-dimensional gel electrophoresis of the S fraction proteins of *E. coli* MC4100 and MC4100 $\Delta$ ibpA/B isolated at 50 °C (4 h) and after recovery time (1 h) at 37 °C. One hundred micrograms of proteins was loaded on each gel. DnaK, IbpA and IbpB were identified by Western blotting of identical gels (not shown). The presented gels were stained with Coomassie brilliant blue. Conditions of the experiment were as described in the legend to Fig. 5. Levels of DnaK were estimated by densitometry.



**Fig. 9.** Immunodetection of DnaK in the soluble fractions and aggregated proteins isolated from *E. coli* MC4100 and MC4100- $\Delta$ ibpA/B. Cells were heat shocked at 50 °C for 4 h, as described in the legend to Fig. 5, and aliquots of the total cellular extracts, S and soluble (CP) fractions, containing 24  $\mu$ g, 6  $\mu$ g and 24  $\mu$ g protein, respectively, were subjected to 0.1% SDS-15% PAGE and immunoblotted with anti-DnaK serum.

## DISCUSSION

According to the current model, based on *in vitro* studies, the function of ATP-independent sHsps is crucial under the depletion of ATP level caused by heat stress. sHsps hold non-native proteins, preventing them from irreversible aggregation until conditions permissive for refolding by ATP-dependent DnaK/DnaJ and GroEL/GroES are restored (Ehrnsperger *et al.*, 1997; Veinger *et al.*, 1998; Lee & Vierling, 2000). In this work we investigated the function of IbpA and IbpB

proteins *in vivo*, analysing the formation and removal of denatured and aggregated proteins (S fraction) in *E. coli*. We showed that the overproduction of IbpA, IbpB or both sHsps resulted in stabilization of the S fraction after heat shock (Fig. 1b). Thus, overproduction of sHsps causes the opposite effect to that observed in cells with excess ATP-dependent chaperones (DnaK/DnaJ, GroEL/GroES systems) which prevent S-fraction formation (Kędzierska *et al.*, 1999). These *in vivo* data are in agreement with the model described above. From the analysis of protein content of the S fraction (Fig. 1a) formed at 45 °C (15 min), we concluded that IbpA and IbpB may exhibit different affinities for unfolded proteins. The precursor of periplasmic  $\beta$ -lactamase is an example of unfolded protein, which *in vivo* accompanied IbpA but not IbpB in the fraction of protein aggregates. Translocation of the  $\beta$ -lactamase precursor to the periplasmic space requires GroEL/GroES chaperone machinery. It is also suggested that DnaK/DnaJ and SecB may cooperate with GroEL/ES in this transport (Lamiet *et al.*, 1990; Wild *et al.*, 1996). In general, chaperones prevent complete folding of precursor proteins in the cytoplasm and hold them in a loose, 'translocation-competent' conformation prerequisite for the transport of proteins across membranes. It seems that stable interaction of IbpA with partly unfolded  $\beta$ -lactamase precursor inhibits its chaperone-assisted translocation to the periplasm since the growth of *E. coli* cells overproducing IbpA, in the medium supplemented with ampicillin, required an increased level of the precursor (Fig. 2).

When IbpA and IbpB were produced together, both sHsps were present in fractions of aggregated proteins and absent in the soluble protein fraction. It is interesting that in the absence of IbpA, most of the IbpB remained in a soluble fraction and only 2% of total IbpB colocalized with the aggregated proteins (Fig. 3). The observed influence of IbpA on localization of IbpB suggests that mixed IbpA-IbpB complexes may be formed. The formation of such complexes has been described by Studer & Narberhaus (2000).

It is remarkable that after a temperature change from 30 °C to 42 °C the cellular level of IbpA/B increases almost 10-fold, whereas the amount of DnaK and GroEL is only twofold higher (Mogk *et al.*, 1999). This indicates that IbpA/B are particularly important at high temperatures compared to other chaperones that function also under physiological conditions. However, we demonstrated that when *E. coli* cells were exposed to high temperatures for a relatively short period (15 min at 45 °C), protein aggregates could be eliminated by chaperones without the participation of sHsps. Moreover, we showed here that heat inactivation of Fda, one of the S-fraction proteins, was less efficient in *E. coli* cells lacking IbpA/B. Recently, it was found that DnaK/DnaJ, but not GroEL/GroES, were essential for the reactivation of heat-denatured Fda *in vivo* (Kędzierska *et al.*, 2001). Taken together, these data suggest that in the WT cells IbpA/B proteins may slightly inhibit the Fda refolding by DnaK/DnaJ, probably by blocking the

DnaK/DnaJ-binding sites in denatured proteins. A similar effect was reported *in vitro*: the rate of refolding of thermolabile malate dehydrogenase and lactate dehydrogenase denatured in the presence of DnaK/J and incubated with GroEL/ES after heat shock was higher than that of the enzymes bound to IbpB during denaturation and incubated subsequently with the DnaK/J–GroEL/ES systems (Veinger *et al.*, 1998). We demonstrated that IbpA/B were required for the suppression of protein aggregation and for the elimination of the S fraction formed during extended (2–4 h) treatment at 50 °C (Figs 6 and 7). Analysis of the protein content of the S fraction indicated that upon extreme heat stress IbpA/B did not protect any particular proteins from aggregation but suppressed protein aggregation in general (Fig. 8a, b). Removal of proteins from the S fraction was not specific either (Fig. 8c, d). However, we found that after prolonged exposure to 50 °C the mutant  $\Delta$ *ibpA/B* strain contained a higher amount of DnaK than the WT strain and this excess was localized in the S fraction (Figs 8 and 9). The increased amount of DnaK in the fraction of aggregated proteins might be a direct consequence of the lack of the IbpA/B: hydrophobic segments of unfolded proteins, usually recognized and bound by IbpA/B, would become available for DnaK. Increased binding of DnaK by the unfolded proteins would in turn cause a decrease in the level of soluble DnaK, which, according to the model of regulation of heat-shock response (Tomoyasu *et al.*, 1998), would result in higher activity of  $\sigma^{32}$  and intensified production of DnaK. Such an overall increase in the DnaK level is indeed observed in the mutant cells (Fig. 9).

The damage caused by heat treatment at 50 °C comprises degradation of ribosomes and inactivation of macromolecular synthesis (Neidhardt & VanBogelen, 1987); thus, after long exposure to 50 °C, when the function of IbpA/B becomes important for cell survival, the level of IbpA/B cannot be increased. Therefore, IbpA/B may have to be strongly induced immediately after a temperature upshift, even though their function is dispensable in the case of relatively short-term heat stress. This may thus provide an explanation for the apparently paradoxical result that the IbpA/B proteins are very strongly induced on heat shock but that  $\Delta$ *ibpAB* strains show no significant phenotype at normal heat-shock temperatures.

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