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Roles of the *Escherichia coli* Small Heat Shock Proteins IbpA and IbpB in Thermal Stress Management: Comparison with ClpA, ClpB, and HtpG In Vivo

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We have constructed an *Escherichia coli* strain lacking the small heat shock proteins IbpA and IbpB and compared its growth and viability at high temperatures to those of isogenic cells containing null mutations in the *clpA*, *clpB*, or *htpG* gene. All mutants exhibited growth defects at 46°C, but not at lower temperatures. However, the *clpA*, *htpG*, and *ibp* null mutations did not reduce cell viability at 50°C. When cultures were allowed to recover from transient exposure to 50°C, all mutations except Δ*ibp* led to suboptimal growth as the recovery temperature was raised. Deletion of the heat shock genes *clpB* and *htpG* resulted in growth defects at 42°C when combined with the *dnaK756* or *groES30* alleles, while the Δ*ibp* mutation had a detrimental effect only on the growth of *dnaK756* mutants. Neither the overexpression of these heat shock proteins nor that of ClpA could restore the growth of *dnaK756* or *groES30* cells at high temperatures. Whereas increased levels of host protein aggregation were observed in *dnaK756* and *groES30* mutants at 46°C compared to wild-type cells, none of the null mutations had a similar effect. These results show that the highly conserved *E. coli* small heat shock proteins are dispensable and that their deletion results in only modest effects on growth and viability at high temperatures. Our data also suggest that ClpB, HtpG, and IbpA and -B cooperate with the major *E. coli* chaperone systems in vivo.

Living organisms respond to stressful environmental conditions by increasing the production of specific proteins which alleviate or reduce damage incurred by the cell. In *Escherichia coli*, temperature increase upregulates two groups of heat-shock proteins (Hsps) that are transcribed by the $E\sigma^{32}$ and $E\sigma^{E}$ holoenzymes. The σ^{32} regulon is implicated in the management of cellular stress in the cytoplasm, whereas $E\sigma^{E}$-transcribed proteins are specifically upregulated in response to stress in the periplasm or the cell envelope (reviewed in reference 13). While the identity and cellular function of most members of the σ^{E} regulon remain unclear, a great deal of effort has been directed at understanding the regulation of σ^{32} and the role of $E\sigma^{32}$-transcribed Hsps in the maintenance of heat-shocked cells. Most members of the σ^{32} regulon have been classified as either molecular chaperones or ATP-dependent proteases (13). Molecular chaperones, which include the DnaK-DnaJ-GrpE and GroEL-GroES systems, facilitate the proper folding of newly synthesized polypeptides and help thermally damaged proteins regain a biologically active conformation (14). Heat shock proteases, such as ClpP, Lon, and HflB, apparently degrade misfolded proteins that cannot be rescued by chaperone action (10). The signal responsible for induction of the heat shock response is believed to be an increase in the intracellular concentration of unfolded and misfolded proteins (13). This can be caused by temperature increase or other stresses, including phage infection; the presence of organic solvents, heavy metals, and certain antibiotics; and the production of aggregation-prone proteins (25, 27, 37).

The DnaK-DnaJ-GrpE and GroEL-GroES systems are the best-characterized molecular chaperones of *E. coli*. Based on in vitro studies and homology to eukaryotic proteins, other members of the σ^{32} regulon are also believed to perform a molecular chaperone function in vivo. These include the Clp ATPases (ClpB, ClpX, and ClpY), the Hsp90 homolog HtpG, and the small Hsps (sHsps) IbpA and IbpB (13, 30). To date, relatively little is known about the cellular functions of these "minor" chaperones. It has been shown that *clpB* null mutants exhibit growth defects at 44°C and undergo a higher rate of killing at 50°C than the wild type (31). Nevertheless, ClpB overexpression does not enhance the viability of wild-type *E. coli* at 55°C (8). While *htpG* null mutants also display growth defects at 44°C (4), little is known about the in vivo function of HtpG, except for a possible involvement in secretion (29, 36). IbpA and IbpB have been found in association with thermally aggregated host proteins (20) and recombinant protein inclusion bodies (2), but knowledge of their in vivo function is limited, since the construction and characterization of an *ibp* mutant have not been previously reported. Despite the fact that the Clp ATPase ClpA is not itself an Hsp, it displays molecular chaperone activity in vitro (40) and provides substrate specificity to the heat shock protease ClpP (17). While a *clpA* null mutation was previously found to have no effect on growth at 42°C (16), other aspects of the role of ClpA in thermal stress management have not been examined.

In this report, we have characterized the effects of deletion or overexpression of the sHsps IbpA and -B on the growth and viability of heat-shocked *E. coli* cells. Results were compared to the effects of deletion or overproduction of ClpA, ClpB, or HtpG. We further investigated the influence of manipulation of the intracellular concentration of these minor chaperones in *dnaK756* and *groES30* genetic backgrounds. We show that IbpA and -B are dispensable in *E. coli* and that ClpB, HtpG, and IbpA and -B cooperate with the major chaperone systems in the management of thermal stress in vivo.

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype and/or description[a]	Source or reference[b]
Top10	F′ λ$^-$ *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi1 gyrA96 relA1* ϕ*80*Δ*lacZ*ΔM15Δ(*lacZYA-argF*) *U169 deoR*	Invitrogen
XL1-blue	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*] (Tet^r)	Stratagene
JCB495	MC1000 *recD*	C. Manoil
CC160	MC1000 *dam*	C. Manoil
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory stock
SG1126	SG1101 *clpA*::*kan*	11
JGT4	MC4100 *clpA*::*kan*	P1(SG1126) × MC4100→Neo^r Str^r Lac$^-$
MC1000ΔclpB	MC1000 Δ*clpB*::*kan*	31
JGT3	MC4100 Δ*clpB*::*kan*	P1(MC1000ΔclpB) × MC4100→Neo^r Str^r Lac$^-$
JCB23	MC1655 F$^-$ *lacZ*::Tn*10 zba315*::*kan* Δ*htpG1*::*lacZ*	4
JGT11	MC4100 *zba315*::*kan* Δ*htpG1*::*lacZ*	P1(JCB23) × MC4100→Neo^r Str^r Lac$^+$
JGT1	JCB495 Δ*ibp1*::*kan*	LinTr(pBRΔibp::kan 14-3) × JCB495→Neo^r Amp^s
JGT17	MC4100 Δ*ibp1*::*kan*	P1(JGT1) × MC4100→Neo^r Str^r Lac$^-$
CG800	C600 *leu dnaK756 thr*::Tn*10*	S. van der Vies
JGT20	MC4100 *dnaK756 thr*::Tn*10*	P1(CG800) × MC4100→Tet^r Str^r Ts44 Lac$^-$
CG712	B178 *zjd*::Tn*10 groES30*	34
JGT6	MC4100 *zjd*::Tn*10 groES30*	P1(CG712) × MC4100→Tet^r Str^r Ts44 Lac$^-$
JGT44	MC4100 *zjd*::Tn*10*	P1(CG712) × MC4100→Tet^r Str^r Lac$^-$
SG1162a	SG1101 *clpP*::*cat*	11
JGT19	MC4100 *clpP*::*cat*	P1(SG1162a) × MC4100→Chl^r Str^r Lac$^-$
JGT31	MC4100 *dnaK756 thr*::Tn*10 clpA*::*kan*	P1(SG1126) × JGT20→Neo^r Lac$^-$
JGT32	MC4100 *dnaK756 thr*::Tn*10* Δ*clpB*::*kan*	P1(MC1000ΔclpB) × JGT20→Neo^r Lac$^-$
JGT30	MC4100 *dnaK756 thr*::Tn*10 zba315*::*kan* Δ*htpG1*::*lacZ*	P1(JCB23) × JGT20→Neo^r Lac$^+$
JGT34	MC4100 *dnaK756 thr*::Tn*10* Δ*ibp1*::*kan*	P1(JGT1) × JGT20→Neo^r Lac$^-$
JGT27	MC4100 *zjd*::Tn*10 groES30 clpA*::*kan*	P1(SG1126) × JGT6→Neo^r Lac$^-$
JGT38	MC4100 *zjd*::Tn*10 groES30* Δ*clpB*::*kan*	P1(CG712) × JGT3→Neo^r Ts44 Lac$^-$
JGT39	MC4100 *zjd*::Tn*10 groES30 zba315*::*kan* Δ*htpG1*::*lacZ*	P1(CG712) × JGT11→Neo^r Ts44 Lac$^+$
JGT28	MC4100 *zjd*::Tn*10 groES30* Δ*ibp1*::*kan*	P1(JGT1) × JGT6→Neo^r Lac$^-$
BT66	MC4100 *malG*(Am) *malT*^{c}-Tn*10 secB*::Tn*5* (F′ *lacpro lacI*^{q})	B. Traxler
JGT43	MC4100 *clpP*::*cat* (F′ *lacpro lacI*^{q})	F′(BT66) × JGT19→Chl^r Lac$^+$ Neo^s Tet^s
JGT47	MC4100 *zjd*::Tn*10* (F′ *lacpro lacI*^{q})	F′(JGT43) × JGT44→Tet^r Lac$^+$ Chl^s
JGT61	MC4100 *dnaK756 thr*::Tn*10* (F′ *lacpro lacI*^{q})	F′(JGT43 × JGT20→Tet^r Lac$^+$ Chl^s
JGT49	MC4100 *zjd*::Tn*10 groES30* (F′ *lacpro lacI*^{q})	F′(JGT43 × JGT6→Tet^r Lac$^+$ Chl^s

[a] Amp, ampicillin; Neo, neomycin or kanamycin; Chl, chloramphenicol; Tet, tetracycline; Str, streptomycin. Superscripts r and s indicate resistance and sensitivity, respectively. Lac refers to the formation of blue colonies on M9 plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG.

[b] P1 transductions are represented as P1(donor) × recipient→phenotypes used for selection and screening. Linear transformations (LinTr) are represented as LinTr(plasmid) × recipient→phenotypes used for selection and screening. F′ mating is represented as F′(donor) × recipient→phenotypes used for selection and screening. Ts44 indicates that the selected cells were unable to form colonies at 44°C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth conditions. Relevant characteristics of the bacterial strains and plasmids used in this study are described in Tables 1 and 2. Top10 and XL1-blue cells were transformed with plasmid DNA by electroporation; all other transformations were performed by the RbCl method (Promega). Routine growth was carried out at 30°C in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol, 34 μg/ml; carbenicillin, 100 μg/ml; neomycin or kanamycin, 50 μg/ml; and streptomycin, 50 μg/ml.

Plasmid constructions. All enzymes and kits were used according to the manufacturer's recommendations. PCR amplifications were performed with the Expand high-fidelity kit (Boehringer Mannheim) and primers purchased from Gibco BRL. PCR products from genomic DNA were purified with the Qiaquick PCR purification kit (Qiagen). All other PCR products and DNA restriction fragments were purified after agarose gel electrophoresis by using the Qiaquick gel extraction kit (Qiagen). Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen). Ligations were carried out with the Rapid DNA ligation system (Boehringer Mannheim) and verified by restriction analysis or sequencing with the PRISM ready reaction dyedeoxy terminator cycle sequencing kit (Applied Biosystems). Ligation products were maintained in either XL1-blue or Top10, and CC160 was used for purification of plasmids requiring restriction with enzymes blocked by *dam* methylation.

Plasmid pBRibp was constructed by ligation of a 2.9-kbp *Hin*dIII-*Pvu*II fragment from pMON18003 to a pBR322 backbone digested with the same enzymes. This plasmid encodes the entire *ibp* operon surrounded by 500 to 1,000 bp of flanking genomic DNA. The majority of the operon was removed by digestion of pBRibp with *Bst*XI (Fig. 1). The large fragment was blunted with T4 polymerase, dephosphorylated with shrimp alkaline phosphatase (Boehringer Mannheim), and ligated to a neomycin phosphotransferase gene isolated on an *Sma*I fragment from pBSL14. Plasmid pBRΔibp::kan14-3, which carries the kanamycin resistance cassette transcribed in the opposite orientation of the *ibp* operon, was selected for further manipulations.

The cloning vector pTG10 was used as a backbone to generate a series of expression plasmids similar to the previously described plasmids pDnaK/J, pGroESL, and pσ^{32} (Table 2). Plasmid pClpA was generated by insertion of a *Bam*HI-*Pst*I fragment from pWPC3 into the *Bcl*I-*Pst*I backbone of pTG10. Plasmid pClpB was constructed by ligation of a *Bam*HI-*Sph*I fragment from pclpB into the same sites of pTG10. Plasmid pHtpG was constructed by ligation of a *Bcl*I-*Sal*I fragment from pBJ5 to the *Bam*HI-*Sal*I backbone of pTG10. The *ibp* operon was amplified by PCR with plasmid pMON18003 as a template and the primer BP-SphI (5′-GCCCCCTCAGTGCATGCAATAGACC), which hybridizes before the promoter region, and primer AB-HindIII (5′-ATCGGTGAAG AAGCTTTGCCTT), which binds between the putative transcription terminator and *orfA* (Fig. 1). The PCR-amplified *ibp* operon was cloned into the pT7blue blunt vector system (Novagen), verified by restriction digests and DNA sequencing, and inserted into pTG10 as a *Hin*dIII fragment. Since the *lac* promoter is very weak compared to heat shock promoters at high temperatures, increased chaperone expression from these plasmids can be considered to mostly result from an increase in gene dosage. The sole exception is pσ^{32}, which contains the *rpoH* gene under control of the *lac* promoter only in order to avoid known

TABLE 2. *E. coli* plasmids used in this study

Plasmid	Description[a]	Source or reference
pMON18003	pUC19 derivative carrying a 5.2-kbp fragment of the *E. coli* chromosome containing the *ibp* operon (Amp^r)	2
pBRibp	pBR322 derivative encoding the *ibp* operon and surrounding genomic DNA (Amp^r)	This study
pBSL14	Encodes the gene for neomycin phosophotransferase flanked by multiple restriction sites (Neo^r)	1
pBRΔibp::kan14-3	pBRibp derivative with most of the *ibp* operon removed by *Bst*XI digestion and a Neo^r cartridge from pBSL14 inserted at this location (Amp^r Neo^r)	This study
pWPC3	pUC19 derivative encoding the *clpA* gene (Amp^r)	11
pclpB	pBR322 derivative encoding the *clpB* gene (Amp^r)	31
pBJ5	pBR322 derivative encoding the *htpG* gene (Amp^r)	4
pT7blue	pUC19-derived cloning vector (Amp^r)	Novagen
pTb-ibp	pT7blue derivative encoding a PCR-amplified *ibp* operon (Amp^r)	This study
pTG10	pACYC184-derived cloning vector (Chl^r)	9
pDnaK/J	pTG10 derivative encoding the *dnaKJ* operon under control of the native promoter (Chl^r)	A. A. Gatenby
pGroESL	pTG10 derivative encoding the *groE* operon under control of the *lac* and native promoters (Chl^r)	9
$p\sigma^{32}$	pTG10 derivative encoding the *rpoH* gene under control of the *lac* promoter only (Chl^r)	33
pClpA	pTG10 derivative encoding the *clpA* gene under control of the native promoter (Chl^r)	This study
pClpB	pTG10 derivative encoding the *clpB* gene under control of the native promoter (Chl^r)	This study
pHtpG	pTG10 derivative encoding the *htpG* gene under control of the native promoter (Chl^r)	This study
pIbp	pTG10 derivative encoding the *ibp* operon under control of the *lac* and native promoters (Chl^r)	This study

[a] Amp, ampicillin; Neo, neomycin or kanamycin; Chl, chloramphenicol. Superscript r indicates resistance.

stability issues (35). Overexpression of all proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and/or Western blotting (data not shown and reference 33).

Strain constructions. An *ibp* null mutant was created by digestion of pBRΔibp::kan14-3 with *Sca*I and transformation of the linear DNA into the *recD* mutant strain JCB495. Putative recombinants were selected on the basis of kanamycin resistance and ampicillin sensitivity. The presence of the mutation was confirmed by PCR analysis of chromosomal DNA, which was isolated by the method of Marmur (21) followed by phenol extraction. JGT1, a homologous recombinant containing the Δ*ibp1*::*kan* mutation, was used for further studies.

Strains were constructed by standard techniques (23). P1 transduction was used to create a panel of chaperone mutants isogenic to *E. coli* MC4100 (Table 1). Transductants were selected at 30°C on LB plates containing the appropriate antibiotics. An F′ episome bearing the $lacI^q$ allele was conjugated into several strains with selection on M9 lactose agar plates containing the appropriate antibiotics and no amino acids, except for 100 μg of threonine per ml in the case of cells carrying the *thr*::Tn*10* mutation (Table 1).

Growth studies and viability measurements. Growth studies were performed with 125-ml shake flasks containing 25 ml of LB medium supplemented with 0.2% glucose and the appropriate antibiotics. A New Brunswick G76 water bath was used for culturing cells at 44°C and above in order to maintain precise temperature control (±0.2°C). Growth rates were determined by recording the culture A_{600} at various time points. Cultures were inoculated at the ratios indicated below to obtain A_{600} readings below 0.1 at the initial time point. For typical growth experiments, seed cultures were grown for 20 h at 30°C and diluted 50-fold into supplemented medium prewarmed to the indicated temperatures.

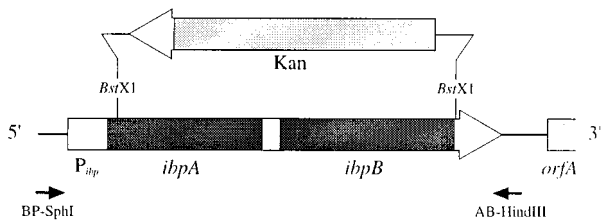

FIG. 1. Structure of the *ibp* operon. An *ibp* null mutant was constructed by insertion of a kanamycin resistance cartridge (Kan) between the indicated *Bst*XI sites. This operation removes about 60% of the *ibpA* open reading frame and the entire *ibpB* gene. The presence of the Δ*ibp1*::*kan* mutation in *E. coli* JGT1 and JGT17 was confirmed by PCR analysis of chromosomal DNA with the primer set shown. The same primers were used for PCR amplification of the *ibp* operon. The figure is not drawn to scale.

To examine the effect of chaperone overexpression on the growth of *dnaK756* and *groES30* mutants, the cells were grown at 30°C to mid-exponential phase and induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and temperature shift to 37°C for 1 h. The cells were diluted 25-fold into fresh medium held at 44 or 46°C, and growth was monitored for up to 24 h. For spot test experiments, 10-μl samples of cultures grown overnight at 30°C were aliquoted onto LB agar plates supplemented with chloramphenicol and IPTG. The plates were incubated for 24 h at 30, 37, 42, or 44°C, and growth was estimated by visual inspection. The ability of the cells to recover from heat shock at 50°C was examined by transfer of cultures grown to mid-exponential phase at 30 to 50°C for 1 h. Thereafter, culture aliquots were diluted 10-fold into fresh medium prewarmed to the indicated temperatures. The data presented were obtained from simultaneous cultures of the various strains. All experiments were repeated on two or more separate occasions to confirm the results. The data shown are the averages from duplicate cultures when indicated.

Thermotolerance at 50°C was determined by measuring CFU. For experiments involving chaperone mutants, cells growing exponentially at 30°C were transferred to 50°C. Plasmid-bearing cells were grown at 30°C to mid-exponential phase, induced by the addition of 1 mM IPTG and temperature upshift to 37°C for 1 h, and transferred to 50°C. In all cases, serial dilutions of cells were spread onto agar plates containing the appropriate antibiotics. The numbers of colonies formed after overnight incubation at 30°C were obtained from duplicate cultures. Experiments were repeated on at least two separate occasions.

SDS-PAGE fractionation analysis. To examine the effects of chaperone mutations on the aggregation of host proteins, cultures growing in mid-exponential phase at 30°C were transferred to 42, 46, or 50°C for 1 h, and culture samples were collected. SDS-PAGE analysis of the cellular fractions was performed as described previously (33). Briefly, culture samples were passaged through a French pressure cell at 10,000 lb/in^2, and soluble and insoluble materials were separated by centrifugation at 30,000 × *g* for 12 min. Soluble proteins were concentrated by methanol-chloroform precipitation (39). Aliquots of soluble and insoluble cellular fractions corresponding to identical absorbance units were fractionated by reducing SDS-PAGE and visualized by Coomassie brilliant blue staining.

RESULTS

Construction of an *ibp* knockout strain and isogenic Hsp mutants. IbpA and IbpB are highly homologous 16-kDa proteins displaying more than 50% identity at the amino acid level (2). The *ibp* operon, which lies at 82.5 min on the *E. coli* genetic map, contains a σ^{32}-regulated promoter followed by the *ibpA* and *ibpB* genes in succession (Fig. 1) (2, 5). Directly after the transcription terminator lies an open reading frame

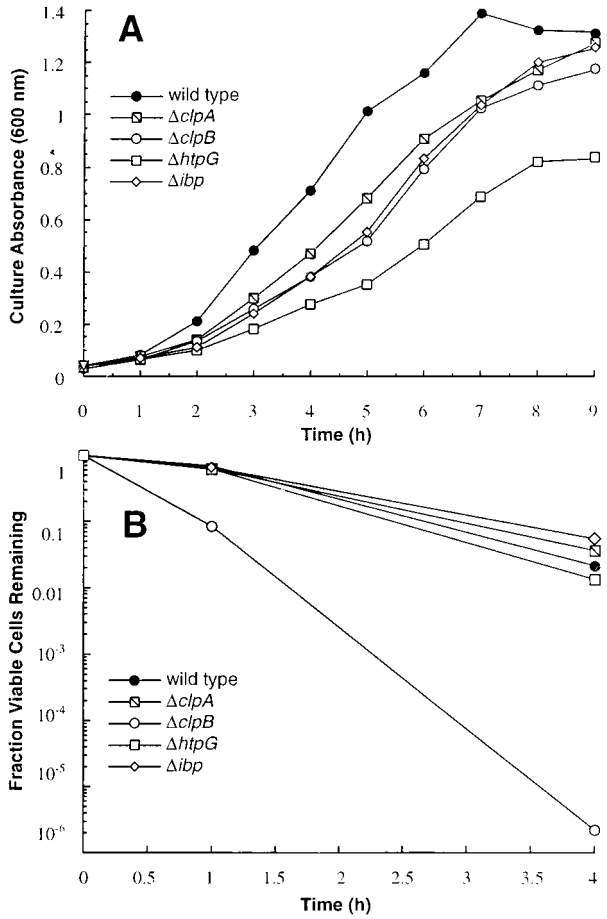

FIG. 2. Growth and viability of chaperone mutants at high temperatures. (A) Stationary-phase cells grown at 30°C were diluted directly into fresh medium held at 46°C. Average variation (coefficient of variation [CV]) between duplicate cultures was 6.2%. (B) Cells containing the indicated mutations were grown to mid-exponential phase at 30°C and shifted to 50°C. Data shown represent the ratio of viable cells after high-temperature incubation to that of viable cells before the shift to 50°C. The average CV for duplicate cultures was 23%.

(*orfA*) of unknown function. To gain further insight into the cellular function of IbpA and -B, we constructed an *ibp* null mutant by inserting a kanamycin resistance cartridge between the two *Bst*XI restriction sites located within the operon (Fig. 1). The resulting deletion, Δ*ibp1*::*kan*, removes most of the *ibpA* gene and all of the *ibpB* gene without disturbing *orfA*. Since the kanamycin resistance gene is transcribed in the opposite orientation of the *ibp* operon, polar effects are not expected. P1 transduction was next used to create a panel of MC4100 derivatives containing mutations in the genes encoding DnaK (*dnaK756*), GroES (*groES30*), ClpA (*clpA*::*kan*), ClpB (Δ*clpB*::*kan*), ClpP (*clpP*::*cat*), HtpG (Δ*htpG1*::*lacZ*), and IbpA and -B (Δ*ibp1*::*kan*) (see Table 1 for further details).

The *E. coli* sHsps are dispensable for growth at high temperatures. We first compared the growth of the *ibp* null mutant to that of isogenic Δ*clpA*, Δ*clpB*, and Δ*htpG* mutants over the 30 to 46°C temperature range. Fresh medium prewarmed to various temperatures was inoculated with cells grown overnight at 30°C. While the growth of all mutants was indistinguishable from that of the wild type at temperatures up to 45°C (data not shown), growth deficiencies were readily apparent when the temperature was raised to 46°C (Fig. 2A). Under these conditions, the specific growth rates of the *clpB* and *htpG* mutants were 30 to 40% lower than that of the wild type. Although it was previously found that the growth of Δ*clpB* and Δ*htpG* mutants is impaired at 44°C (4, 31), the 2°C difference in temperature for full expression of the growth-deficient phenotype in these genetic backgrounds is likely explained by variations in host strains, growth medium, or other experimental conditions. The Δ*ibp* cells grew at a rate similar to that of the Δ*clpB* cells at 46°C. More surprisingly, we reproducibly found that the specific growth rate of the *clpA* mutant was 80% that of the wild type, despite the fact that ClpA is not an Hsp.

Differential influence of chaperones on basal thermotolerance. We next examined the viability of the various mutants following incubation at 50°C. For these experiments, cells growing exponentially at 30°C were shifted to 50°C, and the number of viable cells remaining after 1 and 4 h was determined by plating culture aliquots at 30°C. In agreement with previous studies (31), the death rate of the Δ*clpB* strain was about 3.5-fold higher than that of the wild type and was comparable to that of an isogenic *dnaK756* mutant (data not shown). In contrast, cellular viability was not affected by the presence of the Δ*clpA*, Δ*htpG*, Δ*ibp* mutation (Fig. 2B). We further observed that colonies formed overnight by Δ*clpB* cells exposed to 50°C for 1 h were smaller and heterogeneous compared to those formed by other minor chaperone mutants. From these observations, it is clear that individual Δ*clpB* cells which remain viable after incubation at 50°C are severely compromised.

We also investigated whether overproduction of various chaperones would improve the viability of *E. coli* cells exposed to lethal temperatures. For these experiments, *E. coli* JGT47, an MC4100 derivative containing an F′ episome encoding the *lacI*^{q} gene, was transformed with a series of pTG10-derived plasmids encoding DnaK-DnaJ, GroEL-GroES, σ^{32}, ClpA, ClpB, HtpG, or IbpA and -B (see Table 2 and Materials and Methods for details). Basal thermotolerance at 50°C was determined as described in Materials and Methods. Whereas GroEL-GroES overexpression had a 7-fold beneficial effect, higher intracellular concentrations of DnaK-DnaJ, ClpA, ClpB, or IbpA and -B led to 5- to 10-fold reductions in viability (data not shown). To examine the rapid loss of viability of ClpB cells at 50°C in more detail, the experiments described above were repeated with JGT51, an MC4100 Δ*clpB* derivative carrying an F′ *lacI*^{q} episome. In this genetic background, only pClpB was able to restore viability to control levels. Cell viabilities in all other strains were 4 to 6 orders of magnitude less than those in pClpB transformants (data not shown).

ClpA, ClpB, and HtpG—but not IbpA and -B—are required for optimal recovery from exposure to high temperatures. Because growth and survival at high temperatures may involve different pathways from that of recovery following exposure to extreme stress, we examined how chaperone mutations influenced the ability of *E. coli* to recover from transient incubation at 50°C. For this purpose, the various mutants were grown to mid-exponential phase at 30°C, shifted to 50°C for 1 h, and diluted into fresh medium held at 37, 42, or 45°C. At 37°C, all strains except for the Δ*clpB* mutant recovered similarly to the wild type (data not shown). When cultures were transferred to 42°C, Δ*clpB* cells were unable to recover for several hours, and an increased growth lag was observed in Δ*htpG* cells (Fig. 3A). The latter effect was much more obvious when the temperature was raised to 45°C (Fig. 3B). Under these conditions, the Δ*clpA* mutant also displayed a pronounced lag in recovery. However, the Δ*ibp* strain behaved comparably to the wild type except for a lower final culture density. It should finally be noted that Δ*clpB* cultures began to grow slowly after 4 to 6 h of incubation, depending on recovery temperature, and reached a

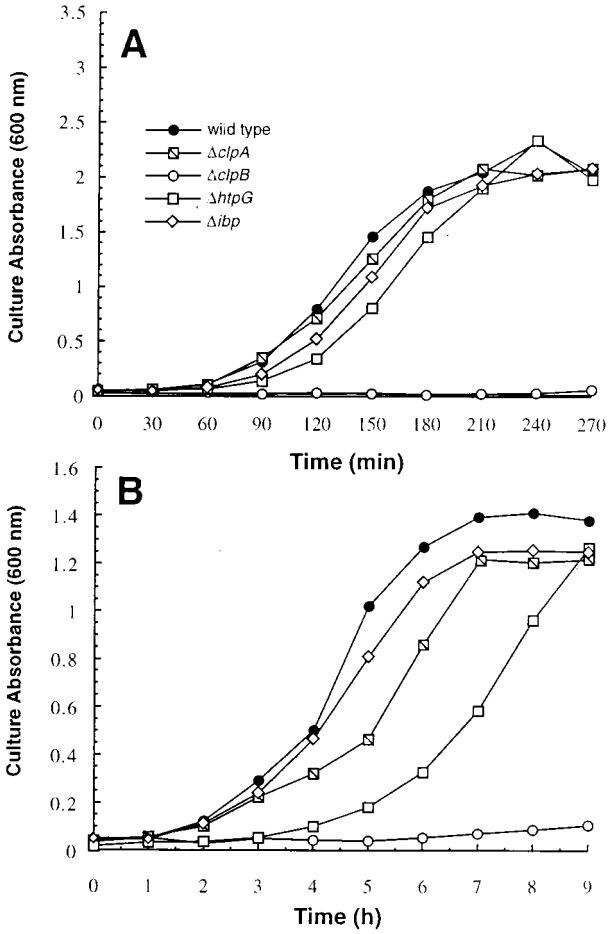

FIG. 3. Recovery of chaperone mutants following transient incubation at 50°C. Cells grown to mid-exponential phase at 30°C were shifted to 50°C for 1 h and diluted into fresh medium held at 42°C (A) or 45°C (B). The average coefficient of variation for duplicate cultures was 7.2%. Note the difference in scales for culture absorbance and incubation time.

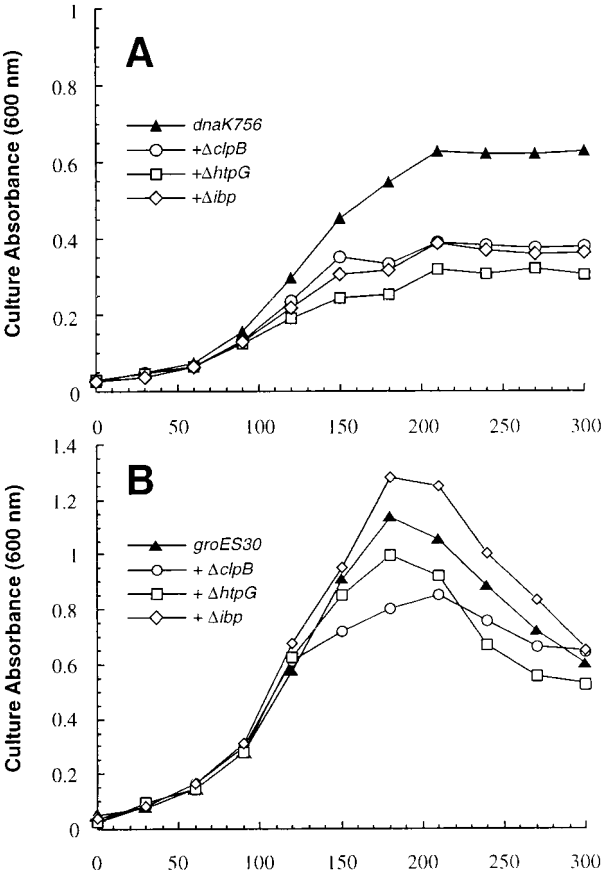

FIG. 4. Effect of double chaperone mutations on growth at 42°C. (A) *dnaK756* single or double mutants grown overnight at 30°C were diluted into fresh medium held at 42°C. The average coefficient of variation (CV) for duplicate cultures was 5.9%. (B) *groES30* single or double mutants were grown overnight at 30°C and diluted into fresh medium held at 42°C. The average CV for duplicate cultures was 6.8%. Note the difference in scale for culture absorbances.

density similar to that of the wild type after 24 h (data not shown). Since the viability of *clpB* mutants is greatly reduced after 1 h of incubation at 50°C (Fig. 2B) and viable cells exhibit morphological changes, both processes are likely to account for the extreme behavior of this strain.

The Hsps ClpB, HtpG, and IbpA and -B cooperate with the DnaK-DnaJ-GrpE and GroEL-GroES systems in stress management. To investigate the possibility that ClpA, ClpB, HtpG, or IbpA and -B cooperate with DnaK-DnaJ-GrpE and/or GroEL-GroES at high temperatures, the various null mutations were introduced into MC4100 derivatives carrying either the *dnaK756* or *groES30* allele, and the growth of the double mutants was characterized at 42°C. Whereas the *clpA* deletion had no detrimental effect on the growth of *dnaK756* or *groES30* cells at 42°C (data not shown), the combination of the *dnaK756* allele with the Δ*clpB*, Δ*htpG*, or Δ*ibp* mutations exerted a clear deleterious effect on cell growth, since all double mutants only reached half of the maximum turbidity of *dnaK756* control cells (Fig. 4A). The simplest explanation for this behavior is that the double mutants experience a higher degree of cellular damage and lose the ability to replicate more rapidly.

The effect of minor chaperone deletions was less dramatic in the *groES30* background. The absorbance of *groES30* control cultures declined 3 h after transfer to 42°C, and the Δ*ibp* and Δ*htpG* double mutants displayed similar growth patterns (Fig. 4B). However, we reproducibly found in several independent experiments that the Δ*ibp groES30* double mutant grew to a slightly higher turbidity than the control, while Δ*htpG groES30* cells exhibited the opposite behavior (Fig. 4B). The Δ*clpB* mutation had the most detrimental effect in the *groES30* background. This double mutant only reached 70% of the maximal turbidity of *groES30* control cultures before gradual lysis occurred. We additionally found that the basal thermotolerance levels of *groES30* cells at 50°C were identical to that of the wild type and that the Δ*clpB* mutation was the only null mutation to reduce the viability of *groES30* cells held at 50°C for 1 h (data not shown). Similar experiments were not performed with *dnaK756* cells, since the death rate of this mutant is similar to that of Δ*clpB* cells.

Overexpression of the minor chaperones does not restore the growth of *dnaK756* or *groES30* mutants at high temperatures. To determine whether an increase in the intracellular concentration of ClpB, HtpG, or IbpA and -B could compensate for the deleterious effects of the *dnaK756* or *groES30* mutations on growth at high temperature, the pTG10-derived series of chaperone expression plasmids were introduced into MC4100 *dnaK756* (JGT61) or *groES30* (JGT49) derivatives

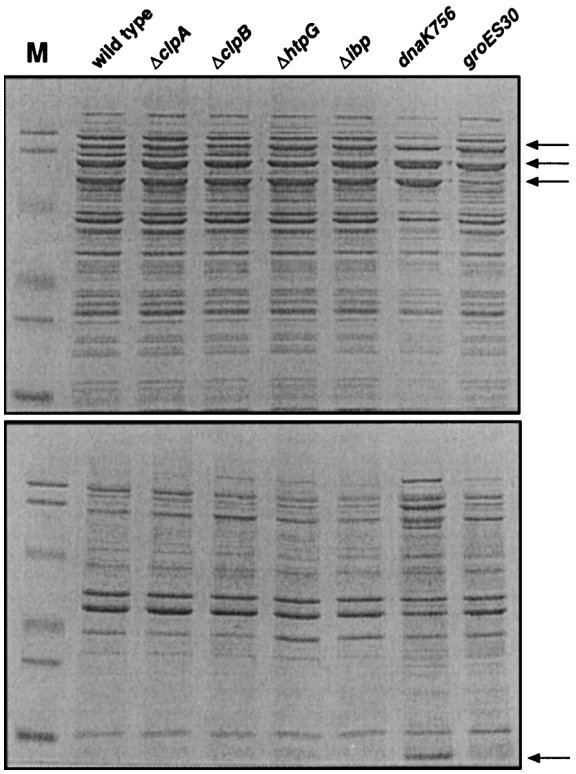

FIG. 5. Effect of chaperone mutations on host protein aggregation at 46°C. Cells containing the indicated mutations were grown to mid-exponential phase at 30°C and shifted to 46°C for 1 h. Soluble (upper gel) and insoluble (lower gel) cellular fractions from identical absorbance units (A_{600}) of culture were separated by reducing SDS-PAGE (12.5% polyacrylamide). Insoluble fractions were loaded at three times the absorbance units of soluble fractions. Markers (M [Bio-Rad]) correspond to the molecular masses 104, 81, 47.7, 34.6, 28.3, and 19.2 kDa. The positions of the following proteins are indicated by arrows: upper gel from top to bottom, ClpA and ClpB, DnaK and HtpG, and GroEL; lower gel, GroES, IbpA, and IbpB. Gels were digitized with a Sharp JX-325 high-resolution scanner and the NIH Image 1.60 software for PowerPC.

conjugated with an F′ episome carrying the *lacI*^{q} allele (Tables 1 and 2). Transformants were tested for growth at high temperatures either in liquid culture or by their ability to grow on LB agar plates, as described in Materials and Methods. Under both sets of experimental conditions, the only plasmids capable of restoring growth of *dnaK756* or *groES30* cells at lethal temperatures (i.e., above 42°C) were pDnaK/J and pGroESL, respectively (data not shown).

Mutations in the minor chaperones do not result in wholesale aggregation of host proteins at high temperature. To directly test the in vivo chaperone activity of ClpA, ClpB, HtpG, and IbpA and -B, we examined whether their deletion would affect the aggregation of host proteins in heat-shocked cells. Single chaperone mutants grown to mid-exponential phase at 30°C were transferred to 46°C for 1 h, and culture samples were fractionated by SDS-PAGE following separation into soluble and insoluble fractions. Although wholesale aggregation of host proteins does not take place in *dnaK756* or *groES30* mutants at 42°C (12), an obvious increase in the amount of insoluble proteins was seen in *dnaK756* cells and, to a lesser extent, in *groES30* cells at 46°C (Fig. 5). Overproduction of Hsps was readily apparent in the *dnaK756* mutant, in which gross aggregation occurred at the expense of the soluble protein. A similar phenotype was not observed in any of the minor chaperone mutants following incubation at 46°C (Fig. 5) or 50°C (data not shown). Finally, obvious synergistic effects between the *dnaK756* and *groES30* mutations and the various minor chaperone deletions could not be detected by SDS-PAGE when these experiments were repeated with the double mutants (data not shown).

DISCUSSION

In this study, we show that the sHsps IbpA and IbpB are dispensable for normal *E. coli* growth at temperatures as high as 45°C, but that their absence leads to growth defects at 46°C (Fig. 2A). Although the cellular roles of IbpA and -B are unknown, it has been shown in vitro that sHsps and Hsp90s can maintain partially folded proteins in a conformation that can be reactivated through interactions with Hsp70s (6, 7, 15, 38). These findings have led to the proposal that sHsps and Hsp90s act as reservoirs of thermally denatured or otherwise stress-damaged proteins, thereby facilitating the reactivation of misfolded proteins upon removal of the insult. The results shown in Fig. 3 are consistent with the idea that the *E. coli* Hsp90 homolog HtpG plays an important role in cell recovery following exposure to lethal temperatures, but also indicate that IbpA and -B are not absolutely required for this process. Thus, it appears that the role of IbpA and -B in protein reactivation following stress is relatively minor or that alternative cellular pathways can compensate for the absence of sHsps. We also found that overproduction of the *ibp* operon did not improve cell viability at 50°C, although overexpression of sHsps from other organisms can improve *E. coli* thermotolerance (24, 28). Taken together, these results suggest that the mode of action of the bacterial sHsps may differ from that of their eukaryotic homologs.

Interestingly, we observed that *clpA* null mutants are defective for growth at 46°C despite the fact that ClpA is not an Hsp. This protein also appears to play a role in cellular recovery from transient incubation at 50°C (Fig. 3). Since the *clpP* and *clpX* genes are part of an Eσ^{32}-transcribed operon (11), it would be reasonable to assume that the ClpXP protease plays a more essential role in *E. coli* survival at high temperatures than ClpAP (10). Nevertheless, the results in Fig. 2 and 3 suggest that ClpAP, or ClpA itself, is also important in heat-shocked cells.

Although a role of ClpB in thermotolerance was reported by Squires et al. (31), the mechanisms responsible for the rapid death of Δ*clpB* cells at 50°C remain unclear. The unique role of ClpB in thermotolerance is further highlighted by our observations that (i) high intracellular concentrations of other molecular chaperones or all Hsps cannot compensate for the deleterious effect of the Δ*clpB* mutation at 50°C (data not shown) and (ii), unlike *dnaK756* cells, Δ*clpB* mutants do not show an obvious increase in host protein aggregation upon incubation at 50°C, even though both mutants exhibit nearly equivalent death rates at this temperature. ClpB and its yeast homolog, Hsp104, have been implicated in the clearance of thermally denatured proteins in *E. coli* (19, 26). It is possible that *E. coli* ClpB plays a vital role in stress recovery through this mechanism.

Among the chaperone mutants tested, *groES30* cells exhibited a unique and somewhat paradoxical behavior. In contrast to all other strains, the turbidity of *groES30* cultures declined after prolonged incubation at 42 or 46°C (Fig. 4 and data not shown), suggesting that the GroE chaperonins are required to maintain cellular integrity and/or cell viability at these temperatures. However, the same mutation did not have an adverse effect on viability or turbidity when the incubation temperature was raised to 50°C, a temperature at which the growth of

groES30 cells is halted (data not shown). A possible explanation for these results is that the GroE chaperonins play a key role in cell division. This hypothesis is in agreement with a recent report suggesting that GroEL and GroES are important in the folding of DapA, an enzyme involved in the synthesis of the cell wall precursor diaminopimelic acid (22). It should finally be noted that the intracellular levels of GroEL were greatly reduced in *groES30* cells (Fig. 5). Interestingly, *groES619* cells, but not *groEL140* mutants, exhibit a similar behavior (32). Although the GroEL140 protein is known to interact suboptimally with GroES, it is still able to associate with the cochaperonin (3). Since the mutations in both GroES30 and GroES619 map in the mobile loop region, which plays a key role in the formation of GroEL-GroES heterooligomers (18), complex formation between GroEL and GroES may be severely reduced or completely abolished in *groES30* and *groES619* strains. Thus, it is possible that interactions with GroES are required to confer stability to GroEL.

To investigate the possible interplay between the Clp ATPases, HtpG, IbpA and -B, and the DnaK-DnaJ-GrpE and GroEL-GroES systems, we characterized the growth of isogenic double chaperone mutants at 42°C. Although the *clpB*, *htpG*, and *ibp* deletions did not affect cell growth at this temperature, all mutations exerted a deleterious effect in the *dnaK756* background. These data suggest that minor heat shock chaperones cooperate with the DnaK-DnaJ-GrpE system in thermal stress management. The fact that the Δ*clpB* and Δ*htpG* mutations, but not the Δ*ibp* deletion, affected the growth of *groES30* cells further suggests that while ClpB and HtpG interact with the GroEL-GroES system, the bacterial sHsps do not. These results are in agreement with recent biochemical data showing that IbpB-bound malate dehydrogenase and lactate dehydrogenase are specifically transferred to the DnaK-DnaJ-GrpE system but that the GroEL-GroES chaperonins do not interact directly with IbpB-released proteins (38). Overall, our findings are consistent with the idea that ClpB, HtpG, and IbpA and -B function as molecular chaperones in vivo. However, their overexpression could not restore the growth of *dnaK756* or *groES30* mutants at or above 44°C. While it remains possible that minor chaperone overexpression may partially suppress other phenotypes of *dnaK* or *groES* mutants, it is obvious that they are not interchangeable with DnaK-DnaJ-GrpE or GroEL-GroES. Thus, the putative chaperone activities of ClpB, HtpG, and IbpA and -B are likely to be of a specialized nature in heat-shocked cells. A more precise examination of the roles of ClpA, ClpB, HtpG, and IbpA and -B in cellular protein folding is in progress.

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