Isolation and Characterization of Point Mutations in the *Escherichia coli* *grpE* Heat Shock Gene

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The *Escherichia coli* *grpE* gene (along with *dnaK*, *dnaJ*, *groEL*, and *groES*) was originally identified as one of the host factors required for phage λ growth. The classical *grpE280* mutation was the only *grpE* mutation that resulted from the initial screen and shown to specifically block the initiation of λ DNA replication. Here we report the isolation of several new *grpE* missense mutations, again using phage λ resistance as a selection. All mutants fall into two groups based on their temperature-dependent phenotype for λ growth. Members of the first group (I), including *grpE17* and *grpE280*, which was obtained again, are resistant to λ growth at both 30 and 42°C. Members of the second group (II), including *grpE25*, *grpE66*, *grpE103*, *grpE13a*, *grpE57b*, and *grpE61*, are sensitive to λ growth at 30°C but resistant at 42°C. All mutations are recessive, since an *E. coli* *grpE* null mutant strain carrying these mutant alleles on low-copy-number plasmids are sensitive to infection by the λ *grpE*⁺ transducing phage. Both group I and group II mutants are temperature sensitive for *E. coli* growth above 42°C. The nucleotide changes were identified by sequencing analyses and shown to be dispersed throughout the latter 75% of the *grpE* coding region. Most of the amino acid changes occur at conserved residues, as judged by sequence comparisons between *E. coli* and other bacterial and yeast GrpE homologs. The isolation of these new mutations is the first step toward a structure-function analysis of the GrpE protein.

The *Escherichia coli* DnaK chaperone machine is composed of three member proteins: DnaK (the homolog of eukaryotic Hsp70), DnaJ (the homolog of eukaryotic Hsp40), and GrpE. These proteins were initially identified about 20 years ago as host factors required for phage λ growth (13, 14). Subsequently, these proteins were shown to be essential for *E. coli* growth as well. DnaK, DnaJ, and GrpE belong to the heat shock protein family, whose expression is transiently induced under various stress conditions, such as heat, ethanol treatment, viral infection, and exposures to heavy metals (33). Earlier observations, combined with ample genetic and biochemical studies from different fields, have led to the appreciation of a more generalized chaperone function for the DnaK, DnaJ, and GrpE proteins (10, 16). The three proteins work together as a team to carry out a variety of cellular functions, including replication of phage λ DNA, the *E. coli* chromosome, and the P1 and F plasmids (14), autoregulation of the heat shock response (11, 25, 45, 48), protection of other proteins from misfolding or aggregation (23), reactivation of damaged proteins under stress conditions (41, 44, 54), facilitating protein export by maintaining polypeptides in a translocation-competent conformation (38), and proteolysis (43).

DnaK, DnaJ, and GrpE are highly conserved in nature. DnaK is 50% identical at the amino acid sequence level to the eukaryotic Hsp70 proteins. It has two functional domains, the N-terminal 44-kDa ATPase domain and the C-terminal 24-kDa substrate-binding domain. A highly conserved DnaJ protein family has also emerged during the last few years. DnaJ has four distinct domains: the highly conserved N-terminal J region, followed by a short Gly/Phe-rich region, a Cys-rich region of four CXXCXGXG motifs, and the less conserved C-terminal domain (3). In *E. coli*, *dnaK* and *dnaJ* are not

single-gene families, since at least one homolog of each has been discovered recently (21, 42, 49). For *grpE*, the long-suspected homolog in eukaryotic cells has been identified recently in mitochondria of the fungi *Saccharomyces cerevisiae* and *Neurospora crassa* (19, 50) and shown to be an essential gene whose product interacts with mitochondrial Hsp70 (2).

The individual activities of DnaK, DnaJ, and GrpE, as well as their combined action as a chaperone team, have been best characterized in the well-defined λ DNA replication system reconstituted by using highly purified phage λ - and *E. coli*-encoded replication proteins (30, 56). The λ O protein binds to the defined *ori* λ sequences and attracts λ P complexed with the DnaB helicase. DnaJ stabilizes this preprimosomal complex and tags it for recognition by DnaK. In the presence of GrpE, 10-fold less DnaK protein is required for efficient *in vitro* λ DNA replication (56).

DnaK and DnaJ are bona fide chaperones which interact with various target polypeptide substrates. GrpE acts only as an accessory factor for DnaK. DnaK has been shown to possess a very weak ATPase activity (approximately 1 ATP hydrolyzed per 10 min per monomer) (26, 57). The presence of both the DnaJ and GrpE cohorts synergistically stimulates DnaK's ATPase up to 50-fold, with DnaJ specifically accelerating the hydrolysis of bound ATP and GrpE accelerating the release of bound nucleotides (26). The hydrolysis of ATP causes conformational changes in DnaK which may help release DnaK from its bound substrates, thus allowing DnaK to recycle more efficiently (27). Recently, Palleros et al. (36) suggested that in the absence of the DnaJ and GrpE proteins, exchange of ADP to ATP bound to DnaK triggers the release of substrate from DnaK.

There exists both *in vivo* and *in vitro* evidence which supports an intimate physical interaction between DnaK and GrpE: (i) extragenic suppressors of the *grpE280* temperature-sensitive mutation which map in the *dnaK* gene can be isolated (20), (ii) the two proteins can be cross-linked by glutaraldehyde

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TABLE 1. Bacterial strains, phages, and plasmids used

Strain, phage, or plasmid	Genotype and description	Reference or source
Strain		
B178	W3110 <i>galE sup</i> ⁺	12
C600	<i>supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21</i>	Our collection
DH5 α	<i>recA1</i>	Bethesda Research Laboratories
DA259	C600 Δ <i>grpE</i> :: Ω -Cam ^r <i>thr</i> ::Tn10	1
BW452	DA259 (pBW401)	This work
BW496	DA259 (pWSK29/ <i>grpE</i> 25)	This work
BW506	DA259 (pWSK29/ <i>grpE</i> 280)	This work
BW509	DA259 (pWSK29/ <i>grpE</i> 103)	This work
Phage		
λ cI ⁻	λ cI60; clear-plaque former	Our collection
434cI ⁻	Clear-plaque former	Our collection
λ <i>grpE</i> ⁺	λ <i>imm int xis att cI857 grpE</i> ⁺	39
Plasmids		
pDK124	pBluescript SK(+) carrying 600 bp of the <i>grpE</i> ⁺ open reading frame obtained by PCR	This work
pEMBL/ <i>grpE</i> 280	1.7-kbp <i>grpE</i> 280 with flanking sequences cloned in pEMBL8(+) vector	Our collection
pBW375	pEMBL/ <i>grpE</i> ⁺	This work
pBW401	pWSK29 with 1.3-kbp <i>grpE</i> ⁺ from pBW375 cloned into the <i>SalI</i> and <i>EcoRI</i> sites	This work
pGp1-2	Kan ^r	46
pEMBL8(+)	Amp ^r Tet ^r	6
pWSK29	Amp ^r , low-copy-number vector	51

and can be coprecipitated in the presence of antibody against one or the other (20, 35), and (iii) the GrpE protein can be purified on the basis of its retention on a DnaK affinity column. The DnaK-GrpE complex is stable in the presence of 2 M KCl, suggesting that the interaction is very hydrophobic in nature. The complex, however, is efficiently destroyed in the presence of ATP (55). It has been shown in vitro that GrpE probably binds to a conserved, exposed loop (around codons 28 to 33) near the nucleotide-binding site of DnaK, because deletion of this loop inhibits formation of the DnaK-GrpE complex (5). This finding is consistent with the in vivo result, namely, that the mutant DnaK756 protein, which has a Gly-to-Asp change at codon 32, has a lower affinity for GrpE (5, 20).

GrpE is essential for bacterial growth at all temperatures; however, it is dispensable in certain *dnaK* mutant backgrounds (1). Further analysis showed that it is not the lack of DnaK function per se that allows *E. coli* to tolerate the deletion of the *grpE*⁺ gene. Rather, it is the presence of various extragenic suppressors of the *dnaK* mutation that somehow compensate for the deficiency in both DnaK and GrpE functions (1).

To gain a better understanding of how GrpE carries out its function(s), we isolated several new *grpE* missense mutations, using phage λ resistance as a selection. The analyses of these *grpE* mutations and the biochemical characterization of the defects of the mutant proteins should furnish insights into the mechanism of action of the DnaK chaperone machine.

MATERIALS AND METHODS

Strains and media. The bacterial strains, phages, and plasmids used in this study are listed in Table 1. L broth and L agar supplemented with the appropriate antibiotics (when necessary) were used for the growth of the *E. coli* strains as described by Miller (31).

Construction of pBW401. Two cloning steps were involved in obtaining a low-copy-number plasmid carrying the wild-type *grpE* gene. First, the 900-bp *Bam*HI-*Eco*RI fragment from pEMBL8(+)/*grpE*280 containing part of the *grpE* open reading frame and its 3' flanking region was replaced by a 480-bp

*Bam*HI-*Eco*RI fragment from plasmid pDK124 to obtain pBW375, resulting in a wild-type *grpE* gene cloned in the pEMBL8(+) vector. Second, the 1.3-kbp *SalI*-*Eco*RI fragment from pBW375, containing the entire *grpE*⁺ open reading frame and its 5' flanking region, was moved into the low-copy-number plasmid pWSK29 (51). The resulting plasmid, pBW401, which has the *grpE*⁺ gene under the transcriptional regulation of a T7 promoter and its own natural promoter, was used in this study for mutagenesis purposes. The *grpE*⁺ open reading frame of pBW401 was confirmed to be wild type by sequencing and comparison with that of wild-type *grpE*⁺ from the *E. coli* chromosome (data not shown).

Plasmid mutagenesis with NH₂OH. Ten micrograms of pBW401 plasmid DNA were treated with 40 mM hydroxylamine (NH₂OH) in 50 mM sodium phosphate buffer (pH 6.0) at 72°C for 60 to 90 min, and the reaction was stopped with TNE buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris HCl [pH 8.0]). The mutagenized plasmid DNA was ethanol precipitated and transformed into *E. coli* DH5 α at 37°C on L-ampicillin (100 μ g/ml) plates. The DNA (designated pBW401*) extracted from the pooled transformants was used for the isolation of *grpE* mutations.

Random PCR mutagenesis. One nanogram of *Xmn*I-linearized pBW401 DNA was used in each PCR as described by Leung et al. (24). The reverse primer, 5'-AACAGCTATGAC CATG-3', and the -40 primer, 5'-GTTTTCCAGTCAC GAC-3', were used to amplify the 1.4-kbp fragment containing the *grpE* gene and flanking DNA regions. After experimenting with different conditions, we used a dGTP/dATP ratio of 5 and an otherwise normal PCR protocol to perform the mutagenesis with a total of 35 cycles. The 1.4-kbp PCR DNA products were digested with *SalI* and *Eco*RI to yield a ~1.3-kbp fragment, which was cloned back into pBW401 treated with the same restriction enzymes to replace the corresponding wild-type *grpE*⁺ DNA fragment. The resulting plasmid pool was subsequently screened for the presence of *grpE* mutations.

DNA sequencing. The nucleotide changes caused by the various *grpE* mutations were identified by using a double-stranded DNA cycle sequencing protocol from GIBCO BRL

as instructed by the manufacturer, with appropriate ^{32}P -end-labeled primers complementary to both strands of the *grpE* DNA sequence.

Phage burst size analysis. Bacteria were grown at 30°C in L broth supplemented with ampicillin (100 µg/ml) and 0.2% maltose to an optical density at 595 nm of approximately 0.25. Phage λCI^- was added to the culture at a multiplicity of infection of 0.05, i.e., one phage per 20 bacteria. After incubation at room temperature for 30 min, the cells were pelleted, washed with 10 mM MgSO_4 twice, and resuspended in the same volume of L broth. The cell cultures were divided into two aliquots. One was kept at 30°C, and the other was kept at 42°C. Fifteen-microliter aliquots were removed every 15 min, vortexed with 1 drop of chloroform, diluted, and spotted onto an *E. coli* C600 lawn to determine the number of plaque-forming units (PFU) at each time point.

Induction and identification of the mutant *grpE* gene products. The *grpE* null mutant bacterial strain (DA259), carrying various mutant *grpE* plasmids, was transformed with a second plasmid, pGp1-2, that carries the T7 RNA polymerase gene 1 cloned under the inducible control of the p_L promoter (repressed by the thermolabile *cI857* repressor) (46). Cells were grown in L broth supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) at 30°C to an optical density at 595 nm of 0.4 and then shifted to 42°C for 30 to 45 min to induce synthesis of the T7 RNA polymerase. Following induction of the T7 polymerase, the cell cultures were shifted back to 30°C. At various time points, aliquots were taken, pelleted, and resuspended in 100 µl of H_2O per ml of cell culture, and 15-µl aliquots were loaded per well of a sodium dodecyl sulfate (SDS)-15% polyacrylamide gel (22).

RESULTS

Isolation of new *grpE* point mutations. Initially we attempted to directly isolate single-copy *grpE* mutations which confer temperature sensitivity for bacterial growth at 42°C or λ resistance mutations in the *E. coli* chromosome by P1-localized mutagenesis (18), using a closely linked drug resistance marker (*Cam*^r), but were unsuccessful. Perhaps the open reading frame of *grpE* (0.6 kbp) is too small for efficient *in vivo* mutagenesis and this is why originally only one *grpE280* mutation was isolated (40). Consistent with this possibility, a more recent attempt to isolate mutations by using a screen for the high constitutive expression of heat shock proteins resulted in the isolation of many *dnaK* and *dnaJ* mutations but failed to yield any in *grpE*, which was also an expected target (53). Therefore, we set out to construct a wild-type *grpE*-carrying plasmid and perform plasmid mutagenesis in order to increase the probability of obtaining mutations in the *grpE* gene.

Mutagenized plasmids, obtained by either NH_2OH treatment or random PCR mutagenesis as described in Materials and Methods, were transformed into the *grpE* null strain DA259 and incubated at 30°C overnight on L-ampicillin plates. Amp^r transformants were pooled, and approximately 10^7 cells were spread onto L-ampicillin plates preseeded with a mixture of 4×10^7 λCI^- and 4×10^7 434 CI^- phages. The plates were incubated at either 37 or 42°C for 24 h. Colonies were streaked on L-ampicillin plates to obtain single colonies free of phage. They were then checked for phage resistance by cross-streaking against both λCI^- and 434 CI^- . Plasmid DNA was extracted from those candidates that were resistant to both phages and retransformed into DA259. Lawns of the transformants were spot tested with a dilution series of λCI^- and 434 CI^- to confirm their mutant phenotype.

The rationale behind using both λCI^- and 434 CI^- phages

FIG. 1. Temperature-sensitive growth phenotypes of two *grpE* mutants. The *grpE* null strain, DA259, was transformed with pWSK29 vector (A), pBW401 (B), pWSK29/*grpE280* (C), or pWSK29/*grpE25* (D). Cultures of transformants were diluted serially 10-fold, spotted on L-agar plates, and incubated overnight at 30, 37, 42, or 43°C.

was to minimize the occurrence of bacterial mutants that simply fail to adsorb λCI^- phage. This scheme greatly facilitated the efficiency of obtaining *grpE* mutations. The reason for incubating the L plates at either 42 or 37°C was to allow the isolation of *grpE* mutants that are temperature sensitive for λ growth. Such temperature-sensitive mutants should be very helpful in the biochemical characterization of their products and facilitate understanding of the mechanism of GrpE protein action in various biological reactions.

Among an initial pool of 57 mutant candidates from a total of three selections, 36 were subsequently shown to be unable to propagate λ phage at either 30 or 42°C (we call these group I mutants). The remaining 21 mutants were temperature sensitive for λ growth, in that they were able to propagate λ phage at 30 or 37°C but not at 42°C (we call these group II mutants). The majority of the mutant candidates were derived through PCR mutagenesis, which proved to be the most efficient way of generating random mutations in the *grpE* gene.

Properties of *grpE* mutations. DA259 (the *grpE* null mutant strain) bacteria bearing any of the 57 mutant plasmids could grow up to 42°C but not at 43°C. The dramatic one-degree difference in cell growth is shown in Fig. 1. The pBW401 plasmid carrying a wild-type *grpE*⁺ gene was able to perfectly complement the Ts⁻ phenotype of DA259 at 43°C (Fig. 1, spot B). However, two mutant plasmids, one from each mutant group, could complement the growth defect of DA259 only up to 42°C (spots C and D). Other plasmid mutants display a similar growth phenotype, as summarized in Table 2.

To quantify the phage λ -resistant phenotype of the isolated mutants, relative plaque-forming ability was determined by spotting serial dilutions of λCI^- and λgrpE^+ transducing phages onto lawns of DA259 bacteria carrying various mutant plasmids. The numbers of plaques formed overnight at 30 or 42°C were compared with those obtained with DA259 bacteria

TABLE 2. Plating efficiency of phages and temperature sensitivity of various *E. coli* *grpE* mutant strains

Mutant plasmid ^a	Plaque-forming efficiency in indicated <i>dnaK</i> ⁺ <i>grpE</i> Δ background ^b				Cell growth			
	λ cI ⁻		λ grpE ⁺					
	30°C	42°C	30°C	42°C	30°C	37°C	42°C	43°C
pBW401	+	+	+	+	+	+	+	+
Group I								
2 (2)	– (<10 ⁻⁶)	– (<10 ⁻⁸)	+	+	+	+	+	–
17 (8)	– (<7 × 10 ⁻⁶)	– (<10 ⁻⁸)	+	+	+	+	+	–
Group II								
25	+	– (<10 ⁻⁸)	+	+	+	+	+	–
66 (8)	+	– (<10 ⁻⁸)	+	+	+	+	+	–
103	+	– (<10 ⁻⁸)	+	+	+	+	+	–
61	+	– (<10 ⁻⁸)	+	+	+	+	+	–
61a	+	+	+	+	+	+	+	–
61b	+	+	+	+	+	+	+	+/-
13	+	– (<10 ⁻⁸)	+	+	+	+	+	+/-
13a	+	– (<10 ⁻⁸)	+	+	+	+	+	–
13b	+	+	+	+	+	+	+	–
57	+	– (<10 ⁻⁸)	+	+	+	+	+	+
57a	+	+	+	+	+	+	+	+
57b	+	– (<10 ⁻⁸)	+	+	+	+	+	+/-

^a Numbers in parentheses indicate the number of times the same mutation has been isolated.

^b Numbers in parentheses are the relative plaque-forming efficiencies of different mutants compared with the wild type. The relative plaque-forming efficiencies were determined by spot testing serial dilutions of the indicated phages onto cell lawns and calculating the ratio of PFU (mutant/wild type). +, relative plaque-forming efficiency within 10 to 100% of wild-type efficiency or cell growth similar to wild-type growth; –, no visible plaques (<10⁻⁵) or colonies (<10⁻⁵); +/-, colony size and colony-forming efficiency reduced compared with the wild type.

carrying the wild-type plasmid, arbitrarily set at 1. The data shown in Table 2 are with those *grpE* mutations obtained that result in single amino acid changes (see below and Fig. 4). The plaque-forming efficiency of λ cI⁻ phage on all mutant bacteria was 10⁸-fold lower than that on wild-type bacteria at 42°C. At 30°C, two mutants, carrying *grpE2* (identical to *grpE280*; see below) or *grpE17*, exhibit a 10⁶-fold-lower plaque-forming efficiency than the rest of the mutants as well as the wild type. This result suggests that all *grpE* mutants isolated, except those carrying *grpE2/280* and *grpE17*, are temperature sensitive for phage λ growth.

A common feature of all *grpE* mutants is that the λ grpE⁺ transducing phage is able to propagate at both 30 and 42°C. This finding suggests that all *grpE* mutations obtained are recessive to *grpE*⁺ and agrees with the observation that the wild-type strain B178, when transformed with any of the mutant plasmids, displays no growth defects and is sensitive to λ cI⁻ phage at 30, 37, 42, or 43°C. Given that *grpE* is an essential *E. coli* gene under all growth conditions (1), it is not surprising that no *trans*-dominant *grpE* mutations were isolated.

To quantify the effects of the *grpE* mutations on λ growth, burst size curve analyses were performed for three of the mutations, *grpE2/280*, *grpE25* and *grpE103*, at 30 and 42°C. The burst size curves obtained provide a more dynamic view of how the different mutants behave upon λ phage infection. As shown in Fig. 2A, a negligible amount of λ phage progeny was observed on *grpE2/280* mutant bacteria (BW506) at either 30 or 42°C, during a time period long enough for one cycle of λ DNA replication and assembly to occur in wild-type cells (BW452). On the contrary, the temperature-sensitive mutant *grpE25* (BW496) showed normal phage production within the normal time period at 30°C but none at 42°C. A second mutant, *grpE103* (BW509), displayed a similar pattern in terms of temperature-sensitive λ growth at 42°C but exhibited a longer latent period (~15 to 30 min), as well as a smaller burst size at 30°C than wild-type bacteria (Fig. 2B). The difference

between *grpE25* and *grpE103* temperature-sensitive mutants at 30°C is interesting and may reflect differences in the mechanism of interfering with λ DNA replication in vivo.

Mutant *grpE* gene products and sequence analyses of *grpE* mutations. To identify the *grpE* missense mutations, we examined the protein products of all 57 putative candidates. Because plasmid pBW401 was constructed in such a way that *grpE* is under the control of the T7 RNA polymerase/promoter system (for the convenience of protein overproduction), the plasmid-borne mutant *grpE* genes can be overexpressed in a *grpE* null background in a temperature-dependent manner. Figure 3 shows the overproduction of the GrpE2/280 (group I) and GrpE25 (group II) mutant proteins compared with wild-type GrpE, indicated by the arrowhead. In a similar way, we screened other mutant candidates and found that 34 of them overexpress protein products that have mobilities identical or very similar to that of wild-type GrpE, indicating that they encode full-length products. Some of these mutant GrpE proteins may have charged amino acid residue changes that would explain their slightly different mobility on SDS-polyacrylamide gels. No products were detected (even by [³⁵S]Met labeling) for the remaining 23 mutant candidates following overexpression. Eleven of these were sequenced and shown to have mutations that result in amino acid changes in the *grpE* open reading frame ranging from one to multiple hits or 1-bp deletions that cause protein truncations or alter the open reading frame (data not shown). Therefore, these 23 mutant candidates were not considered further.

On the basis of information presented above, we sequenced all 34 mutant candidates that coded for full-length polypeptides to identify those resulting in single amino acid alterations. The results are summarized in Fig. 4. Although the majority (20 candidates) have single amino acid changes in their open reading frames, they constitute only five different point mutations: *grpE2/280*, *grpE17*, *grpE25*, *grpE66*, and *grpE103*. The names designate the serial numbers of the first sequenced candidate of each type of mutation. The fact that the *grpE17*-

FIG. 2. Burst size curves of phage λ infecting *grpE* null strains containing various plasmids at 30 and 42°C. (A) BW452 (*grpE*⁺), BW506 (*grpE2/280*), and BW496 (*grpE25*); (B) BW452 (*grpE*⁺) and BW509 (*grpE103*). Panels A and B represent two independent experiments with the wild type as a positive control.

like and *grpE66*-like mutations were isolated eight times each indicates that saturation of mutant types may have been reached (Table 2). It is worth mentioning again that *grpE280*, which results in a Gly-to-Asp change at codon 122, was also selected, having been shown by sequencing to carry the same change as *grpE2*. This result confirms the effectiveness of the original selection scheme.

In addition to the single-hit mutations described above, PCR also generated nine double hits, three triple hits, one quadruple hit, and an interesting five-amino-acid in-frame internal deletion, from codons 87 to 91 (*grpE75*). Since it would be difficult to explain the phenotypes of multiple-hit mutations, we separated some of the double and multiple mutations by exchanging the appropriate DNA domains with wild-type *grpE*⁺ and examined the phenotypes of the separated single mutations. The single-point mutations obtained by such gene manipulations were named after the original mutant isolate, followed by *a* or *b*, depending on the order of the amino acid change in the *grpE* open reading frame, in order to distinguish them from the single-hit mutations.

Phenotypically, *grpE13a* (H154R) is temperature sensitive for λ and cell growth, identical to the parental *grpE13* mutation (H154R, D163G). Similarly, the *grpE57b* (L131P) mutation exhibited the original phenotype displayed by *grpE57* (E42G, L131P). In contrast, the *grpE13b* (D163G) mutation behaved essentially like the wild type (Table 2). In contrast to the

clear-cut phenotypes of the *grpE13* and *grpE57* mutations, *grpE61* (K176E, V190A) exhibited an additive effect compared with the single *grpE61a* (K176E) and *grpE61b* (V190A) mutations. Neither *grpE61a* nor *grpE61b* alone showed any apparent defect in terms of phage plating and cell growth even at higher temperatures (Table 2). It is interesting that codons 176 and 190 are highly conserved and located within segment V of the sequence homology (see below and Fig. 5), yet a change (even a change at position 176) in either of them does not seem to affect significantly phage or bacterial growth.

DISCUSSION

In this study, we report the isolation and phenotypic characterization of several new *grpE* missense mutations. The locations and natures of these mutations are summarized in Fig. 4, along the *grpE* open reading frame. The genetic screen was designed based on the fact that *grpE* is one of the few host factors that are required for the propagation of lambdoid phages. The single missense mutations in the *grpE* gene were identified by their ability to produce full-length mutant gene products and by sequencing analysis. From the genetic screen used, we obtained two kinds of *grpE* missense mutations: group I, which blocks λ growth at either 30 or 42°C, and group II, which blocks λ growth at 42 but not 30°C. Mutation *grpE2* in group I turns out to be identical to the classical *grpE280*

FIG. 3. Identification of the mutant *grpE* gene products. A T7 RNA polymerase/promoter expression system (46) was used to over-express pWSK29-encoded mutant GrpEs in a *grpE* null mutant background (DA259). Cells were grown at 30°C until early log phase, shifted to 42°C for 30 min, and then shifted back to 30°C for 1 h before harvest. A parallel culture for each strain was grown continuously at 30°C as an uninduced control. Cell extracts were electrophoresed on an SDS–15% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: 0, purified GrpE wild-type protein; 1, 3, 5, and 7, uninduced cell extracts; 2, 4, 6, and 8, induced cell extracts for each strain as indicated.

mutation, an expected result since the *grpE280* allele was also isolated on the basis of its λ -resistant phenotype. All mutations are recessive to wild-type *grpE*⁺ because they are sensitive to the λ *grpE*⁺ transducing phage and there are no apparent phenotypes in terms of cell growth and λ propagation when the mutant plasmids are transformed into B178 wild-type bacteria.

Both group I and group II mutations are temperature sensitive for cell growth in that they allow bacterial growth at 42 but not 43°C. The different behaviors of these *grpE* mutations in cell growth versus λ growth at 42°C do not necessarily mean that GrpE functions differently in cell growth compared with λ replication. Rather, they may reflect a differential need for GrpE in the two situations. For example, mutant bacteria can manage to grow with mutant GrpE protein that is less active than the wild type. However, the low activity of these same mutant GrpEs may not suffice in λ DNA replication, a situation that necessitates a great deal of DnaK, DnaJ, and GrpE chaperone action in a very short time (14, 15). Perhaps the DnaK chaperone protein is not recycled fast enough by these mutant GrpE proteins to keep up with the fast replication of λ DNA. The fact that the isolated *grpE* mutations are found distributed throughout the open reading frame, instead of clustering in certain areas, supports the notion that GrpE may have a relatively cohesive structure rather than the distinct functional subdomains of DnaK and DnaJ; i.e., perhaps most of the information residing in the GrpE molecule is required for its full activity (10).

Using the Genetics Computer Group sequence analysis software, a database search was conducted (37). The alignment of the *E. coli* GrpE amino acid sequence with sequences of seven other bacterial homologs and the yeast protein is shown in Fig. 5, with the consensus sequence shown below the alignment. They each exhibit a ~30% amino acid identity and 50 to 60% similarity with the *E. coli* GrpE protein. Although

FIG. 4. The location of the *grpE* missense mutations in the *grpE* open reading frame. The entire bar represents the GrpE open reading frame, composed of 197 amino acids. The shaded areas labeled with beginning and ending codon numbers are highly conserved segments numbered I to V. The numbers below the bar indicate the mutated codons. The altered nucleotide and the resulting amino acid changes are given below together with their corresponding allele names. The boldface residues are the conserved amino acids determined by the sequence comparison in Fig. 5. The dashed line above the open reading frame indicates the putative position of the computer-predicted coiled-coil region; however, the ends of the putative coiled-coil region are not well defined.

FIG. 5. Comparison of the amino acid sequences of GrpE proteins (or homologs) from *E. coli* (Eco) (28), *B. subtilis* (Bsu) (52), *B. burgdorferi* (Burg) (47), *Lactococcus lactis* (LacIa) (7), *C. trachomatis* (Ctra) (8), *Clostridium acetobutylicum* (Cace) (32), *Staphylococcus aureus* (Staph) (34), *Streptomyces coelicolor* A3(2) (Strep) (4), and the yeast *S. cerevisiae* (Yeast) (19). The number at the end of each line indicates the position of the last amino acid shown. Highlighted residues are those that are conserved in at least seven of nine sequences. The asterisks under the consensus sequence indicate the positions of the most conserved missense mutations discussed in this work (in parentheses). The analysis was performed with the Genetics Computer Group sequence analysis program (9, 17).

sequence homology exists almost throughout the entire length of the protein, there are five distinct, highly conserved segments, which we have numbered I to V (Fig. 4 and 5). Segment I spans codons 62 to 79, segment II spans codons 94 to 105, segment III spans codons 122 to 139, segment IV spans codons

146 to 157, and segment V spans codons 175 to 193, the codon numbers referring to those of the *E. coli* GrpE protein. When the amino acid changes caused by the *grpE* missense mutations were compared with the consensus sequence, five of them, *grpE66* (L102P), *grpE2/280* (G122D), *grpE13a* (H154R),

grpE25 (G177S), and *grpE61* composed of *61a* (K176E) and *61b* (V190A), were changes occurring at very conserved residues (asterisks in Fig. 5) located in the highly conserved segments II, III, IV, and V, respectively. Although the other three mutations, *grpE103* (E53G), *grpE17* (L127P), and *grpE57b* (L131P), do not result in changes of conserved amino acid residues, nevertheless they are all located next to highly conserved ones, Glu at codon 54 for *grpE103*, Val at codon 126 for *grpE17*, and Leu at codon 130 for *grpE57b*. The overall good correlation between the locations of the *grpE* missense mutations obtained in this study and the conserved residues revealed through sequence homology comparisons strongly suggests that these conserved amino acids are important for GrpE function.

Because there is no available information on GrpE's secondary structure, we entered the GrpE amino acid sequence into a computer program that predicts the probability of coiled-coil secondary structure based on the context of the amino acid sequence (29). All four bacterial protein sequences entered, namely, those from *E. coli*, *Bacillus subtilis*, *Borrelia burgdorferi*, and *Chlamydia trachomatis*, were predicted to possess a possible coiled-coil region between *E. coli* GrpE codons 40 and 85, which span the conserved segment I (Fig. 4). One of the missense mutations, *grpE103*, falls in the middle of this predicted coiled-coil region. When the Glu residue of codon 53 was substituted with that of Gly found in *grpE103*, the resulting sequence showed a 30% reduced probability of forming the coiled-coil structure compared with wild-type GrpE. Coiled-coil regions have been postulated to facilitate protein-protein interactions. Previously, GrpE had been shown to form a salt-resistant but ATP-sensitive complex with DnaK and to be a dimer by glutaraldehyde cross-linking (35). It will be interesting to determine whether the mutant GrpE103 protein is deficient in DnaK-GrpE interaction and/or dimerization.

Another interesting feature of the distribution of the *grpE* mutations is that the only two mutations (*grpE2/280* and *grpE17*) that block λ growth at all temperatures are located only within the conserved segment III, while all other mutations are temperature sensitive for λ growth and are spread throughout the open reading frame, either before or after *grpE280*. This result may imply that codons around amino acid 122 in *E. coli* are very crucial for GrpE's function. However, the significance of such mutant distributions must await the in vitro biochemical characterization of the defects of each mutant protein.

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