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Two Classes of Extragenic Suppressor Mutations Identify Functionally Distinct Regions of the GroEL Chaperone of *Escherichia coli*

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The GroES and GroEL proteins of *Escherichia coli* function together as the GroE molecular chaperone machine to (i) prevent denaturation and aggregation and (ii) assist the folding and oligomerization of other proteins without being part of the final structure. Previous genetic and biochemical analyses have determined that this activity requires interactions of the GroES 7-mer with the GroEL 14-mer. Recently, we have identified a region of the GroES protein that interacts with the GroEL protein. To identify those residues of the GroEL protein that interact with GroES, we have exploited the thermosensitive phenotype of strains bearing mutations at one or the other of two GroEL-interacting residues of GroES. We have isolated, cloned, and sequenced six suppressor mutations in *groEL*, three independent isolates for each *groES* mutant. Changes of only three different amino acid substitutions in GroEL protein were found among these six *groEL* suppressor mutations. On the basis of a number of in vivo analyses of the chaperone activity of various combinations of *groES* mutant alleles and *groEL* suppressor alleles, we propose that an amino-proximal region of the GroEL protein which includes amino acid residues 174 and 190 interacts with GroES and that a carboxyl-proximal region which includes residue 375 interacts with substrate proteins.

The *groE* locus of *Escherichia coli* was originally identified through the isolation of bacterial mutants unable to support growth of bacteriophage λ or T4 (6, 11, 25, 26; for a review, see reference 33). Subsequent studies revealed that the *groE* operon consists of two genes: the upstream *groES* gene which encodes the 10,368-M_r GroES protein and is required for bacteriophage λ head assembly and the downstream *groEL* gene which encodes the 57,259-M_r GroEL protein and is required for bacteriophage λ and T4 head assembly (27a; for a review, see reference 33). Both the *groEL* and the *groES* genes are essential for viability at all temperatures (9), although the precise role of their products in the physiology of *E. coli* is not yet completely understood. One activity that is by now well established for the GroEL and GroES proteins is their molecular chaperone function, namely, their ability to assist in the folding of a variety of protein substrates without being part of the final structure (7). Whether or not this rather general activity fully describes their function in the cell is not yet clear, but a recent study demonstrates the importance of this activity: Horwich et al. (13) isolated a *groEL* mutant strain which encodes a Glu→Lys substitution of residue 461 of the GroEL protein and confers a temperature-sensitive growth phenotype on the cells. From their studies, those researchers concluded that a specific set of cytoplasmic proteins depend on GroEL function to achieve their native conformations.

The number of different mutations generated from the original bacteriophage resistance selection is apparently small, since among 17 independent *groE* isolates there are only four different *groEL* alleles (32) and three different *groES* alleles (19). Two of these *groEL* mutant products have been purified and characterized biochemically (1, 35). The biochemical analysis of the GroEL140 mutant protein, carrying a Ser→Phe substitution at residue 201 (32), showed that it is reduced in its ability to interact with wild-type GroES protein (1). Interestingly, evidence of a reduced interaction was also found for the GroEL673 mutant protein with Gly→Asp substitutions at residues 173 and 337 (35). However, the very different forms of biochemical analyses used to characterize the mutant proteins preclude a refined comparison of activities for the two proteins. Nevertheless, both GroEL140 and GroEL673 mutants were obtained by utilizing resistance to bacteriophage λ as a selection, so it is not unreasonable to assume that parallel defects in function might be found for the two proteins.

The *groES* mutants isolated from the selection described above have also proven to be useful in elucidating the function of the GroES protein. Previous studies had already established that the two wild-type *groE* gene products interact both in vivo and in vitro (reviewed in reference 33). Recent work has extended this conclusion by showing that all of the *groES* mutations fall in a region that by means of biophysical analyses has been established to be a mobile domain of the GroES protein (19). In the presence of GroEL, this GroES mobile domain is significantly reduced in its mobility. Furthermore, it has been determined that peptides corresponding to the GroES mobile domain do not compete with substrate peptides for binding to GroEL. Thus, it has been proposed that GroES and substrate proteins interact with different regions of the GroEL protein (19).

These conclusions have recently been supported by the demonstration that the effects of GroES and folding protein on the activity of GroEL are opposing (23). From their data, Martin et al. propose that the mechanism of folding occurs by means of a competition between substrate protein and GroES for binding to GroEL, the binding of one lowering the affinity for binding of the other. As the substrate protein achieves a continually more folded state, the affinity for binding of GroES

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will eventually be greater than that of the substrate protein, and a complete release of substrate would then be assured.

Earlier genetic studies had demonstrated that it was possible to isolate *groEL* mutants that suppress the thermosensitive growth of certain *groES* mutant bacteria (27). These studies formed the genetic basis for the idea that the GroEL and GroES proteins physically interact (27). However, these original isolates were never analyzed at the DNA sequence level. Given that we now have identified the region of the GroES protein that interacts with GroEL, it is possible that such *groEL* suppressor mutants might identify regions of the GroEL protein that interact with GroES. With the specific goal of defining at the DNA sequence level what these *groEL* mutations are, we have repeated the original isolation procedure to obtain *groEL* suppressor mutations (27), using specially constructed *groES* mutant derivatives that facilitate the cloning and subsequent sequencing of the entire *groE* operon from these strains onto multicopy plasmids. Here, we present the DNA sequences of the isolated *groEL* suppressor mutations. Furthermore, we analyze and compare the chaperone activities in vivo of the various mutant pairs with respect to two different protein substrates; i.e., chaperone function with respect to the bacteriophage λB protein is examined by assessing the ability of the GroE proteins to support bacteriophage λ growth, and chaperone function with respect to DnaA protein is examined by determining the ability of the proteins to suppress the temperature-sensitive phenotype of the *dnaA46* allele (8, 15). Finally, we test the *groEL* suppressor mutations for allele specificity with respect to the *groES* mutant strain from which they were derived.

MATERIALS AND METHODS

Media. Luria-Bertani (LB) broth or LB agar media were prepared by the method of Miller (24); however, 5 g of NaCl per liter was used in all cases except in the growth and phenotypic analysis of the *dnaA46* mutant strain OFB24 or its isogenic wild-type parent strain OFB187, for which 10 g of NaCl per liter was used. The following supplements and antibiotics were added when appropriate: ampicillin (50 μg/ml) and methicillin (500 μg/ml), spectinomycin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), and thymine (1 μg/ml).

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this work are described in Table 1. The bacterial strains used to isolate and subsequently clone the *groEL* suppressor mutations were derived as described previously (32) from *groES* mutant strains isolated by Georgopoulos et al. (11).

DNA manipulations. Plasmid DNA was prepared by the alkaline lysis procedure of Ish-Horowicz and Burke (14) or by using the Magic Miniprep kit of Promega. *E. coli* transformations were performed as described by Cohen et al. (5). DNA fragments were prepared by using the GeneClean kit protocol (Bio 101) or the Magic DNA CleanUp kit protocol (Promega). Analytical restriction endonuclease digests and ligation reactions were carried out as described by Maniatis et al. (20) or according to the manufacturer's instructions. Plasmid constructions are described in Table 1.

Cloning *groE* mutations. The *groE* mutations were cloned by means of RecA-mediated homologous recombination between the chromosome and plasmid pJZ512, as previously described (32). They were subsequently transferred to other plasmids by standard in vitro manipulation techniques (Table 1). A complete description of the plasmid constructions is available upon request.

DNA sequencing. Double-stranded sequencing of the pJZ512 derivatives bearing the cloned *groE* mutant alleles was performed on alkali-denatured plasmid templates. The sequencing reactions were carried out according to the United States Biochemicals protocol, using Sequenase version 2.0.

Temperature sensitivity analysis. The strains were transformed with the appropriate plasmids. Subsequently, three or four of these fresh transformants were streaked onto LB agar plates (containing antibiotics or supplemented with thymine where appropriate) and incubated at the temperatures indicated in Tables 3 and 4. Bacterial growth was monitored at 24 and 48 h.

Bacteriophage spot tests. The abilities of various bacteriophages to grow on the various strains tested were determined as previously described (32).

RESULTS

Isolation of suppressor mutations in *groEL*. We have previously identified at the DNA sequence level two different *groES* mutations that display thermosensitive growth. These mutations result in Gly→Asp amino acid substitutions at either residue 23 (*groES42*) or residue 24 (*groES619*) of GroES (19). Strains OFB1072 and JZ620 bear one or the other of these *groES* mutations (Table 1) and also have a Kan^r cassette inserted immediately downstream of the $groEL^+$ gene in the chromosome. Cultures of the two strains were spread on LB agar plates and incubated at the nonpermissive temperature, 43°C, until colonies were observed, usually after 48 h.

These temperature-resistant (Ts^+) colonies may be the result of four different kinds of mutations, namely, (i) true $groES^+$ revertants, (ii) pseudorevertants that map within the *groES* gene, (iii) extragenic suppressors that map in the *groEL* gene, or (iv) extragenic suppressors that map elsewhere on the chromosome. To identify directly members of the third class of revertants, we tested all Ts^+ revertants for resistance to bacteriophage T4. This screen is based on the earlier observation that growth of bacteriophage T4 is not affected by mutations in *groES* but that mutations in *groEL* can affect the ability of T4 bacteriophage to form plaques (reviewed in reference 33). In this way, we hoped to identify those Ts^+ revertants that retained the original *groES* mutation but had acquired an additional, compensatory mutation in the *groEL* gene. It should be noted here, however, that while we have focused on the extragenic suppressors that met the requirement of blocking bacteriophage T4 growth, there are probably additional *groEL** mutations that allow growth of the *groES* mutant strains at the nonpermissive temperature but retain the ability to support the growth of bacteriophage T4.

We also tested the ability of other bacteriophages to propagate on our isolates, including bacteriophage λ and a number of ε mutants of T4 bacteriophage. The T4 bacteriophage ε mutants were isolated as plaque formers on bacterial strains bearing various *groEL* mutations (10, 11, 27). The mutations present in the various T4 ε bacteriophage mutants have been mapped to gene *31* and identified at the DNA sequence level (18; see also footnotes to Table 2).

Examples of the phenotypically distinguishable isolates obtained from our selection are summarized in Table 2. Among those isolates derived from each *groES* mutant on which wild-type bacteriophage T4 was unable to form plaques, we found that, whereas growth of bacteriophage λ on one class of isolates (A) was supported, on another class (B) it was still blocked.

Cloning and DNA sequence analysis of the *groEL* suppressor mutations. The *groE* operon was cloned from one class A

TABLE 1. Strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
B178	*galE groE*$^+$	Our collection
DH5α	F$^-$ φ80d*lac*ZΔM15 *endA1 recA1 hsdR17* ($r_K^-m_K^-$) *supE44 thi-1* λ$^-$ *gyrA96 relA1* Δ(*lacZYA-argF*)*U169*	16
CG2246	B178 *zjd*::Tn*10 groEL673*	Our collection
CG2242	B178 *zjd*::Tn10 *groEL59*	Our collection
CG2241	B178 *zjd*::Tn*10 groEL44*	Our collection
OFB1072	B178 *groES619 zje*::Kan^r	Our collection
JZ484	B178 *zjd*::Tn*10 groES42*	This study
JZ558	Class B Ts$^+$ derivative of OFB1072; *groES619 groEL** G375S	This study
JZ560	Class A Ts$^+$ derivative of OFB1072; *groES619 groEL** V190I	This study
JZ564	Class B Ts$^+$ derivative of OFB1072; *groES619 groEL** G375C	This study
JZ620	B178 *zjd*::Tn*10 groES42 zje*::Kan^r	This study
JZ628	B178 *zjd*::Tn*10 groE*$^+$	This study
JZ648	B178 *zjd*::Tn*10 groES619*	This study
JZ661	Class A Ts$^+$ derivative of JZ620; *groES42 groEL** V174F	This study
JZ662	Class B Ts$^+$ derivative of JZ620; *groES42 groEL** G375S	This study
JZ665	Class B Ts$^+$ derivative of JZ620; *groES42 groEL** G375S	This study
OFB24	W1485 *thi leu thyA deoB* or *deoC recA56 srl*::Tn*10 dnaA46*	Our collection
OFB187	W1485 *thi leu thyA deoB* or *deoC recA56 srl*::Tn*10*	Our collection
OFB280	(F$^-$) W1485 *leu thi thyA deoB* or *deoC zjd*::Tn*10 zje*::Kan^r	Our collection
Bacteriophages		
λb_2cI	Clear-plaque former	Our collection
T4 D_o	Wild-type T4 bacteriophage	18
T4ε1	T4 D_o mutant isolated on *groEL44*	18
T4ε711	T4 D_o mutant isolated on a pseudorevertant of *groES7*	18
T4ε9725	T4ε1 (double) mutant isolated on a pseudorevertant of *groES97*	18
Plasmids derived from:		
pGB2 (4)		
pJZ512	pGB2::*Eco*RI-*Hin*dIII Δ*groE* chromosomal fragment from pOFX60; Spc^r	32
pJZ549	pJZ512::*groES*$^+$ *groEL*$^+$, by in vivo recombination from OFB280; Spc^r Kan^r	This study
pJZ576	pJZ512::*groES619 groEL** V190I, by in vivo recombination from JZ560; Spc^r Kan^r	This study
pJZ577	pJZ512::*groES619 groEL** G375S, by in vivo recombination from JZ558; Spc^r Kan^r	This study
pJZ597	pJZ512::*groES42 groEL** V174F, by in vivo recombination from JZ661; Spc^r Kan^r	This study
pJZ598	pJZ512::*groES42 groEL** G375S, by in vivo recombination from JZ662; Spc^r Kan^r	This study
pJZ599	pJZ512::*groES42 groEL** G375S, by in vivo recombination from JZ665; Spc^r Kan^r	This study
pJZ619	pJZ512::*groES42 groEL** V190I, by *Ksp*I-*Xho*I fragment substitution between pJZ576 and pJZ597; Spc^r Kan^r	This study
pACYC184 (3)		
pJZ643	pACYC184 Δ(*Fsp*I-Tet^r-*Fsp*I); Cm^r	This study
pJZ644[a]	pJZ643::p*lac groEL*$^+$, by cloning from pJZ549; Cm^r Kan^r	This study
pJZ645[a]	pJZ643::p*lac groEL** V174F, by cloning from pJZ618; Cm^r Kan^r	This study
pJZ646[a]	pJZ643::p*lac groEL** V190I, by cloning from pJZ576; Cm^r Kan^r	This study
pJZ647[a]	pJZ643::p*lac groEL** G375C, by cloning from pJZ579; Cm^r Kan^r	This study
pJZ648[a]	pJZ643::Kan^r, by cloning the Kan^r cassette from pUC-4K; Cm^r Kan^r	This study
pBR322 (2)		
pMC9	pBR322::*lac*I^q	31
pOF12	pBR322::8.1-kb *Eco*RI chromosomal fragment containing *groES*$^+$ and *groEL*$^+$; Amp^r Tet^r	8
pOFX60	pOF12 Δ(*groES groEL*), 4-kb *Bst*XI deletion; Amp^r Tet^r	32
pJZ541	pOFX60::*groES619 groEL*$^+$, by in vivo recombination from OFB1072; Amp^r Tet^r Kan^r	19
pJZ579	pOFX60::*groES619 groEL** G375C, by in vivo recombination from JZ564; Amp^r Tet^r Kan^r	This study
pJZ618	pOFX60::*groES619 groEL** VI74F, by *Ksp*I-*Xho*I fragment substitution between pJZ597 and pJZ541; Amp^r Tet^r Kan^r	This study
pJZ631	pBR322::*groES619 groEL** VI90I, by cloning the *Eco*RI-*Hin*dIII *groE* fragment from pJZ576 into pBR322; Amp^r	This study
pJZ632	pBR322::*groES619 groEL** G375S, by cloning the *Eco*RI-*Hin*dIII *groE* fragment from pJZ577 into pBR322; Amp^r	This study
pJZ633	pBR322::*groES42 groEL** VI74F, by cloning the *Eco*RI-*Hin*dIII *groE* fragment from pJZ597 into pBR322; Amp^r	This study
pJZ634	pBR322::*groES42 groEL** VI90I, by cloning the *Eco*RI-*Hin*dIII *groE* fragment from pJZ619 into pBR322; Amp^r	This study
pUC-4K	pUC4 derivative with a Kan^r cassette	30

[a] A complete description of these plasmid constructions is available upon request.

TABLE 2. Plaque-forming abilities of *E. coli groES groEL* double-mutant strains at 37°C

groE strain	*groES* allele (mutation)	*groEL* allele	Suppressor class	Plaque-forming ability of bacteriophage[a]:				
				Wild-type T4	T4ε1[b]	T4ε711[c]	T4ε9725[d]	λb_2cI
JZ560	*groES619* (G24D)	*groES619* suppressor, *groEL** V190I	A	−	−	+	+	+
JZ558	*groES619* (G24D)	*groES619* suppressor, *groEL** G375S	B	−	−	+	+	−
JZ564	*groES619* (G24D)	*groES619* suppressor, *groEL** G375C	B	−	−	+	+	−
OFB1072	*groES619* (G24D)	Wild-type *groEL*$^+$		+	+	+	+	−
JZ661	*groES42* (G23D)	*groES42* suppressor, *groEL** V174F	A	−	−	+	+	+
JZ662	*groES42* (G23D)	*groES42* suppressor, *groEL** G375S	B	−	−	+	+	−
JZ620	*groES42* (G23D)	Wild-type *groEL*$^+$		+	+	+	+	−

[a] +, efficiency of plating of 1 and good plaque size; −, no visible PFU ($<10^{-5}$) with the exception of occasional bypass (ε) mutants for bacteriophage T4.
[b] Bacteriophage T4ε1 bears a mutation in gene *31* resulting in a Leu-35→Ile change in gp31.
[c] Bacteriophage T4ε711 bears a mutation in gene *31* resulting in a ΔGlu-28 change in gp31.
[d] Bacteriophage T4ε9725 bears mutations in gene *31* resulting in Leu-35→Ile and Gly-38→Asp changes in gp31.

revertant strain and two class B revertant strains derived from each *groES* mutant, by means of RecA-mediated homologous recombination, onto a multicopy plasmid, as previously described (32). The DNA sequences of the entire *groE* operons were then determined from the resulting plasmid clones. The results, shown in Fig. 1, indicate that the mutations in *groEL* corresponding to the two different classes are localized to two distinct regions of the *groEL* gene. The class A mutant derived from *groES619* (strain OFB1072; Table 1) results in the Val-190→Ile substitution in GroEL, and that derived from *groES42* (strain JZ620; Table 1) results in the Val-174→Phe change in GroEL; both suppressor strains now support bacteriophage λ growth. These suppressor mutations lie close to a number of our previously described *groEL* mutations, which were selected by their inability to support the growth of bacteriophage λ and subsequently shown to be unable to grow at the nonpermissive temperature of 43°C (32). Thus, we find that *groEL* mutations in this same region not only suppress the thermosensitive phenotype conferred by the *groES* mutations alone but also support bacteriophage λ growth when combined with the resident *groES* mutation.

Surprisingly, all four class B double mutants sequenced, those that still block the growth of bacteriophage λ, have mutations that result in a change at only amino acid residue 375 of GroEL. The change is either a Gly→Ser substitution derived from both the *groES619* and the *groES42* strains or a Gly→Cys substitution derived from the *groES619* strain. (Note that for *groES42*, two independent isolates bearing the same *groEL** suppressor mutation were obtained.) This is the same region of the *groEL* gene in which two other mutations, unable to support bacteriophage λ growth, had previously been mapped (32). These previously identified *groEL* mutant genes, *groEL515* and *groEL764*, both bear the same mutation that results in a change at amino acid residue 383 and show no Ts bacterial growth phenotype.

Such a high level of redundancy of suppressor mutations strongly suggests that there are only very few *groEL* mutations that are able to fulfill our stringent selection criteria. On the basis of the phenotypes of the suppressor mutants combined with our DNA sequence information, we hypothesize that on the one hand the class A isolates represent allele-specific suppressors of the particular *groES* mutation from which they are derived, and thus the mutations might be localized within the *groEL* gene to a region of GroES-GroEL protein-protein interaction. On the other hand, the class B mutations might represent changes that alter GroEL activity, perhaps in a region of the GroEL protein that is involved in substrate-GroEL interaction. To test this hypothesis, it would be necessary to demonstrate allele specificity with respect to the class A isolates and to explore exactly what kind of alteration is

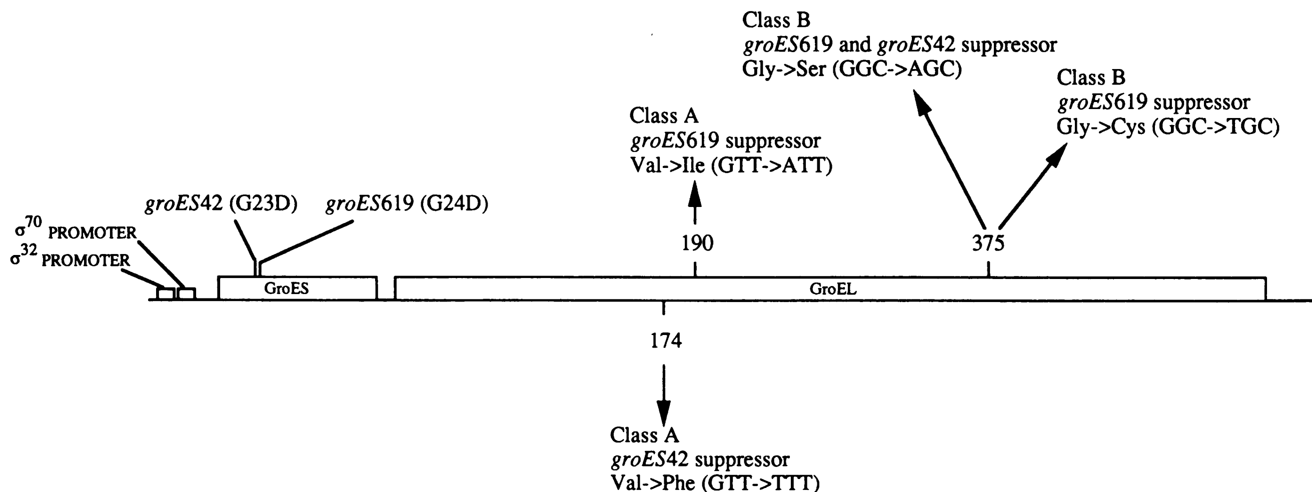

FIG. 1. Schematic overview of the *groEL* suppressor mutant alleles. Numbers refer to the GroEL codons mutated, and the amino acid changes are given. DNA sequences of the mutations are given in parentheses. The *groES* strains from which the *groEL* mutants were derived are as indicated. G23D, G-to-D mutation at position 23; G24D, G-to-D mutation at position 24.

TABLE 3. Suppression of temperature sensitivity of *E. coli* OFB24 bearing the *dnaA46* allele[a]

groE alleles on multicopy plasmid		Plasmid	Growth at 42°C[b]
groES	*groEL*		
groES619 (G24D)	*groES619* suppressor, *groEL** V190I	pJZ631	+
groES619 (G24D)	*groES619* suppressor, *groEL** G375S	pJZ632	+
groES619 (G24D)	*groES619* suppressor, *groEL** G375C	pJZ579	+
groES619 (G24D)	Wild type	pJZ541	+
groES619 (G24D)	*groES42* suppressor, *groEL** V174F	pJZ618	−
groES42 (G23D)	*groES42* suppressor, *groEL** V174F	pJZ633	−
groES42 (G23D)	*groES619* suppressor, *groEL** V190I	pJZ634	+
Wild type	Wild type	pOF12	+

[a] Results were obtained with LB agar supplemented with thymine and appropriate antibiotics.

[b] +, growth identical to that of the isogenic wild-type *dnaA*$^+$ strain (OFB187); −, no growth observed at the nonpermissive temperature of 42°C, whereas growth is equivalent to that of the wild-type *dnaA*$^+$ strain at 37°C.

represented by the class B mutants. The following results represent our attempts, using genetic analyses, to analyze these aspects of protein function for the mutant proteins.

Multicopy suppression of the temperature-sensitive growth of *dnaA46* bacteria. It has been established previously that the functionality of various thermosensitive proteins, including DnaA mutant proteins (8, 15) as well as those encoded by certain mutations in metabolic genes (29), can be restored in vivo when multiple copies of the *groES* and *groEL* genes are present. While the mechanism of this suppression is not fully understood, it is likely that it is due to the chaperone function of the GroES and GroEL proteins, whose overproduction may result in either a higher percentage of correctly folded structures of the mutant polypeptides at the nonpermissive temperature or less aggregation. To analyze the ability of the pairs of GroES and GroEL mutant proteins isolated in this study to function as chaperones and to explore allele specificity of the *groEL** mutations, we determined whether multicopy plasmids bearing various combinations of *groES* and *groEL* mutations could also suppress the Ts phenotype of *dnaA46*. This analysis required that mutant *groEL* genes be paired with one of the two *groES* mutant alleles on the plasmids. The pairs of *groE* alleles were then inserted into a pBR322-derived vector, and the growth of the plasmid-bearing *dnaA46* strains at the nonpermissive temperature was assessed. The results for the various *groES-groEL* mutant pairs tested are summarized in Table 3.

In this analysis of multicopy suppression of the Ts phenotype of *dnaA46*, we did not observe the allele specificity that we had hypothesized for the class A *groEL* suppressor mutations. However, it is quite clear that the two class A GroEL* mutant proteins are distinctly different in their ability, in combination with mutant GroES protein, to function as chaperones with respect to the DnaA46 substrate protein. Thus, the GroEL* mutant with a change at amino acid residue 190 is able to work with both GroES mutant proteins, but the GroEL* mutant with a change at amino acid residue 174 does not work with either GroES mutant protein with respect to *dnaA46* suppression. Also, the class B GroEL* mutant with the Gly→Ser change at residue 375 can work with either GroES mutant protein to suppress the Ts phenotype of *dnaA46*. Since the class A suppressor strains support the growth of bacteriophage λ, whereas the class B suppressor strains do not, the opposing results with respect to *dnaA46* suppression clearly demonstrate that the chaperone activities of the various GroE mutant proteins are not identical with respect to their various protein substrates; λB protein, the protein target of GroE chaperone function for bacteriophage λ (11), interacts differently with the GroE chaperones than does DnaA protein.

Exploring allele specificity of the *groEL* suppressors by using multicopy plasmids. We and others have previously shown that increasing the copy number of the *groES* gene, in combination with the wild-type *groEL* gene, can alter the phenotypes associated with the same mutant gene when present in a single copy (19). Additionally, given the essentiality for bacterial growth of both the *groES* and the *groEL* genes (9), it may be futile to attempt to construct the various combinations of mutants on the chromosome, in that they may not be viable. For these reasons, we decided to construct plasmids bearing only *groEL** mutant genes. This arrangement also allows us to be certain that the phenotypes observed are dependent solely on the *groEL** allele present on the various plasmids and are not due to any other, secondary mutations.

Because the same mutation encoding the Gly→Ser substitution at amino acid residue 375 in GroEL* was obtained from both *groES619* and *groES42*, we already knew that the suppression by that mutation was not allele specific. We constructed a set of plasmids bearing the other mutant *groEL* alleles as well as the wild-type gene. The vector used was a pACYC derivative (Table 1). The plasmid-borne *groEL* alleles are under *lac* promoter control. Tight regulation of the expression of the *groEL* genes on the plasmids was provided by a second compatible plasmid, pMC9, bearing the *lacI*^{q} gene (Table 1). This arrangement also allowed expression of the plasmid-borne *groEL* genes to be regulated independently of the growth temperature. Table 4 summarizes the abilities of the various *groEL* alleles to suppress the Ts phenotype of the *groES* mutants. Also analyzed was the ability of the *groEL* mutants to function with strains that are wild type with respect to the chromosomal *groES*$^+$ gene but bear various *groEL* mutations (32). This last set of strains provided the means to analyze the ability of the various mutant *groEL* gene products provided in *trans* to function with wild-type GroES encoded on the chromosome.

The results for the *groES* mutant strain transformants presented in Table 4 show that, whereas the *groES* mutant strains transformed with the vector alone or the vector bearing the wild-type *groEL*$^+$ gene show very limited growth at the nonpermissive temperature, the *groES* mutant strains transformed with plasmid DNA bearing any of the three *groEL** mutant genes can grow at the nonpermissive temperature. Thus, in this respect, there is no allele specificity between the particular *groEL** mutation and the *groES* mutation. The results for the transformed strains bearing chromosomal *groEL* mutations indicate that the GroEL proteins encoded by the plasmid-borne *groEL** suppressor alleles are able to function with the wild-type *groES*$^+$ gene. This is exemplified by strain CG2241, which, when transformed with any of the suppressor-bearing plasmids, can grow as well as the wild-type strain at the nonpermissive temperature. Interestingly, some of the chromosomal mutations demonstrate dominance over the plasmid-encoded alleles, i.e., strain CG2246 remains unable to grow at the nonpermissive temperature in the presence of the suppressor-bearing plasmids.

In contrast to the suppression of Ts bacterial growth, however, there is a clear distinction with respect to bacteriophage λ growth (Table 5). For the *groES42* allele, only the class

TABLE 4. Temperature sensitivity of *E. coli groES* or *groEL* mutant strains bearing multicopy plasmids with various *groEL* mutated genes[a]

groE strain	Mutation(s)	Growth at 43°C of *groE* mutant strain bearing plasmid[b]:									
		pJZ644 (*groEL*$^+$)		pJZ645 (groEL* V174F)		pJZ646 (groEL* V190I)		pJZ647 (groEL* G375C)		pJZ648 (vector)	
		Without IPTG	With IPTG	Without IPTG	With IPTG	Without IPTG	With IPTG	Without IPTG	With IPTG	Without IPTG	With IPTG
CG2246	*groEL673* (G173D, G337D)	+	+++	±	±	−	−	−	−	−	−
CG2242	*groEL59* (S201F)	ND[c]	ND	±	+++	±	++	±	++	±	±
CG2241	*groEL44* (E191G)	+	+++	++	+++	+	+++	±	+++	−	−
JZ648	*groES619* (G24D)	−	−	+	+++	+	+++	++	+++	±	±
JZ484	*groES42* (G23D)	−	−	++	+++	+	+++	+	+++	−	−
JZ628	None (wild type)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a] Note that all of these strains also bear plasmid pMC9; thus, expression of the *groEL* alleles present on the plasmids is inducible with IPTG (isopropyl-β-D-thiogalactopyranoside). Plasmid constructions are described in Table 1.
[b] Growth on LB agar with or without IPTG as indicated and supplemented with appropriate antibiotics. +++, growth equivalent to that of the wild type in terms of both CFU and colony size; ++, 10-fold-fewer CFU or colony size reduced relative to that of the wild-type strain or both; +, 10^2-fold-fewer CFU relative to the wild-type strain; ±, very marginal level of growth relative to the wild type; −, no growth observed ($<10^{-5}$ CFU).
[c] ND, not determined; no transformants of CG2242 were obtained with plasmid pJZ644 despite attempts executed in parallel with successful transformation of this strain with the other plasmids listed here and with plasmid pJZ644 in the other strains listed here.

A *groEL** allele resulting in a Val→Phe substitution at amino acid residue 174 of GroEL suppresses growth (although it should be noted that the suppression is only marginally improved over that observed for the wild-type *groEL*$^+$ gene in multiple copies). However, it is clear that the other *groEL** alleles combined with the *groES42* mutation do not support bacteriophage λ growth at all. These results differ from those obtained for the *groES619* mutation, for which plaque formation was observed to be optimal in the presence of the *groEL** allele encoding the Val→Ile substitution at amino acid residue 190, and there is also suppression with both the codon 174-substituted GroEL* protein and the codon 375-substituted (Gly→Cys) GroEL* protein, but no suppression is observed with the wild-type *groEL*$^+$ gene. Finally, none of the *groEL** suppressor mutations support bacteriophage λ growth in combination with the wild-type *groES*$^+$ gene (Table 5, results for strain CG2241).

We also analyzed these *groEL** mutant-carrying plasmids with respect to their ability to suppress *dnaA46* in the absence of multiple copies of *groES*. The presence of multiple copies of both *groES*$^+$ and *groEL*$^+$ genes had previously been shown to be required for the restoration of activity of the DnaA mutant protein (8). This analysis would allow us to determine whether any of the GroEL* suppressor proteins had acquired the capacity to function in the presence of lower levels of GroES protein than required by wild-type GroEL protein. We found that none of the plasmids suppressed the thermosensitive phenotype of *dnaA46* (results not shown). Thus, these GroEL* suppressor proteins still require the presence of elevated levels of GroES for suppression of the *dnaA46* Ts phenotype.

DISCUSSION

We have identified two regions of the GroEL protein in which changes suppress the thermosensitive defect of two different *groES* mutant strains. The more amino-terminal region of GroEL, identified in this study by the class A mutants, includes amino acid residues 174 and 190, while the carboxyl-terminal region, identified by the class B mutants, consists of amino acid residue 375 only. The distinction between these two classes of *groEL* suppressor mutations is that while all the *groES-groEL* double mutants can grow at temperatures nonpermissive for the *groES* mutant strains, those with *groEL** suppressor mutations encoding changes at amino acid residue 174 or 190 of the GroEL protein support the growth of bacteriophage λ, whereas the suppressor mutations encoding a change at codon 375 do not support bacteriophage λ growth.

Previous work has identified similar regions of GroEL protein as important for function: the *groEL* mutants isolated on the basis of inability to support the growth of bacteriophage λ had mutations either in a region spanning residues 173 to 201

TABLE 5. Plaque-forming abilities of bacteriophage λ on *E. coli groES* or *groEL* mutant strains bearing multicopy plasmids with various *groEL* genes[a]

groE strain	Mutation	Plaque-forming ability of bacteriophage λ at 37°C on *groE* mutant strain bearing plasmid[b]:					Comments
		pJZ644 (*groEL*$^+$)	pJZ645 (groEL* V174F)	pJZ646 (groEL* V190I)	pJZ647 (groEL* G375C)	pJZ648 (vector)	
CG2241	*groEL44* (E191G)	+	−	−	−	−	
JZ648	*groES619* (G24D)	−	±	+	±	−	Plaque size largest on strain JZ648(pJZ646)
JZ484	*groES42* (G23D)	±	±	−	−	−	Plaque size larger and more distinct on strain JZ484(pJZ645) than on strain JZ484(pJZ644)
JZ628	None (wild type)	+	+	+	+	+	Plaque size reduced on strain JZ628 with plasmid pJZ645, pJZ646, or pJZ647

[a] Note that these strains also bear plasmid pMC9, so expression of the *groEL* allele on the plasmids is inducible with IPTG.
[b] Cells were grown on LB agar with IPTG and supplemented with appropriate antibiotics. +, efficiency of plating of 1 and good plaque size; ±, efficiency of plating of 0.01 to 1 and small plaque size; −, no visible PFU ($<10^{-4}$).

or at residue 383 (32). However, among these earlier-isolated mutants, only the strain with an amino acid alteration at codon 383 was suppressed by λ*B* mutations, whereas the others were suppressed by mutations in λ*E* (11). While the λB morphogenetic protein specifically interacts with GroEL proteins (34), the mechanism of suppression by mutations in the λ*E* gene is through an indirect effect; namely, the λ*E* mutants are slowed in assembly, allowing bacteriophage morphogenesis to still occur in the partially defective GroEL mutant strains (11). This evidence suggests that changes in the GroEL carboxyl-terminal region identified here affect substrate binding activity, whereas changes in the more amino-terminal region of GroEL affect GroEL-GroES interaction.

An ATP-binding pocket composed of amino acid residues 164 to 172 and 241 to 251 and the region around Tyr-477 has recently been proposed, with the tyrosine residue identified as binding ATP on the basis of cross-linking studies with the ATP analog $8N_3$-ATP (22). While these amino acid residues do not overlap with those identified by the mutants isolated here, or those previously isolated and described above, there is an overlap of the amino-terminal region with an ATPase consensus sequence described by Martel et al. (21) which spans amino acid residues 168 to 189 of GroEL.

Interestingly, not all of the amino acid residues identified by the suppressor mutations here are conserved. While codons 174 and 375 are conserved in the human homolog (P1 protein) and the wheat homolog (Rubisco binding protein) to *E. coli* GroEL, codon 190 is Ile in P1 protein and Glu in the wheat GroEL homolog but Val in *E. coli* GroEL (12, 17).

The fact that the results obtained with respect to bacteriophage λ growth are not identical to those obtained with respect to suppressing mutated DnaA protein function demonstrates quite clearly that in vivo there are differences in chaperone activity on different protein substrates; the codon 375 mutation presumably abolishes chaperone activity of GroEL with respect to λB protein but is as efficient as wild-type GroEL with respect to DnaA. On the other hand, the codon 190 mutation apparently suppresses differently from the codon 174 mutation, with the mutation at codon 190 being more effective at suppressing the mutant DnaA defect. According to the folding model of Martin et al. (22), one can propose that the mutations able to compensate for the defect in GroES in our Ts mutant strains are quite specific; the competition for binding to GroEL by GroES and substrate protein most likely places a tight constraint on the affinity of binding of GroES to GroEL, in order for the appropriate balance of binding release by GroES and substrate protein to GroEL to take place in the cell. Thus, while all of the suppressor mutations identified here have met these criteria sufficiently well for survival at elevated (heat shock) temperatures, we can also detect changes in the "affinity balance" when we look at specific substrates (here, bacteriophage λB protein and DnaA).

By using bacteriophage T4, we have explored another aspect of the functionality of the GroEL mutant proteins. Among the previously identified *groEL* mutants, only the change in codon 201 blocked the ability of wild-type bacteriophage T4 to grow, but, by definition, all of the *groEL* suppressor mutants isolated in this study block wild-type T4 bacteriophage growth. Given the previously postulated similarity in function of T4 gp31 and GroES (18), we might expect that mutations that suppress *groES* mutants would change regions corresponding to those interacting with T4 gp31 as well. Strikingly, wild-type T4 and its ε mutant derivatives behave identically in all of the mutant strains isolated here. Thus, the *groEL* mutations affect T4 in the exact same way. The similarity of GroES-GroEL and gp31-GroEL interactions is clearly displayed; when GroES and GroEL are mutated so that the cells can grow at an elevated temperature, appropriate gp31 and GroEL mutant proteins can also productively interact. We propose that gp31 binds to GroEL in a fashion similar to that of GroES. For bacteriophage λ and T5, this interaction actually allows for productive protein folding in vivo, as demonstrated by the fact that gp31, in multiple copies, restores growth of both of these bacteriophages on *groES* mutant strains (28, 31a).

These findings imply that there might be even more proteins that can at some level bind to GroEL at the GroES site rather than the protein substrate site. Thus, some protein substrates, perhaps among them those that appear to not require the activity of GroES in GroEL-mediated folding reactions, may be able to compete for binding to GroEL at the same site as GroES. Alternatively, certain protein substrates might be able to interact with GroEL at both sites and, even more intriguing, in this way they might be able to assist in their own folding by fulfilling some level of the GroES function. If such complexities of binding are the true state of affairs, the difficulties encountered in attempts to identify protein sequences that are substrates for GroEL are readily understandable.

Finally, the fact that allele specificity was not always demonstrated might be due in part to the position and sequence of the two *groES* mutations that were used to obtain *groEL** suppressor mutations. They both encode the same amino acid substitution, Gly→Asp, in immediately adjacent residues of the GroES protein. It is possible that this close proximity and identical amino acid change allow for some overlap of suppression by the *groEL** mutations isolated here. Biochemical analyses of these GroES-GroEL mutant protein pairs will undoubtedly provide further insights into the mechanism by which the altered GroEL proteins compensate for the defective GroES proteins.

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