This paper is dedicated to the memory of Professor Karol Taylor

Cloning of the groE operon of the marine bacterium Vibrio harveyi using a lambda vector

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\textit{groES} and \textit{groEL} genes encode two co-operating proteins GroES and GroEL, belonging to a class of chaperone proteins highly conserved during evolution. The GroE chaperones are indispensable for the growth of bacteriophage \(\lambda\) in Escherichia coli cells. In order to clone the \textit{groEL} and \textit{groES} genes of the marine bacterium \textit{Vibrio harveyi}, we constructed the \textit{V. harveyi} genomic library in the \textit{λEMBL1} vector, and selected clones which were able to complement mutations in both \textit{groE} genes of \textit{E. coli} for bacteriophage \(\lambda\) growth. Using Southern hybridization, in one of these clones we identified a DNA fragment homologous to the \textit{E. coli groE} region. Analysis of the nucleotide sequence of this fragment showed that the cloned region contained a sequence in 71.7\% homologous to the 3' end of the \textit{groEL} gene of \textit{E. coli}. This confirmed that the \(\lambda\) clone indeed carries the \textit{groE} region of \textit{V. harveyi}. The positive result of our strategy of cloning with the use of the genomic library in \(\lambda\) vector suggests that the same method might be useful in the isolation of the \textit{groE} homologues from other bacteria. The \textit{V. harveyi} cloned \textit{groE} genes did not suppress thermosensitivity of the \textit{E. coli groE} mutants.

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All organisms exposed to high temperature or other environmental stresses respond by the increased synthesis of a group of evolutionarily conserved proteins called heat-shock proteins. These proteins usually represent one of the two groups: molecular chaperones or proteases. The chaperonin GroEL (Cpn60) and co-chaperonin GroES (Cpn10) constitute the GroE chaperone machine, which takes part in the process of folding and assembly of proteins [1]. Because of their chaperonin reactions [1], their importance for growth at all temperatures [2], and the importance of GroEL as a major antigen of pathogenic bacteria, the genes coding these proteins have been cloned from a wide variety of bacteria. Cloning and sequencing of these genes served in the studies oriented at characterization of protein structure and function [3] as well as in the comparative studies on regulation of the heat shock-gene expression [4]. The GroE proteins and regulation of their genes have been studied most intensively in Escherichia coli. The groES and groEL genes form an operon essential for E. coli viability at all temperatures [2]. They were initially identified because mutations in these genes block morphogenesis of several bacteriophages, including bacteriophage \( \lambda \) [5]. The groE operons of E. coli and other bacteria tested are arranged in the order: promoter–groES–groEL, with several bacteria having an additional, monocistronic groEL operon; so far, there is only one case (Mycobacterium bouis) of groES without groEL [4]. In E. coli, the groE operon belongs to the main heat shock regulon, regulated by the sigma 32 factor [6]. Under heat shock conditions, the groE genes are efficiently transcribed from a heat shock promoter located upstream of the groES gene by the RNA polymerase cooperating with the sigma 32 factor. The groE operon has a second promoter, located immediately downstream from the heat shock promoter, which can be utilized under normal growth conditions by RNA polymerase cooperating with the vegetative sigma 70 factor [6]. The strategies of regulation of the groE operons in bacteria are diverse and, contrary to the E. coli system, poorly understood [6–8]. Vibrio harveyi is a Gram-negative marine bacterium, belonging to the \( \gamma \) purple subdivision of proteobacteria. Among proteobacterial groE operons, only two (E. coli groE and Haemophilus ducreyi groE) have been characterized with respect to heat shock activation [4]. As an integral element of our studies on regulation of heat shock response in V. harveyi [9], we have undertaken to clone the V. harveyi groE genes.

MATERIALS AND METHODS

**Bacteria and bacteriophages** used in this study are described in Table 1. All E. coli strains were grown on L agar plates or in Luria broth (LB) [10]. The V. harveyi strain was grown in BOSS medium (1% peptone, 0.3% beef extract, 0.1% glycerol, 3% NaCl, pH 7.3). Bacteria used for growth of the \( \lambda \) EMBL1 vector phage and the \( \lambda \) recombinant clones, as well as the lysogenic strains used for preparation of \( \lambda \) packaging extracts, were cultured in NZCYM medium [10]. When appropriate, the following antibiotics were added: ampicillin (50 \( \mu \)g/ml) or tetracycline (10 \( \mu \)g/ml). Stocks of recombinant lambda phages were prepared as described by Sambrook *et al.* [10]. Phages were titered using T agar plates (1% tryptone, 0.5% NaCl, 1.5% agar, pH 7.4) and T top agar (the same as the T agar but with 0.7% agar), according to the standard procedures [10].

**DNA manipulations.** Recombinant DNA techniques were performed by standard protocols [10]. DNA fragments, if necessary, were purified by the glass beads method using the BioRad Prep-A-Gene DNA Purification System. To prepare the \( \lambda \) vector DNA, \( \lambda \) EMBL1 bacteriophage was grown using E. coli NM538 as a host strain, purified by a method including centrifugation in CsCl gradients and then phage DNA was extracted, as described by Sambrook *et al.* [10]. DNA sequencing procedures, using single-stranded templates pre-
Table 1. Bacterial and bacteriophage strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>Vibrio harveyi</em></td>
<td></td>
<td></td>
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<tr>
<td>NM538</td>
<td>supF hadR r&lt;sup&gt;&lt;em&gt;r&lt;/em&gt;&lt;sub&gt;k&lt;/sub&gt;</td>
<td>our collection [35]</td>
</tr>
<tr>
<td>B178</td>
<td>W3110 galE sup&lt;sup&gt;+&lt;/sup&gt;</td>
<td>our collection</td>
</tr>
<tr>
<td>CG2241</td>
<td>B178 groEL44 Tc&lt;sup&gt;R&lt;/sup&gt; Ts</td>
<td>C. Georgopoulos</td>
</tr>
<tr>
<td>CG2244</td>
<td>B178 groEL619 Tc&lt;sup&gt;R&lt;/sup&gt; Ts</td>
<td>C. Georgopoulos</td>
</tr>
<tr>
<td>BL202</td>
<td>B178(Alc&lt;sup&gt;+&lt;/sup&gt; imm&lt;sup&gt;+&lt;/sup&gt; iad)</td>
<td>this work; made by standard lysogen of B178</td>
</tr>
<tr>
<td>BL203</td>
<td>like BL202, but groEL44 Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>this work; made by P1 transduction from CG2241</td>
</tr>
<tr>
<td>BL204</td>
<td>like BL202, but groEL619 Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>this work; made by P1 transduction from CG2244</td>
</tr>
<tr>
<td>JM101</td>
<td>Δ(lac-proAB) supE thi&lt;sup&gt;&lt;em&gt;T&lt;/em&gt;&lt;/sup&gt; lac&lt;sup&gt;l&lt;/sup&gt; lacΔM15traD36 proAB&lt;sup&gt;+&lt;/sup&gt;</td>
<td>our collection [10]</td>
</tr>
<tr>
<td>TZ144</td>
<td>DH5α recA&lt;sup&gt;−&lt;/sup&gt; (pOF39, Ap&lt;sup&gt;+&lt;/sup&gt;) – the pOF39 plasmid carries groEL operon of E. coli</td>
<td>O. Fayet [36]</td>
</tr>
<tr>
<td>BHB2588</td>
<td>recA Su&lt;sup&gt;−&lt;/sup&gt; (λ Dam4 Δb2 red3 imm434 clts Samb&lt;sup&gt;+&lt;/sup&gt;)/λ</td>
<td>R. Wolinowska [10]</td>
</tr>
<tr>
<td>BHB2690</td>
<td>recA Su&lt;sup&gt;−&lt;/sup&gt; (λ Dam4 Δb2 red3 imm434 clts Samb&lt;sup&gt;+&lt;/sup&gt;)/λ</td>
<td>R. Wolinowska [10]</td>
</tr>
<tr>
<td>λEMBL1</td>
<td>cloning vector</td>
<td>R. Wolinowska [35]</td>
</tr>
<tr>
<td>λ 371</td>
<td>λ gt: 8.1 kb EcoRI fragment containing groES&lt;sup&gt;+&lt;/sup&gt; and groEL&lt;sup&gt;+&lt;/sup&gt; of E. coli</td>
<td>O. Fayet [36]</td>
</tr>
<tr>
<td>λ</td>
<td>cl&lt;sup&gt;+&lt;/sup&gt; imm&lt;sup&gt;+&lt;/sup&gt; iad</td>
<td>C. Georgopoulos</td>
</tr>
<tr>
<td>M18mp1/19</td>
<td>cloning vector</td>
<td>our collection [10]</td>
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</table>

pared from M13 hybrid phages, were performed as described by the manufacturers of the T7 Sequencing<sup>TM</sup> Kit (Pharmacia Biotech.). The sequences were analysed by the PC Gene program.

**Construction of the Vibrio harveyi genomic library.** V. harveyi chromosomal DNA was prepared according to Silhavy *et al.* [11] and used to construct genomic libraries in the λEMBL1 vector, following the procedures described by Sambrook *et al.* [10]. The DNA was partially digested with Sau3A under conditions promoting the formation of 10 kb fragments, the digested DNA was purified by phenol extraction and ethanol precipitation, and then ligated with vector DNA cut with *BamHI* and *SalI*. Prior to the ligation, the vector DNA was extracted with phenol and ethanol-precipitated. Ligation mixtures contained 1-2 μg of vector DNA and 0.5-2 μg of chromosomal DNA in final volume of 20 μl. The ligated DNA (5 μl) was packaged *in vitro* into λ phage particles by using extracts from two lysogenic strains, *E. coli* BHB2588 and *E. coli* BHB2690. The phage obtained by the packaging were titered using *E. coli* NM538 as the indicator strain.

**Southern hybridization** was performed using Boehringer Mannheim DIG DNA Labeling and Detection Kit as described by the manufacturer. The HindIII-EcoRI DNA fragment of the plasmid pOF39, containing both groES and groEL genes of *E. coli*, was used as an *E. coli* groEL probe. As a V. harveyi groEL probe, the 1500 kb XbaI-Pst1 fragment of the plasmid pDK1 was used. As a λ probe, the λEMBL1 DNA, digested with *BamHI* and HindIII endonucleases, was used. High stringency conditions were applied in all hybridization experiments.
Complementation tests. To test complementation of E. coli mutants with respect to \( \lambda \) growth, the \( \lambda \) groE\textsubscript{vibrio} clones, isolated from the V. harveyi genomic library in \( \lambda \)EMBL1 vector, were titered on E. coli strains with mutations in groEL and groES genes. Plating was performed at 30\(^\circ\)C, because of the thermostability of the mutants. The efficiency of plating was calculated as the ratio of plaque-forming units (p.f.u.) on a tested strain to the p.f.u. on the E. coli strain NM538.

To test whether the hybrid \( \lambda \) groE\textsubscript{vibrio} phages were able to suppress thermostability of E. coli groE mutants, the mutant bacteria carrying the \( \lambda \) imm\textsuperscript{mnd} prophage were constructed. This was done in two steps: (i) by lysogenization of the wild-type B178 strain, into which (ii) the groEL or groES mutation was transferred by P1 transduction using phage P1L4, essentially as described by Miller [12]. The prophage-bearing bacteria were grown at 30\(^\circ\)C in LB supplemented with tetracycline to \( A_{575} \) of 0.3, and 100 \( \mu \)L of this culture was mixed with about \( 10^5 \) p.f.u. of a tested \( \lambda \) hybrid phage. After 30 min of incubation at room temperature, cells were plated onto LA-agar plates and incubated at 30 and 42\(^\circ\)C. Abundant growth of normal-looking colonies at 42\(^\circ\)C indicated suppression.

RESULTS AND DISCUSSION

Selecting clones carrying V. harveyi groES and groEL genes and their characterization

Bacteriophage \( \lambda \) requires the groES and groEL gene products for its morphogenesis [4]. Since there is a very high degree of conservation among the groE homologues from bacteria [3], we assumed that V. harveyi GroE proteins would be functional in E. coli and enable \( \lambda \) phage to grow in cells with mutations in the groE genes. Based on this assumption, we constructed a V. harveyi genomic library in the \( \lambda \)EMBL1 vector (1.1 \( \times \) \( 10^5 \) p.f.u./ml) and plated it on E. coli CG2244(groES819) strains. Both strains were nonpermissive for growth of the \( \lambda \)EMBL1 vector. We obtained one clone, \( \lambda \) groEL\textsubscript{vibrio1}, growing on the E. coli groEL bacteria and two clones, \( \lambda \) groES\textsubscript{vibrio1} and \( \lambda \) groES\textsubscript{vibrio2}, growing on the E. coli groES bacteria (Table 2). Since the bacterial groES and groEL genes usually form one operon, we expected that the V. harveyi groE genes may have a similar organization, and tested whether the \( \lambda \) groES\textsubscript{vibrio} and \( \lambda \) groEL\textsubscript{vibrio} clones were able to complement mutations in the two groE genes of E. coli. The clones \( \lambda \) groEL\textsubscript{vibrio1} and \( \lambda \) groES\textsubscript{vibrio2} complemented both the groES and groEL mutations (Table 2), which suggests that each of these hybrid phages carries the V. harveyi groES and groEL genes, and that these genes may form one operon. The \( \lambda \) groES\textsubscript{vibrio1} clone, unable to complement the groEL mutation (Table 2), most probably carries only the groES gene of V. harveyi.

In order to confirm the presence of the V. harveyi groE genes in the selected \( \lambda \) hybrid clones, we used Southern hybridization and a probe which contained both groE genes of E. coli. We isolated DNA from the hybrid clones, digested the DNA with the ClaI endonuclease, hybridized with the groE DNA probe and found that a 1500 bp fragment of the \( \lambda \) groES\textsubscript{vibrio2} clone gave a positive signal in hybridization (Fig. 1 A, lane 3, and B, lane 2). The 1500 bp fragment did not hybridize with a probe containing DNA of \( \lambda \) vector only (not shown), which indicated that the whole fragment was derived from the V. harveyi chromosome. A 1200 bp fragment of the clone \( \lambda \) groEL\textsubscript{vibrio1} also gave a positive signal in hybridization with the groE probe (not shown), but for further experiments we chose the \( \lambda \) groES\textsubscript{vibrio2} clone.

The 1500 bp ClaI fragment of the \( \lambda \) groES\textsubscript{vibrio2} clone, which revealed homology to the E. coli groE genes, was subcloned in the M13mp18 and 19 vectors cut with the AccI endonuclease and plasmids pDK1 and pDK2 were obtained, respectively. Using the cloned
Table 2. Growth of the \( \lambda \) groE hybrid clones isolated from \( V. \) harveyi genomic library on the \( E. \) coli groE bacteria

<table>
<thead>
<tr>
<th>( \lambda ) phage</th>
<th>( E. ) coli B178 (p.f.u./ml)</th>
<th>( E. ) coli CG2241 groEL44 (p.f.u./ml)</th>
<th>( E. ) coli CG2244 groSS619 (p.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td>( \lambda ) groEL(_{cibrio1} )</td>
<td>( 3 \times 10^9 )</td>
<td>( 3 \times 10^9 )</td>
<td>( 3 \times 10^9 )</td>
</tr>
<tr>
<td>( \lambda ) groES(_{cibrio1} )</td>
<td>( 1.5 \times 10^8 )</td>
<td>&lt;10</td>
<td>( 3 \times 10^8 )</td>
</tr>
<tr>
<td>( \lambda ) groES(_{cibrio2} )</td>
<td>( 3 \times 10^9 )</td>
<td>( 3 \times 10^9 )</td>
<td>( 3 \times 10^9 )</td>
</tr>
<tr>
<td>( \lambda ) EMBL1 vector</td>
<td>( 1 \times 10^9 )</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*plaque-forming units.

1500 bp fragment as a probe, we found that it hybridized efficiently with an EcoRI fragment of \( V. \) harveyi chromosomal DNA and weakly with an EcoRI fragment of \( E. \) coli DNA.

Figure 1. Southern hybridization with the \( E. \) coli groE DNA as a probe.

A. DNA of the clones: \( \lambda \) groEL\(_{cibrio1} \) (lane 1), \( \lambda \) groES\(_{cibrio1} \) (lane 2), \( \lambda \) groES\(_{cibrio2} \) (lane 3) was digested with 
CniI endonuclease and resolved by agarose electrophoresis. Lane 4 contains the DNA molecular mass marker (\( \lambda \) DNA digested with HindIII). The photograph of the gel is shown. The gel was blotted and hybridized with a HindIII-EcoRI DNA fragment of the pOF39 plasmid, containing the \( E. \) coli groE genes (not shown). The arrow points to the 1500 bp fragment homologous to the groE region (in lane 3). B. DNA of the plasmid pOF39 was digested with HindIII-EcoRI (lane 1), DNA of the \( \lambda \) groES\(_{cibrio1} \) phage was digested with Clal (lane 2), DNA of the plasmid pDK1 was digested with XbaI and PstI (lane 3), chromosomal DNA of \( E. \) coli (lane 4) and \( V. \) harveyi (lane 5) was digested with EcoRI. The DNA was resolved in agarose gel, blotted and hybridized with the HindIII-EcoRI DNA fragment of the pOF39 plasmid. Lane 6 contains the DNA molecular mass marker (\( \lambda \) DNA digested with HindIII, labelled with digoxigenin).
These results confirmed that using the λEMBL1 vector we cloned a fragment of the V. harveyi chromosome homologous to the E. coli groE operon.

![Figure 2. Southern hybridization with a fragment of the V. harveyi groE region as a probe.](image)

The 1500 bp DNA fragment of the clone λ groES_vibrio2 cloned in the pDK1 plasmid, was used in hybridization with: λ groES_vibrio2 DNA digested with CiaI (lane 1); plasmid pDK1 digested with XbaI and PstI (lane 2); E. coli chromosomal DNA digested with EcoRI (lane 3); V. harveyi chromosomal DNA digested with EcoRI (lane 4). Lane 5 contains the DNA molecular mass marker (λ DNA digested with HindIII, labelled with digoxigenin).

To further confirm that indeed we cloned the groE region, we sequenced partially the 1500 bp fragment of V. harveyi DNA cloned in the pDK1 and pDK2 plasmids. The sequence found at one of the ends of the fragment has 71.7% homology with the groESL operon of E. coli, specifically with the nucleotides 1468-1850 of the operon (Fig. 3). Since these nucleotides code for the amino acids 334 through 471 of the E. coli GroEL protein, we assume that the 1500 bp CiaI fragment contains the 3' end of the groEL gene. We conclude that the clone λ groES_vibrio2 carries the groE region of V. harveyi.

Most probably, V. harveyi, like the majority of bacteria, has only one copy of the groEL gene, since a single EcoRI fragment of V. harveyi chromosomal DNA hybridized with the probe containing a fragment of the V. harveyi groEL gene (Fig. 2).

Immunoprecipitation with the anti-serum raised against the E. coli GroES showed that the V. harveyi GroES protein was synthesized in E. coli cells infected with the λ groES_vibrio2, but not in those infected with the vector λEMBL1 phage (not shown). It was possible to demonstrate the presence of the V. harveyi GroES protein due to the fact that the molecular mass of the V. harveyi GroES is lower than that of the E. coli GroES [9]. A similar analysis of the groEL protein was not performed, since the GroEL proteins of V. harveyi and E. coli are indistinguishable by SDS/PAGE [9], and the E. coli groE deletion mutants are not viable [2].

Several approaches to cloning bacterial heat shock genes have been used so far. One is to use the polymerase chain reaction and degenerate primers for conserved regions of the heat shock proteins to synthesize homologous DNA probes; these probes serve subsequently in creating and screening genomic libraries. This methodology was used to clone the groEL genes of Rhizobium meliloti [13] and Lactococcus lactis [14], the dnaK genes of R. meliloti [15], Pseudomonas cepacia [15], Streptomyces coelicolor [16], L. lactis [17], Synechococcus [18], Agrobacterium tumefaciens [19] and the dnaJ genes of Coxiella burnetii [20] and A. tumefaciens [19].

Another method is to screen genomic libraries by Southern hybridization, using probes derived from heterologous organisms. This approach resulted in cloning the groESL operons of A. tumefaciens [21] and Rhodobacter sphaeroides [22], the dnaK genes of Bacillus subtilis [23], Clostridium acetobutylicum [24], Brucella ovis [25], the dnaKJ operons of Borrelia burgdorferi [26] and Bradyrhizobium japonicum [27], and the dnaJ gene of L. lactis [14]. A similar method is to use for screening the oli-
Figure 3. Alignment of the partial sequence of the 1500 bp ClaI fragment of V. harveyi groE region (VHGROE) and of the 3' end of the E. coli groEL gene (ECGROE).

Colon marks identical nucleotides. The V. harveyi sequence is 71.7% homologous to the E. coli sequence. The sequence of the E. coli groE operon was published by Hemmingsen et al. [37].

gonucleotide probes corresponding to the most highly conserved sequences of heat shock proteins, as it was done in the case of the groEL and dnaK genes of Synechocystis [28]. Another approach, used in cloning of the groESL operon of Synechococcus [29] and the dnaK gene of Zymonas mobilis [30], involved immunoscreening of genomic libraries with the antibodies raised against the relevant heat shock proteins.

All the described methods are elaborate and time-consuming therefore it seems that screening of a genomic library in a λ vector just by the virtue of growth on a relevant E. coli mutant is such a simple method that it is
worth trying before making use of more complicated approaches. Use of a genomic library in the \( \lambda \)ZAPII vector and \( E. coli \) dnaJ mutant as a host strain enabled Zubr et al. [31] to clone the dnaK operon of Francisella tularensis. Miyazaki et al. [32] cloned the dnaK gene of \( E. coli \) B and the mutant dnaK genes of \( E. coli \) K12 employing the \( \lambda \)EMBL3 libraries and dnaK and dnaJ \( E. coli \) mutants. To our knowledge, this was the first case of successful use of this approach to clone bacterial groE genes.

Complementation of the \( E. coli \) groE mutant strains with \( V. harveyi \) groE

The function of the \( V. harveyi \) GroE proteins in \( E. coli \) was studied by complementation tests using the \( E. coli \) groES619 and groEL44 mutants, which are unable to grow at 42°C. The mutants were lysogenized with phage \( \lambda \) et\( ^{imm^{4}} \)ind (to prevent bacterial lysis by a superinfecting \( \lambda \) phage), infected with the hybrid clone \( \lambda \) groES\_vibri\_groEL, carrying the groE region of \( V. harveyi \), and plated at the permissive (30°C) and nonpermissive (42°C) temperatures (see Materials and Methods). Unlike the control phage carrying the \( E. coli \) groE genes (\( \lambda \)371 groES\_groEL\_), the hybrid phage failed to restore growth at 42°C of the \( E. coli \) groE mutants.

Suppression of the termosensitive phenotype in the case of heterologous heat shock genes is very often not possible, even in the cases of very high homology between the relevant genes and proteins. For example, the DnaK proteins of \( V. harveyi \) (Lipińska et al., unpublished results), \( Brudyrhizobium japonicum \) [27] or \( Borrelia burgdorferi \) [26] do not complement the \( E. coli \) dnaK mutants with respect to termosensitivity. On the other hand, the DnaK proteins of \( Brucella ovis \) [25], \( Zymonas mobilis \) [30] or \( Francisella tularensis \) [31] are able to suppress termosensitivity of the \( E. coli \) dnaK mutants. Recently, it has been shown that a GroEL protein of \( Rhizobium laguminosarum \) complemented a temperature sensitive mutation in the \( E. coli \) groEL gene at 37°C but not at 43°C [33]. The problem of species-specificity of the heat shock chaperone proteins is unsolved and puzzling, especially when considering the current models explaining the molecular mechanisms of the chaperone function. According to these models, the chaperones bind to their target proteins because they recognize the unfolded or misfolded regions of the polypeptides, but not their specific sequences [1]. The GroE chaperones have been shown to function efficiently in heterologous systems in vitro (reviewed in [34]). What makes the chaperones recognize their targets in vivo is not fully understood.

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