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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[o]

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groES **and** ***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 λ in** ***Escherichia coli*** **cells. In order to clone the** ***groEL*** **and** ***groES*** **genes of the marine bacterium** ***Vibrio harveyi,*** **we constructed the** ***V. harveyi*** **genomic library in the λEMBL1 vector, and selected clones which were able to complement mutations in both** ***groE*** **genes of** ***E. coli*** **for bacteriophage λ growth. Using Southern hybridization, in one of these clones we identified a DNA fragment homologous to the** ***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** ***groEL*** **gene of** ***E. coli.*** **This confirmed that the λ clone indeed carries the** ***groE*** **region of** ***V. harveyi.*** **The positive result of our strategy of cloning with the use of the genomic library in λ vector suggests that the same method might be useful in the isolation of the** ***groE*** **homologues from other bacteria. The** ***V. harveyi*** **cloned** ***groE*** **genes did not suppress thermosensitivity of the** ***E. coli groE*** **mutants.**

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Abbreviation: p.f.u., plaque-forming unit.

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 bateriophage λ [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 bovis*) 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 γ-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 λEMBL1 vector phage and the λ recombinant clones, as well as the lysogenic strains used for preparation of λ packaging extracts, were cultured in NZCYM medium [10]. When appropriate, the following antibiotics were added: ampicillin (50 μg/ml) or tetracycline (10 μg/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 λ vector DNA, λEMBL1 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

Strain	Relevant genotype or description	Source or reference
Vibrio harveyi	wild type	J. Lee
Escherichia coli		
NM538	*supF hsdR* r_k^-	our collection [35]
B178	W3110 *galE sup*$^+$	our collection
CG2241	B178 *groEL*44 Tc^R Ts	C. Georgopoulos
CG2244	B178 *groES*619 Tc^R Ts	C. Georgopoulos
BL202	B178(λcI$^+$ *imm*$^\lambda$*ind*)	this work; made by standard lysogenization of B178
BL203	like BL202, but *groEL*44 Tc^R	this work; made by P1 transduction from CG2241
BL204	like BL202, but *groES*619 Tc^R	this work; made by P1 transduction from CG2244
JM101	Δ(*lac-proAB*) *supE thi/F' lacI*^{q} *lac*Δ*M15traD*36 *proAB*$^+$	our collection [10]
TZ144	DH5α *recA*$^-$ (pOF39, Ap^R) – the pOF39 plasmid carries *groE* operon of *E. coli*	O. Fayet [36]
BHB2688	*recA* Su^-(λ *Eam*4 Δ*b*2 *red*3 *imm*434 cIts *Sam*7)/λ	R. Wolinowska [10]
BHB2690	*recA* Su^- (λ *Dam*15 Δ*b*2 *red*3 *imm*434 cIts *Sam*7)/λ	R. Wolinowska [10]
λEMBL1	cloning vector	R. Wolinowska [35]
λ 371	λ gt::8.1 kb *Eco*RI fragment containing *groES*$^+$ and *groEL*$^+$ of *E. coli*	O. Fayet [36]
λ	cI$^+$*imm*$^\lambda$*ind*	C. Georgopoulos
M13mp18/19	cloning vector	our collection [10]

pared from M13 hybrid phages, were performed as described by the manufacturers of the T7 Sequencing™ 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 *Sau*3A 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 *Bam*HI and *Sal*I. 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* BHB2688 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 *Hin*dIII-*Eco*RI DNA fragment of the plasmid pOF39, containing both *groES* and *groEL* genes of *E. coli*, was used as an *E. coli groE* probe. As a *V. harveyi groE* probe, the 1500 kb *Xba*I-*Pst*I fragment of the plasmid pDK1 was used. As a λ probe, the λEMBL1 DNA, digested with *Bam*HI and *Hin*dIII endonucleases, was used. High stringency conditions were applied in all hybridization experiments.

Complementation tests. To test complementation of *E. coli* mutants with respect to λ growth, the $\lambda groE_{Vibrio}$ clones, isolated from the *V. harveyi* genomic library in λEMBL1 vector, were titered on *E. coli* strains with mutations in *groEL* and *groES* genes. Plating was performed at 30°C, because of the thermosensitivity 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 λ $groE_{vibrio}$ phages were able to suppress thermosensitivity of *E. coli groE* mutants, the mutant bacteria carrying the λ $cI^{+}imm^{\lambda}ind$ 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°C in LB supplemented with tetracycline to A_{575} of 0.3, and 100 μl of this culture was mixed with about 10^8 p.f.u. of a tested λ hybrid phage. After 30 min of incubation at room temperature, cells were plated onto L-agar plates and incubated at 30 and 42°C. Abundant growth of normal-looking colonies at 42°C indicated suppression.

RESULTS AND DISCUSSION

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

Bacteriophage λ 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 λ phage to grow in cells with mutations in the *groE* genes. Based on this assumption, we constructed a *V. harveyi* genomic library in the λEMBL1 vector (1.1×10^5 p.f.u./ml) and plated it on *E. coli* CG2241(*groEL*44) and *E. coli* CG2244(*groES*619) strains. Both strains were nonpermissive for growth of the λEMBL1 vector. We obtained one clone, λ $groEL_{vibrio1}$, growing on the *E. coli groEL* bacteria and two clones, λ $groES_{vibrio1}$ and λ $groES_{vibrio2}$, 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 λ $groES_{vibrio}$ and λ $groEL_{vibrio}$ clones were able to complement mutations in the two *groE* genes of *E. coli*. The clones λ $groEL_{vibrio1}$ and λ $groES_{vibrio2}$ 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 λ $groES_{vibrio1}$ 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 λ 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 *Cla*I endonuclease, hybridized with the *groE* DNA probe and found that a 1500 bp fragment of the λ $groES_{vibrio2}$ 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 λ vector only (not shown), which indicated that the whole fragment was derived from the *V. harveyi* chromosome. A 1200 bp fragment of the clone λ $groEL_{vibrio1}$ also gave a positive signal in hybridization with the *groE* probe (not shown), but for further experiments we chose the λ $groES_{vibrio2}$ clone.

The1500 bp *Cla*I fragment of the λ $groES_{vibrio2}$ clone, which revealed homology to the *E. coli groE* genes, was subcloned in the M13mp18 and 19 vectors cut with the *Acc*I endonuclease and plasmids pDK1 and pDK2 were obtained, respectively. Using the cloned

Table 2. Growth of the λ *groE* hybrid clones isolated from *V. harveyi* genomic library on the *E. coli groE* bacteria

λ phage	*E. coli* B178 (p.f.u.*/ml)	*E. coli* CG2241 *groEL44* (p.f.u./ml)	*E. coli* CG2244 *groES619* (p.f.u./ml)
λ $groEL_{vibrio1}$	3×10^9	3×10^9	3×10^9
λ $groES_{vibrio1}$	1.5×10^8	< 10	3×10^8
λ $groES_{vibrio2}$	3×10^9	3×10^9	3×10^9
λ EMBL1 vector	1×10^9	< 10	< 10

*plaque-forming units.

1500 bp fragment as a probe, we found that it hybridized efficiently with an *Eco*RI fragment of *V. harveyi* chromosomal DNA and weakly with an *Eco*RI fragment of *E. coli* DNA

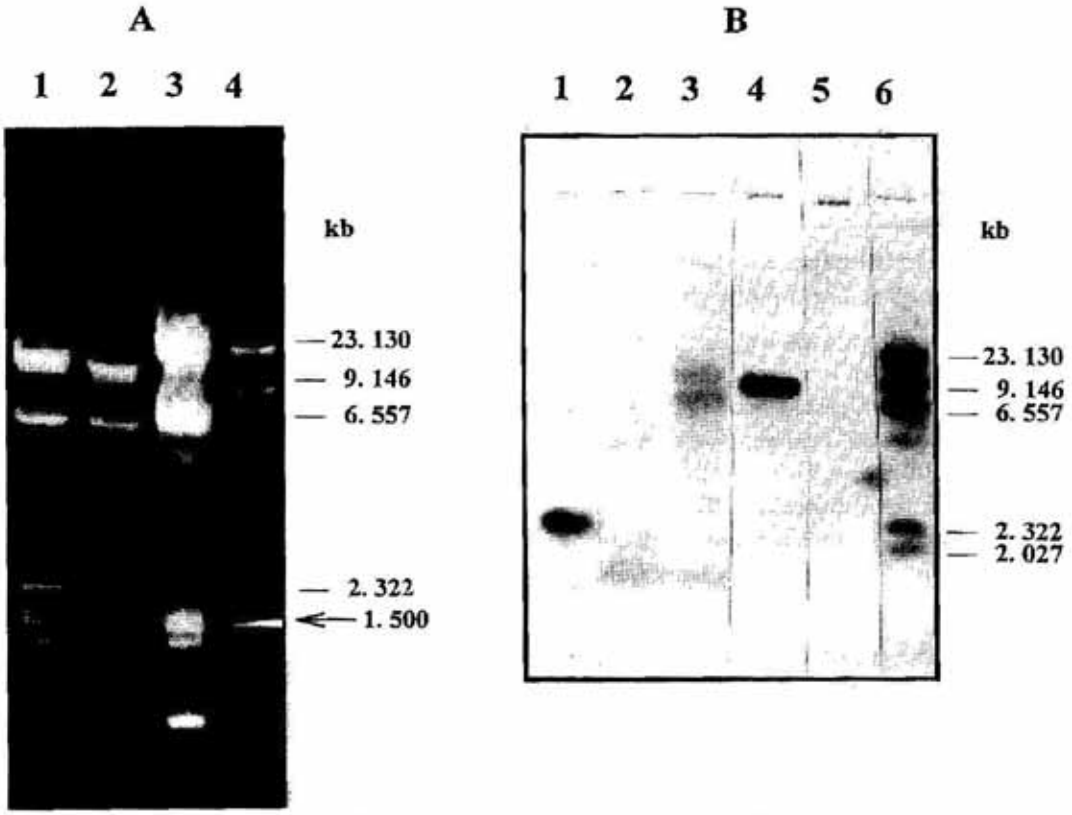

Figure 1. Southern hybridization with the *E. coli groE* DNA as a probe.

A. DNA of the clones: λ $groEL_{vibrio1}$ (lane 1), λ $groES_{vibrio1}$ (lane 2), λ $groES_{vibrio2}$ (lane 3) was digested with *Cla*I endonuclease and resolved by agarose electrophoresis. Lane 4 contains the DNA molecular mass marker (λ DNA digested with *Hin*dIII). The photograph of the gel is shown. The gel was blotted and hybridized with a *Hin*dIII-*Eco*RI 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 *Hin*dIII-*Eco*RI (lane 1), DNA of the λ $groES_{vibrio2}$ phage was digested with *Cla*I (lane 2), DNA of the plasmid pDK1 was digested with *Xba*I and *Pst*I (lane 3), chromosomal DNA of *E. coli* (lane 4) and *V. harveyi* (lane 5) was digested with *Eco*RI. The DNA was resolved in agarose gel, blotted and hybridized with the *Hin*dIII-*Eco*RI DNA fragment of the pOF39 plasmid. Lane 6 contains the DNA molecular mass marker (λ DNA digested with *Hin*dIII, labelled with digoxygenin).

(Fig. 2). These results confirmed that using the λEMBL1 vector we cloned a fragment of the *V. harveyi* chromosome homologous to the *E. coli groE* operon.

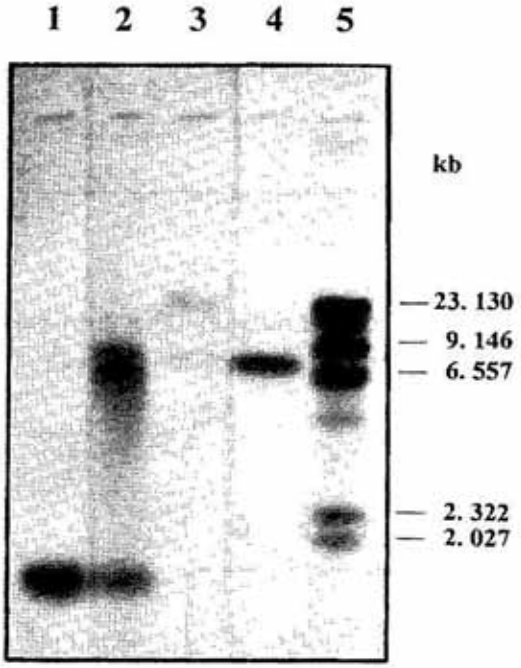

Figure 2. Southern hybridization with a fragment of the *V. harveyi groE* region as a probe.

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 *Cla*I (lane 1); plasmid pDK1 digested with *Xba*I and *Pst*I (lane 2); *E. coli* chromosomal DNA digested with *Eco*RI (lane 3); *V. harveyi* chromosomal DNA digested with *Eco*RI (lane 4). Lane 5 contains the DNA molecular mass marker (λ DNA digested with *Hin*dIII, labelled with digoxygenin).

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 1468–1880 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 *Cla*I 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 *Eco*RI 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-

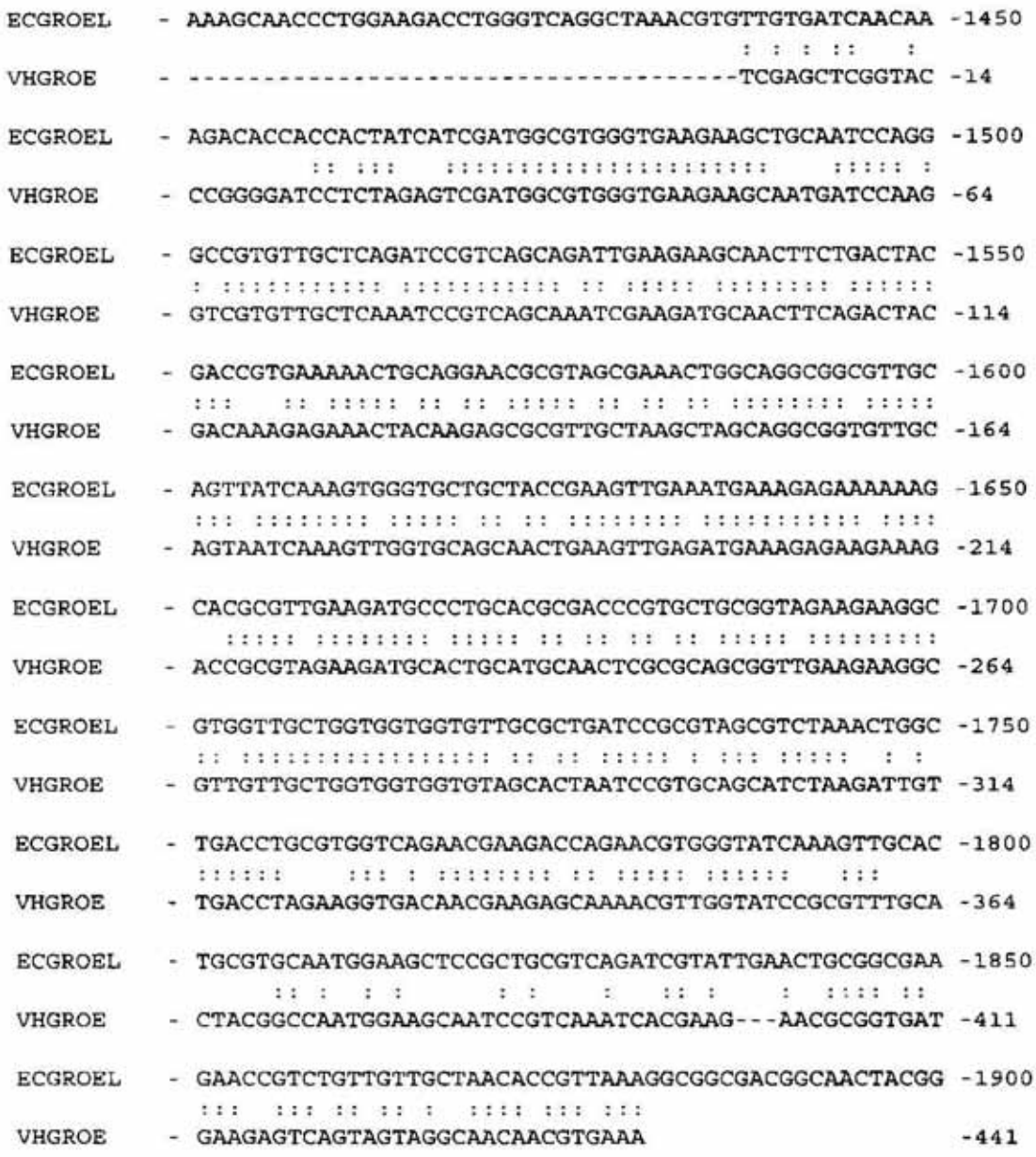

Figure 3. Alignment of the partial sequence of the 1500 bp *Cla*I fragment of *V. harveyi groE* region (VHGROE) and of the 3′ end of the *E. coli groEL* gene (ECGROE).

Colons mark 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 λZAPII vector and *E. coli dnaJ* mutant as a host strain enabled Zuber *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 λEMBL3 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 groES*619 and *groEL*44 mutants, which are unable to grow at 42°C. The mutants were lysogenized with phage λ $cI^{+}imm^{\lambda}ind$ (to prevent bacterial lysis by a superinfecting λ phage), infected with the hybrid clone λ $groES_{vibrio2}$, 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 (λ371 $groES^{+}groEL^{+}$), 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), *Bradyrhizobium japonicum* [27] or *Borrelia burgdorferi* [26] do not complement the *E. coli dna K* mutants with respect to thermosensitivity. On the other hand, the DnaK proteins of *Brucella ovis* [25], *Zymonas mobilis* [30] or *Francisella tularensis* [31] are able to suppress thermosensitivity of the *E. coli dnaK* mutants. Recently, it has been shown that a GroEL protein of *Rhizobium leguminosarum* 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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