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Cloning, sequencing and expression of GroEL-like protein gene of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is an important enteropathogen in regions where much seafood is consumed. Substantial quantity of GroEL-like protein is produced during the heat shock of *V. parahaemolyticus* and located in periplasmic and extracellular fractions. In this study, the GroEL-like protein gene of this pathogen was cloned and sequenced and its properties were analyzed. The open reading frame consisted of 1647 bp, encoded a 57.6-kDa GroEL-like protein of 548 amino acids. The amino acid sequence, hydrophobicity and antigenic pattern of *V. parahaemolyticus* GroEL-like protein were similar to the GroEL proteins of other vibrios, *Escherichia coli* and *Salmonella enterica* Typhi. The *groEL*-like gene of *V. parahaemolyticus* was cloned into the pQE-30 expression vector and the expression of a His-tagged GroEL-like protein was rapidly induced by isopropyl-β-D-thiogalactopyranoside, largely in an insoluble form. The results of this study facilitate the study of the functions of the GroEL-like protein of *V. parahaemolyticus*.

Key words: Base sequence, GroEL protein, heat-shock proteins, *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is an important human enteropathogen, prevalent in Taiwan, Japan and other Asian Pacific countries, that generally inhabits seafood or lives freely in seawater. This pathogen may become a globally important etiologic agent, following the appearance of the first pandemic O3:K6 strains in 1996 and the rapid spread in Asia and to other continents [1].

Environmental stresses influence the physiology and virulence of pathogenic vibrios [2]. In its natural habitat, *V. parahaemolyticus* and other marine vibrios usually live at 20-25°C during the summer and at an even lower temperature during the winter. During infection, *V. parahaemolyticus* is challenged by a temperature upshift from ambient temperature to 37°C or by other stresses. After being heat shocked, *V. parahaemolyticus* produces DnaJ-, GroEL- and GroES-like proteins, with molecular sizes of 47, 62 and 12 kDa, respectively [3]. Production of GroEL-like protein was also demonstrated by Koga et al [4].

Several universal stress or heat-shock proteins have been characterized. These heat-shock proteins normally function in teams; examples are the hsp70 family (DnaK, DnaJ and GrpE) and the hsp60 family (GroEL and GroES) [5]. GroEL is a universal stress protein and usually a higher amount is found in stressed cells than in unstressed cells [6].

As well as exhibiting chaperone activity, GroEL proteins are also associated with the virulence of some pathogenic bacteria. GroEL is also an important immunogen during streptococcal [7] or *Klebsiella pneumoniae* infection [8]. Among the several heat-shock proteins observed, the GroEL-like protein is of special interest in *V. parahaemolyticus*. A large amount of the GroEL-like protein is produced during the heat shock of *V. parahaemolyticus* and being located in the periplasmic and extracellular fractions [3] may result in close interaction with epithelial cells or with the immunological system of human host. A further investigation is required to elucidate the functions of the GroEL-like protein in *V. parahaemolyticus*. In this work, the *groEL*-like gene of *V. parahaemolyticus* was cloned using a polymerase chain reaction (PCR), with primers derived from conserved sequences of *Salmonella enterica* serotype Typhi *groEL* [9], and the cloned gene was sequenced and analyzed.

Materials and Methods

Bacterial strain and cultivation

V. parahaemolyticus ST550, a serotype O4:K13 and Kanagawa phenomenon positive clinical strain isolated in Thailand, was used to clone the *groEL*-like gene in

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this study. The *V. parahaemolyticus* was cultured in tryptic soy agar (Difco Laboratories, Detroit, MI, USA) with the supplement of 3% NaCl at 37°C. For the purification of chromosomal DNA, a single colony of *V. parahaemolyticus* was inoculated into 100 mL Luria-Bertani Broth Medium (Difco)-3% NaCl and cultured at 37°C for 16 hours. *Escherichia coli* strains were cultured in these media without additional NaCl.

DNA techniques

Purification of plasmids and genomic DNA, analysis of DNA by agarose gel electrophoresis, preparation of competent cells and plasmid transformation techniques were as described by Sambrook et al [10]. PCR-amplified products were separated by gel electrophoresis before being purified by a gel band purification kit (Pharmacia, Uppsala, Sweden).

Cloning and sequencing

The *groEL*-like gene of *V. parahaemolyticus* was amplified by PCR using primers (primer-I: 5'-CGCGGATCCG CGATGGACGCTAAAGACGTAAAATTCGG-3'; primer-II: 5'-CACCGTACCCGCCGTACTACATTGC GCCTAGGCGC-3') designed according to the *S. enterica* serotype Typhi *groEL* gene sequence. PCR was performed by a Personal cycler 20 (Biometra biomedizinische analytik Gmbh, Gottingen, Germany) with the following cycle parameters: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and finally, an additional 10 min at 72°C [3].

The 1.6-kb amplified fragment was cloned into pGEM-T Vector (Promega Corp., Madison, WI, USA) by the TA cloning techniques [11] and transformed into *E. coli* JM109 recipient cells.

The DNA sequence of the insert in the recombinant plasmid was determined in both strands with primers for the vector and internal primers. DNA sequencing was performed on an ALFexpress DNA sequencer (Pharmacia) by fluorescence-based dideoxy-sequence reactions. The sequence was verified by repeating the determinations with an ABI Prism dye terminator cycle sequence kit and an ABI model 377-96 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

The Deep Vents DNA polymerase of high PCR fidelity (New England Biolabs, Beverly, MA, USA) was used in these sequencing reactions [12].

Sequence analysis

The sequence determined in this study was compared with homologous sequences from the GenBank Library using BLAST software from the National Center for Biotechnology Information. The amino acid sequence was generated and its properties analyzed by the Expert Protein Analysis System of the Swiss Institute of Bioinformatics. Human leukocyte antigens (HLA) peptide motifs prediction was completed using the world wide web (http://bimas.dcrt.nih.gov/) based on Parker et al 1994 [13]. The B cells epitope prediction of the amino acid sequences were done manually [8].

Expression and analysis of the GroEL-like proteins

The insert from the recombinant plasmid was excised by *Bam*H1 digestion, cloned into the QIAexpressionist pQE-30 expression vector (Qiagen GmbH, Hilden, Germany) and transformed into *E. coli* JM109 [14]. The transformed bacteria were cultured in Luria-Bertani Broth (Difco) supplemented with ampicillin (200 μg/mL) and expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. The bacterial cells were harvested by centrifugation and lysed by a lysozyme treatment [15]. Soluble and insoluble fractions were separated by centrifugation at 10,000 *g* for 15 min at 4°C. The GroEL-like protein was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using an anti-GroEL antibody (Sigma Co., St. Louis, MO, USA) or Penta-His antibody (Qiagen) [3,16]. A Gibco-BRL prestained protein ladder (Gibco-BRL, Gaithersburg, MD, USA) was used as molecular size markers (172.6, 111.4, 79.6, 61.3, 49.0, 36.4, 24.7 kDa).

Accession number

The *groEL*-like gene sequence of *V. parahaemolyticus* reported in this paper was submitted in 2000 to the GenBank Data Library under the accession number AF221845.

Results and Discussion

The open reading frame of the cloned *V. parahaemolyticus groEL*-like gene consisted of 1647 bp and encoded a 57.6-kDa GroEL-like protein (GenBank accession number AAF27528) with deduced amino acid sequence shown in Fig. 1. The properties of the GroEL-like protein of *V. parahaemolyticus* were more similar to those of the *Vibrio* spp. than those of other non-vibrios. Similar results were reported by comparing partial sequences of this gene in different *Vibrio* spp. [17]. This GroEL-like protein was comprised of 548 amino acids with a theoretical isoelectric point (pI) of about 4.68. The

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01   MAAKDVKFGN   DARVKMLEGV   NVLADAVKVT   LGPKGRNVVL   DKSFGAPTIT   KDGVSVAREI
61   ELEDKFQNMG   AQMVKEVASK   ANDAAGDGTT   TATVLAQAIV   NEGLKAVAAG   MNPMDLKRGI
121  DKAVAAAVEQ   LKELSVECND   TKAIAQVGTI   SANSDASVGN   IIAEAMERVG   RDGVITVEEG
181  QALQDELDVV   EGMQFDRGYL   SPYFINNQEA   GSVELENPFI   LLVDKKISNI   RELLPTLEAV
241  AKASRPLLII   AEDVEGEALA   TLVVNNMRGI   VKVAAVKAPG   FGDRRKAMLQ   DIAILTGGTV
301  ISEEIGLELE   KVTLEDLGQA   KRVSITKENS   TIIDGAGEEA   MIQGRVAQIR   QQIEDATSDY
361  DKEKLQERVA   KLAGGVAVIK   VGAATEVEMK   EKKDRVEDAL   HATRAAVEEG   VVAGGGVALI
421  RAASKIVDLE   GDNEEQNVGI   RVALRAMEAP   IRQITKNAGD   EDSVVANNVK   AGEGSYGYNA
481  ATGEYGDMLE   MGILDPTKVT   RSALQFAASV   AGLMITTEAM   VTDLPQKESA   GMPDMGGMGG
541  MGGMGGMM
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Fig. 1. Deduced amino acid sequence of the cloned GroEL-like protein of *Vibrio parahaemolyticus.*

GroEL protein of *Vibrio cholerae* (GenBank accession number AAF95805) consists of 544 amino acids with a molecular mass of 57.2 kDa, and a pI of 4.78, while the GroEL protein of *Vibrio vulnificus* (GenBank accession number AY017169) consists of 547 amino acids with a molecular mass of 57.7 kDa, and a pI of 4.71. A typical structure in GroEL, Gly-Gly-Met repeated at the C-terminus, was observed in the *V. parahaemolyticus* GroEL-like protein sequence [18].

The deduced amino acid sequence of *V. parahaemolyticus* GroEL-like protein (Fig. 1) was compared with those of known bacterial GroEL proteins. A high amino acid identity (84-92%) was observed among all these sequences. The amino acid sequence of this *V. parahaemolyticus* GroEL-like protein exhibited a particularly high identity with the GroEL proteins of *V. cholerae* and *V. vulnificus* (92%) [Table 1], whereas it revealed 91% identity with the deduced protein from the genome of *V. parahaemolyticus* RIMD 2210633, which is the first pandemic O3:K6 strain (Table 1). The alignment of the deduced amino acid sequence of *V. parahaemolyticus* with the GroEL protein sequences of other vibrios, *E. coli* and *S. enterica* Typhi, revealed extensive similarity along the length, verifying the identity of the former as a GroEL homolog.

Table 1. Comparative amino acid sequences of the GroEL-like protein of *Vibrio parahaemolyticus* and other corresponding published bacterial sequences.

Species	GenBank accession number	Identity (%)
Vibrio vulnificus	AAG48876	92
Vibrio cholerae	AAF95805	92
Vibrio parahaemolyticus[a]	NP799230	91
Escherichia coli O157:H7	BAB38547	86
Salmonella typhi	AAA85277	85
Enterobacter intermedius	BAA25209	84
Erwinia aphidicola	BAA25239	84
Klebsiella pneumoniae	BAA25225	84
Pseudoalteromonas haloplanktis	CAB50775	84
Yersinia enterocolitica	S26423	84

[a]The deduced protein from the genome of the first pandemic O3:K6 strain *V. parahaemolyticus* RIMD 2210633.

GroEL proteins are strong HLA, and are associated with inflammatory diseases, such as ankylosing spondylitis [8]. Epitopes with a strong affinity for HLA have been identified using computer software [13]. In this study, the GroEL proteins of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*, *E. coli*, and *S. enterica* Typhi were compared. These pathogens differ in pathogenesis; however, when the motifs for different HLA molecules were analyzed, their GroEL proteins exhibited similar but not identical patterns. All the motifs for the HLA-B27 molecule have an arginine residue in the second position of the nonapeptides [8]. Twenty three arginine residues were present in the GroEL-like protein of *V. parahaemolyticus*; therefore, 23 nonapeptides with affinity for the HLA-B27 molecule, were identified. Among these, 3 showed high affinity, namely residues 57-65 (AREIELEDK, score of 2000; score is the estimation of half time of dissociation of a molecule containing this sequence), residues 284-292 (RRKAMLQDI, score of 1800) and residues 444-452 (LRAMEAPLR, score of 1000). All the GroEL-like proteins of these 3 *Vibrio* spp. exhibited similar motif sequences, except for the nonapeptide residues 117-125 (KRGIDKAVA), in which the *V. cholerae* GroEL-like protein had an isoleucine rather than an alanine residue in the last position and had an affinity score increasing from 600 to 1800. Motif sequences of GroEL-like proteins of these 3 *Vibrio* spp. differed from those of *E. coli* and *S. enterica* Typhi by a single nonapeptide (residues 12-20, ARVKMLEGVNV) with a glutamic acid as a substitute for an arginine.

Three B cell epitopes, corresponding to those of *E. coli*, were identified in the GroEL-like proteins of these *Vibrio* spp. [8]; they were residues 283-291 (DRRKAMLQD), 361-369 (DKEKLQERV) and 390-398 (KEKKDRVED) with only a single amino acid switch in the last 2 sequences (Fig. 2). The arginine and alanine in residue 362 and 394 of the *E. coli* GroEL

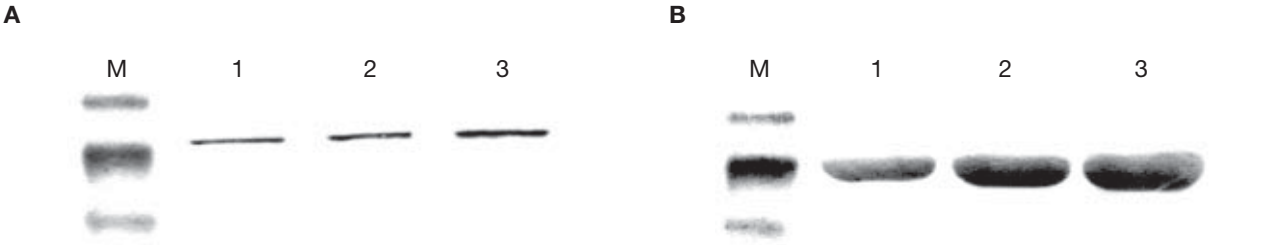

Fig. 2. Expression of the recombinant GroEL-like protein of *Vibrio parahaemolyticus* in soluble **(A)** and insoluble **(B)** fractions. The bacterial culture was induced by isopropyl-β-D-thiogalactopyranoside for 30 minutes (lane 1), 1 hour (lane 2) and 2 hours (lane 3), and the proteins were separated into soluble and insoluble fractions. They were visualized by immunoblotting with Penta-His antibody. Lane M shows the prestained protein ladder markers (Gibco-BRL), 79.6, 61.3, and 49.0 kDa from the top.

were replaced by lysine and aspartic acid, respectively, in the GroEL-like proteins of these 3 *Vibrio* spp. The HLA and B cell epitope analysis shows that the antigenic properties of GroEL-like protein of *V. parahaemolyticus* were similar to other GroEL proteins.

The hydrophobicity plot of the amino acid sequence in the GroEL-like protein of *V. parahaemolyticus* (Fig. 3A) was compared with that in *E. coli* GroEL (Fig. 3B). The sequences exhibited similar hydrophobicity patterns, except that the amino acid residues 120-150 were hydrophilic in *V. parahaemolyticus* GroEL-like protein but hydrophobic in *E. coli* GroEL. The 3-dimensional configuration of the *V. parahaemolyticus* GroEL-like protein was also generated using the Expert Protein Analysis System of the Swiss Institute of Bioinformatics, and was spatially similar to that of the GroEL of *E. coli* (data not shown).

The *groEL*-like gene of *V. parahaemolyticus* was excised by *Bam*H1 digestion and cloned into the QIAexpressionist pQE-30 expression vector and then transformed into *E. coli* JM109. The expression of such His-tagged *V. parahaemolyticus* GroEL-like protein was analyzed and verified by gel electrophoresis followed by immunoblotting with anti-GroEL or Penta-His antibodies. The His-tagged protein was slightly larger than sequence-deduced 57.6-kDa GroEL-like protein (Fig. 2). The expression of the *V. parahaemolyticus* GroEL-like protein was induced by IPTG for 30 min to 5 hours; a large quantity of GroEL-like protein was detected; the quantity was maximum in about 1 hour (Fig. 2). This tagged GroEL-like protein could be conveniently purified using single-step affinity nickel-nitrilotriacetic acid column chromatography [7], and subjected to further physiological or pathological studies.

The IPTG-induced bacterial cultures were lysed and the soluble and insoluble fractions were analyzed. Only a small amount of *V. parahaemolyticus* GroEL-like protein was observed in the soluble fraction,

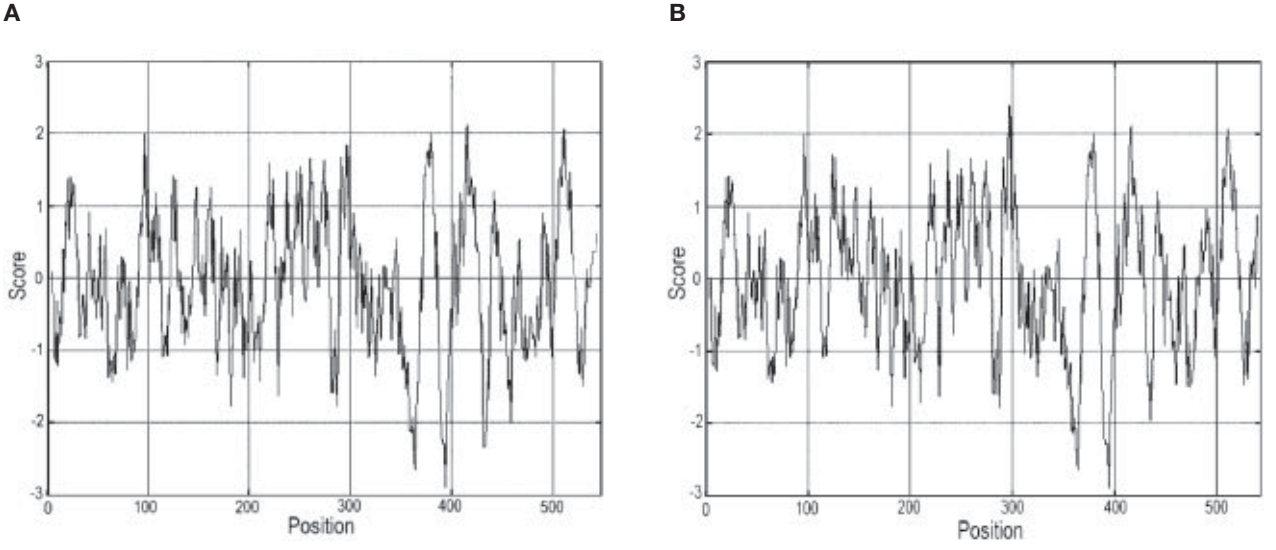

Fig. 3. Comparison of the hydrophobicity of the amino acid sequences of the GroEL-like protein of *Vibrio parahaemolyticus* **(A)** and *Escherichia coli* **(B)**.

while there was a large quantity in the insoluble fraction (Fig. 2). GroEL and DnaK are known to be antagonist controllers of inclusion body formation by promoting and preventing, respectively, the aggregation of proteins in cytoplasm [19]. As expected, the formation of an inclusion body of the over-produced *V. parahaemolyticus* GroEL-like protein was observed whenever DnaK protein was not simultaneously enhanced. The solubilized form may be produced by reducing the IPTG stimulation or by simultaneously over-producing both GroEL and DnaK proteins to obtain the functional *V. parahaemolyticus* GroEL-like protein for further study.

In conclusion, the GroEL-like protein gene of *V. parahaemolyticus* was cloned, sequenced and over-produced by IPTG induction, largely in inclusion body form. The characteristics of this GroEL-like protein were similar, but not identical, to corresponding proteins of other bacterial spp.

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