Cloning, sequencing and expression of a GroEL-like protein gene of 

*Vibrio vulnificus*

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Running title: *groEL* gene of *Vibrio vulnificus*

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ABSTRACT

*Vibrio vulnificus* causes wound infection and septicemia in humans with high mortality via the consumption of contaminated seafood. This study cloned, sequenced and analyzed the general properties of the GroEL-like protein gene of this pathogen. A 1.6 kb amplified DNA fragment from *V. vulnificus* ATCC27562 was cloned into pGEM-T Vector and nucleotide sequenced. The open reading frame consisted of 1,644 bp, and encoded for a 57.7 kDa GroEL-like protein of 547 amino acids. The amino acid sequence of the *V. vulnificus* GroEL-like protein showed high identity (82-92%) with other GroEL proteins, especially those of vibrios, *E. coli* and *S. enterica* Typhi. The amino acid residues 269 to 282 of the *V. vulnificus* GroEL-like protein were significantly more hydrophobic than others, with two basic lysine residues replaced by hydrophobic isoleucine residues. The *groEL*-like gene of *V. vulnificus* was cloned into the pQE-30 expression vector and expression of a His-tagged GroEL-like protein was rapidly induced by isopropyl-β-D- thiogalactopyranoside and confirmed by immunoblotting with anti-GroEL and Penta-His antibodies. The over-produced GroEL-like protein of *V. vulnificus* was present largely in an insoluble form. Results of this report may facilitate the study of responses of *V. vulnificus* to environmental stresses.

Key Words: *Vibrio vulnificus*, GroEL, heat shock protein, nucleotide sequence
INTRODUCTION

*Vibrio vulnificus* causes human wound infection and septicemia, largely a result of occupational activities around seawater. *V. vulnificus* occurs in high numbers in molluscan shellfish, primarily oysters, and its ingestion in raw oysters results in a ca. 60% mortality in those persons who are susceptible to this bacterium (Linkous and Oliver 1999). In addition to humans, *V. vulnificus* is also pathogenic to aquatic and other animals, such as eels, shrimp, mice, etc. (Amaro et al, 1994; Biosca et al, 1999).

The physiology and virulence of pathogenic vibrios are affected by environmental stresses (Parsot and Mekalanos 1990; Yildiz and Schoolnik 1998). In *V. vulnificus*, highly variable responses to environmental stresses are found in different strains. Starvation enhances freeze-thaw resistance and heat tolerance for some strains (Bang and Drake 2002). Other than studies on the viable but nonculturable state, the effect of various stresses on *V. vulnificus* has not been well characterized. Since the presence of the environmental stress mediator ToxRS protein has been shown in *V. vulnificus* (Lee et al, 2000), *V. vulnificus* may also have tightly regulated responses to environmental changes, as do *V. cholerae*, *V. parahaemolyticus* and other vibrios.

Beside their chaperone activity, GroEL proteins are also associated with the virulence of some pathogenic bacteria. The GroEL-like protein of *Actinobacillus actinomycetemcomitans* is found in extracellular material and is strongly toxic for HaCaT epithelial cells (Goulhen et al,
A significant amount of GroEL homolog protein is absorbed on the surface of Helicobacter pylori (Dunn et al, 1997). The GroEL-like protein of Campylobacter rectus stimulates both interleukin-6 and IL-8 secretion by a confluent monolayer of human gingival fibroblast cells (Hinode et al, 1998). During infection, V. vulnificus moves from the environment into the human body and is challenged by a shift-up of incubation temperature. Heat shock proteins like GroEL may be produced and play a role in the pathogenesis of this bacterium. In this study, the groEL-like gene of V. vulnificus was cloned using polymerase chain reaction (PCR) method with primers derived from the conserved sequences of the Salmonella enterica serotype Typhi groEL gene (Lindler and Hayes 1994; Rusanganwa et al, 1992), and the cloned gene was sequenced and analyzed.

MATERIALS AND METHODS

Bacterial strain and cultivation

The type strain V. vulnificus ATCC27562 was used for the cloning of the groEL-like gene in this study. It is a hemolytic strain isolated from a human blood sample (Okada et al, 1987). Bacterial strains were stored in culture broth with 10% glycerol at –85°C. V. vulnificus was cultured in Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI, USA) with a supplement of 3% NaCl, or Luria-Bertani Agar Medium (LA, Difco)-3% NaCl (pH 7.5) at 37°C. For the purification of chromosomal DNA, a single colony of V. vulnificus was inoculated into 100 ml Luria-Bertani Broth Medium (LB, Difco)-3% NaCl and cultured at 37°C for 16 h.
*Escherichia coli* JM109 was 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.* (Sambrook *et al.*, 1989). PCR amplified products were separated by gel electrophoresis and purified by a gel band purification kit (Pharmacia, Uppsala, Sweden).

**Cloning and sequencing**

The *groEL*-like gene of *V. vulnificus* was amplified by PCR using primers (5’-CGCGGATCCGGATGGACGCTAAAGACGTAAAATTCGG, 3’-CGCGGATCCGGTTACATCATGCCGCCCATGCCAC) designed according to the *S. typhi groEL* gene sequence (Lindler and Hayes 1994). PCR was performed by a Personal cycler 20 (Biometra biomedizinische analytik GmbH, Gottingen, Germany) with the following 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 (Wong *et al.*, 1998).

The 1.6 kb amplified fragment was cloned into pGEM-T Vector (Promega Corp., Madison, WI, USA) by the TA cloning techniques (Zhou and Gomez-Sanchez 2000) and transformed into *E. coli* JM109 recipient cells (Sambrook *et al.*, 1989).
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 confirmed 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 (Cline et al, 1996).

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 done using the world wide web (http://bimas.dcter.nih.gov/) based on Parker et al. 1994 (Parker et al, 1994). The B cells epitope prediction of the amino acid sequences were done manually (Cancino-Diaz et al, 1998).

Expression and analysis of the GroEL-like protein

The insert from the recombinant plasmid was excised by BamH1 digestion, cloned into the QIAexpressionist pQE-30 expression vector...
(Qiagen GmbH, Hilden, Germany) and transformed into E. coli JM109 (Chow et al, 2000). 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 for 30 min to five hours. The bacterial cells were harvested by centrifugation and lysed by a lysozyme treatment (Thies et al, 1999). Soluble and insoluble fractions were separated by centrifugation at 10,000g 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)(Wong et al, 1998; Wong et al, 2002). A prestained protein ladder (Gibco-BRL, Gaithersburg, MD, USA) was used as molecular size markers.

Accession numbers

The groEL-like gene sequence of V. vulnificus reported in this paper has been submitted in 2000 to the GenBank Data Library under the accession number AY017169.

RESULTS AND DISCUSSION

A 1.6 kb amplified DNA fragment from V. vulnificus ATCC27562 was cloned into a pGEM-T Vector and nucleotide sequenced (GenBank accession number AY017169). The open reading frame consisted of 1,644 bp and encoded a 57.7 kDa GroEL-like protein. The properties of this V. vulnificus deduced GroEL-like protein were similar to those of other
*Vibrio* species, *S. enterica* serotype Typhi and *E. coli*. The *V. vulnificus* GroEL-like protein consisted of 547 amino acids with a theoretical pI of ca. 4.71. The GroEL protein of *V. cholerae* (GenBank accession no AAF95805) consisted of 544 amino acids, 57.2 kDa, and pI of 4.78, while the GroEL protein of *V. parahaemolyticus* (GenBank accession no AAF27528) consisted of 548 amino acids, 57.6 kDa, and a pI of 4.68. A typical structure of GroEL molecules, a Gly-Gly-Met repeat at the C-terminus, was found in the *V. vulnificus* groEL-like sequence (Fig. 1).

The deduced amino acid sequence of the *V. vulnificus* GroEL-like protein was compared to known bacterial HSP60 or GroEL protein sequences. It is highly similar to the groEL sequences of *V. vulnificus* YJ016 and CMCP6 which were released after the completion of our present study (Table 1). High amino acid identity (82-92%) was found in all other sequences, and the amino acid sequence of the *V. vulnificus* GroEL-like protein showed especially high identity with GroEL proteins of *V. cholerae* (91%) and *V. parahaemolyticus* (92%) (Table 1). Alignment of the deduced amino acid sequence of *V. vulnificus* with GroEL protein sequences of other vibrios, *E. coli* and *S. enterica* Typhi (Fig. 1) showed extensive similarity throughout its length, confirming its identity as a GroEL homolog.

GroEL proteins are strong human leukocyte antigens (HLA), and are associated with inflammatory diseases, such as ankylosing spondylitis (Cancino-Diaz *et al*, 1998). Epitopes with a strong affinity for HLA have been identified using computer software (Parker *et al*, 1994). In this study,
the GroEL proteins of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, *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 (Cancino-Diaz et al, 1998). Twenty-three arginine residues were present in the GroEL-like protein of *V. vulnificus*; therefore, 23 nonapeptides with affinity for the HLA-B27 molecule, were identified; among these, three showed high affinity, namely residues 57-65 (AREIELEDK, score of 2000, score is the estimation of half time of disassociation 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 three *Vibrio* species 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 increased from 600 to 1800. Motif sequences of GroEL-like proteins of these three *Vibrio* species differed from those of *E. coli* and *S. enterica* Typhi by a single nonapeptide (residues 12-20, ARVKMLEGV) 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 *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* species (Cancino-Diaz et al, 1998); they were residues 283-291 (DRRKAMLQD), 361-369 (DKEKLQERV) and
390-398 (KEKKDRVED) with only a single amino acid switch in the last two sequences (Fig. 1). The arginine and alanine in residue 362 and 394 of the *E. coli* GroEL were replaced by lysine and aspartic acid, respectively, in the GroEL-like proteins of these three vibrios. The HLA and B cell epitope analysis showed that the antigenic properties of GroEL-like protein of *V. vulnificus* were similar to other GroEL proteins.

The hydrophobicity of the GroEL-like protein amino acid sequence of *V. vulnificus* (Fig. 2A) was compared with the *E. coli* GroEL (Fig. 2B). Identical hydrophobicity profile was observed in both sequences, although amino acid residues 269 to 282 of the *V. vulnificus* GroEL-like protein were significantly more hydrophobic than the relative segment of *E. coli* GroEL (Fig. 2B). In this segment, two basic lysine residues were replaced by two hydrophobic isoleucine residues in the *V. vulnificus* GroEL (Fig. 1). GroEL proteins are chaperonins forming ring-shaped oligomeric protein complexes that are of crucial importance for protein folding *in vivo*. The interior cavity of GroEL offers a hydrophilic-like environment to the substrate protein (Betancourt and Thirumalai 1999). The hydrophobic portion of the GroEL protein sequence is involved in the recognition and stabilization of the protein and substrate complexes (Fenton and Horwich 1997; Sparrer *et al.*, 1996). Such enhancement of hydrophobicity of certain segments of the GroEL protein may affect its chaperonin activity.

The three-dimensional configuration of the *V. vulnificus* GroEL-like protein was also generated by the Expert Protein Analysis System of
Swiss Institute of Bioinformatics and showed similar spatial configuration with the GroEL of *E. coli* (data not shown).

The *groEL*-like gene of *V. vulnificus* was excised by *Bam*H1 digestion and cloned into the QIAexpressionist pQE-30 expression vector and transformed into *E. coli* JM109. Expression of the His-tagged *V. vulnificus* GroEL-like protein was analyzed and confirmed by gel electrophoresis followed by immunoblotting with anti-GroEL or Penta-His antibody. It was slightly larger than the deduced 57.7 kDa GroEL-like protein (Fig. 3). Expression of the *V. vulnificus* GroEL-like protein was induced by IPTG for 30 min to two hours, and a high quantity of GroEL-like protein was detected that reached a maximum level in about one hour (Fig. 3).

The IPTG-induced bacterial cultures were lysed and the soluble and insoluble fractions were analyzed. Only small amounts of *V. vulnificus* GroEL-like protein were detected in the soluble fraction, while large amounts were in the insoluble fraction (Fig. 3). GroEL and DnaK are known to be antagonist controllers of inclusion body formation by promoting and preventing, respectively, the aggregation of proteins in the cytoplasm (Carrio and Villaverde 2003). It was thus reasonable to observe the presence of inclusion bodies of the over-produced *V. vulnificus* GroEL-like protein while DnaK protein did not simultaneously enhance inclusion body formation. The solubilized form may be produced by reducing the IPTG stimulation or by simultaneous over-producing both GroEL or DnaK proteins to obtain the functional *V. vulnificus*
GroEL-like protein for further study.

In conclusion, the GroEL-like protein gene of *V. vulnificus* was cloned, sequenced and over-produced by IPTG induction, largely in an inclusion body form. Properties of this GroEL-like protein were similar to other corresponding proteins of other bacterial species.

**ACKNOWLEDGEMENT**

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REFERENCES


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Table 1. Comparison of the amino acid sequence of the GroEL-like protein of *Vibrio vulnificus* with other published bacterial sequences.

<table>
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<tr>
<th>Species</th>
<th>GenBank Accession Number</th>
<th>Identity, %</th>
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<td>AP005342</td>
<td>97</td>
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<tr>
<td><em>Vibrio vulnificus</em> CMCP6</td>
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<td><em>Vibrio parahaemolyticus</em></td>
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<td><em>Vibrio cholerae</em></td>
<td>AAF95805</td>
<td>91</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>BAB38547</td>
<td>84</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>AAK03191</td>
<td>84</td>
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<td><em>Actinobacillus</em></td>
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Legends for Figures

Fig. 1. Alignment of the amino acid sequence of the GroEL-like protein of *Vibrio vulnificus* with the corresponding published sequences of *V. parahaemolyticus* (VP), *V. cholerae* (VC), *Salmonella enterica* serotype *Typhi* (SE) and *Escherichia coli* (EC). Positions that are identical in all five bacterial species are noted with asterisks. Gaps have been introduced to maximize similarity. The hydrophobic amino acid residues 269 to 282 are in bold. The underlined amino acid sequences are the predicted B cell epitopes.

Fig. 2. Hydrophobicity of the amino acid sequences of the GroEL-like protein of *Vibrio vulnificus* (A) and *Escherichia coli* (B). The score represents the hydrophobicity of the amino acid residue in the sequence. Higher the number represent higher hydrophobicity of the residue.

Fig. 3. Expression of the recombinant GroEL-like protein of *Vibrio vulnificus* in the soluble (A) and insoluble fraction (B). The bacterial culture was induced by ITPG for 30 min (lane 1), 1 h (lane 2) and 2 h (lane 3), and the proteins separated into soluble and insoluble fractions (B) and visualized by immunoblotting with Penta-His antibody. Lane M contains the prestained protein ladder markers (Gibco-BRL), 79.6, 61.3 and 49.0 kDa, from top.
Fig. 1
Fig. 2
Fig. 3