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Expression analysis *groE* gene under high pressure conditions from deep-sea piezophilic *Shewanella violacea*

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The *groESL* operon of the deep-sea piezophilic and psychrophilic bacterium *Shewanella violacea*, was cloned and sequenced by screening lambda phage library. The molecular masses of GroES and GroEL proteins were calculated to be 10,213 and 57,079 Da, respectively. Expression studies by Northern blot analysis under high pressure conditions showed that the amount of groESL mRNA was increased by elevated pressure conditions. Primer extension analysis showed that five transcription initiation sites were existed. Out of five promoters, the site at the most upstream (P1) was the *Escherichia coli* s^{32}-like promoter. No consensus promotes were found in the remained four promoters, suggesting the novel promoters specific for the high pressure conditions.

Key words *Shewanella violacea*, heat shock protein, *groESL* operon, molecular chaperone

Introduction

The deep-sea environment is a unique habitat characterized by extremely high pressure and low temperature and microorganisms living there have adapted to this extreme environment. We have identified several piezophilic bacteria isolated from deep-sea mud samples [1]. *Shewanella violacea* strain DSS12 is one of the piezophilic organisms isolated from the Ryukyu Trench at a depth of 5110 m, and it grows optimally at 30 MPa and 8°C [1, 2]. We are interested in examining the molecular mechanisms of gene regulation in piezophilic bacteria at high pressure. In our previous studies, a pressure-regulated operon, controlled by elevated pressure at the level of transcription, was cloned from this strain [3]. A variety of environmental stresses induce the synthesis of a set of highly conserved proteins called heat shock proteins (HSPs) [4]. These proteins play important roles in assisting the folding, assembly and degradation of proteins, not only under stress conditions, but also under normal growth conditions. Although many reports have described the HSPs of mesophilic and thermophilic bacteria, little is known about the mechanisms of the response of piezophilic bacteria to not only high temperature conditions, but also high pressure conditions [5]. One of HSPs, the GroES and GroEL proteins are thought to be evolutionarily conserved with those of mesophilic and thermophilic bacteria. Thus, in order to understand piezoresponse of these stress proteins in the deep-sea bacterium, it is important to clone *groESL* operon from the strain. In this paper, we describe the cloning of *groESL* operon from this strain, and analysis of its sequence, and it is demonstrated that expression of *groESL* is positively controlled at the transcriptional level by elevated pressure. Our results suggest that σ^{32} may contribute to pressure-regulation in this piezophilic bacterium.

Materials and Methods

Bacterial strains and culture conditions

S. violacea strain DSS12 [1, 2] and *E. coli* strain JM109 were used in this study. *S. violacea* cells were grown at atmospheric pressure and 8°C with vigorous shaking in Marine Broth 2216 medium (Difco, Sparks, MD, USA). Cells of *E. coli* strain JM109 were grown at 37°C with vigorous shaking in Luria-Bertani medium.

Isolation of the *groESL* operon from *S. violacea*

First in order to isolate *groES* genes, we prepared a hybridization probe for the gene. Based on the amino acid sequences of the gene from several gram-negative bacteria (*E. coli*, *Pseudomonas putida*, *Vibrio cholerae*, and *Yersinia pestis*) were aligned, conserved sequences, two synthetic

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degenerate oligonucleotide primers (data not shown), were designed and synthesized to amplify part of the *groES* gene from *S. violacea*. A fragment of approximately 300 bp amplified by PCR and expected to contain part of the *rpoB* gene was cloned into the pCR2.1 vector and its nucleotide sequence was determined. To clone the complete *groESL* operon, the partial *groES* gene fragment was labeled with digoxygenin (DIG) in PCR as a hybridization probe for plaque hybridization. Chromosomal DNA isolated from *S. violacea* was partially digested with *Sau*3AI. These fragments were inserted into the *Bam*HI site of lambda DASH II (Stratagene Co., La Jolla, CA, USA). Then, *in vitro* packaging of the ligated DNA was performed using GIGAPACK III XL packaging extracts (Stratagene Co.) according to the manufacturer's instructions. The DSS12 λ phage library was screened for plaque hybridization with the *groES* probe and a positive clone was obtained. The positive clone containing the *groES* gene was purified by several single-plaque isolation steps. The insert in the λ phage was amplified by long PCR and was subcloned into the pCR-Blunt vector (Invitrogen Co., Carlsbad, CA, USA). For sequencing of these cloned fragments, the random shotgun sequencing method was used with a DNA sequencer model 377 (Perkin-Elmer/Applied Biosystems Co., Foster City, CA, USA).

Northern hybridization analysis

Total cellular RNA was prepared from *S. violacea* cells cultivated at 0.1, 10, 30, and 50 MPa, using Sepasol RNA I (Nakalai Tesque, Japan) as described previously [6]. The RNA pellet was dissolve in diethyl pyrocarbonate-treated water, quantified by spectrophotometry, and stored at -80°C. Northern blot analysis was performed by the method reported previously [6]. Probes for Northern blot analysis were constructed by the PCR DIG Probe Synthesis Kit(Boehringer Mannheim Co.).

Primer extension analysis

Total cellular RNA was prepared from *S. violacea* cells cultivated at 0.1, 10, 30, and 50 MPa, using Sepasol RNA I (Nakalai Tesque, Japan) as described previously [6]. Primer extension analysis was carried out according to the procedure previously reported [7], using the following synthesized oligonucleotides primers, ES-1, 5'-ACC ACG GGT ATC GGT AAA GCG TAG ATC AAC-3' and ES-2, 5'-GGA TTG AGA CGT GCT GTT CTT TAC CAC GGG-3'. Transcripts were analyzed on a 5% polyacrylamide gel under denaturing conditions The sequencing ladder was generated using the same primers as those used in the dideoxy sequencing reactions.

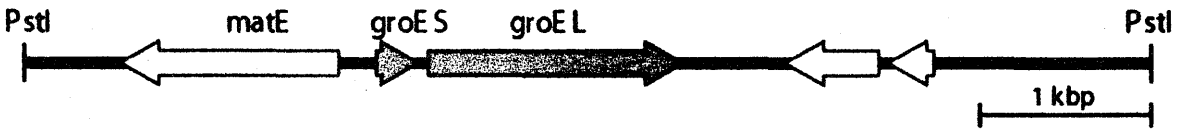

Fig. 1 Restriction map of the genomic region containing the *groES* and *groEL* genes in *Shewanella violacea*.

Results and discussion

Structural analysis of the *rpoBC* genes of *S. violacea*

By screening the λ phage library, a fragment containing *groES* followed by *groEL* was obtained (Fig. 1). Sequence analysis showed that the gene organization and orientation of the fragment containing *groESL* operon is identical with those in *E. coli*. Therefore, *groESL* oepron is likely to be transcribed as an operon, as reported for *E. coli*. The open reading frames of the *groES* and *groEL* genes consist of 291 and 1,638 bp, respectively. The *groES* gene encodes a protein consisting of 96 amino acid residues with a molecular mass of 10,213 Da, whereas the *groEL* gene encodes a protein consisting of 545 amino acid residues with a molecular mass of 57,979 Da (data not shown).

Significant homology was found when comparing the GroESL proteins of *S. violacea* with those of *E. coli* (*groES*, 79.5%; *groEL*, 78.6%), *P. putida* (*groES*, 69.1%; *groEL*, 71.0%), and *Bacillus subtilis* (*groES*, 44.2%; *groEL*, 46.2%). Evolutionarily conserved regions were also found to exist over the predicted amino acid sequences.

Expression studies of the *groESL* genes of *S. violacea*

In order to understand pressure response (piezoresponse) at molecular level of the genes for *groES* and *groEL*, Northern blot analysis of RNAs from the moderately piezophilic bacterium, *Shewanella violacea* grown under varied pressure conditions, was carried out. As shown in lane 1 of Fig. 2, small quantity of mRNA for *groEL* gene was

observed under atmospheric pressure condition (0.1 MPa) and relatively large quantity of mRNA for *groEL* gene was observed under both 30 MPa and 50 MPa (lane 2 and 3of Fig. 2).

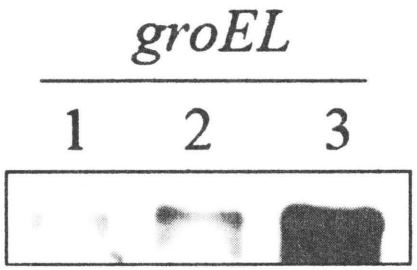

Fig. 3. Different effects of groEL gene in *S. violacea* under the pressure conditions.
Lane: 1, total RNA from 0.1 MPa culture; 2, 30 MPa; 3, 50 MPa.

The result shows that the expression of the gene, *groEL*, was positively controlled at transcriptional level by elevated pressure. The molecular basis for the mechanism in transcription under high pressure conditions may depends upon the natures upstream region (promoter sequence) of the gene for *groESL* operon. It is, therefore, necessary to determine transcription initiation site(s) of the operon and analyze sequence features of the *cis*-elements [8, 9].

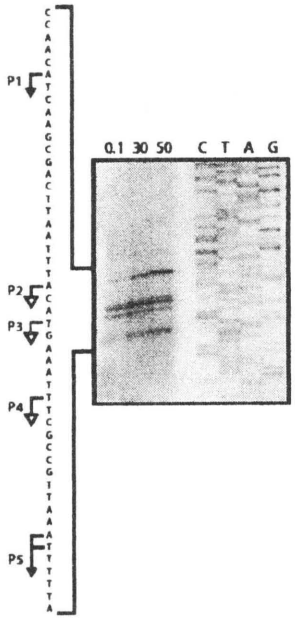

Fig. 3. Primer extension analysis of transcription of the *glnA* gene in *S. violacea* under different pressure conditions.
Total cellular RNA was prepared from cells grown at 0.1, 10, 30 and 50 MPa. The sequencing ladder was generated using the same primers as those used in the dideoxy sequencing reactions. The transcripts are shown by arrows.

Primer extension and promoter analysis of the region upstream of the *groESL* operon

To identify the promoter regions of the *groESL* operon, we determined the transcription initiation sites by primer extension analysis. As shown in Fig. 3, five transcription sites for the *groESL* operon were detected. They were located from 52 to 90 nucleotides upstream of the translational initiation site of *groES*. The levels of these extension products were increased by elevated pressures. The sequences of the -35 and -10 regions of the putative promoter (P1) for *groES* were CCTTG and CCCCATAT, respectively. These are similar to the consensus sequence of the σ^{32}-dependent promoter in *E. coli* [10]. No consensus sequences were not found in the promoter for the transcription initiation site for P2 – P5 (Fig. 4). For future study, detailed sequence analysis of the promoter elements is definitely required to understand molecular mechanisms for transcription under high pressure conditions.

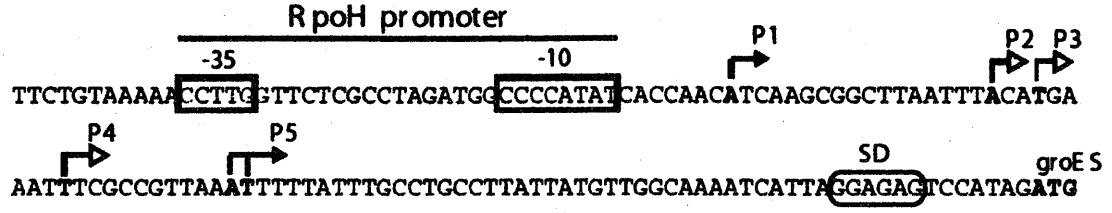

Fig. 4 Nucleotide sequence of the region upstream of the *groESL* operon.
Transcription initiation sites (P1 - P5) are indicated with arrows. The initiation site at the most upstream (P1; the *Escherichia coli* s^{32}-like promoter) is indicated in the box.

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