The groESL Operon of the Halophilic Lactic Acid Bacterium

Tetragenococcus halophila

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The groESL operon of the halophilic lactic acid bacterium Tetragenococcus halophila was cloned by a PCR-based method. The molecular masses of GroES and GroEL proteins were calculated to be 10,153 and 56,893 Da, respectively. The amount of groESL mRNA was increased 3.8-fold by heat shock (45℃), and 4-fold by high NaCl (3–4 M). The Bacillus subtilis σ^A-like constitutive promoter existed in front of groES, and was used under both normal and stress (heat shock and high salinity) conditions.

Key words: heat shock protein; groESL operon; CIRCE; halophile; Tetragenococcus

Heat shock proteins (HSPs) are temporarily overexpressed when cells are exposed to high temperature, high salinity, and various other kinds of environmental stresses. HSPs are widely contributed to protect cells from lethal effects of stresses as molecular chaperones involved in maturation of newly synthesized polypeptides and promotion of refolding and degradation of denatured proteins. Studies on a variety of bacterial genera have demonstrated increased synthesis of GroES and GroEL following exposure to heat shock, low pH, ethanol, and salt, suggesting a role for these proteins in the adaptation to general stress.

Tetragenococcus halophila (formerly known as Pediococcus halophilus) is a moderately halophilic Gram-positive lactic acid bacterium used for brewing of Japanese soy sauce. T. halophila can tolerate high salt concentrations (up to 26% NaCl), and grows optimally in media containing 0.5 to 3.0 M of NaCl. When cultivated in a high salt concentration medium, T. halophila is known to accumulate intracellularly not only Na^+ but also much K^+ and several organic compounds as compatible solutes.

We have much interest in the functions and the expression behavior of T. halophila GroESL under the conditions of high intracellular osmotic pressure and increased hydrophobic interactions in the protein structure. In this paper, we describe the cloning, structural characterization, and transcriptional analysis of the groESL operon of T. halophila. Data on the character of T. halophila GroESL contribute to better understanding of its adaptation mechanism to environmental stresses, especially to high salinity.

T. halophila JCM5888 (ATCC33315) used in this study, was grown at 30℃ in MRS medium (Oxoid, Hampshire, England) containing 1 M NaCl. To identify the groESL locus of the strain, a PCR-based approach was used. Two oligonucleotide primers for degenerative PCR, groel-TS1 and groel-TA1 (Table 1), were constructed based on the amino acid sequence alignment of GroEL proteins from Gram-positive bacteria. PCR was done in a 100-μl volume containing 1 μg genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM of each dNTP, 100 pmol of each primer, and 5 U of Taq polymerase. Amplification was done for 30 cycles (denaturation at 95℃ for 1 min, annealing at 50℃ for 30 sec, and polymerization at 72℃ for 1 min). The amplified 900-bp fragment of the expected size was cloned into Escherichia coli JM109 (Toyobo, Osaka, Japan) using pUC18 and sequenced. The nucleotide sequence of the cloned PCR fragment showed strong similarities to other bacterial groEL homologues. This PCR fragment was labeled with the AlkPhos Direct System (Amersham Pharmacia Biotech, Uppsala, Sweden) and used as a probe for hybridization with chromosomal DNA of T. halophila JCM5888. Southern hybridization was done using Hybrid-N+ (Amersham Pharmacia Biotech) in 6 × SSC, 0.5% SDS and 5 × Denhardt’s solution at 65℃ for overnight. The 1.5-kb and 1.3-kb fragments, which generated a strong hybridization signal with the probe, were cloned by “inverse-PCR” as follows. T. halophila...
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<td>groel-TS1</td>
<td>5'-GT(GC)GAAGATTGGAAAGACGGTTAC-3'</td>
<td>The degenerative oligonucleotide primer corresponding to 287 to 308 of the <em>T. halophila</em> groEL gene.</td>
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<tr>
<td>groel-TA1</td>
<td>5'-CAGTTCTAGTAGGC(GG)CGACCAAC-3'</td>
<td>The degenerative oligonucleotide primer corresponding to 1123 to 1144 of the <em>T. halophila</em> groEL gene.</td>
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<tr>
<td>gros-IS1</td>
<td>5'-CTCTTCAGCACATCTTCTGTG-3'</td>
<td>The oligonucleotide primer corresponding to 356 to 357 of the <em>T. halophila</em> groEL gene.</td>
</tr>
<tr>
<td>gros-IA1</td>
<td>5'-GGTTACCGGTTAATTGGAAAGAAGGACGGTTAC-3'</td>
<td>The oligonucleotide primer corresponding to 594 to 620 of the <em>T. halophila</em> groEL gene.</td>
</tr>
<tr>
<td>grol-IS2</td>
<td>5'-CACCTACGGGCTGGCCCAACATCAGAC-3'</td>
<td>The oligonucleotide primer corresponding to 816 to 835 of the <em>T. halophila</em> groEL gene.</td>
</tr>
<tr>
<td>grol-IA2</td>
<td>5'-GAGATCGTGTGATGCTCAGTGACGTGACATGACCGTTAC-3'</td>
<td>The oligonucleotide primer corresponding to 853 to 875 of the <em>T. halophila</em> groEL gene.</td>
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**Table 1. Oligonucleotide Primers Used in This Study**

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**Fig. 1.** Nucleotide and Amino Acid Sequences of the *T. halophila* groEL Operon.

The deduced amino acid sequences of GroES and GroEL are shown below the nucleotide sequence. The hexamers indicative of promoters (−35 and −10) are underlined. The inverted repeat sequences including CIRCE element and potential Shine-Dalgarno sequence (S.D.) are indicated by arrowheads and double underlined, respectively.
chromosomal DNA was digested completely with HindIII and re-ligated for use as the template. Amplification was done with KOD DNA polymerase (Toyobo), which increased polymerization fidelity. Amplification was done for 30 cycles (denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, and polymerization at 74°C for 1 min). Inverse-PCR reaction with groS-IA1 and groS-IS1 (Table 1) yielded a 1.5-kb amplified fragment. 1.3-kb amplicon was also obtained by the same method with primers groS-IA2 and groS-IS2 (Table 1). These two fragments were cloned into pUC18 and sequenced. The nucleotide sequence reported in this article has been assigned GenBank accession number AB073399. The complete nucleotide sequence of 2,853 bp had two open reading frames (ORFs) encoding putative GroES and GroEL, separated by 60 bp (Fig. 1). An inverted repeat sequence (5′-TTAGCAGTC-N9-GAGTGTCAAA-3′) was identified in front of groES, and the sequence corresponded to the regulatory sequence of CIRCE. In Bacillus subtilis, and Staphylococcus aureus, a repressor protein, HrcA, is known to bind to the CIRCE element, and negatively regulates the expression of dnaK and groESL operon. We have previously characterized the hrcA gene on the T. halophila chromosomal DNA. It can be seen that in T. halophila the CIRCE element and HrcA protein also represent an important system to regulate the expression of the T. halophila groESL operon. Immediately downstream of the groEL, a stem-loop structure, which may be a rho-independent transcription terminator, was found, which had a free energy of -25.8 kcal mol⁻¹.

The deduced amino acid sequence of T. halophila groESL indicated that the gene encoded 95 aa and the calculated molecular mass was 10,153 Da. T. halophila GroESL was high similarities with the GroES proteins of Lactococcus lactis, Lactobacillus zeae, and Lactobacillus helveticus (60%, 52%, and 51% identical, respectively). T. halophila GroEL encoded 535-aa residues in a length with a mass of 56,893 Da. T. halophila GroEL also showed high similarities with the GroEL proteins of those Gram-positive bacteria (72%, 74%, and 73% identical, respectively). The notable feature of GroESL amino acid sequences is a higher content of acidic amino acid residues (aspartic acid and glutamic acid), and the isoelectric points of GroES and GroEL were calculated to be 4.27 and 4.41. These scores are lower than those of other bacteria such as L. lactis (4.84 and 4.68, respectively). The higher content of acidic amino acids are predicted to be an important feature for salinity adaptation since acidic amino acids can bind excess amounts of water and stabilize the protein structure in concentrated salt solutions.

Northern blot hybridization was done with groEL probe to clarify the organization and the expression behavior of the T. halophila groESL operon. Total RNA was isolated from T. halophila cells using an RNeasy Total RNA kit (Qiagen, Chatsworth, CA, USA). The PCR-generated probe, used in cloning the T. halophila groESL operon described above, was labeled with 32P-dCTP. Hybridization was done at 42°C overnight. The relative concentration of groESL transcripts was estimated by autoradiograph densitometry with a BAS 2000 Bio-Imaging Analyzer system (Fuji Photo Film, Tokyo, Japan). The autoradiograph using a groEL probe showed a 2.2-kb transcript. The size of 2.2-kb mRNA could comprise the whole groESL operon, indicating that in T. halophila, groES and groEL are transcribed as a bicistronic operon (Fig. 2). Moreover, the amount of 2.2-kb groESL mRNA was increased 3.8-fold upon 30 min heat shock at 45°C. These data suggested that cloned T. halophila groESL surely belongs to a system of heat shock response.

In addition to heat shock, we analyzed the effects of high salinity on the transcriptional induction of T. halophila groESL by slot blot hybridization using the groEL specific primer labeled with 32P-dCTP. The slot-blot hybridization method is less prone to pipetting errors compared to the Northern blot hybridization, being more relevant in terms of exact measurement. Five micrograms of alkaline-denatured total RNA were transferred to Zeta Probe blotting membranes (Bio-Rad, Hercules, CA, USA) with a Bio-Dot SF microfiltration apparatus (Bio-Rad). Hybridization was done by the same method as the Northern hybridization described above. Relative amounts of transcript were measured by densitometric analysis using a BAS 2000 Bio-Imaging Analyzer sys-
Fig. 3. Transcription of *T. halophila* groESL Gene under NaCl Stress. Transit ®on levels were measured using the groEL probe by slot-blot analysis. The cells were grown at 30°C for 24 h in MRS medium containing 1 M NaCl. Each total RNA was obtained from cells after transfer to the fresh MRS medium containing the indicated concentrations of NaCl and 1 h of incubation. The signal intensity with 1 M NaCl was defined as 1.0.

The transcriptional initiation site of the groESL operon was analyzed by primer extension analysis using the 32P-labeled primer Gro-PE10 (5’-TAC GTC CTG GAC CGA CAG C-3’), which complements nucleotides 131 to 149 of the groES gene. Reverse transcription was done with the AMV Reverse Transcription System (Promega, Madison, WI, USA). The transcription started at T located 76 bases upstream of the start codon of groES, and was induced by heat shock (Figs. 1 and 4). The *B. subtilis* σA-like promoter was identified upstream of the CIRCE element (with a −35 sequence, 5’-TTGCAAA-3’, and −10 sequence, 5’-TAATAT-3’) (Fig. 1). These data suggested that the expression of *T. halophila* groESL operon was governed by this constitutive promoter. Moreover, under the heat shock (45°C) and the salinity (4 M NaCl) conditions, the *T. halophila* groESL used the same transcriptional initiation site (Fig. 4). Since the site was used under both normal and the stress (heat shock, high salinity) conditions, overexpression of *T. halophila* groESL operon against environmental stresses might be governed by the interaction of HrcA protein and the CIRCE element.

To our knowledge, this is the first report about the genetic characterization of groESL operon from moderately halophilic eubacteria. The molecular chaperone DnaK1 from the halotolerant cyanobacterium *Aphanothece halophytica* showed the chaperone activity at 1.0 M NaCl.12) *T. halophila* can grow under higher external salinity conditions than *A. halophytica*,6) so the functional chaperone activity of *T. halophila* GroESL is our great interest. We are now investigating the ATPase and refolding activity of GroESL protein of *T. halophila* in high salinity. The halotolerant molecular chaperone, which has strong folding activity of denatured protein under high salinity, is probably useful for molecular biological applications.

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References