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Fukuda, Daisuke
Laboratory of Microbial technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Watanabe, Maki
Laboratory of Microbial technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Aso, Yuji
Laboratory of Microbial technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Sonomoto, Kenji
Laboratory of Microbial technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

他

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Cloning and regulatory analysis of *groESL* operon from halophilic lactic acid bacterium *Tetragenococcus halophila*

Daisuke FUKUDA, Maki WATANABE, Yuji ASO, Kenji SONOMOTO* and Ayaaki ISHIZAKI

Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6–10–1, Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan
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The *groESL* operon of a halophilic lactic acid bacterium, *Tetragenococcus halophila*, was cloned and sequenced. The nucleotide sequence of 2,853-bp revealed the presence of two open reading frames corresponding to the *groES* and *groEL* genes. The molecular masses of GroES and GroEL proteins were calculated to be 10,153 and 56,893 Da, respectively. They showed high similarities with the corresponding proteins of other lactic acid bacteria such as *Lactobacillus zeae*. CIRCE (Controlling Inverted Repeat of Chaperone Expression) element was identified in the upstream region of *groES*. Northern blot hybridization has demonstrated that the *groES* and *groEL* genes are transcribed as a bicistronic mRNA of 2.2 kb, and transcriptionally induced 3.8-fold by heat shock (45 °C) for 30 min. The amount of *groESL* mRNA was also increased about 4-fold by high NaCl condition. Primer extension analysis indicated that the expression of *T. halophila groESL* was governed by the constitutive promoter both under normal and stress conditions.

INTRODUCTION

Heat shock proteins (HSPs) are temporarily overexpressed when cells are exposed to high temperature, high salinity and other various kinds of environmental stresses (Lindquist and Craig, 1988). HSPs are widely contributed to protect cells from lethal effect of stresses as molecular chaperones involved in maturation of newly synthesized polypeptides and promotion of refolding and degradation of denatured proteins (Hartl, 1996).

In *Escherichia coli*, about 20 heat shock proteins are known (Gross *et al.*, 1991), and two major HSPs families, DnaK and GroESL have been extensively characterized (Liberek *et al.*, 1991). Molecular chaperonin proteins of GroES and GroEL are essential for growth of *E. coli* even under optimum growth conditions, and enhanced synthesis of GroESL was observed by exogenous environmental stresses (Langer *et al.*, 1992). The transcription of heat shock genes in *E. coli* is mainly mediated by σ^{32}-dependent promoters (Cowing *et al.*, 1985). However, in *B. subtilis*, some heat shock genes possess an regulatory inverted repeat sequence, termed CIRCE, in the region around vegetative promoter (Zuber and Schumann, 1994). Studies on other variety of bacteria genera have also 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 against general

* Corresponding author (E-mail: sonomoto@agr.kyushu-u.ac.jp)

stress (Fujita *et al.*, 1998; Homuth *et al.*, 2000). In *Lactococcus lactis*, GroEL and GroES showed similar temporal induction patterns of NaCl stress, resembling those of heat shock (Kilstrup *et al.*, 1997). This has indicated that the GroESL molecular chaperone system plays an important role to adapt to salinity conditions as well as heat shock.

Tetragenococcus halophila (formerly known as *Pediococcus halophilus*) is a moderately halophilic Gram-positive lactic acid bacterium (LAB) used for brewing of Japanese soy sauce. Based on 16S rDNA sequence studies, this bacterium shows close phylogenetic relationship to enterococci and lactobacilli (Collins *et al.*, 1990). Unlike these genera of LAB, *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 (Rölling and Van Verserveld, 1996). When cultivated in a high salt concentration medium, *T. halophila* is known to accumulate intracellularly not only Na^+ but also much amount of K^+ and several organic compounds as compatible solutes (Robert *et al.*, 2000). We have much interest in the functions and the expression behavior of *T. halophila* GroESL under the condition 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 contributes to better understanding of its adaptation mechanism to environmental stresses, especially against high salinity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

T. halophila JCM5888 (ATCC33315^T) used in this study, was grown at 30 °C in MRS medium (Oxoid, Hampshire, England) containing 1 M NaCl. The medium was adjusted to pH 7.5 before sterilization. *Escherichia coli* JM109 (Toyobo, Osaka, Japan) was grown at 37 °C with shaking in Luria–Bertani broth. When the growing was appropriate for clonal selection, X-gal (5-bromo-4-isopropyl-β-D-4-chloro-3-indol-β-D-galactopyranoside), IPTG (isopropyl 1-thio-β-D-galactoside) and ampicillin were added at concentrations of 50, 40, 20 mg/l, respectively.

DNA isolation and manipulation

T. halophila chromosomal DNA was isolated by applying a combination of the two methods as described previously (Marmur, 1961; Berns and Thomas, 1965). Plasmid DNA, pUC18 vector, used for cloning of *T. halophila groESL* operon, was purified from *E. coli* with Mag extractor plasmid extraction kit (Toyobo). Restriction endonuclease digestions, analyses and ligations were performed according to the methods of Sambrook *et al.* *E. coli* competent cells for electroporation were prepared according to the protocol recommended for the Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA). Southern hybridization with nucleotide probes was performed by using the AlkPhos labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacture's protocol.

Cloning of *T. halophila groESL* operon

Two degenerated oligonucleotides (groel-TS1 and groel-TA1) used as PCR primers were designed from GroEL conserved region of other Gram-positive bacteria (Table 1).

PCR was performed in a 100-μl volume containing 1 μg genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 100 pmol of

Table 1. Oligonucleotide primers used in this study.

Names	Sequences	Notes
groel-TS1	5'-G(T/C)GAAGATTTGAAGAACGTTAC-3'	The degenerative oligonucleotide primer corresponding to the 287 to 308 of the *T. halophila groEL* gene.
groel-TA1	5'-CAGTTTCAGTAGC(A/G)GCACCAAC-3'	The degenerative oligonucleotide primer corresponding to the 1123 to 1144 of the *T. halophila groEL* gene.
gros-IS1	5'-CCTTCAACGACATCCAATTCTG-3'	The oligonucleotide primer corresponding to the 536 to 558 of the *T. halophila groEL* gene.
gros-IA1	5'-GGTAACGGATAATGAAAAAATGGAAGC-3'	The oligonucleotide primer corresponding to the 594 to 620 of the *T. halophila groEL* gene.
grol-IS2	5'-GACGGCCGCCAAATCCAGGA-3'	The oligonucleotide primer corresponding to the 816 to 835 of the *T. halophila groEL* gene.
grol-IA2	5'-GACATCGCTGTATTGACTGGTGC-3'	The oligonucleotide primer corresponding to the 853 to 875 of the *T. halophila groEL* gene.

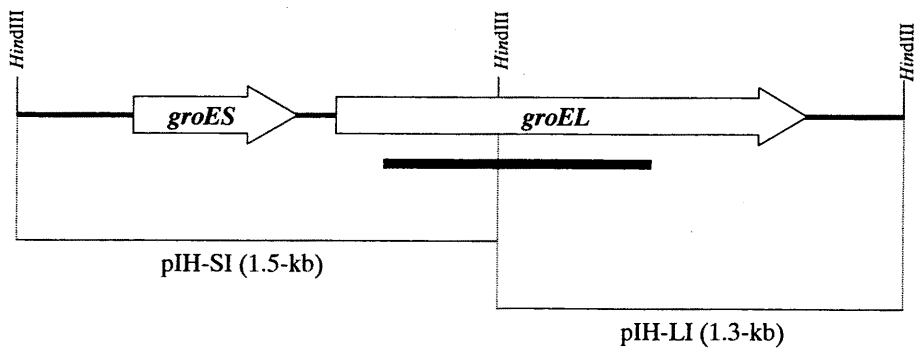

Fig. 1. Genetic organization and restriction map of the *T. halophila groESL*.
The originally amplified region by priming with groel-TA1 and groel-TS1 oligonucleotides is shown with a bold box. Three *Hin*dIII sites which were used in the inverse PCR are also shown. Two bold bar correspond to the coding region of *groES* and *groEL*.

each primer and 5 U of *Taq* polymerase. Amplification was carried out for 30 cycles (denaturation at 95 °C for 1 min, annealing at 50 °C for 30 sec, and polymerization at 72 °C for 1 min). Amplified fragment of an expected size (0.9-kb) was labeled with AlkPhos Direct System (Amersham Pharmacia Biotech) and used as a probe to screen a *T. halophila* genomic library. The 1.5-kb (pIH-SI) and 1.3-kb (pIH-LI) fragments (Fig. 1), which generated a strong hybridization signal with the probe, were cloned by "inverse PCR" as follows (Innis *et al.*, 1990). *T. halophila* chromosomal DNA was digested completely with *Hin*d III and religated for use as the template. 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 same method with primers grol-IA2 and grol-IS2 (Table 1). Inverse PCR was performed with KOD DNA polymerase (Toyobo), which increased polymerization fidelity. The generated fragment was cloned into *Sma*I site of pUC18 and transformed into *E. coli* JM109. The cloned fragments were sequenced with ALF express automated DNA sequencer (Amersham Pharmacia Biotech) and analyzed with the DNASIS program (Hitachi Software Engineering, Tokyo, Japan) and GENETYX-WIN (Software Development, Tokyo, Japan).

Comparison of deduced amino acid sequence and phylogenetic analysis of *T. halophila* GroESL

The amino acid sequence similarities of GroES and GroEL were investigated using BLAST service (Altschul *et al.*, 1997). The multiple alignments of the GroEL amino acid sequences were performed by the program ClustalW and were adjusted manually (Thompson *et al.*, 1994). The phylogenetic tree based on the NJ (Neighbor-joining) method was constructed by Treeview (ver. 1.6) (Saitou and Nei, 1985). Accession numbers of other sequences used for the analysis are as follows; *Bacillus subtilis groEL*, D10972 (Li and Wong, 1992); *Clostridium acetobutylicum groEL*, M74572 (Nerberhaus and Bahl, 1992); *E. coli groEL*, X07850 (Hemmingsen *et al.*, 1988); *Lactobacillus helveticus groEL*, AF031929 (Broadbent, 1998); *Lactobacillus johnsonii groEL*, AF214488 (Walker *et al.*, 1999); *Lactococcus lactis groEL*, AY029215 (Kim and Batt, 1993); *Neisseria gonorrhoeae groEL*, U64996 (Tauschek *et al.*, 1997); *Listeria monocytogenes groEL*, AF335323 (Gahan *et al.*, 2001); *Pseudomonas aeruginosa groEL*, S77424 (Sipos *et al.*, 1991); *Porphylomonas gingivalis pggroEL*, D17398 (Hotokezaka *et al.*, 1994); *Staphylococcus aureus hsp60*, D14711 (Ohta *et al.*, 1993); *Streptococcus pneumoniae groEL*, AF117741 (Kim *et al.*, 2001); *Thermus aquaticus groEL*, U29483 (Mikulik and Benada, 1993); *Vibrio vulnificus groEL*, AY017169 (Wong *et al.*, 2001).

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been assigned GenBank accession number AB073399.

Northern blot hybridization

Total RNA was isolated from *T. halophila* cells using RNeasy Total RNA kit (Qiagen, Chatsworth, CA, USA). RNA samples (5 μg) were denatured with formaldehyde and electrophoresed at 50 V for 1 h on a 1.5% agarose gel containing 20 mM MOPS (morpholine propane sulfonic acid) buffer, pH 8.0 and 2.2 M formaldehyde. After electrophoresis,

capillary transfer to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech) was carried out in 20×SSC (1×SSC; 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0). The PCR-generated probe, used in cloning the *T. halophila groESL* operon described above, was labeled with ^{32}P-dCTP. Northern blot hybridization was carried out at 42 °C for 12 h. The relative concentration of *groESL* transcripts was estimated by autoradiograph densitometry with a BAS 2000 Bio-Imaging Analyzer system (Fuji Photo Film, Tokyo, Japan).

Slot-blot hybridization

Slot-blot hybridization method is less prone to pipetting errors compared to the Northern blot hybridization, being more relevant in terms of exact quantification. Five microgram of alkaline-denatured total RNA was transferred to Zeta Probe blotting membranes (Bio-Rad) with a Bio-Dot SF microfiltration apparatus (Bio-Rad) as specific manufacturer, and treated with UV cross link. Prehybridization and hybridization were carried out by the same method as Northern hybridization described above. Relative amounts of the transcript were also quantified by densitometric analysis using BAS 2000 Bio-Imaging Analyzer system (Fuji Photo Film).

Primer extension analysis

The transcriptional initiation site was determined by primer extension analysis using the ^{32}P-labeled primer Gro-PE10 (5'-TAC GTC CTG GAC CGA CAG C-3') (Asubel *et al.*, 1987), which complements nucleotides 131 to 149 of the *groES* gene. Total RNA was isolated as described above, and reverse transcription was performed with AMV Reverse Transcription System (Promega, Madison, WI, USA). Nucleic acids were precipitated with ethanol and after drying, resuspended in 10 mM Tris, 1 mM EDTA and formamide loading buffer, which were subjected to electrophoresis on a 6% polyacrylamide gel. DNA sequencing reactions were carried out with the same primer, and the sequencing products were electrophoresed under the same condition. The autoradiography was used to visualize the products.

RESULTS

Cloning and nucleotide sequence of the *groESL* locus of *T. halophila*

To identify the *groESL* locus of *T. halophila* JCM5888, 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 highly conserved regions of GroEL proteins from *Lactococcus lactis* (Kim and Batt, 1993), *Lactobacillus zeae* (Murphy and Chassy, 1997) and *Bacillus subtilis* (Li and Wong, 1992). Amplified 900-bp fragment as expected size was cloned into *E. coli* JM109 using pUC18 and sequenced. The nucleotide sequence of the cloned PCR fragment showed high similarities to other bacterial *groEL* homologues. This PCR fragment was labeled and used as a probe for Southern hybridization with chromosomal DNA of *T. halophila* JCM5888. The 1.5-kb and 1.3-kb *Hin*dIII digested fragments were strongly hybridized with the probe and cloned into pUC18 vecter. The complete nucleotide sequence of 2,853-bp revealed the presence of two open reading frames (ORFs) encoding putative GroES and GroEL, separated by a 60bp (Fig. 2). Two putative Shine-Dargano sequences

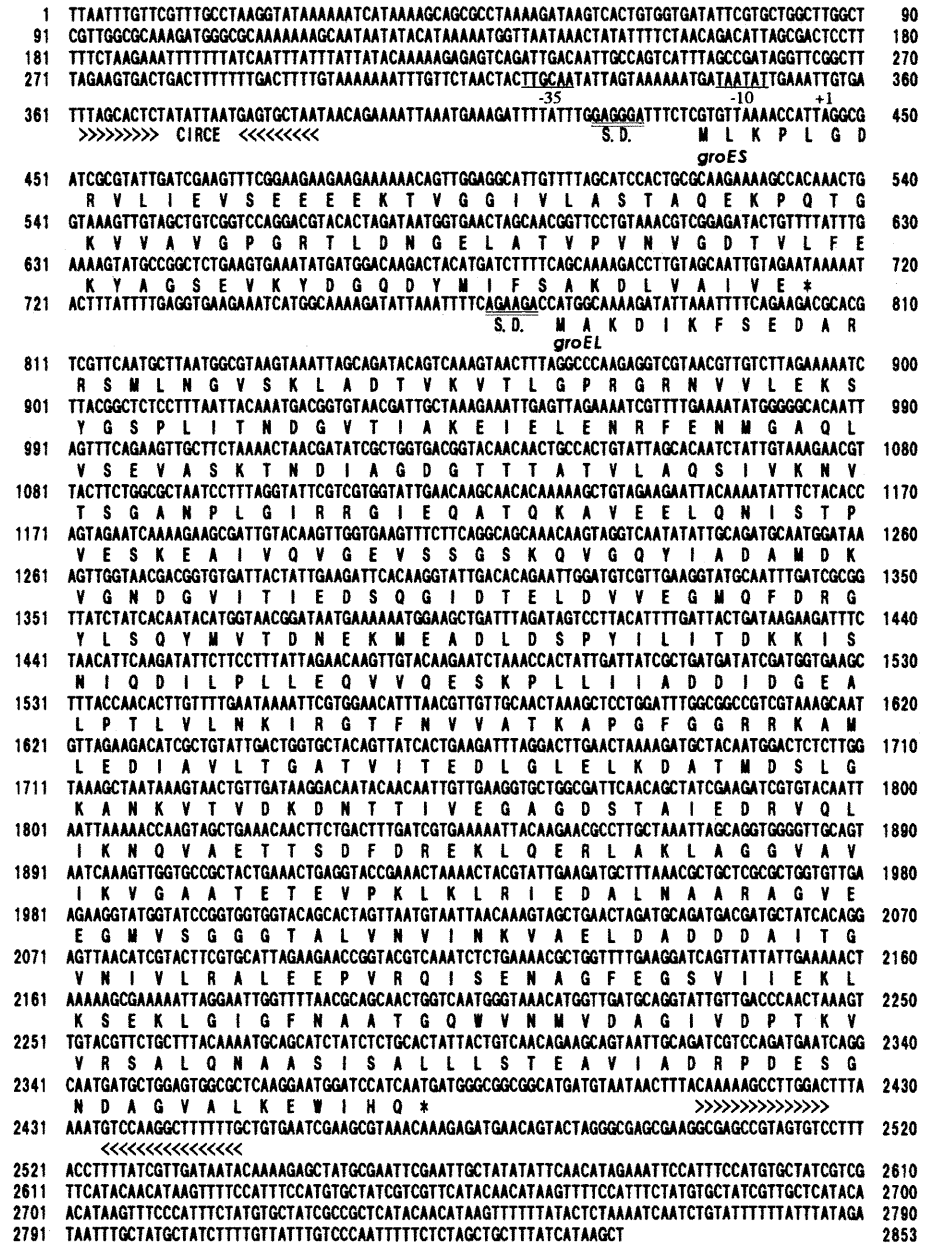

Fig. 2. Nucleotide and amino acid sequences of the *T. halophila groESL* operon.
The deduced amino acid sequences of GroES and GroEL are shown below the nucleotide sequence. The hexamers indicative of promoters (−35 and −10) are underlined. The inverted repeat sequences including CIRCE element and potential Shine–Dargano sequence (S. D.) are indicated by arrowheads and double underlined, respectively.

were found, one (GAGGGA) was upstream of *groES* and the other (AGAAGA) was upstream of *groEL* gene. An inverted repeat sequence (5'–TTAGCACTC–N_9–GAGTGCTAA–3') was identified in front of *groES*. The sequence corresponds to the regulatory sequence of CIRCE, which is involved in the negative regulation of *dnaK* and *groESL* operon in numerous Gram–positive bacteria. Immediately downstream of the *groEL*, stem–loop structure, which may be a rho–independent transcription terminator was found, which had a free energy of -25.8 kcal.mol^{-1}.

Analysis of the deduced amino acid sequence of the *T. halophila groESL* operon

The deduced amino acid sequence of *T. halophila groES* indicated that the gene encoded 95–aa in length and the calculated molecular mass was 10,153 Da (Fig. 2). *T. halophila* GroES showed high similarities with the GroES proteins of *L. lactis*, *L. zeae* and *L. helveticus* (68%, 49% and 48% identical, respectively). *T. halophila groEL* encoded 535–aa residues in a length with a mass of 56,893 Da (Fig. 2). *T. halophila* GroEL also showed high similarities with the GroEL proteins of those Gram–positive bacteria (78%, 72% and 68% identical, respectively). To assess the evolutionary relationship of the cloned *T. halophila* GroEL, phylogenetic tree of 15 bacterial GroEL homologues was constructed as described in Materials and Methods (Fig. 3). Phylogenetic analysis clearly showed that *T. halophila* GroEL belongs to the LAB cluster, closely related to *L. helveticus* and *L. johnsonii*, and separated from *L. lactis*.

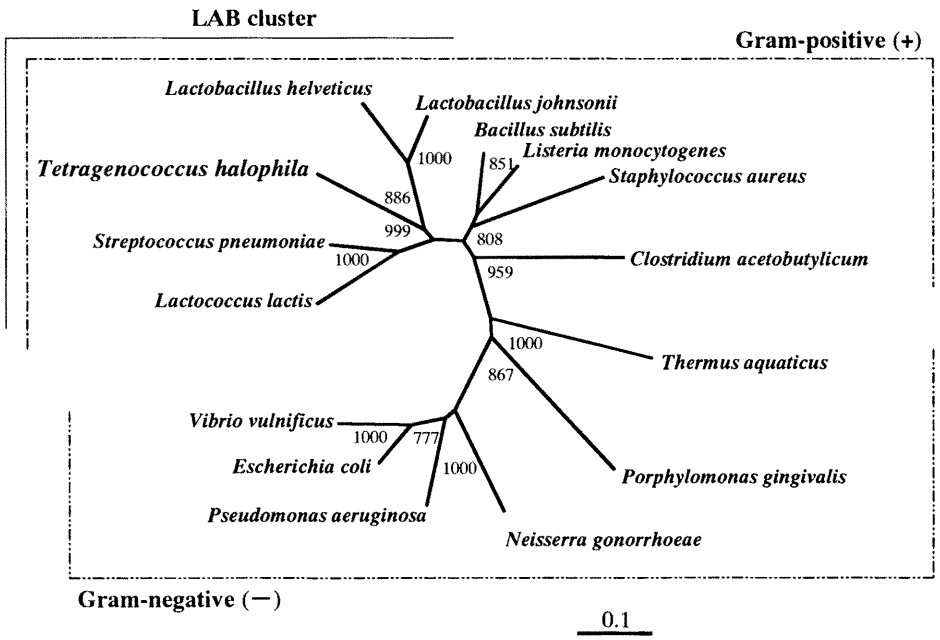

Fig. 3. Phylogenetic analysis of *T. halophila* GroEL and other bacterial GroEL (Hsp60) homologues. A phylogenetic tree was constructed based on the NJ method as described in Materials and Methods.

Heat shock induction of the *T. halophila groESL* operon

Northern blot hybridization was performed with 900–bp *groEL* probe to clarify the organization and the expression behavior of the *T. halophila groESL* operon (Fig. 4). The autoradiograph using *groEL* probe revealed 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. Moreover, the amount of 2.2–kb *groESL* mRNA was increased 3.8–fold upon 30 min heat shock at 45 °C. This data suggested that cloned *T. halophila groESL* surely belongs to a system of heat shock response.

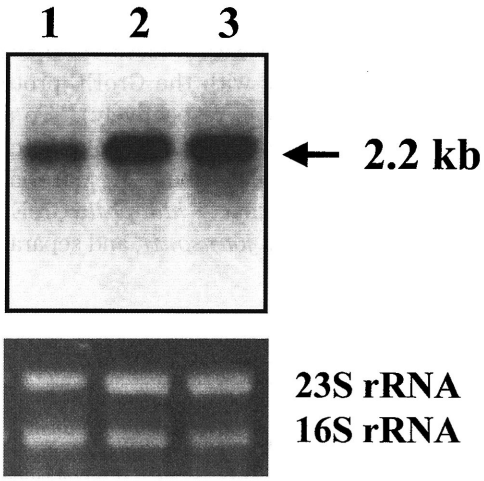

Fig. 4. Northern blot analysis of *groEL* gene expression in *T. halophila*.
Each total RNA was isolated from the cells grown at 30 °C for 24 h in MRS medium containing 1 M NaCl (lane 1) and further incubated for 10 and 30 min after shifting to 45 °C (lanes 2 and 3). Ethidium bromide staining of the gel shows that equal amounts of RNA were loaded.

Salt stress induction of the *T. halophila groESL*

In addition to heat shock, we analyzed the effect of high salinity on the transcriptional induction of *T. halophila groESL* by slot–blot hybridization (Fig. 5). The cells grown in the presence of 1.0 M NaCl for 24 h were transferred to the same fresh medium containing the indicated NaCl concentrations, and incubated for further 1 h. Figure 5 indicates the amount of *groESL* mRNA increased about 4–fold with 3–4 M NaCl, the concentration of which may be critical for cell growth. This indicated that the salt induction of *T.*

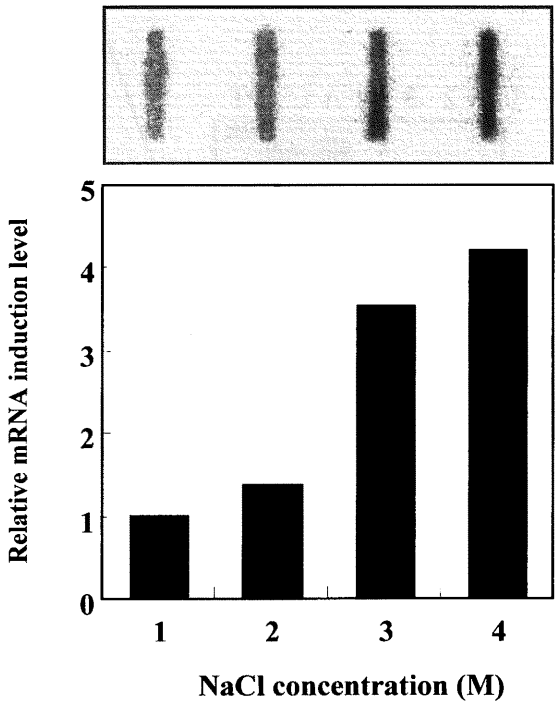

Fig. 5. Transcription of *T. halophila groESL* gene under NaCl stress.
Transcript levels were quantified using the *groEL* probe by slot-blot analysis. The cells were grown at 30 °C for 24 h in MRS medium containing 1 M NaCl. Each total RNA was obtained from cells after transfer to the fresh MRS medium containing the indicated concentrations of NaCl and 1 h of incubation. The signal intensity with 1 M NaCl was defined as 1.0.

halophila groESL is also regulated at the transcriptional level.

Identification of the transcriptional initiation site of the *T. halophila groESL* mRNA.

The transcriptional initiation site of the *groESL* operon was determined by primer extension analysis. The results with primer PE10 are shown in Fig. 6. The transcription started at T located 76-bases upstream of the start codon of *groES*, and was induced by heat shock. The *B. subtilis* σ^A like promoter (Fujita and Sadaie, 1998) was identified upstream of CIRCE element (with a -35 sequence, 5'-TTGCAA-3', and -10 sequence, 5'-TAATAT-3') (Fig. 2). 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* utilized the

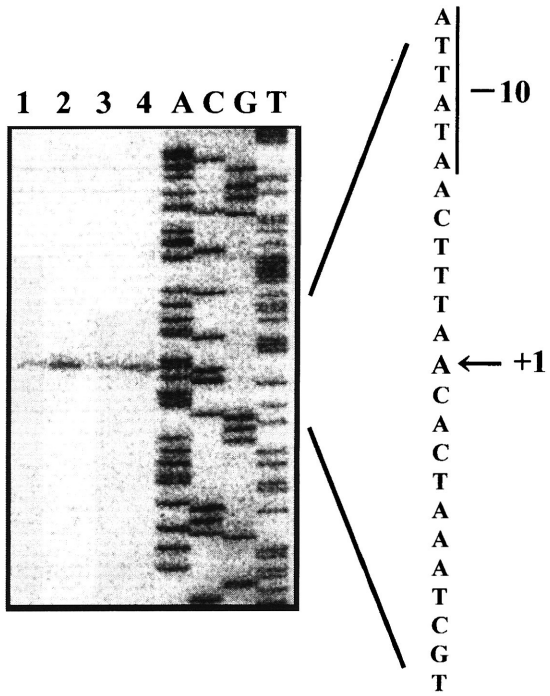

Fig. 6. Determination of the transcriptional initiation site of *groESL* operon by primer extension analysis.
Lanes A, C, G and T indicate the dideoxy termination lanes. Extension products were generated with total RNA isolated from the cells under normal (lane 1 and 3, 30 °C, 1 M NaCl), heat shocked (lane 2, 45 °C, 10 min), and high salinity (lane 4, 4 M NaCl) conditions as described in the legends of Figs. 4 and 5.

same transcriptional initiation site. This indicated that the *T. halophila groESL* used a unique promoter even exposed to the environmental stresses.

DISCUSSION

In this paper, we describe the cloning and nucleotide sequence of *groESL* operon of the halophilic LAB *T. halophila* JCM5888, as well as the expression analysis of mRNA level. In our knowledge, this is the first report about the genetic characterization of *groESL* operon from moderately halophilic eubacteria. The nucleotide sequence of 2,853-bp revealed that the cloned fragments contained two ORFs bearing significant homology to numerous bacterial GroES and GroEL proteins (Fig. 2). A highly conserved inverted repeat sequence, termed CIRCE element, was found in the upstream of *groES* start codon. This element is extensively studied in *B. subtilis* (Zuber and Schumann,

1994) and *S. aureus* (Ohta *et al.*, 1996). We have previously characterized the *hrcA* gene on the *T. halophila* chromosomal DNA (Fukuda *et al.*, 2001). It can seem that in *T. halophila* the CIRCE element and HrcA protein also represent an important system to regulate the expression of *T. halophila groESL* operon.

Recently, GroEL (Hsp60) homologues have been used for determining bacterial deep relationship because of the ubiquity and the high degree of sequence conservation (Kwok *et al.*, 1999; Goh *et al.*, 1996). Phylogenetic analysis clearly showed that the GroEL homologues of LAB formed a well-defined subgroup among the Gram-positive cluster. *T. halophila* GroEL was closely related to *Lactobacillus* species subcluster and separated from *L. lactis* (Fig. 3). This result corresponded to the previous taxonomic analysis based on 16S rDNA sequence (Collins *et al.*, 1990). The data also suggested that the GroEL homologues could be use as DNA target for species-specific identification of LAB.

Northern blot hybridization analysis has revealed that the *groES* and *groEL* genes in *T. halophila* are transcribed as a bicistronic operon, similar to those found other species of bacteria. The size of *groESL* mRNA was 2.2-kb in length (Fig. 4). This corresponded to the size from determined transcriptional initiation site of *groESL* operon to its own rho-independent terminator (Fig. 2). The amount of *groESL* mRNA was increased about 3.8-fold upon heat shock. The data suggested that the induction of *T. halophila groESL* by heat shock was regulated at the transcriptional level like other genus of bacteria. Slot-blot analysis of total RNA confirmed that the transcription of *T. halophila groESL* was induced by high concentration of NaCl (Fig. 5). The increased expression of *groESL* represents that GroESL acts as an important factor in *T. halophila* to adapt to environmental high salinity condition. Primer extension analysis determined a unique transcriptional initiation site of *groESL* operon, preceded by vegetative *B. subtilis* σ^{A} like promoter. The transcriptional initiation site of *groESL* did not change under the stress condition tested (Fig. 6). Since the site was utilized under both normal and the stress (heat shock, high salinity) conditions, we can conclude that overexpression of *T. halophila groESL* operon against environmental stresses is governed by the interaction of HrcA protein and CIRCE element.

The molecular chaperone DnaK1 from halotolerant cyanobacterium *Aphanothece halophytica* showed the chaperone activity at 1.0 M NaCl (Hibino *et al.*, 1999). *T. halophila* can grow under higher external salinity condition than *A. halophytica* (Rölling *et al.*, 1996), so the functional chaperone activity of *T. halophila* GroESL is of great interest. We are now investigating the ATPase and refolding activity of GroESL protein of *T. halophila* under high salinity condition. The halotolerant molecular chaperone, which possesses strong folding activity of denatured protein under high salinity might be useful for molecular biological application.

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