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Cloning and Molecular Characterization of *groESL* Heat-Shock Operon in Methylotrophic Bacterium *Methylovorus* Sp. Strain SS1 DSM 11726

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The *groESL* bicistronic operon of a restricted facultative methylotrophic bacterium *Methylovorus* sp. strain SS1 DSM 11726 was cloned and characterized. It was found to consist of two ORFs encoding proteins with molecular masses of 11,395 and 57,396 daltons, which showed a high degree of homology to other bacterial GroES and GroEL proteins. The genes were clustered in the transcription order *groES-groEL*. Northern blot analyses suggested that the *groESL* operon is transcribed as a bicistronic 2.2-kb mRNA, the steady-state level of which was markedly increased by temperature elevation. Primer extension analysis demonstrated one potential transcription start site preceding the *groESL* operon, which is located 100bp upstream of the *groES* start codon. The transcription start site was preceded by a putative promoter region highly homologous to the consensus sequences of *Escherichia coli* σ^{32}-type heat shock promoter, which functioned under both normal and heat shock conditions in *E. coli*. Heat shock mRNA was maximally produced by *Methylovorus* sp. strain SS1 approximately 10min after increasing the temperature from 30 to 42°C. The *groESL* operon was also induced by hydrogen peroxide or salt shock.

Keywords: *groESL*, Heat-shock operon, *Methylosinus* sp. SS1, Methylotrophic bacteria

Introduction

Organisms rapidly synthesize heat shock proteins (Hsps) when exposed to abrupt shifts in temperature (Lindquist and Craig, 1988; Ang *et al*., 1991; Georgopoulos and Welch, 1993; Becker and Craig, 1994). However, most Hsps are also induced by other environmental stresses, such as, exposure to ethanol, salt, or heavy metals (Segal and Ron, 1998), which suggests that heat shock response is a general stress response. Molecular chaperones and ATP-dependent proteases are prominent among the Hsps (Lindquist and Craig, 1988). Moreover, the primary structures of the Hsp70 (DnaK in *Escherichia coli*) and Hsp60 (GroEL in *E. coli*) families, both molecular chaperone families, are highly conserved (Craig *et al*., 1993; Hendrick and Hartl, 1993; Hartl, 1996).

The chaperonin GroEL (Cpn60) and co-chaperonin GroES (Cpn10) constitute the GroE chaperone, and were originally found to be required for bacteriophage assembly (Tilly *et al*., 1981). GroE chaperone is involved in the folding and refolding of a number of structurally unrelated proteins (Horwich *et al*., 1993; Ewalt *et al*., 1997; Houry, 2001; Hartl and Hayer-Hartl, 2002), is essential for cell viability at all temperatures (Fayet *et al*., 1989), and is found in bacteria, mitochondria and chloroplasts (Ellis and van der Vies, 1991). In most bacteria studied to date, the genes encoding GroES and GroEL are transcribed as a bicistronic operon in the order *groES-groEL* (Fayet *et al*., 1989). Several bacteria are known to have an additional, monocistronic *groEL* operon, e.g., *Synechocystis vulcanus* (Furuki *et al*., 1996), *Rhizobium meliloti* (Rusanganwa and Gupta, 1993), and *Anabaena* sp. (Rajaram *et al*., 2001), whereas several *groE* operons were found in *Rhodobacter sphaeroides* (Lee *et al*., 1997), and *Bradyrhizobium japonicum* (Babst *et al*., 1996).

The regulatory mechanism of heat shock response differs among species. In *E. coli*, the *groE* operon is regulated by alternative sigma factor σ^{32} (Yura *et al*., 1993). Under heat shock conditions, the *groE* genes are efficiently transcribed

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from a heat shock promoter located upstream of *groES* by RNA polymerase cooperating with σ^{32} factor (Cowing *et al*., 1985). Moreover, the *groE* operon has a second promoter, which can be used under normal growth conditions by RNA polymerase acting with vegetative sigma factor, σ^{70} (Zhou *et al*., 1988). In most Gram-positive bacteria, e.g., in *Bacillus subtilis*, and in some Gram-negative species, *groESL* genes are preceded by an inverted repeat (IR) named CIRCE (Controlling Inverted Repeat of Chaperone Expression) (Zuber and Schumann, 1994; Mogk *et al*., 1997). HrcA repressor binds to CIRCE, and thereby, regulates *groE* transcription (Hecker *et al*., 1996; Segal and Ron, 1998).

Methylotrophic bacteria are capable of aerobic growth using carbon compounds containing a single carbon atom (C_1), like methanol, as the sole energy and carbon source, and therefore could serve as biocatalysts for the conversion of methanol to commercially valuable multicarbon compounds like amino acids and cytochromes (Lidstrom and Stirling, 1990). However, little is known about the nature and regulation of stress responses in methylotrophs, although these bacteria have enormous potential applications environmentally and commercially. The *Methylovorus* sp. strain SS1 DSM 11726 (Seo and Kim, 1993) belongs to the β-subdivision proteobacteria and is a Gram-negative, restrinceted facultative methylotrophic bacterium (Bulygina *et al*., 1990; Bratina *et al*., 1992). The exposure of *Methylovorus* sp. strain SS1 to various types of stress, including heat, resulted in the identification of a series of stress proteins (Park *et al*., 2001). The 60- and 70-kDa stress proteins were shown to be immunologically cross-reactive with GroEL and DnaK of *E. coli*, respectively (Park *et al*., 2001). In a previous study, we showed that the *dnaK* locus of *Methylovorus* sp. strain SS1 is composed (in transcription order) of *grpE-dnaK-dnaJ*, and that the transcription start site is preceded by an *E. coli* σ^{32}-type heat shock promoter (Eom *et al*., 2002).

To gain a further understanding of heat shock response in methylotrophic bacteria, we cloned and characterized the *groESL* operon from *Methylovorus* sp. strain SS1. Here, we describe the first detailed characterization of the *groESL* operon in a restricted facultative methylotrophic bacterium.

Materials and Methods

Bacterial strains, phages, plasmids, and growth conditions The bacterial strains, phages and plasmids used in this study are listed in Table 1. *Methylovorus* sp. strain SS1 was cultivated at 30°C in a standard mineral base medium (Kim and Hegeman, 1981) supplemented with 0.5% (vol/vol) methanol. Heat shock for *Methylovorus* sp. strain SS1 was performed by transferring cells growing exponentially at 30°C to 42°C. Other stress conditions were provoked by exposing the cells to a final concentration of 5% (vol/vol) ethanol, 4% (wt/vol) NaCl, or 0.02% (vol/vol) H_2O_2. *E. coli* strains were grown at 37°C on LB agar or in LB broth supplemented with ampicillin (50 μg/ml), if required. For phage infection, 0.2% (wt/vol) maltose and 10 mM $MgSO_4$ were added to the LB medium. Stocks of recombinant phages were prepared as was described by Sambrook *et al*. (1989).

DNA manipulations Chromosomal DNA from *Methylovorus* sp. strain SS1 was isolated from cells at the early stationary growth phase, as described previously (Goldberg and Ohman, 1984). Recombinant DNA techniques, plasmid isolation, and phage lysate preparation were performed using standard protocols (Sambrook *et al*., 1989). The electrotransformation of *E. coli* was carried out using a Gene Pulser apparatus (Bio-Rad, Hercules). DNA fragments, when necessary, were purified using QIAquick Gel Extraction Kits (Qiagen, Valencia, USA). DNA was sequenced by the dideoxy chain termination method (Sanger *et al*., 1977) using a Sequenase DNA sequencing kit (version 2.0, US Biochemical Corp., Cleveland)

Table 1. Bacterial strains, phages and plasmids used in this study

Bacterial strain, phage, or plasmid	Genotype or description	Source or reference
Bacterial strains		
Methylovorus sp. strain SS1	Wild type (DSM 11726)	Seo and Kim (1993)
E. coli		
DH5α	*supE*44Δ*lacU*169(ϕ80*lacZ*ΔM15)*hsdR*17*recA*1*endA*1*gyrA*96*thi*-1-*relA*1	Stratagene
XL1-Blue MRA(P2)	Δ(*mcrA*)183,Δ(*mcrCB-hsdSMR-mrr*)173*endA*1*supE*44*thi*-1*gyrA* 96*relA*1*lac* (P2 lysogen)	Stratagene
Phages and plasmids		
Lambda DASH II	*Bam*HI-digested arms	Stratagene
λE601	λDASH II derivative harboring 11-kb *groE* locus	This study
λE602	λDASH II derivative harboring 16-kb *groE* locus	This study
pGEM-T	3.0-kb cloning vector; Am^r	Promega
pBluescript II KS+	2.9-kb cloning or sequencing vector; Am^r	Stratagene
pGL3-Basic	4.8-kb promoterless, luciferase reporter vector; luc^+, Am^r.	Promega
pSSE1	pGEM-T harboring 587-bp PCR product	This study
pYK100	pBluescript II KS+ harboring 3.0-kb *Eco*RI fragment from λE601	This study
pYK101	pBluescript II KS+ harboring 3.6-kb *Sal*I fragment from λE602	This study
pGL-EP1	pGL3-Basic harboring *groE* promoter in right orientation	This study

or using a PE 377 automated Sequencer (Perkin-Elmer, Norwalk, USA) at the Korean Basic Science Institute. Sequences were analyzed using BLAST programs on the NCBI (National Center for Biotechnology Information) network server.

Amplification of *groEL* DNA by PCR The DNA sequence corresponding to partial GroEL was amplified by PCR from *Methylovorus* sp. strain SS1 genomic DNA using two degenerate primers. The forward primer EF (5' GGYACNACNACNGCNACN GCN 3') and the reverse primer ER (5' RTCRCCRAARCCNGGN GCYTT 3') were synthesized to match the highly conserved region of bacterial GroEL homologues (corresponding to aa 88-94, GTTTATV, and aa 277-283, KAPGFGD, respectively, of *E. coli* GroEL). PCR was conducted using a Fast Air Temp-Cycler FTC-2000 (Daehan Medical Co., Seoul) using the 30 cycles of; 95°C for 20s, 66°C for 15 s, 72°C for 15 s. The PCR product was eluted from agarose gel, labeled with digoxigenin-11-dUTP (DIG), and used as a probe for Southern blot and plaque hybridization.

Southern blot analysis Genomic DNA of *Methylovorus* sp. strain SS1 was digested with *Eco*RI, *Eco*RV, *Nco*I, *Pst*I, or *Sal*I (Promega, Madison, USA), separated by agarose gel electrophoresis, and transferred to Hybond-N+ membranes (Amersham, Arlington Heights, USA) by capillary blotting. Hybridization was carried out at 68°C for 16 h in 5X SSC, 0.02% sodium dodecyl sulfate (SDS), 0.1% N-lauroylsarcosine, and 1% blocking reagent using a DIG-labeled probe. Colorimetric detection of positive bands was performed as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Genomic library construction and screening A *Methylovorus* sp. strain SS1 genomic library was constructed in λDASH II vector (Stratagene, La Jolla, USA). Briefly, *Methylovorus* sp. strain SS1 DNA was partially digested with *Sau*3AI, ligated to λDASH II, packaged with Stratagene Gigapack II Gold packaging extracts, and transduced into *E. coli* XL1-Blue MRA (P2) cells. Plaque replicas were screened with the DIG-labeled probes.

RNA isolation and Northern blot and Slot blot analyses Total RNA was isolated from *Methylovorus* sp. strain SS1, as described previously (Eom *et al*., 2002). DNA contamination was removed by DNase I treatment. For each sample, 10 μg of total RNA was electrophoresed on denaturing 1.2% agarose/formaldehyde gels and transferred to Hybond-N+ membranes over 2 h in a 1 M ammonium acetate solution by using a vacuum blotter (Hoefer Scientific Instruments, San Francisco). Northern blots were subjected to hybridization with ^{32}P-labeled DNA fragments internal to *Methylovorus* sp. strain SS1 *groES* and *groEL*, respectively. The conditions used for hybridization and washing have been described elsewhere (Eom *et al*., 2002). For Slot blotting, we used a slot blotter (PR648, Hoefer Scientific Instruments) according to the manufacturer's instructions.

Primer extension mapping Primer extension mapping was used to determine the transcription start site of the *Methylovorus* sp. strain SS1 *groESL* operon using total RNA, an avian myoblastosis virus reverse transcriptase primer extension system (Promega), and a 21-mer oligonucleotide primer (5'CGCTTCAATTCGCTTGACC AC3'), complementary to nucleotide positions 31 to 51 downstream of the *groES* start codon. The oligonucleotide was end-labeled with [γ-^{32}P]ATP, using a standard technique (Sambrook *et al*., 1989), and hybridized to 10 μg of total RNA isolated from *Methylovorus* sp. strain SS1 cells before and after heat shock treatment for 5 min. The annealed primer was extended at 42°C for 30 min using 10 U of reverse transcriptase. Loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to the reaction and the mixture was loaded onto a 6% polyacrylamide-urea sequencing gel with the corresponding sequencing reaction of the upstream flanking region of the *groES* gene.

Western blot analysis To obtain cell-free extracts of *Methylovorus* sp. strain SS1, bacterial cells were harvested, washed twice with 50 mM Tris-HCl (pH 7.5), and resuspended in the same buffer with 250 mM NaCl. After repeated sonification, protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. For Western blot analysis we used 50 μg of cell lysate. SDS-PAGE was carried out using 12% polyacrylamide gels, from which proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) using a Towbin buffer system (Towbin *et al*., 1979). Proteins were probed with rabbit polyclonal anti-GroEL antibody (Sigma, St. Louis, USA), and bound antibodies were detected by peroxidase-conjugated enhanced chemiluminescence (Amersham).

Promoter assay The activity of *Methylovorus* sp. strain SS1 *groES* promoter was assayed in *E. coli* DH5α transformed with a plasmid (pGL-EP1) carrying *Methylovorus* sp. strain SS1 *groE* promoter. The forward primer EPF (5'CGCGGATCCATGCCCTCTTG3'), corresponding to nucleotide positions 33 to 43 upstream of the transcription start site with artificial *Bam*HI site (underlined and extra nucleotides CGC, and the reverse primer EPR (5'GACGAAT TCTCATTTCAAATC3'), which is complementary to the nucleotide positions 34 to 54 downstream of the transcriptions start site except that the nucleotide G complementary to the position 47 downstream of the transcription start site was replaced by nucleotide T to make artificial *Eco*RI site (underlined), were used to amplify the *Methylovorus* sp. strain SS1 promoter region. The amplied 156-bp fragments were ligated to pBluescript II KS+ to make pBE-3. The pBE-3 was then digested with *Sac*I and *Xho*I and the resulting fragment containing the PCR fragment was ligated to pGL3-Basic digested with the same enzymes to make pGL3-EP1. Luciferase activity was assayed using luciferase assay reagent (Promega) and a luminometer (T2020, Turner Designs, Sunnyvale, USA) according to the manufacturer's instructions.

Nucleotide sequence accession number The nucleotide sequences of *Methylovorus* sp. strain SS1 *groESL* operon was assigned the GenBank accession no. AF152236.

Results and Discussion

Molecular cloning of the *groESL* operon By using the degenerate oligonucleotide primers EF and ER, which were synthesized using the sequence of a highly conserved region of bacterial GroEL-like proteins, a 587-bp DNA fragment was

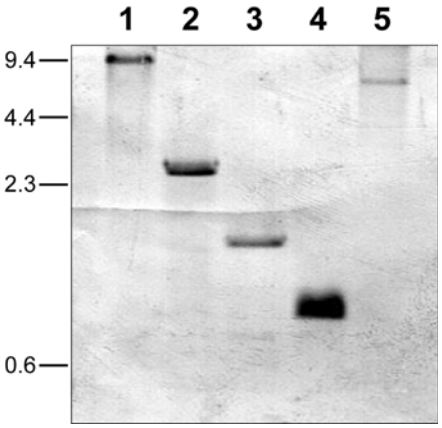

Fig. 1. Southern blot analysis of *Methylovorus* sp. strain SS1 genomic DNA digested with various restriction enzymes. Lanes: 1, *Eco*RI; 2, *Eco*RV; 3, *Nco*I; 4, *Pst*I 5, *Sal*I. A DIG-labeled 587-bp fragment of the *Methylovorus* sp. strain SS1 *groEL* gene was used as a probe. Bars indicate molecular sizes.

amplified by PCR using *Methylovorus* sp. strain SS1 genomic DNA as a template (data not shown), cloned into pGEM-T vector (Promega) to construct pSSE1, sequenced, and found to be 72% identical to the sequence that encodes amino acid positions 88 through 283 of *E. coli* GroEL. Southern blot analysis showed that the DIG-labeled *Methylovorus* sp. strain SS1 *groEL* probe hybridizes to only one fragment formed by the restriction enzyme reactions, which indicates that *Methylovorus* sp. strain SS1 contains a single copy of the *groEL* gene (Fig. 1).

Forty-five positive clones were obtained by plaque hybridization of the lambda library of *Methylovorus* sp. strain SS1 using random-primed probes. Of the positive clones, λE601, which contained an 11-kb insert DNA, was digested with *Eco*RI and subjected to agarose gel electrophoresis for Southern blotting. A 3-kb positive DNA fragment was isolated from the gel and ligated into an *Eco*RI-digested pBluescript II KS+ plasmid (Stratagene). The resulting plasmid is referred to as pYK100 (Fig. 2). A database homology search based on nucleotide sequence analysis of cloned fragment DNA showed that the entire *groES* gene and a portion of the *groEL* gene were present in pYK100. A 3.6-kb *Sal*I DNA fragment from the other clone (λE602), which contained a 16-kb insert DNA, was also cloned into pBluescript and named pYK101 (Fig. 2). pYK101 was found to contain full length genes encoding *Methylovorus* sp. strain SS1 GroEL.

Characterzation of *Methylovorus* sp. strain SS1 bicistronic *groESL* operon Sequence analysis of the relevant regions of pYK100 and pYK101 revealed the presence of two contiguous ORFs (Fig. 2). The first ORF (ORF1) was 318-bp long and encodes a polypeptide of 105 aa with a predicted molecular mass of 11,395 daltons, and has a calculated pI of 5.8. This polypeptide (called *Methylovorus* sp. strain SS1 GroES) exhibited considerable homology to the GroES-like proteins of other bacterial species. In particular, it showed overall identity to GroES homologues of α-subdivision Proteobacteria *Agrobacterium tumefaciens* (Segal and Ron, 1993), β-subdivision Proteobacteria *Nitrosomonas europaea* (Chain *et al.*, 2003), and *Neisseria meningitidis* (Tettelin *et al.*, 2000), and of γ-subdivision Proteobacteria *E. coli* (Tilly and Georgopoulos, 1982) of 54.0%, 68.0%, 66.0%, and 49.5%, respectively.

The start codon of the second ORF (ORF2) was found to be located 46-bp downstream of the stop codon (TGA) of ORF1. ORF2 is 1,623-bp long and encodes a polypeptide of 540 aa with a predicted molecular mass of 57,396 dalton and has a predicted pI of 5.1. The deduced amino acid residues were aligned with the complete sequences of the GroEL homologues of *A. tumefaciens* (66.7% identity), *N. europaea* (74.0% identity), *N. meningitidis* (71.0% identity), and *E. coli* (70.1% identity). However, We could not find out tandem repeats of the Gly-Gly-Met that is found in C-terminus of many bacterial GroEL proteins (McLennan *et al.*, 1993). This analysis indicates that GroEL is more conserved among the species than GroES, and that the GroES and GroEL of *Methylovorus* sp. strain SS1, which belongs to β-subdivision of Proteobacteria, are more closely related to the proteins found in bacteria that belong to the same phylogenetic group.

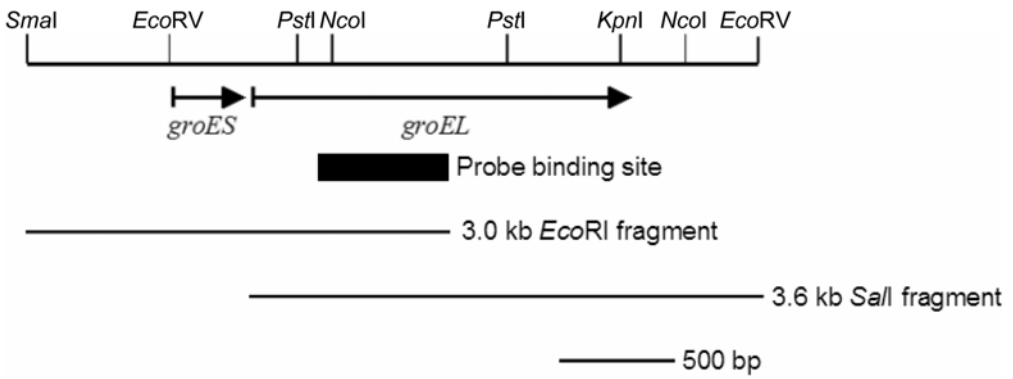

Fig. 2. Restriction maps of the *Methylovorus* sp. strain SS1 *groESL* operon. A 3.0-kb *Eco*RI fragment in pYK100 and a 3.6-kb *Sal*I fragment in pYK101 are indicated below the map. The positions and lengths of ORFs and direction (arrows) were determined by nucleotide sequencing. The probe-binding site is marked under the map.

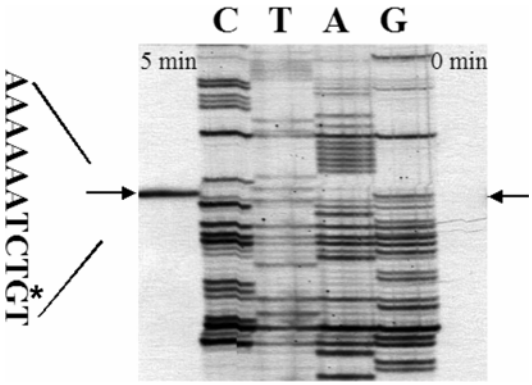

Fig. 3. Mapping of the *groESL* transcription start site. Primer extension products were generated by reverse transcription of total RNA from *Methylovorus* sp. strain SS1 cells heat-shocked for 5 min. Lanes G, A, T and C show the dideoxy-sequencing ladder obtained using the primer used for primer extension. An asterisk shows the transcriptional start site.

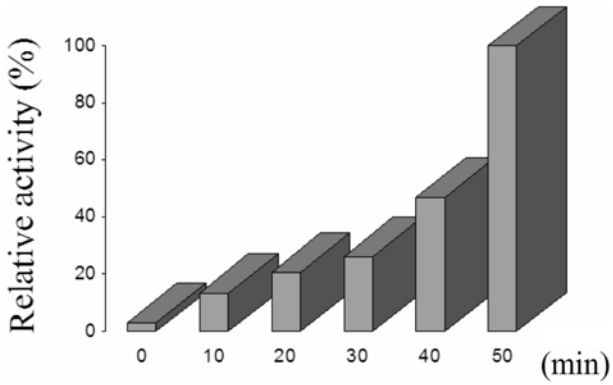

Fig. 4. Activity of *Methylovorus* sp. strain SS1 *groESL* promoter in *E. coli*. *E. coli* cells transformed with pGL-EP1 containing a DNA fragment including the *Methylovorus* sp. strain SS1 *groESL* promoter region were subjected to a temperature shift from 28°C to 37°C over 10-50 min; luciferase activity was measured as described in Materials and Methods. Activity (240.9 relative light units) measured at 50 min after the temperature shift was set as 100%. The activities presented are the mean of three tests.

This further suggests that GroES identity could be useful as an index for studying phylogenetic relationships in Proteobacteria.

Features of the noncoding regions Both ORF1 and ORF2 are preceded by putative ribosome-binding sites (AAGGAGA) (Shine and Dalgarno, 1974), 8 and 10 bp upstream of the AUG start codon, respectively. Primer extension analysis identified a presumed transcription start site corresponding to a G residue located 100 bp upstream of the translation start codon of the *groES* gene (Fig. 3). An analysis of the upstream region of the transcriptional start site revealed the presence of consensus sequences of the -35 (5' CCCTCTTGAA 3') and the -10 (5' ACCCATATC 3') regions found in σ^{32}-type promoters of α-, β-, and γ-subdivision Proteobacteria, such as, in *C. crescentus* (Roberts et al., 1996), *N. europaea* (Iizumi and Nakamura, 1997), and *E. coli* (Cowing *et al*., 1985), respectively. Furthermore, a sequence of dyad symmetry between positions -70 and -35 was identified (5' AAGAGGGCATTGAA-7 bp-TTCAATGCCCTCTT 3'). Stem-loop structures (designated CIRCE elements that control inverted repeat of chaperone expression) have been described in the 5' noncoding regions of many bacterial *groE* and *dnaK* genes (Zuber and Schumann, 1994; Avedissian and Lopes Gomes, 1996; Hecker *et al*., 1996; Segal and Ron, 1996). However, the *Methylovorus* sp. strain SS1 sequence differs from the CIRCE consensus sequence (5' TTAGCACTC-N9-GAGTGCTAA 3'). This indicates that a different mechanism is involved in the translational regulation of the *groESL* operon in *Methylovorus* sp. strain SS1. Analysis of the downstream region of the *groEL* gene revealed an inverted repeat capable of forming a stem-loop structure with a calculated free energy of -103.8 kJ per mol. The repeat was followed by a short run of Ts, suggesting that it may act as a rho-independent transcriptional terminator.

To determine whether the putative σ^{32}-type promoter of the *groESL* operon of *Methylovorus* sp. strain SS1 is functional in *E. coli*, competent *E. coli* cells were transformed with plasmid (called pGL-EP1) containing *Methylovorus* sp. strain SS1 *groE* promoter. Cells were subjected to a temperature shift from 28°C to 37°C for 10-50 min, and luciferase activity was measured (Fig. 4). The *E. coli* cells transformed with pGL-EP1 and induced at 37°C for 10, 20, 30, 40 and 50 min exhibited luciferase activities 4.3-, 6.6-, 8.5-, 15.0-, and 32.4-fold higher than those of cells harboring pGL3-Basic vector stored at 28°C for the corresponding period, respectively. This observation indicates that *Methylovorus* sp. strain SS1 *groESL* promoter is capable of functioning as a heat shock promoter in *E. coli*.

Transcription of the *groESL* bicistronic operon In *Methylovorus* sp. strain SS1, *groES* and *groEL* are separated by a 46-bp intergenic region, which contains no specific promoter sequences, but does contain ribosome binding sites. This suggests that these two genes may be co-transcribed as a single transcript. The *in vivo* transcripts of the *groESL* bicistronic operon were detected by Northern blot analysis. Total RNA was isolated from *Methylovorus* sp. strain SS1 cells grown exponentially at 30°C, subcultured, and subsequently heat-shocked for 5-60 min at 42°C. A ^{32}P-labeled 587-bp-containing *groES* probe hybridized to a single 2.2-kb transcript (Fig. 5). RNA transcripts of the same size were also detected in an experiment using *groEL*-specific probe (data not shown). This indicates in that *Methylovorus* sp. strain SS1 *groES* and *groEL* consist of a bicistronic operon that is transcribed in the order *groES-groEL*. *groESL* mRNA was expressed at a very low level in *Methylovorus* sp. strain SS1 grown at 30°C (Fig. 5. lane 1), which concurs with the result of primer extension analysis (Fig. 3). After shifting *Methylovorus* sp. strain SS1 cells to 42°C, a rapid increase in the *groESL* mRNA level was observed, which reached a maximum after 10 min and then

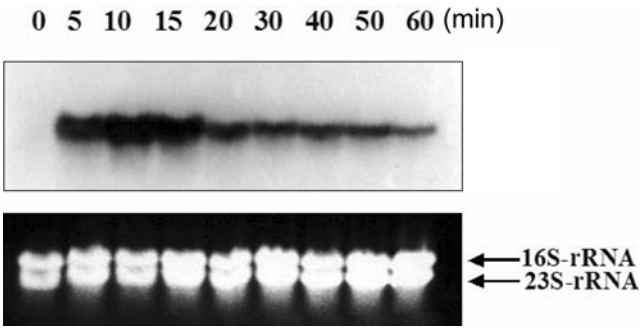

Fig. 5. Northern blot analysis of *groESL* locus genes. Northern blot analysis was performed using a ^{32}P-labeled *groES* probe and total RNAs prepared from cells harvested at the indicated times after transferring cells growing exponentially at 30°C (0 min) to 42°C, as described in Materials and Methods. Each lane contains 10 µg of RNA. The same result was obtained using a *groEL*-specific probe.

decreased from 15 min (Fig. 5). This result shows that *Methylovorus* sp. strain SS1 *groESL* is a typical heat shock operon, and that its expression level is controlled at the transcription level. To confirm Northern blot results, Western blot analysis was conducted, and the results showed that *Methylovorus* sp. strain SS1 produces similar levels of GroEL for 1 h after a temperature shift (Fig. 6), which suggests that the level of *Methylovorus* sp. strain SS1 GroEL may be controlled at the translational and transcriptional levels.

***groESL* operon is a general stress response operon** Slot blot hybridization revealed that *Methylovorus* sp. strain SS1 *groESL* was inducible by oxidative (Fig. 7, lane 4) and salt stresses (Fig. 7, lane 5) in addition to temperature shifts (Fig. 7, lane 1). This implies that the proteins encoded by *Methylovorus* sp. strain SS1 *groESL* operon may function as general shock proteins. Rifampicin (100 µg/ml), when added to culture 2 min before heat-shock treatment, was found to strongly prevent an increase in the *groESL* mRNA level after a temperature shift (Fig. 7, lane 2). This confirms that the observed increase in the amount *Methylovorus* sp. strain SS1 *groESL* operon mRNA was a result of enhanced RNA synthesis under stressed conditions.

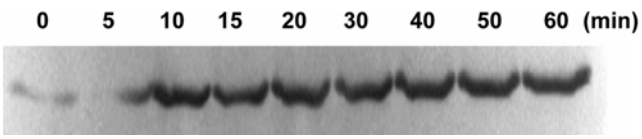

Fig. 6. Immunoblotting of GroEL in *Methylovorus* sp. strain SS1. The expression of *groEL* in cells grown on methanol was analyzed using ECL Western blot protocols after subjecting extracts prepared from cells grown on 0.5% methanol (vol/vol) at 30°C followed by incubation at 42°C for 0, 5, 10, 15, 20, 30, 40, 50, or 60 min to denaturing PAGE (10% acrylamide, 0.1% SDS), as described in Materials and Methods. Extracts from cells incubated at 30°C for 5, 10, 15, 20, 30, 40, 50, or 60 min exhibited the same expression pattern as that of 0 min stage cells.

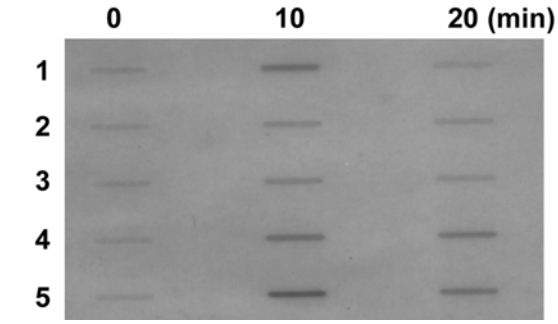

Fig. 7. Transcription of the *groESL* operon under different stresses. Slot blot analysis with total RNAs prepared from cells grown at 30°C followed by incubation at 42°C (lane 1), and cells treated with rifampicin for 2 min at 30°C followed by incubation at 42°C (lane 2), or treated with ethanol (lane 3), H_2O_2 (lane 4), or NaCl (lane 5) were performed using ^{32}P-labeled 587-bp *groEL* probes. 10 and 20 min indicate the times when cells were harvested after being exposed to stress conditions. Five µg of total RNA was applied per slot.

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