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Molecular Cloning and Nucleotide Sequence of the *groEL* Gene from the Alkaliphilic *Bacillus* sp. Strain C-125 and Reactivation of Thermally Inactivated α-Glucosidase by Recombinant GroEL

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The *groEL* gene of the alkaliphilic *Bacillus* sp. strain C-125 was cloned in *Escherichia coli* and sequenced. The *groEL* gene encoded a polypeptide of 544 amino acids and was preceded by the incomplete *groES* gene, lacking its 5′-end. The sequence of the derived amino acids was 87.5% identical to that of *B. subtilis*, 85.4% identical to that of *B. stearothemophilus*, and 60.9% identical to that of *E. coli*. The GroEL protein was expressed in *E. coli*. Purified GroEL protected yeast α-glucosidase from irreversible aggregation at a high temperature and the addition of Mg-ATP was essential for reactivation of the α-glucosidase. The addition of *E. coli* GroES increased recovery of the enzyme activity, indicating that C-125 GroEL could function in coordination with *E. coli* GroES.

Key words: alkaliphilic *Bacillus*; GroES; GroEL

When cells are exposed to various environmental stresses, synthesis of heat shock proteins (Hsps), which are highly conserved, increases in both prokaryotic and eukaryotic organisms.[1] Members of the Hsp 60 family, called chaperonin-60s, are heat shock proteins with subunits of the molecular mass of 60 kDa. The bacterial Hsp 60 is GroEL, named because it is a host protein needed for bacteriophage assembly.[2,3] GroEL acts together with another heat shock protein, GroES. Both are essential for the growth of *Escherichia coli* at temperatures between 17 and 42°C.[4] Mutations in *groES* or *groEL* lead to deficiency in DNA and RNA synthesis,[5] protein export,[6] proteolysis,[7] septation,[8] or coupling of replication between F factor and the chromosome.[9] *In vitro* studies have shown that GroEL and GroES are required for proper folding of many cellular proteins.[10] *In vivo*, a severe conditional deficiency of GroEL leads to defective biogenesis of many newly translated cellular proteins, which may then aggregate or be degraded.[11] As a general model of GroEL function in protein folding, GroEL interacts directly with an unfolded polypeptide, and then GroES binds to the GroEL-polypeptide complex. After folding, the polypeptide is released with consumption of ATP. GroES seems to affect the release by modulation of the ATPase activity of GroEL.[12]

GroEL proteins of mesophilic organisms have been characterized in terms of structure and function, but those of alkaliphiles have not been studied extensively. We describe here the characterization of the *groEL* gene and its product in *Bacillus* sp. strain C-125, which we have been studying as a typical alkaliphile.[13]

Materials and Methods

Bacterial strains and plasmids. Alkaliphilic *Bacillus* sp. strain C-125[14] was the source of the *groEL* gene. *E. coli* strain MV1184 (*araΔ*(*lac-proAB*) *rpsL thi* (*φ*80 *lacZΔ*M15) *Δ*(*srl-recA*)306::Tn10(Tet^r) F′[*traD36 proAB lacI^q lacZΔ*M15]) was used as the host strain for cloning. Plasmids pUC118 and 119 were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

E. coli was grown aerobically at 37°C in LB media, supplemented with ampicillin when appropriate. *Bacillus* sp. strain C-125 was grown in Horikoshi-II medium (pH 9)[15] at 37°C unless otherwise noted.

Molecular cloning of groEL. A pair of oligonucleotides, 5′-ATGGCAAAAGAAATTAAGTTTAGT-3′ and 5′-TTACATCATTCCACCCATACCGCC-3′, which correspond to the 5′- and 3′-ends of the *B. subtilis groEL* gene,[16,17] were used in the amplification of the *B. subtilis groEL* gene by PCR. Template chromosomal DNA was obtained from *B. subtilis* BD224 (*trpC2 thr-5 recE4*). The amplified *groEL* gene was labeled with digoxigenin-11-dUTP with a DIG DNA labeling kit (Boehringer Mannheim Biochemicals, Germany).

The *groEL* gene of *Bacillus* sp. strain C-125 was cloned as follows.[18] Restriction digests of the chromosomal DNA of strain C-125 were analyzed by Southern hybridization with *groEL* amplified by PCR and labeled with digoxigenin as the probe. DNA fragments that hybridized to the probe were purified by preparative agarose gel electrophoresis, ligated with pUC118 or pUC119, and introduced into competent *E. coli* MV1184 cells. The *groEL* clones were screened by colony hybridization.

Hybridization was done in 5 × SSC containing 20% formamide, 2% blocking reagent (Boehringer Mannheim), 0.05% sodium dodecyl sulfate (SDS), and 0.1% sodium *N*-laurolylsarcosine at 42°C overnight. After the membranes were washed in 2 × SSC, hybridization signals were detected with a DNA detection kit (Boehringer Mannheim).

DNA sequencing. Restriction fragments of the *groESL* locus of strain C-125 were subcloned into pUC vectors. Each fragment was sequenced on an ABI DNA sequencer (model 373A, Applied Biosystems Japan, Tokyo).

Purification of GroEL expressed in E. coli. Unless otherwise noted, all purification procedures were done at 4°C. *E. coli* cells carrying pGER2-18 (Fig. 1), which contained the complete *groEL* gene under control of the

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lac promoter, were grown at 37°C for 6 h in 1 liter of LB broth containing 100 μg/ml ampicillin. Then isopropylthio-β-D-galactoside (IPTG) was added to the culture to the final concentration of 1 mM, and the culture was incubated at 37°C for 2 h more for expression of the *groEL* gene.[18] After cells were pelleted at 8000 × *g* for 10 min, cells were washed once in a buffer of 50 mM Tris–HCl (pH 7.5) containing 2 mM EDTA. This buffer was used throughout the purification. The cells were suspended in 50 ml of the buffer containing 200 μM phenylmethanesulfonyl fluoride and disrupted by sonication. After removal of cell debris by centrifugation at 12,000 × *g* for 1 h, the lysate was brought to 40% saturation of ammonium sulfate. After centrifugation at 10,000 × *g* for 15 min, the supernatant was brought to 65% saturation of ammonium sulfate. The precipitate was collected by centrifugation, dissolved in the buffer, and dialyzed against the same buffer. The resultant solution was used in DEAE-Toyopearl column chromatography (1.4 × 7.0 cm) and eluted with a linear gradient of 0 to 1 M NaCl at a flow rate of 0.4 ml/min. The fractions containing GroEL were concentrated to 1.0 ml with a Centriprep-30 apparatus (Amicon Co., Beverly, Massachusetts). The 0.2-ml portions were put on a Superdex 200 HR 10/30 column and fractionated at a flow rate of 0.5 ml/min under control of a FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden).

SDS–PAGE. SDS–PAGE was done on a prepared gel (TEF Co., Nagano, Japan) according to the supplier's instructions. Phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400) were used as molecular mass markers. Protein bands were stained with 0.2% Coomassie Brilliant Blue R-250.

Amino-terminal amino acid sequence. Protein bands on SDS–PAGE were transferred to an Immobilon transfer membrane (Millipore Corp., Bedford, Massachusetts) by a semi-dry transfer cell apparatus (Biorad, Hercules, California). The amino-terminal sequences were identified on an Applied Biosystems 373 protein sequencer.

Reactivation of thermally inactivated α-glucosidase by purified GroEL. GroEL activity was examined with thermally inactivated α-glucosidase.[19] Yeast α-glucosidase (EC 3.2.1.20) was purchased from Boehringer Mannheim. The α-glucosidase (2.2 μM) was inactivated thermally in 0.1 M Tris–HCl (pH 7.5) containing 10 mM KCl at 47°C for 1 h in the presence of purified GroEL (2.6 μM as a 14-subunit oligomer). After the mixture was cooled to 25°C, $MgCl_2$ and ATP were added to the final concentrations of 10 mM and 2.0 mM, respectively, and the solution was incubated at 25°C for 3 h. The enzymatic activity of the α-glucosidase was assayed as described elsewhere with *p*-nitrophenyl-α-D-glucopyranoside as the substrate.[20] The effect of *E. coli* GroES (Takara) was measured by the addition of the protein (final concentration, 2.2 μM as a 7-subunit oligomer) together with $MgCl_2$ and ATP.

Heat shock response. The upper limit of temprature at which strain C-125 grew was 54°C. The organism was first grown at 37°C for 8 h, and then the temperature was shifted to the sublethal temperature of 52°C. Samples were taken every 1 hour and the total crude proteins obtained by sonication were analyzed on 10% SDS–PAGE. Protein bands on the gel were transferred to a Hybond-ECL nitrocellulose membrane (Amersham International plc., Buckinghamshire, England) with a semi-dry transfer cell apparatus (Biorad) according to the supplier's instructions. GroEL was detected by a ECL detection system with anti-rabbit IgG linked with horseradish peroxidase (Amersham). Rabbit antiserum against C-125 GroEL was prepared with use of purified GroEL as described before.[14]

Protein concentration. Protein concentrations were assayed by the method of Bradford[21] with bovine plasma gamma globulin as the standard.

Nucleotide sequence accession number. The nucleotide sequence of the C-125 *groESL* locus has been assigned DDBJ accession number D55630. The nucleotide sequences of the *groESL* operons of *B. subtilis*, *B. stearothermophilus*, and *E. coli* were obtained from GenBank (M81132, L10132, and X07850, respectively).

Results and Discussion

Molecular cloning and nucleotide sequence of the groEL gene

Several DNA fragments of alkaliphilic *Bacillus* sp. strain C-125 that hybridized to the *B. subtilis groEL* gene were cloned into *E. coli* as shown in Fig. 1. In the total nucleotide sequence of the cloned fragments (2540 bp), the *groEL* gene homologue of 1632 bp was preceded by an incomplete *groES* gene homologue (264 bp) lacking its 5'-region (Fig. 2). The derived amino acid sequence of the incomplete *groES* homologue (88 amino acids) was 81.8%, 84.1%, and 51.1% identical to that of *B. subtilis*, *B. stearothermophilus*, and *E. coli*, respectively. The *groEL* homologue encoded a polypeptide of 544 amino acids with the calculated molecular weight of 57,773. The Gly-Gly-Met sequence that has been found in *groEL* proteins of various sources[17] was found at the carboxy terminus. The derived amino acid sequence was 87.5%, 85.4%, and 60.9% identical to that of *B. subtilis*, *B. stearothermophilus*, and *E. coli*, respectively. As described below, the product from the *groEL* homologue reactivated thermally inactivated yeast α-glucosidase in the presence of Mg-ATP, so we concluded that the homologue was *groEL*. Several trials to clone the amino-terminal part of the *groES* gene failed, probably because the region upstream from the *groES* gene is unfavorable to the host organism.

A putative ribosome binding site, AGGAGG, was located 11 bases upstream from the start codon (ATG) of the *groEL* gene. No promoter consensus was observed in the region between *groES* and *groEL*, suggesting that these genes are encoded as a single operon, as in *E. coli* and *B. subtilis*.[16,17,22] A stem-loop structure with T's found 96 bases downstream of the termination codon (TAA) probably functioned as a transcriptional terminator.

Expression of C-125 groEL in E. coli and purification of recombinant GroEL

E. coli cells carrying the recombinant plasmid pGER2-18 (Fig. 1) produced a 58-kDa protein when treated with IPTG. Prolonged induction gave another abundant protein of 53 kDa (data not shown). The amino-terminal sequences of these proteins were MAKDIKFSEDARRXMLRGVDK-LAXAVK and KFGSPLITNDGVTIAKEIELXDAFXN-MGAKLVAE (X, not identified), which corresponded to amino acids 1–27 and 42–75 of the derived amino acid

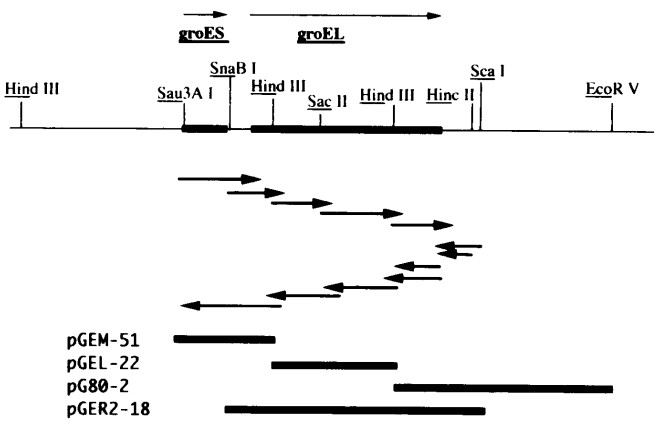

Fig. 1. Restriction Map of the *groESL* Locus and Sequence Strategy.

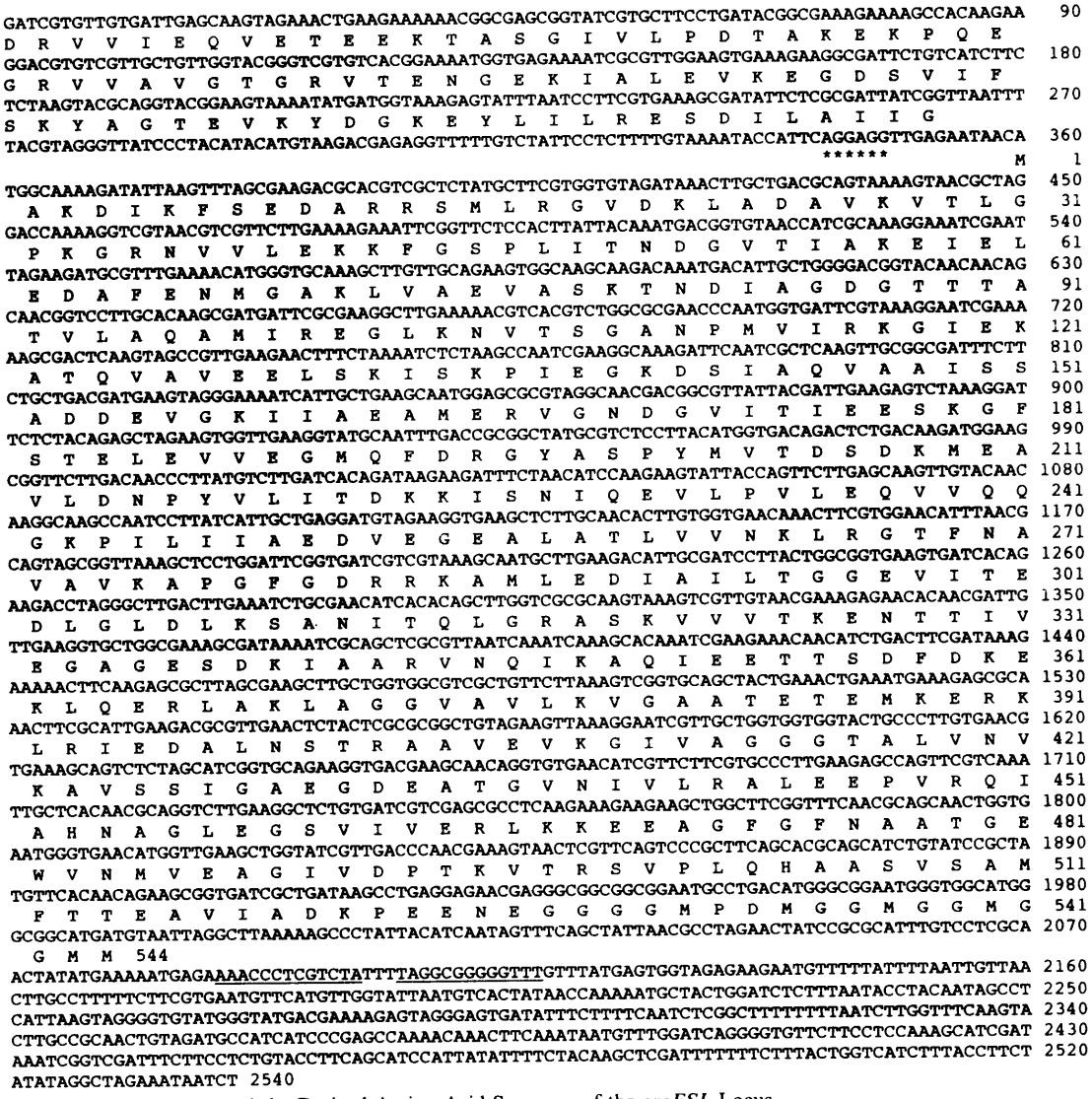

Fig. 2. Nucleotide Sequence and the Derived Amino Acid Sequence of the *groESL* Locus.

A putative ribosomal binding site is indicated by asterisks below the sequence. A stem-loop structure downstream from the *groEL* gene is underlined.

sequence of the *groEL* gene, respectively. The 53-kDa protein was probably a degradation product of GroEL.

C-125 GroEL (58 kDa) was purified from *E. coli* carrying pGER2-18 by ammonium sulfate precipitation, DEAE-Toyopearl chromatography, and gel filtration on Superdex 200 HR 10/30 (Fig. 3).

Reactivation of thermally inactivated α-glucosidase

When incubated at 47°C, yeast α-glucosidase was completely and irreversibly inactivated with formation of protein aggregates,[19] seen as precipitates. In the presence of C-125 GroEL, protein aggregates were not observed, but neither was α-glucosidase activity detected. The enzyme activity was not affected by incubation of the native enzyme with GroEL at 25°C. Addition of GroEL after the formation of the aggregates and subsequent incubation at 25°C did not cause the aggregates to go into solution. These results suggested that GroEL interacted only with the nonnative enzyme. One characteristic of nonnative proteins is that hydrophobic residues otherwise in the interior are exposed.[23] By formation GroEL–enzyme complexes, these exposed hydrophobic residues are probably prevented from inter- and intramolecular aggregation.

The addition of Mg-ATP to the mixture of GroEL and enzyme was required for reactivation of the α-glucosidase (Fig. 4). After the addition of $MgCl_2$ and ATP, enzyme activity recovered over time and reached 30% of the original. No reactivation was observed when GroEL and Mg-ATP were added to the aggregated form of the enzyme (data not shown). Therefore, GroEL alone could protect the enzyme from irreversible aggregation, but was not sufficient for its reactivation. The reactivation and release of thermally inactivated α-glucosidase required Mg-ATP.

The addition of *E. coli* GroES and Mg-ATP to the mixture of GroEL and enzyme gave recovery twice that with Mg-ATP alone. Thus, *E. coli* GroES can act in coordination with C-125 GroEL, although the sequences of *E. coli* and C-125 GroESs were not very similar.

Effects of temperature and pH on the production of GroEL in Bacillus sp. strain C-125

When strain C-125 cells grown at 37°C were next cultured at 53°C, GroEL production increased (Fig. 5). The amount of GroEL reached a maximum within 120 min. This result indicated that production of GroEL was heat-inducible in strain C-125, as in other organisms.

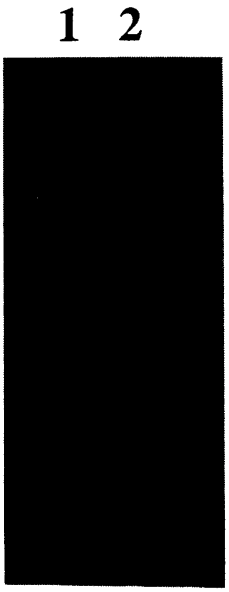

Fig. 3. SDS-PAGE of the Purified GroEL.

Lane 1, purified GroEL (3 μg). Lane 2, phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

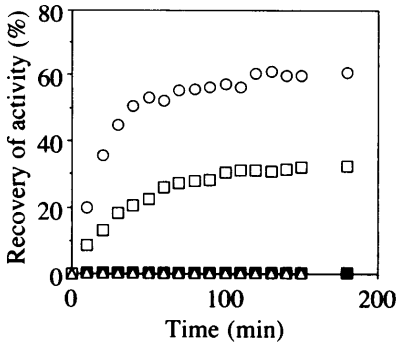

Fig. 4. Reactivation of Thermally Inactivated α-Glucosidase.

α-Glucosidase and GroEL were first incubated in 0.1 M Tris-HCl (pH 7.5) containing 10 mM KCl at 47°C for 1 h. After the mixture was cooled to 25°C, $MgCl_2$ and ATP (open squares), or *E. coli* GroES, $MgCl_2$, and ATP (open circles) were added and reactivation was monitored. No additions (open triangles). Closed squares show results of the control experiment with α-glucosidase alone.

The amount of GroEL was unaffected by pHs in the range of 7.0 to 12.5 (data not shown). Thus, induction of GroEL synthesis did not seem to be required for growth at extreme pHs. Strain C-125 has an efficient sodium proton antiport system to change the intracellular pH toward neutral,[24] which might help the cells survive at extreme pHs.

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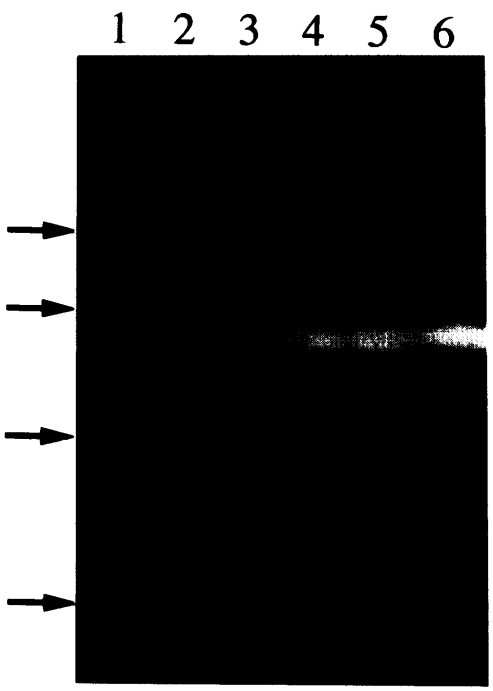

Fig. 5. Production Triggered by a Temperature Shift of GroEL by Strain C-125 Cells (ECL-Western Blot Analysis).

Lane 1, purified GroEL (1 μg); lanes 2 to 6, 0, 1, 2, 3, and 4 h after the temperature shift started (50 μg of total proteins). Positions of the protein standards, phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000), are indicated by arrows.