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Cloning and characterization of *grpE* in *Acetobacter pasteurianus* NBRC 3283

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The *grpE* gene in *Acetobacter pasteurianus* NBRC 3283 was cloned and characterized, to elucidate the mechanism underlying the resistance of acetic acid bacteria to the stressors existing during acetic acid fermentation. This gene was found to be located in tandem with two related genes, appearing on the genome in the order *grpE–dnaK–dnaJ*. A σ^{32}-type promoter sequence was found in the upstream region of *grpE*. The relative transcription levels of *grpE*, *dnaK*, and *dnaJ* mRNA were in the ratio of approximately 1:2:0.1, and the genes were transcribed as *grpE–dnaK*, *dnaK*, and *dnaJ*. The transcription level of *grpE* was elevated by heat shock and treatment with ethanol. Co-overexpression of GrpE with DnaK/J in cells resulted in improved growth compared to the single overexpression of DnaK/J in high temperature or ethanol-containing conditions, suggesting that GrpE acts cooperatively with DnaK/J for expressing resistance to those stressors considered to exist during acetic acid fermentation. Our findings indicate that GrpE is closely associated with adaptation to stressors in *A. pasteurianus* and may play an important role in acetic acid fermentation.

[**Key words:** *Acetobacter*; *grpE*; Stress; Acetic acid fermentation; Heat-shock protein; qRT-PCR]

The function of acetic acid bacteria belonging to Acetobacteraceae, Alphaproteobacteria, is critical for vinegar production. They have high ability to produce acetic acid from ethanol for energy generation. The oxidation of ethanol, catalyzed by membrane-bound alcohol dehydrogenase and acetoaldehyde dehydrogenase, is referred to as acetic acid fermentation (1, 2).

During acetic acid fermentation, acetic acid bacteria are inevitably exposed to various stressors, such as heat, ethanol, and acetic acid. Such stressors inhibit the growth of many other microorganisms; however, acetic acid bacteria are resistant to these stressors and maintain their ability for cell growth. Therefore, elucidating the mechanism underlying the resistance of acetic acid bacteria to these stressors is important for establishing more efficient techniques for fermentation and vinegar production. Thus, in the present study, we investigated the function of molecular chaperones in acetic acid bacteria, which are considered to protect cells against various stressors.

Previously, we reported on the cloning and characterization of the *groESL* and *dnaKJ* in *Acetobacter pasteurianus* NBRC 3283, which is used in traditional vinegar production in Japan (3, 4). These studies showed that, in this bacterium, both GroES/L and DnaK/J play important roles in resistance to various stressors (3, 4). However, the role of GrpE was not included in these studies. GrpE is a kind of heat-shock protein that acts as a co-chaperone in the functional cycle of the DnaK chaperone system. It binds with high affinity to the ATPase domain of DnaK to mediate the dissociation of DnaK-bound ADP (5–11). This activity accelerates the ATP-binding/ADP-release cycle of DnaK and, consequently, the ATP-mediated dissociation of proteins from DnaK. Thus, GrpE is considered to be essential for the proper chaperone activities of the DnaK system (5).

To accurately judge the effect of this chaperone system, it is essential to investigate the relationship between GrpE and DnaK/J. Hence, in the present study, we examined GrpE together with DnaK/J in *A. pasteurianus* NBRC 3283, to further investigate the mechanism underlying stress resistance during fermentation. To elucidate the role of GrpE in acetic acid fermentation, we cloned the *grpE* gene in *A. pasteurianus* NBRC 3283 and characterized the effect on expression of this gene to stressors that normally exist during acetic acid fermentation.

MATERIALS AND METHODS

Bacterial strains, plasmids, probes, and primers *A. pasteurianus* NBRC 3283, which was formerly assigned as *Acetobacter aceti*, was used throughout this experiment. *Escherichia coli* DH5α [*deoR*, *endA1*, *gyrA96*, *hsdR17* ($r_K^-m_K^+$), *recA1*, *relA1*, *supE44*, *thi-1*, Δ(*lacZYA*$^-$*argFV169*), φ80*lacZ*Δ*M15*, F^-] and *E. coli* HB101 [*supE44*, *hsd20* ($r_B^-m_B^-$), *recA13*, *ara-14*, *proA2*, *lacY1*, *galk2*, *rpsL20*, *xyl-5*, *mtl-1*, *thi-1*, *leuB6*] were used as hosts for the cloning experiments. The pGEM®-T EASY VECTOR plasmid (Promega, WI, USA) was used for both cloning and sequencing analysis. The pUC119 plasmid (Takara Bio, Shiga) was used to construct the plasmid used for qRT-PCR analysis. The pMV24 plasmid (*Ap*^{r}, *lacZ*), a shuttle vector between *Acetobacter* and *E. coli* (12), was used for overexpression of the genes in *A. pasteurianus* NBRC 3283. The oligonucleotide primers used in this experiment are listed in Table 1.

Media and culture conditions *A. pasteurianus* NBRC 3283 was grown at 30 °C on YPD medium, which was composed of 10 g yeast extract (Oxoid, Hampshire, UK), 10 g polypeptone (Nippon Seiyaku, Tokyo), and 30 g glucose dissolved in 1 L of distilled water. When necessary, the medium was supplemented with 100 μg/mL ampicillin.

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TABLE 1. Primers used in this study.

Name	Sequence (5′ to 3′)	Also used for
For cloning of *grpE*		
KF1	ATGAGTAAAGTTATTGGTATCGACC	Northern hybridization and RT-PCR
ER1	GGAAGCCTTGGCAACCACCACCATG	Northern hybridization
For Northern blotting		
EF1	GCTGCGTTCAGAGGCTGAAA	
KR1	CAGAACAAGGTGTTTCGG	
JF1	ATGAGTAAAGTTATTGGTATCGACC	RT-PCR
JR1	TTATTCCTTCCCTTCAAA	RT-PCR
For RT-PCR		
EF2	ATGACCCAAGATACAGATCCCACACAGGTCG	
KR2	TCAGGACTTCTTGGTGTCGTTCAC	
For qRT-PCR		
QEF	TCGTGCAAAGCGTGATCTG	
QER	CGTCACGCGCAAATTTCTG	
QKF	CATTGTGTCTGTGTCTGCTA	
QKR	TTGGCTTCAGCGTCCTTC	
QJF	CAGCAGAAGCCAAGTTCA	
QJR	CGCTTTTGTTCGTCCTTCA	
QmF	CTCACGGCTGCTGATGCTT	
QmR	CAAACGTGTAACCGCCTGATG	
For construction of a control plasmid of qRT-PCR		
muF	CCATCGATTGGACCCTGCGGAAATTC	
muR	GGACTAGTCCATATTCGGCCCCGTTAAC	
PUF1	GGACTAGTAGTTTTCGTTCCACTGAGCG	
PUR1	CCATCGATGGGATTTTGGTCATGAG	
For construction of overexpressing strains		
OEEF1	ATGGATCCCTATAGACTAGGCCCTGCTATCCTATCCTG	Construction of a control plasmid of qRT-PCR
OEER1	ATGGATCCAGGCCTGAATGATAAACCTGAGTATGGTG	
OEKF1	ATGAATTCTGACCCGGCAGGCAAGCCGTTTGATGCCAA	
OEJR1	ATGAATTCGCTGTTCGGCTTACGTGTGTCCGCCAGAAA	Construction of a control plasmid of qRT-PCR

E. coli was grown at 37 °C in Luria-Bertani (LB) broth supplemented with 50 μg/mL ampicillin.

General molecular biology techniques General molecular biology techniques, including restriction enzyme digestion, agarose gel electrophoresis, and transformation of *E. coli*, were performed according to standard protocols (13). A ligation kit (Takara Bio, ver.1) was used for standard ligations. Plasmids were extracted by the alkali-SDS method, and chromosomal DNA was prepared by the CTAB method, as described previously (3). PCR amplification was performed with the use of Ex-Taq polymerase (Takara Bio) in a total volume of 100 μL with a 10-ng DNA template and 20 pmol of each primer. The reaction was carried out with 30 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min. Southern blot analysis was performed by using a DIG Labeling and Detection System (Roche Diagnostics, Tokyo) according to the manufacturer's instructions and at a hybridization temperature of 42 °C. Nucleotide sequences were determined by using a CEQ8000 genetic analyzer (Beckman Coulter, CA, USA). Cycle sequencing for nucleotide sequence analysis was carried out with a GenoLab™ DTCS-Quick Start kit (Beckman Coulter) according to the manufacturer's instructions. Sequence data were analyzed with the BLASTP (National Center for Biotechnology Information, MD, USA) and GENETYX-WIN (Genetyx, Tokyo) software programs. Competent cells of *A. pasteurianus* NBRC 3283 were prepared and transformed as described previously (3).

Cloning of the *grpE* in *A. pasteurianus* NBRC 3283 The *grpE* fragment of *A. pasteurianus* NBRC 3283 was located upstream of the previously reported *dnaK* gene [EMBL/DDBJ/GenBank accession no. AB121676 (4)]. We cloned the full sequence of the *grpE* gene by inverse PCR with the primers KF1 and ER1 by using the self-ligated PstI fragment of the chromosomal DNA as a template. The sequences of these primers are shown in Table 1. The nucleotide sequence data reported here were deposited in the EMBL/DDBJ/GenBank databases under the accession number AB491474. PCR amplification and preparation of the self-ligated products were performed as described previously (3).

Northern blot analysis For Northern blot analysis, total RNA was prepared from *A. pasteurianus* NBRC 3283 according to the following procedure. *A. pasteurianus* NBRC 3283 was cultured at 30 °C to mid-log exponential phase (OD_{660} = 0.2), at which point the temperature was raised to 42 °C. At various time intervals after the temperature shift, cells were harvested and total RNA was isolated by means of the hot phenol extraction method described by Brumlik and Storey (14). Northern blot analysis was performed as described previously (3). The 415-bp *grpE* probe, the 879-bp *dnaK* probe, and the 1140-bp *dnaJ* probe were amplified by using the EF1 and ER1, KF1 and KR2, and JF1 and JR1 primers, respectively. The sequences and the location of these primers are shown in Table 1 and Fig. 1, respectively. Prehybridization and hybridization were performed at 42 °C. A digoxigenin labeling and detection kit (Roche Diagnostics) was used to label the probes and detect the bands according to the manufacturer's instruction. CSPD (Roche Diagnostics) was used as the substrate for the digoxigenin-antibody-conjugated alkaline phosphatase, and the chemiluminescence emitted from the hybridized probe was recorded on Polaroid film with an ECL mini-camera (Amersham Biosciences).

RT-PCR analysis For RT-PCR analysis, total RNA was prepared according to the following procedure. *A. pasteurianus* NBRC 3283 was cultured at 30 °C to mid-log exponential phase (OD_{660} = 0.2), at which point the temperature was either raised to 42 °C or ethanol was added to the culture (to a final concentration of 1%). After 10 min, the cells were harvested and treated with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany). Total RNA was then was extracted by use of the RNeasy kit according to the manufacturer's instructions (Qiagen). RNA samples were then treated with DNaseI (Qiagen) to remove contaminating DNA.

After denaturation at 65 °C for 5 min, the RNA samples (1 μg) and 100 units of ReverTra Ace (Toyobo, Osaka, Japan) were added to a reaction mixture composed of 10 units of RNase inhibitor (Toyobo) and random primer (final concentration, 2.08 μm). cDNA was synthesized by incubating this mixture at 30 °C for 10 min, 42 °C for 60 min, and 85 °C for 5 min. The cDNA was then subjected to PCR amplification by using the primer pairs EF2–JR1 (for *grpE–dnaK–dnaJ*), EF2–KR2 (for *grpE–dnaK*), KF1–JR1 (for *dnaK–dnaJ*), and JF1–JR1 (for *dnaJ*) (Table 1 and Fig. 1). PCR amplification was performed with KOD-Plus DNA polymerase (Toyobo, Ltd.) according to the manufacturer's instructions.

Quantitative reverse transcriptase PCR analysis *Cloning of the mutS gene and construction of a control plasmid for calibration of gene expression* The *mutS* gene, which encodes the mismatch repair protein MutS in *A. pasteurianus* NBRC 3283, was obtained by chance from a genome library constructed for another study. This gene was found upstream of *glnD* gene which we focused on. The full sequence

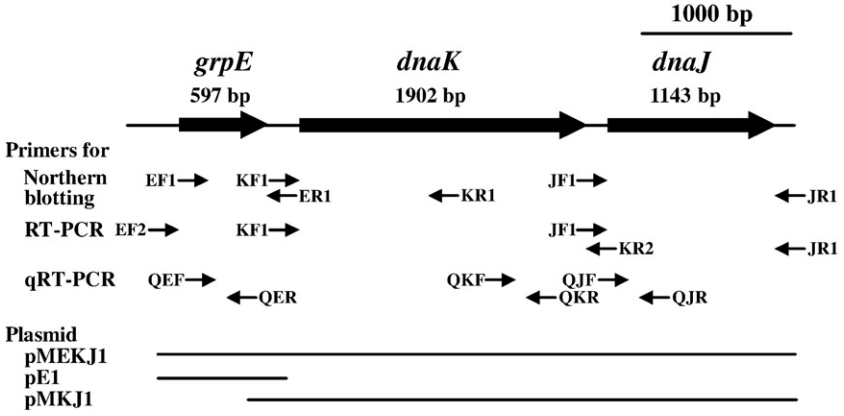

FIG. 1. Genetic map of the *grpE*, *dnaK*, and *dnaJ* regions of *A. pasteurianus* NBRC 3283. The locations of the *grpE*, *dnaK*, and *dnaJ* genes are indicated by large arrows. The locations of the primers used in the Northern hybridization analysis, RT-PCR, and qRT-PCR are indicated by small arrows. The DNA fragments that were inserted in the plasmids used to construct the overexpressing strains are shown as solid lines.