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Contributed Paper

Disruption of the *groEL* Gene Revealed A Physiological Role for Chaperonin in the Thermotolerant Acetic Acid Bacterium, *Acetobacter pasteurianus* SKU1108

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ABSTRACT

In the present study, we aimed to clarify the potential roles, the effect of *groEL* gene disruption and the induction of *groESL* gene expression in the thermotolerant acetic acid bacterium, *Acetobacter pasteurianus* SKU1108. The growth of the *groEL* gene disruptant decreased and the disruptant exhibited a complete loss of toleration ability to stressors compared with a wild type. Complementation of the *A. pasteurianus* SKU1108 *groESL* gene in a *groEL* gene disruptant restored significant toleration ability to stressors. The heat shock promoter was induced by stressors for *groESL* gene expression as shown by a steady increase in the transcription level of the *groESL* gene with elevated temperature, acetic acid and ethanol concentration. Moreover, a CIRCE element found in the upstream region was examined and compared in all known genome sequences of acetic acid bacteria. The results revealed that these consensus sequences of all strains are highly conserved and have evolved phylogenetically.

Keywords: *Acetobacter pasteurianus* SKU1108, CIRCE element, *groEL* gene disruptant, *groESL* operon, stressor

1. INTRODUCTION

One of the interesting microbial strains for studying adaptation under environmental stresses is acetic acid bacterium (AAB), especially *Acetobacter* sp. During acetic acid fermentation, AAB are exposed to unfavorable conditions such as high temperature, acetic acid and ethanol. Although there are many stressors, AAB show relatively normal growth with high resistance to these stressors. Elucidation of the resistance mechanism in AAB against those stressors is important for developing strains with more sophisticated acetic acid fermentation ability.

The adaptation to such various stressors involves the induction of the synthesis of a large number of highly conserved heat shock proteins (HSPs). Major HSPs are DnaK, DnaJ and GrpE (both co-chaperones of DnaK) which form the DnaK chaperone machine working in cooperation with the ClpB chaperone [1, 2]. Additionally, there are GroEL (chaperonin) and GroES (co-chaperonin), which constitute the GroE chaperone machine [1]. Most of these are associated with the bacterial stress-response mechanism. The most intensively studied HSPs are those of the 60 kDa-GroEL families, which are designated as molecular chaperones according to their functions. The GroES and GroEL are essential for growth at all temperatures and are constitutively translated. In cooperation with the 10 kDa-GroES protein, GroEL mediates the correct folding and assembly of many cellular proteins and consequently, prevents misfolding, promotes the refolding and proper assembly of unfolded protein [3, 4].

In *Acetobacter pasteurianus,* the expression of *groESL* operon is transcriptionally regulated by the heat shock transcription activator, alternative sigma factor, that recognizes specific heat shock promoter [5]. A characteristic inverted repeat (IR) sequence (Controlling Inverted Repeat of Chaperone Expression; CIRCE) is detected downstream of the heat shock promoter in the *groESL* operon, and the *hrcA* gene encoding HrcA repressor protein, which functions with the CIRCE, is detected in this bacterium [5, 6]. The highly conserved CIRCE element has been found in numerous phylogenetically distant bacteria such as *Agrobacterium tumifaciens* [7] and *Bacillus subtilis*. [8]. The CIRCE element is involved in negative regulation at the RNA level by affecting mRNA synthesis and/or stability or at the DNA level by acting as an operator for the repressor protein, HrcA [7, 8]. The existence of a conserved CIRCE in the upstream regulatory region of the two most highly conserved proteins GroEL and DnaK suggests that the corresponding genes are also regulated in a conserved way [9].

Acetobacter pasteurianus SKU1108 is a thermotolerant acetic acid bacterium showing high ability for ethanol oxidation and acetic acid production at high temperature [10]. In this study, we aimed to elucidate the function of the *groESL* gene especially the role of this protein in the stress response during acetic acid fermentation. The *groESL* operon of this bacterium was disrupted, and then investigated for its contribution to the bacterial survival mechanism under stress conditions. We also analyzed the regulatory region, the CIRCE heat shock element, including the effect of stressors to induce *groESL* gene expression and also its distribution in AAB.

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Culture Medium and Culture Conditions

The *A. pasteurianus* SKU1108 (thermotolerant strain) used in this study was previously isolated from grape in Thailand [10]. The stock culture was maintained on potato medium (5 g of glucose, 20 g of

glycerol, 10 g of yeast extract, 10 g of polypeptone and 15 ml of potato extract, made up to 1,000 ml with tap water) containing 0.5% $CaCO_3$ and 1.5% agar. YPGD medium (5 g of yeast extract, 5 g of polypeptone, 5 g of glycerol, and 5 g of glucose per 1000 ml of tap water) was used for general cultivation to determine bacterial growth. Acetic acid or ethanol was added aseptically as indicated concentration in the Figure legends. Five milliliters of overnight culture in potato medium was inoculated into 100 ml of YPGD medium in a 500-ml Erlenmeyer flask. Cultivation was performed at 30°C on a rotary shaker at 200 rpm. The growth was periodically measured with a spectrophotometer. *Escherichia coli* DH5α and S17-1 were routinely cultured in Luria-Bertani broth. Ampicillin (50 μg/ml), kanamycin (50 μg/ml) and tetracycline (25 μg/ml) were added to the medium when necessary to maintain the plasmids.

2.2 Cloning and Nucleotide Sequencing of the *groESL* Gene

Molecular cloning of the *groESL* gene was performed by PCR and shotgun cloning into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and pUC119 Vector (Takara, Biomedicals, Tokyo), respectively. The *groESL* gene was amplified by two modified specific primers, GroF1 (5'-AGG AAAAGCCTATGGAAG-3') started at the 97th nucleotide from initiation codon of *groES* and GroR1 (5'-GCTGTGGAAGAAGGC AT-3') started at the 1,216th nucleotide from initiation codon of *groEL* [11]. The PCR was performed by using the Ready-To-Go™ PCR Beads (Amersham Biosciences, Buckinghamshire, UK), in the total volume of 25 μl. The PCR thermal cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min. The obtained 1.4 kb PCR product was recovered from 0.8% agarose gel and introduced into pGEM®-T Easy Vector for PCR cloning. The constructed plasmid was designated pGEM-TgroESL and its nucleotide sequences were determined. The purified 1.4 kb *groESL* gene was labeled with digoxigenin-11-dUTP (Dig-11-dUTP; Roche Applied Science, Penzberg, Germany) by random primed labeling and used as a DNA probe for Southern hybridization. The chromosomal DNA from *A. pasteurianus* SKU1108 was completely digested with several enzymes: *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Sma*I (Thermo scientific, USA). The digested chromosomal DNAs were hybridized with the 1.4 kb *groESL* gene DNA probe. An approximately 3.2 kb *Hind*III DNA fragment showing a positive hybridization signal was recovered from gel slices. The purified 3.2 kb *Hind*III DNA fragment carrying *groESL* gene was cloned into the *Hind*III digested pUC119. The constructed plasmid was designated pUCgroESL. Plasmid pUCgroESL was transformed into *E. coli* DH5α competent cells. Colony hybridization was performed according to the procedure described by Kim *et al.* [12]. Among 200 ampicillin resistant transformants, three positive clones exhibiting positive signals by colony hybridization were obtained and confirmed by PCR amplification with two specific primers. One of these clones was selected for further DNA sequencing.

2.3 Overexpression of *groESL* Gene in *A. pasteurianus* SKU1108

Plasmid pCMgroESL was constructed to overexpress the *groESL* operon (*groES* and *groEL* gene). A 3.2 kb *Hind*III fragment carrying *groESL* gene from *A. pasteurianus* SKU1108 was inserted into a broad host range vector, pCM62 [13]. The *groESL* gene was inserted with the same orientation as P*lac*.

Both Plasmids, pCM62 and pCMgroESL, were individually transferred into *A. pasteurianus* SKU1108 by conjugal transformation and selected on YPGD medium containing 25 µg/ml tetracycline and 0.1% acetic acid. The obtained tetracycline resistant conjugants were designated as SKU1108/pCM62 and SKU1108/pCMgroESL. For growth determination under stress conditions, the *groESL*-overexpressed strain, SKU1108/pCMgroESL and the control strain, SKU1108/pCM62 were pre-cultured in 5 ml of the potato medium and incubated at 30°C with vigorous agitation (200 rpm) for 18 h. The turbidities of the bacterial cell cultures were measured with a spectrophotometer at 540 nm. The cell cultures were inoculated into 100 ml of YPGD medium containing 0%, 4% acetic acid or 5% ethanol in a 500-ml Erlenmeyer flask at an initial absorbance of approximately 0.1. Cultivation was performed at 30°C or 40°C on a rotary shaker (200 rpm). Bacterial growth was periodically measured for 7 d.

2.4 Reverse Transcriptase PCR Analysis

Cells were grown until early exponential phase and subsequently induced with stressors such as high temperature and high concentrations of acetic acid or ethanol. After incubation for various times, total RNA was prepared by the hot phenol method [14]. The concentration of RNA was measured with spectrophotometer at 260 nm. RT-PCR analysis was performed by using an mRNA Selective RT-PCR Kit (Takara Shuzo, Kyoto, Japan) with 0.1 µg of RNA as template and the primer set that is described below. The *groEL* gene was amplified by two specific primers, GroEL-F (5'-GCAGAAA GTTGGCTCGA-3') and GroEL-R (5'-TT CAGGGTAACGGTTTCC-3') started at the 498th nucleotide and the 926th nucleotide from the initiation codon of *groEL*, respectively. The RT-PCR reaction was performed at 40°C for 15 min; PCR consisted of denaturing at 82°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min. The 447 bp *groEL* gene PCR products after 20, 25, 30 and 35 cycles were analyzed by 0.8% agarose gel electrophoresis. Under our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

2.5 Construction of *groEL* Gene Disruptant

The *groEL* gene disruptant was constructed by replacing the internal part of *groEL* gene with non-polar Km^r cassette using a homologous recombination strategy. The procedures may be described briefly as follows. The *Msc*I restriction site, the 679th nucleotide downstream from the ATG start codon of the *groEL* gene on pUCgroESL, was blunt digested and further inserted with a 0.9 kb *Eco*RV DNA fragment carrying a kanamycin resistant cassette from a pTKm plasmid [15] at the same site. The resulting plasmid, pUCgroEL::Km^r was electroporated into *A. pasteurianus* SKU1108 and the *groEL* gene-deficient mutants were selected on YPGD medium containing 50 µg/ml kanamycin. Growth of the *groEL* gene disruptant was observed and compared with the wild type under the following stress conditions: 40°C, 2% acetic acid or 8% ethanol. The procedures for growth determination are described in the *groESL* gene overexpression section.

2.6 Complementation of *groESL* Gene in the *groEL* Gene Disruptant

The pCMgroESL plasmid and pCM62 plasmid were individually transferred into the *groEL* gene deficient mutant by a

conjugation method. The obtained conjugants were selected on YPGD medium containing 25 µg/ml tetracycline and 50 µg/ml kanamycin. The conjugants were designated as DGL5/pCMgroESL and DGL5/pCM62. For plate assay, the turbidity of cells grown on potato liquid medium at 30°C for 18 h was measured and the concentration was adjusted to the same concentration (absorbance of approximately 1.0), and then the cells were subjected to 10-fold dilution with sterilized water. Three µL of serially diluted (10 times) cells were spot-inoculated on YPGD agar plate and incubated at various temperatures for determination of temperature sensitivity. Their abilities to grow were observed after incubation for 3 d. To check sensitivity to acetic acid or ethanol, growth behaviors were also observed on YPGD with and without acetic acid or on YPGD containing 0.5% $CaCO_3$ with and without ethanol addition at 30°C incubated for 3 d and 1 d, respectively.

2.7 Sequence Retrieval and Sequence Alignment of CIRCE Element in 20 AAB Genomes

Four complete genome sequences of AAB, *Acetobacter pasteurianus* IFO 3283-01 (NC_013209), *Gluconobacter oxydans* 621H (NC_006677), *Gluconacetobacter xylinus* NBRC 3288 (NC_016027) and *Gluconacetobacter diazotrophicus* Pal 5 (NC_010125) were obtained from the NCBI FTP website at ftp.ncbi.nlm.nih.gov/genomes/Bacteria/ [6, 16-18]. Sixteen draft genome sequences of AAB, *Acetobacter aceti* NBRC 14818 (BABW01000001-BABW01001488), *Acetobacter pasteurianus* 3P3 (CADQ01000001-CADQ01000101), *Acetobacter pasteurianus* IFO 3191 (BACG01000001-BACG01000306), *Acetobacter pasteurianus* NBRC 101655 (SKU1108) (BACF01000001-BACF01000294), *Acetobacter pasteurianus* subsp. *pasteurianus* LMG 1262 (CADO01000001-CADO01000141), *Acetobacter pomorum* DM001 (AEUP01000001-AEUP01000066), *Acetobacter tropicalis* NBRC 101654 (BABS01000001-BABS01000773), *Gluconacetobacter europaeus* 5P3 (CADS01000001-CADS01000256), *Gluconacetobacter europaeus* LMG 18494 (CADR01000001-CADR01000216), *Gluconacetobacter europaeus* LMG 18890 (CADP01000001-CADP01000321), *Gluconacetobacter hansenii* ATCC 23769 (ADTV01000001-ADTV01000071), *Gluconacetobacter oboediens* 174Bp2 (CADT01000001-CADT01000200), *Gluconacetobacter* sp. SXCC-1 (AFCH01000001-AFCH01000064), *Gluconobacter frateurii* NBRC 101659 (BADZ01000001- BADZ01000145), *Gluconobacter morbifer* G707 (AGQV01000001-AGQV01000019), and *Gluconobacter oxydans* WSH-003 (AHKI01000001-AHKI01000179), were obtained from the DDBJ FTP website at ftp.ddbj.nig.ac.jp/ddbj_database/wgs/ [19-25]. CIRCE elements of all AAB genomes were identified by a homology search of known CIRCE element sequences using the BLASTN [26]. Homologous regions were collected and CIRCE element regions were manually assigned. Nucleotide sequence alignment was carried out using CLUSTALW [27]. We used the MEGA version 5.05 package to generate phylogenetic trees to study the phylogenetic relationship based on CIRCE element nucleotide sequences with the neighbor-joining (NJ) approach and 1000 bootstrap replicates [28]. Consensuses of CIRCE elements were analyzed and displayed in the Web Logo format [29].

2.8 Nucleotide Sequence Accession Number

The nucleotide sequence data reported here have been submitted in the DDBJ database under accession no. AB758433.

3. RESULTS AND DISCUSSION

3.1 Cloning and the Overexpression of *groESL* gene in *A. pasteurianus* SKU1108

The *groESL* operon of thermotolerant acetic acid bacterium, *A. pasteurianus* SKU1108, was cloned and sequenced, as described in Materials and Methods. Sequencing analysis revealed two contiguous open reading frames (ORFs) of 372 nucleotides for the *groES* gene and 1,641 nucleotides for the *groEL* gene (data not shown). The overexpressed *groESL* gene in *A. pasteurianus* SKU1108 enhanced resistance against 4% acetic acid and 5% ethanol (data not shown). Similar results have previously been reported in *A. pasteurianus* NBRC 3283 [5]. In addition, in *A. pasteurianus* NBRC 3283, it has been reported that overexpression of *dnaKJ* [30] and co-overexpression of *grpE* with *dnaKJ* [31] resulted in improved growth compared to the control strain at high temperature or in a medium containing ethanol but not acetic acid. Also, *clpB* mutation of the same strain has been shown to affect growth at high temperature [32]. Thus, such chaperones seem to play important roles in resistance to various stressors. However, unlike NBRC 3283, the *groESL*-overexpressed strain showed only slightly greater growth at 42°C, which is probably because *A. pasteurianus* SKU1108 is originally thermotolerance [10].

3.2 Effect of the *groEL* Gene Disruption on Bacterial Growth

To more clearly analyze the effects of different stresses on the survival of *A. pasteurianus* SKU1108 cells lacking the chaperones GroEL, the genomic *groEL* gene was disrupted by homologous recombination using kanamycin resistant cassette. One kanamycin resistant colony, designated as DGL5, was isolated and confirmed by PCR amplification as well as Southern hybridization compared to the wild type strain (data not shown). The growth curve of *A. pasteurianus* SKU1108 and DGL5 grown in YPGD medium under various conditions were compared as shown in Figure 1. The growth curve under standard condition (30°C in YPGD medium) is shown in Figure1A. Under this condition, the growth pattern of SKU1108 grew better than DGL5. Moreover, the growth was compared between SKU1108 and *groEL* gene disruptant, DGL5, at 40°C (Figure1B), in the presence of 2% acetic acid (Figure1C), and of 8% ethanol (Figure 1D). Under these conditions, SKU1108 exhibited normal growth whereas DGL5 could not grow. The growth of wild type in the medium containing acetic acid exhibited an extended period of lag phase which has also been referred to adaptation period before entering exponential growth phase. Similar results have been reported by Nakano *et al.* [33]. The results suggested that the *groEL* gene disruptant entirely lost its toleration activity to these stressors.

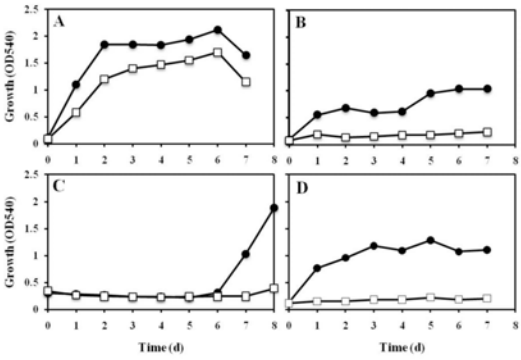

Figure 1. Time course of growth of *A. pasteurianus* SKU1108 (●) and *groEL* gene disruptant, DGL5 (□) grown in YPGD medium. Cells were cultured in 100 ml of YPGD medium at 30°C (A), 40°C (B), YPGD supplemented with 2% acetic acid (C) or 8% ethanol (D) at 30°C. Bacterial growth was determined by using a spectrophotometer at 540 nm.

3.3 Complementation of *groESL* Gene in the *groEL* Gene Disruptant

From the above described results, the *groEL* gene disruptant seems to be deficient in its ability to tolerate high temperature, acetic acid and ethanol. In order to confirm these findings, restoration of *groEL* gene disruption was examined by the conjugational transfer of pCMgroESL carrying *groESL* gene into the *groEL* gene disruptant (DGL5). The growth of *A. pasteurianus* SKU1108, DGL5, DGL5 harboring pCM62 (vector control), and DGL5 harboring pCMgroESL were compared on YPGD medium at various temperatures and also on the same medium supplemented with acetic acid or ethanol as shown in Figure 2. The results showed that the growth of the complemented strain, DGL5/pCMgroESL, was similar to that of the wild type under any conditions, which was contrary to the growth of the *groEL* gene disruptant (DGL5). On agar plate, DGL5 could not grow well over 37°C while the wild type and the complemented strain grew well at 42°C. DGL5 could not grow well under high concentration (over 2.0%) of acetic acid but the complemented strain did well even at 2.5% acetic acid. In the presence of ethanol, DGL5 showed some impairment of growth even at 2% ethanol, while the wild type and the complemented strain did well even at 8% ethanol. These results suggested that the *groESL* gene has an important role in the growth of the SKU1108 strain at high temperature as well as under acetic acid fermentation conditions.

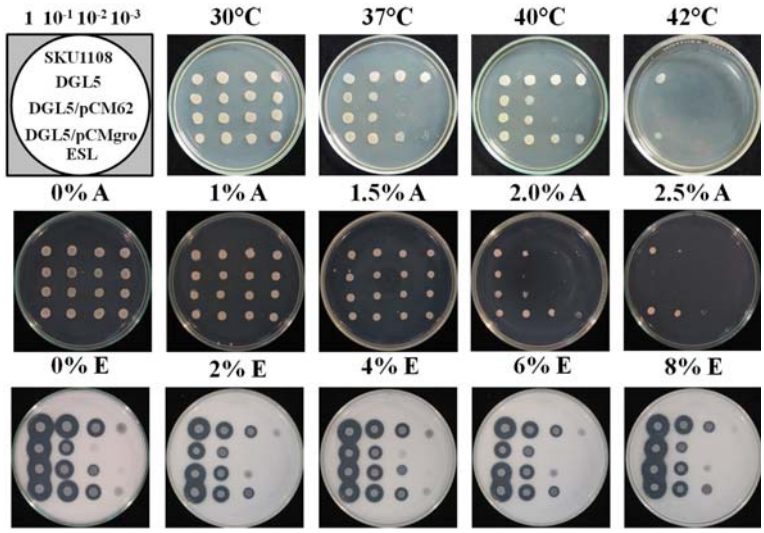

Figure 2. Comparison of growth between *A. pasteurianus* SKU1108 (1108), *groEL* gene disruptant (DGL5), *groEL* gene disruptant harboring pCM62 (DGL5/pCM62) and *groEL* gene disruptant harboring pCMgroESL (DGL5/pCMgroESL). These strains were spot-inoculated on YPGD agar containing 1%, 1.5%, 2% and 2.5% acetic acid; 2%, 4%, 6% and 8% ethanol incubated at 30°C, 37°C, 40°C and 42°C. After incubation for 1-3 d, their ability to grow was observed.

3.4 Induction Analysis of *groESL* Gene by Different Stress Conditions

To elucidate the expression level of *groESL* gene in *A. pasteurianus* SKU1108 at the transcriptional level, RT-PCR was performed with total RNA from cells under different stress conditions and at various time intervals as described in Materials and Methods. The expression level was slightly increased by exposure to stressors such as high temperature (42°C), 4% acetic acid and 5% ethanol (data not shown), which has also been observed in NBRC 3283 by Northern blotting [9].

Furthermore, the effects of mild to strong stressors on induction of the putative heat shock promoter for *groESL* gene expression in *A. pasteurianus* SKU1108 were determined by performing RT-PCR. The structure of the *groESL* gene of *A. pasteurianus* SKU1108 and the schematic representation of amplification of 447 bp *groEL* PCR products with primer GroEL-F and GroEL-R are shown in Figure 3A. Various temperatures and various concentrations of acetic acid and ethanol were used for induction of *groESL* gene expression. The appearance of a band in each cycle and its intensity suggested that the level of *groESL* mRNA expression gradually increased with increased temperature and acetic acid and ethanol concentrations (Figure 3B).

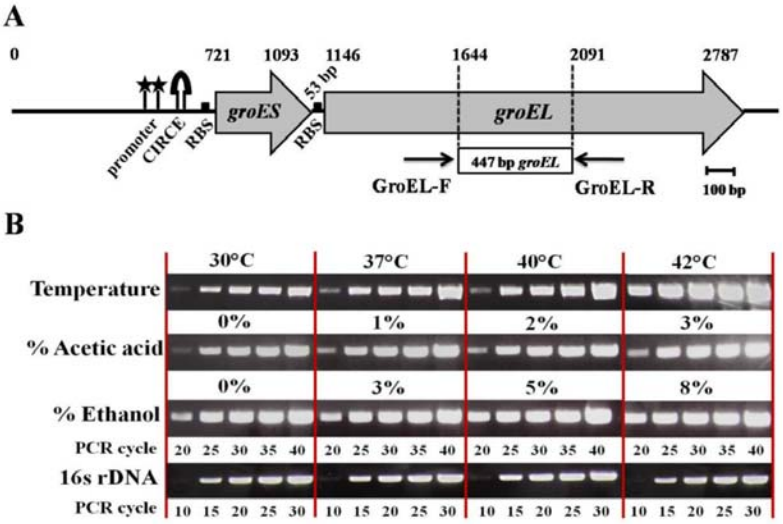

Figure 3. Structure of a DNA fragment carrying the promoter region, structural gene of *groESL* and a schematic representation of amplification of *groEL* PCR products by using primer GroEL-F and GroEL-R (A) and *groESL* gene expression under stress conditions at various temperatures and various concentrations of acetic acid or ethanol (B) *A. pasteurianus* SKU1108 cells were cultured in YPGD medium and incubated at 30°C until the early exponential phase, and then the cells were exposed to various stressors. Total RNA was isolated and subjected to RT-PCR as described in Materials and Methods.

3.5 *In Silico* Analysis of CIRCE Element in AAB

It has been reported that a sequence highly similar to the CIRCE heat shock element, an inverted repeat relating to regulation of *groESL* gene expression, was observed downstream of the promoter in *A. pasteurianus* NBRC 3283 [5]. The sequences of the putative heat shock promoter and the highly conserved regulatory CIRCE element were also found in the upstream region of the *groESL* operon in the *A. pasteurianus* SKU1108 genome (Figure 4A). We compared the CIRCE element detected from all known genome sequences of AAB (four complete and sixteen draft genomes). It was observed that these consensus sequences were conserved in all strains. To clarify the phylogenetic relationships of these CIRCE elements, we aligned these regions (Figure 4B). Consensus of CIRCE elements created by using Weblogo is also shown [29]. Results showed that the pattern of CIRCE elements was highly conserved in the same genera. Phylogenetic analysis revealed that these elements have evolved with the phyletic evolution (Figure 4C). This suggests that the *groESL* operon might play a crucial role in acetic acid fermentation.

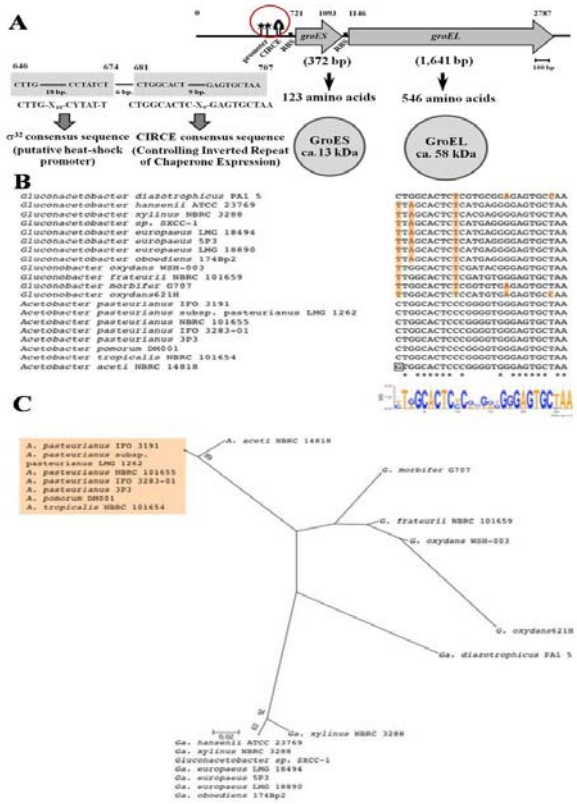

Figure 4. Regulatory region of *groESL* gene in *Acetobacter pasteurianus* SKU1108 (A), alignment of the CIRCE consensus sequences detected from 20 AAB genomes (B), and phylogenetic tree of these consensus sequences (C). The putative -35 and -10 binding sites for *sigma32*-like (Sig32) subunits of RNA polymerase, and CIRCE consensus sequence in the promoter region of the *groESL* gene are located at the 76th and the 41st nucleotide upstream from the initiation codon of the *groES* gene, respectively. Asterisks indicate nucleotides which are identical in all sequences.

4. CONCLUSION

From this study, the *groEL* gene disruptant was successfully constructed from *A. pasteurianus* SKU1108. The *groEL* disruptant clearly exhibited defective responses to all stressors occurring during acetic acid fermentation. The *groESL* gene from *A. pasteurianus* SKU1108 was able to complement the stress response defect of the disruptant. It could be clarify that *groESL* gene has an important role in the growth of the SKU1108 strain at stress conditions. For *groESL* gene expression, the putative heat shock promoter of *A. pasteurianus* SKU1108 could be induced by high temperature as well as high concentration of acetic acid and ethanol. In addition, there are conserved CIRCE in the upstream regulatory region of the *groESL* operon of all AAB. These sequences may be useful for the determination of the phylogenetic relationships among genera of AAB.

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