Stress Resistance Mechanism of *Lactobacillus fermentum* MS79 with Unusually High Resistance to Various Environmental Stresses
Stress Resistance Mechanism of *Lactobacillus fermentum* MS79 with Unusually High Resistance to Various Environmental Stresses
by

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resume
ABSTRACT

In previous studies 108 vaginal *Lactobacillus* spp. were isolated from Korean and several strains which showed high antimicrobial activity, high detergents MIC, antibiotics MIC were selected. In this study *Lactobacillus* sp. MS79 which showed high resistance to various environmental stresses was selected for further study. MS79 was identified as *L. fermentum* using PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and 16S rDNA sequencing. MS79 maintained high viability (10^7 CFU/ml) for two weeks at low temperature (4ºC). When the cells were exposed to various stresses: acid (pH 3.0), heat (60ºC), ethanol (10%), dehydration (in the dessicator containing 10 g CaCl₂), oxidative stress (0.01% H₂O₂), osmotic stress (2 M NaCl), freeze-thaw, *L. fermentum* MS79 maintained high viable cell number: 10^5 – 10^6 CFU/ml under osmotic (2 M NaCl) and ethanol stress (10%) after three days, under dehydration and oxidative stress after eight hours. After being stored one month at room temperature freeze-dried cells maintained viable cell number of 10^5 CFU/ml. To our surprise, when *L. fermentum* MS79 was transferred to a fresh acidic MRS (pH 3.0) viability increased
twofold. By following the growth of the MS79 regained growth rapidly with almost no lag phase. Morphological changes after being exposed to various stresses were observed using scanning electron microscopy. As a result, MS79 maintained high viability with morphological changes. Cell surface hydrophobicity measurements of stressed and unstressed cells were compared. Unstressed cells surface of MS79 were hydrophobic, whereas stressed cell surface of MS79 altered to be hydrophilic in acid stress, osmotic stress, starvation, long term storage in low temperature. The surface protein by procedure was subjected to SDS-PAGE and the results were considered that stressed cells of MS79 were missing hydrophobic proteins and cells were hydrophilic. In order to determine the mechanism of stress resistance at the molecular level we attempted to clone \textit{rpoE} gene from MS79. Initially we cloned partial \textit{rpoE} (directed RNA polymerase, delta subunit) using degenerate primers. \textit{RpoE} has been known to play a role in adaptation to environmental stress and the cellular morphology in other bacterium during growth. The cloned \textit{rpoE} of MS79 is similar to that of the directed RNA polymerase, delta subunit of \textit{Lactobacillus plantarum}, \textit{Listeria monocytogenes} EGD-e, displaying sequence identity of 68%, and 64%, respectively. Attempts are being made for full sequencing the
rpoE using single primer PCR as well as for comparing protein expression profile between stressed cell and unstressed cell. Southern blot analysis was used to determine copy number of rpoE. We have shown that rpoE gene is induced and requires during survival at extreme osmotic stress using Northern blot analysis. Two transcripts of were observed of the stressed cells and transcripts displayed similar levels of induction. These results show that the extreme osmotic stress response in L. fermentum MS79 is under transcriptional regulation of rpoE and indicate cotranscription of two promoters.
CONTENTS

ABSTRACT ........................................................................ 1

CONTENTS ........................................................................ IV

LIST OF TABLES ............................................................... VIII

LIST OF FIGURES ............................................................. IX

1. INTRODUCTION ............................................................. 1

1.1 Microbial stress responses ........................................... 1

1.2 Alteration of morphology and cell surface and stress response
.............................................................................................. 2

1.3 Alternative sigma factor ................................................. 3

1.4 Extacytoplasmic Function Sigma Factor E (rpoE) ............ 6

2. MATERIALS AND METHODS .......................................... 8

2.1 Bacterial strains and culture condition ......................... 8

2.2 Stress assay ................................................................. 10

2.2.1 Acid, ethanol, oxidative and salt stress .................... 10

2.2.2 Cold stress and starvation; long term storage test
.............................................................................................. 10
2.2.3 Desiccation ........................................... 10
2.2.4 Freezing and thawing stress ....................... 11
2.2.5 Heat stress ........................................... 11
2.2.6 Freeze-drying ........................................ 11
2.3 Molecular identification of selected strain .......... 13
  2.3.1 Isolation of chromosomal DNA from Lactobacillus spp. ......................................................... 13
  2.3.2 Restriction fragment length polymorphism (RFLP) · .............................................................. 14
  2.3.3 16S rDNA sequencing ............................ 17
  2.3.4 Data analysis of sequences ....................... 19
2.4 Observation of morphological changes using Scanning electron microscopy (SEM) ......................... 19
2.5 Comparison of the cell surface hydrophobicity wild and stress cells ............................................. 19
2.6 Cell surface protein analysis .......................... 20
  2.6.1 Preparation of protein ............................... 20
  2.6.2 Electrophoresis of protein ......................... 20
2.7 Isolation of rpoE gene ................................... 21
  2.7.1 Design of rpoE gene primers ...................... 21
2.7.2 Degenerate-PCR and sequencing  ...................... 23
2.7.3 Single primer PCR to obtain whole gene sequence  ⋄
 .................................................................................. 25
2.8 Southern blot analysis  ................................. 27
2.9 Regulation of rpoE gene  ............................... 27
  2.9.1 Total RNA isolation and quantitative analysis  ⋄ 27
  2.9.2 Northern blot analysis  .............................. 28
  2.9.3 Reverse transcriptase Polymerase chain reaction (RT-PCR)  ............................. 28

3. RESULTS AND DISCUSSION  ...................... 30
  3.1 Stress assay  .............................................. 30
    3.1.1 Acid stress  ........................................... 30
    3.1.2 Cold (long term storage), osmotic, freezing drying and oxidative stress  ................... 31
    3.1.3 Heat, ethanol, freezing and thawing and desiccation  ⋄
    ...................................................................... 36
  3.2 Selection and characterization of high stress resistant strain
    ................................................................................ 39
  3.3 Molecular identification of selected strain  .......... 43
  3.4 Comparison of stresses response  .................... 47
3.5 Observation of morphological changes using scanning electron microscopy (SEM) .......................................................... 50
3.6 Comparison of the cell surface hydrophobicity between control and stressed cells ......................................................... 52
3.7 Alteration of cell surface proteins ........................................... 55
3.8 Analysis of rpoE gene sequence of Lactobacillus fermentum MS79 .......................................................... 58
3.9 regulation of rpoE ............................................................... 61
4. CONCLUSION ........................................................................... 64
5. REFERENCES ........................................................................... 66
LIST OF TABLES

TABLE 1. B. subtilis s factors ................................. 5
TABLE 2. Bacterial strains in this study ......................... 9
TABLE 3. Primers in this study ................................. 9
TABLE 4. Stress conditions in this study ....................... 12
TABLE 5. PCR mixture for 16S rDNA amplification .......... 15
TABLE 6. PCR condition for 16S rDNA amplification .......... 15
TABLE 7. Enzyme digestion condition for PCR-RFLP .......... 16
TABLE 8. Degenerate-PCR mixture for rpoE gene ............ 24
TABLE 10. Single primer PCR mixture for rpoE gene .......... 26
TABLE 11. Single primer PCR condition for rpoE gene ........ 26
TABLE 12. MICs against chemicals of L. fermentum MS79
            .................................................................. 42
TABLE 13. Alteration of cell surface hydrophobicity after
            challenge the stresses ........................................ 53
LIST OF FIGURES

FIGURE 1. pGEM-Teay vector  ........................................ 18
FIGURE 2. CLUSTAL W (1.83) multiple sequence alignment for
degenerate primers design  ........................................ 22
FIGURE 3. Experimental scheme for rpoE gene analysis and
regulation ................................................................. 29
FIGURE 4. Survival of Acid stress and long-term storage at low
temperature .......................................................... 33
FIGURE 5. Survival of freeze-drying and osmotic stress ..... 34
FIGURE 6. Survival of oxidative stress  ............................ 35
FIGURE 7. Survival of heat stress and desiccation .............. 37
FIGURE 8. Survival of ethanol stress and freezing and thawing
................................................................. 38
FIGURE 9. Growth curve and morphology of L. fermentum MS79
................................................................. 40
FIGURE 10. Anti-microbial activity against C. albicans of
L. fermentum MS79 .................................................. 41
FIGURE 11. Restriction pattern of PCR-amplified product of 16s
rDNA of MS79 ......................................................... 44
FIGURE 12. 16S rDNA sequence of Lactobacillus fermentum
MS79 ................................................................. 45
FIGURE 13. Sequence homology of Lactobacillus fermentum
MS79 ................................................................. 46
FIGURE 14. Survival of acid stress and osmotic stress ........ 48
FIGURE 15. Survival of starvation and cold storage .......... 49
FIGURE 16. Observation of morphological changes of L. fermentum
MS79 ................................................................. 51
FIGURE 17. Determination of hydrophobicity .................... 54
FIGURE 18. Alteration of surface protein pattern after challenge
stresses of L. fermentum MS79 ............................... 56
FIGURE 19. RpoE (algU) of Pseudomonas fluorescens,
a biocontrol agent ................................. 57
FIGURE 20. Amino acid sequence comparison of a \textit{rpoE} gene
......................................................... 59
FIGURE 21. Amplification of a partial \textit{rpoE} gene and Southern blot
......................................................... 60
FIGURE 22. Total RNA and northern blot analysis under osmotic stress
......................................................... 62
FIGURE 23. Two promoters of \textit{rpoE} and multiple promoters of \textit{algT}
......................................................... 63
1. INTRODUCTION

1.1 Microbial stress responses

In their natural environments, bacteria spend most of their life in a starving or nongrowing state because various growth limiting conditions. Therefore, bacteria have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress. The phenomenon of stress adaptation and protection has been studied more extensively in model microorganisms, food-borne pathogenic bacteria: *Escherichia coli* O157:H7 (Duffy et al. 2000, Jenkins et al. 1988, Ryu and Beuchat 1999, and Leenanon and Drake 2001), *Salmonella typhimurium* (Leyer and Johnson 1993), and *Lasteria monocytogenes* (Farber and Pagotto 1992). But during the last decade the use of the microorganisms considered probiotic (health promoting) has increased dramatically. Given that probiotic microorganisms play a role on promoting and maintaining health (Salminen et al. 1998 and Gardiner et al. 2000) has stimulated considerable interest in incorporating these into functional foods and pharmaceutical products. Therefore, from a commercial point of view,
an inexpensive method for large-scale production of cultures containing high level of viable probiotic cells on a form product application is highly desirable. Specially, some lactic acid bacteria have been shown to provide protection against gastrointestinal disorders or bacterial vaginosis. In recent years, interest has grown in the stress response phenomenon of LAB species (for review, Schmidt, Hertel, 1999; Teixeira et al., 1994; Lorca, Raya, Taranto, & de Valdez, 1998). Understanding the mechanism of stress may lead to the development of culture with improved capacity to survive and function under industrial production conditions. The sensitivity of lactobacilli to various environmental stresses, resulting in structural and physiological injury, makes it difficult to preserve. In spite of this, freezing is commonly used for the production of concentrated starter cultures for the food industry.

1.2 Alteration of morphology and cell surface and stress response

Many factors contribute to bacterial resistance to environment stress. Among these are cell surface structures and appendages, which provide the firstline of defense for a bacterium. The cell envelope plays an
essential role for the bacterium providing a barrier between the cell and
the environment, determining cellular morphology, and maintaining the
structural integrity of the cell. The various environmental stresses like
thermal, dehydration, and osmotic stress involved in the freezing and
thawing process can drastically alter the bacterial surface. Bacterial
surface stress responses play important roles in protein folding, cell
wall biosynthesis and pathogenesis (Miticha et al. 1998).

1.3 Alternative sigma factors

Microorganisms have evolved signal transduction system, which in
response to environmental stresses, control the coordinated expression
of genes involved in cellular defense mechanisms. A common
regulatory mechanism involves the modification of sigma (σ) factors
whose primary role is to bind to core RNA polymerase conferring
promoter specificity (Haldenwang, 1995). Alternative sigma factors
play an important role on regulating the transcription of many genes
that are induced during stationary phase, starvation, and stress
adaptation (Hild et al. 2000, Hengge-Aronis et al. 1999 and Wosten et
al. 1998). In \emph{B. subtilis}, the alternative sigma factor $\sigma^B$ regulates
<table>
<thead>
<tr>
<th>Sigma factor (alternative designation)</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Promoter sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetable-cell factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>σ&lt;sup&gt;43&lt;/sup&gt;(σ&lt;sup&gt;45&lt;/sup&gt;, σ&lt;sup&gt;55&lt;/sup&gt;)</td>
<td>sigA, rpoD</td>
<td>Housekeeping/early sporulation</td>
<td>TTGA 17 TA&lt;sub&gt;7&lt;/sub&gt; AAT</td>
<td>210</td>
</tr>
<tr>
<td>σ&lt;sup&gt;37&lt;/sup&gt;</td>
<td>sigB</td>
<td>General stress response</td>
<td>RGGXTTRA 14 GG&lt;sub&gt;4&lt;/sub&gt; TA&lt;sub&gt;7&lt;/sub&gt;</td>
<td>24</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>Postexponential gene expression</td>
<td>AAATC 15 TX&lt;sub&gt;5&lt;/sub&gt; GYTZT</td>
<td>145</td>
</tr>
<tr>
<td>σ&lt;sup&gt;30&lt;/sup&gt;</td>
<td>sigD, flaB</td>
<td>Chemotaxis/autolysin/flagellar gene</td>
<td>TAAA 15 GCCGATA&lt;sub&gt;7&lt;/sub&gt;</td>
<td>119</td>
</tr>
<tr>
<td>σ&lt;sup&gt;H&lt;/sup&gt;</td>
<td>sigH, spoOH</td>
<td>Postexponential gene expression;</td>
<td>RWAGGAXXT 14 HGAAT&lt;sub&gt;7&lt;/sub&gt;</td>
<td>237</td>
</tr>
<tr>
<td>σ&lt;sup&gt;L&lt;/sup&gt;</td>
<td>sigL</td>
<td>Degradative enzyme gene expression</td>
<td>TGGCAC 5 TTGC&lt;sub&gt;7&lt;/sub&gt; NNNN</td>
<td>59</td>
</tr>
<tr>
<td>Sporulation-specific</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>σ&lt;sup&gt;28&lt;/sup&gt;</td>
<td>sigE, spoIIGB</td>
<td>Early mother cell gene expression</td>
<td>ZHATA&lt;sub&gt;5&lt;/sub&gt; X 14 CATACAH&lt;sub&gt;7&lt;/sub&gt;</td>
<td>252</td>
</tr>
<tr>
<td>σ&lt;sup&gt;spoIAC&lt;/sup&gt;</td>
<td>sigF, spoIAC</td>
<td>Early forespore gene expression</td>
<td>GCATR 15 GGHRA&lt;sub&gt;4&lt;/sub&gt; HTX</td>
<td>291</td>
</tr>
<tr>
<td>σ&lt;sup&gt;G&lt;/sup&gt;</td>
<td>sigG, spoIIIG</td>
<td>Late forespore gene expression</td>
<td>GHATR 18 CATX&lt;sub&gt;5&lt;/sub&gt; HTA</td>
<td>217</td>
</tr>
<tr>
<td>σ&lt;sup&gt;27&lt;/sup&gt;</td>
<td>sigK, spoIVCB:</td>
<td>Late mother cell gene expression</td>
<td>AC 17 CAT&lt;sub&gt;7&lt;/sub&gt; ANNTA</td>
<td>338</td>
</tr>
</tbody>
</table>

<sup>a</sup> The designation for the sigma proteins and their structural genes as well as likely functions of their regulons are listed. Reference for each item can be found in the text. The probable consensus sequences for the holoenzyme forms are aligned at their -10 positions (underlined). The spacer region represents the number of bases between the upstreammost -10 region base that is given and the downstreammost base of the -35 region. The reference for each consensus sequence is listed to its right.

<sup>b</sup> H, A or C; N, A, G, C, or T; R, A or G; W, A, G, or C; X, A or T; Y, C or T; Z, T or G.

**TABLE 1. B. subtilis s factors**

5
1.4 Extracytoplasmic Function Sigma Factor E (rpoE)

Bacterial envelope stress response play important roles in protein folding, cell wall biosynthesis and pathogenesis (Mitika et al. 2003). The alternative sigma factor $\sigma^E$ control the cell envelope stress response. Homologs of rpoE encode proteins that are members of the $\sigma^E$ family, termed extracytoplasmic-function [ECF] $\sigma$ factors that also contribute to stress resistance. In response to the extracellular environment, ECF $\sigma$ factors have been found to regulate gene expression in diverse bacterial species. RpoE homologs have been implicated as critical in a variety of stress responses (Hild et al. 2000). One of the best-studied examples is the role of AlgU in the pathogenicity of Pseudomonas aeruginosa in cystic fibrosis (Govan, J. R. W., and V. Deretic. 1996). The rpoE gene is located in an operon including three downstream genes, rseA, rseB and rseC, and the operon has been shown to be transcribed from two promoters, one of which, P2, is $\sigma^E$-dependent. A role for ECF $\sigma$ factors in the expression of genes enhancing bacterial adaptation to environmental conditions adverse to growth like heat shock (Martin, D. W. et al. 1994, Hiratsu, K. et al. 1995, Huang, X. et al. 1997, and Fernandes, N. D. et al. 1999), oxidative stress (Yu, H. et al. 1996, Wu, Q.-L. et al. 1997, and Fernandes, N. D. et al. 1999), osmotic shock (Bianchi, A. A., and F. Baneyx. 1999), adaptation to cold temperatures and high pressures (Chi, E., and D. H. Bartlett. 1995.), protection against photolysis (Gorham, H. C. et al. 1996), acid stress (Wu, Q.-L. et al. 1997), desiccation resistance (Moreno, S. et al. 1998), antibiotic production during
stationary phase.
2. MATERIALS AND METHODS

2.1 Bacterial strains and culture condition

The bacterial strains used in this study are shown in TABLE 1. Four *Lactobacillus* strains were grown at 37°C in MRS broth to stationary phase. MRS medium contained 20g glucose, 10g of peptone No. 3, 10g of beef extract, 5g of yeast extract, 5g of sodium acetate, 2g of ammonium citrate, 2g of potassium phosphate dibasic (K$_2$HPO$_4$), 1g of tween80, 35mg of FeSO$_4$.7H$_2$O, 575mg of MgSO$_4$, 120mg of MnSO$_4$.7H$_2$O per liter. *Escherichia coli* JM109 was grown on Luria-Bertani broth with aeration. LB medium contained 10g of peptone, 5g of yeast extract, 1ml of 1N NaOH, and 5g of sodium chloride per liter. For antibiotic selection, 100µg ampicillin per ml was added to the medium.
### TABLE 2. Bacterial strains in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus fermentum</em> MS79</td>
<td>Target species</td>
</tr>
<tr>
<td><em>Lactobacillus crispatus</em> KLB46 (AF243167)</td>
<td>Former selected for application</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> KLB12 (AF522394)</td>
<td>Identical species with MS79</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> KLB213</td>
<td>Former industrialization</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>For transformation</td>
</tr>
</tbody>
</table>

### TABLE 3. Primers in this study

<table>
<thead>
<tr>
<th>Designed primers</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uni I</td>
<td>5'-CCCAAGCTTAGAGTTTGATCCTGGCTCAG-3’</td>
</tr>
<tr>
<td>Uni II</td>
<td>5'-ACGCGTCGACAAGGAGGTGATCCAGCC-3’</td>
</tr>
<tr>
<td>rpoE - for</td>
<td>5’-GATCGAAGTTGCTCGTCG-3’</td>
</tr>
<tr>
<td>rpoE - rew</td>
<td>5’-TCATCTGAATCGTAGTCG-3’</td>
</tr>
<tr>
<td>rpoE - s/f</td>
<td>5’-GCATGCATGGTACCATTGTG-3’</td>
</tr>
<tr>
<td>rpoE – s/r</td>
<td>5’-CAAATGGGTACCATGCACGC-3’</td>
</tr>
</tbody>
</table>

**TABLE 3. Primers in this study**

2.2 Stress assay

Environmental stress conditions used in this study are shown in TABLE 4. Cells grown were harvest by centrifugation at 15,000rpm for 5min and washed twice and resuspended with 0.85% NaCl solution, except desiccation. And cells were exposed to various environmental stress conditions. Bacterial survival was determined by taking aliquots of the bacterial suspensions at different time intervals follow by dilution in 0.85% NaCl solution and plating on the MRS agar plate. The colonies were enumerated after incubation at 37°C for overnight.

2.2.1 Acid, ethanol, oxidative and salt stress

Cell resuspension was transferred to acidic MRS (pH3 MRS, adjust HCl) at 37°C to expose to acid stress. MRS containing 10% (v/v) ethanol or 0.01% (v/v) H₂O₂ was used for ethanol stress or oxidative stress, respectively. For salt stress, the medium was supplemented with 2M NaCl.

2.2.2 Cold stress and starvation; long term storage test

Cells collected by centrifugation, washed with 0.85% NaCl solution resuspended in normal MRS medium. The resuspension were stored at 4°C in the refrigerator for two weeks.

2.2.3 Desiccation
Cells of each strain were collected by centrifugation, washed with 0.85% NaCl solution in the sterile 1.8ml microtube. Resuspension was harvested by centrifugation and supernatant was completely removed. Samples were dried in a desiccator over CaCl₂. The desiccator was sealed and the dried cultures were stored at room temperature for 6h.

2.2.4 Freezing and thawing stress

Cell resuspension was transferred to new normal MRS for extreme condition. One-milliliter aliquots of cell suspensions were frozen at -20°C for 24 h and thawed at 37°C for 3h in an incubator. Unfrozen samples were used as controls.

2.2.5 Heat stress

To induce a heat stress response, the culture temperature was shifted from 37°C to 60°C. The samples were treated in a circulating water bath.

2.2.6 Freeze-drying

The samples for freeze-drying experiments were prepared by centrifugation and removal supernatant. All samples in a given experiment were frozen at -70°C for 3 h. The frozen samples were lyophilized for 24 h on a lyophilizer. And the dried samples were rehydrated in water to the same volume for viable counting. The viability of control was immediately determined before freeze-drying.
<table>
<thead>
<tr>
<th>Acid stress</th>
<th>Desiccation</th>
<th>Ethanol stress</th>
<th>Osmotic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3 MRS 37°C</td>
<td>desiccator 10g CaCl₂</td>
<td>10% ethanol MRS 37°C</td>
<td>2M NaCl MRS 37°C</td>
</tr>
<tr>
<td>Cold stress</td>
<td>Starvation</td>
<td>Heat stress</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>normal MRS 4°C, 2 weeks</td>
<td>0.85% NaCl 22°C</td>
<td>normal MRS 60°C</td>
<td>0.01% H₂O₂ MRS 37°C</td>
</tr>
<tr>
<td>Freezing and thawing</td>
<td></td>
<td></td>
<td>normal MRS</td>
</tr>
<tr>
<td>Freeze-drying</td>
<td></td>
<td></td>
<td>freezing : -70°C, 5hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>drying : freeze dryer, 18hrs</td>
</tr>
</tbody>
</table>

**TABLE 4. Stress conditions in this study**
2.3 Molecular identification of selected strain

2.3.1 Isolation of chromosomal DNA from *Lactobacillus* spp.

To isolate genomic DNA of *Lactobacillus* sp. MS79 for PCR, a modified method was used as described by Oh et al. (2002). Briefly, cell was harvested by centrifugation at 15000rpm for 5min and resuspended in high TE buffer (50mM Tris-HCl, 20mM EDTA, and pH 8.0) of 0.8 cultured volumes. Resuspension was vortexed with 0.4mg glass beads (425-600 microns, Sigma Chemical Co., USA) for 3min. At once the sample was mixed with phenol mixture (phenol: chloroform: isoamyl alcohol = 25: 24: 1, Sigma Chemical Co., USA) and centrifuge at 15000rpm for 10min. The supernatant was treated chloroform and aqueous phase was separated by centrifugation at 15000rpm for 10min. DNA was precipitated by adding of 1/10 volume of 3M sodium-acetate (pH5.4) and two volume of ice cold absolute ethanol at -20°C for 20min and followed by washing with 70% ethanol. DNA pellet was dried and resuspended in 40µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 2µl RNase was added to the dissolved DNA solution.
2.3.2 Restriction fragment length polymorphism (RFLP)

For molecular identification, genomic DNA sample of selected strain was performed degenerate-PCR with universal primers (TABLE 3) to amplify 1.6Kb of 16S rDNA. PCR composition and condition were listed in TABLE 5 and TABLE 6. For PCR-RFLP, PCR product was digested with five different restriction endonucleases; *Hinf* I, *Alu* I, *Msp* I, *Hae* III (TaKaRa, Japan) and *Cfo* I (Promega, U.S.A) by using the appropriate reaction condition and incubation time according to the supplier’s recommendation. Reaction mixture of five restriction endonucleases was presented at TABLE 7.
### TABLE 5. PCR mixture for 16S rDNA amplification

<table>
<thead>
<tr>
<th>PCR mixture component</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>template</td>
<td>100ng</td>
</tr>
<tr>
<td>5 × buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 pmol UniI</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 pmol UniII</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>2 unit</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Up to 50 µl</td>
</tr>
</tbody>
</table>

### Thermal condition for degenerate-PCR

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Temperature</th>
<th>Time</th>
<th>35 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-denaturation</td>
<td>94°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>annealing</td>
<td>56°C</td>
<td>1 min 30 sec</td>
<td></td>
</tr>
<tr>
<td>extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>final extension</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 6. PCR condition for 16S rDNA amplification
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>200ng</td>
</tr>
<tr>
<td>10×Restriction enzyme buffer</td>
<td>1μl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>1μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>10μl</td>
</tr>
</tbody>
</table>

**TABLE 7. Enzyme digestion condition for PCR-RFLP**
2.3.3 16S rDNA sequencing

For 16s rDNA sequencing, the same PCR product eluted from agarose gel by using Wizard SV Gel & PCR Clean-up System (Promega, USA) and ligated with pGEM-Teasy vector. Ligation mixture was mixed with 50µl of *E. coli* JM109 competent cells. The mixture was treated at 42°C for 1min 30sec and kept in ice for 1min after storage at 4°C for 30min. The transformed cells were added into 500ml SOC medium and grown for 1h before being plated on LB medium containing with 100µg/ml of ampicillin. To select an appropriate transformed cell, insertional inactivation of *lacZ* gene was used by pGEM-Teasy vector system with X-gal (5-Br-4-Cl-3-Indolyl-bate-D-galactosidate) and IPTG (isopropylthiogalactoside) on the selective plates. Appropriate white colonies were incubated in 5ml of LB broth containing 100µg/ml of ampicillin and culture at 37°C for 12h. To prepare a plasmid from selected cells, QIAprep Spin Miniprep Kit (QIAGEN, USA) was used. Then, cloning vector was digested with restriction enzyme *EcoRI* and was electrophoresis in 1% agarose gel. Vector band of 3 Kb and inset 1.6kb were visualized on agarose gel by UV illuminator. The sequencing process was conducted by sequencing company, Bionex in Hanyang University, Korea.
FIGURE 1. pGEM-Teay vector (Promega, USA)
2.3.4 Data analysis of sequences

The Blastn was used to search databases for 16S rDNA sequences similar to obtained nucleotide sequences. BLAST search (http://www.ncbi.nlm.nih.gov) has information on the comparison and analysis of DNA sequence and of its homology.

2.4 Observation of morphological changes using Scanning electron microscopy (SEM)

The samples were analyzed in a SEM (Hitach-4200, Inha univ., Incheon, Korea) in order to examine the external appearance of the stressed cells and unstressed cells. The samples were washed twice and resuspended with PBS. 5µl of resuspension was transferred onto a cover glass and air-dried at room temperature and after 5h of incubation. Samples were fixed for 3h with 2.5% (wt/vol) glutaraldehyde in phosphate buffered saline and incubated at 4°C for 12 h and after rinsed twice in the same buffer. Samples were dehydrated with ethanol (10, 25, 50, 75, 95, and finally 100%) serially and air-dried at room temperature. Cells coated with gold were examined and photographed with a FE-SEM (Field Emission Scanning Electron Microscopy, Hitach S-4200) operating at 10kV × 18k.

2.5 Comparison of the cell surface hydrophobicity wild and stress cells
The cells were harvested by centrifugation at 15000 rpm for 5 min, washed twice, and resuspended in phosphate buffered saline (PBS, pH 7.0) to approximately $10^9$ CFU ml$^{-1}$. The absorbance of the cell suspension was measured at 600 nm ($A_0$). One milliliter of n-hexadecane was added to 3 ml of cell suspension. The two-phase system was mixed by vortexing for 2 min and the aqueous phase was removed after 20 min of incubation at room temperature. And its absorbance at 600 nm ($A_1$) was measured. The percentage of bacterial adhesion to solvent was calculated as $(1-A_1/A_0) \times 100$. (Bellon-Fontaine et al. 1996)

2.6 Cell surface protein analysis

2.6.1 Preparation of protein

Unstressed and stressed cells were resuspended in 1% SDS for 30 min at 37°C for the isolation of surface protein. After centrifugation at 9000 g for 5 min, the supernatant was mixed with 5× sample buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4% 2-mercaptoethanol, 0.1% bromophenol blue) and boiled at 100°C for 10 min. The samples were centrifuged at 15000 rpm for 10 min and the supernatant was used for electrophoresis.

2.6.2 Electrophoresis of protein

The protein samples were analyzed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V, 80 mA using
12% acrylamide gels. Protein bands were visualized by staining the gels with Coomassie brilliant blue.

2.7 Isolation of rpoE gene

2.7.1 Design of rpoE gene primers

The rpoE gene primers of Lactobacillus fermentum MS79 were designed by comparison of sequences with known rpoE genes of other lactobacilli strains with codon bias of amino acids in consideration using CLUSTALW (1.83). The primers were named rpoE-for (5’-gatcgaagttgctgctgc -3’) and rpoE-rew (5’-tcatctgaatcgtagtcg -3’) (TABLE 3).
FIGURE 2. CLUSTAL W (1.83) multiple sequence alignment for degenerate primers design
pla: RNAP delta-subunit of *L. plantarum*, gasseri: RNAP delta-subunit of *L. gasseri*, john: RNAP delta-subunit of *L. johnsonii*
2.7.2 Degenerate-PCR and sequencing

PCR reaction mixture and PCR parameters were listed in TABLE 8 and TABLE 9. PCR product was approximately ~399 bp and was cloned as mentioned in part of 16S rDNA sequencing (FIGURE 1). For sequence analysis the BlastX was used to search databases for protein amino acid sequences similar to those obtained by translation the DNA sequences.
### TABLE 8. Degenerate-PCR mixture for \textit{rpoE} gene

<table>
<thead>
<tr>
<th>PCR mixture component</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>template</td>
<td>100ng</td>
</tr>
<tr>
<td>5 × buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>1µl</td>
</tr>
<tr>
<td>10 pmol rpoE-for</td>
<td>1µl</td>
</tr>
<tr>
<td>10 pmol rpoE-rew</td>
<td>1µl</td>
</tr>
<tr>
<td>\textit{Taq} polymerase</td>
<td>2unit</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Up to 50µl</td>
</tr>
</tbody>
</table>

### TABLE 9. Degenerate-PCR condition for \textit{rpoE} gene

<table>
<thead>
<tr>
<th>Thermal condition for degenerate-PCR</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-denaturation</td>
<td>94°C</td>
<td>5min</td>
</tr>
<tr>
<td>denaturation</td>
<td>94°C</td>
<td>1min</td>
</tr>
<tr>
<td>annealing</td>
<td>45°C</td>
<td>1min</td>
</tr>
<tr>
<td>extension</td>
<td>72°C</td>
<td>1min</td>
</tr>
<tr>
<td>final extension</td>
<td>72°C</td>
<td>10min</td>
</tr>
</tbody>
</table>

35 cycles
2.7.3 Single primer PCR to obtain whole gene sequence

Single primer PCR allows amplification of unknown regions in chromosome from known sequence. At sufficiently low stringency in annealing step of PCR, a primer will misprime while continuing to bind specifically to its intended site. Conditions can usually be found allowing mispriming sufficiently close to the correct site to permit amplification anchored at the original site. Reamplification with a nested primer and the original outside primer generates a product with unique ends. The resulting size shift can be used to diagnose the correct product, which can then be sequenced from either end. PCR reaction mixture and PCR product parameters were summarized in TABLE 10 and TABLE 11. The bands were eluted by Wizard SV & PCR Clean-up system (Promega, USA). DNA from individual band was subjected to second-PDR to confirm the correct product. Second-PCR condition was equal to that of degerate-PCR (TABLE 8 and TABLE 9). PCR product contained a partial \( rpoE \) gene was cloned by pGEM-Teas vector and sequenced.
<table>
<thead>
<tr>
<th>PCR mixture component</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>template</td>
<td>100ng</td>
</tr>
<tr>
<td>5 × buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>1µl</td>
</tr>
<tr>
<td>10 pmol rpoE-for</td>
<td>1µl</td>
</tr>
<tr>
<td><em>Taq</em> polymerase</td>
<td>2unit</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Up to 50µl</td>
</tr>
</tbody>
</table>

**TABLE 10. Single primer PCR mixture for *rpoE* gene**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-denaturation</td>
<td>94°C</td>
<td>5min</td>
</tr>
<tr>
<td>denaturation</td>
<td>94°C</td>
<td>1min</td>
</tr>
<tr>
<td>annealing</td>
<td>30°C</td>
<td>2min 30sec</td>
</tr>
<tr>
<td>extension</td>
<td>72°C</td>
<td>3min</td>
</tr>
<tr>
<td>final extension</td>
<td>72°C</td>
<td>10min</td>
</tr>
</tbody>
</table>

**TABLE 11. Single primer PCR condition for *rpoE* gene**
2.8 Southern blot analysis

A probe specific for \textit{rpoE} gene was isolated by digestion of construct of pGEM containing partial \textit{rpoE} gene with \textit{EcoRI}, followed by gel elution of a 399 bp fragment to the \textit{rpoE} gene. The probe was labeled by using a nonradioactive digoxigenin-dUTP labeling system (Boehringer Mammheim, Germany). Isolated chromosomal DNA from MS79 was digested with \textit{EcoRI} and \textit{HindIII}, subjected to electrophoresis, and capillary transferred onto a nitrocellulose membrane. The developed filter was hybridized and hybridized to filters in hybridization solution at 65°C. The hybridized probes were immunodetected with anti-digoxigenin-AP, the fragment and visualized with the colorimetric substrates NBT/BCIP as in the DIG DNA labeling and detection Kit (Boehringer Mannheim).

2.9 Regulation of \textit{rpoE} gene

2.9.1 Total RNA isolation and quantitative analysis

Total RNA was isolated in a rapid and simple manner. All the glassware and solutions were pretreated diethylpyrocarbonate (DEPC) to inactive RNase activity. Cells were harvested and resuspended in lysis solution (sodium acetate 2.7g, SDS 5g, EDTA 0.34g per liter of deionized water, pH5.5). The glass beads were added in the cell suspensions and mixture was vortexed for 3min to break cell wall. Directly, the sample was mixed with hot-saturated phenol (pH4.5) and was inverted position for protein and DNA. The supernatant gained by
centrifugation at 15000rpm for 20min was transferred to new tube and was treated with chloroform. The total RNA was precipitated using 3M sodium acetate (pH 5.3) and ice-cold absolute ethanol, subsequently RNA samples were resuspended with RNase free-water. DNA was removed by treatment with RNase free-DNase (Promega, USA). For quantitative analysis of total RNA, each sample without DNA was diluted to 200 fold with DEPC treated-water. Concentration of RNA samples was calculated by A260 nm and was adjusted to 1.5µg/µl.

2.9.2 Northern blot analysis

For Northern blot analysis, 20mg total RNA per sample was fractioned on a 1.8% agarose-formaldehyde gel. RNA was blotted from the gel to nitrocellulose membrane (Amersham), and the RNA was UV cross-linked for 5min. A 0.396-kb PCR product, which carries most of the rpoE coding sequence, was obtained from pGEM using primers rpoE-for and rpoE-rew and then gel purified. The probe was used at a concentration of 10ng/ml in hybridization experiments at 42°C.
FIGURE 3. Experimental scheme for rpoE gene analysis and regulation;
3. RESULTS AND DISCUSSION

3.1 Stress assay

The survivals of four Lactobacilli in stress conditions are shown in FIGURE 4 to FIGURE 8. Survival of MS79 was compared with *L. crispatus* KLB46. KLB46 had been formerly selected for industrialization.

3.1.1 Acid stress

Generally lactobacilli can survive in acidic conditions and contribute to the maintenance of a low vaginal pH (4.0-4.5) (Sharpe, 1981 and Vallor et al., 2001). But we show that survival upon exposure to lethal acidic conditions and the acid tolerance response of *L. fermentum* MS79 and KLB46 in stationary phase. The survival of MS79 in acid stress condition is shown on FIGURE 4(A). MS79 displayed an active acid tolerance response upon exposure to a lethal acidic condition, pH3. To our surprise, when *L. fermentum* MS79 was transferred to a fresh acidic MRS (pH 3.0) viability increased twofold (FIGURE 4 (A)), indicating the importance of induction activation acid tolerance response at lethal pH. Acid stress can be described as the combined biological effect of low pH and acid in the environment as a result of fermentation or preservation. In response to encounters with acids microorganisms have evolved complex, inducible acid survival strategies. Acid tolerance response (ATR) has been described (Bearso
et al., 1997).

3.1.2 Cold (long term storage), osmotic, freeze-drying and oxidative stress

*L. fermentum* MS79 maintained high viability (10^7 CFU/ml) for two weeks at low temperature (4°C) (FIGURE 4 (B)). When the cells were exposed to oxidative stress (0.01% H\textsubscript{2}O\textsubscript{2}) (FIGURE 6) and osmotic stress (2M NaCl), *L. fermentum* MS79 maintained high viable cell number: 10^5-10^6 CFU/ml under osmotic (2M NaCl) after three days (FIGURE 5 (D)), under oxidative stress after eight hours. After being stored one month at room temperature freeze-dried cells maintained viable cell number of 10^5 CFU/ml (FIGURE 5 (C)). But KLB46 was died all in the period of the test. Among the many ways used to preserve food products, increased osmotic pressure, i.e. lowering of water activity (*a_w*), is one of the most widely used. Desiccation or addition of high amounts of osmotically active compounds such as salts or sugars lowers the water activity of the food. The internal osmotic pressure in bacterial cells is higher than that of the surrounding medium. This results in a pressure exerted outwards on the cell wall, called the turgor pressure, which is thought to provide the mechanical force necessary for cell elongation (Csonka, 1989). Therefore, bacterial cells must be able to maintain turgor despite variations in the osmotic pressure of the surrounding medium. The extended use of frozen and chilled (convenience) foods and the increased popularity of fresh or minimally processed food, often
preservative-free, greatly increased the interest in cold adaptation behaviour of microorganisms and food pathogens, in particular. Mechanisms that permit low-temperature growth involve membrane modifications maintaining membrane fluidity and the maintenance of the structural integrity of macromolecules and macromolecule assemblies such as proteins and ribosomes. Microorganisms have developed a number of strategies to maintain their membrane lipids fluid and functional at low growth temperature.
FIGURE 4. Survival of Acid stress (A) and long-term storage at low temperature (B)
FIGURE 5. Survival of freeze-drying (C) and osmotic stress (D)
FIGURE 6. Survival of oxidative stress (E)
3.1.3 Heat, ethanol, freezing and thawing and desiccation

In these stresses, MS79 was not showed distinguished survival differences but the survival of MS79 was higher than that of the KLB46 when challenged to every environmental stress: The final survivals of MS79 have $10^4$~$10^6$, but those of KLB46 have $10^2$~$10^4$. Heat stress test was performed during 60 minutes, as 60°C is extremely high temperature for general microorganism. MS79 maintained $10^3$ CFU, while KLB46 was dead almost (FIGURE 7). Cells contain several targets for the action of heat, and so it can be proposed that the basal heat resistance of microorganisms may be due to the intrinsic stability of macromolecules, i.e. ribosomes, nucleic acids, enzymes and proteins inside the cell and the membrane (Abee., T. et al. 1999).
FIGURE 7. Survival of heat stress (F) and desiccation (G)
FIGURE 8. Survival of ethanol stress (H) and freezing and thawing (I)
3.2 Selection and characterization of high stress resistant strain

*Lactobacillus* sp. MS79 was selected as high stress resistant strain for application and industrialization. The growth curve of two strains after transferring to a fresh medium, the comparison of adaptation to a fresh medium and morphology after sub-culture was made. MS79 had short and rod-shape cell, the colony was round and shiny (FIGURE 9 (B)). Following sub-culture MS79 regained growth more rapidly than KLB46. By following the growth of *L. fermentum* MS79 after transferring to a fresh medium, we found that sub-cultured *L. fermentum* MS79 regained growth rapidly with almost no lag phase (FIGURE 9 (A)). In addition to MS79 had anti-microbial activity (FIGURE 10) and high MICs (Minimal Inhibition Concentration) against chemical like detergent, organic acid, salt (TABLE 12).
FIGURE 9. Growth curve (A) and morphology (B) of *L. fermentum* MS79; by following the growth of *L. fermentum* MS79 after transferring to a fresh medium, sub-cultured *L. fermentum* MS79 regained growth rapidly with almost no lag phase and had anti-microbial activity and high MICs had anti-microbial activity and high MICs
FIGURE 10. Anti-microbial activity against *C. albicans* (arrow) of *L. fermentum* MS79
<table>
<thead>
<tr>
<th>Chemical</th>
<th>MIC</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandelic Acid</td>
<td>5 mg/ml</td>
<td>Average</td>
</tr>
<tr>
<td>Benzalkonium Chloride</td>
<td>16 µg/ml</td>
<td>High</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.9 M</td>
<td>High</td>
</tr>
</tbody>
</table>

TABLE 12. MICs against chemicals of *L. fermentum* MS79
3.3 Molecular identification of selected strain

Based on 16S rDNA sequencing and PCR-RFLP, the selected strain as high resistant one was molecularly identified. Genomic DNA was prepared from the cells and used to amplify the 16S rDNA with universal primer as described in Materials and Methods. MS79 was produced a single band about 1.6 kb long. The amplified 16S rDNAs were digested with five restriction endonucleases and the resulting restriction fragments were size-fractionated by 2.0% agarose gel electrophoresis (Chang et al. 2002). And the bands were compared with that of *L. fermentum* KLB12. Based on PCR-RFLP results, we have found that MS79 belongs to *L. fermentum*. MS79 was further identified by 16S rDNA sequencing. The results showed that MS79 was *L. fermentum* and molecular identity of it based on 16S rDNA sequencing result was the same as 16S rDNA PCR-RFLP data.
FIGURE 11. Restriction pattern of PCR-amplified product of 16s rDNA of MS79; Lane M: 100bp DNA ladder, lanes (a) to (e): *Alu I, cfo I, Msp I, Hae III, Hinf I*, left lane: MS79, right lane: *L. fermentum* KLB12
Origin (1567 bp)

```
1 AGAGTTTGAT CTTGCTCACG GATGAACGCC GGGCGGTGTGC CTAATACATG
51 CAAGTCGAAC GGGTGGGCC AATTGATTGA TGGTGCCTTC ACCTGATTGA
101 TTGTTGTCGC CAACGAGTGAG GGACGGGTGA GTAACAGTA GTAACACCTGC
151 CCGAAAGCGG GGGACAACAT TTGGAAACAG AGTCAATACAC GGCATAACAG
201 CGTTGTTTCGC ATGAACACCG CTTAAAAGAT GGCTTCTGC TATCATTTCT
251 GGATGGACCT GGGTGCATT GTAACACGTA GGTAACCTG C
301 GGGCGCAAGC GGGACAACAT TTGGAAACAG ATGCTAATAC CGCATAACA
351 TCTGAAACTG CGTTGTATCA AAGAAGACAC GTCATAGAGT AACTGTTCAT
401 GCTGAGAATCT TAGGCGTGGT AATGGTACAG TGGAGATTTG GGGCTGTAAG
451 AGAGTCGCGG CAGGTTTTCTA AGTTCTGATGT GAAAGCCTTC GGGCTTAACCG
501 GGGGGCCCGC ACAAGCAGGT GAGCATGTGG TTTAATTCGA AGCTACGCGA
551 CAAACCCGTC CTTGCTCACG GATGAACGCC GGGCGGTGTGC CTAATACATG
601 TTTGTTGTCGC CAACGAGTGAG GGACGGGTGA GTAACACCTGA TGGAACACAG
651 CCGAAAGCGG GGGACAACAT TTGGAAACAG ATGCTAATAC CGCATAACAG
701 TCTGAAACTG CGTTGTATCA AAGAAGACAC GTCATAGAGT AACTGTTCAT
751 CGAAGGCGGC TACCTGGTCT GCAACTGACG CTGAGACTCG AAAGCATG
801 TAGCGAAACAG GATCGATATAT CTTAAAACCA GAAAGTCACG GCTAACTACG TGCCAGC
851 CACGTCGCTG CGTTGTATCA AAGAAGACAC GTCATAGAGT AACTGTTCAT
901 TCTGAAACTG CGTTGTATCA AAGAAGACAC GTCATAGAGT AACTGTTCAT
951 GGGCGCAAGC GGGACAACAT TTGGAAACAG ATGCTAATAC CGCATAACAG
1001 AGAACCCTAC CAGGCTTTGGA CATCTTGGGC CAAACATGAG AATGGGCTG
1051 TTCCCTCGGG AACGCAATGA CAGGTTGTGC ATGGTGCCTG TCGACTCTTG
1101 TCGTGAAGAT TGGTGTAAGA TCCCGAAACG AGCGCAACCC TTGGTTACTG
1151 TGGCCAGCAT TAAAGTTGGC ACTCTAATGGA GACTGGCGGT GAAACTACG
1201 AGAGTGCTGG GAGCGATGTC AGATCTGAGA GCCCTTATTG ACCTGGCTGA
1251 CACAGTGGCT ACAAGTGGG GATAAATGAC TCGCAAAAG CACCGGTAAG
1301 CCGAATCTCT TAAACGCGTT CTGATGTCCG ACTGACGGCT GCAAACCGG
1351 TCGAGAAGAT CAGGATGCGC AGTAAATGCAC CGCGCTGCTGA CAGAGTTTGTA
1401 ACACCAAAAG TCAGTTGGGT AATGCTTATTG GAAGCAGCGG CTTAAGGTGA
1451 CACAGTGGCT ACAGGGCGCA AGACAGCATG CAGGTTGGTA CAGAACTCGG
1501 TGGTGGTTCG AGCCGTCGAA GCTACCTACA AGCTAACAAG TGAGCTGGTG
1551 GCTGATGCAC GCCTCCT
```

FIGURE 12. 16S rDNA sequence of *Lactobacillus fermentum* MS79
*Lactobacillus fermentum* MD-9 16S ribosomal RNA gene, partial sequence
Length = 1567
Score = 3037 bits (1532), Expect = 0.0
Identities = 1561/1568 (99%), Gaps = 2/1568 (0%)

*Lactobacillus fermentum* 16S ribosomal RNA gene, complete sequence
Length = 1540
Score = 3037 bits (1532), Expect = 0.0
Identities = 1538/1540 (99%) Gaps = 4/1540 (0%)

*Lactobacillus cellobiosus* 16S rRNA gene, specimen voucher CECT 562
Length = 1555
Score = 1534 bits (774), Expect = 0.0
Identities = 814/822 (99%), Gaps = 4/822 (0%)

*Lactobacillus fermentum strain* KC5b 16S ribosomal RNA gene, partial sequence
Length = 1525
Score = 1471 bits (742), Expect = 0.0
Identities = 788/798 (98%), Gaps = 4/798 (0%)

**FIGURE 13.** Sequence homology of *Lactobacillus fermentum* MS79
3.4 Comparison of stresses response

The acid, osmotic stress, starvation, cold storage ($4^\circ$C) test were performed to determine the comparison of resistant with three other species (FIGURE 14, 15). As *Lactobacillus* sp. MS79 was identified *Lactobacillus fermentum* MS79, the same species, *Lactobacillus fermentum* KLB12 was added to the test for further studies. In addition to *Lactobacillus plantarum* KLB213 was added because it was known to high stress resistance as an industrial species. The survival rate of KLB46 decreased after 2 days as previously observed in studies performed. The survival rates for KLB213 during stress challenging were better than the survival rates for MS79: osmotic stress and cold storage (FIGURE 14(B), FIGURE 15 (D)). These findings may be attributed to the greater stress tolerance of KLB213. As a result MS79 maintained viability as high as KLB213, sometimes higher than KLB213, and always higher than KLB12. As a result MS79 maintained viability as high as KLB213, sometimes higher than KLB213 which was known for industrial microorganism, and always higher than KLB12, same species with MS79.
FIGURE 14. Survival of acid stress (A) and osmotic stress (B)
FIGURE 15. Survival of starvation (C) and cold storage (D)
3.5 Observation of morphological changes using scanning electron microscopy (SEM)

Given the fact that ECF sigma factors have also been demonstrated to have a role in cell envelope, we explored this further. Scanning electron micrographs revealed that cells challenged to various stress conditions (FIGURE 16). Cells with higher resistance to osmotic stress showed more drastic morphological changes. The rough cell surface with irregular shape and significant alterations of cells of MS79 frequently took over the whole population in osmotic stress. These results suggest that the cells of MS79 are able to sense and endure when environmental stresses are approaching limiting conditions, which involves the uptake of the remaining nutrient from the medium. During early stationary phase and late exponential phase, cells underwent reductive cell division and became more resistant to various stresses. Upon recovery in fresh medium, stationary-phase cultures showed an immediate increase in protein synthesis irrespective of culture age. Colony morphology variants accumulated in stationary-phase cultures.
FIGURE 16. Observation of morphological changes of *L. fermentum* MS79
Control (upper), osmotic stress (lower)
3.6 Comparison of the cell surface hydrophobicity between control and stressed cells

Given the fact that ECF sigma factors have also been demonstrated to have a role in cell envelope, we explored this further. Specially, to demonstrate whether the morphological change of MS79 is correlated with the change in the surface hydrophobicity, the overall cell surface hydrophobicity was investigated by measuring the cell partition with hydrocarbon, in this study. Cell surface hydrophobicity measurements of stressed and unstressed cells were compared. Hydrophobicity was calculated according to formula in FIGURE 17. Unstressed cell surface of MS79 were hydrophobic, 70.6% hydrophobicity and KLB12, KLB46 showed hydrophobic cell surfaces, 98.0%, 99.4%, respectively, either (TABLE 13). However stressed cell surface of MS79 altered to be hydrophilic; 46.0%, 9.2%, 13.4%, 0% in acid stress, osmotic stress, starvation, long term storage in low temperature, respectively (TABLE 13). KLB213 has hydrophilic cell surface, initially, but its alteration surface hydrophobicity was not detectable. The significant differences were found that the stressed cells of MS79 were immediately nearly completely moved to aqueous phase layer, when mixed with n-hexadecane. However, the other species remained in organic phase. These results were considerable as a role for alteration of cell surface hydrophobicity in stress resistance of MS79.
<table>
<thead>
<tr>
<th></th>
<th>KLB12</th>
<th>KLB46</th>
<th>KLB213</th>
<th>MS79</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>98.0 % (H+)</td>
<td>99.4 % (H+)</td>
<td>27.3 % (H-)</td>
<td>70.6 % (H+)</td>
</tr>
<tr>
<td>acid stress</td>
<td>94.7 % (H+)</td>
<td>94.0 % (H+)</td>
<td>21.9 % (H-)</td>
<td>46.0 % (H0)</td>
</tr>
<tr>
<td>osmotic stress</td>
<td>99.0 % (H+)</td>
<td>87.2 % (H+)</td>
<td>22.2 % (H-)</td>
<td>9.2 % (H-)</td>
</tr>
<tr>
<td>starvation</td>
<td>87.0 % (H+)</td>
<td>75.8 % (H+)</td>
<td>22.8 % (H-)</td>
<td>13.4% (H-)</td>
</tr>
<tr>
<td>4°C storage</td>
<td>92.5 % (H+)</td>
<td>95.7 % (H+)</td>
<td>20.9 % (H-)</td>
<td>0% (-8.3) (H-)</td>
</tr>
</tbody>
</table>

TABLE 13. Alteration of cell surface hydrophobicity after challenge the stresses
FIGURE 17. Determination of hydrophobicity

\[
\text{Hydrophobicity (\%) = } \frac{A_0 - A}{A_0} \times 100
\]

- \( A_0 \) = initial O.D.
- \( A \) = O.D. after mixing
3.7 Alteration of cell surface proteins

We studied the protein alteration of the stressed cell surface in osmotic stress of MS79. The surface protein by procedure was subjected to SDS-PAGE and visualized by the Comassie blue staining method. As shown FIGURE 18, control of MS79 displayed four main bands. When the stressed cells were examined, the small size bands were absent. And one band appeared, newly. The results were considered that stressed cells of MS79 were missing hydrophobic proteins and cells were hydrophilic. Membrane-bound anti-sigma factors which release extracytoplasmic Function (ECF) RNA polymerase sigma factors in response to particular extracytoplasmic-inducing cues contributed to stress resistance (FIGURE 19). AlgU (synonyms, AlgT, RpoE, and $\sigma^{22}$) positively regulates its own transcription. Located downstream of algU, the mucABCD genes ensure tight control of AlgU activity in P. aeruginosa (Govan, J., and V. Deretic. 1996.). The mucA gene encodes a transmembrane protein which acts as an anti-sigma factor for AlgU, and mucB codes for a periplasmic protein which is another negative regulator of AlgU (Mathee, K., et al. 1997, Schurr, M. J., et al. 1996., and Xie, Z., et al. 1996).
FIGURE 18. Alteration of surface protein pattern after challenge stresses of *L. fermentum* MS79; Control (A), acid stress (B), osmotic stress (C), starvation (D), 4°C (E)
3.8 Analysis of \textit{rpoE} gene sequence of \textit{Lactobacillus fermentum} MS79

A partial \textit{rpoE} gene fragment (~396 bp) was isolated from \textit{L. fermentum} MS79 by degenerate-PCR with designed primers based on the alignment of known \textit{rpoE} genes from other lactobacilli. The cloned \textit{rpoE} of MS79 is similar to that of the directed RNA polymerase, delta subunit of \textit{Lactobacillus plantarum}, \textit{Listeria monocytogenes} EGD-e, displaying sequence identity of 68\%, and 64\%, respectively.

The \textit{rpoE} gene of \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}), which encodes the extracytoplasmic stress response sigma factor $\sigma^E$, is critically important for the virulence of \textit{S. Typhimurium}. (Southern blot analysis was used to determine copy number of \textit{rpoE}. Isolation method for genomic DNA and southern blot procedure were described in materials and methods. As shown in FIGURE 20, EcoRI and \textit{pstI} digested DNA hybridized with the DIG-labeled \textit{rpoE} probe, showed.)
**DNA-directed RNA polymerase, delta subunit [Lactobacillus plantarum WCFS1]**

Length = 199 Score = 126 bits (317), Expect = 1e-28 Identities = 63/92 (68%), Positives = 69/92 (75%)

Query: 2 ILAYHNEAMAFADLTNEIQQYLGKSDEEIRELRSQFYTDLNVDGSFISLGNDNTWGLRAWY PYESIDEATVGXXXXXXXXXXXXXXXXVNAFLA 277
IL+ H + MAFADLTN +Q YLGKSDEEIRELRSQFYTDLN+DGFSIFSLGDN WGLRAWY P+ESIDEA + VNAFLA
Sbjct: 23 ILSQHGDVMAFADLTNAVQSYLGKSDEEIRELRSQFYTDLNIDGSFISLGDN MWGLRAWY PFESIDEAVI-HTDDDEDEDPRKRRKVNNAFLA 113

**DNA-directed RNA polymerase, delta subunit [Lactobacillus gasseri]**

Length = 186 Score = 103 bits (258), Expect = 1e-21 Identities = 45/67 (67%), Positives = 54/67 (80%)

Query: 2 ILAYHNEAMAFADLTNEIQQYLGKSDEEIRELRSQFYTDLNVDGSFISLGDN WGLRAWY 181
IL + MAFAD+ N +Q +LGKSDEEIREL QFYTD+N DGFSIFSLGDN WLR+W+ PYESIDEA + VNAFLA
Sbjct: 29 ILQDSGKRMAFADIVNAVQFLGKSDEEIREL PQFYTM NTDGFE SMGDNVW ALRSWF 88

**DNA-directed RNA polymerase delta chain [Lactobacillus johnsonii NCC 533]**

Length = 180 Score = 101 bits (251), Expect = 6e-21 Identities = 44/67 (65%), Positives = 53/67 (79%)

Query: 2 ILAYHNEAMAFADLTNEIQQYLGKSDEEIRELRSQFYTDLNVDGSFISLGDN WGLRAWY VPYESIDEPYESIDE 202
IL + MAFAD+ N +Q +LGKSDEEIREL QFYTD+N DGFSIFSLGN WLR+W +PYES+DEPYES+DE
Sbjct: 23 ILQDSGKRMAFADIVNAVQFLGKSDEEIREL PQFYTMNTDGFE SMGHNVW ALRSWF PYESVDE PYESVDE 95

**FIGURE 20. Amino acid sequence comparison of a rpoE gene**
FIGURE 21. Ampification of a partial rpoE gene (A) and Southern blot (B): M, Hind III size marker; lane a, agarose gel picture of rpoE degenerate-PCR product; lanes 1 and 3, EcoR I digested; lanes 2 and 4, pst I digested
3.9 regulation of \textit{rpoE}

To examine the regulation of \textit{rpoE} expression, the cells of MS79 were challenged to osmotic stress (2M NaCl MRS). To ensure survival in changing environments, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stress situations. Regulatory elements that make essential contributions to bacterial survival under stress conditions include the alternative sigma factors RpoS (\(\sigma^R\)) and RpoE (also referred to as AlgU or AlgT in fluorescent pseudomonads). We then proceeded to measure the levels of \textit{L. fermentum} MS79 \textit{rpoE} mRNA before and after osmotic stress by probing Northern blots with an \textit{rpoE} - specific probe. Before isolation \textit{rpoE} mRNA, cells were treated with osmotic stress. The results were described in FIGURE 21, \textit{rpoE} mRNA was induced in osmotic stress condition. No \textit{rpoE} transcripts were detected in normal culture cells (FIGURE 22). Two transcripts of were observed of the stressed cells and transcripts displayed similar levels of induction. These results show that the extreme osmotic stress response in \textit{L. fermentum} MS79 is under transcriptional regulation of \textit{rpoE} and indicate cotranscription of two promoters. In \textit{E. coli}, expression of the \textit{rpoE} gene has been shown to be directed by two promoters, a more upstream \textit{rpoE}-independent P1, and a promoter P2 that is autoregulated by \(\sigma^E\) (Miticka, H., et al. 2003)
FIGURE 22. Total RNA (A) and northern blot analysis (B) under osmotic stress; (a): control, (b): first, (c): second
4. CONCLUSION

In this study, we selected *L. fermentum* MS79 as unusually high resistant strain to diverse environmental stresses. MS79 was identified as *Lactobacillus fermentum* using PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) and 16S rDNA sequencing. MS79 maintained high viability (\(10^7\) CFU/ml) for two weeks at low temperature (4\(^\circ\)C). When the cells were exposed to various stresses: acid (pH 3.0), heat (60\(^\circ\)C), ethanol (10%), dehydration (in the dessicator containing 10g CaCl\(_2\)), oxidative stress (0.01% H\(_2\)O\(_2\)), osmotic stress (2M NaCl), freeze-thaw, *L. fermentum* MS79 maintained high viable cell number: \(10^5 – 10^6\) CFU/ml under osmotic (2M NaCl) and ethanol stress (10%) after three days, under dehydration and oxidative stress after eight hours. After being stored one month at room temperature freeze-dried cells maintained viable cell number of \(10^5\) CFU/ml. To our surprise, when *L. fermentum* MS79 was transferred to a fresh acidic MRS (pH 3.0) viability increased twofold. By following the growth of the MS79 regained growth rapidly with rapidly with almost no lag phase. For further study, the acid, osmotic stress, starvation, cold storage (4\(^\circ\)C) test were performed to determine the comparison of resistant with three other species; *L. crispatus* KLB46, *L. fermentum* KLB12, *L. plantarum* KLB213. Scanning electron micrographs revealed that cells challenged to various stress conditions (FIGURE 16). Cells with higher resistance to osmotic stress showed more drastic morphological changes. The rough cell surface with irregular shape and significant alterations of cells of MS79 frequently
took over the whole population in osmotic stress. These results suggest
that the cells of MS79 are able to sense and endure when environmental
stresses are approaching limiting conditions, which involves the uptake
of the remaining nutrient from the medium. Cell surface
hydrophobicity measurements of stressed and unstressed cells were
compared. Unstressed cell surface of MS79 were hydrophobic, whereas
stressed cell surface of MS79 altered to be hydrophilic in acid stress,
osmotic stress, starvation, long term storage in low temperature,
respectively. The surface protein by procedure was subjected to SDS-
PAGE and the results were considered that stressed cells of MS79 were
missing hydrophobic proteins and cells were hydrophilic. A partial
rpoE gene fragment (~396 bp) was isolated from L. fermentum MS79
by degenerate-PCR and the cloned rpoE of MS79 is similar to that of
the directed RNA polymerase, delta subunit of Lactobacillus plantarum,
Listeria monocytogenes EGD-e, displaying sequence identity of 68%,
and 64%, respectively. Southern blot analysis was used to determine
copy number of rpoE. We have shown that rpoE gene is induced and
requires during survival at extreme osmotic stress using Northern blot
analysis. rpoE mRNA was induced in osmotic stress condition. No
rpoE transcripts were detected in normal culture cells. Two transcripts
of were observed of the stressed cells and transcripts displayed similar
levels of induction. These results show that the extreme osmotic stress
response in L. fermentum MS79 is under transcriptional regulation of
rpoE and indicate cotranscription of two promoters.
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