

Heat-Induced Synthesis of δ^{32} in *Flexibacter chinensis*: The Role and Function in Cell Division

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Abstract

A Sigma factor gene which is important in the global regulation of heat shock responses in prokaryotes, *rpoH*, was isolated from *Flexibacter chinensis* by PCR, sequenced and compared to the *rpoH* genes of a variety of other organisms. The *rpoH* gene was 98% similar to other previously characterized genes. A mutant of *rpoH* was produced (JR102) which had a reduced growth rate at both low and high temperature. JR102 also produced filaments when grown at temperatures greater than the organism's optimum growth temperature. During the stationary phase when the wild-type cell size was reducing, the cell size of JR102 did not change, suggesting that cells division was inhibited in the mutant. The results presented in this paper suggest that *F. chinensis* subjected to starvation and other stresses reduces its cell size by miniaturization or cell division to conserve energy. Anything which prevents the formation of these miniaturized cells reduces the chances of the bacterium surviving under stress conditions.

Keywords: *Flexibacter chinensis*, δ^{32} , Heat shock protein, *RpoH*, SOS response

Introduction

The heat shock response in *Escherichia coli* depends primarily on the increased synthesis and stabilization of otherwise scarce and unstable sigma32 (*rpoH* gene product), which is required for the transcription of heat shock genes [1]. *RpoH* (δ^{32}) was originally identified in *Escherichia coli* as a sigma factor that responds to heat shock. In response to a sudden increase in temperature or other stresses, the levels of RpoH rise transiently, inducing transcription of a subset of genes encoding heat shock proteins (HSPs). HSP includes DnaK, DnaJ and GrpE, and GroEL and GroES, and proteases.

Although RpoH and HSPs were identified as part of the heat shock response, these proteins are present at low temperature and play important roles in cellular processes under non stress conditions [2].

They constitute the two major chaperone systems of *E. coli*. They are important for cell survival, since they play a role in preventing aggregation and refolding proteins.

The heat shock response is positively controlled at transcriptional level by product of RPOH gene. Because of its rapid turn over the cellular concentration of sigma32 is very low under steady state conditions and is limiting for heat shock gene transcription. The shut off

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of the heat shock response occurs as a consequence of declining sigma 32 levels and inhibition of sigma 32 activities [3].

In this study, the role of the δ^{32} dependent heat shock regulon on cell size of *F. chinensis* was investigated. For this purpose, mutant strains of *F. chinensis* was generated which were deficient in the expression of the first alternative sigma factor as a regulator of heat shock chaperons and protease enzymes.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains used were, the *Flexibacter chinensis* strain and the *Escherichia coli* strains including ML30, TG1, S17-1 and the plasmids used, were listed in Table 1.

Bacterial Growth Media and Conditions

All bacterial strains were routinely grown in Luria Broth (10 g/l Bacto tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) or on Luria Agar (10 g/l bacto tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l Agar).

Plasmid Mobilization

The biparental method has been described by Wolk *et al.* [4] but was used with slight modifications. For the mobilization of recombinant plasmid pLRPOH32, *E. coli* S17-1 was used as the donor for the transfer of the recombinant plasmid, pLRPOH32 into *F. chinensis*. The plasmid *RP4* is an *incP* type plasmid which is integrated in the chromosome of *E. coli* S17-1 and the plasmid, pLYLO3, contains an *oriT* (transfer origin) from *PK2*, an *incP1* plasmid, which is recognized by *IncPa* plasmids, such as *RP4*, but not by *Incp β* plasmids. The recombinant plasmid, pLRPOH32, was transferred into the *E. coli* S17-1 by transformation and ampicillin resistant cells were isolated on LB plates containing 100 μ g/ml ampicillin. The donor strain, *E. coli* S17-1, was grown to mid exponential phase in LB containing ampicillin as a selective agent for construct (Erythromycin resistance is not expressed in *E. coli* strains) at 37°C and *F. chinensis* as the recipient in LB containing kanamycin as a selective agent at 30°C. Both donor and recipient strains were harvested by centrifugation, mixed together (1:1 ratio), and approximately 10^9 cells were spotted onto LB agar plates without antibiotics. After incubation for 18 hr at 30°C, the cells were scraped off the plates, diluted in LB and plated onto LB agar containing 200 μ g

Erythromycin to select for transconjugants. The plates were incubated for 2 to 3 days at 30°C. Fifteen erythromycin-resistant colonies were single recombinants with disruption of the target gene at one site which also resistant to ampicillin. A single recombinant clone carrying pLRPOH38 in the chromosome was named *F. chinensis* JR 102.

Sequencing

The dideoxy chain termination DNA sequencing method was carried out. Samples were prepared and sent to MWG-Biotech Co. (Germany).

Southern blot protocol

The DNA was digested and separated on a 0.7% agarose gel, then incubated in 0.25 M HCl at 20°C for 20 min, in denaturation solution (50 ml 10 M NaOH and 87.66 g/l NaCl in 1 liter distilled water) for 45 min and in neutralization solution (121.14 g/l Tris and 87.66 g/l NaCl in 1 liter distilled water) for 30 min. The transfer buffer (20 x SSC) contained 175.3 g/l NaCl and 88.2 g/l Tri-sodium citrate in 1 liter distilled water.

The southern hybridization method was carried out using the 32 P-labeled probe (Amersham,UK) and immobilized DNA on the membrane was using the method of Sambrook *et al.* [11].

Preparation of Random-Labeled DNA Probes

A commercially available random-primed DNA labelling kit (Boehringer Mannheim) was used to label the DNA. The DNA was purified by phenol: chloroform extraction, precipitated and resuspended in TE buffer (50 mM Tris pH 8.0, 5 mM EDTA, 50 mM NaCl). The method was carried out per the manufacturer's instructions.

Polymerase Chain Reaction Technique (PCR)

The PCR technique was used to amplify the *rpoH* gene, using two primers, 5'-GGGGAATTCTG / TGTTA / CAACCAGC / GCCAGA / GCGCGCTG / TC-3' and 5'-GGGGGATCCCGA / CTGTTGTCGG / CCTGAC / AGAGGAGC-3'. A total of 35 cycles consisted of 94°C for 45 sec, 55°C for 1 min and 72°C for 45 sec was repeated.

Viable Count

The viable count was determined using a surface spread plate technique. Samples (1 ml) were taken from the

Table 1. Strains, plasmids used in this study

Strains & Plasmids	Description	Source
<i>Strains</i>		
<i>Flexibacter chinensis</i>	Wild type strain 34	Warwick U.
<i>Flexibacter chinensis.JR101</i>	rpoH::pLYLO3	In this study
<i>E. coli</i> ML30	Wild type	Warwick U.
<i>E. coli</i> TG1	<i>K12,Δ(lac pro)SupEthia-l'hsDd5,F traD36ProA⁺B⁺lacIqZΔm15</i>	(Warwick U.)
<i>E. coli</i> S17-1	RP4-TC::Mu-Km::Tn7 Tcr,Smr,Kmsres-,mod+	(Warwick U.)
<i>Plasmids</i>		
pBluescript II KS+	High copy cloning vector, Ap ^r	Stratagene
pIC19H	High copy cloning vector, Ap ^r	Warwick U.
pKNG101	Suicide vector, SacB, Sm ^r , Ap ^r	Warwick U.
pKRP10	Cassette carrier vector, Cm ^r , Ap ^r	Warwick U.
pLYLO3	Suicide vector, Er ^r ,Ap ^r	Warwick U.
pMOSBlue	PCR cloning vector, Ap ^r	Amersham International(PLC)
JC2926	Conjugational plasmid, Tc ^r , Cm ^r	Warwick U.
RP4	Conjugational Plasmid, Ap ^r , Km ^r , Tc ^r	Warwick U.
PMRPOH32	0.403 kb PCR product of <i>rpoH</i> gene cloned into pMOS Blue	This study
PLRPOH32	0.422 kb <i>BamHI</i> fragment subclone of pMRPOH32 into pLYLO3p	This study

flasks and serial dilutions prepared as a 10-fold serial dilution in Quarter-strength Ringers solution (2.25 g/l NaCl, 0.12 g/l CaCl₂, 0.05 g/l NaHPO₄ and 0.105 g/l KCl in 1 liter Distilled water). 100 µl of the diluted samples were spread on duplicate plates. The plates were incubated at 30°C for at least 48 hr. Plates were counted manually by using an illuminated colony counter. The results were expressed as cfu/ml.

Total Counts

The total count was determined using a Coulter counter ZM (Coulter Euro Diagnostics GMBH). The data were analyzed using Coulter channelyzer software to estimate the size distribution. The samples were diluted in an isotonic buffer containing 0.4% (v/v) glutaraldehyde to fix the cells. Total count is expressed as total particles/ml. The software also determines mean cell size and volume.

Transmission Electron Microscopy

Transmission electron microscopy was used after samples were negatively stained with phosphotungstic acid. Samples were placed onto a Formvar-coated copper grid (100 segment mesh; Agar scientific) for 30 sec. After drying, the grid was negatively stained with

one drop of 1% (w/v) phosphotungstic acid. The samples were examined using the Jeol JEM-100S transmission electron microscope with an 80 kV accelerating voltage. Photographs were taken using Kodak Panasonic film, which was developed in Kodak D-19 developer at 20°C for 3 min and fixed in Kodak fixer. Final pictures were printed on Kodak Veribrom paper.

Results

Construction of a Vector, Carrying *rpoH* Gene

The 0.4 kb DNA fragment resulting from the PCR was purified and cloned into the pMOSBlue vector. The construct was named pMRPOH32. Restriction digestion of pMRPOH32 with *EcoRI* and *BamHI* resulted in two bands, one 2.9 kb in size which represented the vector and one of 0.4 kb size which was the *rpoH* gene (Fig. 1). DNA sequencing analysis showed that the *rpoH* gene region upstream was highly conserved and similar to the *rpoH* of other organisms. In the next step, the 0.4 kb *BamHI* fragment subclone of pMRPOH32 was cloned into the linearized and dephosphorylated pLYLO3 (which also digested with *BamHI*). This construct named pLRPOH32 and transformed into *E. coli* S17-1 (Table 1).

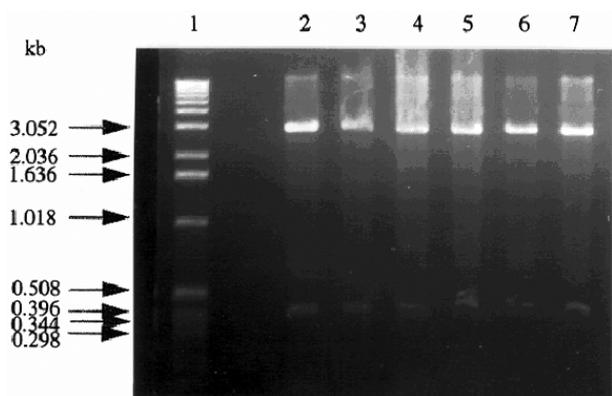


Figure 1. Restriction digestion of pMRPOH32. Lane 1; λ ladder of 1 kb DNA as a molecular size marker. Lane 2 to 7; pMRPOH32 digested with *EcoRI* and *BamHI*.

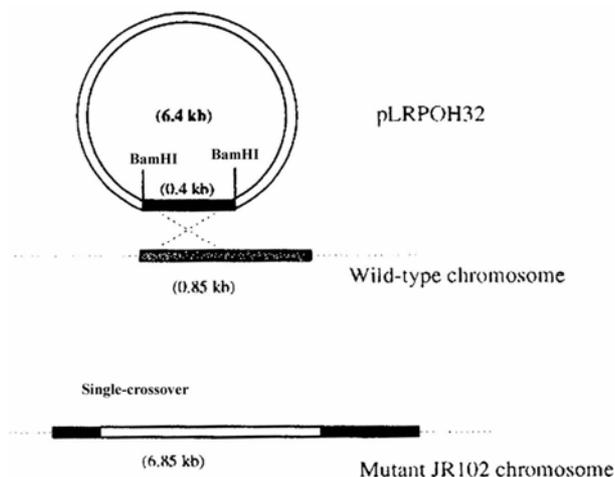


Figure 2. Schematic illustration of integration of pLRPOH32 in the *F. chinensis* wild type chromosome.

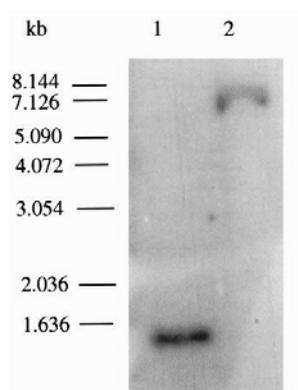


Figure 3. Southern blot analysis. Chromosomal DNA was digested with *EcoRV*. Lane 1; wild-type *F. chinensis*, Lane 2; the single recombinant strain *F. chinensis* JR102.

Analysis of rpoH Mutant Strain

Southern blot analysis of the *F. chinensis* strain JR102 was carried out to confirm the interruption of the *rpoH* gene in the chromosome by a single crossover event between disrupted internal parts of the *rpoH* gene cloned in pLYLO3 and the intact *rpoH* gene in the chromosome of *F. chinensis*. (Fig. 2) Chromosomal DNA was purified from *F. chinensis* wild type and JR102 and the subclone of pLRPOH32 was used as a probe. Chromosomal DNA from both the wild type and JR102 were digested with *EcoRV*. Southern blot analysis showed that the probe hybridized with a band in strain JR102 approximately 6 kb larger than the wild-type (Fig. 3). The probe hybridized with the wild-type chromosomal DNA digested with *EcoRV* with a band slightly less than 1.636 kb, while there was no hybridization at this site with strain JR102. The probe hybridized with mutant chromosomal DNA digested with *EcoRV* slightly larger than 7.126 kb. The result also proved that there was no wild-type copy of *rpoH* gene in strain JR102 due to the result of single crossover recombination.

Growth Characteristics of Mutant Strain

The growth rate of wild type and strain JR102 were investigated in Luria broth at 20°C, 30°C and 37°C using changes in optical density as measure of growth. Strain JR102 grew more slowly and had a longer lag phase than the wild type at both 20°C and 30°C. At 30°C, JR102 did not show a stable stationary phase and the optical density began to decline immediately, as a result of cell lysis or of cell miniaturization. The photomicrographs of both the wild type and strain JR102 was carried out after 24 hr incubation at the different temperatures. Samples were directly applied to slides, fixed; Gram stained and observed using a Zeiss microscope at 600 times magnification. At 20°C, there was little difference between the cell size of the wild-type and strain JR102. There was little visible evidence of a change in cell size over a 48 hr incubation period at 20°C for strain JR102. At 30°C (Fig. 4) the cell size was different in the mutant and wild type organisms after 24 hr incubation with the cells of strain JR102 being longer than the wild-type cells. The change in cell size with increasing temperature shows that the longer size must be a direct result of the temperature affecting the mutant.

The growth experiments, particularly at 30°C, showed that δ^{32} , the gene product of *rpoH* was absolutely necessary for cell viability and microphotography showed that considerable changes in cell

morphology occurred in strain JR102 lacking the δ^{32} , when grown at higher temperatures. Both strains were grown at different temperatures (20°C and 30°C) and the total count, viable count and cell size were measured for and incubation period of up to 60 hr. The results are shown in Figure 5 for the wild-type, and show that although the viable count at 30°C declined slightly faster than at 20°C, there was no significant difference in the total cell counts at either temperature. At 30°C the cell size was slightly greater than that at 20°C the reduction in cell size between 20 and 60 hr of incubation was slightly faster at 30°C than at 20°C.

Strain JR102 showed considerable differences in total count, viable count and cell size compared to the data obtained for the wild type strain. Figure 6 shows that cell viability declined more quickly in the wild type so that after 40 hr incubation the viable count had declined below the limits of detection at 30°C, although for the wild-type viable counts were still high even after 60 hr incubation. At 20°C the viable count for strain JR102 did not show a similar decline however it did not reach the same maximum size as for the wild type strain. The total cell count at 30°C and 20°C did not show similar decline so the decrease in cell viable counts was not due to cell lysis. The total count at 20°C was slightly higher than at 30°C unlike with the wild type strain where the counts were very similar. At both temperatures the total counts were lower for the wild type at the same temperature. The cell size declined rapidly at 20°C after about 10 hr of incubation and failed to reach the same maximum size as the wild type. At 30°C the cell size did not decrease and reached a maximum size almost identical to that reached by the wild type. Again this demonstrates a close link between cell size reduction and maintenance of cell viability under starvation conditions. In this case the cells are entering the stationary phase of growth. These results also clearly demonstrate that a mutation in the *rpoH* gene of *F. chinensis* results in inhibition of cell division.

Discussion

The *rpoH* gene has been mapped immediately downstream of a cell division operon in *E. coli* and the genes which map in this region are the cell division genes *ftsY*, *ftsE*, *ftsX* and *ftsS*, the heat shock regulatory gene *rpoH*, the lipoprotein biogenesis gene *fam-715*, and another essential gene, *dnaM*. The *rpoH* gene lies immediately downstream of the last gene *ftsX* of a cell division operon and is transcribed in the same direction [6]. It is possible that in *E. coli* the mutation in the *rpoH* gene affects cell division because of the overlapping of *rpoH* and *ftsX* gene but this is considered unlikely.

However as we do not know the location of the *rpoH* gene in *F. chinensis*, it seems likely that *rpoH* mutants reported here could be located immediately after the promoters of a cell division operon, and could therefore have affected cell division not directly through the involvement of *rpoH*.

One possible suggestion why the mutant of *rpoH* gene results in filament formation is the role of the *Lon* gene. The *Lon* protease was the first ATP-dependent protease to be identified and characterized in detail. It has the ability to hydrolyze casein and a variety of other protein substrates [7]. It is known that one class of UV-sensitive mutants which form long non-separated filaments and die after treatment, have *Lon* mutations. Suppressors of the UV-sensitivity of *Lon* mutations can be isolated at two sites in *E. coli*, *sulA* and *sulB* (*sfiA* and *sfiB*) [8].

It has been reported that *sulB* is an allele of *ftsZ*. A *sulB* mutation leads to an altered *ftsZ* gene product which is slightly temperature sensitive. This altered *ftsZ* gene product is resistant to the *SulA* inhibitor which allows cells division after induction of the SOS response [9].

Lon protease is produced at a detectable level in normally growing cells. Its synthesis is increased transiently when cells are subjected to a heat shock stress. A consensus sequence for this is found in the promoter region of the *Lon* gene [8]. It is known that the *Lon* protease is the part of the δ^{32} regulon which plays an important role in protein degradation and cells lacking the protease show a 50% decrease in ATP dependent degradation of abnormal proteins [10]. In the absence of the *Lon* protease two proteins which are substrates for *Lon* are synthesized at a high level. In *E. coli*, mutation in the *Lon* gene produces two detectable phenotypes [11]. One of these has a mucoid appearance due to the over-production of the *RcsA* protein. This is a natural substrate for the *Lon* protease and is a positive regulator of capsular polysaccharide synthesis.

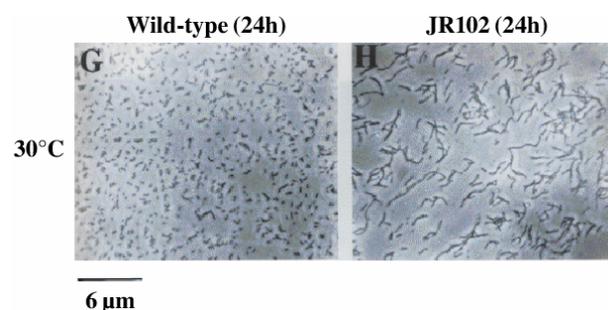


Figure 4. The photomicrographs. G and H shows the wild-type and JR102 strain cells after 24 hr incubation at 30°C respectively.

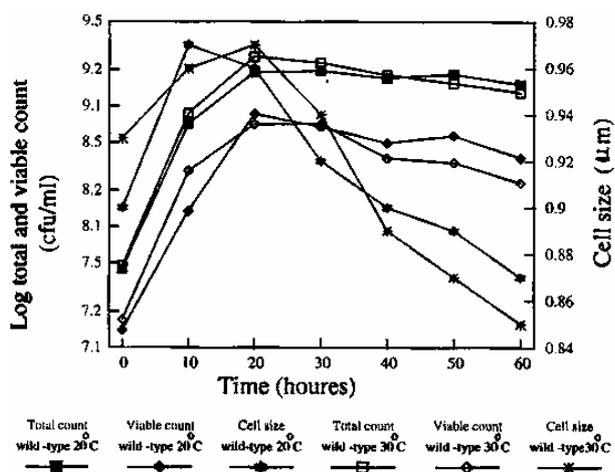


Figure 5. The total counts, viable counts and cell size of wild-type *F. chinensis*. *F. chinensis* wild-type cells were grown with shaking at 20°C and 30°C in Luria broth from an initial viable count of 10⁷ cfu/ml. Viable counts were determined on nutrient agar plates after incubation at 30°C for 48 hr. The total counts and cell size were determined using a CellFactors analyzer after dilution of the samples in the appropriate electrolyte solution to which 0.4% (v/v) glutaraldehyde had been added to fix the cells.

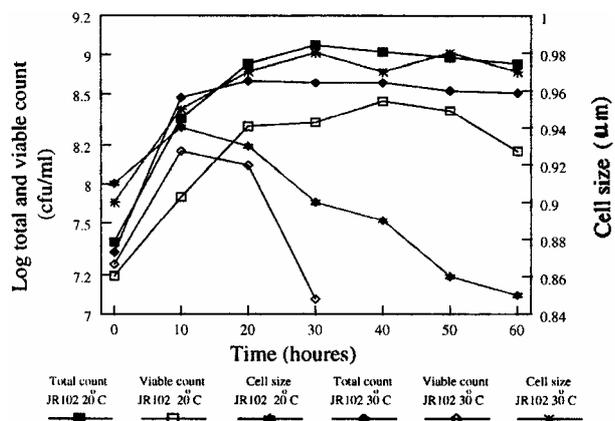


Figure 6. The total counts, viable counts and cell size of *F. chinensis* strain JR102. *F. chinensis* strain JR102 cells were grown with shaking at 20°C and 30°C in Luria broth from an initial viable count of 10⁷ cfu/ml. Viable counts were determined on nutrient agar plates after incubation at 30°C for 48 hr. The total counts and cell size were determined using a CellFactors analyser after dilution of the samples in the appropriate electrolyte solution to which 0.4% (v/v) glutaraldehyde had been added to fix the cells.

The other phenotype is sensitive to DNA damaging agents. DNA damage induces the synthesis of a set of genes which is regulated by the LexA repressor following induction of the SOS response. One of the

induced genes, *sulA*, is a reversible inhibitor of cell division. Over-production of SulA leads to elongation of cells due to the inhibition of cell septation through the interaction with the cell division protein FtsZ [10]. This protein accumulates in annular rings at the points of septation for correct septum formation. *SulA* interacts with *FtsZ* and forms a *SulA-FtsZ* complex which prevents the proper formation of the septum. This inhibition is reversible as degradation of either free *SulA* or the *SulA-FtsZ* complex leads to the formation of a new septum [11].

Lon mutants which are exposed to stress and have the SOS response induced can repair their DNA normally. However, they are unable to recover from transient filamentation and instead they form long non-viable filaments. It has been suggested that following DNA damage, the RecA protein (which belongs to the SOS regulon) is activated. The RecA protein in turn inactivates the LexA protein which is a repressor of SulA synthesis in normally growing cells. After the inactivation of this repressor, the SulA protein accumulates in the absence of the LexA protein. In wild-type cells the Lon protease degrades SulA and cell division will resume. However in the mutants, *SulA* will react with *FtsZ* and will prevent septum formation. These results occur in the transient inhibition of cell division and consequently lethal filamentation of the cells [11].

All things considered, although the *rpoH* mutants produced here had phenotypes similar to other mutants the exact mechanism of the formation of these filaments remains to be elucidated. However, the purpose of producing these mutants was to investigate the effects of such mutations on bacterial survival. The inability to miniaturize leads to loss of survivability once the cell enters the stationary or starvation state. Again the molecular mechanisms behind this enhanced death rate remain to be investigated.

References

1. Nonaka G., Blankschien M., Herman C., Gross C.A., and Rhodius V.A. Regulon and promoter analysis of the *E. coli* heat shock factor, δ^{32} , reveals a multifaceted cellular response to heat stress. *Genes and Development*, **20**: 1776-1789 (2006).
2. Bittner A.N. and Oke V. Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. *J. of Bacteriology*, **188**: 3507-3515 (2006).
3. Aresene F, Tomoyasu T., Bukau B. The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.*, (2000).
4. Wolk C.P., Vonshak A., Kehoe P., and Elhai J. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing

- filamentous cyanobacteria. *Proceeding of the National Academy of the Sciences USA*, **81**: 1561-1565 (1984).
5. Sambrook J., Fritsch E.J., and Maniatis T. *Molecular Cloning; A Laboratory Manual*. 2nd Ed., Cold Spring Harbor Laboratory Press, New York (1989).
 6. Crickmore N. and Salmond G.P.C. The *Escherichia coli* heat shock regulatory gene is immediately downstream of the cell division operon: The *fam* mutation is allelic with *rpoH*. *Molecular and General Genetics*, **205**: 535-569 (1986).
 7. Miller C.G. Protein degradation and proteolytic modification. In: Cutris R., Ingraham J.L., Lin E.C.C., Low K.B., Magasanik B., Reznikoff W.E., Riley M., Schaechter M., and Umbarger H.E., Fredrick C., and Neidhardt F.C. (Eds.), *Escherichia coli and Salmonella*. **1**, 1382-1399. Washington DC, USA (1996).
 8. Gottesman S. Genetic of proteolysis in *Escherichia coli*. *Annual Review of Genetics*, **23**: 163-198 (1989).
 9. Lutkenhaus J.F. Coupling of DNA replication and cell division: *sulB* is an allele of *ftsZ*. *Journal of Bacteriology*, **154**: 1339-1346 (1983).
 10. Gross C.A. Function and regulation of the heat shock proteins. In: Neidhardt F.C., Curtis R., Ingraham J.L., Lin E.C.C., Low K.B., Magasanik B., Reznikoff W.E., Riley M., Schaechter M., and Umbarger H.E. (Eds.), *Escherichia coli and Salmonella*. **1**, 1382-1399. Washington DC, USA (1996).
 11. Gottesman S. and Maurizi M.R. Regulation by proteolysis: Energy-dependent proteases and their targets. *Microbiological Reviews*, **56**: 592-621 (1992).