Regulation of *clpQ*$^+$*Y*$^+$ (*hslV*$^+$*U*$^+$) Gene Expression in *Escherichia coli*

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Abstract: The *Escherichia coli* ClpYQ (HslUV) complex is an ATP-dependent protease, and the *clpQ*$^+$*Y*$^+$ (*hslV*$^+$*U*$^+$) operon encodes two heat shock proteins, ClpQ and ClpY, respectively. The transcriptional (op) or translational (pr) *clpQ*$^+$*::lacZ* fusion gene was constructed, with the *clpQ*$^+$*Y*$^+$ promoter fused to a *lacZ* reporter gene. The *clpQ*$^+$*::lacZ* (op or pr) fusion gene was each crossed into lambda phage. The λ*clpQ*$^+$*::lacZ*$^+$ (op), a transcriptional fusion gene, was used to form lysogens in the wild-type, *rpoH* or/and *rpoS* mutants. Upon shifting the temperature up from 30 ^{o}C to 42 ^{o}C, the wild-type λ*clpQ*$^+$*::lacZ*$^+$ (op) demonstrates an increased β-galactosidase (βGal) activity. However, the βGal activity of *clpQ*$^+$*::lacZ*$^+$ (op) was decreased in the *rpoH* and *rpoH rpoS* mutants but not in the *rpoS* mutant. The levels of *clpQ*$^+$*::lacZ*$^+$ mRNA transcripts correlated well to their βGal activity. Similarly, the expression of the *clpQ*$^+$*::lacZ*$^+$ gene fusion was nearly identical to the *clpQ*$^+$*Y*$^+$ transcript under the *in vivo* condition. The *clpQ*^{m1}*::lacZ*$^+$, containing a point mutation in the -10 promoter region for RpoH binding, showed decreased βGal activity, independent of activation by RpoH. We conclude that RpoH itself regulates *clpQ*$^+$*Y*$^+$ gene expression. In addition, the *clpQ*$^+$*Y*$^+$ message carries a conserved 71 bp at the 5' untranslated region (5'UTR) that is predicted to form the stem-loop structure by analysis of its RNA secondary structure. The *clpQ*^{m2}*Δ40::lacZ*$^+$, with a 40 bp deletion in the 5'UTR, showed a decreased βGal activity. In addition, from our results, it is suggested that this stem-loop structure is necessary for the stability of the *clpQ*$^+$*Y*$^+$ message.

Key Words: Promoter activity, Gene regulation, *E. coli*, ClpYQ (HslUV), ATP-dependent protease, 5' stem-loop structure, mRNA stability.

INTRODUCTION

The *E. coli clpQ*$^+$*Y*$^+$ operon, encoding an ATP-dependent protease, was originally discovered as part of the heat shock apparatus [1]. In the *clpQ*$^+$*Y*$^+$ operon, the first gene, *clpQ*$^+$, encodes a small peptidase of 19 KDa, and the second gene, *clpY*$^+$, encodes an ATPase large subunit of 50 KDa [1-3]. In the presence of ATP, the ClpYQ complex forms an active protease with an $Y^6Q^6Q^6Y^6$ configuration [2, 3]. Biochemical and genetic analyses have shown that the cellular proteins, SulA, RcsA, and RpoH are degraded by the ClpYQ protease [4-11], and that ClpY interacts with SulA in the yeast two-hybrid assays [12]. It was recently reported that Cpx-mediated TraJ degradation by ClpYQ is a stress-mediated response [13]. The most recent aspects for the study of the biological activity of the ClpYQ ATP-dependent protease is reviewed in Wu *et al*., 2008 [14].

Heat shock responses are typically observed in *E. coli* (reviewed in [15-17]). Upon heat shock, protein misfolding leads to a cascade of intracellular protein synthesis, usually dependent on a sigma factor, i.e., σ^{32}, for their gene expression. The σ^{32} consensus binding sequences are found in the upstream region of the *clpQ*$^+$*Y*$^+$ operon and its mRNA production is increased upon heat shock induction (Fig. **1A**) [1]. However, no empirical experiment was performed to directly identify that the RpoH (σ^{32}) is responsible for the heat shock induction of *clpQ*$^+$*Y*$^+$. In addition, the transcriptional start point of the *clpQ*$^+$*Y*$^+$ gene lies 71 bases upstream from the *clpQ*$^+$ start codon (Fig. **1A**). An untranslated region (UTR) upstream of this mRNA contains a 20 bp inverted repeat (IR) sequence 5'CCCCGTACTTTTGTACGGGG3', which is unique for the *clpQ*$^+$*Y*$^+$ operon (Fig. **1A**). In addition, from the wild bacterial genome, the 5'UTR of *clpQ*$^+$*Y*$^+$ also exists in other bacterial species (Fig. **1B**).

In this study, we analyzed *clpQ*$^+$*Y*$^+$ mRNA production in wild-type (wt) cells versus sigma-factor deficient mutants using a λ*clpQ*$^+$*::lacZ*$^+$ fusion gene, in which a chromosomal *clpQ*$^+$*Y*$^+$ promoter is fused with a *lacZ*$^+$ reporter. We demonstrated that the expression of the *lacZ* reporter gene is induced in the wild-type cells but not in *rpoH*-deficient mutants under the heat shock induction. The *clpQ*^{m1}*::lacZ*$^+$ fusion gene, carrying a C to T mutation in the -10 promoter site for RpoH-binding, showed a decreased gene expression. No additional sigma factor was identified for an activation of its gene expression. Thus, through a genetic analysis, the *clpQ*$^+$*Y*$^+$ promoter is *in vivo* recognized by σ^{32}. In addition, a stem-loop structure (IR) at the 5' end of the UTR of *clpQ*$^+$*Y*$^+$ mRNA is shown to be necessary for *clpQ*$^+$*Y*$^+$ gene expression.

MATERIALS AND METHODOLOGY

Materials and Media

Bacterial strains and phages used in this study are listed in Table **1**. LB complex media was purchased from Difco

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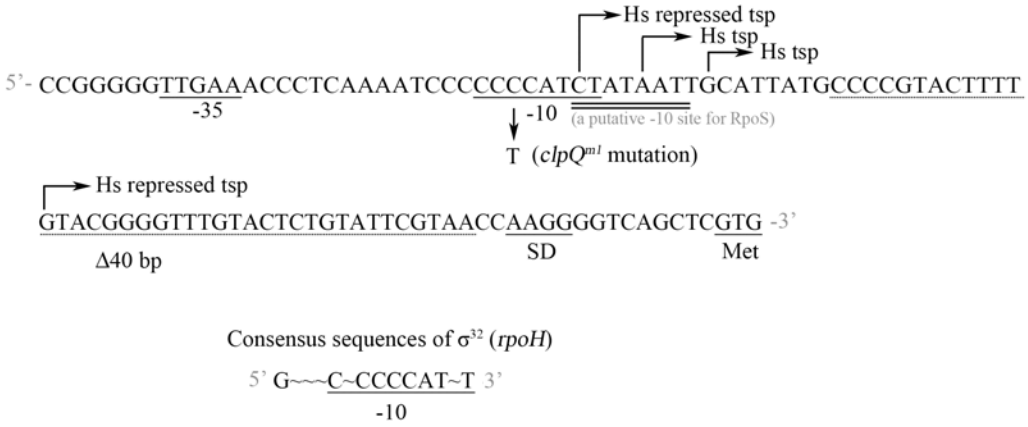
(**A**)



(**B**)

E. coli	5' ------CCCCGTACTTTTGTACGGGGTTTGTACTCTGTATTCGTAACCAAGGGGTCAGCTC**GTG**-3'
S. flexneri	5'-------CCCCGTACTTTTGTACGGGGTTTGTACTCTGTATTCGTAACCAAGGGGTCAGCTC**GTG**-3'
S. enterica	5'---GCCCCGTACGCCGTACGGGGCCGCAATTCAGCATTAGTAACCAAGGGGTCTGCTC**GTG**-3'
K. pneumonia	5' GGCCCCGCGGACAGCGCGGGGCCCCTGTTCCGCTTTTGCAACCAAGGGGTCTGCTC**GTG**-3'
Enterobacter sp.	5'----GCCCTGAGTATCAGCTCAGGGCATCTCTTTCGAATTTGTAACCAGGGGGTCTGCTC**GTG**-3'
Serratia sp.	5'-CCTCGTAGTGTCTGCGCAAGCAGATACTGCGAGGGCTTCTTTCCGTCTTCAACGAGGGTCTGCTC**GTG**-3'
Y. pestis.	5'--CTCCGTACTGTTTTTTAACAGGCAGTACGGGGACTCATTCAGTCTGCAACGAGGGTCTGCTC**GTG**-3'
Erwinia sp.	5'---GCCCCACACTGACGTGTGGGGCCGT-------CTTTTTGACTGTAACCAAGGGGTCTGCCC**GTG**-3'
Citrobacter sp.	5'---- GCCCCGTACCGCGTGCGGGGCCTGT---ATTCTGCATTTGTAACCAAGGGGTCTGCTC**GTG**-3'

Fig. (1). (**A**) Nucleotide sequences of the promoter region of the $clpQ^+Y^+$ operon [1]. The transcriptional start site is shown by arrows. The consensus -10 and -35 sequences recognized by σ^{32} are underlined. The SD region and the start codon GTG of $clpQ^+Y^+$ are as specified. The $clpQ^{m1}$ carries a C to T nucleotide substitution in the region of the RpoH -10 promoter element. The dotted line indicates the deletion of 40 bps in the leader sequence in front of the translational start codon in the $clpQ^{m2}\Delta 40$::$lacZ^+$ fusion gene. (**B**) The conserved regions of sequences from the 5'UTR of the $clpQ^+Y^+$ genes were aligned among the different bacterial species. The bacteria are present mainly in the *Enterobacteriaceae* family, i.e. *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica subsp.* Enteritidis, *Klebsiella pneumonia*, *Enterobacter sp.*, *Serratia proteamaculans*, *Yersinia pestis*, *Erwinia tasmaniensis* and *Citrobacter koseri.* The sequence that forms a hairpin structure is underlined and the IR sequence is highlighted. The Shine-Darlgarno sequence is boxed and the translational start site is in bold.

and the bacteria were grown in LB broth, with an addition of the appropriate antibiotics as required. Supplements were added as the following final concentrations when needed: 100 μg ampicillin (Ap) ml^{-1}; 40 mg X-gal ml^{-1}. The Taq DNA polymerase, restriction endonucleases, and other enzymes were obtained from Takara, New England BioLabs and Biolab (Taipei). Chemicals were obtained from Sigma or Wako.

P1 Transduction and Other General Methods

P1*vir*, λ derivative phages, and transduction were prepared as described by Silhavy *et al*. [23]. Mutant strains were constructed by P1 transduction by procedures as described by Miller [24]. For plasmid constructions, *E. coli* XL-1 blue competent cells were used. Plasmid transformations were performed as described by Chung *et al*. [25]. Plasmid purification was performed according to the instructions of manufacturers (Viogene).

Primers, Construction of Plasmids and DNA Sequencing

The forward (F) and reverse (R) primers used in this study were listed below with the restriction enzyme site underlined and the substituted nucleotides bold. F1: 5'-CG GAATTCCCGGGGGTTGAAA-3', F2: 5'-CCGGAATTC AGCCGTACCAGGATCTGCTGCAAACTCCTG-3', F3: 5'-CCGAATTCCCGGGGGTTGAAACCCTCAAAATCCC-CC**T**CATCTATAATTGCATTATGCCCCGTA-3', F4: 5'-CC ATCTATAATTGCATTATGCCAAGGGGTCAGCT-3', F5: 5'-GGGGTACCCATCTATAATTGCATTATGCCC-3', F6: 5'-GGGGTACCTTTGTACTCTGTATTCGTAAC-3', the F7: 5'-GGGGTACCCCAAGGGGTCAGCTCGTGACAA-3', F8: 5'-GGGGTACCCATCTATAATTGCATTATGCCCCGTA CTTTT**TTTTTTTTT**TTTGTACTCTGTATTCGTAACCAA GGGGTCAG-3', F9: 5'-GGGGTACCCATCTATAATTGC ATTATGGGGGCATGTTTTCATGCCCCTTTGTACTCTG TATTCGTAACCAAGGGGTCAG-3', R1: 5'-GCGGGATCC ACATGGCCGTTACGGC-3', R2:5'-CATAATGCAATTAT AGATGG-3', R3: 5'-CGCGGATCCGCATCCGCAGTACC GCCCGCAAAG-3'. R4: 5'-CCCAAGCTTTTACGCTTTGT AGCTTAATT-3'. All the plasmids, used in this study, were listed in Table **2**. Plasmids pSY1 [$clpQ^+Y^+$ promoter (141 bps)-$lacZ^+$, in pRS415], pSY2 [$clpQ^+Y^+$ promoter (141 bps)-*lacZ*' in pRS414], pSY3 [$clpQ^+Y^+$ promoter (478 bps)-$lacZ^+$, in pRS415] and pSY4 [$clpQ^+Y^+$ promoter (478 bps)-

Table 1. The *E. coli* Strains and Phages Used in this Study

Strains or Phages	Description	References or Derivation
Strains		
MC4100	F$^-$ *araD*139Δ(*argF-lac*)U169 *rpsL*150 *relA1 fibB*5301 *deoC*1 *ptsF*25 *rbsR*	[18]
KY1429	MC4100 *rpoH6*(*Am*) *zhf*-50*::*Tn10	[19]
RH90	MC4100 *rpoS-*359*::*Tn10	[20]
SM25	MC4100 *rpoH rpoS*	[21]
HY20001	MC4100 λ*clpQ$^+$::lacZ$^+$* (op-141 bps)	This work
HY20002	MC4100 λ*clpQ$^+$::'lacZ* (pr-141 bps)	This work
HY20003	MC4100 λ*clpQ$^+$::lacZ$^+$* (op-478 bps)	This work
HY20004	MC4100 λ*clpQ$^+$::'lacZ* (pr-478 bps)	This work
HY20005	*rpoS* λ*clpQ$^+$::lacZ$^+$* (op-141 bps)	This work
HY20006	*rpoH* λ*clpQ$^+$::lacZ$^+$* (op-141 bps)	This work
HY20007	*rpoH rpoS* λ*clpQ$^+$::lacZ$^+$* (op-141 bps)	This work
HY20017	*rpoH* λ*clpQ::'lacZ* (pr-141 bps)	This work
HY20019	MC4100 λ*clpQ^{m2}Δ40::lacZ$^+$* (op)	This work
HY20020	MC4100 λ*clpQ^{m1}::lacZ$^+$* (op-141 bps)	This work
HY20021	*rpoH* λ*clpQ^{m1}::lacZ$^+$* (op-141 bps)	This work
HY20022	MC4100 λ*clpQ^{m1}::'lacZ* (pr-141 bps)	This work
HY20023	*rpoH* λ*clpQ^{m1}::'lacZ* (pr-141 bps)	This work
AC3112	*lon clpQY cpsB::lacZ*	[10]
Phages		
λRS45 phage (Φ)	*imm21 ind$^+$ bla'-lacZ^{sc}*	[22]
λ*clpQ$^+$::lacZ$^+$* (op-141 bps)	carrying *clpQ$^+$Y$^+$* promoter (141 bps) with *lacZ$^+$*	This work
λ*clpQ$^+$::'lacZ* (pr-141bps)	carrying *clpQ$^+$Y$^+$* promoter (141 bps) with *'lacZ*	This work
λ*clpQ$^+$::lacZ$^+$* (op-478 bp)	carrying *clpQ$^+$Y$^+$* promoter (478 bps) with *lacZ$^+$*	This work
λ*clpQ$^+$::'lacZ* (pr-478)	carrying *clpQ$^+$Y$^+$* promoter (478 bps) with *'lacZ*	This work
λ*clpQ^{m1}::lacZ$^+$* (op-141)	carrying a C to T mutation in -10 promoter (op)	This work
λ*clpQ^{m1}::'lacZ* (pr-141)	carrying a C to T mutation in -10 promoter (pr)	This work
λ*clpQ^{m1}Δ40 :: lacZ$^+$* (op)	carrying a 40 bp internal deletion in the 5'UTR (op)	This work

lacZ', in pRS414] were constructed as follows. The plasmid, pWPC80 [9], was used as a template for an amplification of a promoter region of *clpQ$^+$Y$^+$*. The PCR was used to amplify the genes surrounding the upstream region, including the *clpQ$^+$Y$^+$* promoter, the Shine-Dalgarno (SD) site, and the first thirteen amino acids of *clpQ$^+$*, and the resulting DNA fragment was fused in-frame to *lacZ'* in pRS414 [22], a translational *lacZ'* fusion (pr) plasmid [22]; this upstream region was also cloned separately in front of *lacZ$^+$* in pRS415 plasmid with a transcriptional *lacZ$^+$* fusion (op). Two sets of primers were used. Set 1: F1 and R1. Set 2: F2, R1. The PCR conditions were performed according to the manufacturer's instructions, and then the PCR products were purified with Viogene Gel-M kits. Both pRS414 and pRS415 plasmids were cut with *Eco*RI and *Bam*HI and ligated with the DNA fragments treated with the same restriction enzymes. The resulting ligated mixtures were then transformed into the *E. coli* XL-1 blue, and the transformants were selected on ampicillin agar plates. Plasmids isolated from colonies grown on the selective media were subjected to restriction enzyme analysis, followed by DNA sequencing. To construct *clpQ^{m1}::lacZ$^+$*, with a C to T mutation, a set of primers was

Table 2. The Plasmids Used in this Study

Plasmids	Description	References
pRS415	*bal*-Tl4-*Eco*RI-*Sma*I-*Bam*HI-*lacZ*$^+$	[22]
pRS414	*bal*-Tl_4- *Eco*RI-*Sma*I-*Bam*HI *lacZ*'	[22]
pWPC80	pACYC184 with *clpQ*$^+$*Y*$^+$ and its surrouding sequences	[9]
pWF1	pACYC184(Tet^r)-*clpQ*$^+$*clpY*$^+$	[9]
pSY1	*clpQ*$^+$*Y*$^+$ promoter (141 bps)-*lacZ*$^+$ in pRS415	This work
pSY2	*clpQ*$^+$*Y*$^+$ promoter (141 bps)-*lacZ*' in pRS414	This work
pSY3	*clpQ*$^+$*Y*$^+$ promoter (478 bps)-*lacZ*$^+$ in pRS415	This work
pSY4	*clpQ*$^+$*Y*$^+$ promoter (478 bps)-*lacZ*' in pRS414	This work
pSY5	*clpQ*^{m1}*::lacZ*$^+$ in pRS415	This work
pSY6	*clpQ*^{m1}*::lacZ*' in pRS414	This work
pSY7	pRS415-*clpQ*^{m2}*Δ40::lacZ*$^+$	This work
pSY8	pRS414-*clpQ*^{m2}*Δ40::lacZ*'	This work
pBAD33	*ori* (pACYC184, Cm^r) *ara*C P_{BAD}	[26]
pSY9	pBAD33-*clpQ*$^+$	This work
pSY10	pBAD33-*ΔIR-clpQ*$^+$	This work
pSY11	pBAD33-*ΔL-clpQ*$^+$	This work
pSY12	pBAD33-*(S-IR)-clpQ*$^+$	This work
pSY13	pBAD33-*(R-IR)-clpQ*$^+$	This work
pBAD24	*ori* (pBR322, Amp^r) *ara*C P_{BAD}	[26]
pSY14	pBAD24-*clpY*$^+$	This work

used as follows: F3 and R1. Similar procedures as described above were used to clone the PCR fragment carrying *clpQ*^{m1} upstream of *lacZ*. Two resulting plasmids, *clpQ*^{m1}*::lacZ*$^+$ in pRS415 and *clpQ*^{m1}*:: lacZ*' in pRS414, were designated pSY5 and pSY6, respectively. Several PCR steps were then used to construct a *clpQ*^{m2}*Δ40:: lacZ*$^+$, with a 40 bp deletion in the internal region of UTR upstream of the fusion gene. To construct a *clpQ*^{m2}*Δ40* mutation, a two-step PCR was used to amplify the *clpQ*$^+$ promoter and its adjacent region. The upstream region primers were F2 and R2. The downstream primers were F4 and R3. Two overlapping PCR fragments were then used as the template and were amplified by the primers of F2 and R3. The resulting DNA fragment with a 40 bp deletion within the leader sequences (as shown in Fig. **1A**) was cloned into pRS415 and pRS414 at *Eco*RI-*Bam*HI sites, and the resulting plasmids were designated pRS415-*clpQ*^{m2}*Δ40::lacZ*$^+$ (pSY7) and pRS414-*clpQ*^{m2}*Δ40:: lacZ*' (pSY8), respectively.

Plasmids pBAD33-*clpQ*$^+$ (pSY9), pBAD33-*ΔIR-clpQ*$^+$ (pSY10) and pBAD33-*ΔL-clpQ*$^+$ (pSY11) were constructed as described below. Using the pWF1 [9] as the template, the various DNA fragments surrounding the upstream region of *clpQ*$^+$ gene and the upstream region alone were separately PCR-amplified. The resulting DNA fragments were cloned in series into the pBAD33 at the *Kpn*I-*Hin*dIII sites, and the cloned genes were all under the pBAD promoter control. Consequently, the plasmid pBAD33-*clpQ*$^+$carries the upstream 71 nucleotides from the translational start site of *clpQ*$^+$ and *clpQ*$^+$ itself. The F5 and R4 primers were used. The plasmid pBAD33-*ΔIR-clpQ*$^+$, which contains 35 nucleotides upstream of the GTG initiative codon of *clpQ*$^+$ and *clpQ*$^+$ itself under the pBAD promoter, was constructed, using F6 and R4 primers to amplify a DNA fragment of *ΔIR-clpQ*$^+$. The plasmid pBAD33-*ΔL-clpQ*$^+$ was constructed, in which the 15 nucleotides upstream of the first GTG codon and *clpQ*$^+$ itself were under the pBAD promoter control. To generate *ΔL-clpQ*$^+$, F7 and R4 primers were used. In addition, the plasmid pBAD33-*(S-IR)-clpQ*$^+$ (pSY12) was constructed, which carries the substituted T sequences at the 5' end of the UTR of *clpQ*$^+$ (indicated with bold in the primer F8), using the F8 and R4 primers. Plasmid pBAD33-*(R-IR)-clpQ*$^+$ (pSY13) carries the substituted nucleotides at the 5' end of UTR of *clpQ*$^+$, resulting in a newly introduced secondary structure. The F9 and the R4 primers were used for generating the DNA fragment of *(R-IR)-clpQ*$^+$. The plasmid pBAD24-*clpY*$^+$ (pSY14) was constructed as follows. Using the pWF1 as the template, the *clpY*$^+$ was amplified, and the resulting DNA fragment was cloned into the pBAD24 at the

*Eco*RI-*Hin*dIII cutting sites [10, 26]. All the constructed plasmids with the correct DNA insert were identified by restriction enzyme mapping as well by DNA sequencing.

Construction of λ*clpQ::lacZ* Fusion Gene

Plasmids pSY1 (*clpQ*$^+$*:: lacZ*$^+$, op-141), pSY2 (*clpQ*$^+$*::lacZ*', pr-141), pSY3 (*clpQ*$^+$*:: lacZ*$^+$, op-478) and pSY4 (*clpQ*$^+$*::lacZ*', pr-478) were made. These four plasmids were transformed into the *E. coli* XL-1 blue, and the resultant transformants (Ap^r) were selected on ampicillin plates. The transformant cells were then infected with the lambda derivative λRS45 and selected for blue plaques on X-gal plates leading to *clpQ*$^+$*::lacZ*$^+$ transcriptional or *clpQ*$^+$*::'lacZ* translational fusion genes transferred into λRS45Φ [22]. The derivative λRS45Φ*clpQ::lacZ*$^+$ (or λ*clpQ*$^+$*::'lacZ*) fusion gene was made with different upstream length of the *clpQ*$^+$*Y*$^+$ promoter. Each phage lysate was used to infect *E. coli* and the resultant single phage lysogen was selected. A single phage lysogen was identified by measurement of βGal levels of five lysogens, and those with the lowest expression value were selected as the single lysogens.

The βGal Assays

The βGal activities were assayed as described by Miller [24]. The cells were grown to log phase, and activity was measured by the βGal assay. All of the βGal activity was determined in triplicate for each assay. Each analysis was repeated at least three times.

Northern Hybridization Assays

The cells were grown in 5 ml of broth to exponential phase at 30 ^{o}C (OD_{600} about 0.5). At each time point, total RNA was isolated by Qiagene RNeasy Mini kits. Equal amounts of total RNA (3 μg each) were separated under denaturing conditions in a 1% agarose-2.1 M formaldehyde-MOPS [morpholinopropane-sulfonic acid] gel, stained with ethidium bromide, and blotted onto nitrocellulose (Schleicher & Schuell) with 20X SSC. The DNA probes were labeled with DIG-dUTP by random priming (Roche). The DNA fragments used for probes were obtained by PCR amplification. The 401 bp DNA fragment corresponding the *clpQ*$^+$ structural gene was used as a probe for *clpQ*$^+$ mRNA, and a 723 bp DNA fragment containing a *lacZ*$^+$ gene was used as a probe for the *clpQ*$^+$*::lacZ*$^+$ mRNA. The membrane was hybridized with the appropriate probe and washed. The signals were produced by an addition of the anti-DIG antibody with fluorescent alkaline phosphatase conjugate and CDP-star (NEB BioLabs) to the membrane and visualized by an exposure to an X-ray film.

The *cpsB::lacZ* Assays and the Western-blot Analysis for ClpQ

Cells, AC3112 (*lon*, *clpQY*, *cpsB::lacZ*) [10] carrying plasmids pBAD33-*clpQ*$^+$, pBAD33-*ΔIR-clpQ*$^+$, pBAD33-*ΔL-clpQ*$^+$, pBAD33-*(S-IR)-clpQ*$^+$, or pBAD33-*(R-IR)-clpQ*$^+$ in combination with pBAD24-*clpY*$^+$ plasmids were grown overnight on LB with ampicillin (50 μg/ml) and chloramphenicol (12.5 μg/ml) with an addition of arabinose (0.5 %) to induce the P_{BAD} promoter. The overnight cultures were inoculated 1:100 into fresh media with the above indicated ingredients and were grown into log phase, and then half of the cells were immediately subjected to the βGal assays for the *cpsB::lacZ* expression. The other half of the log cells were harvested and subjected to the western blot analysis. The polyclonal ClpQ antibody was used to detect the ClpQ protein, which was separately encoded by the above three plasmids pBAD33-*clpQ*$^+$, pBAD33-*ΔIR-clpQ*$^+$ and pBAD33-*ΔL-clpQ*$^+$. The procedure was adopted as described previously [12]. The western blots were developed by the enhanced chemiluminescence (Pierce).

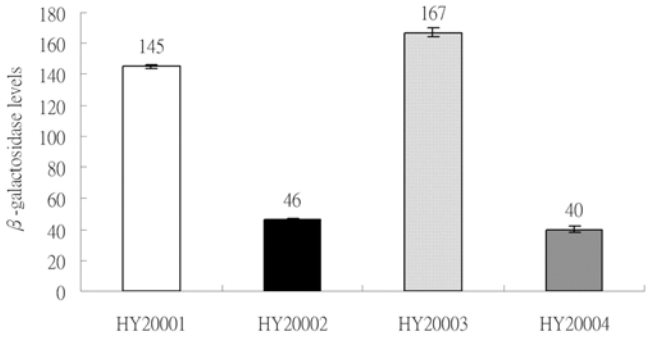


Fig. (2). Expression of two sets of λ*clpQ*$^+$*::lacZ* gene fusion, in a transcriptional or a translational fusion type, with two different lengths of the *clpQ*$^+$ promoter. The βGal activities are represented as bars, from left to right, HY20001 (op-141 bp), HY20002 (pr-141 bp), HY20003 (op-478 bp) and HY20004 (pr-478 bp). The lysogens were cultivated on LB media to log phase at 30 ^{o}C, and the enzyme assays were performed in triplicate at least three times.

RESULTS

The Expression of *clpQ*$^+$*::lacZ* Fusion Gene in λRS45

To monitor the expression of the *clpQ*$^+$*Y*$^+$ genes in *E. coli*, two sets of *clpQ*$^+$*::lacZ* fusion genes were made; in each one, the *clpQ*$^+$*Y*$^+$ promoter is in front of *lacZ*. Both fused genes have the *lacZ* gene under the *clpQ*$^+$*Y*$^+$ promoter control, but they carry the different length of the upstream sequence in either a transcriptional or a translational fusion type. After transferring these four *clpQ*$^+$*::lacZ* fusion gene into the λRS45, the resulting phages, λ*clpQ*$^+$*::lacZ*$^+$ (op-141 bp), λ*clpQ*$^+$*::'lacZ* (pr-141 bp), λ*clpQ*$^+$*::lacZ*$^+$ (op-478 bp), and λ*clpQ*$^+$*::'lacZ* (pr-478 bp), were separately lysogenized into a *lacZ*-deficient strain (MC4100). These four newly lysogens were isolated, namely, HY20001, HY20002, HY20003 and HY20004, correspondingly. The βGal activity was then measured for the each single phage lysogen. Despite the two different sizes of the *clpQ*$^+$*Y*$^+$ promoter fragments (141 bps or 478 bps), the two lysogens in a transcriptional type (HY20001 and HY20003) had an almost equal βGal activity of *lacZ* gene fusion (Fig. **2**); similarly this was observed in a translational type (HY20002 and HY20004) (Fig. **2**). Thus, only one set of lysogens (the op and pr *lacZ* fusion gene with an identical length of the upstream *clpQ*$^+$*Y*$^+$ promoter) would be used for the further analysis.

ClpQ$^+$Y$^+$ Promoter-Mediated Basal and Heat Shock-Induced Gene Expression Requires RpoH

It was shown that *clpQ*$^+$*Y*$^+$ promoter has the RpoH (σ^{32})-binding site [1]. However, a putative -10 site for an

RpoS(σ^{38})-binding was partially overlapped with the similar RpoH recognition site (Fig. **1A**). Therefore, to further identify that RpoH and/or RpoS have effects on $clpQ^+Y^+$ gene expression, the $\lambda clpQ^+::lacZ^+$ (op-141 bp) was lysogenized into the wild-type strain MC4100 [18], and mutant strains KY1429 (*rpoH*) [19], RH90 (*rpoS*) [20], and the double mutant SM25 (*rpoH rpoS*) [21]. The four resulting lysogens all carried $\lambda clpQ^+::lacZ^+$ (op-141 bp) as a prophage. The basal and heat induction of the $clpQ^+::lacZ^+$ gene expression were measured among these four lysogens: HY20001 (the wild-type), HY20005 (*rpoS*), HY20006 (*rpoH*) and HY20007 (*rpoS rpoH*). HY20001 and HY20005 have identical βGal activity at 30 °C (Fig. **3**). In contrast, HY20006 (*rpoH*) and HY20007 (*rpoS rpoH*) show a decreased βGal activity (Fig. **3**). At 42 °C, the wild-type HY20001 and the HY20005 (*rpoS*) mutant have increased βGal activity, whereas HY20006 (*rpoH*) and HY20007 (*rpoS rpoH*) maintain a basal expression (Fig. **3)**. Thus, RpoH, but not the RpoS, appears to control the $clpQ^+::lacZ^+$ gene expression, and the $clpQ^+::lacZ^+$ expression increases under the heat induction.

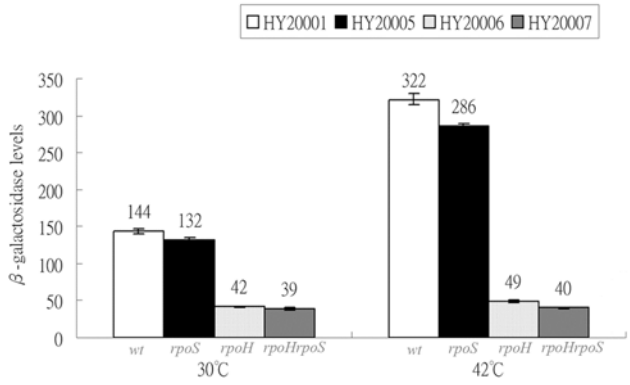


Fig. (3). Expressions of $\lambda clpQ^+::lacZ^+$ in the wild-type and the various sigma- factor deficient strains. HY20001 (the wild-type), HY20005 (*rpoS*), HY20006 (*rpoH*) and HY20007 (*rpoH rpoS*) are as indicated at 30 °C or 42 °C. The lysogens were cultivated at 30 °C first for log growth and then shifted to 42 °C for 20 min.

The mRNA Transcripts of $clpQ^+::lacZ^+$ and $clpQ^+Y^+$ in Wild-Type and RpoH/RpoS Mutants

Northern blot analyses were used to demonstrate that the $clpQ^+::lacZ^+$ mRNA expression is well correlated with its βGal levels in the above bacterial strains. Total RNA was isolated from the wild-type cells (HY20001), *rpoS* (HY20005), *rpoH* (HY20006), and *rpoH rpoS* (HY20007) double mutants at 30 °C. Using a $lacZ^+$ probe, significant RNA transcripts of $clpQ^+::lacZ^+$ were observed in the wild-type and the *rpoS* mutant, whereas only a minor transcript was detected in the σ^{32}-deficient mutant, HY20006, and in the double mutant *rpoS rpoH*, HY20007 (Fig. **4A**). Similar results were observed using a $clpQ^+$ probe to detect the $clpQ^+Y^+$ mRNA production among the wild-type and the various sigma-factor deficient strains (Fig. **4B**).

Expression of $clpQ^{m1}::lacZ$, with a C to T Mutation in the -10 RpoH-biding Site

To reversely show that a specific mutation at the promoter region of $clpQ^+Y^+$ also has an effect on its gene expression, a C to T cis-mutation in the -10 promoter region was made in both pRS414-$clpQ^{m1}::lacZ$' and pRS415-$clpQ^{m1}::lacZ^+$ plasmids. The $clpQ^{m1}$ has a nucleotide substitution, 5'C~CCTCATCT3', in the -10 promoter region distinct from the consensus sequence for the RpoH-binding, 5'C~CCCCATCT3' (Fig. **1**). The two promoter mutant plasmids carrying $clpQ^{m1}::lacZ$ (op or pr fusion) were separately transferred into λRS45, and the resulting phages were designated $\lambda clpQ^{m1}::lacZ^+$ (op) and $\lambda clpQ^{m1}::'lacZ$ (pr), respectively. These two phages were then lysogenized into the wild-type and the *rpoH* mutant, and the four resultant lysogens were HY20020 (op), HY20021 (op, *rpoH*), HY20022 (pr), and HY20023 (pr, *rpoH*). As a control, a lysogen that carries the wild-type $\lambda clpQ::'lacZ$ (pr-141 bps) in the *rpoH* background was also made and named HY20017. The βGal activity was then measured for all the single phage lysogens. As shown, the expression of $clpQ^{m1}::lacZ$ (op or pr fusion) was decreased 1.7-fold as compared to that of the wild-type $\lambda clpQ^+::lacZ$ (Fig. **5**) [compare HY 20020 to HY20001 (op) at 30 °C (Fig. **5A**), and HY20022 to HY20002 (pr) at 30 °C in (Fig. **5B**)]. As shown before, the wild-type $clpQ^+::lacZ$ (op or pr fusion) increased its expression at 42 °C [see HY20001 at 42 °C and at 30 °C (Fig. **5A**) and HY20002 at 42 °C and at 30 °C in (Fig. **5B**)]. But the gene fusion was not activated in the *rpoH* mutant (see HY20006 at 30 °C and at 42 °C and HY20017 at 30 °C and at 42 °C). However, $clpQ^{m1}::lacZ$ (op or pr fusion) was not highly induced at 42 °C [see HY20020 at 42 °C and at 30 °C (Fig. **5A**) and HY20022 at 42 °C and at 30 °C (Fig. **5B**)]. In addition, in the *rpoH* mutant, $clpQ^{m1}::lacZ$ (op or pr fusion) was expressed at a basal or at a slightly lower level than its expression in the wild-type cells [(Fig. **5A**) and (Fig. **5B**), compare HY20021 to HY20020 and compare HY20023 to HY20022, at 30 °C or 42 °C]. Thus, $clpQ^{m1}$, carrying a C to T mutation, has a negative effect on the $clpQ^+Y^+$ promoter activity, and it is not activated by the RpoH.

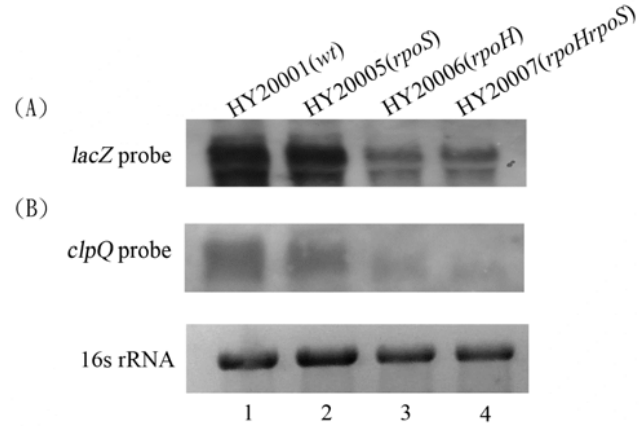


Fig. (4). Northern blots; the total RNA were probed with a DNA fragment of $lacZ^+$ (**A**), or $clpQ^+$ (**B**). The HY20001 (the wild-type), HY20005 (*rpoH*), HY20006 (*ropS*), and HY20007 (*rpoH rpoS*) mutants, each carrying $\lambda clpQ^+::lacZ^+$, were hosts for the RNA isolation. Each lane contains 3 μg RNA for the analysis. The ethidium-bromide stained 16S rRNA was used for normalization of the gel loading.

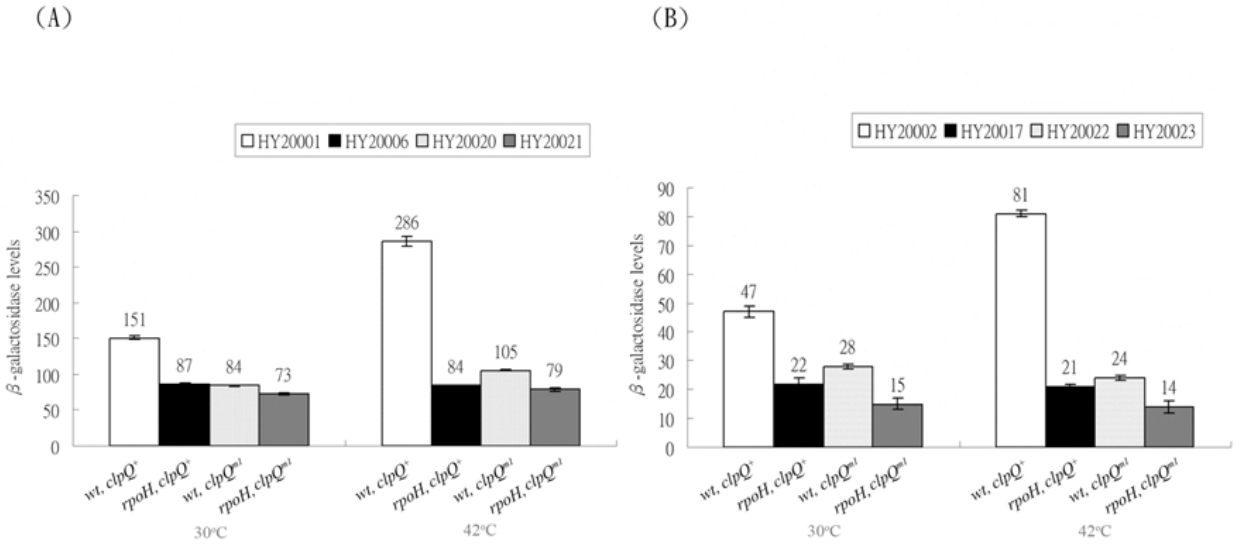


Fig. (5). Expression of the λ*clpQ*⁺*::lacZ* (op or pr fusion) and the λ*clpQ*^{m1}*::lacZ* (op or pr fusion) in the wild-type or the *rpoH* mutant at 30 °C or 42 °C. (**A**) The βGal activities of HY20001 (*clpQ*⁺*::lacZ*⁺, op), HY20006 (*rpoH*, *clpQ*⁺*::lacZ*⁺, op), HY20020 (*clpQ*^{m1}*::lacZ*⁺, op), and HY20021 (*rpoH*, *clpQ*^{m1}*::lacZ*⁺, op) are as indicated. (**B**) In contrast, the βGal activities of HY20002 (*clpQ*⁺*::'lacZ*, pr), HY20017 (*rpoH*, *clpQ*⁺*::'lacZ*, pr), HY20022 (*clpQ*^{m1}*::'lacZ*, pr) and HY20023 (*rpoH*, *clpQ*^{m1}*::'lacZ*, pr) are also listed.

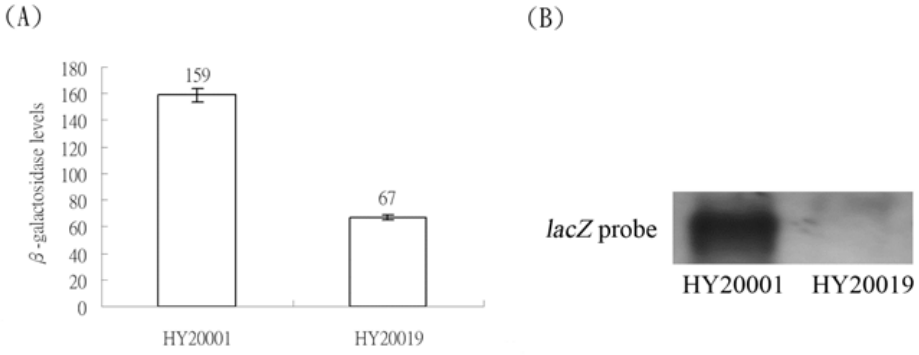


Fig. (6). (**A**) Expression of the λ*clpQ*⁺*::lacZ*⁺ in HY20001 and expression of λ*clpQ*^{m2}*Δ40::lacZ*⁺, with a 40 bp deletion in the internal leader sequences, in HY20019. (**B**) The northern blot of the mRNAs of these two fusion genes, using the 723 bp of *lacZ*⁺ as a probe.

Expression of *clpQ*^{m2}*Δ40::lacZ*⁺, with a Deletion of 40 bps in the 5'UTR

The first transcriptional start site of the *clpQ*⁺*Y*⁺ operon was mapped 71 bp upstream from the translational start site. An UTR is present upstream from an initiation codon for the *clpQ*⁺*Y*⁺ operon. It was logically speculated that this UTR might have an effect on *clpQ*⁺*::lacZ*⁺ gene expression. To test this possibility, a *clpQ*^{m2}*Δ40bp* mutation, with a 40 bp deletion of the internal region of the UTR, was constructed in the *lacZ*⁺ gene fusion [see (Fig. **1**)]. This deletion mutant, *clpQ*^{m2}*Δ40::lacZ*⁺, a transcriptional fusion type, was crossed into the λ phage, and the resulting phage λRS45*clpQ*^{m2} *Δ40::lacZ*⁺ (op) was lysogenized into the wild-type cells, MC4100. The newly formed lysogen, HY20019, displayed a significant decrease in βGal activity, consistent with its mRNA production by northern blot analysis (Fig. **6**). In addition, the *clpQ*^{m2}*Δ40bp::'lacZ* (pr) mutant in a protein fusion construct (pRS414) had much lower βGal activity, and no blue plaques could be selected on the X-gal plates when crossed into the λ phage (data not shown).

Functional Activity of Inverted Repeat Sequence in the 5' UTR of *clpQ*⁺*Y*⁺

Most single strand RNA form a secondary structure. Utilizing an *in silico* M-Fold program (http://frontend.bioinfo.rpi.edu/applications/mfold/) [27], a predicted stem-loop structure, including the IR sequence, with a calculated ΔG = -24.6 kcal/mol was identified in the untranslated leader region (UTR) upstream of the *clpQ*⁺*Y*⁺ structural gene (Fig. **7A-a**). To demonstrate that the stem-loop structure is necessary for the expression of *clpQ*⁺*Y*⁺, DNA fragments with different lengths of the leader sequence upstream of *clpQ*⁺ were cloned into plasmid pBAD33, and all of the *clpQ*⁺ constructs, with different upstream sequences, were under the heterologous pBAD promoter control (induced by arabinose). Three different plasmids were constructed and designated pBAD33-*clpQ*⁺, pBAD33-*ΔIR-clpQ*⁺ and pBAD33-*ΔL-clpQ*⁺, and the secondary structures of the 5' regions of their *clpQ*⁺ mRNA were predicted (Fig. **7A-a**, **b**, **c**.) To evaluate the physiological activity of the above three plasmids encoded ClpQ(s), we used the AC3112 strain for an

assay of a functional ClpQ. Since AC3112, a *lon clpQY* mutant, carries a *cpsB::lacZ,* it is expressed a higher βGal activity due to a stable activator, RcsA [9-11]. When the AC3112 carries both pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ plasmids, it has a decreased βGal activity of the *cpsB::lacZ,* due to an instability of RcsA, targeted by the ClpYQ under the arabinose induction. AC3112 cells that carry pBAD33 and pBAD24, as a negative control, had higher βGal activities of *cpsB::lacZ.* However, while cells carrying plasmids pBAD33-*ΔIR-clpQ*$^+$ or pBAD33-*ΔL-clpQ*$^+$, in the presence of pBAD24-*clpY*$^+$, each bacterial strain had much higher βGal activity from its *cpsB::lacZ* (Fig. **7B**). Western blot analysis was then used to show that ClpQ was accumulated lower in the cells carrying plasmids pBAD33-*ΔIR-clpQ*$^+$ or pBAD33-*ΔL-clpQ*$^+$, as compared to the cells carrying the wild-type pBAD33-*clpQ*$^+$ (Fig. **7C**). To further identify that the instability of *clpQ*$^+$ mRNA leads to a loss of the functional ClpQ, a northern blot analysis was used to detect the *clpQ*$^+$ messages in all of the above strains. As shown, the mRNA levels of *ΔIR-clpQ*$^+$ or *ΔL-clpQ*$^+$ were much lower than those of *clpQ*$^+$ (Fig. **7D**). In addition, the stability of the wild-type *clpQ*$^+$ and *ΔIR-clpQ*$^+$ mRNAs was measured at different time points after the addition of rifampin to the culture media to block the re-initiation of transcription. Results show that the message of the wild-type *clpQ*$^+$ is much more stable than that of the *ΔIR-clpQ*$^+$ (Fig. **7E**).

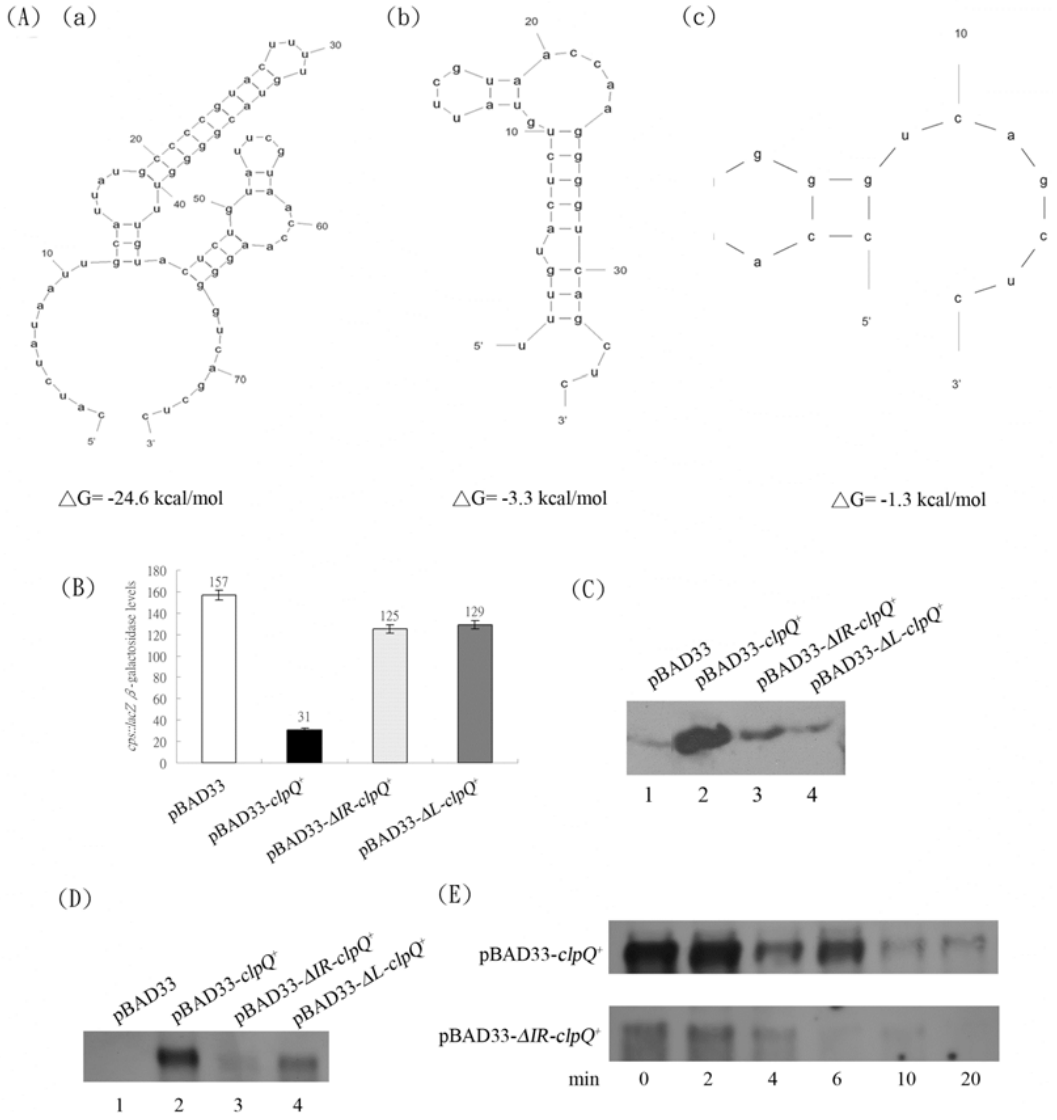


Fig. (7). (**A**) The secondary structures of the 5' UTR region of the mRNA from (**a**) *clpQ*$^+$ with ΔG = -24.6 Kcal/mole (**b**) *ΔIR-clpQ*$^+$ with ΔG = -3.3 Kcal/mole and (**c**) *ΔL-clpQ*$^+$ with ΔG = -1.3 Kcal/mole. (**B**) The βGal of *cpsB::lacZ* in the presence of pBAD33, pBAD33-*clpQ*$^+$, pBAD33-*ΔIR-clpQ*$^+$, or pBAD33-*ΔL-clpQ*$^+$, while ClpY was induced. (**C**) The western blot of ClpQ, expressed, in series, from the above plasmids. The loading was normalized by the readings of OD_{600}. (**D**) The mRNA of *clpQ*$^+$, which individually expressed from the above plasmids. The growth condition was as indicated earlier for the addition of antibiotics and 0.5 % arabinose, and the loadings were normalized by 16S rRNA (data not shown). (**E**) The decay of mRNAs of *clpQ*$^+$ and *ΔIR-clpQ*$^+$; the remaining amount of mRNA at different time points, after an addition of rifampin (at a final concentration, 200 μg/ml).

The Stem-loop Secondary Structure (IR) of 5'UTR of *clpQ⁺Y⁺* Responsible for its mRNA Stability

To further verify that a stem-loop structure at the 5' end of the leader sequences is responsible for the stability of *clpQ*⁺ mRNA, the substituted nucleotides were placed at the 5'UTR with or without a disruption of its stable secondary structure. As described earlier, the two new plasmids, pBAD33-*(S-IR)-clpQ*⁺ and pBAD33-*(R-IR)-clpQ*⁺, were constructed. In these constructs, the *(S-IR)-clpQ*⁺ gene has replaced the nucleotides on the 5' end of UTR, with a ΔG= -10.1 kal/mol, that destabilized a stem-loop structure (Fig. **8A-a**); in contrast, the pBAD33-*(R-IR)-clpQ*⁺ gene has replaced the nucleotides on the 5' end of UTR, with a lower ΔG= -24.6 kal/mol, that stabilized a stem-loop structure. AC3112 cells, which carry pBAD24-*clpY*⁺ and pBAD33-*(S-IR)-clpQ*⁺, show a higher βGal activity, as compared to that of cells carrying pBAD24-*clpY*⁺and pBAD33-*clpQ*⁺ (Fig. **8B**). In contrast, cells that carry pBAD24-*clpY*⁺and pBAD33-*(R-IR)-clpQ*⁺ show the decreased expression of *cpsB::lacZ* (Fig. **8B**). AC3112, which carries pBAD33-*ΔIR-clpQ*⁺ and pBAD24-*clpY*⁺, has higher βGal activity of the *cpsB::lacZ* expression as that of cells with pBAD33 and pBAD24 (Fig. **8B**). In the northern blot analysis, the level of *(S-IR)-clpQ*⁺ message was much lower than that of the *clpQ*⁺ and was more similar to that of the *ΔIR-clpQ*⁺; in contrast, the message of *(R-IR)-clpQ*⁺ was similar to that of the wild-type *clpQ*⁺ (Fig. **8C**).

DISCUSSION

Using a *clpQ⁺::lacZ*, a gene fusion, the results presented here show that *clpQ⁺::lacZ* has similar βGal activity in constructs containing either a long or a short region of the upstream sequence of the *clpQ⁺Y⁺* promoter. We have shown that the further sequence upstream of the *clpQ⁺Y⁺* promoter has no significant effects on its gene expression. Yet, as usual, a higher βGal level was detected with the *clpQ⁺::lacZ⁺* transcriptional fusion as compared to the translational fusion, and it is accordingly due to a stronger SD region of the LacZ⁺ itself in the transcriptional gene fusion [22].

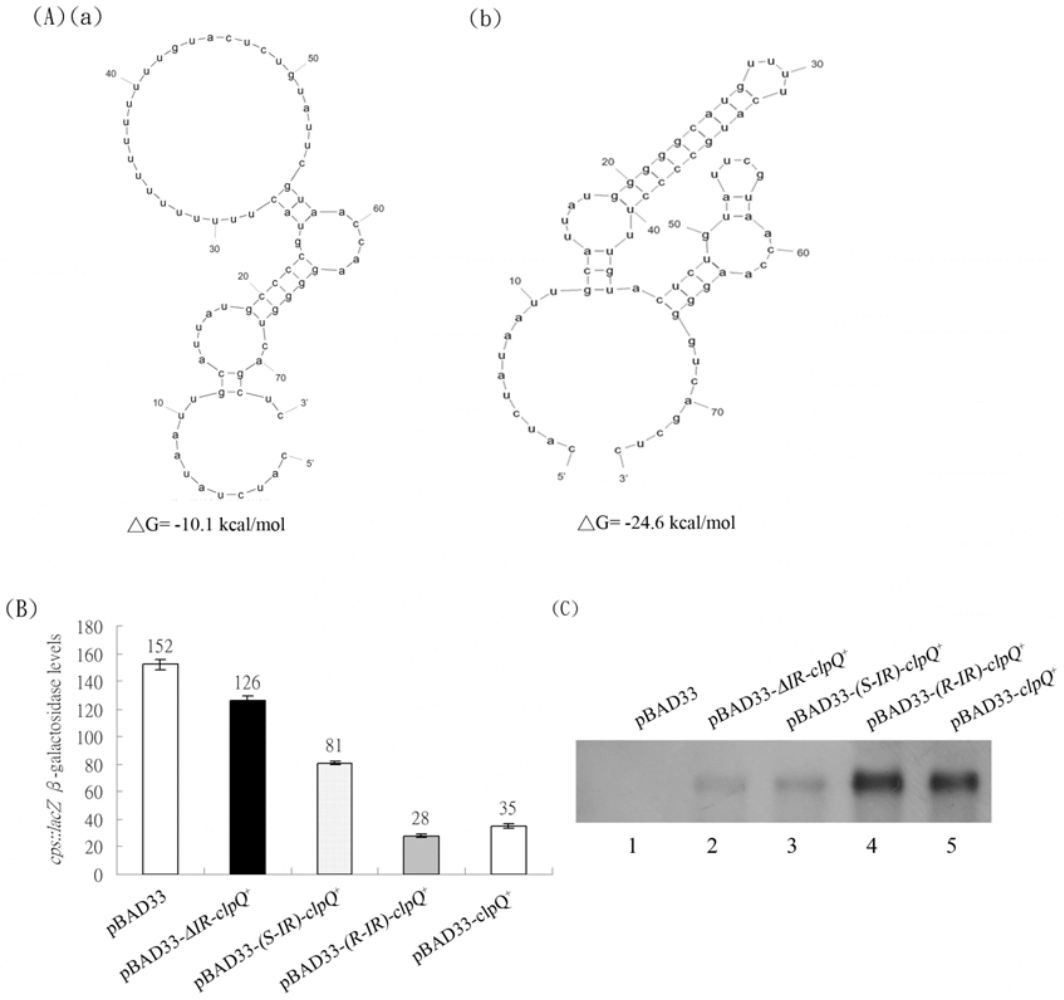


Fig. (8). (**A**) The secondary structures of 5'-UTR in (**a**) (*S-IR*)-*clpQ*⁺ with ΔG = -10.1 kcal/mol and (**b**) (*R-IR*)-*clpQ*⁺ with ΔG = -24.6 kcal/mol. (**B**) The βGal activities of *cpsB::lacZ*, in the presence of ClpY, the ClpQ was induced from pBAD33-(*S-IR*)-*clpQ*⁺ or pBAD33-(*R-IR*)-*clpQ*⁺, as compared to the control pBAD33-*clpQ*⁺and pBAD33-*ΔIR-clpQ*⁺. (**C**) The mRNAs of *clpQ*⁺ from the pBAD33-*(S-IR)-clpQ*⁺ and pBAD33-*(R-IR)-clpQ*⁺ as compared to those from the pBAD33-*clpQ*⁺and pBAD33-*ΔIR-clpQ*⁺.

Coincidentally, *clpQ*$^+$*Y*$^+$ and *clpQ*$^+$*::lacZ* (op or pr fusion) genes have increased expression when the temperature is up-shifted from 30 ^{o}C to 42 ^{o}C. Little of *clpQ*$^+$*Y*$^+$ or *clpQ*$^+$*::lacZ*$^+$ mRNA was observed in the *rpoH* mutant. In addition, a *clpQ*^{m1}*::lacZ*$^+$, with a C to T mutation in the RpoH -10 consensus sequences, was shown to have decreased βGal activity, independent of a functional RpoH; there was also no heat shock induction in *clpQ*^{m1}*::lacZ* (op or pr fusion). Using the transcriptional start site as a primer initiating site in the RT-PCR, the end-product for the transcripts of *clpQ*^{m1}*::lacZ*$^+$, was less accumulated as compared to those of the wild-type *clpQ*$^+$*::lacZ*$^+$ (data not shown). However, no signal was detected for the transcripts of both fusion genes, while using an annealing primer complementary to the nucleotides upstream of the transcriptional start site (data not shown). These results indirectly implied that both *clpQ*$^+$*::lacZ*$^+$ and *clpQ*^{m1}*::lacZ*$^+$ should have the identical transcriptional start site. In addition, we also made an additional mutation in the *clpQ*$^+$*Y*$^+$ promoter region at the possible -10 site for the RpoS binding [see in the (Fig. **1**)], and this mutation has no effects on an activity of the *clpQ*$^+$*Y*$^+$ promoter (data not shown). Therefore, we have experimentally shown that *clpQ*$^+$*Y*$^+$ gene expression is mediated by the RpoH factor.

The deletion of the IR or UTR in the *ΔIR-clpQ*$^+$, *ΔL-clpQ*$^+$ and the *clpQ*^{m2}*Δ40::lacZ* gene fusion leads to decreased *clpQ*$^+$ mRNA. In addition, the mRNAs of *ΔIR-clpQ*$^+$ mutants, without a stem-loop region at the 5'-UTR, decayed much faster. However, no significant sequences were observed in the 5' UTR of the *clpQ*$^+$*Y*$^+$ messages acting as a signal for targeting by the RNase E degradosome (an AU rich or A/GAUU/AU) [28, 29] or the RNase III (A/UNAGA/UG) [30]. We also found that a functional ClpQ is not affected by a higher temperature (data not shown); thus, there is no thermo-sensitive segment in the *clpQ*$^+$ message. However, while obliterated the IR in the 5' UTR of *clpQ*$^+$*Y*$^+$ mRNA but retained its stem-loop structure, a stable mRNA was present. In *E. coli*, it has been reported that the 5' stem-loop structure stabilizes the mRNA of *papA* (pyelonephritis-associated pili gene) [31] and *ompA* (outer membrane protein) [32, 33] under physiological conditions, and it was later found that *ompA* is being regulated by the small *micA* RNA [34]. Currently, a model is emerging of small RNA sequences that are involved in gene regulation [35]. In some cases, an Hfq (a host factor for RNA phage Qβ phage replication) [36], RNA-binding protein is also involved in it [35]. It stabilizes sRNAs against decay and also helps an association with their mRNA target [35]. However, no such effects were found for the *clpQ*$^+$*::lacZ*$^+$ fusion gene expression (our own data). Besides, in the wild bacterial genome, the 5' UTR was also found in the *clpQ*$^+$*Y*$^+$ mRNA transcripts mainly in the *Enterobacteriaceae* family. These bacteria all have the conserved sequences of the 5' UTR of *clpQ*$^+$*Y*$^+$ mRNA for a predicted secondary stem-loop structure. However, via the bioinformatics, the conservation of sequence of the small RNA is not sufficient for a searching of a regulation of the target gene; a more sophisticated algorithm is needed [37]. It is noteworthy that this is the first example in the ATP-dependent proteases to demonstrate that the 5' stem-loop structure itself participates in the stability of its downstream mRNA. It is of interest to determine whether this stem-loop structure in the 5'UTR plays a role for its gene expression under certain physiological condition and also whether there is a regulation of *clpQ*$^+$*Y*$^+$ by the other regulatory proteins or via the small RNA.

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ABBREVIATIONS

UTR = Untranslated leader region

op = Transcriptional

pr = Translational

RBS = Ribosome-binding site

wt = Wild-type

βGal = β-galactosidase

nt = Nucleotide(s)

bp = Base pair(s)

kb = 1000bp

LB = Luria-Bertani

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