http://dx.doi.org/10.5012/bkcs.2013.34.8.2419

Cloning, Over-expression, and Characterization of YjgA, a Novel ppGpp-binding Protein

Gopalsamy Gnanasekaran,[†] SangO Pan,[‡] Wontae Jung,[§] Kwangjoon Jeong,[†] Jae-Ho Jeong,[#] Joon Haeng Rhee,[†] Hyon E. Choy,[#] and Che-Hun Jung[†,§,*]

[†]Department of Molecular Medicine, Clinical Vaccine R&D Center, Chonnam National University, Hwasun 519-809, Korea
[‡]Jeollanamdo Institute of Natural Resources Research (JINR), Jangheung 529-852, Korea
[§]Department of Chemistry, Chonnam National University, Gwanjgu 500-757, Korea. [*]E-mail: jungch@chonnam.ac.kr
[#]Department of Microbiology, Chonnam National University Medical School, Kwangju 501-746, Korea
Received May 3, 2013, Accepted May 20, 2013

Guanosine-5'-diphosphate 3'-diphosphate (ppGpp) serves as alarmone in bacterial stringent responses. In this study, an affinity column was constructed by immobilizing ppGpp to NHS-Sepharose for isolating ppGpp-binding proteins. A novel ppGpp-binding protein, YjgA, was isolated and characterized by MALDI-TOF MS (matrix-assisted laser desorption ionization-time-of-flight mass spectrometry) coupled with two-dimensional gel electrophoresis. YjgA and truncated forms of YjgA were cloned and over-expressed in BL21 (DE3). The binding affinity of YjgA to ppGpp was determined by equilibrium dialysis. The interaction of YjgA with ppGpp was very specific, considering that the dissociation constant of YjgA with ppGpp was measured as 5.2 ± 2.0 μM, while the affinities to GTP and GDP were about 60 and 30 times weaker than ppGpp. Expression of *yjgA* gene in *Escherichia coli* K-12 MG1655 was examined by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR results revealed that *yjgA* was expressed from early to late stationary phase. The *yjgA* deletion mutant exhibited decreased cell number at stationary phase compared to parent strain and the over-expression of YjgA increased the cell number. These results suggested that YjgA might stimulate cell division under stationary phase. In most prokaryotic genome, about half of the protein candidates are hypothetical, that are expected to be expressed but there is no experimental report on their functions. The approach utilized in this study may serve as an effective mean to probe the functions of hypothetical proteins.

Key Words : YjgA, ppGpp, Stringent responses, Stationary phase, Hypothetical protein

Introduction

Prokaryotes show stringent response to various nutritional changes, including amino acids (Haseltine and Block, 1973), carbon (Flardh *et al.*, 1994), nitrogen (Silberbach *et al.*, 2005) and phosphate (Bougdour and Gottesman, 2007) limitation. Cashel and Gallant first discovered guanosine-5'-diphosphate 3'-diphosphate (ppGpp) accumulation in bacteria during nutrient starvation (Cashel and Gallant, 1969), and RelA and SpoT serve as ppGpp synthetases in *E. coli* (Cashel *et al.*, 1996). ppGpp binds to the β and β' subunits of core RNA polymerase, inhibits stable RNA biosynthesis (rRNA and tRNA) during growth inhibition (Ryals *et al.*, 1982; Toulokhonov *et al.*, 2001), and arrests cell cycles (Ferullo and Lovett, 2008). A complete absence of ppGpp creates its own phenotypic features including multiple amino acid requirements, poor survival of aged culture, aberrant cell division, morphology, and immotility (Potrykus and Cashel, 2008). Importantly, ppGpp influences the pattern of overall gene expression that requires for survival in stress conditions (Song *et al.*, 2004). In *E. coli*, ppGpp acts as a global regulatory affects on gene expression through its influence on the levels of global regulators such as IHF, RpoS and RpoH (Lange *et al.*, 2005). The absence of ppGpp impairs or severely delays the accumulation of RpoS, a stress response sigma factor (Gentry *et al.*, 1993). In addition to transcription, ppGpp regulates translation by interacting with EF-G and IF2 (Mitkevich *et al.*, 2010). ppGpp is involved in the acid stress response *via* lysine decarboxylase Ldc1 (Kanjee *et al.*, 2011), phosphate metabolism *via* polynucleotide phosphorylase (Gatewood and Jones, 2010), and replication *via* DnaG primase (Maciag *et al.*, 2010). With such pleotropic eects, ppGpp seems to be a major regulator of bacterial growth rate (Potrykus *et al.*, 2011). In spite of the diverse functions connected to ppGpp, the mechanism of ppGpp regulation remains controversial (Vrentas *et al.*, 2008).

The first genome structure was announced in 1995 and more than 1,000 genome sequences are currently available in public domain (Karin *et al.*, 2010). Although the whole genome provides rich information on metabolic pathways and their regulations, about a half of proteins in most genomes are annotated as hypothetical proteins (Bhatia *et al.*, 1997). Hypothetical proteins, also referred as 'putative', 'uncharacterized' or 'unknown' proteins, are those whose existence can be predicted but no experimental reports on their functions are available. Even for *Escherichia coli* K-12, the most studied organism, there are still about 1,500 genes that have not been experimentally categorized (Koonin and Galperin, 2003). The functions of hypothetic proteins are

probed by an affinity proteomics approach (Park *et al.*, 2012; Choi *et al.*, 2008; Park *et al.*, 2008). In which, affinity columns are utilized to isolate proteins with specificity and the functions of hypothetical proteins are further studied by single crystal structure studies.

YjgA is a hypothetical protein. It has been reported that YjgA is present in a 50S ribosomal preparation (Jiang *et al.*, 2006). In which, YjgA was identified as a component of the ribosomal fraction, but it was not even clear that YjgA was a ribosome-binding protein. No other characterization has been reported. In this study, a novel ppGpp-binding protein, YjgA, identified by MALDI-TOF MS, was cloned, over-expressed, and characterized. YigA shows a high affinity to ppGpp. The *yjgA* deletion and over-expressing mutants were constructed for investigating the role and phenotype. This study provides a novel ppGpp-binding protein and an effective mean to study the functions of hypothetical proteins.

Materials and Methods

Bacterial Strains and Media. The bacterial strains and plasmids used in this work are listed in Table 1. Oligonucleotide primers for *yjgA*, truncated *yjgA*, deletion mutant *yjgA*, and RT-PCR are listed in Table 2. Bacteria were grown with constant agitation at 37 °C in Luria Bertani (LB) medium, VB minimal medium supplemented with 0.2% glucose (Vogel and Bonner, 1956), and phosphate rich medium (5.6 g/L KH_2PO_4, 37.8 g/L K_2HPO_4, 10 g/L yeast extract, 15 mg/L thiamine, after autoclaving filtered 50 mL of 40% (w/v) glucose and 10 mL of 0.1 M magnesium acetate were added). When required, 100 μg/mL ampicillin (Amp), 50 μg/mL kanamycin (Kn), 30 μg/mL chloramphenicol (Cm), 0.5 mM Isoprophyl-β-D-thiogalactoside (IPTG) were added. Solid media were prepared by adding 1.5% agar to LB broth.

Preparation of ppGpp. Ribosomes were isolated from *Escherichia coli* (Spedding, 1990). The ribosome concentration was determined by measuring the absorbance at 260 nm. Guanosine tetra-phosphate (ppGpp) was synthesized in a reaction containing 50 mM Tris acetate (pH 8.0), 15 mM magnesium acetate, 60 mM potassium acetate, 27 mM ammonium acetate, 1 mM DTT, 0.2 mM EDTA, 4 mM ATP, 2 mM GDP and 50 units of ribosome (Krohn and Wagner, 1995). ppGpp was purified by DEAE-Sephadex column (2.5 × 20 cm) pre-equilibrated with 50 mM Tris-HCl, pH 7.4 containing 0.1 M LiCl. After applying ppGpp reaction

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characters	Source or reference
Strains		
Escherichia coli K-12	MG1655	ATCC
ppGpp°	Δ*relA* Δ*spoT* Cm^R Kn^R	Laboratory collection
BL21 (DE3)	hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1)	Laboratory collection
DH5α	F^- *recA*1 restriction negative	Laboratory collection
Plasmids		
pET21a (+)	Ori-pBR322, Amp^R, N-terminal His-tag	Novagen
pKD3	bla FRT Cm FRT Ori-R6K	Datsenko & Wanner
pKD4	bla FRT Kn FRT Ori-R6K	Datsenko & Wanner
Red Helper plasmids (pKD46)	bla PBAD gam bet exo pSC101 Ori-TS	Datsenko & Wanner

Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Kn^R, kanamycin resistance; Tc^R, tetracycline resistance.

Table 2. Primers for *yjgA*, truncated *yjgA* and deletion *yjgA*

Protein	Primer	Sequence 5' → 3'
YjgA	YjgA (R)	ATA***CTCGAG***CCCTTCGTTCTCCGCTAACTCG
	YjgA (F)	ACG***CATATG***ACTAAGCAGCCCGAAGACT
YjgA15	YjgA15 (F)	TCG***CATATG***GACATCGAAGACGAAGACGATG
	YjgA15 (R)	ATA***CTCGAG***CCCTTCGTTCTCCGCTAACTCG
YjgA20	YjgA20 (F)	CGC***CATATG***GACGATGAAATTATCTGGGTCAG
	YjgA20 (R)	ATA***CTCGAG***CCCTTCGTTCTCCGCTAACTCG
ΔYjgA	YjgA::Kn (F)	TATCAGGCCTACAGGTGCACCGTATCCGGCAAACCATACTCA
		CATCAACAACGAAAATTATG***TGTAGGCTGGAGCTGCTTC***
	YjgA::Cm (R)	GTTTTTCAGTGCGCATCTTAACTGGTAACATTAGCCTCTTTTTTT
		AAGGAGCCTGAGATGC***ATATGAATATCCTCCTTA***
RT-PCR	YjgA (F)	GGCGTTTTAGCTCCTCGGCATC
	YjgA (R)	CAGCGCGACGTAGAGCCTATTC
	16s rRNA (F)	AGTCAGATGTGAAATCCCCGGG
	16s rRNA (R)	TCAAGGGCACAACCTCCAAGTC

mixture, the column was washed with 10 column volume of the equilibrium buffer, and ppGpp was eluted with a gradient (10 column volume with buffer B: 50 mM Tris-HCl, pH 7.4, 0.5 M LiCl) at the flow rate of 0.5 mL/min. ppGpp was detected by UV absorbance at 252 nm. The purity and quantity of ppGpp was further confirmed by an HPLC (The HPLC conditions are described in the binding study part below.).

Identification of ppGpp-Binding Proteins. A ppGpp affinity column was prepared by immobilizing ppGpp on *N*-hydroxysuccinimidyl-Sepharose (Sigma Co.), following the manufacturer's instructions. The column (1 × 5 cm) was equilibrated with binding buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$). The *E. coli* cells were resuspended in 5 volumes of lysis buffer (20 mM Tris-HCl buffer, pH 7.5 and 1 mM PMSF), and ruptured by sonication (Vibra-cell sonicator, SONICS). The supernatant was separated from the pellets by centrifugation at 12,000 g for 30 min at 4 °C. The remaining pellet was treated with lysis buffer containing 1% Brij-35 and the solubilised proteins were isolated by centrifugation as above. The protein fractions were applied to ppGpp column and the unbound proteins were washed with 10 column volume of binding buffer. The bound proteins were eluted with 20 mM Tris-HCl, pH 7.5 containing 10 mM $MgCl_2$ and 1 M NaCl. The ppGpp-binding proteins were then resolved on two-dimensional gel electrophoresis (O'Farrell, 1975) and the individual protein spots were analyzed by MALDI-TOF MS (Jensen *et al.*, 1997). The ppGpp-binding proteins were also identified by MudPIT method (Washbum *et al.*, 2001). The same procedures were repeated with GDP affinity column as a control.

Cloning and Over-Expression of YjgA, a ppGpp-Binding Hypothetical Protein.

Primer Design and Cloning: The oligonucleotide primers for *yjgA* and truncated *yjgA* (*yjgA*15, the first 15 amino acids removed from *yjgA*; *yjgA*20, the first 20 amino acids removed from *yjgA*) were designed as in Table 2. The *yjgA* and truncated *yjgA* genes were amplified from genomic DNA of *E. coli* K-12 MG1655 by polymerase chain reaction (PCR). The plasmid pET 21a (+) and amplified products were digested by restriction endonuclease *Nde* I and *Xho* I and carried out ligation. The plasmid constructs were transferred to competent cells of DH5α *E. coli*. The plasmids were transferred to *E. coli* expression strains BL21 (DE3) for over-production (Ausubel *et al.*, 1998). The cloned *yjgA* and truncated *yjgA* genes were verified by sequence analysis.

Isolation of YjgA and Truncated YjgAs: The transformed BL21 (DE3) was grown with shaking at 200 rpm in 1 L LB medium containing 100 μg/mL ampicillin at 37 °C until OD_{600} reaches 0.5-0.7. The cultures were induced with 0.5 mM IPTG and further incubated for 4 h at 37 °C. The cells were harvested by centrifugation at 8600 g for 20 min, resuspended in lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA with freshly added 1 mM PMSF and 1 mM DTT), and disrupted by sonication. The suspension was centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was applied to the Ni-NTA column pre-equilibrated with 20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0. The unbound proteins were removed with 10 column volume of the same buffer. YjgAs' were eluted with a linear gradient using elution buffer (20 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). The buffer was changed by HiPrep 26/10 desalting column equilibrated with 50 mM Tris-HCl, pH 7.5. YjgAs' were further purified by Mono Q HR 10/10 ion exchange column using a linear gradient with 50 mM Tris-HCl, pH 7.5 and 50 mM Tris-HCl, 1 M NaCl, pH 7.5.

Binding Study of Nucleotides with YjgA and Truncated YjgA. The binding affinities of YjgA and truncated YjgA with ppGpp, guanosine-5'-triphosphate (GTP) and guanosine diphosphate (GDP) were determined by equilibrium dialysis by using micro-equilibrium dialyzer (Harvard Bioscience) as manufacturer's instruction. The concentrations of ppGpp, GTP and GDP were determined by HPLC (Shimadzu class VP, Japan) using an amino column (Phenomenex). The nucleotides were eluted at a flow rate of 0.5 mL/min in gradient mode that made up of low ionic (20 mM KH_2PO_4, 5% ACN, pH 7.5) and high ionic (500 mM KH_2PO4, 5% ACN, pH 7.5) buffers.

Construction of *yjgA* Deletion Mutant. Target specific gene deletion was accomplished by Datsenko and Wanner. The primer was designed with an internal overlap with the resistance marker (pKD3 or pKD4) and external overlap with the target knockout (*yjgA*) gene (Table 2). PCR amplification was performed by using pKD3 (cat) and pKD4 (kan) as the template with forward and reverse primer. The PCR products were purified and transformed into electrocompetent cells of *E. coli* K-12 MG1655 carrying pKD46 (Red helper plasmid) by electroporation. The mutants were confirmed by PCR using upstream yjgA forward primer (ATACTCGAGCCCT TCGTTCTCCGCTAACTCG) and common test primers of chloramphenicol reverse (GATCTTCCGTCACAGGTAGG) and kanamycin reverse (CGGCCACAGTCGATGAATCC) primers.

Quantitative Expression of *yjgA*. Expression of y*jgA* in *E. coli* K-12 MG1655 and the mutant *E. coli* lacking ppGpp synthesis (ppGpp°) was analysed using reverse transcription polymerase chain reaction (RT-PCR), following Qiagen's instructions. Gene-specific primers for *yjgA* (190 bps) and 16S rRNA (250 bps) used in this study were listed in Table 2. Expression of y*jgA* in serine hydroxamate (Ser-HX) and α-methylglucoside (α-MG) treated *E. coli* was also determined. The wild type *E. coli* cells were grown in LB medium at 37 °C until OD_{600} reaches 0.5 and separately treated with 2.5% α-MG and 2 mM Ser-HX and monitored growth for 24 h. The total RNA was extracted (Qiagen RNeasy mini kit) at various time points and *yjgA* expression was quantified.

Results

Preparation of ppGpp Column and Identification of ppGpp-Binding Proteins. ppGpp was synthesized from 4 mM ATP, 2 mM GDP and isolated ribosomes. It was then

Table 3. Binding affinities of YjgA

Protein	Ligand	Dissociation constant (Kd)
YjgA	ppGpp	5.2 ± 2.0 μM
	GTP	300 ± 280 μM
	GDP	180 ± 150 μM
YjgA20	ppGpp	7.6 ± 2.4 μM

purified on DEAE-Sephadex column by a linear gradient of LiCl and the purity was confirmed by HPLC. The ppGpp and GDP affinity columns were constructed and ppGpp- and GDP-binding proteins were identified by proteomics approaches (for the list of proteins exclusively bound to the ppGpp column, manuscript in preparation).

Cloning and Purification of YjgA and Truncated YjgAs. The recombinant 6His-tagged YjgA proteins were over-expressed and isolated by Ni-NTA column and MonoQ ion exchange chromatography (data not shown). The protein concentration was measured at 280 nm using molar extinction coefficients of YjgA (17,990), YjgA15 (12,490) and YjgA20 (12,490), respectively.

Binding Study. The binding affinity of YjgA to ppGpp, GTP and GDP were studied by equilibrium dialysis. The nucleotides were analyzed by HPLC with an amino (NH_2) column. ppGpp, GTP and GDP were eluted at 36.4 min, 26.6 min and 21.3 min, respectively. As shown in Table 3, the dissociation constant of YjgA with ppGpp was measured as 5.2 ± 2.0 μM. The interactions with GTP and GDP were 60 and 30 times weaker than with ppGpp, which indicated the interaction of YjgA with ppGpp was specific. YjgA20 also showed the similar dissociation constant like wild type YjgA, which suggested that the first 20 N-terminal amino acids had a minimal role on ppGpp binding. Initial effort to grow a single crystal of YjgA had failed. Molecular modelling of YjgA suggested that the N-terminal amino acid might not have a solid conformation and thereby hindered crystal growth. An effort to solve the crystal structure to elucidate the ppGpp-binding site is in progress using the truncated forms of YjgA.

Effects of *yjgA* Deletion on Vegetative Growth. In order to elucidate cellular functions of YjgA, a deletion mutant for *yjgA* gene was constructed. The growths of the wild-type K12 strain and the deletion mutant were measured in LB broth. Both strains showed the similar growth until early stationary phase and the growth of the deletion mutant decreased at the stationary phase (Fig. 1). The growth impairment was quite significant, considering that the cell number for the wild type at the stationary phase was more than 10 times higher than the deletion mutant (Fig. 1(b)). The cell growth was also measured in VB minimal media and the mutant strain showed an impaired growth as in LB broth (data not shown). This suggested that YjgA increased cell proliferation at the stationary phase.

Effects of *yjgA* Over-Expression on Cell Growth. In order to cross-examine the role of YjgA in cell proliferation, a plasmid based mutant was created, by introducing plasmid

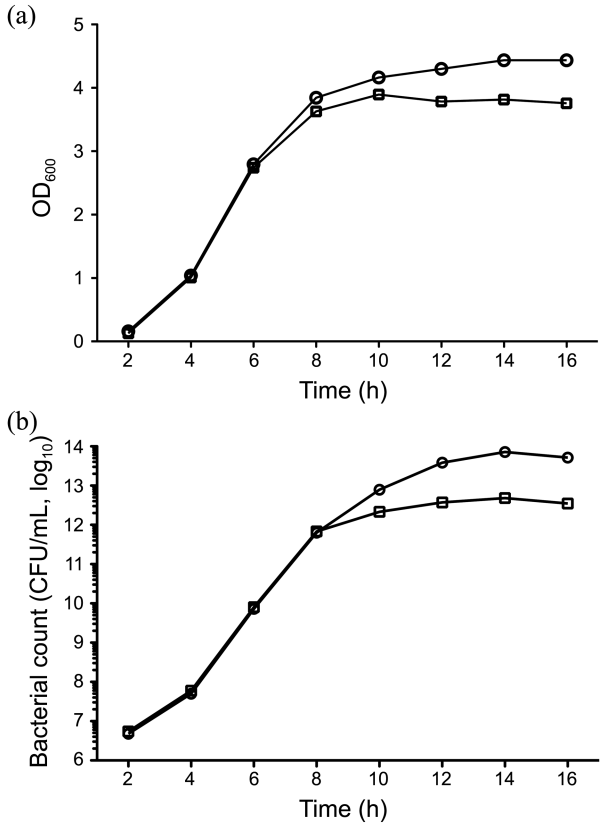

Figure 1. yjgA deletion mutant strain showed retarded growth at stationary phase. The cells were grown in LB at 37 °C. Bacterial growth was compared by measuring OD_{600} (A) and by total viable cell counts (B) between Δ*yjgA* (□) and its parental *E. coli* K-12 MG1655 (●) wild type strain. For the dense cultures with high obsorbance, the OD_{600} was measured after proper dilutions.

pET 21a carrying *yjgA* gene to wild type K12 strain. The cells were induced with 0.2 mM IPTG at various time points for YjgA expression and the cell growths were compared. Over-expression of YjgA resulted in increased proliferation of *E. coli* compared to the K12 control (Fig. 2). The strain over-expressing YjgA showed about 50 times higher number of cells (2.8×10^8 CFU/mL when induced at OD_{600} ~0.6) compared to the control (4.5×10^6 CFU/mL) at 14 h, corresponding to the observation of OD_{600}. IPTG itself hampered *E. coli* growth significantly and earlier induction by IPTG resulted in lower number of cells, as compared in Fig. 2(a) and 2(b). As a result, the growth difference became less significant when the strains were induced at 0 h (data not shown). Together with the knockout experiment, the function of YjgA is certainly to increase cell numbers.

***yjgA* Expression at the Stationary Phase.** The *yjgA* expression was analyzed using RT-PCR to understand the relationship between ppGpp synthesis and *yjgA* expression. The growth curves of the wild type *E. coli* in LB was determined by OD_{600} (Fig. 3(a)) and the *yjgA* gene expression was observed after 4 h (Fig. 3(b)), which clearly showed that *yjgA* was expressed at early stationary phase. To investigate the role of ppGpp in *yjgA* expression, the wild type strain was treated separately with α-MG and Ser-HX at OD_{600} ~0.5

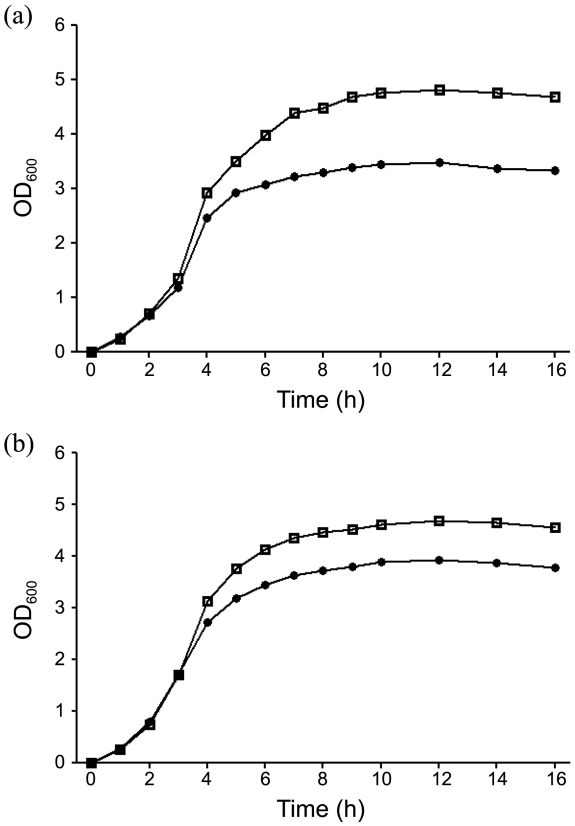

Figure 2. YjgA overexpressed cells showed better growth in stationary phase. The cells were induced with IPTG at OD_{600} ~0.6 (a) and 3 h (b). Bacterial growths of *E. coli* K-12 MG1655 (●) and *yjgA*::pET transformed *E. coli* (□) were compared.

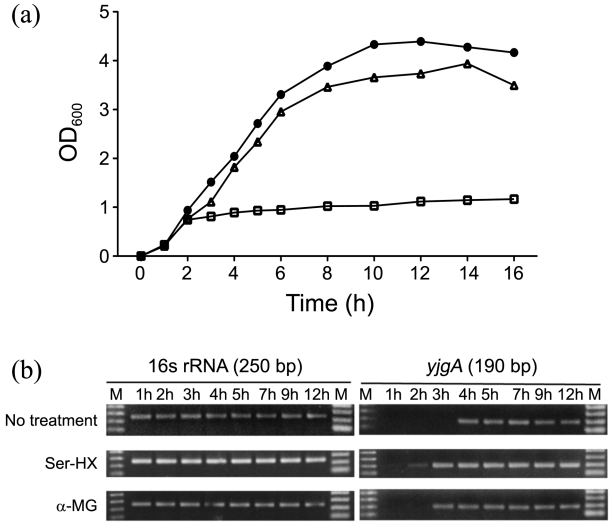

Figure 3. *yjgA* expression under conditions inducing intracellular ppGpp synthesis. Bacterial growth (a) was examined after adding Ser-HX (□) and α-MG (△) OD_{600} ~0.5 (about 1.5 h) and compared with mock (●) treatment. The *yjgA* transcription (b) was measured as described in Materials and method.

(about 1.5 h). α-MG and Ser-HX were used to elicit the ppGpp synthesis in *E. coli*. Ser-HX immediately arrested cell growth, while α-MG slowed down the cell growth

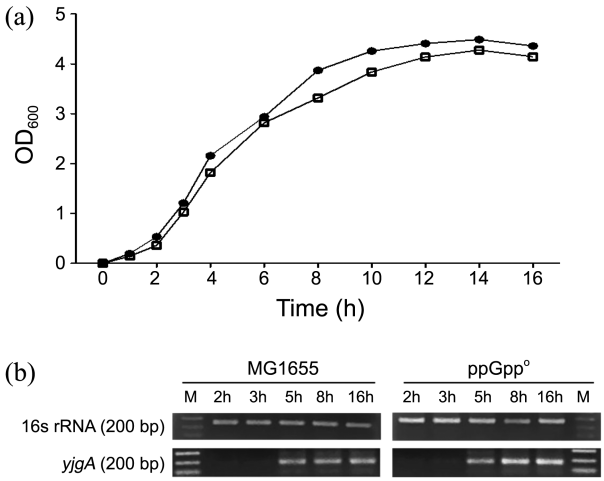

Figure 4. Bacterial growth curve of *E. coli* K-12 MG1655 (●) and ppGpp° (□) strains (a) and *yjgA* expression at various time points (b). The *yjgA* transcription was measured by RT-PCR as described in Materials and method. 16S rRNA was used as an internal control.

slightly (Fig. 3(a)). The Ser-HX- and α-MG-treated cells expressed *yjgA* immediately at OD_{600} ~0.75 (2 h) and ~1.08 (3 h), respectively (Fig. 3(b)) while the wild-type *E. coli* cells expressed *yjgA* at OD_{600} ~1.75 (4 h). This shift of gene expression suggested that the energy deficiency (by α-MG) and amino acid starvation (by Ser-HX) caused *yjgA* expression. In order to further examine the role of ppGpp in yjgA expression, The *yjgA* expression was also examined with a ppGpp negative strain, lack of RelA and SpoT responsible for ppGpp synthesis (Fig. 4(b)). Surprisingly, the *yjgA* expression was intact in ppGpp negative mutant compared to wild type strain. This suggested that the control of *yjgA* expression was independent of ppGpp. The yjgA expression in catabolite response protein (CRP)-negative mutant strain was examined, and the gene expression was also independent to CRP (data not shown).

Discussion

In this study, a ppGpp affinity column was constructed and ppGpp-binding proteins were isolated and characterized by MALDI-TOF MS and MudPIT method. Among the identified proteins, a hypothetical protein, YjgA, was further characterized. YjgA specifically bound to ppGpp compared to GDP and GTP. Therefore, affinity proteomics approaches seemed to be effective for probing the function of hypothetical proteins. Considering that about a half of the deduced genes from genome studies were hypothetical, affinity proteomics might be important for studying the functions of the hypothetical proteins.

In addition to a tight binding of YjgA with ppGpp, the *yjgA* deletion mutant exhibits significant growth defect at stationary phase compared to parent strain. On the contrary, the over-expression of YjgA stimulated the cell growth. Therefore, the cellular function of YjgA seemed to be related with cell division especially at stationary phase. The

mechanism how cell growth was sustained or stimulated at stationary phase by YjgA required further studies. ppGpp is synthesized at stationary phase and the link between ppGpp binding to YjgA and the cellular function of YjgA as an activator for cell proliferation also requires further attentions.

RT-PCR results reveal that *yjgA* is exclusively expressed from the early stationary phase. Although the ppGpp-inducing agents, α-MG and Ser-HX, trigger *yjgA* expression, a ppGpp negative strain also expresses *yjgA* gene in a growth specific manner just like wild type strain. Therefore, *yjgA* expression seems to be regulated independently to ppGpp. ppGpp expression was also independent to CRP. The study on the regulation of *yjgA* expression may lead us a novel regulatory mechanism for gene expression at stationary phase.

This is the first report ever describing properties of a hypothetical protein, YjgA. That is, YjgA specifically binds with ppGpp, YjgA is a stationary phase protein, and YjgA increases cell number at stationary phase. When nutrients are exhausted, that condition leads to growth retardation, extensive reprogramming of physiology, and the essential gene expression for cell survival in stationary phase (Chang *et al.*, 2002). The regulation of *yjgA* gene expression, however, seems to be ppGpp independent. The mechanism how cell growth is stimulated or maintained by YjgA, together with the regulation mechanism for *yjgA* expression, will be important for further studies.

Acknowledgments. This research was supported by National Research Foundation of Korea (NRF) (2009-0073887, 2009-0070099) and by Special Research Program, CNU (2009).

References

1. Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Current Protocols in Molecular Biology*; Chanda, V. B., Ed.; John Wiley and Sons Inc.: Boston, MA, 1998.
2. Bhatia, U.; Robison, K.; Gilbert, W. *Science* **1997**, *276*, 1724-1725.
3. Bougdour, A.; Gottesman, S. *Proc. Natl. Acad. Sci.* U. S. A. **2007**, *104*, 12896-12901.
4. Cashel, M.; Gallant, J. *Nature* **1969**, *221*, 838-841.
5. Cashel, M.; Gentry, D. R.; Hernandez, V. J.; Vinella, D. In *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology; Neidhardt, F. C., Ingraham, J. C., Brooks Low, K., Magasanik, B., Schaechter, M., Umbarger, H. E., Eds.; American Society for Microbiology Press: Washington, DC., 1996; pp 1458-1496.
6. Chang, D. E.; Smalley, D. J.; Conway, T. *Molecular Microbiology* **2002**, *45*(2), 289-306.
7. Choi, J. W.; Lee, J.; Nishi, K.; Kim, Y. S.; Jung, C. H.; Kim, J. S. *J. Mol. Biol.* **2008**, *377*, 258-267.
8. Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci.* U. S. A. **2000**, *97*, 6640-6645.
9. Ferullo, D. J.; Lovett, S. T. *PLoS Genetics* **2008**, *4*, e1000300.
10. Flardh, K.; Axberg, T.; Albertson, N. H.; Kjelleberg, S. *J. Bacteriol.* **1994**, *176*, 5949-5957.
11. Gatewood, M. L.; Jones, G. H. *J. Bacteriol.* **2010**, *192*, 4275-4280.
12. Gentry, D. R.; Hernadez, V. J.; Nguyen, L. H.; Jensen, D. B.; Cashel, M. *J. Bacteriol.* **1993**, *175*, 7982-7989.
13. Haseltine, W.; Block, R. *Proc. Natl. Acad. Sci.* U. S. A. **1973**, *70*, 1564-1568.
14. Janssen, G. M. C.; Maassen, J. A.; Moller, W. In *Ribosomes and Protein Synthesis: A Practical Approach*; Spedding, G., ed.; IRL Press: Oxford, UK, 1990; pp 51-68.
15. Jensen, O. N.; Podtelejnikov, A. V.; Mann, M. *Anal. Chem.* **1997**, *69*, 4741-4750.
16. Jiang, M.; Datta, K.; Walker, A.; Strahler, J.; Bagamasbad, P.; Andrews, P. C.; Maddock, J. R. *J. Bacteriol.* **2006**, *188*, 6757-6770.
17. Kanjee, U.; Gutsche, I.; Ale xopoulos, E.; Zhao, B.; El Bakkouri, M.; Thibault, G.; Liu, K.; Ramachandran, S.; Snider, J.; Pai, E. F.; Houry, W. A. *EMBO J.* **2011**, *30*, 931-944.
18. Karin, L.; Dave, W. U.; Trudy, M. W. *Microbiology* **2010**, *156*, 603-608.
19. Koonin, E. V.; Galperin, M. Y. Sequence-Evolution-Function: Computational approaches in comparative genomics; *Kluwer Academic*, Boston, MA, 2003.
20. Krohn, M.; Wagner, R. *Anal. Biochem.* **1995**, *225*, 188-190.
21. Lange, R.; Fischer, D.; Hengge-Aronis, R. *J. Bacteriol.* **1995**, *177*, 4676-4680.
22. Maciag, M.; Kochanowska, M.; Lyzen, R.; Wegrzyn, G.; Szalewska-Palasz, A. *Plasmid* **1995**, *63*, 61-67.
23. Mitkevich, V. A.; Ermakov, A.; Kulikova, A. A.; Tankov, S.; Shyp, V.; Soosaar, A.; Tenson, T.; Makarov, A. A.; Ehrenberg, M.; Hauryliuk, V. *J. Mol. Biol.* **2010**, *402*, 838-846.
24. O'Farrell, P. H. *J. Biol. Chem.* **1975**, *250*, 4007-4021.
25. Park, H. M.; Park, J. H.; Choi, J. W.; Lee, J.; Kim, B. Y.; Jung, C. H.; Kim, J. S. *Acta Crystallogr. D. Biol. Crystallogr.* **2012**, *68*, 920-926.
26. Park, S. Y.; Lee, S. H.; Lee, J.; Nishi, K.; Kim, Y. S.; Jung, C. H.; Kim, J. S. *J. Mol. Biol.* **2008**, *376*, 1426-1437.
27. Potrykus, K.; Cashel, M. *Ann. Rev. Microbiol.* **2008**, *62*, 35-51.
28. Potrykus, K.; Murphy, H.; Philippe, N.; Cashel, M. *Environ. Microbiol.* **2011**, *13*, 563-575.
29. Ryals, J.; Little, R.; Bremer, H. *J. Bacteriol.* **1982**, *151*, 1261-1268.
30. Silberbach, M.; Huser, A.; Kalinowski, J.; Puhler, A.; Walter, B.; Kramer, R.; Burkovski, A. *J. Biotechnol.* **2005**, *119*, 357-367.
31. Song, M.; Kim, H. J.; Kim, E. Y.; Shin, M.; Lee, H. C.; Hong, Y.; Rhee, J. H.; Yoon, H.; Ryu, S.; Lim, S.; Choy, H. E. *J. Biol. Chem.* **2004**, *279*, 34183-34190.
32. Spedding G. Ribosomes and Protein Synthesis: A Practical Approach; Oxford: IRL Press; 1990.
33. Toulokhonov, I. I.; Shulgina, I.; Hernandez, V. J. *J. Biol. Chem.* **2001**, *276*, 1220-1225.
34. Vogel, H. J.; Bonner, D. M. *J. Biol. Chem.* **1956**, *218*, 97-106.
35. Vrentas, C. E.; Gaal, T.; Berkmen, M. B.; Rutherford, S. T.; Haugen, S. P.; Vassylyev, D. G.; Ross, W.; Gourse, R. L. *J. Mol. Biol.* **2008**, *377*, 551-564.
36. Washburn, M. P.; Wolters, D.; Yates, J. R., 3rd. *Nat. Biotechnol.* **2001**, *19*, 242-247.