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

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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 (Bougour 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; Touloukhonov 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 effects, 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 KH₂PO₄, 37.8 g/L K₂HPO₄, 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

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**Table 1. Bacterial strains and plasmids**

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<thead>
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<th>Strain or plasmid</th>
<th>Relevant characters</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<td>MG1655</td>
<td>ATCC</td>
</tr>
<tr>
<td>ppGpp</td>
<td>ΔrelA ΔspoT Cm⁺ Kn⁺</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>hsdS gal (λcl857 ind1 Sam7 nin5 lacUV5-T7 gene1)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α</td>
<td>F⁻ recA₁ restriction negative</td>
<td>Laboratory collection</td>
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<td><strong>Plasmids</strong></td>
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<td>pET21a (+)</td>
<td>Ori-pBR322, Amp⁺, N-terminal His-tag</td>
<td>Novagen</td>
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<td>pKD3</td>
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<td>Datsenko &amp; Wanner</td>
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<tr>
<td>Red Helper plasmids (pKD46)</td>
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<td>Datsenko &amp; Wanner</td>
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</tbody>
</table>

Amp⁺, ampicillin resistance; Cm⁺, chloramphenicol resistance; Kn⁺, kanamycin resistance; Te⁺, tetracycline resistance.

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

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<th>Protein</th>
<th>Primer</th>
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<td></td>
<td>YjgA (F)</td>
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<td>YjgA15</td>
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<td>TCGCATAATGGACATCGGAAGAGAAGAGATG</td>
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<td>ATATCAGGCCCTTCGTTCTCCTGGTTAACTCG</td>
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<td>YjgA20</td>
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<td>YjgA20 (R)</td>
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<tr>
<td>ΔYjgA</td>
<td>YjgA::Kn (F)</td>
<td>TATCAAGGCTCACAGGGTCACGGCTAGCACGAAACAATAATGATTGTAGGGCTGGAGCGTTC</td>
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<td></td>
<td>YjgA::Cm (R)</td>
<td>AAGGAGGCGCGAGGATGACGATAATGGATGGCTGAGATG</td>
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<td>RT-PCR</td>
<td>YjgA (F)</td>
<td>GCCGTTTTGACTCCTCAGGATCAT</td>
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<tr>
<td></td>
<td>YjgA (R)</td>
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<tr>
<td>16s rRNA (F)</td>
<td>AGTCAGATGGAAACTCCAGG</td>
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<tr>
<td>16s rRNA (R)</td>
<td>TCAAGGGCGCACACCTCAAGG</td>
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</table>

Amp⁺, ampicillin resistance; Cm⁺, chloramphenicol resistance; Kn⁺, kanamycin resistance; Te⁺, tetracycline resistance.
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 MgCl₂). 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 MgCl₂ 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 identified by MALDI-TOF MS (Jensen et al., 1997). The cloned 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* 420, 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 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 identified by sonication. The supernatant was applied to the Ni-NTA column pre-equilibrated with 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 MgCl₂ 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 identified by MALDI-TOF MS (Jensen et al., 1997). The cloned 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.

**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 OD₆₀₀ reached 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 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 instructions. The concentrations of ppGpp, GDP 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 KH₂PO₄, 5% ACN, pH 7.5) and high ionic (500 mM KH₂PO₄, 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 transferred 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 (ATACTCGAGGCTTCGTTCGCTCAACTCG) and kanamycin reverse (CGGCCACAGTCGATGAATCC) primers.

**Quantitative Expression of yjgA.** Expression of *yjgA* 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 *yjgA* 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 OD₆₀₀ 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
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 (NH₂) 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 cellular functions of YjgA, a deletion mutant for 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 cellular functions of YjgA, a deletion mutant for YjgA was created, by introducing plasmid forms of YjgA.

**Expression at the Stationary Phase.** The yjgA gene 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 OD₆₀₀~0.5

**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 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 × 10⁶ CFU/mL when induced at OD₆₀₀~0.6) compared to the control (4.5 × 10⁶ CFU/mL) at 14 h, corresponding to the observation of OD₆₀₀~0.6. 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.

**Table 3. Binding affinities of YjgA**

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<th>Protein</th>
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<th>Dissociation constant (K_d)</th>
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<tr>
<td>YjgA</td>
<td>ppGpp</td>
<td>5.2 ± 2.0 µM</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>300 ± 280 µM</td>
</tr>
<tr>
<td></td>
<td>GDP</td>
<td>180 ± 150 µM</td>
</tr>
<tr>
<td>YjgA20</td>
<td>ppGpp</td>
<td>7.6 ± 2.4 µM</td>
</tr>
</tbody>
</table>

**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 OD₆₀₀ (A) and by total viable cell counts (B) between ΔyjgA (△) and its parental *E. coli* K-12 MG1655 (●) wild type strain. For the dense cultures with high absorbance, the OD₆₀₀ was measured after proper dilutions.
(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 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.

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References


