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Doctoral Dissertation

Biochemical Analysis of GTP-binding Proteins of Obg/Era family in *Bacillus subtilis* and Functional Characterization of YqeH

枯草菌における GTP 結合蛋白質の生化学的研究及び
YqeH 蛋白質の機能解析

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Biochemical Analysis of GTP-binding Proteins of Obg/Era family in *Bacillus subtilis* and Functional Characterization of YqeH

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Abstract

GTP-binding proteins are ubiquitous proteins that involve in pivotal cellular processes such as signal transduction, cell cycle progression, and protein translation. Bacterial genome sequencing has revealed a novel family of P-loop GTPases that are often essential for growth, and involved in biogenesis of the 30S or 50S ribosomal subunits. These GTPases are represented by the *Bacillus subtilis* Obg and *Escherichia coli* Era, encompassing Obg, Era, YqeH, YsxC, YlqF, YphC and YloQ. However, correlations between their GTPase activities and ribosome assembly or maturation remain largely unknown. We found these proteins possess intrinsic GTPase activities that capable of hydrolyzing the bound-GTP to GDP, except for YsxC. Obg, Era and YlqF revealed a slow rate of GTPase activities, comparable to those of *E. coli* Era and its homologs. However, YqeH and YphC showed unexpectedly high GTPase activities. Moreover, we provide the first experimental evident that the GTPase activities of Obg and Era were enhanced by the 50S and 30S ribosomal subunit, respectively.

YqeH is a member of this Obg/Era GTPase family, with its function remains to be uncovered. I present results showing that YqeH is involved in the 30S subunit biogenesis in *B. subtilis*, in which reduction in the 70S ribosome and accumulation of the free 50S subunit were observed in YqeH-depleted cells. Interestingly, no free 30S subunit accumulation was evident. Consistent with the theory that YqeH is involved in 30S subunit biogenesis, a precursor of 16S rRNA and its degradation products were detected. YqeH overexpression did not compensate for growth defects in mutants devoid of Era and *vice versa*. The findings strongly suggest that YqeH and Era function at distinct checkpoints during 30S subunit assembly. In addition, the involvement of YqeH in 30S assembly was further supported by its interaction with S4, an assembly initiator of the 30S subunit. *B. subtilis yqeH* is classified as an essential gene due to the inability of the IPTG-dependent P_{spac}-*yqeH* mutant to grow on PAB agar plates in the absence of IPTG. However, in our experiments, the P_{spac}-*yqeH* mutant grew in PAB liquid medium without IPTG supplementation, albeit at an impaired rate. This finding raises the interesting possibility that YqeH participates in assembly of the 30S ribosomal subunit as well as other cellular functions essential for growth on solid media.

Key words: bacterial GTPase, ribosome assembly, ribosome biogenesis, YqeH
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CHAPTER 1 GENERAL INTRODUCTION

1.1 GTP-binding protein

GTP-binding proteins (GTPases) are found in all domains of life and involve in regulation of various essential processes such as cell cycle progression, signal transduction, protein translation, and vesicular trafficking (Bourne *et al*., 1991; Kjeldgaard *et al*., 1996 and Sprang, 1997). The first human oncogene *ras*, which encodes a small GTPase Ras with ~20 kDa that modulates normal cell growth and differentiation, was identified in 1981. Specifically, mutations that impaired or disrupt Ras GTPase ability have been implicated in more than 30% of all human cancer (Bos, 1989). On the other hand, a few GTPases in bacteria had been characterized, including translation factors IF-2, EF-Tu and EF-G, cell division protein FtsZ, and signal recognition particle components FtsY/FfH (Caldon et al., 2001). Bacterial genome sequencing has led to the identification of a novel family of P-loop GTPases, known as the Obg/Era family, which is often essential for growth, and possibly involved in biogenesis of the 30S or 50S ribosomal subunits (Caldon et al., 2001; Brown, 2005; Comartin and Brown, 2006).

GTP-binding proteins bind specifically to guanine nucleotides GTP and GDP, and possess intrinsic GTPase activity. The protein exists in three conformations: the transient empty state (inactive), the active GTP-bound state, and inactive GDP-bound state (Fig. 1A). Binding of GTP molecule initiates protein conformational change, enabling the protein to dock to its downstream target(s), and subsequently, activates specific cellular signaling cascade. Subsequently, the bound-GTP is hydrolyzed to GDP, and the GTP-binding protein is released from its targets due to induced conformational change of GDP-bound form. Interconvertion between the active and inactive forms is a unique property of GTP-binding protein as a binary switch (Bourne *et al*., 1991). For eukaryotic small GTPase Ras, the GTP

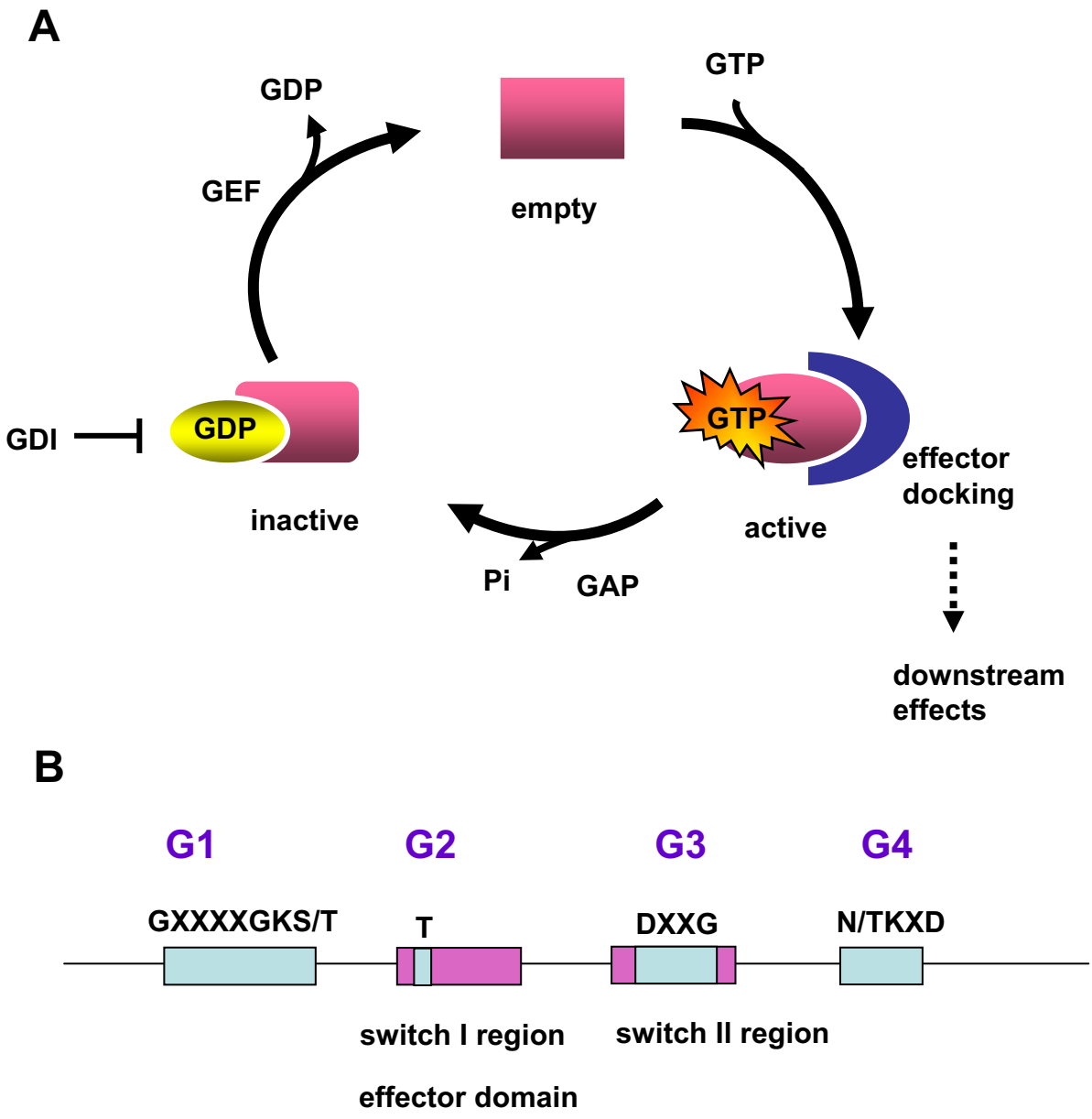


Fig. 1 The GTPase cycle and the conserved GTP-binding domains. (A) GTPase displays three conformations: transient empty form, the active GTP-bound and inactive GDP-bound forms. Binding of GTP molecule initiates protein conformational change, enabling the protein to dock to its downstream effector, and subsequently, activates specific cellular signaling cascade. In eukaryotic Ras, the most well studied small GTPase that involves in regulation of cell cycle, the Ras bound-GTP is hydrolyzed to GDP, assisted by GAP. GDI acts by blocking GDP-dissociation. Finally, with the enhancement of GEF, the bound GDP is released from the protein, leaving the transient empty protein is now ready to bind a new GTP molecule. (B) For GTPases of Obg/Era family, the four conserved GTP-binding domains involved in guanine nucleotide binding are indicated as blue boxes and the consensus sequences are indicated. The switch I and switch II are the regions show significant conformational differences in the GTP-bound and GDP-bound forms, are depicted as purple boxes.

hydrolysis and GTP/GDP exchange occurs at low rates *in vitro*, but are modulated *in vivo* by three regulators: GTPase activating protein (GAP), guanine nucleotide exchange factor (GEF) and guanine nucleotide dissociation inhibitor (GDI). The interconversion between GTP-bound and GDP-bound forms enables GTPases to function as molecular switches in a broad variety of biological processes (Bourne et al., 1990; Hall, 1990).

All GTPases are characterized by at least four highly conserved amino acid sequences motif (G1, G2, G3 and G4), known as GTP-binding domain (Fig. 1B), which interact with GTP/GDP molecule or effector proteins (Bourne *et al*., 1991; Caldon *et al*., 2001). G1 domain (consensus: GXXXXGKS), is responsible for the binding of α and β-phosphate groups of the nucleotides. The G2 domain is unique to each GTP-binding protein, and known as the effector-binding domain, specifically interacts with target protein (Lowy, 1993). The conserved 'threonine' of this domain involved in the Mg^{2+} binding. The G3 domain (consensus: DXXG), with conserved aspartic acid (D) that coordinates with Mg^{2+}, and glycine (G) that hydrogen-bonded to the γ-phosphate. G4 domain (consensus: NKXD) is important for the recognition of the guanine ring (Pandit and Srinivasan, 2003; Brazzolotto et al., 2006).

1.2 GTP-binding proteins of Obg/Era family

1.2.1 Background

The Obg/Era family consists of Obg, Era, YqeH, YsxC, YlqF, YphC and YloQ, which are widely conserved from bacteria to higher eukaryotes. Obg was first discovered in genome of *Bacillus subtilis* in an operon downstream of *spoOB*, and was predicted as an essential gene for vegetative growth and initiation of sporulation (Trach and Hoch, 1989). Era was found in *Escherichia coli* based on its sequence homology to the GTP-binding domain of yeast Ras, and is capable of binding GTP molecule (Ahnn et al., 1986). Using the conserved

GTP-binding domain of *E. coli* Era as a probe, our laboratory had identified six bacteria GTP-binding proteins in *B. subtilis*, Obg, Bex, YqeH, YlqF, YsxC, and YphC, which are essential for growth (Morimoto et al., 2002) (Fig. 2).

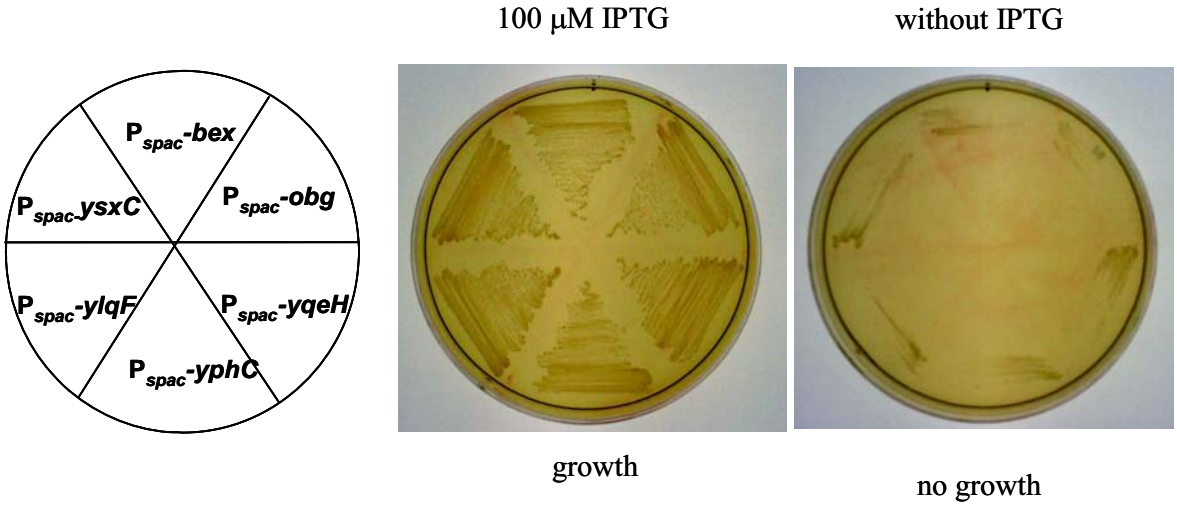


Fig. 2 Growth properties of IPTG-inducible conditional null mutants of P_{spac}-*bex,* P_{spac}-*obg,* P_{spac}-*yqeH,* P_{spac}-y*phC,* P_{spac}-*ylqF,* P_{spac}-*ysxC* on a PAB agar plate in the presence and absence of IPTG. Photographs were taken after incubation at 37°C for 12 h.

While studies of Era and Obg have been started nearly two decades ago, investigation of the balance of the GTPases of Obg/Era family, including YloQ, have been post-genomic. YloQ is a circular permuted GTPase, in which its depletion resulted in altered ribosome profile, with accumulation of free ribosomal subunits at the expense of intact 70 S ribosomes (Campbell et al., 2005). YloQ counterpart in *E. coli* has been shown to interact with 30S subunit (Daigle and Brown, 2004). Therefore, YloQ is included as a member of the Obg/Era family. The position and conserved amino acid of GTP-binding domain of the seven members of Obg/Era family are shown in Fig. 3. Note that YqeH, YloQ, and YlqF display circular permuted G4-G1-G2-G3 motif, different from the classical G1-G2-G3-G4 motif of Obg, Era, YlqF, and YphC. Anand et al. (2006) search for all possible circular permutations after artificially permuting the classical GTPases, and subjecting them to profile Hidden Markov Model searches. These profiles were subjected to search against Swissprot/TrEMBL

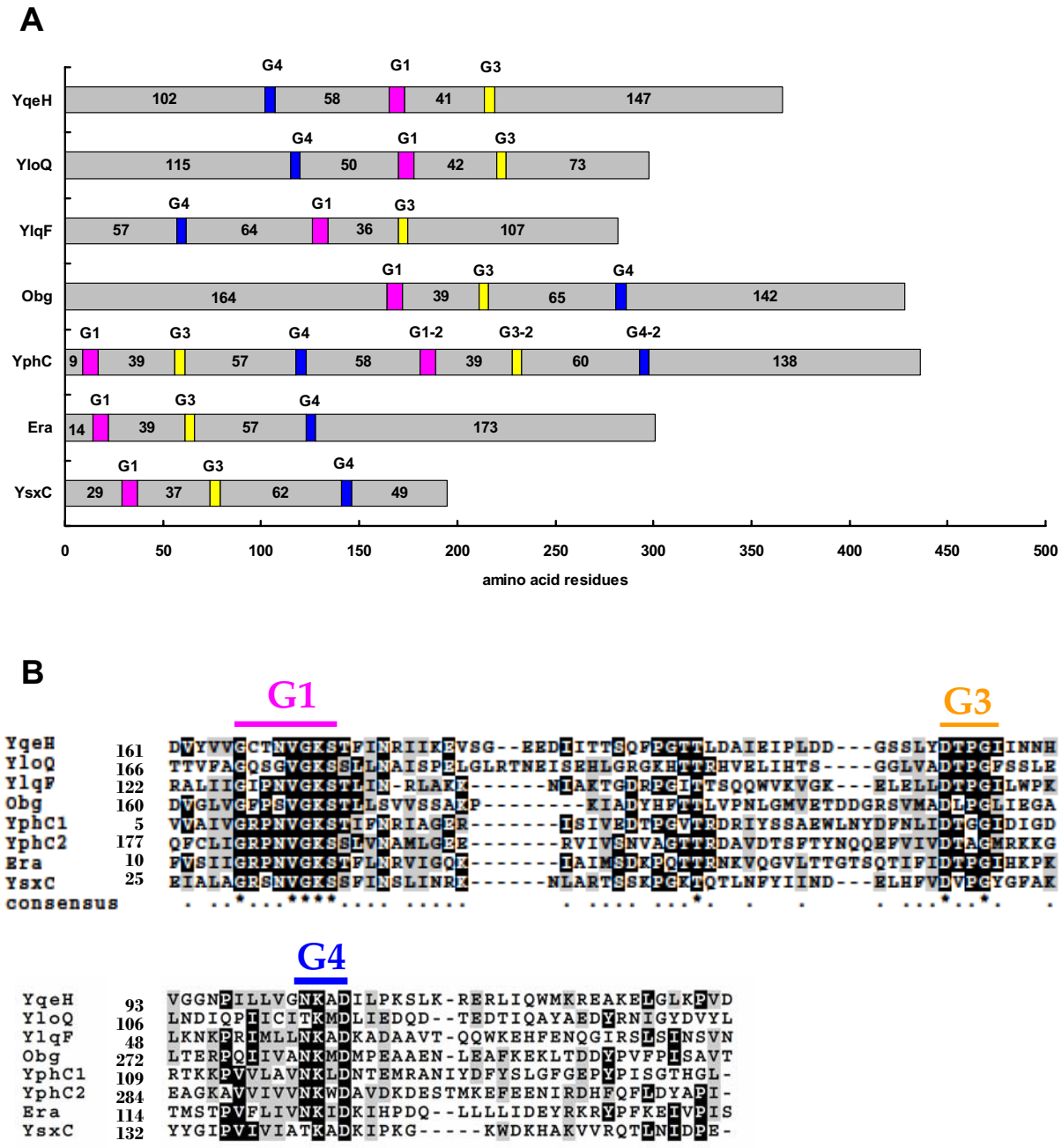


Fig. 3 Position of the GTP-binding domain of the members of Obg/Era family (A) GTPase are characterized by four conserved amino acid sequences in the GTP-binding domain, known as G1, G2, G3 and G4 sequence motifs. G2 domain is the effector binding domain, which is not well conserved. The number in between the GTP-binding domains indicates amino acid residues separating the conserved motifs. Conserved sequence spacing between G1 and G3 domains (range 36-42 aa) is a typical feature within a GTP-binding protein subfamily. (B) Multiple alignment of conserved amino acid sequences of GTP-binding domain of the GTPases using ClustalW 1.82, and shading with BoxShade3.21 (http://wwww.ch.embnet.org/software/BOX_form.html). Black shading indicates residues that are completely conserved, and gray shading indicates conservative substitutions.

sequences database. As a result, the G4-G1-G2-G3 motif was found as the only possible circular permutation that can exist in nature. Notably, among these proteins, a zinc finger domain (in both YqeH and YloQ) or oligonucleotide/oligosaccharide-binding (OB)-fold structure (YloQ only) that proposed to bind RNA are exist, suggestive of the potential RNA-binding proteins. It is believed that circular permuted GTPases possess a novel means to couple RNA-binding with guanine nucleotides binding function, or *vice versa* (Anand et al., 2006).

On the other hand, YphC has two GTP-binding domains, arranged in tandem. Since the sequences of the two domains are more similar to each other than to other GTPases, it is likely that ancient gene duplication, rather than a fusion of evolutionary distinct GTPases, gave rise to this feature (Leipe et al., 2002). Study of the *Thermotoga maritima* EngA (YphC homolog) suggests that the GTPase cycles of the two domains are regulated differentially (Robinson et al., 2002). Furthermore, biochemical analysis showed that inactivation of nucleotide-binding of G-domain 1 (GD1) dramatically reduced the GTP-hydrolysis of EngA, but inactivation of GD2 had no effect (Robinson et al., 2002).

Fig. 4 gives a summarizing overview on the occurrence of individual GTPase of Obg/Era family in the genome of different species. Among these, Obg, Era, YphC, YsxC, and YloQ are conserved universally in bacteria. However, YlqF and YqeH are mainly restricted to low-GC Gram-positive bacteria, and higher eukaryotes, without any counterpart in *E. coli*.

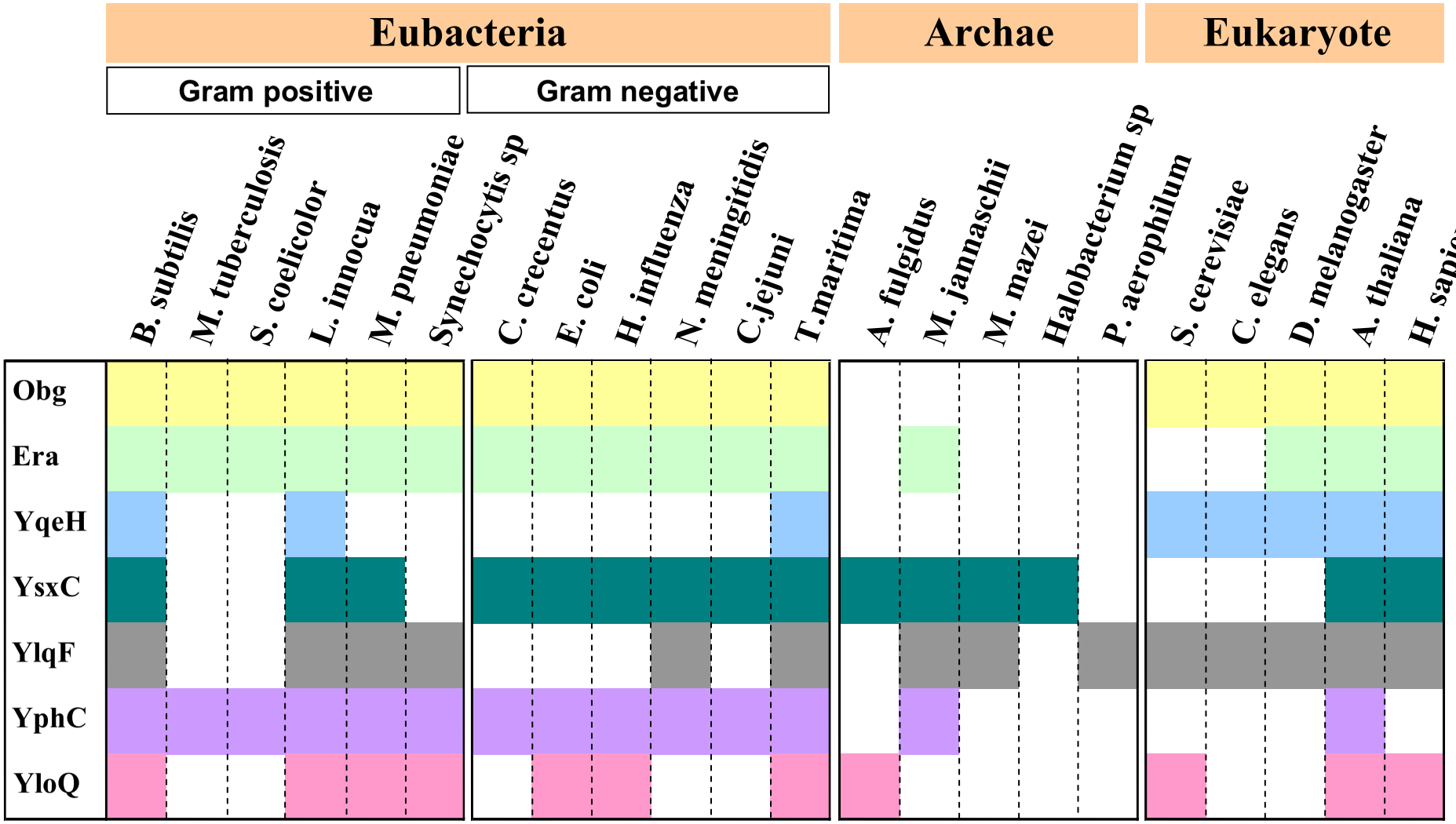


Fig. 4 Orthologs of the GTP-binding proteins of Obg/Era family of *B. subtilis.* Obg, Era and YphC are widely conserved among the eubacteria kingdom, but YqeH is unique because its homologs are found predominantly in eukaryotes. The orthologs search was performed using Microbial Genome Database (MBGD, http://mbgd.genome.ad.jp) and NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/)

1.2.2 Obg/Era family and the biogenesis of ribosomal subunits

Recently, accumulating evidence showed that the GTPases of Obg/Era family plays important roles in the assembly or maturation of 30S and 50S ribosomal subunits. *B. subtilis* YlqF and *E. coli* ObgE (ortholog of *B. subtilis* Obg) are non-ribosomal factors required for the maturation of the 50S ribosomal subunit. Depletion of YlqF or ObgE results in an altered ribosome profile displaying a reduced level of 70S ribosome and emergence of the 50S subunit precursor (Jiang et al., 2006; Matsuo et al., 2006). Furthermore, YlqF associates with the free 50S subunit *in vivo* (Uicker et al., 2006; Matsuo et al., 2006), binding to a region between the A and P sites, located at helices 38, 81 and 85 of 23S rRNA (Matsuo et al., 2006). Clearly, YlqF is involved in the late stages of 50S subunit biogenesis. ObgE preferentially interacts with the free 50S subunit *in vivo*, indicative of direct involvement in 50S formation (Sato et al., 2005; Jiang et al., 2006). Similarly, a recent study demonstrates accumulation of 50S precursors in *B. subtilis* cells depleted of YsxC or YphC (Schaefer et al., 2006).

On the other hand, Era and YloQ are non-ribosomal factors that participate in assembly of the 30S subunit. *E. coli* Era is the most extensively characterized GTPase of the Obg/Era family. However, Era mutants display a variety of phenotypes (Lerner and Inouye, 1991; Britton et al., 1998; Sayed et al., 1999; Meier et al., 2000; Inoue et al., 2003), thus making it difficult to ascertain the precise function of the protein. Recently, Sharma and colleagues (2005) reported a plausible cryo-electron microscopic map of the Era-30S complex of *Thermus thermophilus*. Era localization is mapped to a site within the 30S subunit, which overlaps the ribosomal protein S1 binding site. Binding of Era to the 30S subunit induces a conformation that inhibits 50S subunit association. Therefore, Era modulates the final stage of 30S assembly, and is replaced by S1, thus defining the completion of the 30S subunit assembly. *B. subtilis* YloQ and its *E. coli* ortholog, YjeQ, additionally associate with the 30S subunit (Daigle and Brown, 2004; Levdikov et al., 2004；Campbell et al., 2005). The GTPase

activity of YjeQ is enhanced several fold upon addition of the 30S subunit (Daigle and Brown, 2004; Himeno et al., 2004).

YqeH is a member of the Obg/Era family, whose function remains obscure. The 41 kDa protein possesses a central localized circular permuted GTP-binding domain with a G4-G1-G2-G3 motif (Leipe et al., 2002; Anand et al., 2006) and an N-terminal putative zinc finger domain with a conserved $CXGCGX_nCXRC$ motif (Levdikov et al, 2004). The zinc finger domain is suggested to bind rRNA, which may be important for the protein function (Anand et al., 2006). However, the C-terminus displays poor homology with proteins of known function. A recent study by Uicker et al. (2007) showed that YqeH depletion results in slow growth and decreased mature 16S rRNA, suggesting a potential role in proper ribosome assembly in *B. subtilis*. The GTPases of Obg/Era family in *B. subtilis* and their predicted functions are summarized in Table 1.

At present, the regulation of GTPases, which involves GEF and GDI are yet to be identified in bacteria. However, promising findings were obtained recently, where the 50S and 30S ribosomal subunits are found to accelerate the GTPase activity of *Bacillus subtilis* YlqF (Matsuo et al., 2006) and *Escherichia coli* YjeQ (Daigle and Brown, 2004; Himeno et al., 2004), respectively. Unlike p120 RasGAP that contains 1047 amino acid residues, with a molecular mass of 120 kDa (Sprang, 1997), the 30S and 50S ribosomal subunits with a respective of ~900 kDa and ~1600 kDa, are apparently act as GAPs in bacteria. The molecular mechanisms underlying the involvement of such a huge ribonucleoprotein complexes in the GTPase activation of YlqF and YjeQ, remain to be unraveled.

Table 1 Summary of the GTPases of Obg/Era family in *B. subtilis* and its orthologs that are implicated in the biogenesis of ribosomal subunit

Protein	Synonym	Characteristic	ribosome interaction	Predicted function	References
Obg	ObgE, CgtA	slow GTPase activity accumulation of premature 50S	50S enhanced Obg GTPase activity 50S, predominantly in GTP-γS form	late step 50S assembly repressor of stringent response	This study Sato et al., 2005 Raskin et al., 2007
Era	Bex	slow GTPase activity slow growth accumulation of premature 30S C-terminal KH domain	30S enhanced Era GTPase activity *E. coli* Era cofractionated with 30S	late step 30S assembly partially complement *rbfA* role in 30S assembly	This study Sharma et al., 2005 Inoue et al., 2003
YqeH	-	high GTPase activity circularly permuted G domain significant reduced 30S N-terminal zinc finger	no interaction was observed	assembly of 30S proper assembly of ribosome	This study Uicker et al., 2007
YlqF	RbgA	slow GTPase activity circularly permuted G domain accumulation of premature 50S	pre-50S, where its GTPase activity is enhanced	late step 50S assembly	Matsuo et al., 2006
YsxC	YihA	nearly undetectable GTPase activity	binds to 70S binds to 50S *in vitro*	? late step 50S assembly	This study Matsuo, unpublished data Schaefer et al., 2006
YphC	Der, EngA	High intrinsic GTPase 2 adjacent G domains C-terminal KH-like domain accumulation of premature 50S	binds to 50S *in vitro*	late step 50S assembly	This study Schaefer et al., 2006
YloQ	CpgA RsgA, YjeQ	slow GTPase circularly permuted G domain cells morpholgy change reduced 30S N-terminal OB-fold C-terminal zinc finger	*E. coli* YjeQ associated to 30S YjeQ GTPase is stimulated by 30S	biosynthesis of cell envelope	Daigle et al., 2004 Himeno et al., 2004 Ledikov et al., 2004 Campbell et al., 2005 Cladiere et al., 2004; 2006

1.3 Biogenesis of the ribosome

1.3.1 The bacterial ribosome

In log phase bacteria, ribosomes account for a half of the cell dry mass (Bremer and Dennis, 1996), and the precisely regulated and energetically efficient process during ribosome biogenesis is a prerequisite for cells survival. To date, our understanding on the ribosome biogenesis at the molecular level, still lagged far behind DNA replication and transcription, owing to its complexity, which involve coupling of rRNA transcription with r-proteins synthesis, and non-ribosomal assembly factors and proteins chaperons that yet to be identified. The bacterial 70S ribosome is the core translation machine for protein synthesis in the cell. It composed of 50S and 30S subunits, which are ribonucleoproteins, comprising about 66% rRNA and 34% ribosomal proteins or r-proteins (Lewin, 2000). In *E. coli*, the 50S subunit is consists of ~34 r-proteins, and 30S subunit, with ~21 r-proteins. The 16S, 23S, 5S rRNAs and tRNAs are encoded by 10 *rrn* operons in *B. subtilis,* whereas 7 is found in *E. coli.* During ribosome biogenesis, the rRNA is co-transcribed as a 30S primary transcript, concurrent with the rRNA processing and ribosomal assembly events such as ribonucleases cleavage, proteins binding, a series of rRNA conformation changing, and base modification, which involved many non-ribosomal assembly factors (Williamson, 2003; Maki et al, 2003; 2005)

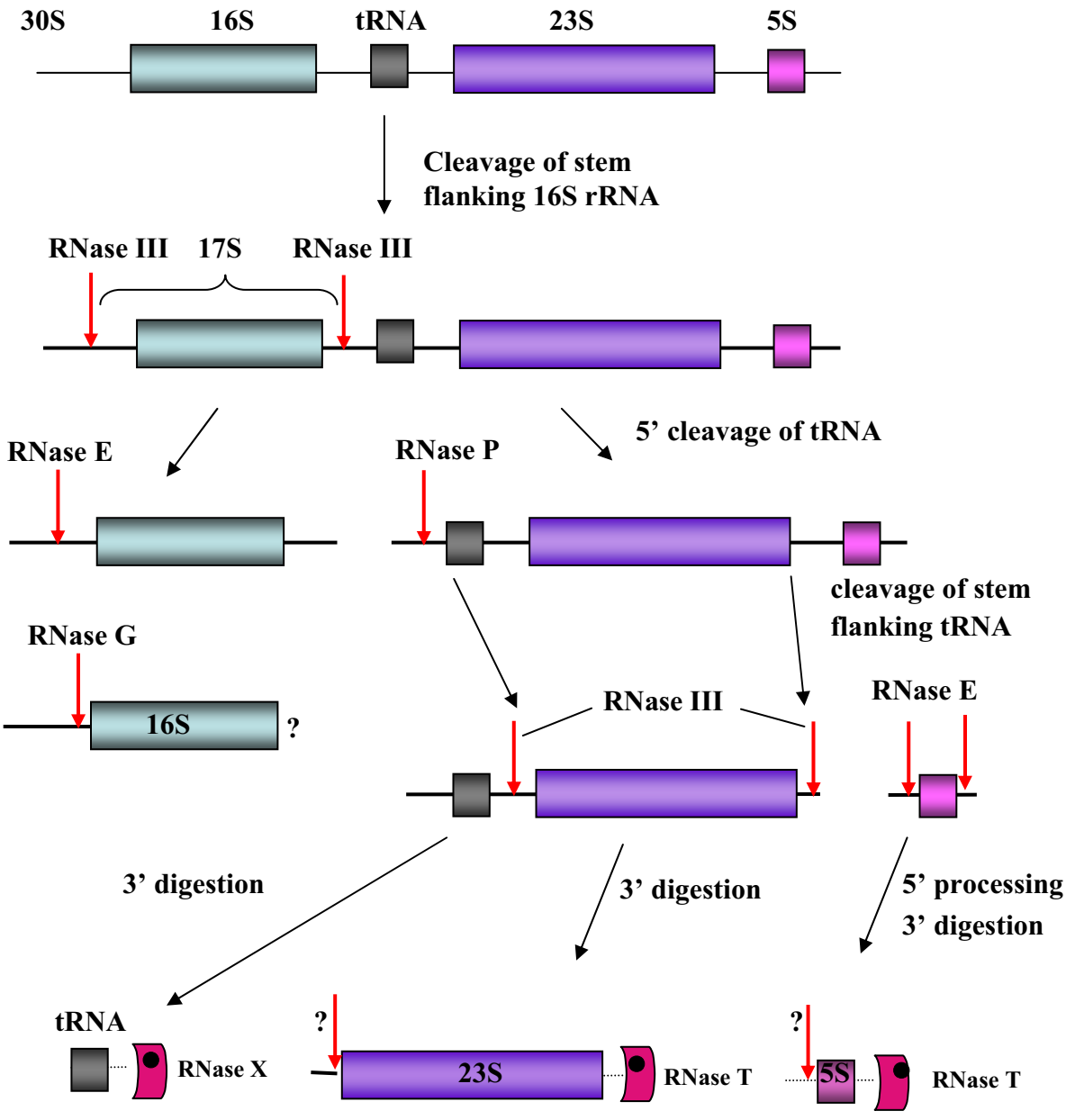
1.3.2 The rRNA processing

The processing of rRNA in *E. coli* involves two main aspects: 1) trimming of the rRNAs to yield the mature molecules found in active ribosomes and 2) base modification of rRNAs such as methylation and pseudouridylation (Nierhaus, 2004).

The mature rRNAs are processed from a polycistronic precursor rRNA, originally transcribed as a 30S primary transcript, and rRNA stepwise trimming involves RNA exonucleases and endonucleases. The mature rRNAs are generated by sequential

endonuclease cleavages, with some are generated by exonuclease digestion (Fig. 5). 5' processing of *E. coli* 23S and 5S rRNA is assumed to be endonucleolytic as no 5'-3' exonuclease has been identified in *E. coli* (Lafontaine and Tollervey, 2001). The mechanism of 3' processing of the *E. coli* 16S rRNA is not known. The compact nature of the RNA structures of ribosomal subunits reveals clear problems for the biogenesis pathway. To allow access to processing, rRNA modification and assembly factors are strictly coordinated during ribosome synthesis. Final folding of the rRNA must be prevented until late in the pathway, maintaining key regions of the pre-RNAs in a relatively loose structure. Finally, the rRNA must be refolded into the mature structure

In *B. subtilis*, the ribonucleases that responsible for maturation 5' end of 16S is known as RNase J1 (YkqC), but the ribonuclease for maturation of 3' end is not yet identified (Britton et al., 2006). It is known that maturation of 16S rRNA occurs in the context of a preribosomal particle, which is altered when ribosomal proteins are missing (Nierhaus, 1991; Li et al., 1999; Kaczanowska and Ryden-Aulin, 2004). Moreover, in *E. coli*, as the final steps of rRNA maturation are likely to occur after the subunits assembly into 70S ribosome, whereas mutations in genes involved in ribosome assembly *per se* often lead to indirect effects on rRNA processing (Charollias et al., 2003; 2004; Hage and Alix, 2004).

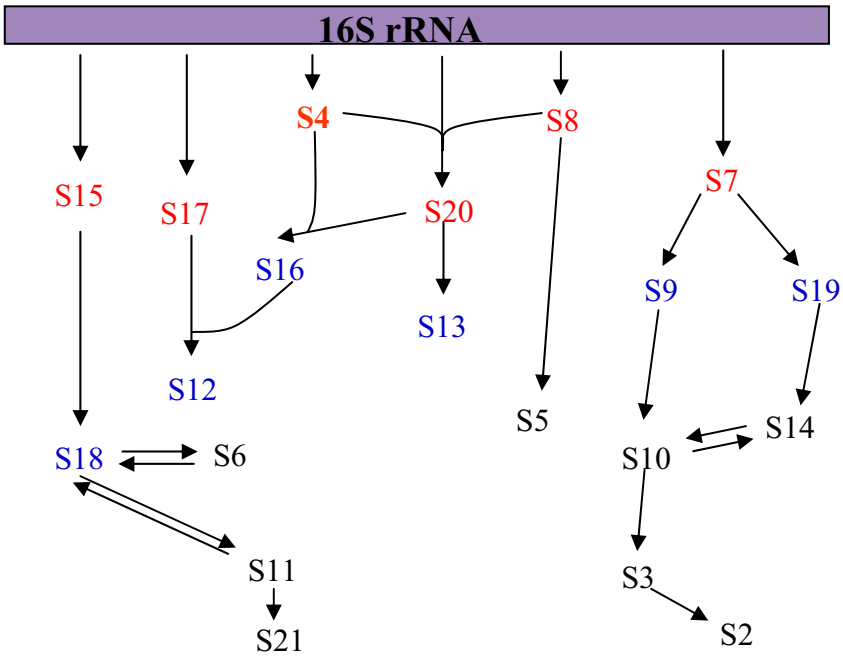


Modified from Lafontaine and Tollervey (2001)

Fig. 5 Pathways of rRNA processing in *E. coli*. The 30S primary transcript is practically not found in wild type cells, but only in RNase III deficient–strain. RNase III cleaves in spacer sequences bordering 16S and 23S. This sequential processing occurs from 5'to 3' direction, compatible with the hypothesis that at least some processing steps are concomitant with assembly of the ribosome. RNase III cleavages yield precursor species of rRNAs. The endonucleases are depicted as red arrow, and exonucleases are depicted as . The final processing at the 3'end of 16S involves enzyme that is yet to be identified. 5' processing of *E. coli* 23Sand 5S rRNA is assumed to be endonuclease as 5'-3' exonuclease has not been identified in *E. coli*.

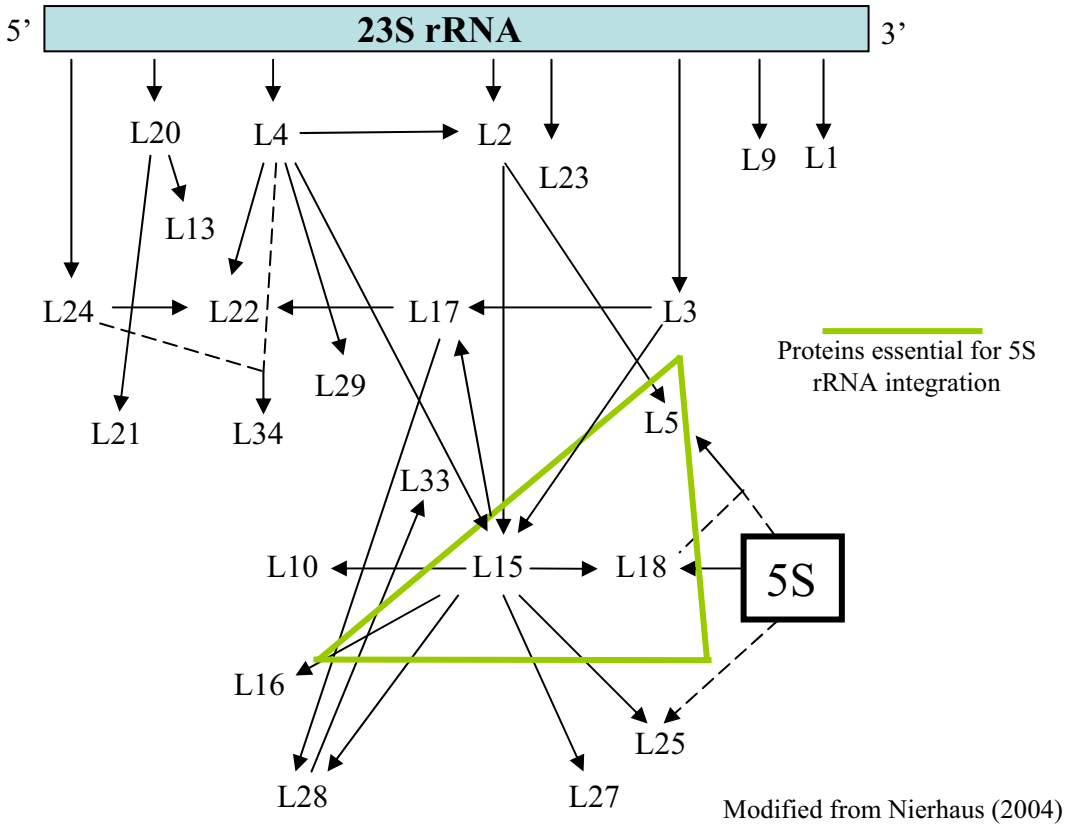
1.3.3 Sequential assembly of the ribosomal proteins

Ribosomal subunit assembly is an ordered process that relies on each of the ribosomal proteins being incorporated into the growing complex at the appropriate timing. The first *in vitro* ribosome reconstitution is achieved using *E. coli* 30S subunit, by incubating the mature 16S rRNA and a mixture of 30S ribosomal-proteins (Traub and Nomura, 1968). However, the reaction requires high concentrations of salt and Mg^{2+}, and incubation at 40 ^{o}C, which involves non-physiological conditions. Nevertheless, incubation with precursor of 16S rRNA produces 30S subunit that is functional inactive (Wireman and Sypherd, 1974). Thus, the observed self-assembly character *in vitro* does not preclude the existing of non-ribosomal factors that play a fundamental role in ribosomal subunit assembly and maturation *in vivo* (Dammel and Noller, 1995; Bylund et al., 1998; Inoue et al., 2003). Since the assembly of the ribosomal subunits in *B. subtilis*, is less known compared to *E. coli*. The model of *E. coli* is used as reference, Fig. 6 and 7 show the assembly map of 30S and 50S ribosomal subunits, respectively.



Modified from Stagg et al. (2003)

Fig. 6 The Nomura assembly map of 30S ribosomal subunit in *E. coli*. There are three basic pathways of the proteins binding: the S15 pathway, the S4/S8 pathway and the S7 pathway. Proteins were added sequentially to the RNA in the order that they appear in the assembly map, primary binding proteins are depicted in red, secondary (blue) and tertiary (black)



Modified from Nierhaus (2004)

Fig. 7 Assembly map of the 50S ribosomal subunit in *E. coli*. L5, L15 and L18 inside the green triangle lines are important for mediating the binding of 5S rRNA to 23S rRNA

1.4 Objectives of this study

The focus of my research can be mainly divided into two topics:

1.4.1. Biochemical analysis of GTPases of Obg/Era family

Despite emerging evidence suggests that GTPases of Obg/Era family is involved in biogenesis of the 30S or 50S ribosomal subunits, the biochemical properties of these proteins remain largely unexplored. To understand how a GTPase works, *in vitro* biochemical study of the GTP-binding and GTPase activities are prerequisite to get an insight of the protein properties. We demonstrated that all members of Obg/Era family bind specifically to GTP and GDP molecules (Morimoto et al., 2002). To date, the GTPase activity of YqeH, Era, YsxC and YphC of *B. subtilis* are yet to be established. Moreover, in spite of the known GTPase activities of Obg, YlqF and YloQ, the precise correlations between the GTPase characteristic, ribosome interaction and assembly remain to be elucidated.

I found these proteins possess intrinsic GTPase activities that capable of hydrolyzing the bound-GTP molecule to GDP, except for YsxC. Obg, Era and YlqF revealed a slow rate of GTPase activities, comparable to those of *E. coli* Era and its homologs. However, YqeH and YphC showed unexpectedly high GTPase activities that may distinguish them from the former group. Moreover, in addition to YlqF that its GTPase activity is stimulated by 50S ribosomal subunit (Matsuo et al., 2006), we also provide the first experimental data that the GTPase activities of Obg and Era were enhanced by the 50S and 30S ribosomal subunit, respectively.

1.4.2. Functional characterization of YqeH

YqeH is a member of the Obg/Era family, whose function remains to be elucidated. A recent study by Uicker et al. (2007) showed that YqeH depletion results in slow growth and decreased mature 16S rRNA, suggesting a potential role in proper ribosome assembly in *B.*

subtilis. Here, we present detailed functional analysis of YqeH. We observed a reduction in the 70S ribosome level, and accumulation of the free 50S subunit, but not free 30S, in YqeH-depleted cells. Consistent with the alleged involvement of YqeH in 30S subunit biogenesis, a precursor of 16S rRNA and its degraded products were detected. However, the reduction of free 30S subunit was not observed in Era-depleted cells. Indeed, YqeH overexpression did not compensate for growth defects occurring due to Era depletion and *vice versa.* Our findings strongly suggest that both YqeH and Era are involved in 30S subunit biogenesis, but play separate roles at distinct checkpoints during 30S assembly.

On the other hand, yeast two hybrid analysis showed that YqeH interacted with S4, a component of the 30S subunit that is essential for growth in *B. subtilis*. S4 intimately involved in both ribosome regulation and functions. In *E. coli*, S4 binds to the precursor of 16S rRNA, and serves as one of the two nucleation sites during the assembly of 30S subunit (Nowotny et al., 1988). The YqeH mutant proteins, YqeH-S173N and -S193P, which lost interaction with S4 may serve as a powerful tool to genetically study the functional consequences of this YqeH-S4 interaction.

CHAPTER 2

In vitro biochemical characterization of the GTPases of Obg/Era family

2.1 INTRODUCTION

Despite emerging evidence suggests that GTPases of Obg/Era family is involved in biogenesis of the 30S or 50S ribosomal subunits, the biochemical properties of these proteins remain to be uncovered. The members of Obg/Era family encompassed seven GTPases such as Obg, Era, YqeH, YsxC, YlqF, YphC and YloQ, which are essential for cell growth. Among these, YqeH, YlqF and YloQ revealed an unusual circular permuted GTP-binding domain of G4-G1-G2-G3 motif, different from the canonical G1-G2-G3-G4 motif of the translational factors such as initiation and elongation factors or small GTPases Ras.

To understand how a GTPase works, *in vitro* biochemical study of the GTP-binding and GTPase activities are prerequisite to get an insight into the protein properties. Using nucleotides-binding competition assays, we demonstrated that all members of the Obg/Era family bind specifically to GTP and GDP molecules, as suggested by their conserved GTP-binding domain (Morimoto et al., 2002). To date, the GTPase activities of YqeH, Era, YsxC and YphC of *B. subtilis* have not been experimentally proven. Moreover, in spite of the reported GTPase activities of *B. subtilis* Obg (Welsh et al., 1994), YloQ (Campbell et al., 2005) and *E. coli* Era (Sood *et al*., 1994), the precise correlation of a GTPase interacts with ribosome subunit, and how it triggers ribosome assembly remain to be elucidated. To further quantify the biochemical properties of these proteins, the kinetic parameters of their GTPase activities, such as GTP-hydrolysis rate constant, k_{cat} and Michaelis constant, K_m were determined.

I found these proteins possess intrinsic GTPase activities that capable of hydrolyzing the bound-GTP molecule to GDP, except for YsxC. Obg, Era and YlqF revealed a slow rate of GTPase activities, comparable to those of *E. coli* Era and its homologs. However, YqeH and

YphC showed unexpectedly high GTPase activities that may distinguish them from the former group. Moreover, in addition to YlqF that its GTPase activity is stimulated by 50S ribosomal subunit (Matsuo et al., 2006), we also provide the first experimental evident showing that the GTPase activities of Obg and Era were enhanced by the 50S and 30S ribosomal subunits, respectively.

2.2 MATERIAL AND METHODS

2.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2 and Table 3

2.2.2 Primers

Primers used in this study are listed in Table 4

2.2.3 General materials

Most of the restriction enzymes, DNA polymerases and other DNA-modifying enzymes used were purchased from Takara Shuzo Co., Ltd.

2.2.4 Media and buffers

All media and buffers were sterilized before use, either by autoclaving at 121^{o}C for 15 min or filter-sterilization. In one-liter media preparation, the final volume was adjusted with distilled H_2O before sterilization.

Luria-Bertani broth (LB broth)
per liter

Bacto-tryptone (Difco)	10 g
Bacto-yeast extract (Difco)	5 g
NaCl	5 g

For solid medium preparation, 18 g of agar powder was added prior to autoclaving.

Penassay broth (PAB)
per liter

Antibiotic medium 3 (Difco)	17.5 g

For solid medium preparation, 18 g of agar powder was added prior to autoclaving. For growing *Bacillus subtilis* CRK6000 strain, the following essential compounds were added to PAB at ~55^{o}C before use:

per liter PAB:

Adenine	20 mg
L-Methionine	50 mg
Guanosine	20 mg
L-Histidine	50 mg

Table 2 Strains used in this study

Strain	Genotype	Source
B. subtilis		
168	*trpC2*	Pasteur stock
LPC101	*trpC2 bex*ΩpMutinT3-`*bex*-His6	this study
LPC102	*trpC2 yqeH*ΩpMutinT3-`*yqeH*-His6	this study
LPC103	*trpC2 ysxC*ΩpMutinT3-`*ysxC*-His6	this study
LPC104	*trpC2 ylqF*ΩpMutinT3-`*ylqF*-His6	this study
LPC105	*trpC2 yphC*ΩpMutinT3-`*yphC*-His6	this study
E. coli		
DH5α	F^- ϕ80d *lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17 (r_K^- m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 λ^- *gyr*A96 *rel*A1	laboratory stock
BL21(DE3)	F^- *omp*T *hsd* S_B(r_B^- m_B^-) *gal dcm* (DE3)	Novagen

Table 3 Plasmids used in this study

Plasmid	Genotype	Source
Used for construction of His fusion GTP-binding protein mutants of *B. subtilis*		
pMutinT3	ColE1 *ori* P_{spac}-*lacZ Amp Erm*	Vagner *et al.,* 1998
pMT301	pMutinT3-`*bex*-His_6	this study
pMT302	pMutinT3-`*yqeH*-His_6	this study
pMT303	pMutinT3-`*ysxC*-His_6	this study
pMT304	pMutinT3-`*ylqF*-His_6	this study
pMT305	pMutinT3-`*yphC*-His_6	this study
Used for purification of GTP-binding proteins		
pET29b	pBR322 *ori* P_{T7} *lacI* His_6 *Kan*	Novagen
pTM291	pET29b-*era*	Morimoto et al., 2002
pTM299	pET29b-*yqeH*	Morimoto et al., 2002
pTM293	pET29b-*ysxC*	Morimoto et al., 2002
pTM298	pET29b-*ylqF*	Morimoto et al., 2002
pTM294	pET29b-*yphC*	Morimoto et al., 2002

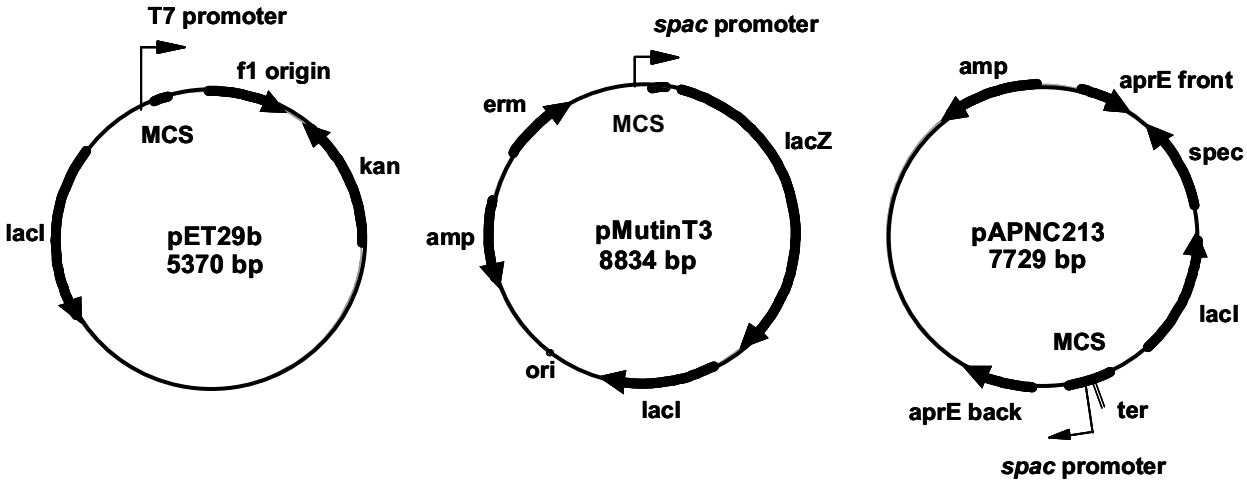


Fig. 8 Structure of the plasmid vectors used in this study. (A) pET-29b vector for purification of protein with a C-terminal His-fusion. (B) pMutinT3, a chromosomal DNA integrated vector for construct of targeted gene with C-terminal His-fusion, regulated by IPTG-inducible promoter to replace its endogenous gen in *B. subtilis.* (C) pANPC213 vector, is used to place a gene into *aprE* locus of *B. subtilis* chromosome, in which gene expression is under exclusive control of IPTG-induction.

MCS	multi-cloning site
lacZ	*E. coli* gene encoding β-galactosidase
lacI	*E. coli* gene encoding β-galactosidase
ori	plasmid origin of replication
amp	ampicillin-resistance gene for selection in *E. coli*
erm	erythromycin-resistance gene for selection in *B. subtilis*
kan	kanamycin-resistance gene for selection in *B. subtilis*
spec	spectinomycin-resistance gene for selection in *B. subtilis*
spac	IPTG-inducible promoter controlled by LacI

Table 4 Primers used in this study

Primer	Restriction site	Primer sequence
Used for constructs of His fusion GTP-binding protein mutants of *B. subtilis*		
bex-F	*Bgl* II	CGCAGATCTGTAACTGACCACCCTGAACG
yqeH-F	*Bgl* II	CGCAGATCTTTGCTATATGCCGAATGAGC
ysxC-F	*Bgl* II	CGCAGATCTAGTCTGAGCGTGAAGCATGG
ylqF-F	*Fba* I	ATGCTGCTGCTGATCACTTGTCGGTTTGAGACTGGC
yphC-F	*Fba* I	ATGCTGCTGCTGATCACTGGATTATGCGCCAATCC
pET-R1[a]	*Bgl* II	GCCGAGATCTTAGTTATTGCTCAGCGGTGG
pET-R2[b]	*Fba* I	ATGCTGCTGCTGATCATAGTTATTGCTCAGCGGTGG
pHV-F		TGTAAAACGACGGCCAGTGTGTGGAATTGTGAGCGG
pHV-R		CAGGAAACAGCTATGACCAGTGTATCAACAAGCTGG
Used for purification of His fusion GTP-binding protein mutants from *E. coli*		
yqeH-F0	*NdeI*	TGTCATATGGAAAAGGTTGTTTGTATCGGG
yqeH-R0	*Bam*HI	GGAGGATCCAGAATTAATGAACGCCGAAC
pET-Seq F		CCGCGAAATTAATACGACTC
pET-R		GCAGCCGGATCTCAGTGGTG

[a] reverse primer used for amplification of inserts paired with *bex*-F, *yqeH*-F and *ysxC*-F
[b] reverse primer used for amplification of inserts paired with *ylqF*-F, *yphC*-F
The underlined sequences correspond to restriction enzyme digestion site; bolded sequences denoted the codon for introduced single point mutation

2.2.5 General recombinant techniques and DNA Sequencing

Recombinant DNA techniques, including restriction endonuclease digestion, DNA precipitation, agarose gel electrophoresis, DNA ligation, DNA transformation by electroporation, and `mini-prep` scale of plasmid isolation was performed in accordance with standard protocols (Sambrook and Russell, 2001). All PCR products were purified with polyethylene glycol prior to ligation reactions. Polymerase chain reaction (PCR) was performed using AmpliTaq DNA polymerase (Applied Biosystems). Nucleotide sequences of cloned products were determined using Big Dye^{R} Terminator version 3.1 Cycle Ready kit (Applied Biosystems) and analyzes on ABI 3100 automated sequencer.

2.2.6 Transformation of *B. subtilis* 168

Transformation of *B.subtilis* was performed according to the method of Davidoff-Abelson and Dubnau (1971). Overnight culture of *B. subtilis* 168 incubated at 30^{o}C was used to inoculate 5 ml of MDCH medium (Section 2.4) at initial optical density, OD_{600} of 0.1 and grew at 37^{o}C. OD_{600} was measured at regular hourly intervals throughout the growth cycle, until it reached stationary phase, at OD_{600} of 1.2 – 1.3. Fresh prepared 5 ml MD medium was added to the culture, and the cells were further grown for another 1 h to promote competency. Plasmid DNA was added to 500 μl of the cell suspension and re-incubate at 37^{o}C, 30 min. LB plate containing 0.5 μg/ml erythromycin was used for selection of transformants.

a. Phosphate citrate buffer
10x concentrated stock solution:
per liter

K_2HPO_4	107 g
KH_2PO_4	60 g
Trisodium citrate dihydrate	10 g

The 10x stock solution was diluted to 1x concentration by adding sterile distilled H2O.

b. MD medium

The following compounds were combined to prepare 10 ml of MD medium:

1x phosphate citrate buffer	9.2 ml
1M MgSO4	0.4 ml
Potassium aspartate (100 mg/ml)	0.1 ml
Ferric ammonium citrate (2.2 mg/ml)	0.05 ml
L-tryptophan (5 mg/ml)	0.25 ml
Glucose (50 %, w/v)	0.03 ml

c. MDCH medium

The following ingredients were added to 10 ml of MD medium to prepare MDCH medium:

10 % casein hydrolysate	0.1 ml
100 x trace elements solution	0.1 ml

100x trace elements solution

per liter

$CaCl_2$	0.55 g
$FeCl_2.6H_2O$	1.35 g
$MnCl_2.4H_2O$	0.1 g
$ZnCl_2.6H_2O$	0.17 g
$CuCl_2.2H_2O$	0.043 g
$CoCl_2.6H_2O$	0.06 g
$Na_2MoO_4.2H_2O$	0.06 g

TKE buffer

Tris-HCl, pH 8.0	10 mM
KCl	100 mM
EDTA, pH 8.0	20 mM

GTP-binding buffer

Tris-HCl, pH 8.0	50 mM
KCl	50 mM

Dithiothreitol	2 mM
$MgCl_2$	5 mM
Glycerol	10 % (w/v)

Antibiotic solutions

Antibiotics were added to the media at ~55°C after autoclaving. Antibiotic concentrations used for stock and selection are stated as in Table 5:

Table 5: Antibiotic solutions

Antibiotic	Abbreviation	Stock conc.	Selective conc.	Solute
Ampicillin	Amp	10 mg/ml	50 μg/ml	H_2O
Chloramphenicol	Crm	25 mg/ml	5 μg/ml	99% ethanol
Erythromycin	Erm	5 mg/ml	0.5 μg/ml	99% ethanol
Kanamycin	Kan	10 mg/ml	10 μg/ml	H_2O

2.2.7 Construction of His-tagged mutants in *B. subtilis*

Using the primers listed in Table 4, a short fragment of each gene (about 300 bp length), was individually amplified from plasmids pTM291(*bex*-His), pTM299 (*yqeH*-His), pTM293 (*ysxC*-His), pTM298 (*ylqF*-His), and pTM294 (*yphC*-His). The PCR primers contained either *Bgl*II (*bex*, *yqeH*, *ysxC*) or *Fba*I (*ylqF* and *yphC*) restriction sites. Amplified PCR products were digested with *Bgl*II or *Fba*I, and cloned into *Bam*HI site of pMutinT3. *Bgl*II or *Fba*I are ioschizomers of *Bam*HI. DNA sequences of inserts were determined by sequencing prior to transformation of *B. subtilis* 168 (Table 4). The growth rate of each transformants was determined as follows. Single colony of His-tagged mutants was streaked on LB agar plate containing erythromycin, precultured overnight at 30°C. The cells were inoculated at initial optical density, OD_{600} of 0.01 in 10 ml LB medium, and grew at 30°C. OD_{600} was measured at regular hourly intervals throughout the growth cycle, until it reached stationary phase, i.e.

when OD_{600} reached 1.0 and above.

2.2.8 Purification of His-tagged proteins

E. coli BL21(DE3)pLysS cells harbouring plasmid of pET29b-*bex*, pET29b-*yqeH,* pET29b-*ysxC* pET29b-*ylqF,* and pET29b-*yphC* were grown at 30^{o}C overnight in 10 ml LB medium, and inoculated to 500 ml LB broth supplemented with 20 mg/ml of kanamycin. When the culture reached an OD_{600} of 0.6, the cells were induced with 1 mM of IPTG and incubate for additional of 3 h under the same condition. The cells were washed in 20 ml prechilled binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9), resuspended in 10 ml of the same buffer and disrupted by sonication (10 sec. pulse, 30 sec. interval) at chilled condition. The cell debris was removed by centrifugation (8,000 rpm, 5 min) and the supernatant was filtered through 0.45 μm-pore size membrane. The crude protein was applied to an equilibrated His-bind resin (1.0 ml). The column was washed with 15 ml binding buffer supplemented with a total of 15 mM imidazole and subsequently, the resin-bound protein was eluted with 3 ml each of wash buffer (60 mM imidazole) and elute buffer (1 M imidazole). The protein concentrations were determined by Bio-Rad protein assay. Aliquot of 3 μl each of protein fractions was subjected to SDS-PAGE analysis and the protein fractions store at -70^{o}C in the presence of 15% glycerol.

2.2.9 Nucleotides-binding competition assay

50 pmol of purified His-tagged protein was incubated with 4 μCi (= 1.3 pmol) of [α-^{32}P] GTP (3,000 Ci/mmol; NEN Life Science Products) in 20 μl of GTP-binding buffer, 10 minutes on ice. Bound radioactive-labeled GTP was cross-linked to proteins by UV light treatment (254 nm, 1 J/cm^{2}). Excessive unbound [α-^{32}P]GTP was eliminated by filtering through a Microcon centrifugal filter (Millipore). For reactions with competing nucleotides, 40 μM (= 800 pmol)

of each nonradioactive nucleotides (GTP, GDP, GMP, ATP, UTP or CTP) was mixed with [α-^{32}P]GTP (with ratio about 600 :1), before addition of the protein. Radioactive-labeled protein-GTP complexes were resolved by SDS-PAGE. The gel was vacuum-dried and exposed to Imaging Plate BAS 2500 for 90 min (Fuji Film).

2.2.10 Assay of GTPase activity

B. subtilis His-fusion proteins were overexpressed from *E. coli* BL21, and purified as described in section 2.2.9. Protein concentrations were determined with the Bradford method using a BSA standard curve. 30S ribosomal subunit from *B. subtilis* wild-type cells was prepared by differential ultracentrifugation, in which the free 30S subunit was isolated via dissociation of 70S ribosome under low Mg^{2+} condition, as described in a previous report (Matsuo et al., 2006). GTPase activity was measured at 30^{o}C using the method of Welsh et al. (1994), with slight modifications. 0.7 μM purified protein (70 pmol) was incubated with 40 μM GTP (4000 pmol) mixed with 0.12 pmol (0.72 μCi equivalent) of radioactive-labeled [γ-^{32}P]GTP (6000 Ci/mmol; Perkin Elmer), in the presence and absence of 0.2 μM 30S subunit (20 pmol). The reaction mixture (100 μl) contained 50 mM Tris, pH 8.0, 8 mM $MgCl_2$, 160 mM KCl, 0.1 mM EDTA, 10% glycerol and 100 μg/ml BSA, and the enzymatic reaction was triggered by the addition of GTP. At the indicated times, 20 μl aliquots were removed and quenched with 100 μl of 6% active charcoal slurry in 50 mM KH_2PO_4, pH 7.5. Samples were mixed well, and the charcoal pelleted by centrifugation. Free P_i released in the supernatant was measured with Cerenkov counting, whereas GTP and GDP molecules selectively bound to the charcoal. To obtain the kinetic rate constant, k_{cat}, for each protein, the GTPase activities at five different GTP concentrations (ranging from 1-40 μM) were measured, and k_{cat} values derived from the Lineweaver-Burk plots.

2.2.11 GTPγS binding assay

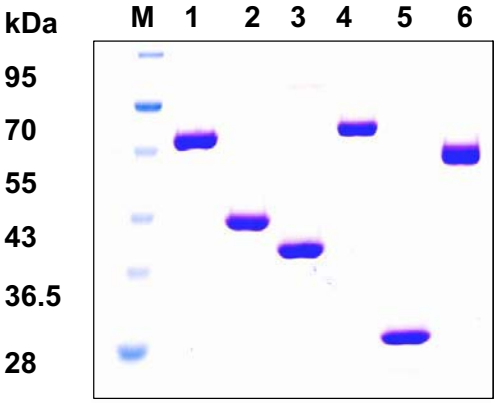
In 30 μl reaction, 1.4 μM YqeH or its derivatives was individually incubated at 37 °C in binding buffer (50 mM Tris, pH 7.6, 0.1 mM EDTA, 160 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol and 100 μg/ml BSA) and 1-100 μM GTP, 0.6 pmol(0.27 x 10^5 Bq) GTPγS ([^{35}S]GTPγS, 9.25 x 10^6 Bq/pmol; Perkim Elmer), for 15 min. 20 μl sample was removed and rapidly filtrate onto 25-mm 0.45 μM nitrocellulose filters (Millipore) by vacuum suction. The filter membrane was immediately washed with 2 ml of ice-cold Wash buffer (50 mM Tris, pH 7.6, 0.1 mM EDTA, 160 mM KCl, 1.5 mM $MgCl_2$). The filter membrane was air-dried before the YqeH.GTPγS complex was measured with liquid scintillation counter (Beckman).

2.3 RESULTS

2.3.1 Purification of the His-tagged proteins from *E. coli* cells

To analyze the GTP-binding and GTPase activities of the proteins of Obg/Era family, including all members of Obg/Era family, *E. coli* BL21(DE3)pLysS cells bearing each of the pET29b derivatives (Table 3) were grown in LB medium at 30^{o}C. When OD_{600} of the culture reached 0.6, 1 mM IPTG was added to induce the protein expression and incubation was continued for 3 hrs. The proteins were then purified using the Ni^{2+} column as described in section 2.9. Subsequently, to remove the high salt and imidazole in the elute buffer, buffer change to GTPase buffer was performed using PD10 column (Amersham Bioscience). All proteins were subjected SDS-PAGE. As shown in Fig. 9, molecular masses of the apparent His-tagged proteins agreed with their predicted masses from the amino acid sequences, except for YqeH-His proteins. YqeH-His and its derivatives migrated as a band with a molecular mass slightly higher than the mass calculated from amino acid sequences. This difference may reflect an imperfect spherical shape of the protein or alternatively, due to other unknown

reasons. Moreover, the YqeH protein was confirmed by Edman sequencing on the first 10 amino acid that located at its N-terminal (data not shown).



Lanes 1: Obg-His (57.5 kDa)
2: Bex-His (41.4 kDa)
3: YlqF-His (39.1 kDa)
4: YphC-His (56.3 kDa)
5: YsxC-His (28.1 kDa)
6: YqeH-His (45.1 kDa)

Fig. 9 SDS-PAGE analysis of purified His-tagged proteins. The respective predicted molecular mass of proteins is stated in parenthesis. All proteins were purified from *E. coli* BL21(DE3)pLysS cells containing respective pET-29b derivatives. 1 μg of each fusion protein was loaded on a SDS-polyacrylamide gel and stained with Coomasive Brilliant Blue after electrophoresis.

2.3.2 Confirmation of no interference of His fusion with biological functions of proteins

Prior to purification of these fusion proteins, the possible interference of His-tag fusion with the biological function of these proteins (Bex-His, YqeH-His, YsxC-His, YlqF-His, and YphC-His) was tested in *B. subtilis* cells. Therefore, the IPTG-inducible His-fusion proteins mutants for these proteins were constructed with pMutinT3. For instance, the case for *ysxC*-His is shown as below (Fig. 10).

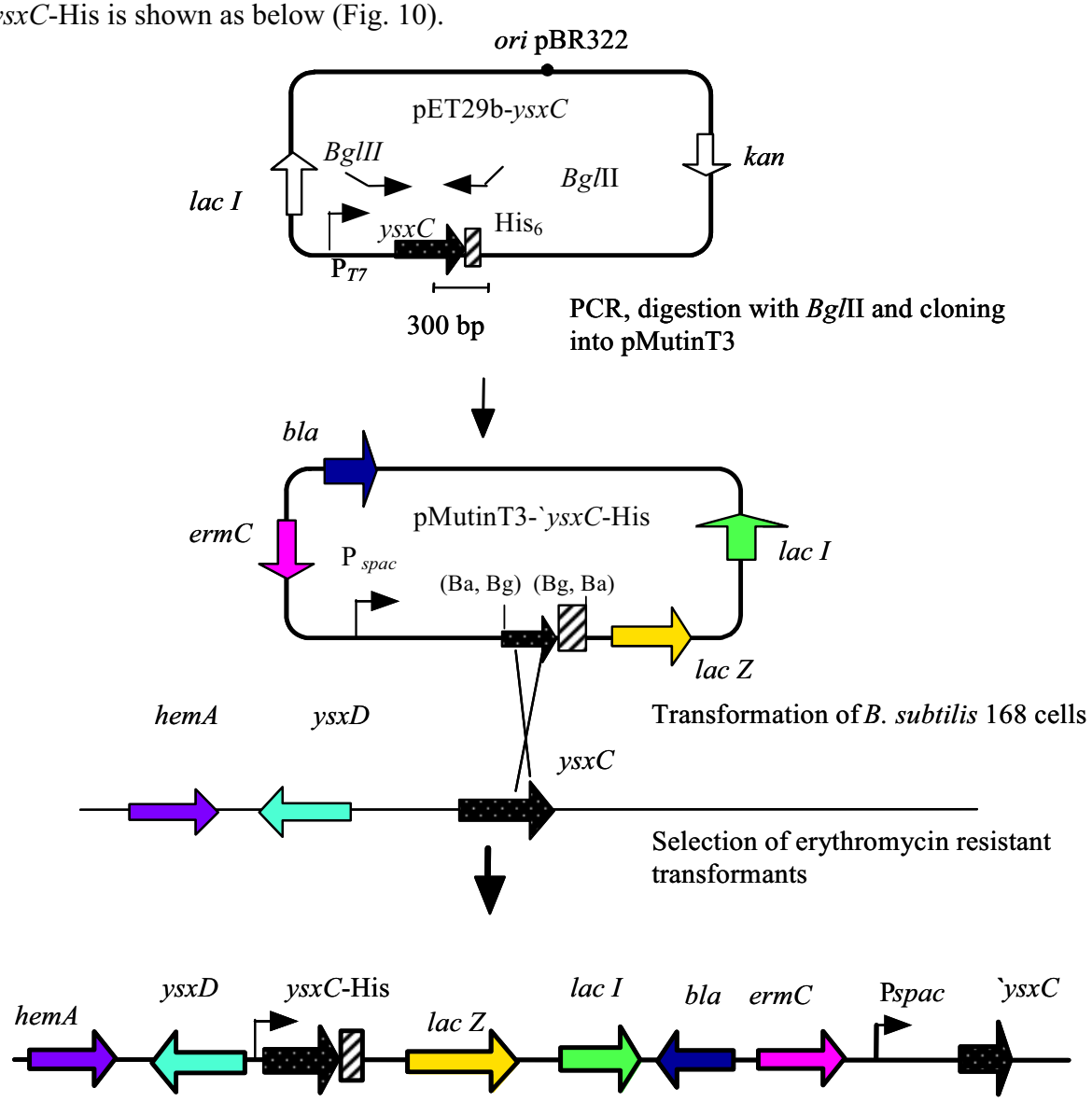


Fig. 10 Construct of *ysxC*-His mutant of *B. subitlis*. A short fragment (about 300 bp) of *ysxC*-His gene was amplified by PCR from pET29b-*ysxC* followed by digestion with *Bgl*II(Bg). Primers used in PCR are shown by bent arrows with *Bgl*II. The *Bgl*II fragment was inserted into the *Bam*HI (Ba) site of the pMutinT3. The resultant plasmid was integrated into *B. subtilis* chromosome by Campbell-type recombination. Using the same transformation, *bex*-His, *yqeH*-His, *ysxC*-His, and *ylqF*-His mutants were obtained, except for *yphC*-His.

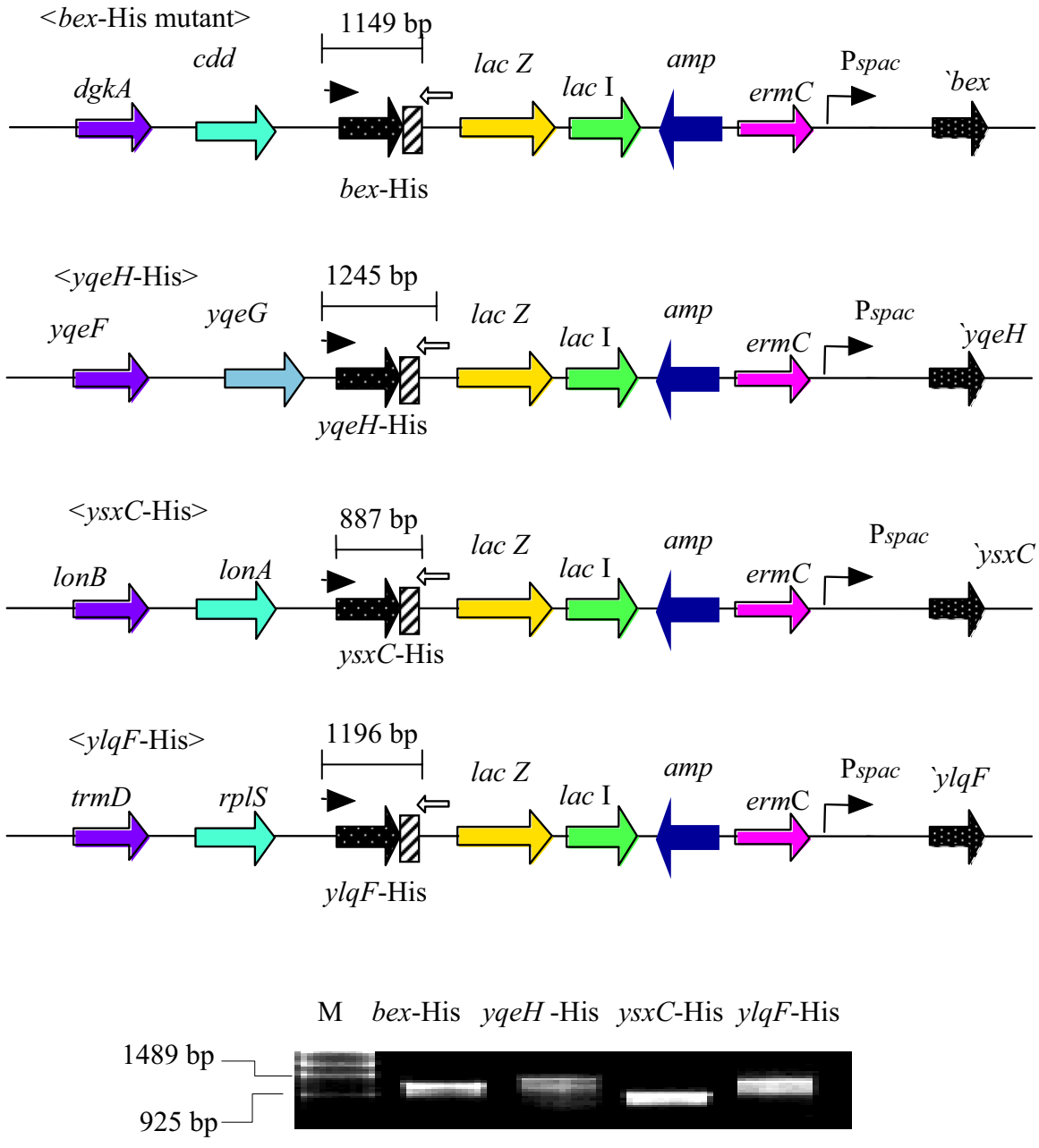


Fig. 11 Confirmation of plasmid integration for His-fusion mutants. Structure of the integration site is schematically shown for each mutant. Proper integration of the plasmid was confirmed by PCR with a primer set specific to each mutant and annealed to pMutinT3. Lengths of DNA fragments expected by PCR are also indicated. Lower panel shows the results of PCR with standard DNA marker.

Endogenous genes encoding GTPases were individually replaced by His-fusion gene in pMutinT3 vector by Campbell-type recombination (Fig. 10). The proper plasmid integration of the mutants was confirmed by PCR and sequencing (Fig 11). To examine if the fused His tag may affect the nature properties of the GTPase which may directly interfere with its biological activity, the mutants were grown in LB medium at 37°C and their growth curves were compared to the wild-type cells. These mutants (*bex*-His, *yqeH*-His, *ysxC*-His, and *ylqF*-His) showed similar doubling time to that of the wild-type cells, indicating the His-tag fusion proteins could function normally. From triplicate measurements, the doubling time of cells was 39 ± 2 min for wild type, 38 ± 2 min for *bex*-His mutant, 39 ± 2 min for *yqeH*-His mutant, 39 ± 2 min for *ysxC*-His mutant, and 40 ± 2 min for *ylqF*-His mutant under these conditions (Fig. 12).

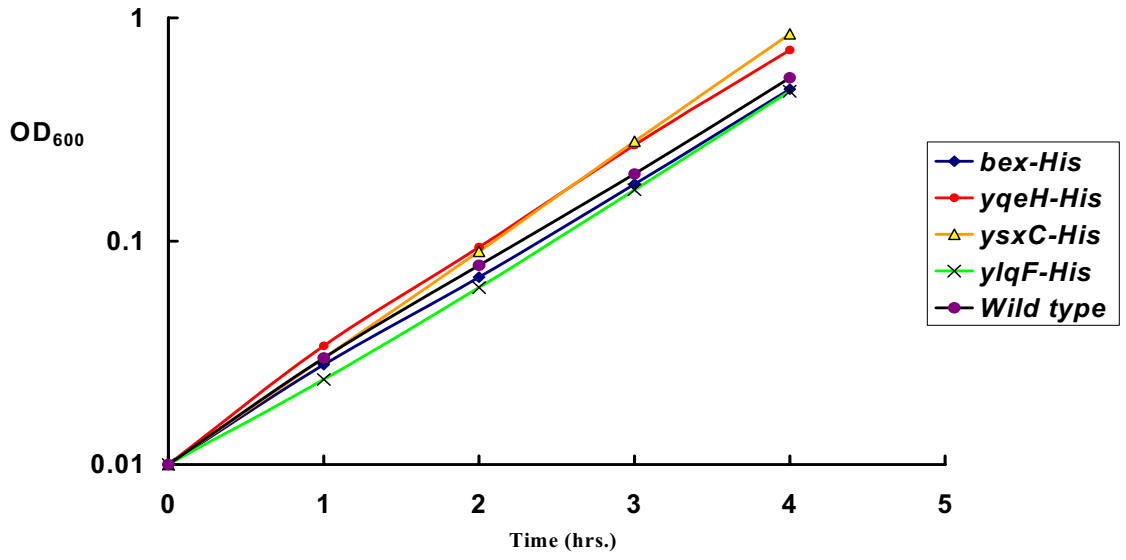


Fig. 12 His-fusion mutants grew with nearly the same rate as the wild type cells. These results indicated that His-tag fusion did not interfere with the biological activities of the GTP-binding proteins

However, *yphC*-His mutant was not obtained, probably the C-terminal fused His-tag could have adverse effects to the biological activity of YphC. Integration of pMutinT3 disrupts the operon structure of genes and would decrease the expression of genes

downstream of the integration site (Fig. 11). Since no obvious inhibition was observed in growth of each His-tagged mutant in media without IPTG supplementation, lowered expression of these genes had no problems for cell growth.

2.3.3 All proteins of Obg/Era family binds specifically to GTP and GDP

Using the conserved GTP-binding domain of *E. coli* Era as probes, we have identified six essential GTPases in *B. subtilis*, such as Obg, Era, YqeH, YsxC, YlqF, and YphC, and renamed them as the Obg/Era family. Obg had been demonstrated to bind GTP and GDP molecules (Welsh *et al*., 1994), but for the other five proteins, their guanine nucleotides binding specificity have not been proven. In this experiment, the His tag of YphC was removed by thrombin digestion since *yphC*-His mutant in *B. subtilis* was lethal. These proteins were incubated with [α-^{32}P]GTP and the nucleotide bound to the proteins were fixed by UV cross-linking. As shown in Fig. 13, all the proteins were found to bind [α–^{32}P]GTP (lane 1).

The association of these proteins with several nucleotides such as GTP, GDP, GMP, ATP, CTP, and UTP were tested by examining their ability to compete with the binding of [α-^{32}P]GTP. We found that the binding of [α–^{32}P]GTP was not affected in the presence of excess amount of competitor nucleotides such as GMP, ATP, UTP or CTP (Fig. 13; lanes 4, 5, 6, and 7, respectively); except for YqeH-His protein, where it showed weak affinity to GMP. In contrast, the binding of [α-^{32}P]GTP was apparently decreased in the presence of GDP (lane 3). Taken together, I found that all GTPases tested bind specifically to GTP and GDP molecules, but not to other nucleotides such as GMP, ATP, CTP or UTP, consistent with the *in silico* suggestion of their conserved GTP-binding domain. Therefore, Bex, YqeH, YlqF, YsxC, and YphC are proven as GTP-binding proteins.

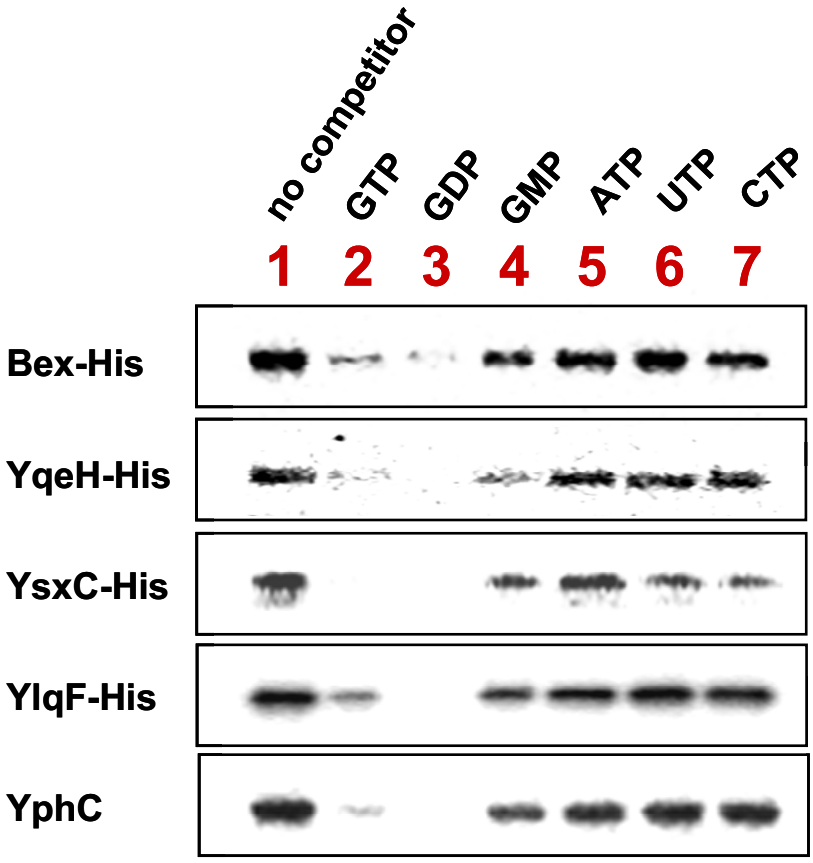


Fig.13 All five proteins bound specifically to GTP and GDP molecules *in vitro*. Nucleotide-binding competition assays were performed by mixing of [α-^{32}P]GTP with unlabelled competitor nucleotides (ratio 1:600), followed by addition of each of the proteins in GTP-binding buffer and incubation on ice for 10 min. Bound [α-^{32}P]GTP was cross-linked to proteins by UV light. Radioactive-labeled protein-GTP complexes were resolved by SDS-PAGE and radioactivity was detected with Imaging Plate and BAS 2500.

2.3.4 Kinetic parameters of the GTPase activities of members of Obg/Era family

To date, the GTPase activities of *B. subtilis* Era, YqeH, YsxC and YphC have not been experimentally characterized, except for Obg (Welsh et al., 1994) and YlqF (Matsuo et al., 2006). Therefore, the kinetic parameter of each protein was further quantitatively characterized, including K_m, *kcat* and *kcat*/K_m. Michaelis constant, K_m expresses the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$),

alternatively, the enzyme-substrate affinity. *kcat* is rate constant of GTP hydrolysis, *kcat*/K_m measures the enzyme efficiency to bind and hydrolyze GTP.

In this experiment, [γ-^{32}P] GTP is converted to GDP and $^{32}P_i$ upon hydrolysis, in which [γ-^{32}P] GTP and GDP would selectively bind to the active charcoal. Free $^{32}P_i$ released in supernatant was quantified with Cerenkov counting. As control, background free Pi was subtracted without addition of GTPase. Fig. 14 showed the representative result of GTPase activities of proteins in 20 μM GTP reaction. We demonstrated that the five purified wild type proteins are able to hydrolyze GTP *in vitro,* except for YsxC. YlqF revealed very slow intrinsic GTPase activity, and it was incubated at 37^{o}C for ease of experimental measurement. YqeH showed the highest GTPase activity among these proteins, followed by YphC, Era, Obg and YlqF.

To obtain the kinetic rate constants K_m and k_{cat} for each protein, the GTPase activity at different five GTP concentrations ranged 1–40 μM were measured. The K_m and k_{cat} values derived from Lineweaver-Burk plots (Fig.15) are summarized in Table 8. Data shown is an average of three independent experiments. For ease of GTPase activities comparison, the enzyme efficiencies are represented as histogram (Fig. 16). The rate constant, *kcat* obtained for each of the proteins in increasing order are as follows: YlqF (0.015 min^{-1}), Obg (0.021 min^{-1}), Era (0.039 min^{-1}), YphC (0.463min^{-1}) and YqeH (0.934 min^{-1}). Two proteins, YqeH and YphC, showed unexpectedly high GTP hydrolysis rate, distinct from the group of YlqF, Obg and Era. The intrinsic GTPase activity of YsxC was relatively low to produce an adequate experimental signal in our GTPase assay. In such case, it has not been possible to determine either K_m or V_{max} accurately for YsxC.

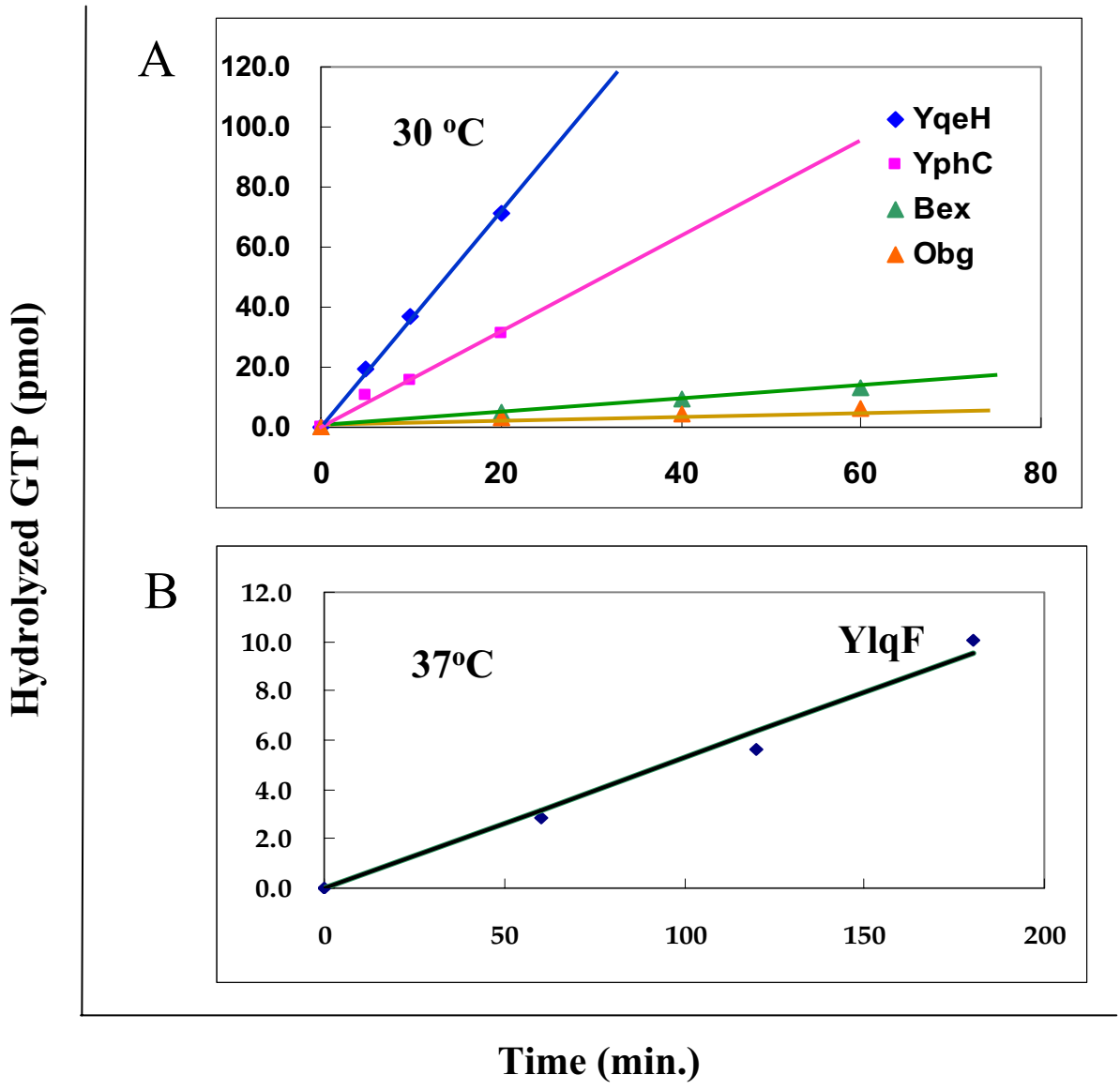


Fig.14 Time course of GTPase activities for various GTPases. 0.7 μM each of the protein was incubated in reaction 20 μM GTP, sampling at the indicated times. The amount of free Pi released from the hydrolysis of [γ-^{32}P]GTP was quantified using Cerenkov counting. Data shown is an average of three independent experiments. YlqF possesses slow intrinsic GTPase activity, the reaction was performed at 37°C for ease of measurement.

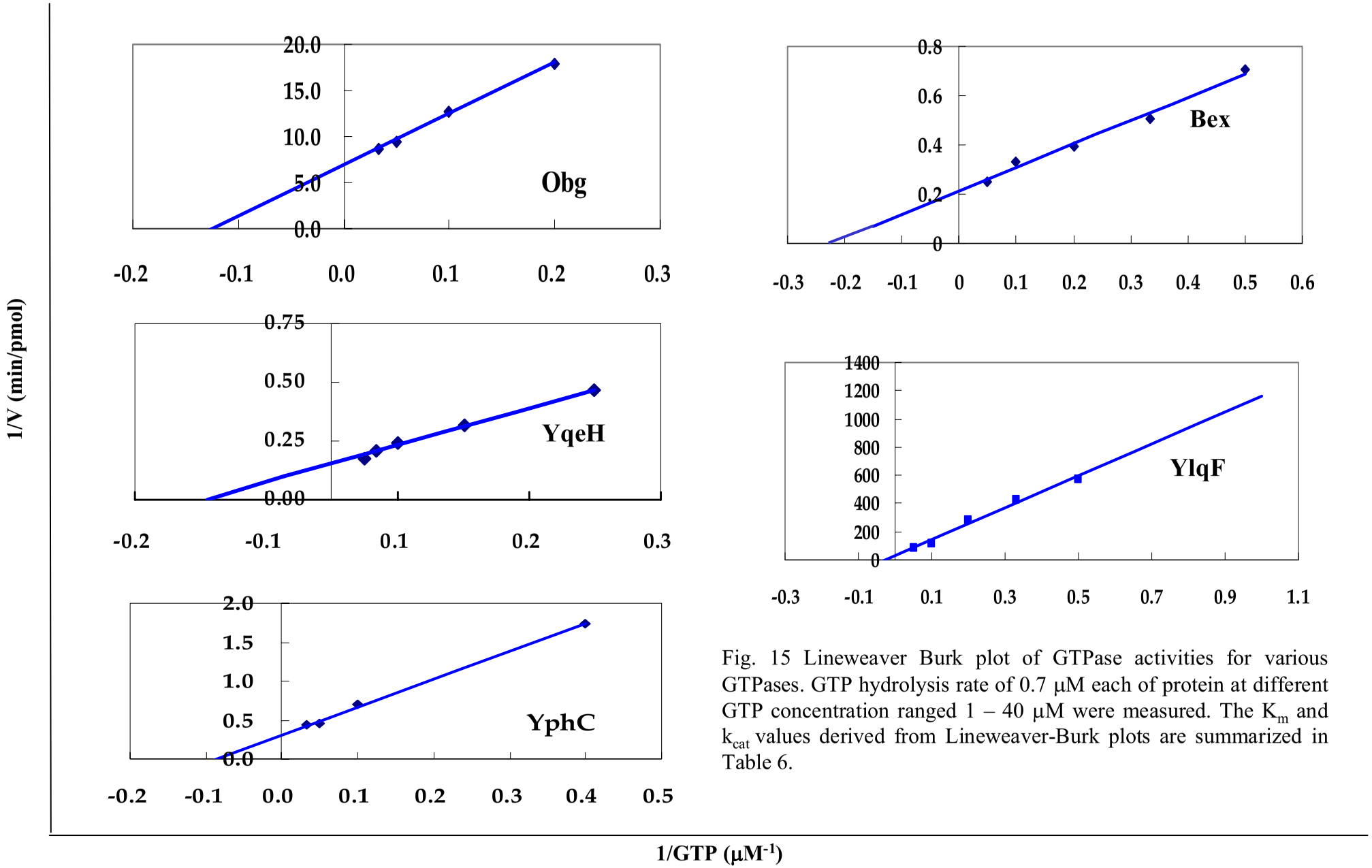


Fig. 15 Lineweaver Burk plot of GTPase activities for various GTPases. GTP hydrolysis rate of 0.7 μM each of protein at different GTP concentration ranged 1 – 40 μM were measured. The K_m and k_{cat} values derived from Lineweaver-Burk plots are summarized in Table 6.

Table 6 Summary of kinetic parameters of the GTPase activities

Protein	K_m (μM)	V_{max} (pmol/min)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)	Relative k_{cat}/K_m
Obg	8.11	0.145	0.021	2.55×10^{-3}	2.83
Era	5.24	0.398	0.039	7.50×10^{-3}	8.31
YphC	11.60	3.244	0.463	40.0×10^{-3}	44.30
YqeH	10.35	6.539	0.934	90.2×10^{-3}	100.00
*YlqF	4.03	0.067	0.015	3.62×10^{-3}	4.01
YsxC	below detection limit				

Data shown is an average of three independent experiments. The GTPase assays were conducted at 30°C, except for YlqF* at 37°C, due to its low intrinsic GTPase activity

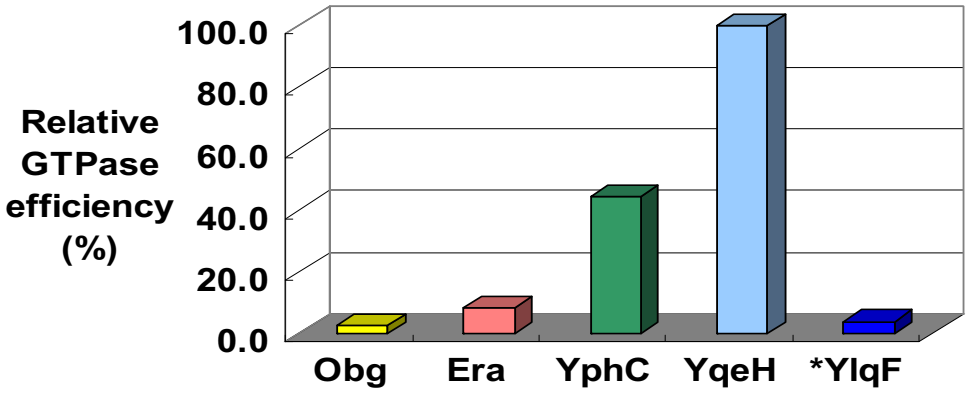


Fig. 16 Relative GTPase efficiencies, *kcat*/K_m for the members of Obg/Era family. The GTPase activity of each protein arranged in the increasing order is YlqF, Obg, Era, YphC and YqeH. YsxC with its GTPase activity below detection limit, was precluded.

2.3.5 The GTPase activities of Obg and Era were individually stimulated by 50S and 30S ribosomal subunits, respectively

Since some GTPases of the Obg/Era family is associated with ribosome subunits, thus, the effect of ribosomal subunit toward the GTPase activities of each protein was examined. In addition of YlqF that its slow intrinsic GTPase activity was enhanced by 50S ribosomal subunit (Matsuo et al., 2006), I found that the GTPase activity of Obg was also enhanced by 50S subunit, with an increase about 5-fold (Fig. 17A), consistent with the reports that Obg orthologs cosediment with 50S subunit, either in *E. coli*, *C. crecentus* or *Vibrio harveyi* (Lin et al., 2004; Wout et al., 2004; Sato et al., 2005; Sikora et al., 2006). On the other hand, the slow intrinsic GTPase activity of Era was stimulated nearly 15-fold by 30S subunit (Fig. 17B). To the best of my knowledge, this is the second protein that shows its GTPase activity was accelerated by 30S subunit, after *E. coli* YjeQ (Daigle and Brown, 2004; Himeno et al., 2004). Likewise, both Era and YjeQ of *E. coli* are known to associate with 30S ribosomal subunit (Sayed et al., 1999; Daigle and Brown, 2004). For the case of YqeH, YphC and YsxC, their GTPase activities remained unaffected in the presence of either 30S or 50S subunit (Fig. 17C, D & E). Interestingly, both YqeH and YphC possess high intrinsic GTPase activities, which are comparable to the ribosome-accelerated GTPase activities of Obg and Era, suggesting that YqeH and YphC are relatively efficient GTPases, with high GTP hydrolysis rate compared to Obg or Era. YsxC showed almost abolished GTPase activity even in the presence of 30S or 50S subunit.

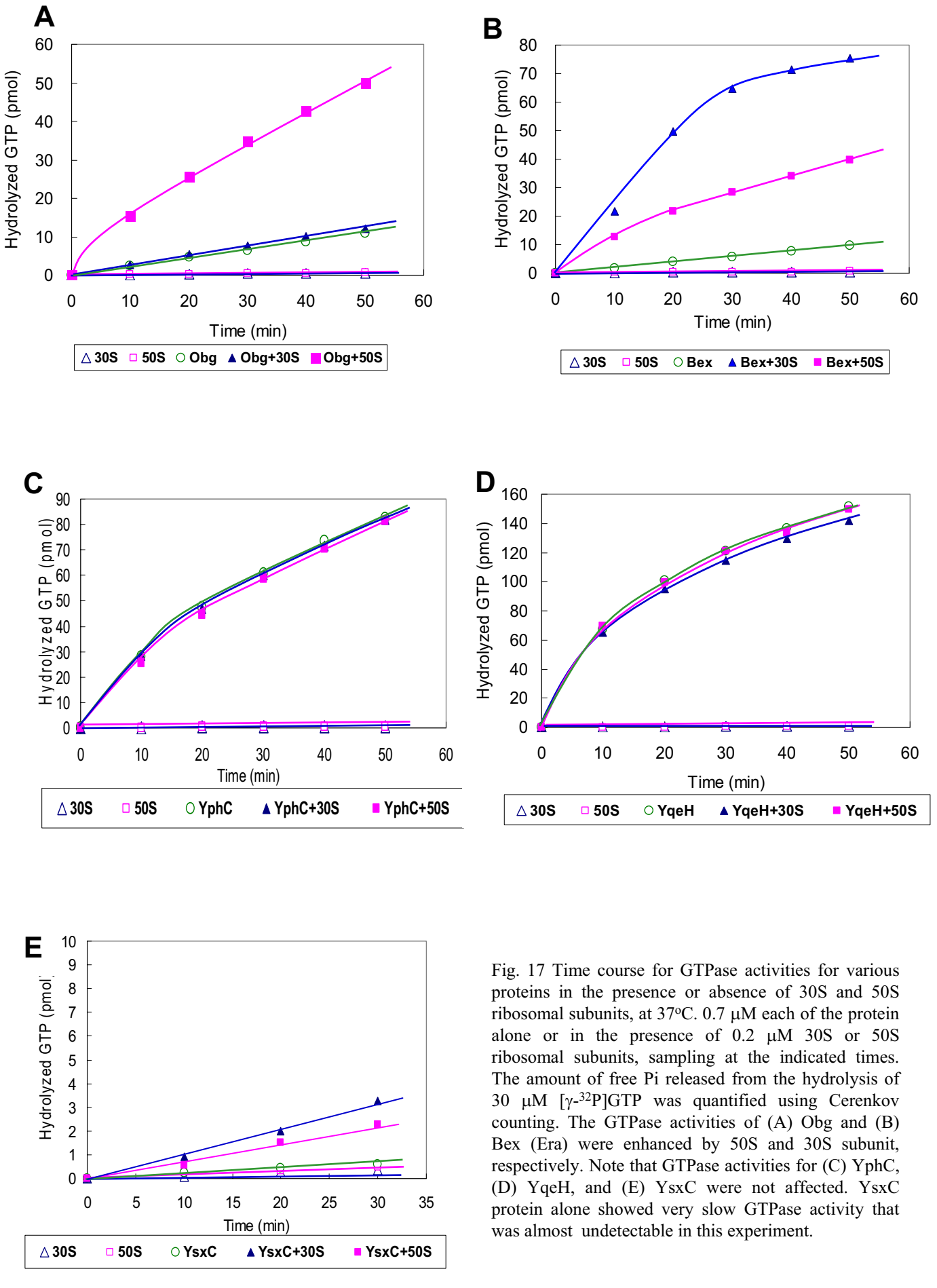


Fig. 17 Time course for GTPase activities for various proteins in the presence or absence of 30S and 50S ribosomal subunits, at 37°C. 0.7 μM each of the protein alone or in the presence of 0.2 μM 30S or 50S ribosomal subunits, sampling at the indicated times. The amount of free Pi released from the hydrolysis of 30 μM [γ-^{32}P]GTP was quantified using Cerenkov counting. The GTPase activities of (A) Obg and (B) Bex (Era) were enhanced by 50S and 30S subunit, respectively. Note that GTPase activities for (C) YphC, (D) YqeH, and (E) YsxC were not affected. YsxC protein alone showed very slow GTPase activity that was almost undetectable in this experiment.

2.4 DISCUSSION

We demonstrated here that five proteins of Obg/Era family, Obg, Era, YqeH, YphC and YlqF are able to hydrolyzed GTP *in vitro,* except for YsxC. Among these, Era, Obg and YlqF exhibited a slow intrinsic GTP hydrolysis rate and GTP dissociation constant, K_m in micromolar range. These kinetic parameters are comparable to those of *E. coli* Era*, C. crecentus* CgtA and eukaryotic Ras. However, YqeH (*kcat* =0.934 min^{-1}) and YphC (*kcat* =0.463 min^{-1}) showed relatively high GTPase activities, with respective 20 and 10 folds higher than that of Era (*kcat* = 0.039 min^{-1}). On the other hand, when compared to the *kcat* of proteins with high GTPase activities such as 10.2 min^{-1} for *E. coli* TrmE (Yim et al., 2003), the GTPase activity of YqeH is much lower. Therefore, the properties of YqeH and YphC are distinct from the Obg/Era family and TrmE.

B. subtilis Obg, Era, YlqF and *E. coli* YjeQ showed GTPase activities that were accelerated by ribosomal subunits, shared one common feature. These proteins revealed a slow rate of intrinsic GTP-hydrolysis activities, suggesting that they are ineffective GTPase catalysts, required active participation of either 50S or 30S subunits, which acts as GAP to achieve fast GTP hydrolysis. A GAP function is demonstrated by elongation factor Tu (EF-Tu). Its almost unmeasurable intrinsic GTPase activity is stimulated dramatically by the mRNA-primed 70S ribosome, with the C-terminal domain of the L7/L12 protein of the 50S subunit presumably acting as an EF-Tu GAP (Liljas and Alkaradaghi, 1997). The single, highly conserved arginine residue in the C-terminal domain of L7/12 is not essential for the activation, excluding an “arginine finger”-type mechanism reported for Ras superfamily (Vetter and Wittinghofer, 1999). Rather, L7/12 seems to function by stabilizing the GTPase transition state of EF-G, in a similar way as the Gα subunits of the heterotrimeric G proteins are activated by regulators of G protein signaling (RGS), by accelerating the conformational

change for intrinsic GTPase reaction (Sprang, 1997).

On the other hand, the molecular mechanisms for the acceleration of GTPase activities of Obg and Era by 50S and 30S subunit, respectively, and their functional consequences, remain to be elucidated. Further studies by isolating the precursors of ribosomal subunit of Obg- or Era-depleted cells, and identification of the r-proteins that are missing, will provide clues in which step the ribosomal assembly are inhibited, respectively. In addition, cross-linking of the GTPase to the ribosomal subunit and RNA footprinting to detect the binding sites of GTPase to the respective rRNA may shed light on the consequences of ribosome-GTPase interaction.

YlqF is one of the most advance studied GTPases of Obg/Era family, is known to associate with the premature 50S subunit and participates in the late step of 50S subunit assembly, (Uicker et al., 2006; Matsuo et al., 2006; 2007). Only the GTP-bound YlqF is able to bind to the 50S subunit, where its GTPase activity was enhanced, and the GDP-bound YlqF is subsequently dissociate from the 50S subunit. The GTP-hydrolysis of YlqF was suggested to trigger a conformational change in the 23S rRNA that allowed the incorporation of late assembly proteins such as L16 and L27 into the premature 50S particle (Matsuo et al., 2007). Further analysis on the conformational changes of 23S rRNA, before and after association of GTP-bound YlqF, may serve as key question to support this hypothesis.

YqeH revealed an intrinsic GTPase activity with a $kcat/K_m$ of more than 30-fold higher than that of YlqF. In addition, no association of YqeH to 30S or 50S subunits was detectable so far, which may imply that the regulation of GTPase activity for YqeH is distinguished from that of Obg/Era family. Furthermore, the transient GTP-bound form of YqeH, owing to its high GTPase activity, make it impossible to detect a ‘transient’ interaction with 30S intermediates, if any, using the sucrose density gradient ultracentrifugation or gel filtration methods.

In yeast, Bms1 is a GTPase that required for the biosynthesis of 40S ribosomal subunit, and has an intramolecular GAP in C-terminus with a novel GTPase activating domain (Karbstein et al., 2005). Moreover, previous work has implicated two amino acid residues, arginine and glutamine are being directly involved in the GAP-activated GTP hydrolysis for Ras, Rho and Rac (Sprang, 1997). The C-terminus of YqeH exhibits an arginine-rich region, it is therefore of interest to investigate the possible GAP domain, as its N and C-terminus are domains with unassigned functions.

On the other hand, YsxC exhibited almost undetectable GTPase activity in our experimental condition. Two groups of scientists who work on YsxC (Brown, pers. comm., 2003; Wicker-Planquart, pers. comm., 2007) also encountered the similar property for YsxC protein. At present, we could not rule out the low GTPase activity of YsxC observed *in vitro*, reflecting the nature of YsxC that required a GAP for GTP hydrolysis *in vivo*, or due to the protein instability after overexpression and purification from *E. coli*.

CHAPTER 3

Functional Characterization of YqeH

3.1 Introduction

B. subtilis YqeH is a unique GTP-binding protein, as its homologs are widely found among the Gram positive bacteria and higher eukaryotes. However, study of YqeH or its homologs has not been reported to date. Here, we present a detailed functional analysis of YqeH. I found YqeH is involved in the 30S ribosomal subunit biogenesis. A reduction in both 70S ribosome and free 30S subunit was observed, consistent with the presence of 16S rRNA precursor and its degraded products in YqeH-depleted cells. Moreover, the 30S subunit formation was closely dependent on the YqeH expression level in cell.

I focused on the functional analysis of YqeH, and introduced single point mutations, aiming to generate dominant negative forms of YqeH which partially lost its functions, by site-directed mutagenesis. *In vitro* biochemical characterization showed that YqeH-S193P and YqeH-S173N lost severely their intrinsic GTPase activities but maintained 70% and 50% of their GTP-binding abilities, respectively. Interestingly, replacement of the endogenous YqeH with either YqeH-S173N or S193P was found to be lethal. Therefore, it is interesting to investigate the consequences of overexpression these mutant proteins to compete with the biological activity of endogenous YqeH *in vivo,* and observe for interesting cell phenotypes in the near future. In future work, a slower GTPase activity of these mutants may help to get a 'snapshot' on the possible identification of downstream effectors of YqeH.

Yeast two hybrid analysis showed that YqeH interacted with S4, a component of the 30S subunit that is essential for cell growth in *B. subtilis*. S4 possesses multiple roles which intimately involved in both ribosome regulation and functions. In *E. coli*, S4 binds to the precursor of 16S rRNA, and serves as one of the two nucleation sites during the assembly of 30S subunit (Nowotny et al., 1988). Moreover, S4 is shown to act as a general

anti-termination factor in transcription reactions *in vitro* (Torres et al., 2001). S4 also regulates its own synthesis by negative feedback mechanism, binds to the α operon mRNA that encodes S11, S13, and L17 in addition to S4 (Deckman and Draper, 1985). Furthermore, mutations in S4 have been shown to alter the translational accuracy of the ribosome (Rosset and Gorini, 1969). At present, the confirmation of YqeH-S4 interaction using an alternative method is underway. The YqeH mutant proteins that did not interacted with S4, such as S173N and S193P were identified. These mutant proteins maintained GTP-binding ability but their GTPase activities were abolished, suggesting that YqeH in GDP-bound form interacted with S4. I am looking forward to study the mechanism of YqeH-S4 interaction, by using these mutants to compete with the endogenous YqeH, in order to elucidate their possible direct roles in regulating the biogenesis of 30S subunit. Collectively, our results suggest that YqeH is important for the biogenesis of 30S ribosome subunit.

3.2 MATERIALS AND METHODS

3.2.1 Strains, plasmids and primers

Microorganism strains, plasmids and primers used are listed in Tables 7, 8 and 9, respectively.

Table7 Strains of microorganism

Strain	Genotype	Source
B. subtilis		
CRK6000	*purA16 met5B hisA3 guaB*	laboratory stock
TMO309	*purA16 metB5 hisA3 guaB* *yqeH*:: pTM101 (P_{spac}-*yqeH erm*)	Morimoto et al., 2002
LPC106	*purA16 metB5 hisA3 guaB* *yqeH*:: pPC202 (P_{spac}-*yqeH* T168V *erm*)	this study
LPC107	*purA16 metB5 hisA3 guaB* *yqeH*:: pPC203 (P_{spac}-*yqeH* S173N *erm*)	this study
LPC108	*purA16 metB5 hisA3 guaB* *yqeH*:: pPC204 (P_{spac}-*yqeH* S193N *erm*)	this study
TMO309C	*purA16 metB5 hisA3 guaB* *yqeH*:: pTM101 (P_{spac}-*yqeH erm*), pNO41GW	Matsuo, unpublished
TMO309B	*purA16 metB5 hisA3 guaB* *yqeH*:: pTM101 (P_{spac}- *yqeH erm*), pNO41GW-*era*	Matsuo, unpublished
TMO309Q	*purA16 metB5 hisA3 guaB* *yqeH*:: pTM101 (P_{spac}-*yqeH erm*), pNO41GW-*yqeH*	Matsuo, unpublished
TMO101	*purA16 metB5 hisA3 guaB* *era*:: pTM101 (P_{spac}-*era* *erm*)	Morimoto et al., 2002
TMO101C	*purA16 metB5 hisA3 guaB* *era*:: pTM101 (P_{spac}-*era* *erm*), pNO41GW	Matsuo, unpublished
TMO101B	*purA16 metB5 hisA3 guaB* *era*:: pTM101 (P_{spac}-*era* *erm*), pNO41GW-*era*	Matsuo, unpublished
TMO101Q	*purA16 metB5 hisA3 guaB* *era*:: pTM101 (P_{spac}-*era* *erm*), pNO41GW-*yqeH*	Matsuo, unpublished

Strain	Genotype	Source
E. coli		
DH5α	F$^-$ ϕ80d *lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17 (r_K^- m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 λ^- *gyr*A96 *rel*A1	laboratory stock
BL21(DE3)	F$^-$ *omp*T *hsd* S_B(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
S. cerevisiae		
PJ69-4a	*MATa, trp1-901, leu2-3, 112, ura3-52,his3-200, gal4Δ, gal80Δ, LYS2::GAL1 -HIS3, GAL2-ADE2, met2::GAL7-lacZ*	James et al., 1996
PJ69-4α	*MATa, trp1-901, leu2-3, 112, ura3-52,his3-200, gal4D, gal80D, LYS2::GAL1 -HIS3, GAL2-ADE2, met2:: GAL7-lacZ*	James et al., 1996

Table 8 Plasmids

Plasmid	Genotype	Source
Used for purification of GTP-binding proteins		
pET29b	pBR322 *ori* P_{T7} *lacI* His_6 *Kan*	Novagen
pTM299	pET29b-*yqeH*	Morimoto et al., 2002
pPC202	pET29b-*yqeH* T168V	this study
pPC203	pET29b-*yqeH* S173N	this study
pPC204	pET29b-*yqeH* S193P	this study
Used for yeast two-hybrid analysis		
pGADT7	*GAL4* AD, *LEU2*, amp^r, HA epitope	Clontech
pGBT9	*GAL4* DNA-BD, *TRP1*, amp^r	Clontech
Used for YqeH and Era functional complementation test		
pPC201	pAPNC213 with full length *yqeH* (1098 bp) and SD	this study
pPC202	pAPNC213 with full length yqeH T168V and SD	this study
pTM101	pAPNC213 with full length *era* (903 bp) and SD	Morimoto et al., 2002
pNO41	multicopy plasmid with constitutively expressed promoter	Ogasawara et al., 1984
pNO41GW	Gateway version of pNO41	K. Tanaka
pNO41GW-*era*	full length-era (903 bp) with SD region	Matsuo, unpublished
pNO41GW-*yqeH*	full length-*yqeH* (1098 bp) with SD region	Matsuo, unpublished

Table 9 Primers

Primer	Restriction site	Primer sequence
Used for purification of His fusion GTP-binding protein mutants from *E. coli*		
yqeH-F0	*NdeI*	TGTCATATGGAAAAGGTTGTTTGTATCGGG
yqeH-R0	*Bam*HI	GGAGGATCCAGAATTAATGAACGCCGAAC
rpsD-F	*NdeI*	GCGCATATGATGGCTCGCTATACAGGTCC
rpsD-R	*XhoI*	GCGCTCGAGGGAACCGCGTGGCACCAGACGAGAGTA GAACTCAACG
pET-Seq F		CCGCGAAATTAATACGACTC
pET-R		GCAGCCGGATCTCAGTGGTG
Used for constructs of IPTG-inducible P*spac-yqeH* mutants in *B. subtilis*		
domF	*Bam*HI	CGCGGATCCCGAAAAGGGCACATTCAGTG
domR	*Eco*RI	CCGGAATTCTTAAATTAATGAACGCCGAAC
YqeH T168V-F		GTTGTCGGATGT**GTA**AATGTAGGAAAG
YqeH T168V-R		CTTTCCTACATT**TAC**ACATCCGACAAC
YqeH S173N-F		GTAGGAAAG**AAC**ACCTTTATTAACC
YqeH S173N-R		GGTTAATAAAGGT**GTT**CTTTCCTAC
YqeH S193P-F		GATATTATTACAACA**CCT**CAATTTCCTG
YqeH S193P-R		CAGGAAATTG**AGG**TGTTGTAATAATATC

[a] reverse primer used for amplification of inserts paired with *bex*-F, *yqeH*-F and *ysxC*-F
[b] reverse primer used for amplification of inserts paired with *ylqF*-F, *yphC*-F
Underlined sequences correspond to restriction enzyme digestion site; bolded sequences denoted the codon for introduced single point mutation

Used for yeast two-hybrid screening

pGBT9-F	AGACAGTTGACTGTATCG
pGBT9-R	CAAGCTAATTCCGGGCGA
prey-F	CTATTCGATGATGAAGATACCCCACCAAACCC
prey-R	GCAAGTTCACTTCAACTGTGCATCGTGCACCAT
pDONR-F	TCGCGTTAACGCTAGCATGGATCTC
pDONR-R	GTAACATCAGAGATTTTGAGACAC
*yqeH*gw-F	AAAAAGCAGGCTCGATGGAA AAGGTTGTTTGTATCG
*yqeH*gw-R	AGAAAGCTGGGTCCTAAATTAATGAACGCCGAAC

Primer	sequences
rpsD-gwF	AAAAAGCAGGCTCGATGGCTCGCTATACAGGTCC
rpsD-gwR	AGAAAGCTGGGTCGAATTAACGAGAGTAGAACTCAACGA
ytrP-gwF	AAAAAGCAGGCTCGATGGTAGAACAAACTAAAGATC
ytrP-gwR	AGAAAGCTGGGTCCTATTTTATTGAGTCATGAATCATC
nupC-gwF	AAAAAGCAGGCTCGATGAAGTATTTGATTGGG
nupC-gwR	AGAAAGCTGGGTCCTAGTAAATCAAGCCCACAATTGC
[1]*cca*-gwF	AAAAAGCAGGCTCGATGGAAAAAGTTTTTATCAAAG
cca-gwR	AGAAAGCTGGGTCTCTTAATGTTGACCGCATG
[2]*trmU*-gwF	AAAAAGCAGGCTCGGTGAATGAAATGGAAAAACG
trmU-gwR	AGAAAGCTGGGTCTTTATACGTACCACAATTTTG
[3]*rnc*-gwF	AAAAAGCAGGCTCGATGTCAAAACACTCACATTATA
rnc-gwR	AGAAAGCTGGGTCGGATTTTATTGTTTCGTATGG
[4]*trmD*-gwF	AAAAAGCAGGCTCGATGAAAATCGATTTTTTGACG
trmD-gwR	AGAAAGCTGGGTCCTATTCTTTTTCCCATTCAG
[5]*cspR*-gwF	AAAAAGCAGGCTCGGTGAACATTGTGGCATTAC
cspR-gwR	AGAAAGCTGGGTCTTTCACTATTTTAAATCACGG
[6]*rnpA*-gwF	AAAAAGCAGGCTCGTTGAAGAAGCGAAATCGTTT
rnpA-gwR	AGAAAGCTGGGTCGTGATGCCTACTTTGACG
[7]*yqeT*-gwF	AAAAAGCAGGCTCGTTGAAATGGTCCGAATTAAGC
yqeT-gwR	AGAAAGCTGGGTCCTATTTTTTCGCAATAATGCTGAC
[8]*yabR*-gwF	AAAAAGCAGGCTCGATGTCGATTGAAGTTGGCAGC
yabR-gwR	AGAAAGCTGGGTCCTATCCTCTTCTTGCTCCGCGCC

[9]*truB*-gwF	AAAAAGCAGGCTCGATGGTTAACGGAGTTCTCC
truB-gwR	AGAAAGCTGGGTCCTATTGTTCGCTTTTTTGCATCAATAC
[10]*ylqE*-gwF	AAAAAGCAGGCTCGATGACAAAGCGATGGTTTAATG
ylqE-gwR	AGAAAGCTGGGTCCTATTCGTCTATTAACCCTTCC

Underlined sequences correspond to partial of the attB1 and attB2 sites for Gateway Technology recombination for forward and reverse primer, respectively
The superscript numbering (from 1 to 10) denoted genes that related to ribosomal RNA processing or modification

3.2.2 Association and dissociation buffers

Diethyl pyrocarbonate (DEPC)-treated water was used to avoid potential RNA degradation throughout the experiment. DEPC and proteinase inhibitors, PMSF, aprotinin, leupeptin and pepstatin A, were purchased from Sigma-Aldrich, and the RNase inhibitor was from Takara Bio Inc. Proteinase and RNase inhibitors were added to the buffers for cell lysis, sucrose gradient density centrifugation and rRNA analyses at concentrations specified by the manufacturers. Association (20 mM Tris, pH 7.6, 8 mM $MgCl_2$, 30 mM NH_4Cl and 2 mM β-mercaptoethanol) and dissociation (20 mM Tris, pH 7.6, 1.5 mM $MgCl_2$, 120 mM NH_4Cl and 2 mM β-mercaptoethanol) buffers were used for ribosome profile analyses.

3.2.3 Sucrose gradient ribosome profile analyses

Glycerol stocks of TMO309 and TMO101 were streaked on Penassay Antibiotic medium 3 (PAB) agar supplemented with 50 μM and 100 μM IPTG, respectively, displayed a similar doubling time to the wild-type strain. Colonies cultured overnight of TMO309 were inoculated into two 300 ml aliquots of fresh PAB medium supplemented with 0 and 20 μM IPTG at OD_{600} of 0.01. Cells were grown at 37°C with agitation until OD_{600} of 0.3 (Fig. 20) or 0.5 (Fig. 21). Wild-type *B. subtilis* was used as the control. The cells were harvested by centrifugation at 11,900 x *g* for 5 min. at 4°C. Cell pellets were washed with chilled 10 ml

association buffer, resuspended in 4 ml of the same buffer, and subjected to three passages through a French pressure cell (8000 psi). After the removal of cell debris, an aliquot of supernatant equivalent to 18 A_{260} units was layered over 12 ml of 10-50% sucrose gradient. The gradient was centrifuged at 192,100 x *g* at 4°C for 4.5 h. Ribosome profiles in the gradients were examined by monitoring absorbance at 254 nm (BioComp Gradient Station with ATTO UV monitor AC-5200). The relative ratio of free 30S to 50S under dissociation condition was calculated from areas under the absorbance tracing of the respective ribosomal subunit peaks.

3.2.4 Immunoblotting

Proteins were separated by SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Bioscience). The following antibody dilutions were used: anti-YqeH, 1:7000 (Morimoto et al. 2002), and anti-rabbit IgG-HRP conjugated secondary antibody, 1:7000 (BioRad 170-6515). Chemiluminescent signals (ECL kit, Amersham) were detected using X-ray film (Fuji Film, Japan).

3.2.5 rRNA analyses

B. subtilis CRK6000 and TMO309 cells were grown in PAB liquid medium at 37°C, in the absence of IPTG. Cells were lysed as described in ribosome profile analyses. Total rRNA was extracted using Isogen reagent (Nippon Gene), following the manufacturer's instructions. The RNA concentration was quantified with a UV spectrometer at an absorbance of 260 nm. Total rRNA (1 μg) from wild-type and TMO309 cells was resolved on a 1.0% agarose gel in 1x MOPS buffer with 2.5 M formaldehyde at 120 V for 3 h. Separated RNA was transferred to a Hybond-N membrane (Amersham Biosciences) by capillary transfer in 10x SSC buffer for 12-18 h. RNA was fixed to the membrane by UV cross-linking (UV Stratalink, 1200 μJoule),

and the transfer efficiency confirmed by methylene blue staining.

RNA probes for Northern hybridization were transcribed *in vitro* with T7 RNA polymerase and a digoxigenin-labeled mix kit (Roche), according to the manufacturer's instructions. Residual genomic DNA and oligonucleotides used to generate RNA were removed by incubation with DNase I (Amersham Biosciences). The oligonucleotides are listed below:

Table 10 DNA oligonucleotides used to generate RNA probes for Northern blotting

Oligo	Sequence
Probe 1	
16S-F	CTGTAACTGACGCTGAGGAGCG
16S-R	TAATACGACTCACTATAGGGCGACATGCACCACCTGTCACTCTGC
Probe 2	
5' p16S-F	ATCGCACTGCGATGTGCGTATC
5' p16S-R	TAATACGACTCACTATAGGGCGATGGATCACAGGTTAAGTTC
Probe 3	
3' p16S-F	AAGGATATTATACGGAATAT
3' p16S-R	TAATACGACTCACTATAGGGCGAAAACTAAACAAGACAGGGAACG

Underlined nucleotides depicted the binding region of T7 RNA polymerase

The rRNA hybridization reactions were performed in high SDS hybridization buffer [0.75 M NaCl, 0.075 M sodium citrate; 50% formamide, 0.5 M sodium phosphate buffer, pH 7.2, 0.1% N-lauroylsarcosine, 7% SDS, and 2% blocking reagent (Roche)] at 68^{o}C for 16-18 h, followed by two 15 min washes in 2x SSC at room temperature. The membrane was washed twice at 68^{o}C with 0.1x SSC for 15 min, and rinsed for 5 min in wash buffer (100 mM maleic acid, 150 mM NaCl and 0.3% Tween 20). After incubation in 1% blocking reagent for 30 min, membranes were probed with a sheep anti-DIG alkaline phosphatase conjugate (1:10,000 dilution) for 1 h. Unbound antibody was removed by two 15 min washes with wash buffer. The membrane was equilibrated in detection buffer (100 mM Tris-HCl, pH 7.5, 100

mM NaCl, and 50 mM $MgCl_2$) for 5 min, followed by incubation with chemiluminescent substrate CSPD ready-to-use (Roche). Hybridized bands were detected with X-ray film (Fuji Film) after 1-10 min exposure.

3.2.6 Function complementation tests

Invitrogen's Gateway Technology was applied for the entire cloning, according to the manufacturer's instructions. Full-length *yqeH* and *era* with the respective Shine-Dalgarno regions were individually cloned into the entry plasmid, pDONR201, generating pDONR-*yqeH* and pDONR-*era*, respectively, using the method as previously described (Ishikawa et al., 2006). The following primers were used for amplification: *yqeH*-F (AAAAAGCAGGCTCGTTCAGTGGGAGGAGTAAGAA) and *yqeH*-R (AGAAAGCTGGGTCTTCTTAAATTAATGAACGCCG) for *yqeH*; and *era*-F (AAAAAGCAGGCTCGGGCGCATTTTCATCGGAGGA) and *era*-R (AGAAAGCTGGGTCAGAGATTATATTCGTCCTC) for *era*. The underlined sequences depict the *att* sequence required for the recombination reaction. pNO41, a multicopy plasmid with a constitutively active promoter（Ogasawara et al., 1984), was converted to a Gateway destination vector, pNO41GW, by ligating a *Hind*III and *Bgl*II digested cassette containing *attR* sites flanking the *ccdB* gene (Invitrogen) into the vector pre-digested with HindIII and BamH1. LR recombination between the entry clones, pDONR-*yqeH* or pDONR-*era*, and the destination vector, pNO41GW, led to the generation of pNO41GW-*yqeH* and pNO41GW-*era*, respectively (Fig. 18).

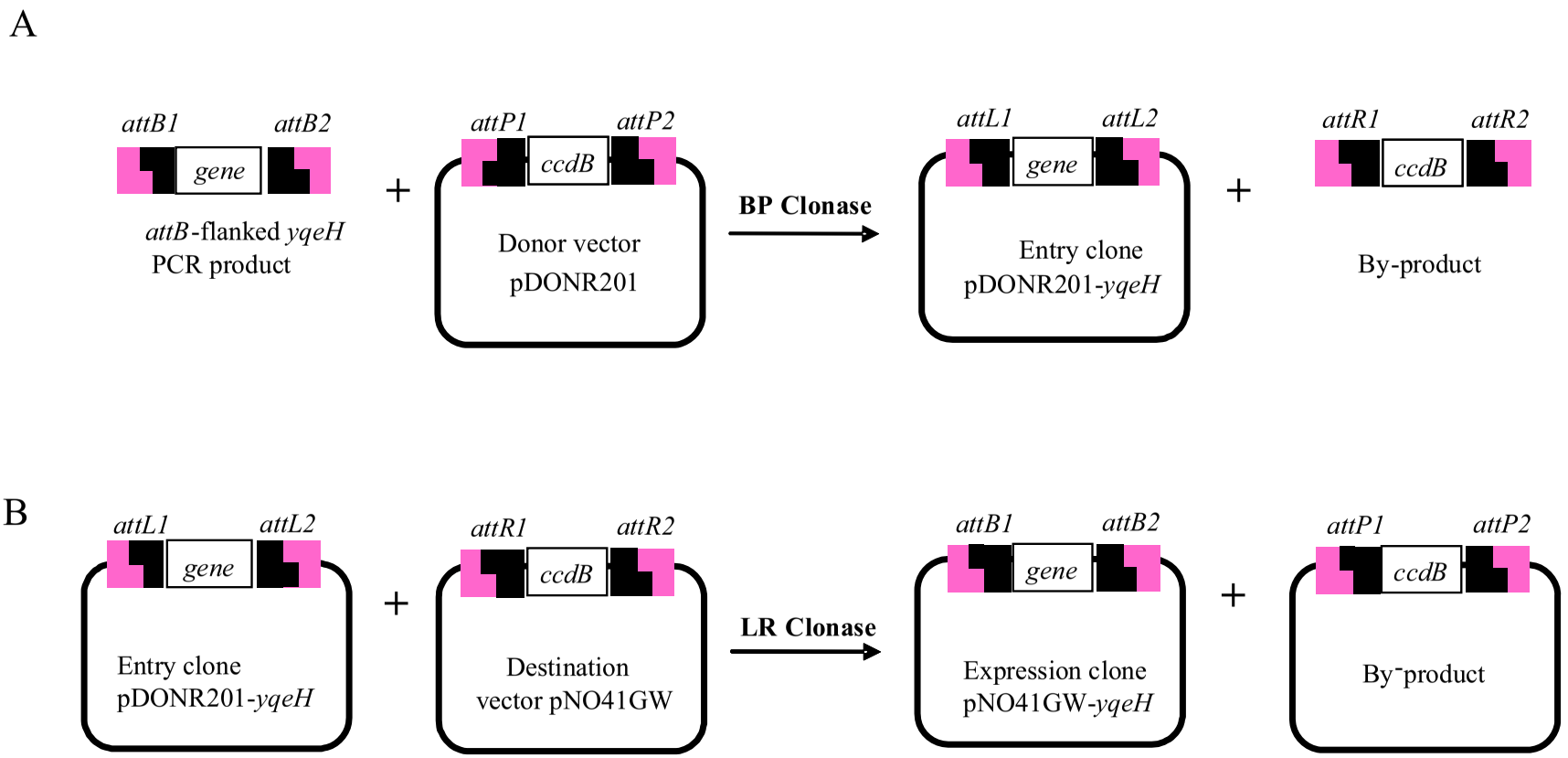


Fig. 18 Construct of constitutively expressed *yqeH* vector, pNO41GW-*yqeH* using Gateway Technology. The method involves lambda recombination system to aid transfer of heterologous DNA (PCR product flanked by modified *att* sites) between vectors, which involves two recombination reactions. (A) BP reaction: catalyzed by BP clonase, facilitates recombination of an *attB* substrate (*attB1-yqeH-attB2*) with an *attP* substrate (pDONR201) to create an *attL*-containing entry clone (pDONR201-*yqeH*) (B) LR reaction: catalyzed by LR clonase, facilitates of an *attL* substrate (pDONR201-*yqeH*) with an *attR* substrate (pNO41GW) to create an *attB*-containing expression clone (pNO41GW-*yqeH*).

3.2.7 Construction of single point mutated proteins of YqeH by site-directed mutagenesis

Primers used to generate the single point mutated *yqeH* such as YqeH-T168V, -S173N, -S193P are shown in Table 9. The PCR ligation products of *yqeH* were cloned into the chromosomal integration vectors pAPNC213, with portion genes of *aprE* front and back, is graphically illustrated (Fig. 8). Plasmids used to replace the endogenous *yqeH* genes by the tetracycline resistance gene, were constructed as follows. The upstream and downstream regions of *yqeH* were amplified, digested at the *Bam*HI/*Xho*I sites or *Xho*I/*Hin*dIII sites introduced in the primers, ligated using the *Xho*I sites, and inserted between the *Hin*dIII and *Bam*HI sites of pBR322. Then, an *Xho*I fragment containing the *tet* gene derived from pBEST307 (Itaya, 1992) was inserted into the *Xho*I site of the resultant plasmids in the same direction as the *yqeH* gene. The pET29b system (Novagen) was used for purification of these YqeH mutant proteins for subsequent GTPase activity analyses.

3.2.8 Yeast two-hybrid screening

3.2.8.1 Media

Sterile components such as glucose, adenine sulfate dihydrate, 3-AT (3-amino-1,2,4-triazole), 10x dropout (DO) solutions, and X-α-gal were added to the autoclaved medium only when the medium was cooled to ~55^{o}C.

a. YPDA medium

per liter

Bacto peptone (Difco)	20 g
Yeast extract (Difco)	10 g
Agar (for plates only)	20 g
40 % (w/v) Glucose *	50 ml

0.2 % (w/v) Adenine sulfate dihydrate *	15 ml

The final volume was adjusted to 935 ml with H_2O prior to autoclaving. The medium was cooled to ~55^{o}C before addition of *components, i.e. sterile 40 % glucose stock and adenine.

b. SD medium

Synthetic dropout (SD) is a minimal medium used in yeast transformation to isolate for specific phenotypes of interest. SD medium is prepared by combining a minimal SD base (providing a nitrogen base and a carbon source) with a stock of "dropout "(DO) solutions that contains a specific mixture of amino acids and nucleotides.

per liter

Yeast nitrogen base (Difco)	6.7 g
Agar (for plates only)	20 g
40% (w/v) glucose	50 ml
1 M 3-AT	2 ml
Appropriate 10x dropout supplements	100 ml

The SD media with appropriate DO supplements used as selection markers are:

bait *yqeH*	SD/-Trp
genomic library	SD/-Leu
low-stringency agar	SD/-Trp/-Leu
medium-stringency agar	SD/-Trp/-Leu/-His + 5 mM 3-AT

c. **10x dropout (DO) supplements**

per liter

Component	Abbreviation	Weight / g
L-Isoleucine	Ileu	0.3
L-Valine	Val	1.5
L-Adenine sulfate dihydrate	Ade	0.2
L-Arginine	Arg	0.2
L-Histidine	His	0.2
L-Leucine	Leu	1.0
L-Lysine	Lys	0.3
L-Methionine	Met	0.2
L-Phenylalanine	Phe	0.5
L-Threonine	Thr	2.0
L-Tryptophan	Trp	0.2
L-Tyrosine	Tyr	0.3
L-Uracil	Ura	0.2

d. **1 M 3-AT (3-amino-1, 2, 4-triazole)**

1.68 g of 3- AT was diluted in 20 ml deionized H_2O and filter sterilize, finally store at 4^oC.

e. **X-α-gal solution (20 mg/ml in DMF)**

X-α-gal (5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside) was diluted at 20 mg/ml in dimethylformamide (DMF), and store at -20^oC in the dark

3.2.8.2 Suppression of bait autonomous transcriptional activity

Yeast transformation was performed by the lithium acetate method, in accordance with the manufacturer's instructions (Yeast Protocol Handbook 2000, Clontech). To examine the leaky His3 expression level of the system, yeast host PJ69-4a bearing DNA BD-*yqeH* and PJ69-4α were grown on SD/-Trp/-His plates with different concentration of 3-AT, ranging from 1, 2, 5 to 10 mM. Finally, 5 mM 3-AT was used for the yeast two hybrid screening.

3.2.8.3 Yeast mating

Since the function of YqeH is implicated on the ribosome biogenesis, the ribosomal proteins encoding genes and rRNA-related processing genes were screened for potential YqeH-interactants at the one to one basic. Gateway Technology was applied in which YqeH was fused to pGBT9GW as bait protein, and a total of 52 ribosomal proteins from ribosome 50S and 30S subunits and 10 rRNA processing or modification related enzymes (Table 3), were individually fused to pGAD424GW as prey. In-frame fusion of insert of the resultant plasmids was confirmed by sequencing. The bait (*trp1*) and the prey (*leu2*) were transformed into yeast strains PJ69-4a and PJ69-4α, respectively (James et al., 1996). Briefly, yeast mating was conducted overnight in YPDA media (1% yeast extract, 2% Difco peptone, 2% glucose and 0.02% adenine) in a 96-well flat bottom microtiter plate. After mating, cells were harvested,

washed with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and spotted on a synthetic complete (SC) agar plates lacking leucine and tryptophan for selection of *Leu2* and *Trp1* diploid cells. The selected diploid cells were then cultured in SC broth lacking leucine and tryptophan medium for overnight. The next day, cells were washed SC broth lacking leucine, tryptophan and histidine, cells aliquot was diluted into a series of 10 folds, and 4 µl each was spotted on SC agar lacking leucine, tryptophan and histidine. The medium was also supplemented with 5 mM 3-aminotriazole to inhibit autonomous activation of the *His3* reporter gene. Cells growth was observed for duration of 7 days, at 30^{o}C.

3.3 RESULTS

3.3.1 YqeH involves in the biogenesis of 30S ribosomal subunit

3.3.1.1 Domains organization of *B. subtilis* YqeH

YqeH is a protein of 41 kDa, may consider as a large GTP-binding protein when compare to that of the Ras, with a molecular mass about 22 kDa. It revealed a central-localized circular permuted GTP-binding domain, with G4-G1-G2-G3 motifs (Leipe et al., 2002; Anand et al., 2006), as opposed to the common G1-G2-G3-G4 motifs observed in Obg and Era of the same family, or translation factors such as IF-2 and EF-G. YqeH possesses an N-terminal putative zinc finger, which belongs to the 'treble cleft' family; with conserved CXGCGX$_n$CXRC residues and predicted secondary structure similar to those of the ribosomal proteins S14 and L24, which is predicted to bind rRNA (Anand et al., 2006). However, its C-terminus shows poor homology with protein of known function, and is composed of nearly 20% of positively-charged amino acids, such as arginine, lysine and histidine (Fig. 19). Using native PAGE, the purified YqeH protein exists predominantly as a monomer, with a small percentage of dimers (data not shown).

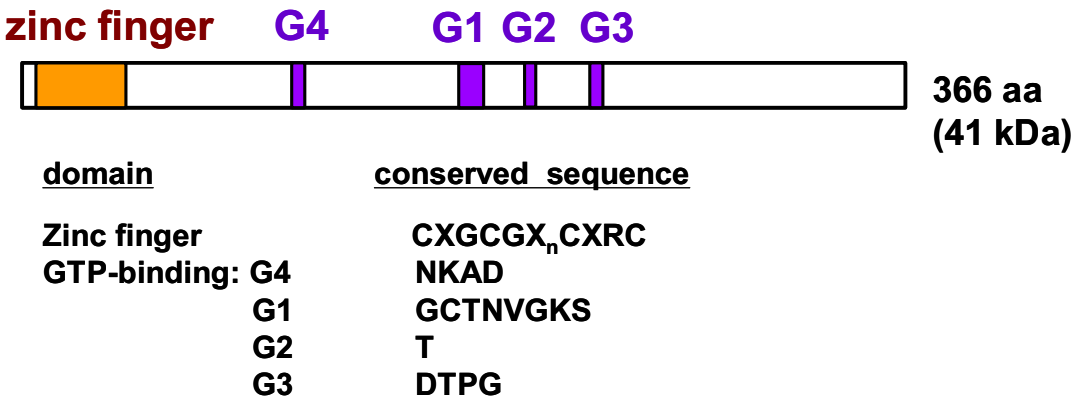


Fig. 19 Domains organization of *B. subtilis* YqeH. YqeH possesses permuted GTP-binding domain, with G4-G1-G2-G3 motifs and an N-terminal putative zinc finger. The four cysteines and conserved residues coordinate to zinc ligand, forming the zinc finger motif CXGCGXnCXRC are depicted.

3.3.1.2 YqeH-depleted cells display a perturbed ribosome profile

We initially examined the ribosome profiles of YqeH-depleted cells using TMO309 in which *yqeH* is under control of the *spac* promoter (Morimoto et al., 2002). Cells were grown in PAB medium alone or supplemented with IPTG, and the respective crude cell lysates were separated by sucrose density gradient centrifugation under high Mg^{2+} conditions to maintain the 70S ribosome (Fig. 20A). The growth doubling time of TMO309 cells in the presence of 20 μM IPTG (35 min) was comparable to that of wild-type cells (30 min). Moreover, the distribution patterns of free 30S and 50S subunits, and the 70S ribosome were similar to those in wild-type cells. However, in the absence of the inducer, IPTG, the growth rate was reduced (doubling time, 48 min), and we observed a reduction in the 70S ribosome and 30S subunits, indicating that depletion of YqeH results in perturbation of the ribosome profile, similar to other members of the Obg/Era family.

The *yqeH* gene has been classified as 'essential' due to the inability of the IPTG-dependent P_{spac}-*yqeH* mutant to grow on PAB agar in the absence of IPTG (Morimoto et al., 2002; also see Fig. 23). However, in our experiments, the P_{spac}-*yqeH* mutant grew in PAB liquid medium with no IPTG supplementation, albeit at an impaired rate, although YqeH was not detectable by Western blotting (Fig. 23B). This difference may be attributed to variations in growth conditions, such as agitation and aeration. It is possible that in addition to 30S subunit assembly, YqeH has other essential cellular functions yet to be identified, which is required for growth on solid media.

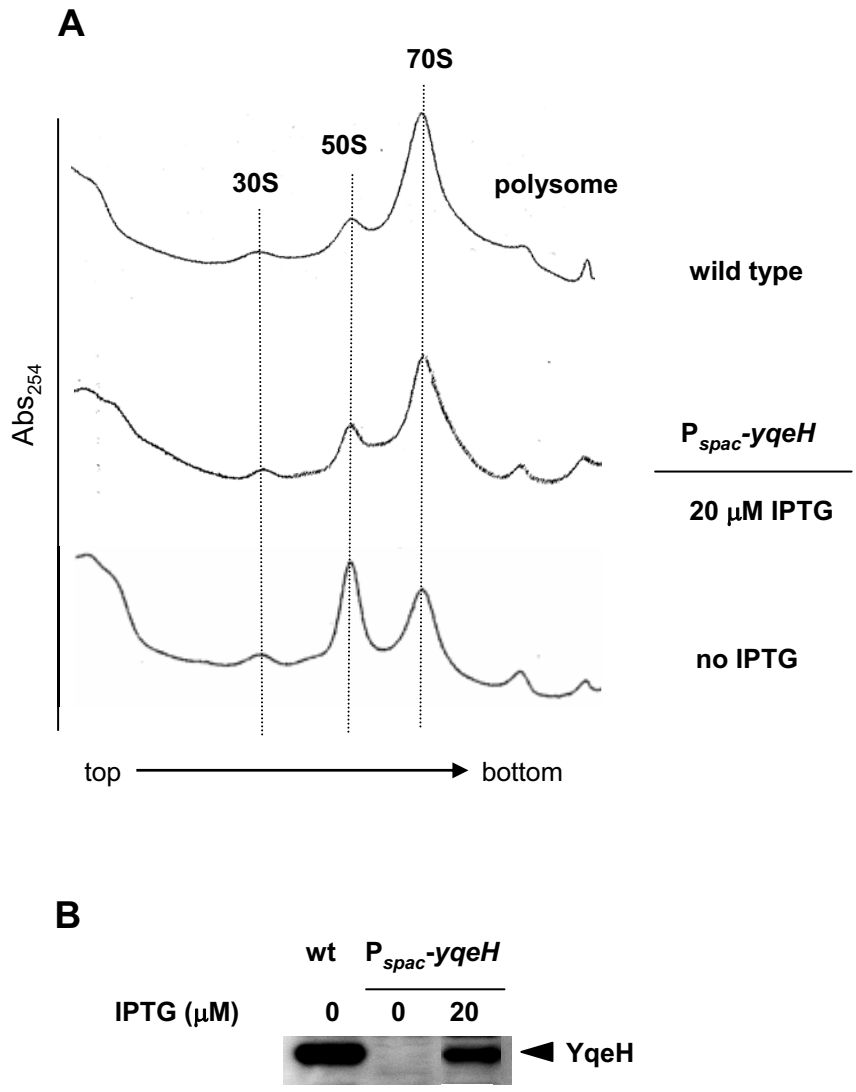


Fig. 20 The ribosome profile is perturbed in YqeH-depleted cells. (A) The ribosome profiles of P_{spac}-*yqeH* cells (TMO309) in the presence and absence of IPTG were compared to those of wild-type cells. Cells were grown at 37°C, and harvested at OD_{600} of 0.3. An equal amount of 18 A_{260} units of crude cell extract was layered over the 10-50% sucrose gradient under association conditions, and centrifuged at 66,800 x *g* for 14 h. Ribosome profiles in the gradients were examined by monitoring absorbance at 254 nm. (B) Western blotting of the whole cell protein in cells. Equal amount (20 μg each) of proteins were loaded for SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-YqeH antibody to detect the protein expression.

3.3.1.3 YqeH depletion leads to a decrease in the free 30S subunit level

Increased accumulation of the free 50S subunit was not accompanied by a corresponding increase in the free 30S subunit in YqeH-depleted cells. To explain this finding, we analyzed the ribosome profiles under high (association) and low (dissociation) Mg^{2+} conditions. The association buffer favors formation of the 70S ribosome, where the predominant 70S ribosome may reflect the actual ribosome profile in a cell. In contrast, the dissociation buffer induces the 70S ribosome to separate into free 50S and 30S, thus enabling quantification of the total subunit amounts. The IPTG-dependent P_{spac}-*era* mutant (Morimoto et al., 2002) was additionally used for comparison, since the Era ortholog in *E. coli* is implicated in 30S subunit assembly (Sayed et al., 1999; Inoue et al., 2003; Inoue et al., 2006).

As shown in Fig. 21A, cell growth was more severely impaired in Era-depleted cells (doubling time of 87 min), compared to YqeH-depleted cells (doubling time of 48 min). The data support a more critical role of Era, which is widely conserved in bacteria, compared to YqeH that is mainly restricted to low-GC gram-positive bacteria. The ribosome profiles of wild-type and Era- or YqeH-depleted cells were compared with equal amounts of crude cell extracts. In association buffer (Fig. 21B), YqeH-depleted cells displayed a decrease in the 70S ribosome and accumulation of the free 50S subunit, but a similar peak representing the free 30S subunit, compared to wild-type cells. In contrast, Era depletion resulted in the accumulation of both free 50S and 30S subunits, and reduction in the 70S ribosome level. Replicate results consistently revealed a slight sedimentation shift of the 30S peak in Era-depleted cells. Under dissociation conditions (Fig. 21C), the peaks representing 30S and 50S levels were similar in Era-depleted and wild-type cells.

On the other hand, consistent with the low level of free 30S in YqeH-depleted cells under association condition, the 30S subunit peak was obviously lower than those of wild-type and Era-depleted cells, although the reduction, about 30%, is apparently milder

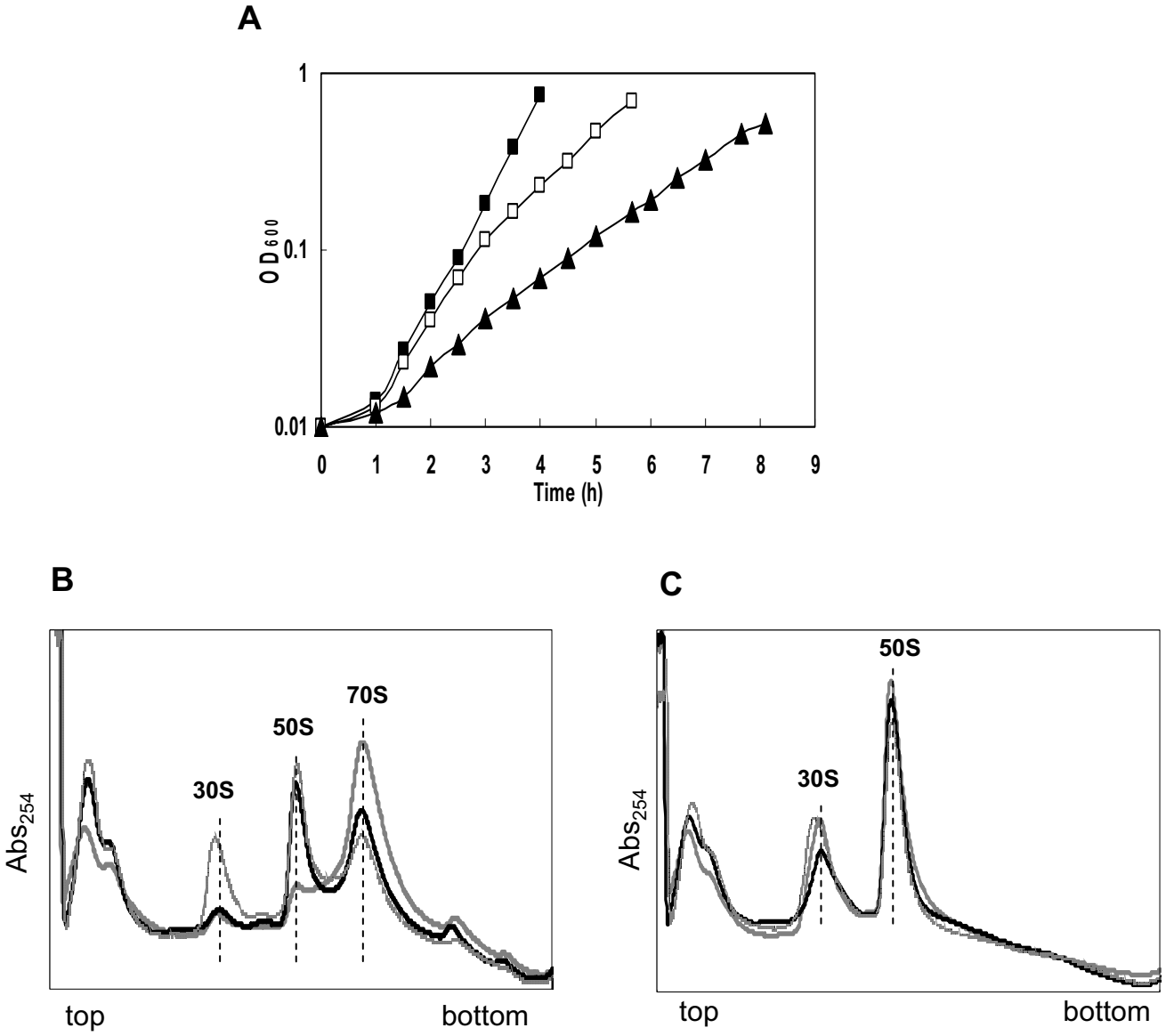


Fig. 21 YqeH depletion results in accumulation of free 50S, but not free 30S subunit (A) Growth curves of *B. subtilis* wild-type (■), P*spac-yqeH* (□) and Pspac-*era* (▲) cells in PAB liquid medium without IPTG supplementation. Ribosome profiles of YqeH-depleted cells (depicted in black), compared to wild-type CRK6000 (depicted in gray) and Era-depleted cells (depicted in dots) in association (B) and (C) dissociation buffers. Cells were harvested at OD_{600} of 0.5. An equal amount of 18 A_{260} units of crude cell extract was applied for comparison.

than that expected from the ratio of the 50S peak (free subunits) and the 70S peak (mature subunits) under association condition. This difference would be, at least partly, due to mature 30S subunits in polysome fractions not clearly visible in our centrifugation under association condition. Moreover, at the top fraction of the ribosome profiles (Fig. 21B and 21C), YqeH-depleted cells revealed a remarkably higher peak compared to that of the wild type. Indeed, the whole cell lysates for wild type and YqeH-depleted cells were pretreated with DNase I to remove chromosomal DNA or any residual DNA, which may also contributed to the absorbance on 254 nm before subjected to sucrose gradient density gradient. Therefore, the peak on the top fraction might consist of predominantly degraded products of unassembled 30S intermediates, such as residual 16S rRNA and 30S-related ribosomal proteins. These results indicate that the rate of 30S biogenesis is reduced, compared to that of 50S, and/or the free 30S subunit is specifically destabilized in YqeH-depleted cells.

3.3.1.4 Accumulation of 17S rRNA and degradation of 16S rRNA in YqeH-depleted cells

Next, to gain an insight into the mechanism of reduction of the free 30S subunit in YqeH-depleted cells, rRNA was analyzed by Northern blotting. Following hybridization of total RNA with a probe complementary to mature 16S rRNA (Probe 1, Fig. 22A and 22B), we observed a decrease in mature 16S rRNA and accumulation of pre-16S rRNA in exponentially growing cells. As schematically presented in Fig. 22B, the pre-16S rRNA is cleaved by endoribonuclease RNase III to yield 17S rRNA. The final maturation of 16S rRNA involves the removal of 76 and 67 nucleotides from the 5' and 3' ends, respectively. In *B. subtilis*, the former process involves endoribonuclease RNase J1 (YkqC), but the corresponding RNases for the latter are currently unknown (Britton et al., 2007). Hybridization using probes 2 and 3, designed to specifically bind to the unprocessed 5' and 3' ends of pre-16S, respectively, confirmed the presence of unprocessed 16S rRNA in YqeH-depleted cells (Fig. 22C).

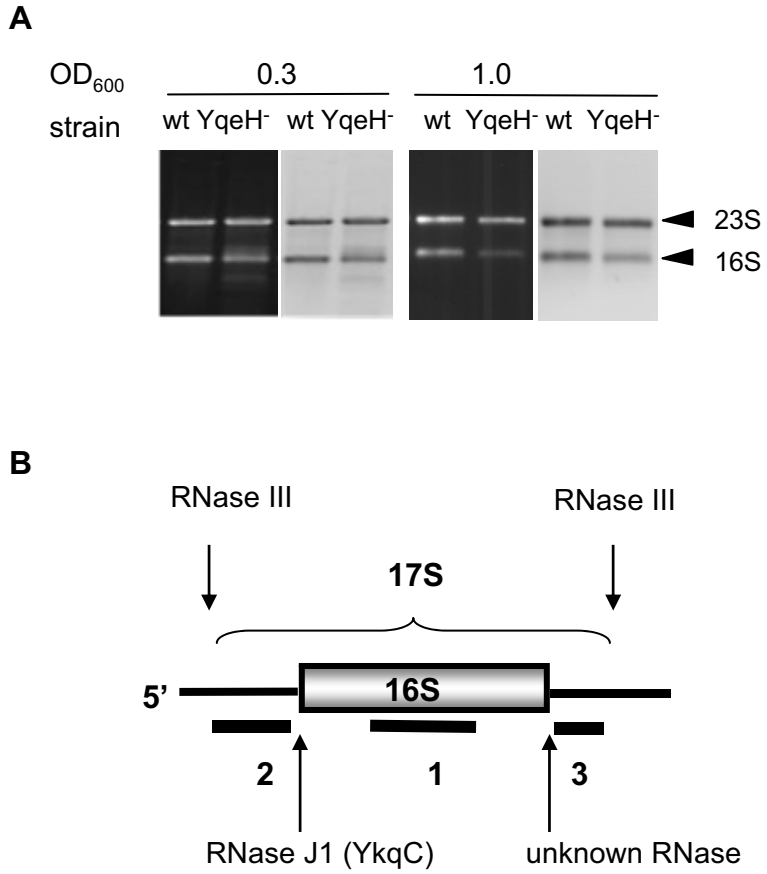


Fig. 22 Accumulation of 16S rRNA precursor and degraded products in YqeH-depleted cells. (A) Ethidium bromide staining of 1 μg each of total rRNA extracted from wild type (wt) and YqeH-depleted (YqeH$^-$) cells, separated on non-denaturing 1.0 % agarose gel. The cells were harvested at OD_{600} of 0.3 and 1.0 respectively. For ease of band intensities comparison, the resolved rRNAs are shown in pair; in which the negative images are shown on the left. (B) Schematic representation of pre-16S rRNA processing in *B. subtilis*. The positions of digoxigenin-labeled RNA probes used in Northern blotting are shown as solid bars. (C) Northern blotting of total rRNA (1 μg each) from wild-type and YqeH-depleted cells at the designated OD_{600}. The positions of mature 16S rRNA, precursors of 16S (pre-16S), and degraded 16S rRNA are indicated.

Maturation of 16S rRNA is believed to occur after association of the 30S and 50S subunits (Srivastava and Schlessinger, 1990). Accumulation of 17S rRNA due to impairment of 30S or 50S subunit biogenesis has been reported in *E. coli* cells depleted or inactivated for Era (Inoue et al., 2003), ObgE (Sato et al., 2005; Jiang et al., 2006), and Der, a YphC ortholog in *E. coli* (Hwang and Inouye, 2006). Thus, 17S rRNA accumulation in YqeH-depleted cells supports the involvement of this protein in 30S subunit biogenesis. In addition, we observed bands resulting from the degradation of premature and/or mature 16S rRNA in YqeH-depleted cells, suggestive of 30S degradation. Interestingly, premature 16S rRNA and degraded products were not detected in stationery phase cells (OD_{600} of 1.0) in which ribosome biogenesis activity is suppressed. Taken together, our results suggest that YqeH participates in the assembly of nascent 30S particles, and plays a minor role, if any, in recycling of the 30S subunit after the completion of translation.

3.3.1.5 Distinct roles of YqeH and Era in 30S ribosomal subunit assembly

In view of the finding that both YqeH and Era are involved in 30S subunit assembly, we were prompted to investigate whether the functions of Era overlap with those of YqeH. We cloned *yqeH* and *era* into a multicopy pNO41GW vector with a constitutively expressed promoter, and the resulting plasmids were individually transformed into P_{spac}-*yqeH* or P_{spac}-*era* cells. Cells were grown in PAB agar in the absence and presence of IPTG. However, overexpression of Era could not compensate for growth inhibition due to YqeH depletion, and *vice versa* (Fig. 23). Moreover, the slower growth rate in PAB liquid medium due to depletion of YqeH or Era was not recovered upon overexpression of the other (data not shown).

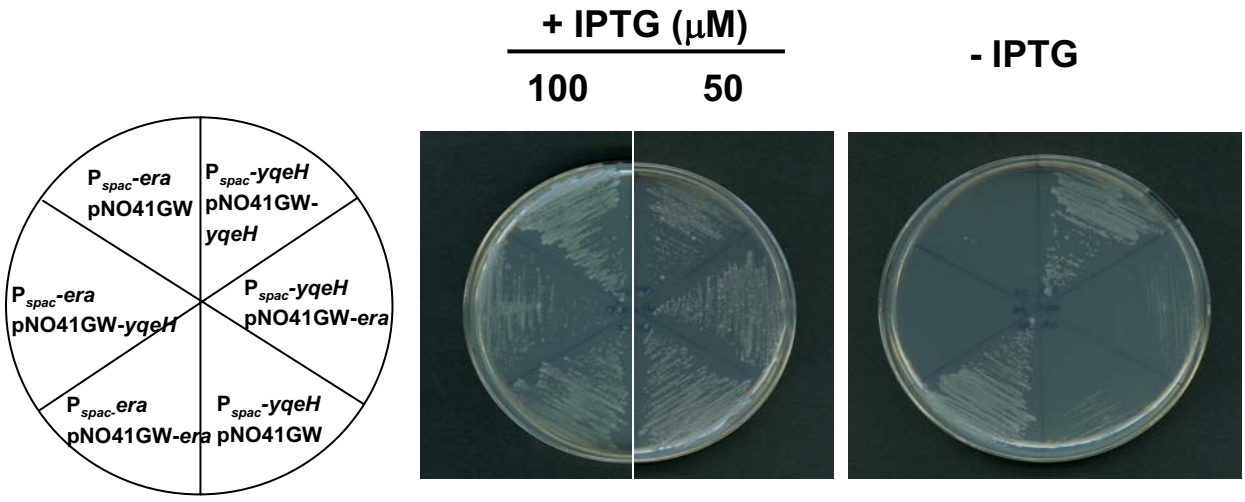


Fig. 23 YqeH and Era possess non-redundant functions that overexpression of YqeH could not compensate for growth inhibition due to Era depletion, and *vice versa*. (A) Growth properties of P*spac-yqeH* (pNO41GW), P*spac-yqeH* (pNO41GW-*yqeH*), P*spac-yqeH* (pNO41GW-*era*), P*spac-era* (pNO41GW), P*spac-era* (pNO41GW-*era*), and P*spac-era* (pNO41GW-*yqeH*) cells on a PAB agar plate in the presence and absence of IPTG. Photographs were taken after incubation at 30°C for 26 h.

3.3.2 Construct of YqeH mutant proteins for study *in vitro* and *in vivo*

3.3.2.1 Generation of YqeH mutant proteins by ligation PCR

In the functional study of small GTPase Ras, the dominant active and negative mutants are widely used to identify its upstream regulators and downstream effectors. These mutants inhibit the activities of the endogenous GTPase by competing for binding to its interactants. In subsequent experiments, I focus on the functional characterization of YqeH. Therefore, with references to the dominant active and negative mutants of Ras superfamily such as Ras, Rac, Rho proteins, single amino acid mutation was introduced to the GTP-binding domain and switch I region of *yqeH* (Ridley, 2000). YqeH mutant proteins T168V, S173N and S193P were individually generated by introducing single point mutation using ligation PCR, where the primers were designed to create the specific point mutations, are stated in Table 3. The generating of pAPNC213-*yqeH* construct for YqeH mutant protein, where the single point mutated protein was placed under the control of *spac* promoter, is illustrated in Fig. 24. YqeH-T168V was generated by substituting threonine with valine of G1 domain (GCT^{168} NVGKS). The G1 domain is also referred as P-loop, which interacts with α and β-phosphate groups of guanine nucleotide (Pandit and Srinivisan, 2003). For YqeH-S173N, introducing of asparagine (N) to replace serine (S) that is required for guanine nucleotide-binding, will interfere with GTP/GDP-bindings (Ridley, 2000). Likewise, in YqeH-S193P, substitution of serine (S) with proline (P) in the effector domain may disrupt the proper interaction of YqeH with its interactants.

The dominant active and negatives properties could only be determined by transforming these genes to replace the endogenous *yqeH in vivo*, and analyze the phenotypes of the transformants. Transformation of YqeH-T168 to replaced endogenous YqeH produced mutants showed similar growth rate to that of wild type cells either in LB or minimal media such as CG-glucose (data not shown). However, the mutant for YqeH-S173N could not be

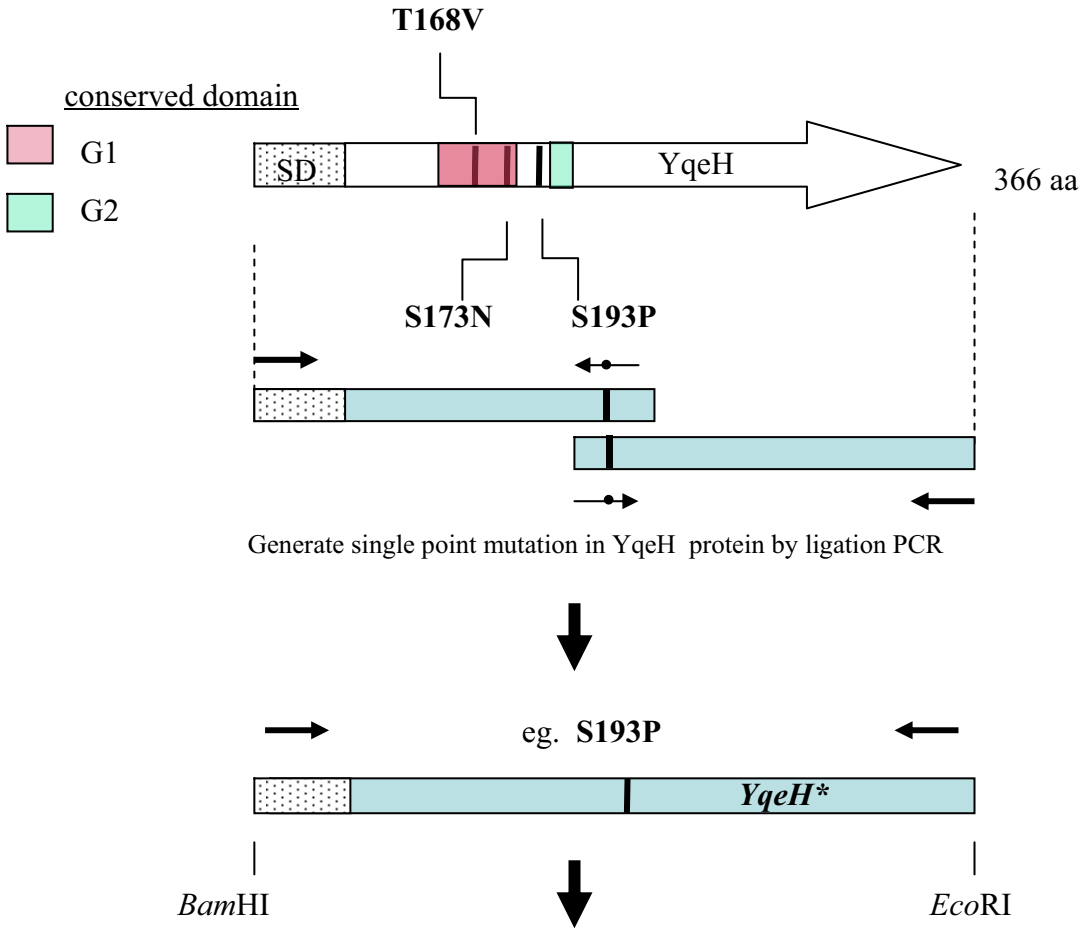


Fig. 24 Constructs for introducing single point mutated *yqeH* in the *aprE* locus of *B. subtilis* chromosome. The IPTG-dependent YqeH mutant proteins were integrated to the *aprE* locus by homologous recombination and subsequently, the endogenous gene was disrupted with a tetracycline cassette. SD, Shine Dalgarno region

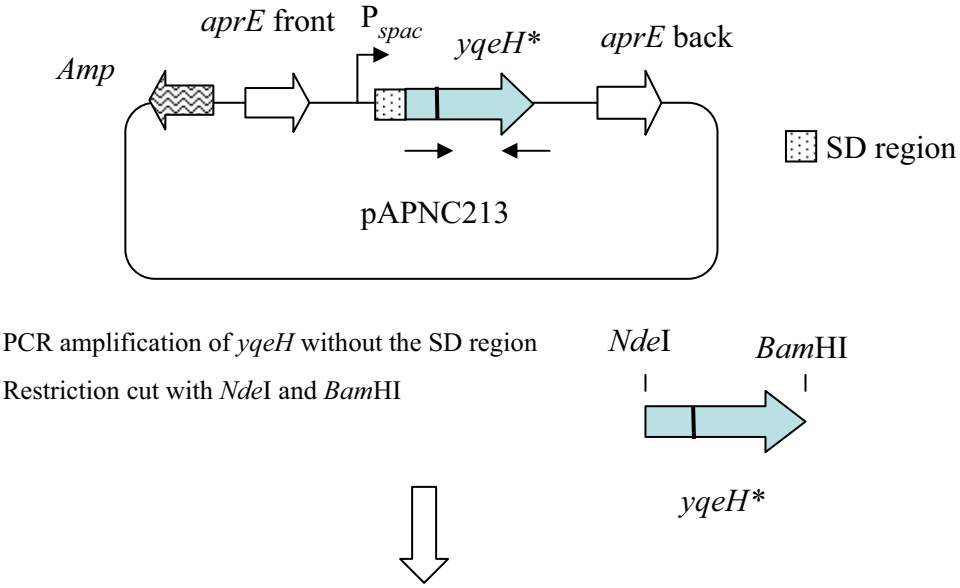


Fig. 25 Constructs for purification of YqeH mutant proteins, pET29b-*yqeH**-His. The full-length YqeH* was amplified from the pAPNC-*yqeH** vector, without the SD region. The fragment was then cloned into the pET29b vector with a C-terminus fused His_6, to generate pET29b-*yqeH**-His_6 for mutant proteins purification

YqeH* is a single point mutated protein of YqeH

obtained, suggesting that YqeH-S173N caused lethality in *B. subtilis*. YqeH-S193P mutant gave small colonies after 2 days incubation at 37^{o}C, indicating that the cells growth were severely retarded. For subsequent biochemical studies of these mutant proteins, such as GTPase activities and GTP-binding abilities, the pET29b-*yqeH**-His6 vectors were generated for proteins purification, as shown in Fig. 25.

3.3.2.2 Comparison of the GTPase and GTP-binding activities of YqeH mutants T168V, S173N and S193P to that of the wild type protein

In view of the high intrinsic GTPase activity of YqeH that distinguished from the members Obg/Era family, we are prompted to ask if the result showed *bona fide* intrinsic GTPase activity of YqeH, but not arises from contaminants of *E. coli* during protein purification. Therefore, we biochemically analysed the YqeH wild type and mutant proteins using the GTPase assay. Moreover, the biochemical properties of these proteins may explain the reasons YqeH-S173N and -S193P expression caused lethality in *B. subtilis* cells.

YqeH-T168V, -S173N and -S193P showed severely impaired GTPase activities compared to that of the wild type YqeH (Fig. 26A). The YqeH-T168V protein, with a valine in place of threonine 168 that is essential for guanine nucleotide contacts, showed impaired GTPase activity with a diminish of 70% activity compare to that of the wild type, as predicted from the constitutive active Ras mutant. On the other hand, YqeH-S173N and S193P exhibited almost no GTPase activities. To gain an insight into this, we proceed to examine the GTP-binding abilities of these proteins, with a non-hydrolysable GTPγS, an analog for GTP. GTPγS-binding results showed YqeH-T168V maintained similar binding ability to that of the wild type. YqeH-S173N and -S193P revealed a reduced GTP-binding activity, with about 70% and 50%, respectively compared to the wild type (Fig. 26C). Collectively, these results indicated that abolished GTPase activities of YqeH-S173N and S193P, may explain the cause

of cell lethality (section 3.3.6). Thus, the GTPase activity is important for the proper functions of YqeH *in vivo*. Furthermore, these data also suggest that the GTPase activity observed is a measure of the intrinsic GTPase activity of YqeH, but not arises from contaminating protein of *E. coli* or artifact due to the His_6-fusion.

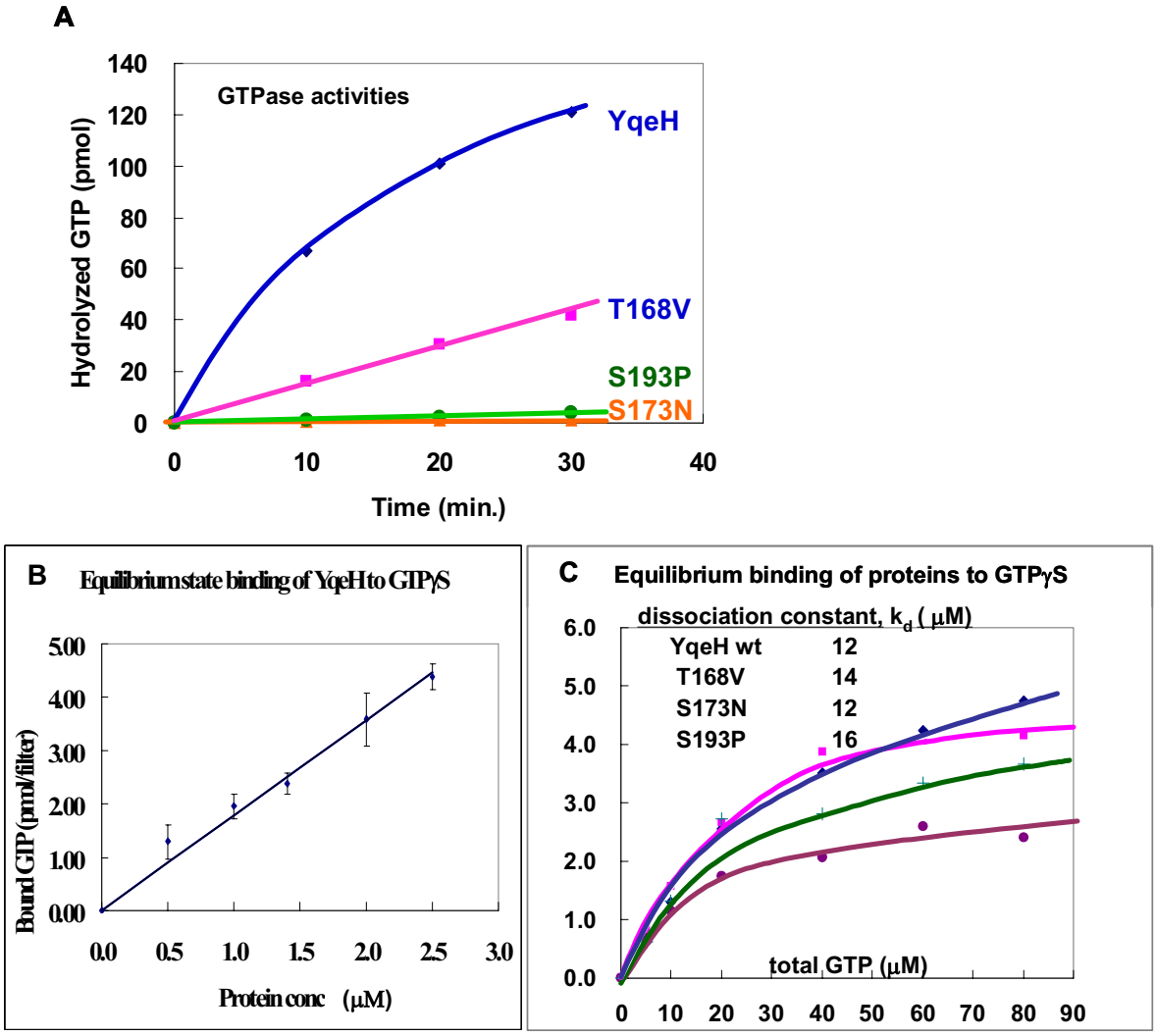


Fig. 26 Comparison of GTPase and GTP-binding activities of wild type YqeH and its derivatives (A) GTPase activities as a function of time. YqeH-T168V showed severely impaired GTPase activity compared to that of the wild type. The GTPase activities for YqeH-S173N and –S193P were almost abolished. (B) Linear correlation of the formation of protein-GTPγS complexes to the amount of protein used in the reaction. (C) GTP-binding ability of proteins with GTPγS as determined with filter-binding assay. YqeH mutant proteins T168V showed almost similar GTP-binding ability to that of the wild type, however, YqeH-S173N and S193P proteins showed impaired GTP-binding ability.

3.3.2.3 Identification of S4, a ribosomal protein of 30S subunit as YqeH interactant

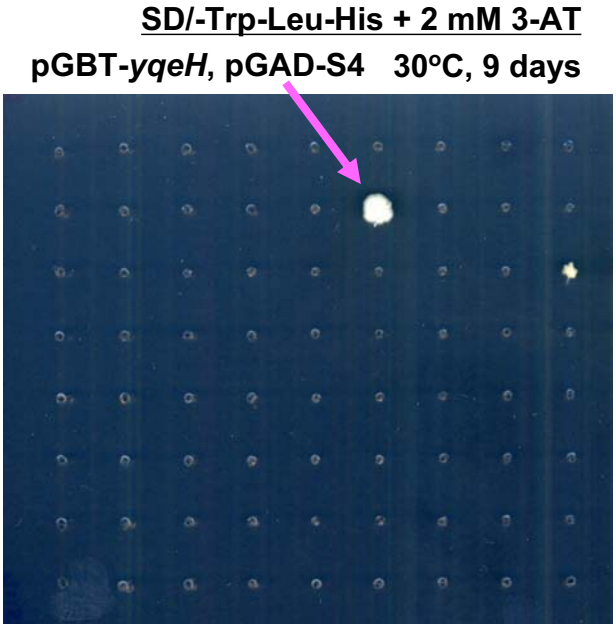
Since all members of Obg/Era family are implicated to involve in biogenesis of the ribosomal subunits, we are prompted to investigate if YqeH may interact with the ribosomal subunit through direct interaction with ribosomal proteins, rRNA modification enzymes such as endonucleases, base-methylases or uridine-pseudouridine isomerases. Yeast two hybrid system was applied to detect the possible interaction of YqeH with these proteins. *yqeH* was fused to pGBT9GW as a bait protein, and a total of 53 ribosomal proteins from 50S and 30S subunits, and RNA modification genes such as *trmD, trmU, rncS, rnpA, cca, cspR, yqeT, yabR, truB, ylqE,* were also used as preys and monitored at the one to one basis.

From a total of 65 candidates screened, we found that YqeH interacts specifically with RpsD, a 30S ribosomal protein which also known as S4 (Fig. 27A & B). The interactions were observed in the presence of 2 mM and even at 5 mM 3-amino-1, 2, 4-triazole (3-AT), which suppress the autonomous transcriptional activity of both bait and prey in the SD medium lacking of His, indicating that the YqeH-S4 interaction is strong. At least four independent experiments were conducted and the results were reproducible. DnaA protein that is known for self-interacting was applied as a positive control. Likewise, to confirm that the YqeH-S4 interaction is specific, the other five GTPases (Obg, Era, YsxC, YlqF and YphC) were individually fused as bait, and examine for S4 interaction (data not shown). We found that S4 interacts specifically to YqeH. It is known that S4 initiates the assembly of 30S ribosomal subunit by binding to the 5'-terminal of 16S rRNA (Nowotny et al., 1988). In *E. coli,* S4 is an autoregulator that translationally represses the mRNA transcribed from the alpha operon encoding for ribosomal proteins S13, S11, S4, α subunit of RNA polymerase and L17 (Deckman and Draper, 1985). However, in *B. subtilis*, S4 is monocistronic that located distant from the remainder genes of α operon on the *B. subtilis* genome (Boylan et al., 1989), suggesting that the regulation of S4 and expression may be different in these two bacteria. In

A

positive control: pGAD-*dnaA* vs. pGBD-*dnaA*

pGAD	L1	L10	L20	L31	S3	S11	S19	TrmD
pGAD GW	L2	L11	L21	L32	S4	S12	S20	TrmU
YqeH	L3	L13	L22	L33 A	S5	S13	S21	+ cont.
T168V	L4	L14	L23	L33 B	S6	S14	DnaA	YqeT
S173N	L5	L15	L24	L34	S7	S15	Cca	YabR
S193P	L6	L16	L27	L35	S8	S16	CspR	TruB
dmut 1	L7/12	L17	L29	L36	S9	S17	RncS	YlqE
dimut 2	L9	L19	L30	S2	S10	S18	RnpA	Ytrp



B

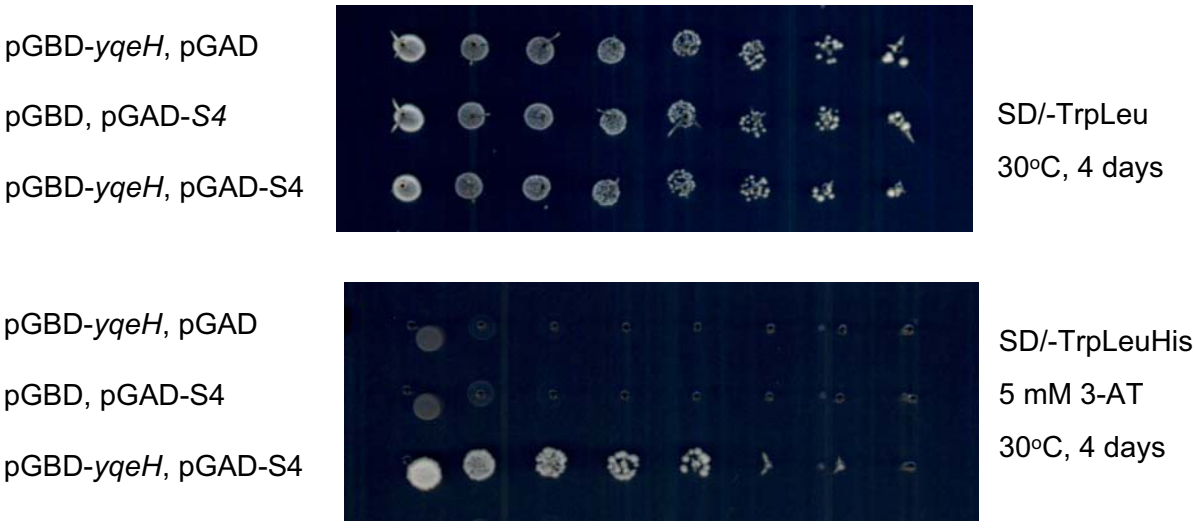


Fig. 27 Screening of YqeH interactants using yeast two hybrid. (A) YqeH was fused to pGBT9 as bait, a total of 53 r-proteins from 50S and 30S subunits, and 11 RNA processing proteins (RNases, methylases, pseudouridylases) were used as preys, and monitored at the one to one basis. YqeH interacts specifically with S4. (B) Confirmation of S4, a 30S ribosomal protein as the interactant of YqeH. Upper panel shows growth of diploid cells (pGBD-*yqeH* and pGAD-S4) in SD media lacking of tryptophan and leucine. Lower panel shows the diploid cells were further selected in SD media lacking of histidine. Only diploid cells with YqeH-S4 interaction could grow in this media.

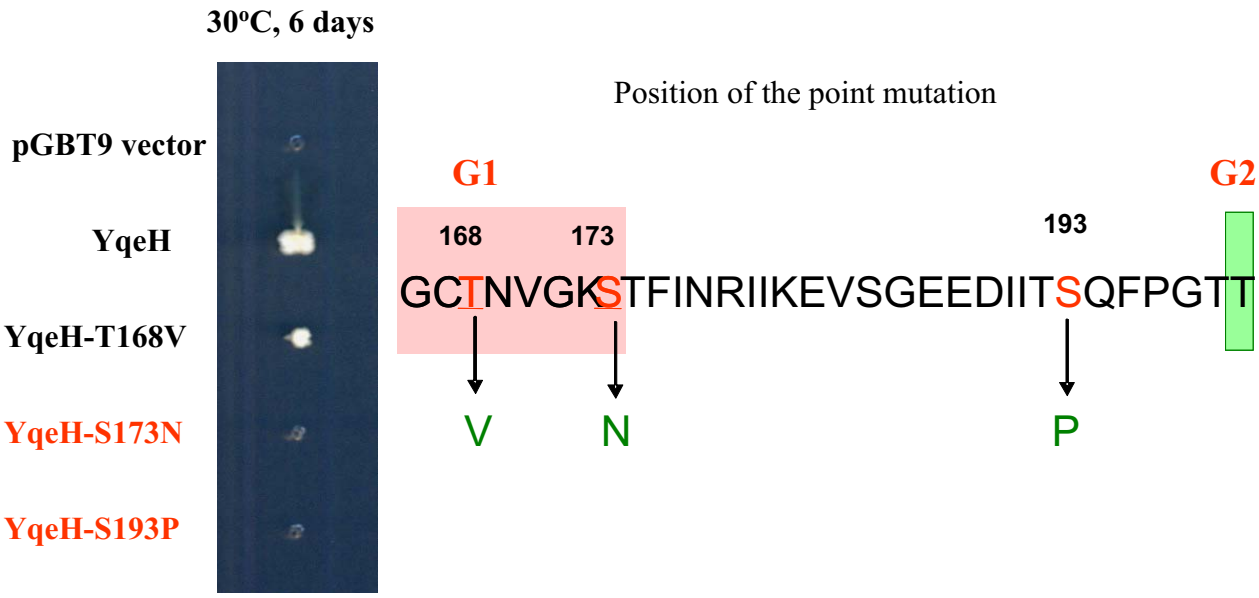


Fig. 28 Single point mutated YqeH-S173N and –S193P lost their interactions with S4. The mutant proteins were generated by ligation PCR . The positions of the amino acid substitution in the G1 (pink box) and effector domain, which includes G2 (green box) domain are shown

E. coli, S4 is also found to be tightly bound to the RNA polymerase, lead to increase in terminator readthrough in transcription of rrn operons, occurs especially when excess ribosomal proteins are in excess over rRNA *in vivo* (Torres et al., 2001).

In addition, we identified two YqeH mutants, YqeH-S173N and S193P that lost their interactions with S4 (Fig. 28). YqeH-S173N and S193P caused lethality in *B. subtilis* cells, and showed almost diminished GTPase activities (Fig. 26A), may serve as powerful tool to uncover the function of YqeH in near future. Furthermore, to know if the *B. subtilis* S4 shared any functional homology to the *E. coli* S4, the amino acid sequence homologies of the two proteins were analysed (Fig. 29). We found that these two proteins are highly conserved, with 49% sequence identities and 67% similarities. The 16S rRNA-binding domain located at the N-terminal of *E. coli* S4 is also conserved in *B. subtilis* S4, with a 72% homology (Grundy and Henkin, 1990). Taken together, high homology between these two proteins, implying that *B. subtilis* S4 may function as its counterpart in *E. coli*, which initiate the assembly of 30S ribosomal subunit. Further experiments including the YqeH-S4 binding to the precursors of 16S rRNA, is required to rule out the precise function of YqeH.

```
eco MARYLGPKLKLSRREGTDLFLKSGVRAIDTKCKIEQAPGQHG-ARKPRLSDYGVQLREKQ
bsu MARYTGPSWKLSRRLGISLSGTG-----KELEKRPYAPGPHGPGQRKKLSEYGLQLQEKQ
    **** **. ***** * .*  ..     . * *** ** .::     :**:**:**:***
eco KVRRIYGVLERQFRNYYKEAARLKGNTGENLLALLEGRLDNVVYRMGFGATRAEARQLVS
bsu KLRHMYGVNERQFRTLFDKAGKLAGKHGENFMILLDSRLDNVVYKLGLARTRRQARQLVN
    *:*::*** *****. :.:*.:* *: ***:: **:.*******::*:. ** :*****.
eco HKAIMVNGRVVNIASYQVSPNDVVSIREKAKKQSRVKAALELAEQREKPTWLEVDAGKME
bsu HGHILVDGSRVDIPSYLVKPGQTIGVREKSRNLSIIKESVEVNN--FVPEYLTFDAEKLE
    * *:*:* *:*.** *.*.:.:.  :***::: * :* ::*: : *    :* .** *:*
eco GTFKRKPERSDLSADINEHLIVELYSK
bsu GTFTRLPERSELAPEINEALIVEFYSR          : 16S rRNA-binding domain
    ***.* ****:*:.:*** ****:**:
```

Fig 29 Homology comparison of the amino acid sequences between *E. coli* and *B. subtilis* S4 using ClustalW. Both proteins share 49% identities and 67% similarities with a 4%. Note the presence of 16S rRNA-binding domain in *E. coli* is depicted in purple box.

3.3.2.4 S4 did not affect the GTPase activity of YqeH

Next, to find out if YqeH-S4 interaction has any functional consequences on the GTPase property of YqeH, the GTPase assays were repeated in the presence of purified S4, 30S ribosomal subunit, or both. S4 may act as an upstream regulator of YqeH, such as GTP-activator protein (GAP) that enhanced the GTPase activity, alternatively; guanine nucleotide dissociation inhibitor (GDI) which may inhibit its activity by slow down the release of bound-GDP and replace with GTP molecules. The results show that addition of either free S4 or 30S ribosomal subunit did not significantly affect the YqeH GTP hydrolytic activities (Fig. 30). Free S4 or the S4 in the 30S ribosomal subunit (in the presence of 16S rRNA) do not act as a regulatory protein of YqeH. Instead, these results suggest that S4 is most probably an effector of YqeH, in a yet to be identified signaling cascade that involves in biogenesis of the 30S subunit. The experiments were also conducted with YqeH mutant proteins such as S173N and S193P, where addition of 30S, S4 or both, showed no influence on their GTPase activities.

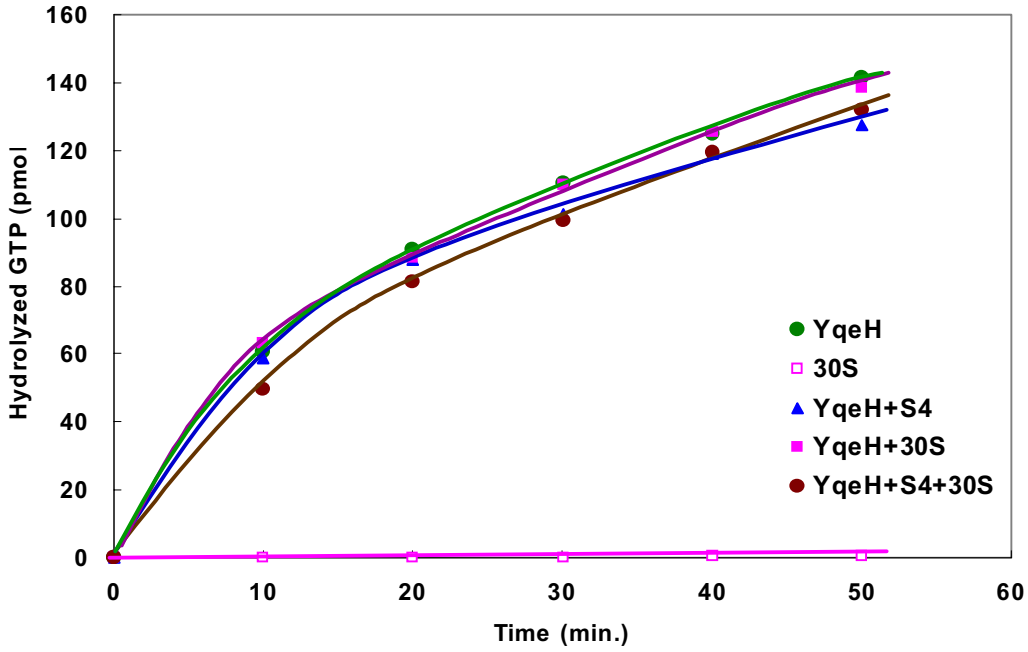


Fig. 30 Time course for YqeH GTPase activities in the presence or absence of S4 and 30S ribosomal subunits, at 37°C. 0.7 μM of YqeH alone or in the presence of 0.2 μM S4 or 30S ribosomal subunits, sampling at interval of 10 minutes. The amount of free Pi released from the hydrolysis of 40 μM [γ-^{32}P]GTP was quantified using Cerenkov counting. Notably, the GTPase activity of YqeH was not significantly affected in the presence of either S4 or 30S subunit.

3.4 DISCUSSION

YqeH participates in the biogenesis of 30S ribosomal subunit

Initial *in vitro* ribosome reconstitution in *E. coli* is achieved by incubating mature 16S rRNA and a mixture of 30S ribosomal proteins (Traub and Nomura, 1968). However, the reaction requires high concentrations of salt and Mg^{2+}, and a temperature of 40^{o}C, which are non-physiological conditions. Thus, the observed self-assembly *in vitro* does not preclude the existence of non-ribosomal factors that play a fundamental role in ribosomal subunit assembly and maturation *in vivo* (Dammel and Noller, 1995; Bylund et al., 1998; Inoue et al., 2003). Several studies confirm that bacterial GTP-binding proteins of the Obg/Era family participate in the late stages of 30S or 50S subunit assembly (Sato et al., 2005; Sharma et al., 2005, Uicker et al., 2006; Jiang et al., 2006; Matsuo et al., 2006; Schaefer et al., 2006). Here we present results demonstrating that YqeH, a member of the Era/Obg family, plays an important role in 30S subunit biogenesis.

A stable precursor of 30S with slightly reduced molecular mass accumulated in Era-depleted cells in *B. subtilis*, consistent with the theory that this protein is involved in final maturation of the 30S subunit (Sharma et al., 2005). In contrast, no accumulation of free 30S was observed in YqeH-depleted cells, despite a reduction in 70S ribosome, accompanied by an increase in the free 50S subunit level, strongly suggesting that YqeH is involved in 30S subunit assembly, in a different way from that of Era. In addition, we detected degradation products of 16S rRNA in YqeH-depleted cells in parallel with accumulation of 17S rRNA. Thus, depletion of YqeH appears to result in both retardation of 30S assembly and destabilization of the premature 30S subunit. During biogenesis of the 30S subunit, pre-16S rRNA interacts with ribosomal proteins and non-ribosomal factors to form pre-ribosomal intermediates (Gulli et al., 1995). In YqeH-depleted cells, a proportion of the newly synthesized 30S intermediates may be impaired, thus rendering the pre-16S rRNA more

susceptible to RNase degradation. YqeH may act as a RNA chaperon and restores the impaired rRNA structure to its proper conformation. The final maturation of 16S rRNA possibly occurs in the context of the 70S ribosome (Srivastava and Schlessinger, 1990), and is altered when a stable pre-ribosomal particle is unable to form (Nierhaus, 1991; Li et al., 1999; Kaczanowska and Ryden-Aulin, 2004). While we cannot rule out the possibility that accumulation of pre-16S rRNA in YqeH-depleted cells is a direct consequence of YqeH depletion, it is more conceivable that the sequential assembly of ribosomal proteins to form 30S subunit is primarily affected, and thus, pre-16S rRNA accumulation is a secondary effect.

To establish the precise role of YqeH in 30S subunit biogenesis, it is essential to determine when the protein is incorporated into the premature subunit and how it reforms the structure of the subunit. However, we have not yet succeeded in detecting direct interactions between YqeH and the premature or mature 30S subunit *in vivo*. *In vitro* GTPase analyses showed that YqeH exhibits high intrinsic GTPase activity relatively to other members of Obg/Era family, and remains unaffected by the addition of 50S or 30S subunit. Thus, identification of intra-molecular GAP domain that modulates the GTPase activity of YqeH should facilitate our understanding of the functions of YqeH.

In *C. crescentus*, a temperature-sensitive mutant of Obg with a defect in 50S subunit assembly or stability was isolated. The defect occurred at both the permissive and restrictive temperatures, suggesting that this is not the essential function of Obg (Datta et al., 2004). Recent studies show that *Vibrio cholera* Obg is dispensable in a *relA* deletion mutant and essentially acts as a repressor of the stringent response by regulating SpoT activity to maintain low ppGpp levels when bacteria grow in nutrient-rich conditions (Raskin et al., 2007). *B. subtilis yqeH* is classified as an essential gene due to the inability of the IPTG-dependent P_{spac}-*yqeH* mutant to grow on LB or PAB agar plates in the absence of IPTG. However, in our experiments, the P_{spac}-*yqeH* mutant grew in the PAB liquid medium without IPTG

supplementation, albeit at an impaired rate. This finding raises the interesting possibility that YqeH functions not only in ribosomal assembly, but has other essential cellular roles to maintain effective growth on solid media.

YqeH is conserved predominantly in Gram positive bacteria and even higher eukaryotes. In view of its important roles in the 30S subunit biogenesis, prompted me to ask why YqeH homologs are not conserved in Gram negative bacteria such as *E. coli,* or in Archae? By comparing the 30S subunit of *E. coli* and *B. subtilis,* differences in the constitution of r-proteins and the processing of pre-16S rRNA, may explain at least partly, the reason why YqeH is not conserved. Firstly, in *E. coli*, r-protein S1 is essential for cell viability, involves in tmRNA-mediated quality control of polypeptide synthesis. This process requires S1 and a specific RNA acting as both a transfer and messenger RNA (tmRNA) to rescue and recycle the stalled ribosome and target the incomplete protein for degradation (Saguy et al., 2007). However, S1 is not presence in *B. subtilis,* which implies a different mechanism may be used to release the stalled ribosome. Secondly, the 17S rRNA, a precursor of 16S rRNA of *E. coli* has an untrimmed 115 nt in 5' and 33 nt at 3'end, respectively, but 17S precursor with untrimmed 5'end of 76 nt, and 67 nt at the 3'end exists in *B. subtilis*. Moreover, the rRNA processing of either 16S or 23S in both bacteria involves endoribonucleases and exoribonucleases that are quite diverse. For instance, RNases E and G are endoribonucleases, important for maturation of 5' end of 16S rRNA in *E. coli* (Li et al., 1999). However, in *B. subtilis*, this process involves an exoribonuclease RNase J1, via a 5' to 3' exonucleolytic pathway (Mathy et al., 2007). These data collectively suggests variants in the biogenesis of 30S ribosomal subunit for both Gram positive and negative bacteria, thus, the role of YqeH may replace by other protein(s) in *E. coli*, which show low amino acid sequences homology to YqeH.

Proposed model for YqeH-S4 interaction

GTPases are known to acts as molecular switches that exhibit different characters in their GTP- and GDP-bound forms. For instance, the bacterial translation factor EF-Tu binds 100-fold more strongly to tRNA in its GTP-bound than in its GDP-bound forms (Romero et al., 1985). Yeast two hybrid result showed YqeH interacts specifically with S4, but not its derivatives YqeH-S173N or S193P (Fig. 28) that showed severely reduced GTPase rate but maintained the GTP-binding ability, suggesting YqeH in GDP-bound form interacts with S4. YqeH-S173N and S193P may serves as potential dominant negative mutants to unveil the functions of YqeH. Taken together, these data strongly suggested that the efficient GTPase activity is pivotal for the proper function of YqeH. Intriguingly, the reason why predominantly GDP-bound YqeH interacts with S4, but not the active GTP-bound form, remains a myth to be uncovered. This interaction will be reconfirmed using affinity pull down of the YqeH-S4 complex, conducted in the GTP- and GDP-enriched condition, and observe for the change in binding affinity.

Indeed, most probably YqeH did not interact with S4 as a component of 30S subunit or 70S ribosome, because no association of YqeH to ribosome is observed under sucrose gradient density ultracentrifugation or gel filtration method (data not shown), or alternatively, if the YqeH interaction to 30S intermediates is transient, it becomes undetectable under the experimental conditions. Moreover, anti-YqeH immunoblotting result of fractionated sucrose density gradient showed that YqeH cosedimented at the top fraction, together with messenger RNA or tRNA, but not the 30S subunit or 70S ribosome (data not shown). In contrast, Era that is implicated in the late step assembly of 30S, was found to be associated with 30S subunit (Sayed et al., 1999; Sharma et al., 2005). Furthermore, the GTPases that involve in the late step assembly of 50S subunit, such as Obg, YlqF, YsxC, and YphC, are cosedimented with 50S precursors (Sato et al., 2005; Jiang et al., 2006; Matsuo et al., 2006; Schaefer et al.,

2006).

In view of 1) no significant accumulation of 30S precursors or intermediates in YqeH-depleted cells, compared that of Era-depleted cells 2) YqeH interacts specifically with S4, a r-protein that initiates the 30S subunit assembly, and 3) 70S ribosome with mature 30S was reduced in YqeH-depleted cells in association buffer, but the amount of free 30S subunit is similar to that of the wild type cells (Fig. 21B). The free 30S peak may consist of pool of recycled 30S upon completion of the translation and premature 30S. No significantly change in the amount, implying that YqeH-depletion does not affect the pool of recycled 30S, in contrast, the mature 30S formation is remarkably affected. All these results collectively suggest that YqeH involves in an early step of 30S subunit assembly, relatively to Era.

In vitro reconstitution assay of 30S subunit was conducted by incubation of a set of purified r-proteins with mature 16S rRNA. S4 makes extensive contacts with a five-way junction formed by helices 3, 4, 16, 17 and 18 of 16S rRNA (Fig. 31) and nucleates the assembly of the 5' domain (Brodersen et al., 2002). However, a precursor of 16S rRNA with untrimmed 5' and 3' end, instead, produced 30S subunit that is not functional active (Wireman and Sypherd, 1974). Therefore, I am tempted to know if YqeH-binding to the precursor of 16S rRNA, is a prerequisite for the subsequent incorporation of S4 to 16S rRNA, because the rRNA transcription is coupling with the sequential assembly of r-proteins, which is strictly coordinated *in vivo*.

A model for YqeH-S4 interaction that plays a key role in the binding of pre-16S rRNA at early assembly of 30S subunit is presented (Fig. 32). In *B. subtilis*, the *rrn* operon is transcribed as a 30S primary transcript, concurrent with the cleavages by endoribonuclease RNase III to yield the pre-16S and pre-23S. The final maturation involves removal of 76 and 67 nucleotides from the 5' and 3' ends, respectively, which may occur at the late step of 30S assembly or even in the 70S ribosome context (Kaczanowska and Ryden-Aulin, 2004; Britton

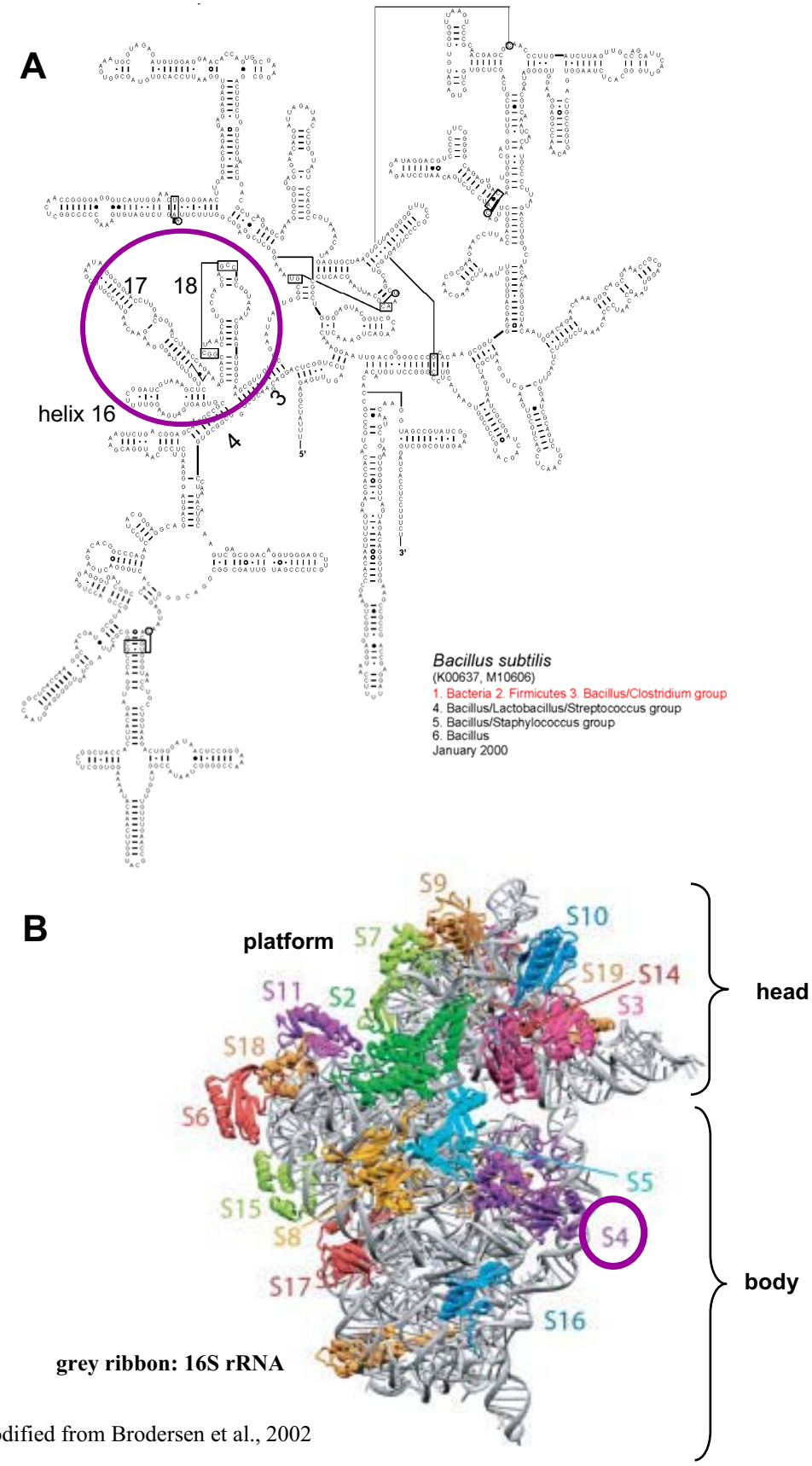


Fig. 31 Binding sites of S4 to the 16S rRNA of 30S ribosomal subunit. (A) S4 binds to helices 3, 4, 16, 17 and 18 of 5'domain of 16S rRNA, and nucleates the initiation of 30S subunit assembly (B) the overview 3D structure of 30S subunit of *E. coli* , with the binding positions of the r-proteins shown.

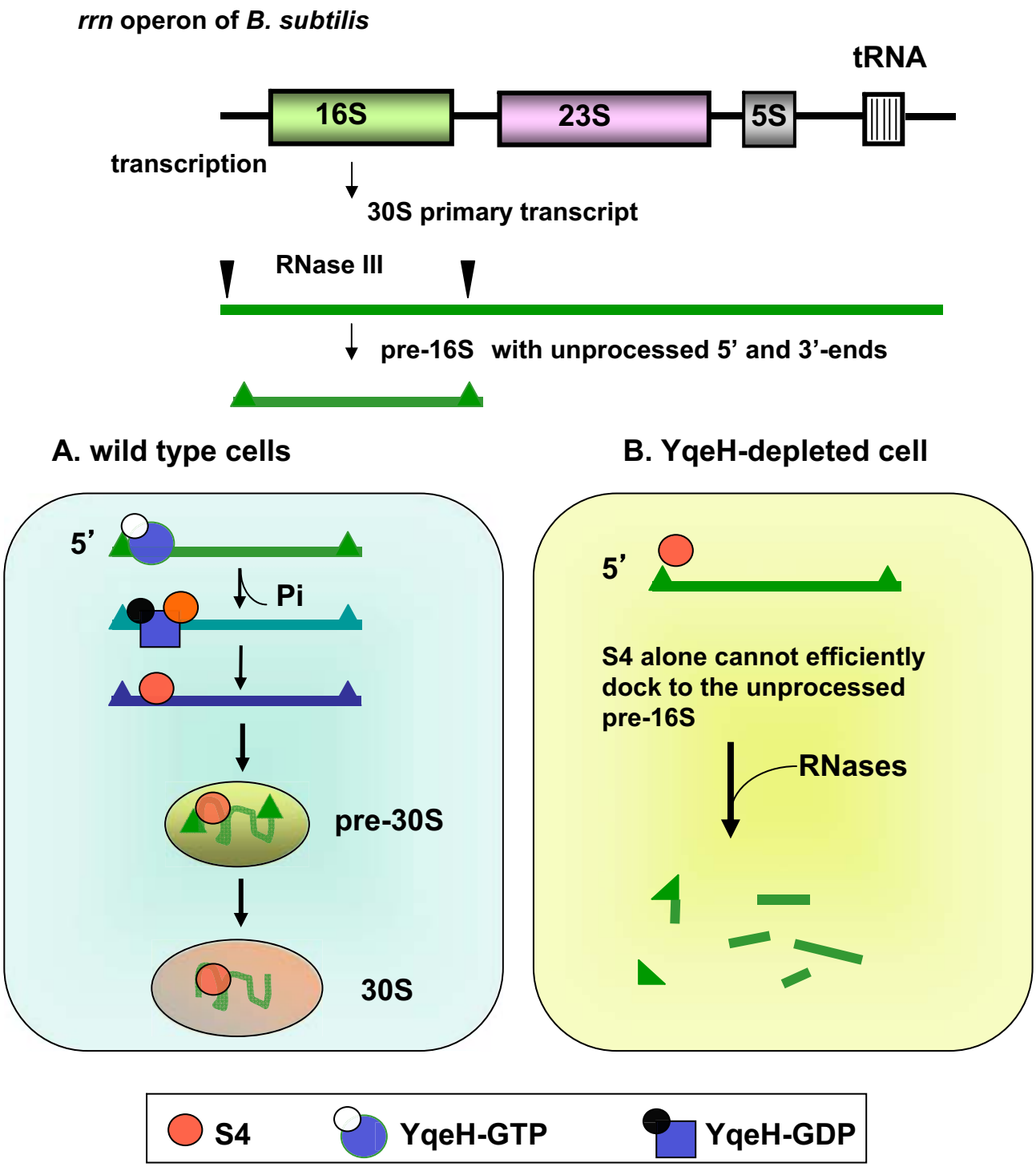


Fig. 32 Proposed model for YqeH-S4 interaction during biogenesis of the 30S subunit. In *B. subtilis*, the *rrnB* operon is transcribed as a 30S primary transcript, concurrent with the cleavages by endoribonuclease RNase III to yield the pre-16S, pre-23S and pre-5S. (A) Firstly, the GTP bound-YqeH, presumably bind to the pre-16S rRNA through its zinc finger domain that is implicated for rRNA-binding. Subsequently, the GTPase activity of YqeH is triggered, followed by an rearrangement of the 16S rRNA conformation, which is now ready for the recruitment of S4 to the 5' domain, through interaction with YqeH. Once S4 is recruited into the 16S rRNA, the sequential assembly of 30S r-protein is initiated, concomitant with the trimming and base modification of pre-16S. 30S intermediate grows into mature 30S subunit, ready for association with 50S subunit to form active 70S ribosome. Color differences of the pre-16S rRNA indicated that confirmation changes upon binding of YqeH and S4 (B) In YqeH-depleted cells, S4 alone may not dock to the unprocessed pre-16S rRNA. Thus, initiation of sequential assembly of 30S subunit is inhibited. As a consequence, the loosely-folded pre-16S rRNA is subjected to cellular RNases degradation.

et al., 2006). First, the GTP bound-YqeH, presumably bind to the pre-16S rRNA through its zinc finger domain that is implicated for rRNA-binding (Anand et al., 2006). Subsequently, the hydrolysis of bound-GTP of YqeH triggers rearrangement of 16S rRNA conformation, which is now ready for the recruitment of S4 to the 5' domain, through interaction with YqeH. Once S4 is recruited into the 16S rRNA, the sequential assembly of 30S r-protein is initiated, concomitant with the trimming and base modification of pre-16S. 30S intermediate grows into mature 30S subunit, ready for association with 50S subunit to form active 70S ribosome.

On the other hand, in YqeH-depleted cells, S4 alone may not dock to the unprocessed pre-16S rRNA. In 16S rRNA precursor, the unprocessed 5' and 3' ends sequences are base-paired and form a long helix, seem to be functionally inactive (Nierhaus, 2004). Therefore, I speculate that in the absence of YqeH, the S4 binding sites in pre-16S rRNA is in improper fold condition, or the unprocessed 5' end may form a hairpin loop adjacent to the S4 binding site, which inhibit the recruitment of S4 to the 16S rRNA. Therefore, initiation of sequential assembly of 30S-related r-proteins is inhibited. As a consequence, the loosely-folded pre-16S rRNA is subjected to cellular RNases degradation. *In vitro* RNA-protein binding analysis, where purified S4 is incubated with mature 16S or 16S rRNA precursors, in the absence or presence of YqeH, may serve as key models to elucidate the biological significant of YqeH-S4 interaction.

CHAPTER 4 SUMMARY AND FUTURE WORK

Quantitative biochemical analyses show that all proteins of Obg/Era family possess intrinsic GTPase activities that capable of hydrolyzing the bound-GTP molecule to GDP, except for YsxC. Obg, Era and YlqF formed a group of proteins with slow rate of GTPase activities, however, YqeH and YphC showed unexpectedly high GTPase activities that distinguished from the former group. In this study, we also provide the first experimental evident that the GTPase activities of Obg and Era were enhanced by the 50S and 30S ribosomal subunits, respectively. These results are consistent with the previously reports showed that Obg and Era of *E. coli* cosedimented with 50S (Wout et al., 2004; Sato et al., 2005) and 30S subunits (Sayed et al., 1999), respectively

We observed a reduced 70S ribosome, and accumulation of the free 50S subunit, but not free 30S, in YqeH-depleted cells. Consistent with the alleged involvement of YqeH in 30S subunit biogenesis, a precursor of 16S rRNA and its degraded products were detected. Era is another GTPase that is proposed to involve in the late stages of 30S subunit assembly. Era-depletion caused an accumulation of a stable 30S precursor in the cells. Indeed, YqeH overexpression did not compensate for growth defects occurring due to Era depletion and *vice versa.* Our findings strongly suggest that both YqeH and Era are involved in 30S subunit biogenesis, but play separate roles at distinct checkpoints during 30S assembly.

Although YqeH is widely conserved in Gram positive bacteria and higher organisms such as yeast, fruitfly, plant and human, none of the studies of YqeH homologs has been reported to date. In yeast, Bms1 is a GTPase required for biosynthesis of 40S ribosomal subunit (Karbstein et al., 2005). The C-terminal domain of Bms1 acts as an intramolecular GTPase-activating protein (GAP), with a novel GAP motif. C-terminal of YqeH is domain with arginine-rich, potentially acts as an ‘arginine finger’ that catalyze the GTPase activity,

as observed in p120 RasGAP (Sprang, 1997). Moreover, GTPase assay of YqeH was repeated with addition of either purified S4, or purified S4 together with 30S subunit, both condition revealed no influence on the GTPase activity of YqeH. Therefore, I am interested to analyze if YqeH possesses an intramolecular GTPase activator domain, by expressing the YqeH truncated proteins with N- or C-terminal deletions, and their GTPase activities were compare to the full length-YqeH. In addition, these deletion variants of YqeH will also be tested for their ability to provide trans-acting GAP activity to Era or YlqF.

Since YqeH is involved in biogenesis of the 30S subunit, it is conceivable that YqeH may interact with the 16S rRNA or 30S-related ribosomal proteins. A zinc finger domain in N-terminal of YqeH is implicated as a putative RNA-binding motif. Therefore, the potential of YqeH binding to the 16S rRNA or its precursors can be determined by formaldehyde cross-linking of the His-tagged YqeH to the rRNA *in vivo*, affinity pull down of the complexes and confirmed with primer extension. The YqeH binding regions on the 16S rRNA, if any, can be further determined by RNA footprinting.

Furthermore, S4 was identified as YqeH interactant, a ribosomal protein of 30S subunit that acts as an initiator of 30S assembly. I also generated YqeH mutants S173N and S193P that did not interact with S4. Because YlqF and Obg that co-sedimented with 50S (Matsuo et al., 2006; Sato et al., 2005), and YjeQ that co-sedimented with 30S subunit (Daigle and Brown, 2004; Himeno et al., 2004) show slow intrinsic GTPase activities, the severely reduced GTPase activity of YqeH mutants that may be useful for 'capturing' potential transient YqeH interactants and effectors, which could not be isolated using wild type YqeH. Understanding the functional significance of YqeH-S4 interaction in *B. subtilis*, may provide the key insight into the functions of *yqeH,* and thus the biogenesis of 30S subunit in bacteria or even in 40S subunit of higher eukaryotes.

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