Microbiology (2002), **148**, 3539–3552 Printed in Great Britain

Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*

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GTP-binding proteins are found in all domains of life and are involved in various essential cellular processes. With the recent explosion of available genome sequence data, a widely distributed bacterial subfamily of GTP-binding proteins was discovered, represented by the *Escherichia coli* Era and the *Bacillus subtilis* Obg proteins. Although only a limited number of the GTP-binding proteins belonging to the subfamily have been experimentally characterized, and their function remains unknown, the available data suggests that many of them are essential to bacterial growth. When the complete genomic sequence of *B. subtilis* was surveyed for genes encoding GTP-binding proteins of the Era/Obg family, nine such genes were identified. As a first step in elucidating the functional networks of those nine GTP-binding proteins, data presented here indicates that six of them are essential for *B. subtilis* viability. Additionally, it is shown that the six essential proteins are able to specifically bind GTP and GDP *in vitro*. Experimental depletion of the essential GTP-binding proteins was examined in the context of cell morphology and chromosome replication, and it was found that two proteins, Bex and YqeH, appeared to participate in the regulation of initiation of chromosome replication. Collectively, these results suggest that members of the GTP-binding Era/Obg family are important proteins with precise, yet still not fully understood, roles in bacterial growth and viability.

Keywords: bacterial GTPase, molecular switch protein, bacterial growth, regulation of initiation of chromosome replication

INTRODUCTION

GTP-binding proteins are found in all domains of life and are involved in various essential processes such as cell cycle progression, signal transduction, protein translation and vesicular trafficking (Bourne *et al.*, 1991; Kjeldgaard *et al.*, 1996; Sprang, 1997). Binding and hydrolysis of GTP results in reciprocal conformational changes of the GTP-binding proteins, and the GTP- and GDP-bound forms define the active and inactive states, respectively (Bourne *et al.*, 1991). With the recent expansion of available genome sequence data, a subfamily of widely distributed bacterial GTP-binding proteins was found, as represented by the *Escherichia coli* Era and the *Bacillus subtilis* Obg proteins (Caldon *et al.*, 2001). Although the *E. coli era* gene was named according to sequence similarity to the GTP-binding domain of the yeast Ras protein (Ahnn *et al.*, 1986), GTP-binding proteins of the Era/Obg family are now recognized as a subfamily separate from Ras, a small GTP-binding protein that is anchored in the eukaryotic membrane and is involved in signal transduction (Bourne *et al.*, 1990). Members of this subfamily have been identified in archaea and eukaryotes, but their function remains undetermined (Zuber *et al.*, 1997; Britton *et al.*, 1998; Devitt *et al.*, 1999; Maddock *et al.*, 1997; Sazuka *et al.*, 1992; Schenker *et al.*, 1994; Sommer *et al.*, 1994).

Among bacterial GTP-binding proteins of the Era/Obg family, the *E. coli* Era and the *B. subtilis* Obg proteins have been well studied, but their precise roles are still poorly defined. The Era protein has been shown to be

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The primer sequences used for PCR in this study are shown as supplementary data on *Microbiology* Online (http://mic.sgmjournals.org).

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Table 1. Bacterial strains

Strain	Genotype	Source/reference
B. subtilis		
CRK6000	*purA16 metB5 hisA3 guaB*	Moriya *et al.* (1990)
TMO101	*purA16 metB, hisA3 guaB bex*::pTM101 (P*spac–bex erm*)	This study
TMO102	*purA16 metB5 hisA3 guaB obg*::pTM102 (P*spac–obg erm*)	This study
TMO303	*purA16 metB5 hisA3 guaB yphC*::*tet*, *amyE*::P*spac–yphC cat*	This study
TMO104	*purA16 metB5 hisA3 guaB ysxC*::pTM104 (P*spac–ysxC erm*)	This study
TMO005	*purA16 metB5 hisA3 guaB thdF*::pTM005 (Δ*thdF* P*spac–gidA erm*)	This study
TMO006	*purA16 metB5 hisA3 guaB ynbA*::pTM006 (Δ*ynbA* P*spac–ynbB erm*)	This study
TMO007	*purA16 metB5 hisA3 guaB yyaF*::pTM007 (Δ*yyaF* P*spac–rpsF erm*)	This study
TMO108	*purA16 metB5 hisA3 guaB ylqF*::pTM108 (P*spac–ylqF erm*)	This study
TMO208	*purA16 metB5 hisA3 guaB ylqF*::pTM208 (P*spac–ylqF erm*)	This study
TMO309	*purA16 metB5 hisA3 guaB yqeH*::*tet aprE*::P*spac–yqeH spec*	This study
E. coli		
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock
BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* Δ(*srl–recA*)*306*::Tn*10*(DE3)pLysS	Novagen

essential for cell growth in *E. coli*, *Salmonella typhimurium* and *Streptococcus mutans* (March *et al.*, 1988; Takiff *et al.*, 1989; Gollop & March, 1991; Anderson *et al.*, 1996; Sato *et al.*, 1998). Era binds to 16S rRNA and contains an RNA-binding KH domain at the C terminus (Meier *et al.*, 1999; Sayed *et al.*, 1999). In *E. coli*, depletion of Era at 27 °C has been shown to cause cell filamentation (Gollop & March, 1991) and a mutation in the GTP-binding domain was found to suppress temperature-sensitive chromosome partitioning mutations, suggesting that Era is a cell-cycle checkpoint regulator (Britton *et al.*, 1997, 1998; Britton & Lupski, 1997). The *B. subtilis* and *Streptomyces* spp. Obg proteins are essential for vegetative growth and initiation of sporulation (Trach & Hoch, 1989; Okamoto *et al.*, 1997; Okamoto & Ochi, 1998; Vidwans *et al.*, 1995; Welsh *et al.*, 1994). Furthermore, *B. subtilis* Obg has been found to be essential for stress activation of transcription factor σ^B and has been shown to cofractionate with ribosomes, together with regulators of σ^B activity (Scott & Haldenwang, 1999; Scott *et al.*, 2000). The existence of Obg is not restricted to bacteria that undergo cellular differentiation. The *Caulobacter crescentus* Obg homologue (CgtA) has been shown to be indispensable for growth (Maddock *et al.*, 1997). Very recently, the *E. coli* homologue YhbZ (renamed ObgE) has been reported to be an essential gene involved in chromosome partitioning (Kobayashi *et al.*, 2001). In addition to Era and Obg, a GTP-binding protein encoded by the *engA* gene of *Neisseria gonorrhoeae* has been suggested to be essential for growth (Mehr *et al.*, 2000). *B. subtilis* YsxC is also an essential GTP-binding protein (Arigoni *et al.*, 1998; Pragai & Harwood, 2000) and the depletion of its homologue (YihA) in *E. coli* caused defective cell division (Dassain *et al.*, 1999). Thus, experimental results are accumulating to suggest that many GTP-binding proteins of the Era/Obg family are essential for bacterial growth, often related to cell cycle progression such as chromosome replication and partitioning, and cell division and cellular differentiation.

When the complete genome sequence of *B. subtilis* was surveyed for genes encoding GTP-binding proteins of the Era/Obg family, we identified in addition to *obg* and *ysxC*, homologues of the *E. coli era* and *N. gonorrhoeae engA* genes, as well as five additional family members. As a first step in elucidating a functional network of those nine GTP-binding proteins, we found that six of them are essential for *B. subtilis* viability. *In vitro* studies demonstrated that the six essential proteins specifically bind GTP and GDP. Furthermore, we analysed the effect of depletion of these proteins in the *B. subtilis* cells on cell morphology and chromosome replication, and found that Bex (a homologue of *E. coli* Era) and YqeH appeared to participate in the regulation of initiation of chromosome replication.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains were grown in antibiotic medium 3 (Difco) supplemented with adenine and guanosine (final concentration 20 μg ml^{-1}) at 30 °C. *E. coli* strains were grown in LB medium. When necessary, antibiotics were added to final concentrations of 5 μg tetracycline ml^{-1}, 0·5 μg erythromycin ml^{-1} and 100 μg spectinomycin ml^{-1} in the *B. subtilis* culture, and 20 μg kanamycin ml^{-1} in the *E. coli* culture. Since the addition of antibiotics affected the growth rate slightly, cells were grown without antibiotics for the flow cytometry analysis.

Plasmids. Table 2 summarizes the characteristics of the plasmids used in this study. Primers used for PCR are shown as supplementary data at http://mic.sgmjournals.org

Derivatives of pMUTINT3 (Vagner *et al.*, 1998; Moriya *et al.*, 1998) or pMUTINNC were used to construct knockout or conditional-null mutants of the *B. subtilis* genes. The native chromosomal gene of *E. coli lacZ* contains three LacI binding sequences; O1, O2 and O3 (Oehler *et al.*, 1994). The O1 sequence was introduced as a LacI binding site in pMUTINT3.

Table 2. Plasmids constructed in this study

Plasmid	Vector	Insert*	Primers†	Source
pMutinT3				Moriya *et al*. (1998)
pMutinNC				This study
pTM001	pMUTINT3	239 bp internal fragment of *bex* (5 to 243)	BEXF2, BEXR	This study
pTM003	pMUTINT3	248 bp internal fragment of *yphC* (5 to 252)	PHCF2, PHCR	This study
pTM005	pMUTINT3	243 bp internal fragment of *thdF* (13 to 255)	THDF2, THDR	This study
pTM006	pMUTINT3	281 bp internal fragment of *ynbA* (5 to 285)	NBAF2, NBAR	This study
pTM007	pMUTINT3	285 bp internal fragment of *yyaF* (13 to 297)	YAFF2, YAFR	This study
pTM008	pMUTINT3	228 bp internal fragment of *ylqF* (12 to 239)	LQFF2, LQFR	This study
pTM009	pMUTINT3	243 bp internal fragment of *yqeH* (13 to 255)	QEHF2, QEHR	This study
pTM101	pMUTINT3	283 bp fragment containing the SD sequence and initiation codon of *bex* (−40 to 243)	BEXF1, BEXR	This study
pTM102	pMUTINT3	206 bp fragment containing the SD sequence and initiation codon of *obg* (−45 to 161)	OBGF1, OBGR	This study
pTM104	pMUTINT3	221 bp fragment containing the SD sequence and initiation codon of *ysxC* (−53 to 168)	SXCF1, SXCR	This study
pTM108	pMUTINT3	272 bp fragment containing the SD sequence and initiation codon of *ylqF* (−33 to 239)	LQFF1, LQFR	This study
pTM208	pMUTINNC	272 bp fragment containing the SD sequence and initiation codon of *ylqF* (−33 to 239)	LQFF1, LQFR	This study
pDLT3				This study
pAPNC213				This study
pBR322				Toyobo
pTM303	pDLT3	1346 bp containing the SD sequence and coding region of *yphC* (−31 to 1315)	PHCF1, PHCR2	This study
pTM309	pAPNC213	1179 bp containing the SD sequence and coding region of *yqeH* (−33 to 1146)	QEHF1, QEHR2	This study
pTM403	pBR322	2575 bp fragment *yphC* (−357 to 21)–*tet*–*yphC* (1283 to 1660)	PHBF, PHBR, GPSF, GPSR	This study
pTM409	pBR322	2619 bp fragment *yqeH* (−424 to −4)–*tet*–*yqeH* (1026 to 1404)	QEGF, QEGR, ARDF, ARDR	This study
pET29b				Novagen
pET28b				Novagen
pTM291	pET29b	903 bp *bex* coding region (1 to 903)	BEXF0, BEXR0	This study
pTM282	pET28b	1287 bp *obg* coding region (1 to 1287)	OBGF0, OBGR0	This study
pTM293	pET29b	585 bp *ysxC* coding region (1 to 585)	SXCF0, SXCR0	This study
pTM294	pET29b	1308 bp *yphC* coding region (1 to 1308)	PHCF0, PHCR0	This study
pTM298	pET29b	846 bp *ylqF* coding region (1 to 846)	LQFF0, LQFR0	This study
pTM299	pET29b	1098 bp *yqeH* coding region (1 to 1098)	QEHF0, QEHR0	This study

* Numbers in parentheses indicate the nucleotide numbers of each gene inserted.

† Primers used to PCR amplify the inserts.

We further inserted the O2 sequence and the sequence between O1 and O2 in pMUTINNC (Fig. 1a). Additional mutations were introduced to make the O1 sequence perfectly palindromic, further increasing its affinity for the LacI repressor. To create a new LacI binding sequence, primers O2F and OidR were used to amplify from pMUTIN1 (Vagner *et al*., 1998) the following 111 bp fragment: 5′-AAATGTGAGCAGTAACAACCTCTGCTAAAATTCCT-GAAAAATTTTGCAAAAAGTTGTTGACTTTATCTA-CAAGGTGTGGCATAATGTGTGGAATTGTGAGCGC-TCACAATT-3′ (underlined nucleotides indicate the LacI binding sequences). The amplified fragment was cloned into the *Hinc*II site of pTZ18 (Toyobo), excised by *Sma*I and *Hin*dIII digestion, and cloned between the *Nru*I and *Hin*dIII sites of pMUTINT3.

The integration vectors pDLT3 (Fig. 1b) and pAPNC213 (Fig. 1c) were constructed and used to integrate a target gene fused with the *spac* promoter into the *amyE* or *aprE* locus of the *B. subtilis* chromosome. To obtain pDLT3, pMUTINT3 was digested with *Cla*I and *Bpu*1102I, blunted by the Klenow enzyme, and circularized to remove the *lacZ* gene. Then, a *Tth*111I–*Bgl*II fragment containing the *lacI* gene and *spac* promoter was inserted between the *Bam*HI and *Bpu*1102I sites of pDLd (Nanamiya *et al*. 1998). To obtain pAPNC213, the *lacI* gene was amplified from pMUTINT3 using primers LACIF and LACIR, followed by digestion at the *Bam*HI and *Hin*dIII sites within the primers. A fragment containing the *spac* promoter was obtained by digesting pMUTINNC with *Bgl*II and *Sph*I. These two fragments were cloned between the *Hin*dIII and *Sph*I sites of pRB373 (Bruckner, 1992) to obtain

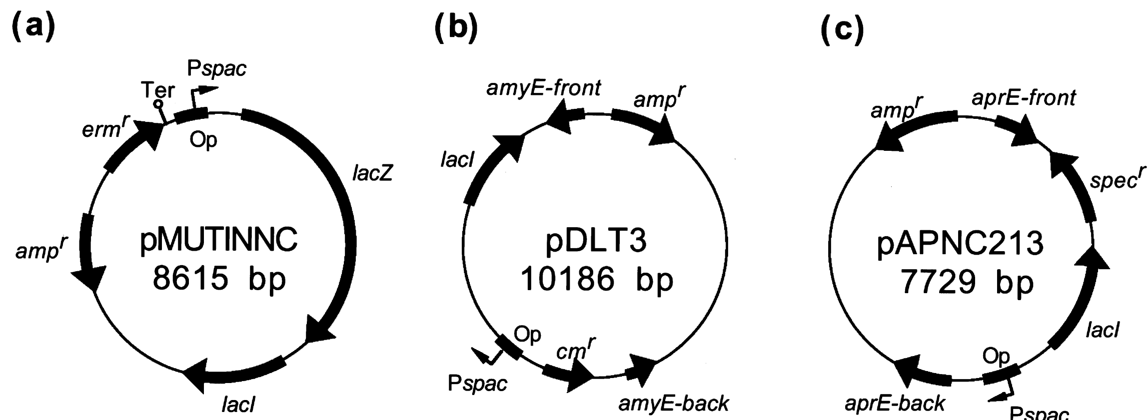


Fig. 1. Structure of plasmid vectors constructed in this study. Location of genes and the *spac* promoter on pMUTINNC (a), pDLT3 (b) and pAPNC213 (c) is shown schematically. Selection of *B. subtilis* transformants was carried out using erm^R, cm^R or $spec^R$. *E. coli* transformants were selected with amp^R. The *lacI* gene regulates P*spac,* and the *lacZ* gene is a reporter gene for the expression of the integrated region of *B. subtilis*. The *amyE*-front and *ameE*-back sequences were used to integrate the DNA fragment between them into the *amyE* locus of the *B. subtilis* chromosome, and *aprE*-front and *aprE*-back sequences into the *aprE* locus. Ter indicates the transcriptional terminator and Op indicates the LacI binding sequence.

pNC213. A cassette for the P*spac–lacI* promoter was obtained from pNC213 by digestion with *Hin*dIII and *Eco*RI. The upper and lower portions of the *aprE* gene were amplified using primer sets APREUF–APREUR and APREDF–APREDR, respectively. These products were digested with *Mun*I/*Hin*dIII and *Hin*dIII/*Nru*I, respectively, ligated using the *Hin*dIII sites, and inserted between the *Eco*RI and *Nru*I sites of pBR322. Then, the cassette for P*spac–lacI* derived from pNC213 was inserted between the *Hin*dIII and *Eco*RI (introduced in the APREDF primer) sites of the resultant plasmid. Finally, a spectinomycin resistance gene of *Staphylococcus aureus* was amplified from pJL62 (LeDeaux & Grossman, 1995) using primers SPCF and SPCR, digested with *Hin*dIII and cloned into the *Hin*dIII site of the plasmid in the same direction as that of the *lacI* gene.

Plasmids pTM403 and pTM409, used to replace the *yphC* or *yqeH* genes by the tetracycline resistance gene, were constructed as follows. The upstream and downstream regions of *yphC* or *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 *yphC* or *yqeH* gene.

Purification of the GTP-binding proteins. To express the GTP-binding proteins with a histidine tag (His_6) at the C terminus, the coding sequences of Obg, Bex, YphC, YsxC, YlqF and YqeH (from the initiation codon to the C-terminal amino acid codon, except the termination codon) were amplified by PCR and cloned into the pET28b or pET29b plasmid (Novagen) to obtain pTM282, pTM291, pTM293, pTM294, pTM298 and pTM299, respectively. *E. coli* BL21(DE3)pLysS derivatives (Novagen) containing each of the plasmids were grown at 30 °C in 500 ml LB medium and the his-tagged GTP-binding proteins were purified according to the pET system protocol (Novagen). When the culture reached OD_{600} 0·6, IPTG was added to a final concentration of 1 mM. The cells were grown 3 h more and then harvested by centrifugation. Collected cells were washed with binding buffer (0·5 M NaCl, 5 mM imidazole, 20 mM Tris/HCl, pH 7·9) and resuspended in 20 ml of the same buffer. The cells were broken by sonication on ice and the lysate was centrifuged at 39 000 ***g*** for 30 min at 4 °C. The supernatant fraction was applied to a Ni^{2+}-NTA resin column (Novagen), and the column was washed with 30 ml binding buffer followed by 10 ml washing buffer (0·5 M NaCl, 20 mM imidazole, 20 mM Tris/HCl, pH 7·9). The GTP-binding protein in the column was eluted with 10 ml binding buffer containing 60 mM imidazole. The resin-bound YphC–His_6 in the Ni-column was cleaved with 2 U thrombin protease in cleavage buffer (20 mM Tris/HCl, pH 8·0, 150 mM KCl, 2·5 mM $CaCl_2$), incubated at 4 °C overnight. Purified proteins were stored at −80 °C after addition of 10 % (v/v) glycerol.

GTP-binding assay. GTP-binding activity was analysed essentially following the procedure of Lin *et al.* (1999). Purified Bex–His_6, YlqF–His_6, YphC, YsxC–His_6 or YqeH protein (50 pmol) was added to 1·3 pmol [4 μCi ($1·5 \times 10^5$ Bq)] of [α-^{32}P]GTP [3000 Ci ($1·1 \times 10^{14}$ Bq) $mmol^{-1}$; NEN Life Science] in 20 μl GTP-binding buffer (50 mM Tris/HCl, pH 8·0, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 10 %, v/v, glycerol) (Sullivan *et al.*, 2000) and incubated on ice for 10 min. The bound radioactive labelled GTP was cross-linked to the proteins by UV light (254 nm, 1 *J* cm^{-2}). Excess unbound [α-^{32}P]GTP was eliminated with a Microcon centrifugal filter (Millipore). For reactions with competing nucleotides, 40 μM nonradioactive nucleotide (GTP, GDP, GMP, ATP, UTP or CTP) was mixed with [α-^{32}P]GTP (with a ratio about 600 : 1) prior to protein addition. Radiolabelled protein–GTP complexes were separated by SDS-PAGE. After electrophoresis, the gel was dried and exposed to an imaging plate for 1 h and signals were detected by BAS2500 (Fuji Film).

Immunoblotting. Rabbit polyclonal antibodies raised against purified Bex–, Obg–, YphC–, YsxC–, YlqF– and YqeH–His_6 proteins were obtained from Takara Shuzo. Preparation of cell lysates from exponentially growing *B. subtilis* cells and separation of proteins were carried out as described previously (Hassan *et al.*, 1997). Proteins were blotted on a Hybond-P

PVDF membrane (Amersham Pharmacia), and the membrane was incubated with rabbit polyclonal antibodies. After the membrane was treated with a second antibody (goat anti-rabbit IgG–horseradish peroxidase conjugate), signals were detected by the ECL-Plus enhanced chemiluminescence system (Amersham Pharmacia).

Fluorescence microscopy. Cell morphology and nucleoid distribution were examined as described under fluorescence microscopy after DAPI (4′,6-diamino-2-phenyl indole) staining (Hassan *et al.*, 1997).

Flow cytometry. Chloramphenicol was added at a concentration of 200 μg ml^{-1} to exponentially growing cells, which were then incubated for 5 h to ensure both inhibition of new rounds of replication initiation and completion of ongoing replication. The cells were fixed with ethanol and treated as described previously (Løbner-Olesen *et al.*, 1989), and the number of replication origins per cell was measured with a Bryte HS flow cytometer (Bio-Rad).

Measurement of DNA/protein ratio. Nucleic acid and protein fractions were extracted from cells by Schneider's method (Herbert *et al.*, 1971) with some modifications as described elsewhere (Kadoya *et al.*, 2002). Briefly, cells were suspended in 0·6 M perchloric acid and heated at 70 °C for 15 min. The soluble fraction was recovered by centrifugation and used as the nucleic acid fraction. The remaining insoluble materials were resuspended in 1 M sodium hydroxide and heated at 95 °C, followed by centrifugation. The soluble fraction was used as the protein fraction. DNA and protein concentrations in these fractions were determined by colorimetric methods described by Burton (1956) and the Lowry method

RESULTS

GTP-binding proteins of the Era/Obg family in *B. subtilis*

GTP-binding proteins are characterized by four localized conserved amino acid sequences in the GTP-binding domain, known as the G1, G2, G3 and G4 sequence motifs. The G1 (G/AXXXXGKT/S), G3 (DXXG) and G4 (NKXD) motifs are involved in GTP-binding and hydrolysis, and are well conserved in all subfamilies. The G2 motif is specific to each subfamily and is often responsible for interaction with effector molecules that regulate the balance between the GTP- and GDP-bound forms (Bourne *et al.*, 1991).

To begin examination of Era/Obg family members in the *B. subtilis* genome, we searched all ORFs of *B. subtilis* by PSI-BLAST (Altschul *et al.*, 1997) using the amino acid sequence of the GTP-binding domain of *E. coli* Era as a probe. We identified twelve related ORFs, including those encoding ribosomal function proteins IF2, LepA and YlaG. Further analysis using the twelve proteins identified as probes detected EF-Tu and EF-G. The predicted amino acid sequences of the detected GTP-binding domains were aligned by CLUSTALW (Thompson *et al.*, 1994) and their phylogenetic tree was constructed using TreeView (Fig. 2a). The resultant tree indicated that the five ribosomal function proteins and the remaining nine form distinct subfamilies, leading us to conclude that the *B. subtilis* genome contains nine GTP-binding proteins that are structurally related to Era and Obg. The amino acid sequences of the GTP-binding domain of these nine proteins are shown in Fig. 2b and the location of the domain in each protein is shown schematically in Fig. 2c. The YphC protein contains two GTP-binding domains. Three motifs (G1, G3 and G4) were well conserved, and a conserved threonine was identified in G2 among the ten GTP-binding domains (Fig. 2b), while amino acid sequences outside of the GTP-binding domains were unique to each protein.

Using unique amino acid sequences from each GTP-binding protein, homologues in other organisms were clearly identified (Table 3). Bex and YphC are homologues of *E. coli* Era and *N. gonorrhoeae* EngA, respectively. Bex, Obg, YphC, ThdF and YyaF seem to be conserved essentially in all eubacteria, although Bex and ThdF are missing in *Chlamydia* and *Mycobacterium*, respectively (see also the COG database at NCBI; http://www.ncbi.nlm.nih.gov/COG/). Furthermore Obg, ThdF and YyaF are widely conserved across all eukaryotes tested. In addition, YyaF was conserved in all archaea whose genomes have been sequenced. By contrast, YsxC, YlqF and YnbA are not conserved universally in bacteria, although they are widely distributed among the three domains of life. Interestingly, YqeH was found in only a limited number of bacteria, but is common in higher organisms including humans.

Six GTP-binding proteins are essential for viability in *B. subtilis*

The Obg and YsxC proteins have been shown to be essential for *B. subtilis* growth (Trach & Hoch, 1989; Arigoni *et al.*, 1998; Pragai & Harwood, 2000). In addition, homologues of Bex and YphC have been reported to be essential in other bacteria (March *et al.*, 1988; Takiff *et al.*, 1989; Gollop & March, 1991; Anderson *et al.*, 1996; Sato *et al.*, 1998; Mehr *et al.*, 2000). To determine whether they are also essential in *B. subtilis* and to discover whether other GTP-binding proteins play an essential role, we created knockouts by Campbell-type plasmid integrations. To this end, the N-terminal internal portions of each of the nine Era/Obg family members were amplified by PCR and cloned into integration vector pMUTINT3. Then we transformed *B. subtilis* CRK6000 cells with the pMUTINT3 derivatives and transformants were selected in the presence of IPTG to guarantee the expression of downstream genes. Transformants were easily obtained with the plasmids containing the *thdF*, *ynbA* and *yyaF* knockout sequences (Fig. 3d, e and f); proper integration of the plasmids into the genome of the transformants was confirmed by PCR amplification of fused sequences of the target gene and the integrated plasmid (data not shown). These results indicate that the ThdF, YyaF and YnbA proteins are not required for growth in LB medium. On the other hand, our failure to obtain knockout mutants of *bex*, *ylqF*, *yphC* and *yqeH* strongly suggested that they are essential genes in *B. subtilis*.

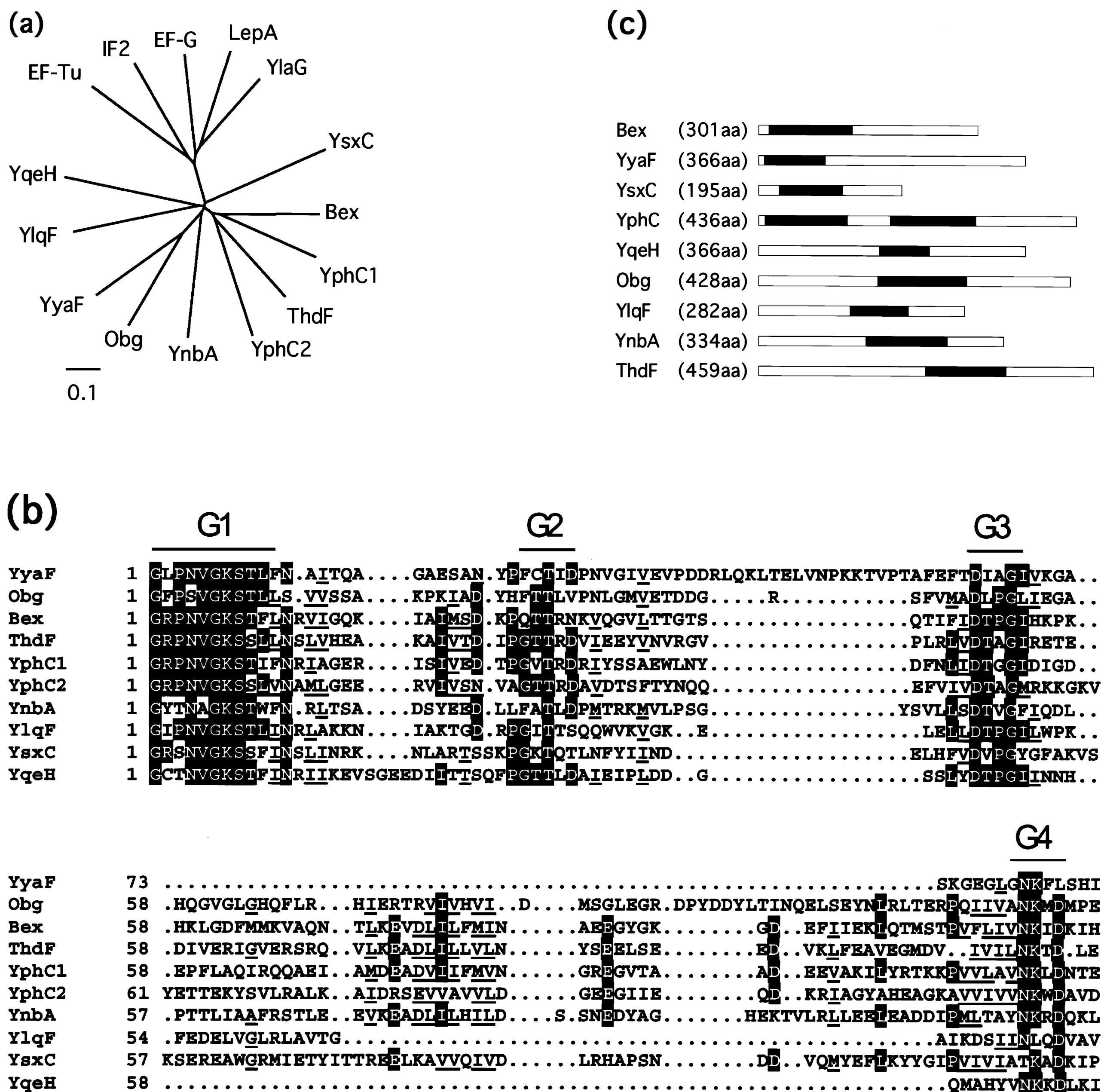


Fig. 2. (a) Phylogenetic tree of the GTP-binding domain of the GTP-binding proteins of the Era/Obg family, as related to ribosomal function. The scale for branch length is shown below the tree. (b) Multiple amino acid sequence alignment of the GTP-binding domain of the GTP-binding proteins of the Era/Obg family in *B. subtilis*. The G1, G2, G3 and G4 motifs are indicated. All sequences were obtained from SubtiList (http://genolist.pasteur.fr/SubtiList/). (c) Location of GTP-binding domain (shown in black) in each GTP-binding protein.

Therefore, we constructed *B. subtilis* cells in which the expression of *bex*, *ylqF*, *yphC* or *yqeH* is under the control of the *E. coli* LacI-repressible and IPTG-inducible *spac* promoter. We also constructed IPTG-dependent mutants of *obg* and *ysxC* in the same manner. The genomic sequence of *B. subtilis* suggests that *bex*, *obg* and *ylqF* are transcribed as a single transcriptional unit (Fig. 3a, b and g) and that *ysxC* is co-transcribed with the preceding *lonA* gene (Fig. 3c). For these genes we inserted the pMUTINT3 plasmids containing the SD sequence and the N-terminal portion of each gene in front of the initiation codon. As expected, the growth of the resultant mutants became IPTG-dependent, except for the *ylqF* mutant. However, when pMUTINT3 was replaced by the pMUTINNC in which the LacI binding sequence had been manipulated to have a higher affinity for the repressor, the growth of the *ylqF* mutant also became IPTG dependent.

Table 3. *B. subtilis* Era/Obg family GTP-binding proteins that are conserved in other species

Orthologues of *B. subtilis* GTP-binding proteins were obtained using BLAST. Accession numbers are described in this table except for *B. subtilis* and *E. coli*.

Species/strain	Bex	Obg	YphC	YsxC	YlqF	YqeH	ThdF	YyaF	YnbA
Synechocystis sp. PCC6803	NP_441951	NP_440268	NP_441526		NP_441269		NP_441216	NP_441270	NP_441353
Mycoplasma pneumoniae	NP_110257	NP_110252	NP_110163	NP_110169	NP_110345		NP_109696	NP_109714	
Mycoplasma genitalium	NP_073060	NP_073056	NP_072998	NP_073003	NP_073112		NP_072668	NP_072684	
Mycobacterium tuberculosis H37Rv	NP_216880	NP_216956	NP_216229					NP_215628	NP_217241
Ureaplasma urealyticum	NP_078328	NP_078298	NP_078218	NP_078100	NP_078431	NP_078305	NP_077848	NP_078434	
T. maritima	NP_228656	NP_227914	NP_229245	NP_229266	NP_228557	NP_228657	NP_228080	NP_229045	NP_228337
E. coli	Era	ObgE	YfgK	YihA			ThdF	YchF	HflX
Helicobacter pylori J99	NP_223184	NP_223008	NP_223491	NP_224193			NP_224063	NP_223234	
Methanococcus jannaschii				NP_247293	NP_248468			NP_248333	NP_248118
Pyrococcus abyssi				NP_127295	NP_127069			NP_127199	NP_126762
Saccharomyces cerevisiae		NP_012038			NP_010623	NP_010921		NP_013736	NP_009581
Caenorhabditis elegans		NP_493334			NP_492276	NP_496845	NP_493573	NP_493349	NP_505523
Arabidopsis thaliana	AAL38371	NP_197358	NP_187815	NP_565543		AAL38787	NP_190329		NP_177924
	NP_176001	NP_200604							
Drosophila melanogaster	AAF54886	AAF52655			AAF56060	AAL48812	AAL49166	AAF46520	AAL49248
Homo sapiens	BAB56112	CAC04015		XP_039952	AAH04409	AAH04894	AAH19261	CAB66481	CAA74749

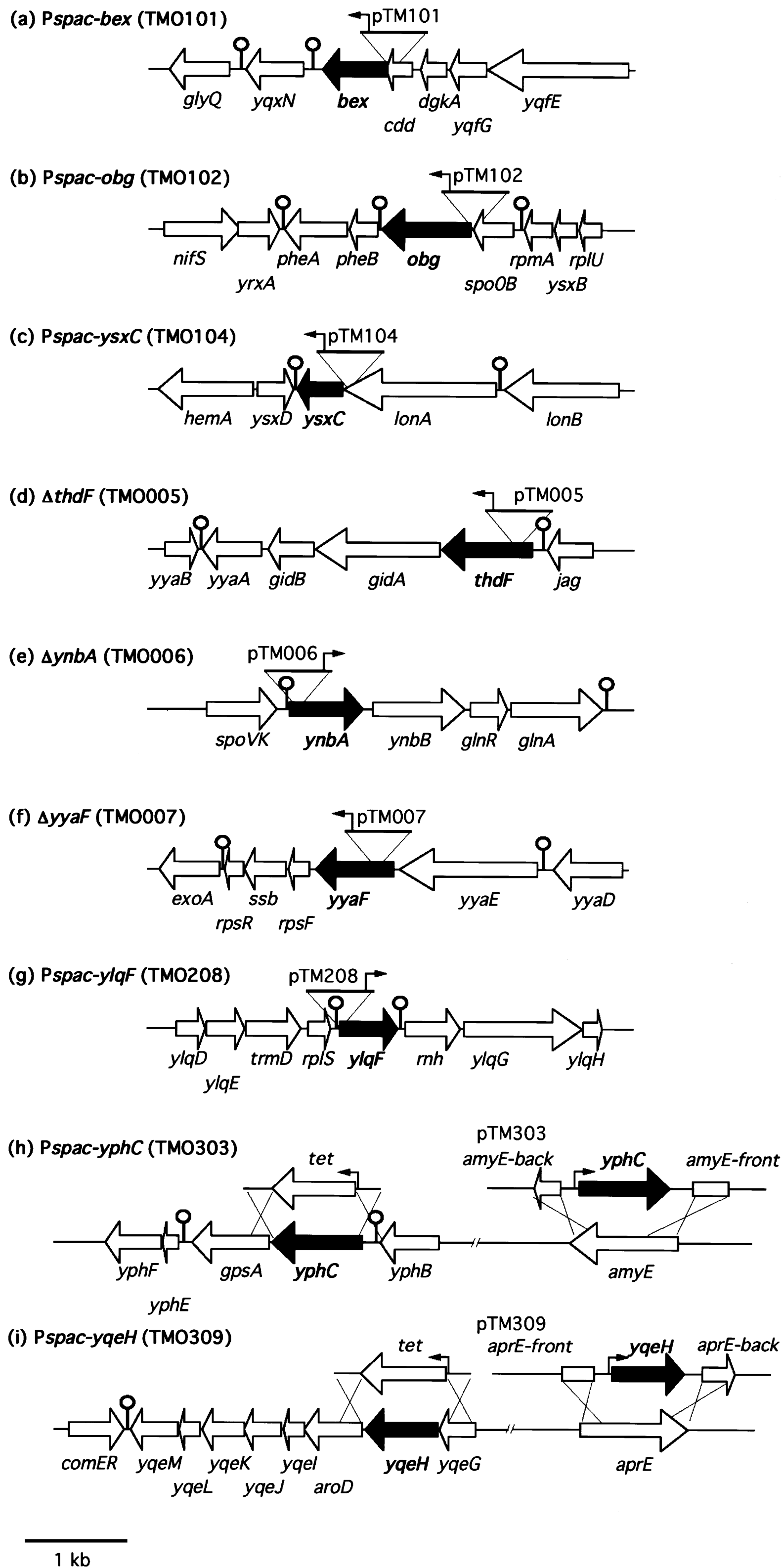


Fig. 3. Operon structure and mutagenesis strategy for the GTP-binding proteins of the Era/Obg family. Location and direction of transcription of genes surrounding the GTP-binding protein genes on the *B. subtilis* chromosome are shown by arrows. The stem–loop structure indicates a possible transcriptional terminator. Insertion site of the pMUTIN derivatives in each mutant is shown by inverted triangles. Small arrows indicate the direction of P*spac* in the integrated plasmids or that of the *tet* promoter. In conditional null mutants, P*spac*–*yphC* and P*spac*–*yqeH*, *yphC* or *yqeH* were fused to P*spac* and integrated into the *amyE* or *aprE* locus, and then the original *yphC* or *yqeH* gene was replaced with the *tet* gene.

yphC and *yqeH* are the first genes of operons containing two and seven genes, respectively (Fig. 3h and i). Therefore the artificial control of their expression by integration of pMUTINT3 should cause a polar effect on the expression of downstream genes. To avoid this, we first cloned the complete *yphC* and *yqeH* genes downstream of the *spac* promoter in pDLT3 or pAPNC213 and integrated them into the *amyE* or *aprE*

loci, respectively, through transformation of wild-type cells with linearized plasmid DNA and selection for chloramphenicol or spectinomycin resistance (Fig. 3h and i). Then, each original gene was replaced by the *tet* gene and the expression of downstream genes were maintained by the *tet* promoter to obtain IPTG-dependent mutants. This work showed that Bex, YphC, YqeH and YlqG are essential in *B. subtilis*.

These essential *B. subtilis* GTP-binding proteins bind specifically to GTP and GDP *in vitro*

Specific GTP/GDP binding and intrinsic GTPase activities have been demonstrated for *B. subtilis* Obg, *C. crescentus* CgtA, and *Thermotoga maritima* TrmE *in vitro* (Welsh *et al*., 1994; Lin *et al*., 1999; Yamanaka *et al*., 2000), so we next analysed the GTP/GDP binding activities for Bex, YlqF, YphC, YqeH and YsxC proteins. Test proteins were fused to a histidine tag (His_6), expressed in *E. coli* and purified to homogeneity (Fig. 4a). Since *B. subtilis* cells with His-tagged replacement genes grew normally except in the case of *yphC* (data not shown), we used purified His-tagged proteins for the GTP-binding assays except for the case of YphC, in which thrombin digestion was used to remove the His-tag before the GTP-binding assay (Fig. 4a).

Purified proteins were incubated with [α-^{32}P]GTP and the bound GTP was fixed to the proteins by UV cross-linking. When reaction products were separated by SDS-PAGE, radiolabelled GTP was clearly detected for all the proteins tested (Fig. 4b). The bindings were greatly reduced when an excess of unlabelled GTP or GDP was added to the reaction mixtures (Fig. 4b). In contrast, the addition of ATP, CTP or UTP did not affect the formation of the GTP–protein complexes. These results indicate that all of the tested proteins have the ability to bind specifically to GTP and GDP.

The effect of depletion of these essential GTP-binding proteins on cell morphology and chromosome replication

Next we used *B. subtilis* cells to analyse the effect of depletion of these essential GTP-binding proteins on cell morphology and chromosome replication, to begin understanding whether they are involved in regulating cell cycle progression. IPTG-dependent mutants were cultivated in antibiotic medium 3 with 100 μM IPTG to OD_{600} 0·4, and cells were washed and diluted in the same medium without IPTG. Immunoblotting indicated that the amount of each tested protein decreased to less than 10 % of control levels after approximately four generations in the absence of IPTG, which is the point at which the growth rate of the mutant cells started to decrease. Microscopic examination of Bex- and YqeH-depleted cells cultivated for nine generations without IPTG revealed that cells became 1·5 to 2 times longer compared to wild-type, and nucleoid distribution dispersed (Fig. 5b and g). On the other hand, cell length increased more than threefold and cell shape was abnormally curved in Obg-, YphC-, YsxC- and YlqF-depleted cells. Furthermore, nucleoid condensation was observed in these cells (Fig. 5c, d, e and f). No further changes in the morphology of cells and nucleoids were observed after a longer period of depletion.

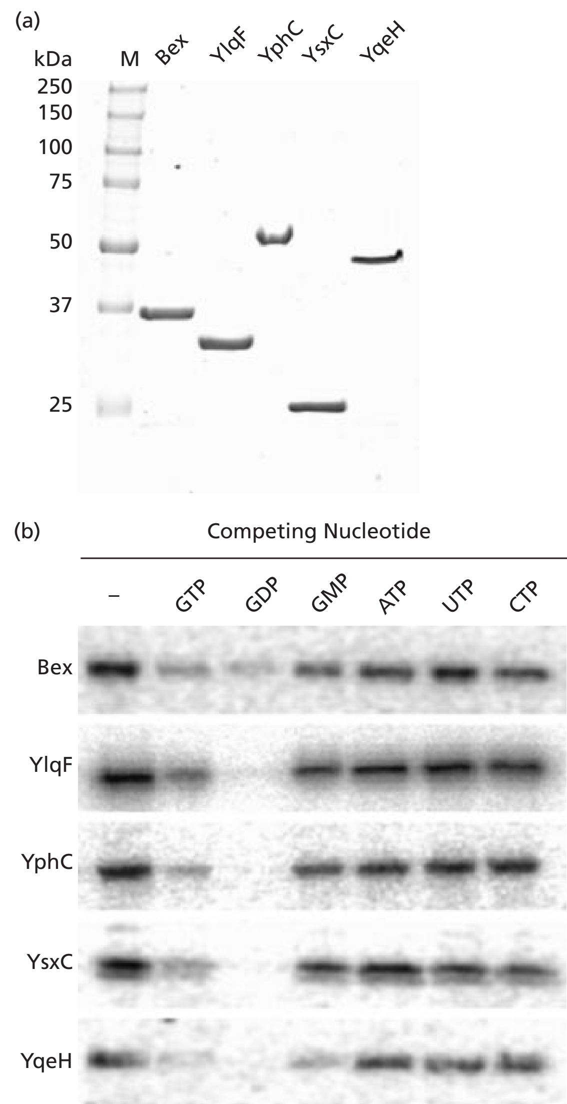


Fig. 4. (a) SDS-polyacrylamide gel showing the purification of the GTP-binding proteins. M, molecular marker; other lanes contain his-tagged GTP-binding proteins. Five micrograms of each protein were applied to the SDS-PAGE gel and stained with Coomassie blue after electrophoresis. (b) Autoradiography of [α-^{32}P]GTP bound to Bex, YlqF, YphC, YsxC and YqeH, in the absence of nucleotide competitor and in the presence of unlabelled GTP, GDP, GMP, ATP, UTP or CTP.

Although *B. subtilis* cells generally tend to form chains, which can make flow cytometry difficult to interpret, our parent strain (CRK6000) does not form chains under usual cultivation conditions (Ogura *et al*., 2001). Therefore, we used flow cytometry to determine the effect of depletion on the number of replication origins present in

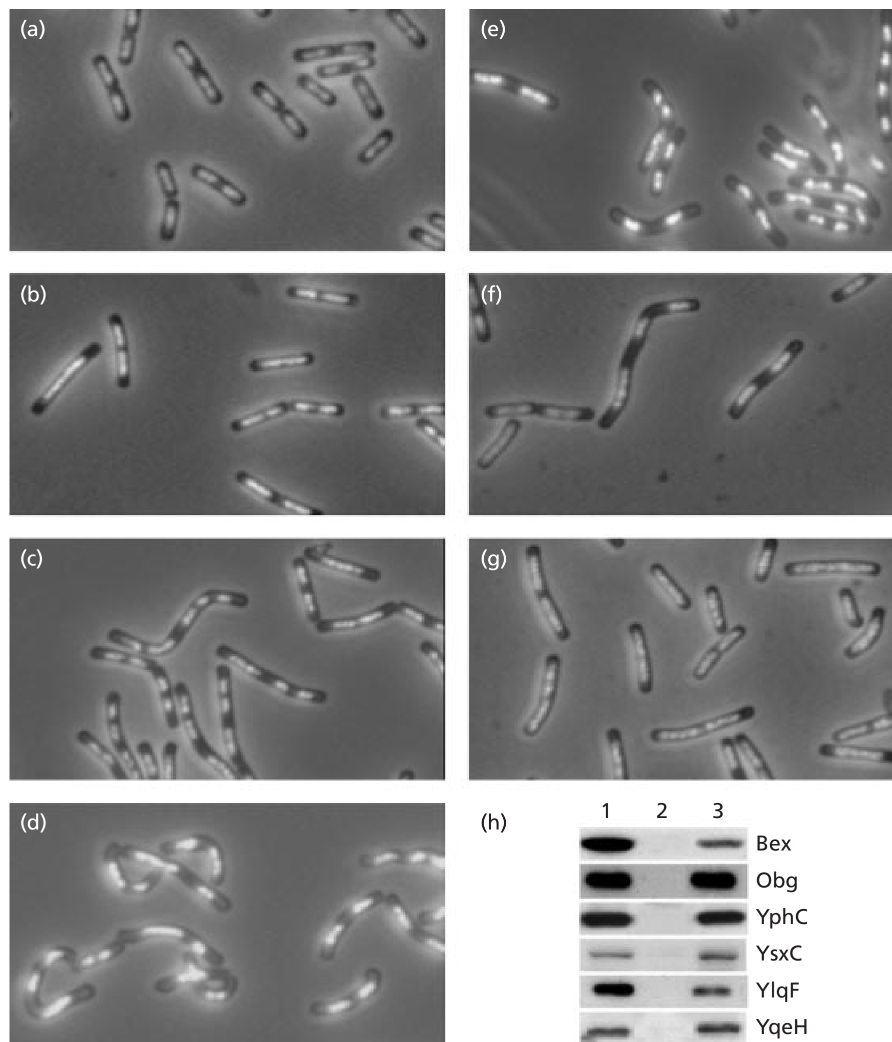
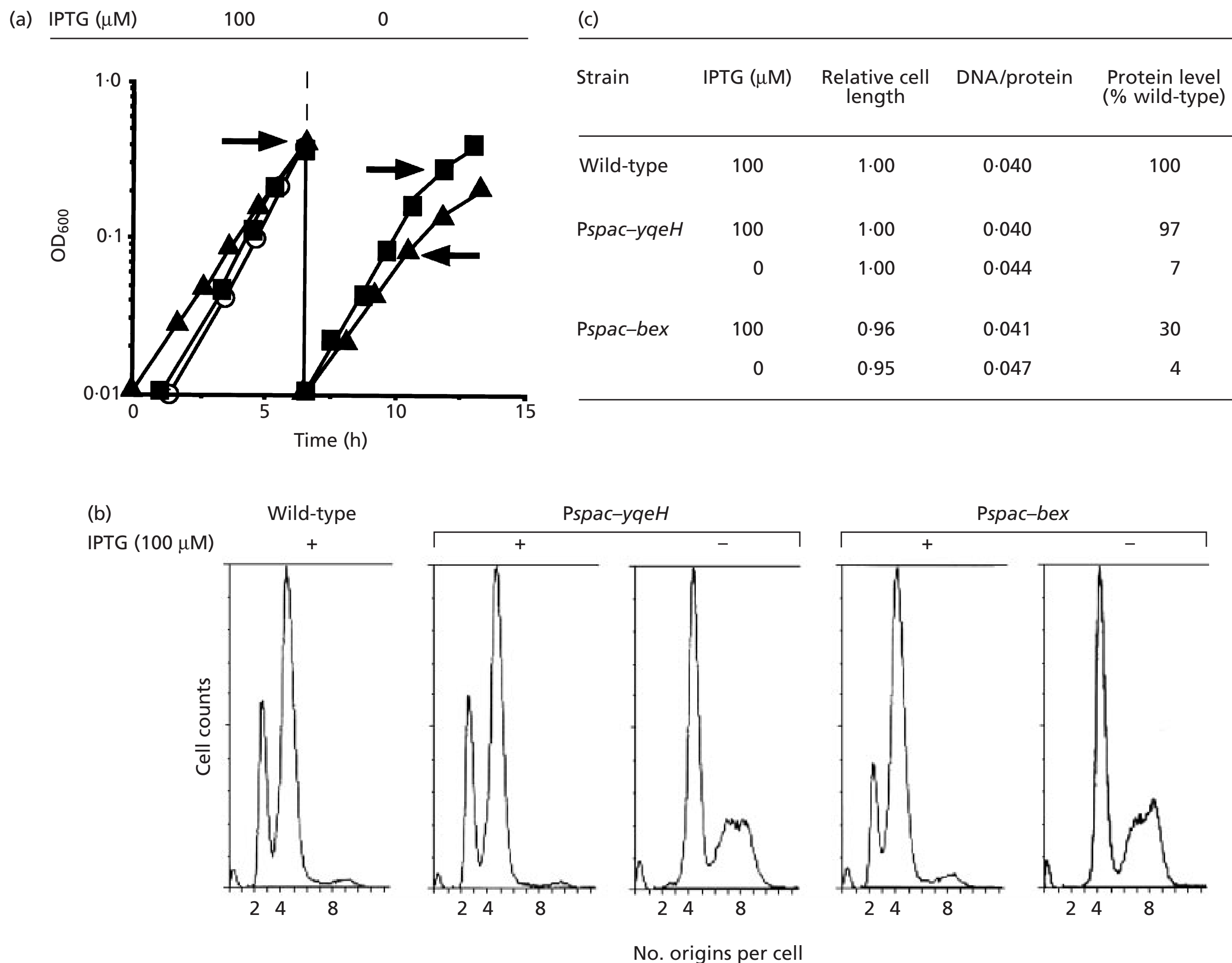


Fig. 5. Fluorescence micrographs of the conditional null mutant cells. Cells were incubated for nine generations without IPTG. Cell morphology and nucleoid distribution were examined under fluorescence microscopy after DAPI staining. (a) wild-type, (b) P*spac–bex*, (c) P*spac–obg*, (d) P*spac–yphC*, (e) P*spac–ysxC*, (f) P*spac–ylqF*, (g) P*spac–yqeH*. Immunoblotting (h) shows the amount of each GTP-binding protein in a fixed amount of whole protein of wild-type cells (lane 1) and in the corresponding conditional null mutants after growth for nine generations without IPTG (lane 2) or grown in the presence of 100 μM IPTG (lane 3).

individual cells. To avoid the effect of cell elongation, the mutant cells were grown without IPTG for three to five generations before chloramphenicol was added to inhibit new rounds of initiation. Incubation was continued for 5 h to complete ongoing replication without cell division. Then the number of fully replicated chromosomes in each cell (equivalent to the number of replication origins at the time of the drug addition) was measured with a flow cytometer. We found that depletion of Bex and YqeH affected DNA replication, shifting the number of replication origins from 2–4 (wild-type cells and the mutant cells in the presence of IPTG) to four or more (Fig. 6b). Although the amount of Bex and YqeH decreased to 7 and 4 % of the wild-type level, respectively, at the time of chloramphenicol addition, histograms of cell length (light scatter in flow cytometry) indicated the lack of cell elongation in the mutant cells (Fig. 6c). These results suggested that the depletion of Bex or YqeH induces an increase in initiation of chromosome replication. Consistent with this finding, the amount of DNA per protein in the *bex* and *yqeH* mutant cells grown in the absence of IPTG increased by 15 % and 8 %, respectively, compared to that of wild-type cells (Fig. 6c). Even in the presence of IPTG, the growth rate of the *bex* mutant was slightly slower and the amount of the Bex protein decreased compared to that of wild-type cells (Fig. 6a and Fig. 5h). In agreement with the decreased Bex level, a slight decrease in the number of cells having two replication origins was observed in *bex* mutant cells in the presence of IPTG, supporting the involvement of Bex in the regulation of initiation of chromosome replication.



Strain	IPTG (μM)	Relative cell length	DNA/protein	Protein level (% wild-type)
Wild-type	100	1·00	0·040	100
P*spac–yqeH*	100	1·00	0·040	97
	0	1·00	0·044	7
P*spac–bex*	100	0·96	0·041	30
	0	0·95	0·047	4

Fig. 6. Effect of YqeH or Bex depletion on chromosome replication. (a) Growth curves of P*spac–yqeH* and P*spac–bex* cells grown in the presence and absence of IPTG. Arrows indicate the time when cells were collected for flow cytometry analysis. The growth curve of the wild type cells in the presence of IPTG is also shown as the control. ○, wild-type; ▲, P*spac–bex*; ■, P*spac–yqeH.* (b) Flow cytometry of the cells grown in the presence and absence of IPTG. DNA histograms of cells after incubation for 5 h in the presence of chloramphenicol are shown. (c) GTP-binding protein level, average cell length and DNA–protein ratio of the mutant cells collected for flow cytometry. Relative amounts of Bex or YqeH in a fixed amount of whole protein from the mutant and wild-type cells were determined by quantitative immunoblotting analysis. The relative value in the mutant cells to that in wild-type cells is shown. The light scatter distribution of each cell culture was measured by flow cytometry and the mean value was divided by that of wild-type cells to obtain the relative cell length. The DNA–protein ratio was determined as described in Methods. The average from four independent experiments is shown.

Conversely, the conditional null mutants of *obg*, *ylqF*, *yphC* and *ysxC* did not show any change in DNA replication as measured by flow cytometry (data not shown).

DISCUSSION

Here we report that of the nine GTP-binding proteins of the Era/Obg family we identified in *B. subtilis*, six are essential for cell growth. The essential nature of Obg and YsxC in *B. subtilis* has been previously reported, and Bex and YphC had been found to be essential for growth in other bacteria, which we were able to replicate in *B. subtilis*. Furthermore, we identified two more essential GTP-binding proteins of the Era/Obg family: YlqF and YqeH. Specific GTP/GDP-binding activity has been previously demonstrated for Obg, and we showed the activity in the remaining five proteins. Quantitative immunoblotting analysis indicated that the six essential proteins are relatively abundant in vegetative *B. subtilis* cells. The mean number of molecules of each protein per cell was estimated to be 6000, 3000,

1000, 1000, 2000 and 7000 for Obg, Bex, YsxC, YlqF, YqeH and YphC, respectively (data not shown).

Three of the identified Era/Obg family members were found to be dispensable for normal growth in *B. subtilis*: ThdF, YnbA, and YyaF. The homologue of ThdF in *E. coli* (TrmE) was reported to be involved in the biosynthesis of the hypermodified nucleoside 5-methylaminomethyl-2-thiouridine (found in the wobble position of some tRNAs) and dispensable in certain genetic backgrounds (Cabedo *et al.*, 1999). The *E. coli* counterpart of *ynbA* (called *hflX*) encodes a regulatory subunit of the CII repressor of lambda phage. However, there is no data to suggest that *B. subtilis ynbA* might relate to phage function. Although dispensable in *B. subtilis*, *yyaF* is conserved in all micro-organisms including archaea and yeast (COG database in NCBI). Furthermore, the YyaF homologue has been identified in the genomes of higher organisms. Therefore this protein could also play an important role in cell growth. Although phenotypes of the *thdF*, *ynbA* and *yyaF* disruptants were not studied in this work, their preliminary characterization by the Japanese and European consortia for functional analysis of the *B. subtilis* genome (Ogasawara, 2001) is available in BSORF (http://bacillus.genome.ad.jp/) and Micado (http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl) databases.

Based on the observed phenotypes of cells in which each protein was depleted, the six essential GTP-binding proteins were classified into two groups. Depletion of Bex or YqeH induced an excess initiation of DNA replication, suggesting that these proteins negatively control initiation of chromosome replication. Both positive and negative regulators of replication initiation have been reported previously in *E. coli*. An overproduction of the replication initiator protein DnaA resulted in overinitiation (Atlung & Hansen, 1993). Sequestration of newly duplicated hemimethylated origins into the cell membrane by the SeqA protein (Lu *et al.*, 1994; Onogi *et al.*, 1999) and inactivation of the DnaA protein by the DnaN and Hda proteins (Katayama *et al.*, 1998; Kato & Katayama, 2001) are known negative regulatory mechanisms for suppression of initiation. Recently, we reported that DnaA levels in the *B. subtilis* cell could also act as a positive regulatory system for the initiation of chromosome replication (Ogura *et al.*, 2001). However, no negative regulatory system controlling chromosome replication has been previously identified in *B. subtilis*. Thus the finding that Bex and YqeH negatively regulate replication initiation is an important step towards understanding the regulatory network of chromosome replication in *B. subtilis*. In *E. coli*, a mutation in *era* (homologue of *B. subtilis bex*) was reported to suppress several temperature-sensitive lethal alleles of genes involved in chromosome partitioning. On the other hand, the mutation did not suppress any of the cell division and DNA replication initiation mutations (Britton *et al.*, 1998). The suppression of the defect in chromosome partitioning by the *era* mutant might be due to overinitiation of replication. The genes encoding the Bex and YqeH homologues are located side by side in *T. maritima*. Powers & Walter (1995) demonstrated that the GTPases FtsY and Ffh interact directly and regulate their GTPase activities reciprocally in *E. coli*. Such direct coupling between Bex and YqeH in *B. subtilis* will be an interesting model for future study.

The depletion of Obg, YphC, YsxC, and YlqF resulted in cell elongation, abnormal cell curvature and nucleoid condensation without apparent change in the DNA histogram upon flow cytometry analysis. Although we did not fully examine the various roles of these proteins in a direct manner, our observations are compatible with the previous observation that depletion of the Obg homologue (ObgE) or YsxC homologue (YihA) in *E. coli* caused defects in cell division (Kobayashi *et al.*, 2001; Dassain *et al.*, 1999). In *B. subtilis*, it has been proposed that Obg is involved in the initiation of chromosome replication (Kok *et al.*, 1994). However, our results do not support this hypothesis, necessitating further work in this area.

The complete genome sequences of diverse organisms have revealed the existence of more unknown genes than had been anticipated. Our work revealed that many GTP-binding proteins of the Era/Obg family play essential but unknown functions in the growth of *B. subtilis*. Our results together with previously published reports indicate that many Era/Obg family members are conserved and play important roles in different facets of bacterial function. Thus, as we look towards the future, the GTP-binding proteins of the Era/Obg family will be an important protein family whose members require further study to elucidate their precise roles in bacterial growth and health.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) (to N. O.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Grants-in-Aid for Scientific Research (B) (to S. M. and N. O.) from the Japan Society for the Promotion of Science, and by a Grants-in-Aid for Scientific Research for Research Fellow from the Japan Society for the Promotion of Science (to T. M.).

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Received 18 April 2002; revised 21 June 2002; accepted 2 July 2002.