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The ObgE/CgtA GTPase influences the stringent response to amino acid starvation in *Escherichia coli*

Nicole S. Persky, Daniel J. Ferullo, Deani L. Cooper, Hayley R. Moore and Susan T. Lovett*
Department of Biology and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA, USA.

Summary

The stringent response is important for bacterial survival under stressful conditions, such as amino acid starvation, and is characterized by the accumulation of ppGpp and pppGpp. ObgE (CgtA, YhbZ) is an essential conserved GTPase in *Escherichia coli* and several observations have implicated the protein in the control of the stringent response. However, consequences of the protein on specific responses to amino acid starvation have not been noted. We show that ObgE binds to ppGpp with biologically relevant affinity *in vitro*, implicating ppGpp as an *in vivo* ligand of ObgE. ObgE mutants increase the ratio of pppGpp to ppGpp within the cell during the stringent response. These changes are correlated with a delayed inhibition of DNA replication by the stringent response, delayed resumption of DNA replication after release, as well as a decreased survival after amino acid deprivation. With these data, we place ObgE as an active effector of the response to amino acid starvation *in vivo*. Our data correlate the pppGpp/ppGpp ratio with DNA replication control under bacterial starvation conditions, suggesting a possible role for the relative balance of these two nucleotides.

Introduction

Bacterial responses to stressful conditions play crucial roles in cell survival. Understanding how cells respond to these conditions can help to ultimately control their persistence and pathogenicity (Jain *et al*., 2006). Among the best-studied stress responses is the stringent response to amino acid starvation (Jain *et al*., 2006; Potrykus and Cashel, 2008). This response is characterized by the accumulation of ppGpp and pppGpp (reviewed in Cashel *et al*., 1996).

Under conditions of amino acid starvation, the presence of uncharged tRNAs signals RelA to synthesize pppGpp and ppGpp from GTP or GDP, respectively, and ATP (reviewed in Potrykus and Cashel, 2008). These reactions occur with similar speeds (Cochran and Byrne, 1974; Cashel, 1975), but because of the excess of GTP in *Escherichia coli*, RelA produces primarily pppGpp, which is then converted to ppGpp. Because of the abundance of ppGpp over pppGpp in the stringent response (see Potrykus and Cashel, 2008), ppGpp is thought to be the biologically relevant nucleotide, although the effects of each nucleotide are often not distinguished. An enzyme named Gpp (or GppA) is one factor that hydrolyses pppGpp to ppGpp (Somerville and Ahmed, 1979). However, there remains at least one unidentified pppGpase in *E. coli* (Somerville and Ahmed, 1979). The most marked outcome of the general rise in pppGpp and ppGpp, collectively known as '(p)ppGpp', in the stringent response is an arrest of cell growth, manifest by the cessation of DNA replication and cell division, a reduced cell size, decrease in stable RNA synthesis and an increase in expression from amino acid biosynthesis genes (Schreiber *et al*., 1991; 1995; Barker *et al*., 2001; Cashel *et al*., 1996, Potrykus and Cashel, 2008 and references therein). For cells to resume growth, it is necessary for them to decrease levels of ppGpp and/or pppGpp in the cell. This is accomplished by the ppGpp hydrolase, SpoT (Cashel *et al*., 1996). SpoT also can act as a (p)ppGpp synthetase and under other stressful conditions, SpoT may substitute for RelA in the synthesis of (p)ppGpp (Xiao *et al*., 1991; Seyfzadeh *et al*., 1993; Spira *et al*., 1995; Vinella *et al*., 2005; Battesti and Bouveret, 2006).

Several observations have indicated a connection between ObgE (Obg in *E. coli*) and the stringent response. Obg is a small protein conserved from bacteria to humans that is essential in all bacterial species tested to date, suggesting an important role for this enzyme within the cell (Obg has also been named 'CgtA' for 'conserved GTPase' but we conform to genetic nomenclature rules that give precedent to names that occur first in the literature). ObgE has been shown to have very weak GTPase activity (Tan *et al*., 2002) and a variety of cellular functions that have yet to be entirely defined. ObgE is

Accepted 8 June, 2009. *For correspondence. E-mail lovett@brandeis.edu; Tel. (+1) 781 736 2497; Fax (+1) 781 736 2405.

required for chromosome segregation (Kobayashi *et al*., 2001; Foti *et al*., 2007), cell division (Kobayashi *et al*., 2001; Foti *et al*., 2005; 2007), recovery from DNA replication inhibition (Foti *et al*., 2005), and has effects on the levels of mature ribosomes in *E. coli* (Sato *et al*., 2005). ObgE has also been shown to interact with ribosomal complexes (Wout *et al*., 2004; Jiang *et al*., 2007) and in *Bacillus subtilis* appears to play an important role in the general stress response (Scott and Haldenwang, 1999; Kuo *et al*., 2008), although it is not clear whether this role will be conserved in *E. coli* (Scott and Haldenwang, 1999). Two crystal structures of Obg have been solved, one from *Thermus thermophilus* that clearly shows the three domains of this protein (Kukimoto-Niino *et al*., 2004), and a second from *B. subtilis* (Buglino *et al*., 2002) with the C-terminal domain deleted. In the *B. subtilis* structure ppGpp was found in half of the GTPase active sites (Buglino *et al*., 2002). The accidental crystallization of Obg with ppGpp may indicate a high affinity for ppGpp, or may have been a consequence of overexpression of Obg in exhausted media (Buglino *et al*., 2002). Initial studies in *B. subtilis* suggested that Obg did not have an especially high affinity for ppGpp (Buglino *et al*., 2002). However, affinity constants have never been measured for Obg and ppGpp, and the relevance of this binding has not been confirmed.

Deficiencies of Obg/CgtA in *Vibrio cholerae* and *E. coli* under certain conditions appeared to cause ppGpp accumulation constitutively and, in *Vibrio cholerae*, expression of a subset of genes known to be induced in the stringent response (Raskin *et al*., 2007; Jiang *et al*., 2007). Jiang *et al*. (2007) also showed that when (p)ppGpp accumulates, ObgE bound less to ribosomal complexes. Finally, in both *E. coli* and *Vibrio cholerae*, Obg/CgtA interacts with SpoT, the ppGpp synthetase and hydrolase (Wout *et al*., 2004; Raskin *et al*., 2007). However, the biological relevance of this interaction remains unknown and there are no reports of Obg/CgtA alteration of SpoT activity. These studies indicate a connection between ObgE and the stringent response and that the stringent response has an effect on ObgE localization (Jiang *et al*., 2007). To date, ObgE has not been shown to have an effect on the cell's response to amino acid starvation, which is the subject of our current study.

We investigate here the influence that ObgE activity has on the stringent response in *E. coli* and show that it is an *in vivo* effector of the response to amino acid starvation. We show that ObgE binds to ppGpp in *E. coli* with biologically relevant affinity, suggesting that this molecule is an *in vivo* ligand of ObgE, competitive with other nucleotides. We also show that ObgE changes the ratio of ppGpp to pppGpp in the cell during a response to amino acid starvation. ObgE subsequently affects cell survival and the control of DNA replication during the stringent response. By correlating the ratio of pppGpp/ppGpp to DNA replication inhibition, our data provide potential downstream effects of changes in the relative levels of two signalling molecules often thought of as one.

Results

ObgE binds to ppGpp with a similar affinity as GDP

Given the potential connections between ppGpp and ObgE, we first investigated the direct binding of ppGpp to ObgE to determine if the strength of the interaction was sufficient to yield biological effects.

Binding of Obg to ppGpp was measured using inhibition experiments. ObgE and its homologues from a variety of species exhibit very slow hydrolysis of GTP under standard multiple turnover conditions [all around 1–2 phosphates produced/enzyme per hour](Welsh *et al*., 1994; Lin *et al*., 1999; Tan *et al*., 2002; Wout *et al*., 2004). Because of Obg's unusually slow hydrolysis rate, multiple turnover conditions could not be accurately utilized for inhibition experiments. Instead, single turnover experiments using excess ObgE over substrate were performed. Hydrolysis rates of GTP at varying concentrations of ObgE were determined similar to a technique used by Peluso *et al*. in 2001 (Peluso *et al*., 2001). This technique allowed the determination of the half saturation constant, $K_{1/2}$, of the GTPase activity of ObgE. (For enzymes with a rate-limiting chemical step, $K_{1/2}$ is equivalent to the dissociation constant, K_d.) Utilizing inhibition assays, the K_i's of ObgE binding to GDP and ppGpp could then be determined.

Single turnover GTPase assays were performed with ObgE in excess of substrate, [γ-^{32}P]-GTP. GTP hydrolysis was followed over a time-course of 2.5 h by separating [γ-^{32}P]-GTP from ^{32}P on thin layer chromatography (TLC). A representative experiment is shown in Fig. 1A. The fraction of radioactivity remaining in the GTP form was graphed as shown in Fig. 1B, and the k_{obs} for this reaction was fit the graph (see *Experimental procedures* and Supporting information). By varying concentrations of ObgE, the $K_{1/2}$ was determined to be 14 $\pm$ 6 μM. From this information we were also able to measure the maximal rate constant, $k_{max,}$ for ObgE as 0.9 $\pm$ 0.2 products per enzyme per hour (see *Experimental procedures* and Supporting information).

Inhibition experiments were then performed by holding ObgE and substrate concentrations constant at 3.5 μM ObgE and 0.0032 μM of [γ-^{32}P]-GTP, respectively, and then adding GDP or ppGpp as inhibitors. These curves were then fit to determine the apparent K_i for GDP and ppGpp (see *Experimental procedures* and Supporting information). GDP and ppGpp appeared to bind to ObgE with exactly the same affinity, with apparent K_i's of 1.6 $\pm$ 0.4 μM and 1.6 $\pm$ 0.5 μM respectively (see Fig. 1C

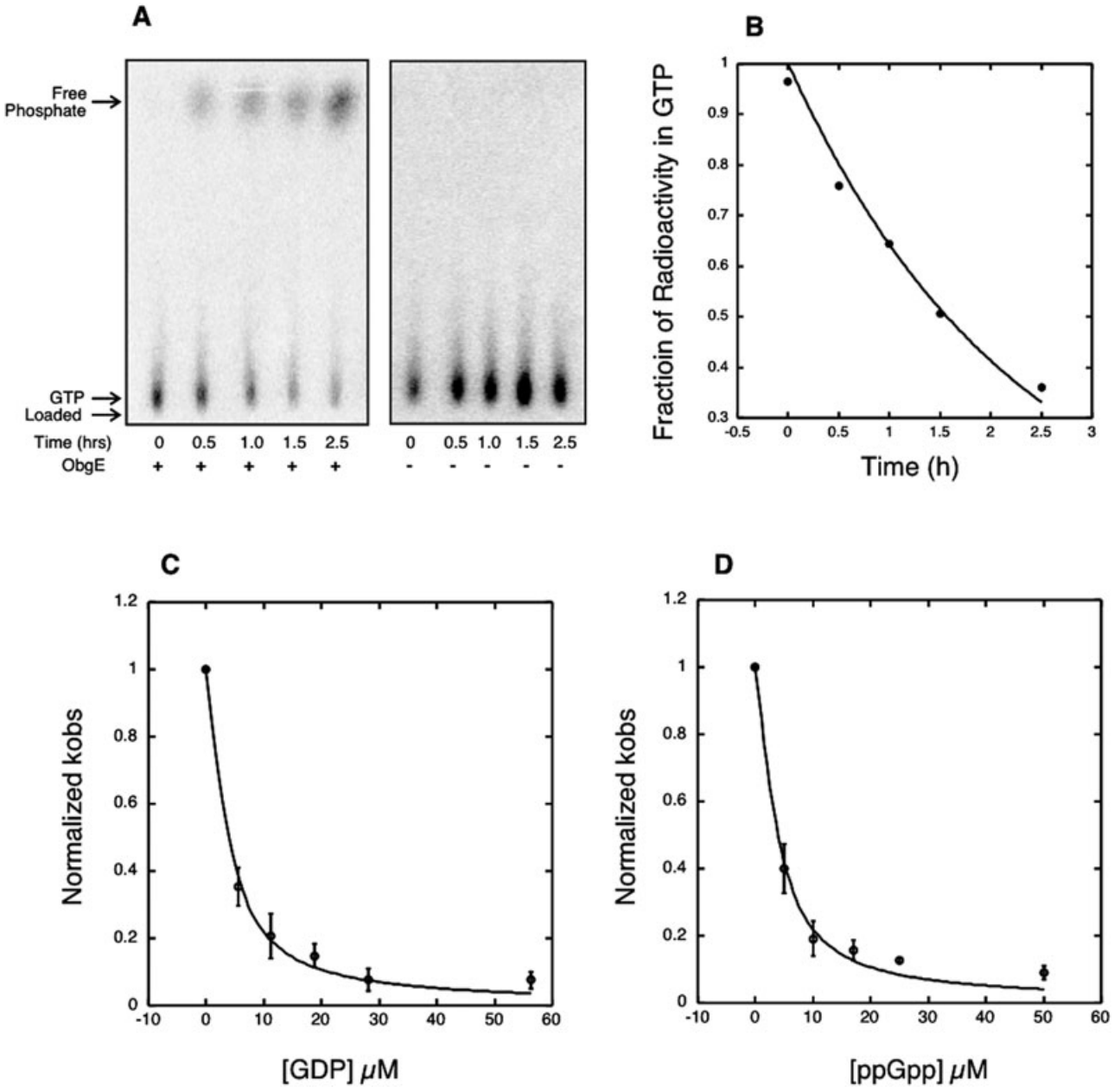

Fig. 1. ObgE GTPase and inhibition experiments.
A. Representative thin layer chromatography plates for GTPase experiments showing the uninhibited ObgE GTPase (left panel), and buffer control (right panel).
B. A representative fit to GTPase data.
C. The average of three GDP inhibition experiments, with the standard deviation indicated as error bars. The curves were originally plotted individually to attain an average and standard deviation for the values of K_i observed.
D. The average of three ppGpp inhibition experiments fit as with GDP.

and D). The *in vivo* concentrations of GTP, GDP and ppGpp during the *E. coli* life cycle all well exceed the $K_{1/2}$ and K_i for ObgE binding (ppGpp can reach concentrations in the hundreds of micromolar) (Buckstein *et al.*, 2008). The fact that ObgE was shown to bind to ppGpp with affinity in the physiological range argues that ppGpp is a ligand of ObgE *in vivo*.

ObgE affects the ratio of pppGpp to ppGpp in response to amino acid deprivation

To determine if ObgE affects the levels of (p)ppGpp *in vivo* during the stringent response, wild-type cells were compared with those carrying a C-terminal insertion of the transposon Tn*5* in ObgE. This strain contains the deletion

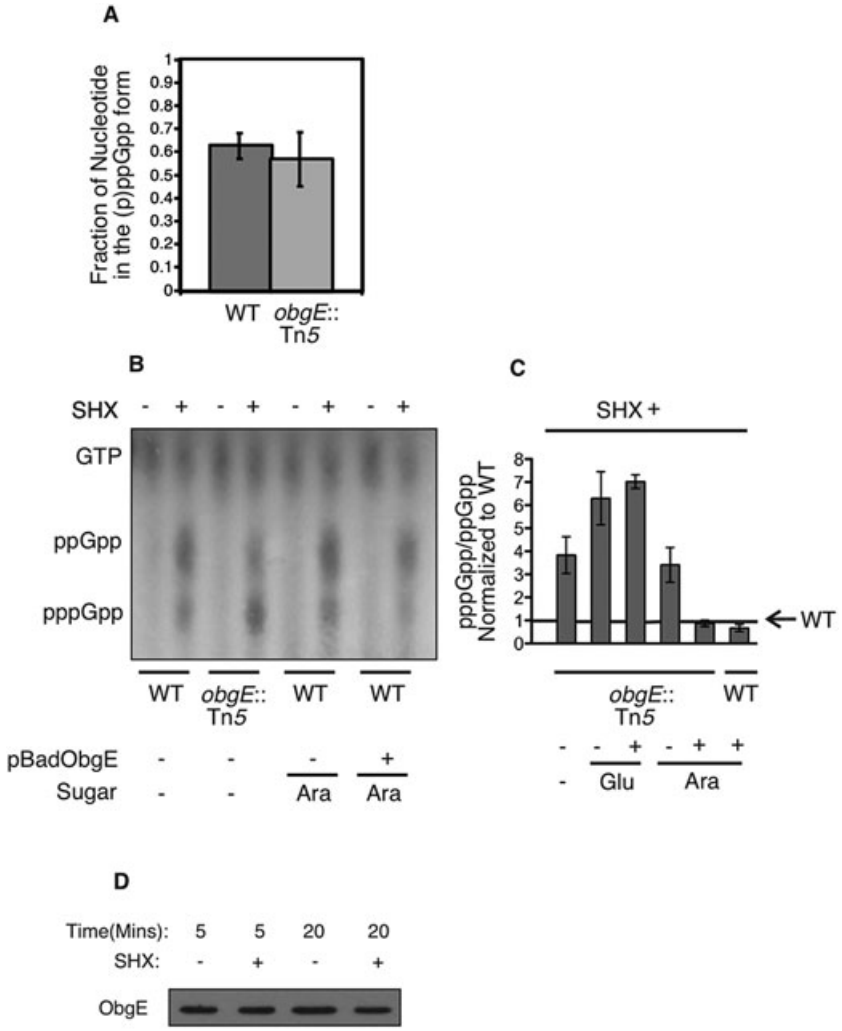

Fig. 2. Levels of (p)ppGpp in cells treated with serine hydroxamate.
A. Fraction of total guanine nucleotides in the (p)ppGpp form for wild-type and *obgE*::Tn*5* mutant cells, averaged from three independent experiments. Error bars represent the standard deviations of the means.
B. Representative thin layer chromatography plate for *in vivo* ppGpp and pppGpp detection, with and without serine hydroxamate treatment. The samples shown (from left to right) are: wild-type (MG1655), *obgE*::Tn5 (STL7742), wild-type (MG1655) grown in the presence of arabinose, wild-type expressing pBADObgE$^+$ (STL7679) grown in the presence of arabinose.
C. Cumulative data averaged over three experiments each showing the ratio of pppGpp/ppGpp following serine hydroxamate treatment, normalized to wild-type (MG1655). Error bars represent standard deviations of the means. The samples shown (from left to right) are: *obgE*::Tn*5* (STL7742), *obgE*::Tn*5* (STL7742) grown in the presence of glucose, pBADObgE$^+$ in an *obgE*::Tn*5* background (STL13897) grown in the presence of glucose (plasmid expression off), *obgE*::Tn*5* (STL7742) grown in the presence of arabinose, pBADObgE$^+$ in an *obgE*::Tn*5* background (STL13897) grown in the presence of arabinose (plasmid expression on), and pBADObgE$^+$ in a wild-type background (STL7679) grown in arabinose (plasmid expression on).
D. Levels of ObgE protein before and after serine hydroxamate treatment. Samples were taken at time points following the addition of serine hydroxamate and ObgE was detected via Western blot using polyclonal anti-ObgE antibody.

of nine amino acids and the addition of 68 at the C-terminal of the protein (Foti *et al.*, 2005). We chose this mutant because it had strong defects in response to replication inhibition, but is otherwise viable (Foti *et al.*, 2005). Both wild-type and *obgE*::Tn*5* strains were grown in Luria–Bertani (LB) medium supplemented with ^{32}P-labelled orthophosphate. The stringent response was induced by the addition of serine hydroxamate (SHX), an inhibitor of serine tRNA synthetase (Tosa and Pizer, 1971), for 20 min. This treatment is known to increase the levels of ppGpp and pppGpp within the cell. The cells were lysed and relative levels of radiolabelled ppGpp, pppGpp, and GTP were detected by TLC analysis. All experiments were performed in triplicate.

To our surprise, although total levels of ppGpp plus pppGpp were not detectably different (Fig. 2A), *obgE*::Tn*5* cells had a substantially increased ratio of pppGpp/ppGpp (Fig. 2B and C). This effect could be completely suppressed by overexpression of wild-type ObgE from an arabinose-induced plasmid promoter (Fig. 2C). This suppression supports the interpretation that any change to the pppGpp/ppGpp ratio observed in *obgE*::Tn*5* mutants in response to amino acid starvation is due to a defect of the ObgE protein itself. Cells containing the *obgE*::Tn*5* mutation and the ObgE$^+$ plasmid were indistinguishable from *obgE*::Tn*5* single mutants when the promoter was repressed by growth in glucose (Fig. 2C), suggesting that the effects are sensitive to the concentration of ObgE in the cell. These results indicate that ObgE may play a role, directly or indirectly, in setting the relative levels of pppGpp to ppGpp during the stringent response in *E. coli*.

ObgE controls cell survival in response to amino acid deprivation

To see if ObgE influences the response to amino acid starvation, we performed Live/Dead fluorescence staining to determine the amount of survivors within the population for wild-type cells and *obgE*::Tn*5* cells. With this procedure, only dead cells with damaged membranes are per-

Table 1. Live/Dead staining, septation and cell length following SHX treatment.

Sample	SHX	Dead (%)	Injured (%)	Septated (%)	Mean length (μM)
Wild-type	–	0.13	0.74	31	3.8
Wild-type	+	0.29	2.1	4	3.0
obgE::Tn*5*	–	2.8	2.9	32	4.6
obgE::Tn*5*	+	13	8.2	2	3.4
relA251	–	0.6	2.7	n.d.	n.d.
relA251	+	3.6	4.8	n.d.	n.d.
obgE::Tn*5 relA*	–	1.8	6.2	n.d.	n.d.
obgE::Tn*5 relA*	+	18	10	n.d.	n.d.

SHX, (+) serine hydroxamate treatment for 90 min; (–) no treatment. n.d., not determined.

meable to staining with propidium iodide (and primarily exhibit red fluorescence) whereas live cells stain with Syto 9 (green fluorescence). Cells that stained strongly with both dyes were considered 'injured' and included in a separate category; our experience suggests that these cells are on the path to death. Whereas wild-type cells have about 98% survival rate after treatment with SHX for 90 min (total cells minus the dead and injured cells), *obgE*::Tn*5* cells drop from a 94% viability (untreated) to 79% (SHX treated) (see Table 1). Mutants in *relA* had a slight decrease in viability and *relA obgE*::Tn*5* double mutants had a loss of viability enhanced relative to the *obgE*::Tn*5* single mutant. The decrease in viability of *obgE*::Tn*5* cells suggests that they are unequipped to process amino acid starvation correctly, and more so in the absence of (p)ppGpp synthesis by RelA.

ObgE protein levels are not largely altered with the initiation of the stringent response

To see if ObgE protein levels were drastically altered during its activity in the stringent response, we used Western blotting to detect ObgE protein levels in wild-type cells with and without serine hydroxamate treatment. A Western blot through 20 min of treatment (the point at which ObgE influences the ppGpp/pppGpp ratio), shown in Fig. 2D, illustrates the lack of major changes in total ObgE protein levels observed. This indicates that ObgE protein levels do not grossly change upon induction of the stringent response. We then altered the protocol to include infrared imaging quantification of our Western blots by lysing the cells and performing quantitative Western blotting on cleared cell lysates, normalized to total protein levels, and using a fluorescent secondary antibody. We found that after 20 min of exposure to serine hydroxamate wild-type cells contained 90 ± 30% of ObgE protein present in untreated cells.

These results are consistent with previous experiments showing only mild changes (twofold) of ObgE RNA levels in wild-type cells during the stringent response to amino acid starvation, although levels of ObgE may be grossly altered in (p)ppGpp null strains (Traxler *et al.*, 2008). These data are in agreement, suggesting that ObgE's functions in the stringent response are not accompanied by large changes in ObgE protein level.

ObgE does not affect the cell's ability to halt growth in response to amino acid deprivation

The poor survival of *obgE*::Tn*5* cells in response to starvation may be consequence of an inability to arrest growth and division in response to increased levels of (p)ppGpp. To test this hypothesis, we looked at the *obgE*::Tn*5* strain's ability to halt growth as measured by OD_{600}, its ability to form division septa in response to amino acid starvation, and cell length. We found that like wild-type cells, *obgE*::Tn*5* cells immediately halted growth as measured by OD_{600} upon the addition of SHX to cultures. As seen in Fig. 3, both wild-type cells and *obgE*::Tn*5* cells, upon SHX induction, no longer increased OD_{600}. In contrast, both strains continued increasing OD_{600} values with time when left untreated, with *obgE*::Tn*5* cells growing slightly slower than wild-type, as has been seen before. We found that *obgE*::Tn*5* cells were able to inhibit septum formation in response to SHX treatment as has been previously reported for wild-type strains (Ferullo and Lovett, 2008). As seen in Table 1, both wild-type and *obgE*::Tn*5* strains growing in early log phase exhibited signs of septation in about 30% of their population. In response to SHX treatment, septating cells in both populations dropped to around 3%. Cell lengths for *obgE*::Tn*5*

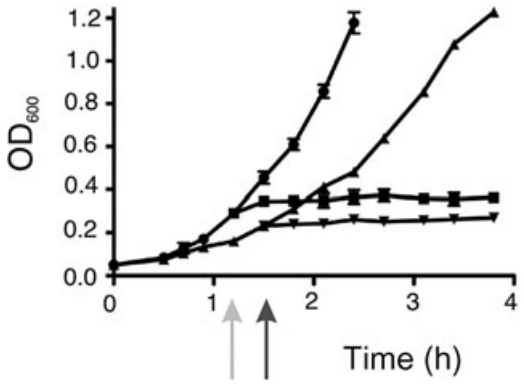

Fig. 3. Growth curves. Wild-type cells and *obgE*::Tn*5* cells were grown with aeration in LB at 37°C. The light gray arrow indicates where SHX was added to wild-type cells, and the dark gray arrow indicates where SHX was added to *obgE*::Tn*5* cells. Strains are shown as follows: untreated wild-type cells (circles), SHX-treated wild-type cells (squares), untreated *obgE*::Tn*5* cells (triangles pointing up) and SHX-treated *obgE*::Tn*5* cells (triangles pointing down).

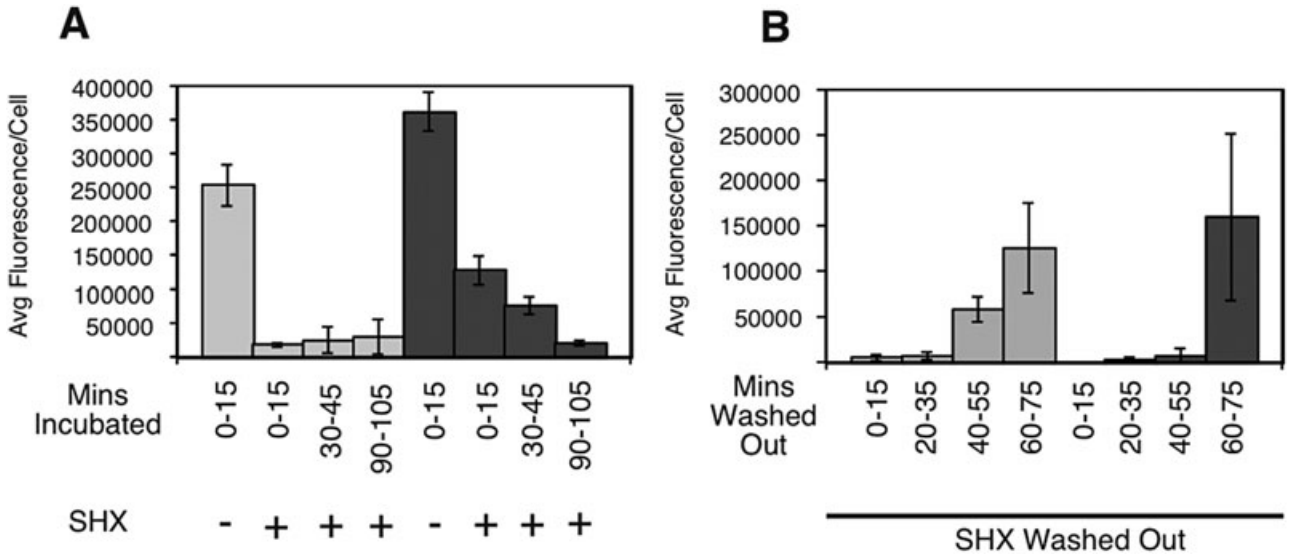

Fig. 4. Arrest and restart of DNA replication in response to SHX treatment and release.
A. EdU-Click fluorescence following SHX treatment for wild-type cells (light grey) and *obgE*::Tn*5* mutant cells (dark grey).
B. EdU-Click fluorescence following SHX removal for wild-type cells (light grey) and *obgE*::Tn*5* mutant cells (dark grey).

were also mildly reduced in response to SHX as has been seen before for stringent wild-type cells (see Table 1), also suggesting a downregulation of growth. Taken together, these data indicate that *obgE*::Tn*5* mutant strains are able to halt growth in response to amino acid starvation and that their lack of survival under these conditions is not correlated with an inability to initiate these responses.

ObgE affects DNA replication control by the stringent response

Our previous study showed that after induction of the stringent response, *E. coli* cells complete ongoing rounds of replication and arrest cell cycle at the level of replication initiation (Ferullo and Lovett, 2008). To determine if ObgE has an effect on DNA replication, newly replicated DNA was followed by labelling with EdU (5-ethyl-2′deoxyuridine) conjugated to fluorescent azides. This assay was previously optimized for use in *E. coli* by our lab for this purpose (Ferullo *et al*., 2009). In this manner, the amount of newly replicated DNA can be correlated to the amount of fluorescence per cell. Untreated and SHX treated cells were pulsed for 15 min at various time points with EdU before fixation, labelled with fluor, and imaged via microscopy. As seen before (Schreiber *et al*., 1995; Ferullo and Lovett, 2008; Ferullo *et al*., 2009), in the presence of SHX, wild-type cells halted their replication. We found that within 15 min of SHX treatment, replicated DNA labelling was virtually undetectable. At this same time point; however, we found that *obgE*::Tn*5* cells continued to incorporate label, indicating ongoing replication (see Fig. 4A and Fig. S1). These data suggest that ObgE is required for the timely inhibition of DNA replication by amino acid starvation. However, by 90–105 min the *obgE*::Tn*5* strains exhibited no detectable replication. This delay in *obgE* mutants was reproducible for multiple isolates.

Interestingly, we found that upon release of these cells from serine hydroxamate wild-type cells regained ability to incorporate label faster than did *obgE*::Tn*5* cells (see Fig. 4B and Fig. S1). By 55 min, wild-type cells had significantly brighter newly replicated DNA labelling than *obgE*::Tn*5* cells. These data indicate that ObgE also impacts the release from the stringent response and the resumption of DNA replication. By 60–75 min *obgE*::Tn*5* cells did reach a wild-type level of DNA replication and were apparently able to recover from replication inhibition. Together, these data indicate that either ObgE is involved in the timing of onset and termination of stringent response DNA replication inhibition or that DNA damage persists that delays completion of replication and its resumption after release.

obgE*::Tn*5 *cells have constitutively high DNA content and exhibit a partial SOS response*

obgE::Tn*5* cells have previously been shown to have longer cell lengths, which we also note here, and higher copy number of the origins of replication (*oriC*) in minimal media as compared with wild-type cells as shown by flow cytometry (Foti *et al*., 2005). To confirm that *obgE*::Tn*5*, like other ObgE mutants (Kobayashi *et al*., 2001; Foti *et al*., 2007), had constitutively higher DNA content in rich medium, we measured DNA content of *obgE*::Tn*5* grown

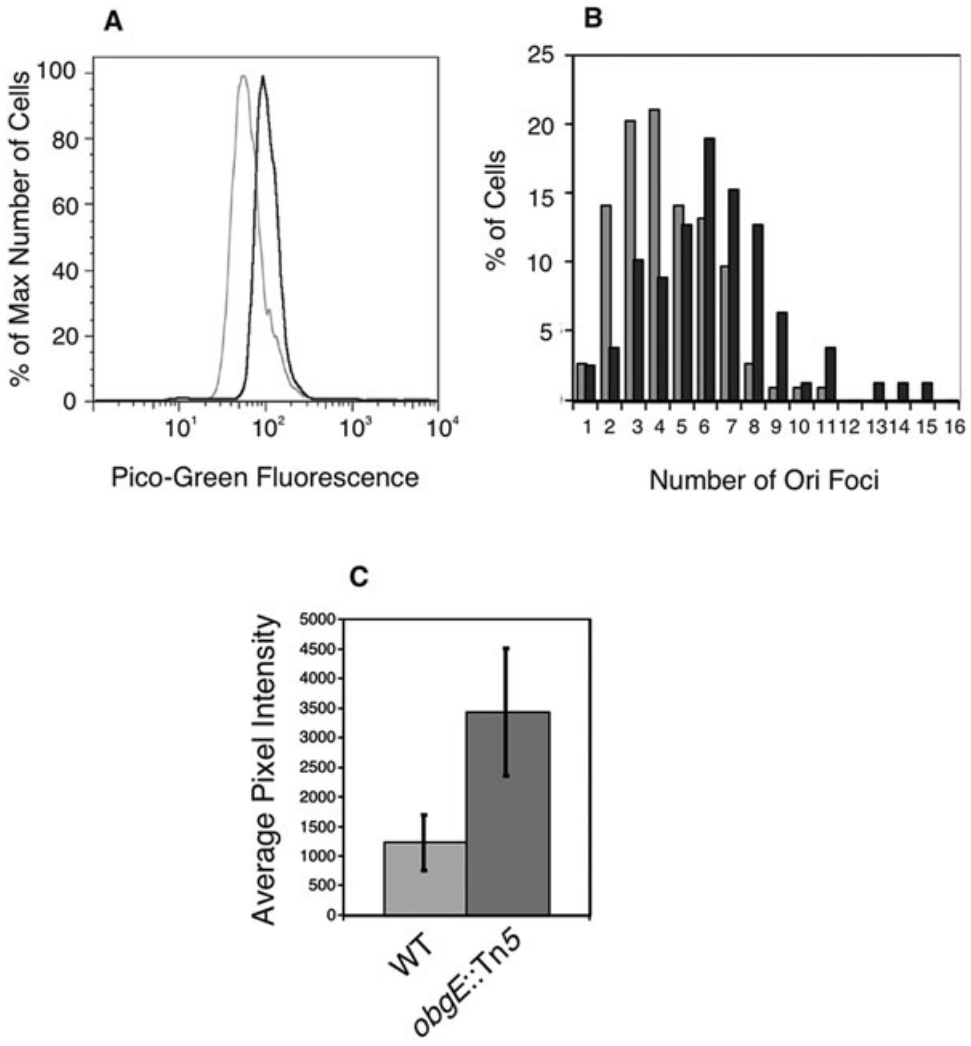

Fig. 5. DNA content and SulA expression measurements.
A. Representative flow cytometry data showing DNA content from 30 000 wild-type and *obgE*::Tn*5* cells labelled with PicoGreen fluorescence. Wild-type cells are shown in light grey and *obgE*::Tn*5* cells are shown in dark grey.
B. Distribution of numbers of oriC foci per cell using a ParS/GFP-ParB system for *obgE*::Tn*5* strains (dark grey) and wild-type strains (light grey).
C. SOS response induction measured by a SulA::mCherry reporter plasmid, averaged over 100 wild-type and *obgE*::Tn*5* cells.

in LB by staining cells with PicoGreen fluorescence detected by flow cytometry. As predicted, *obgE*::Tn*5* cells exhibited constitutively higher DNA content than wild-type cells as indicated by an increase in fluorescence (see Fig. 5A). To confirm this, we used *obgE*::Tn*5* strains containing the *parS* DNA sequences near the origin of replication as a binding site for GFP-ParB expressed from a plasmid and visualized *oriC* regions via microscopy. When compared with wild-type, *obgE*::Tn*5* cells showed more *oriC* foci (see Fig. 5B). On average, wild-type cells showed 4.4 ori foci, and *obgE*::Tn*5* showed 6.3 ori foci. Run-out flow cytometry of cells with rifampicin and cephalexin confirmed higher initiation capacity of *obgE*::Tn*5* (data not shown). Put together, these data indicate that ObgE regulates total DNA content within *E. coli* cells, and that the *obgE*::Tn*5* mutant cells have higher DNA content than do wild-type cells.

One explanation of the replication phenotype of *obgE* mutants is that there may be constitutively high levels of DNA damage. To test this hypothesis, we used strains containing a reporter of the SOS response to DNA damage. This reporter consists of a plasmid containing the promoter for the SulA gene containing a tight LexA binding site highly regulated by the SOS response. When this plasmid is transformed into both wild-type and *obgE*::Tn*5* cells and analysed for mCherry fluorescence, the *obgE*::Tn*5* cells showed significantly higher SulA expression than did wild-type (Fig. 5C), indicating that *obgE*::Tn*5* cells constitutively exhibit a partial SOS response.

Discussion

ObgE binds ppGpp similarly to GDP

We show here that ObgE binds to ppGpp with similar affinity as it binds to GDP. These data correlate with previously published biochemical data for ObgE. The single turnover k_{max} observed here for GTP hydrolysis of 0.9 products per enzyme per hour is very similar to the reported multiple turnover k_{max} of 1.0–1.2 products per enzyme per hour (Tan *et al*., 2002; Wout *et al*., 2004). The $K_{1/2}$ observed here as 14 ± 6 μM matches well with the reported K_d of binding to GTP of 8 μM (Wout *et al*., 2004), and the reported multiple turnover K_m of 18 μM (Tan *et al*., 2002). The K_i of ObgE binding to GDP measured here of 1.6 ± 0.4 μM is very similar to the K_d of binding of *B. subtilis* (Welsh *et al*., 1994) and *Salmonella typhimurium* (Lamb *et al*., 2007) Obg to GDP of 1.7 and 1.4 μM respectively.

The result that GDP and ppGpp bind with the same affinity is consistent with the *B. subtilis* structure of Obg bound to ppGpp (Buglino *et al*., 2002). In the structure, the two phosphates attached to the 3′ carbon of ppGpp appear to make no contacts with the protein, but hang free (Buglino *et al*., 2002). Obg appears to bind to the parts of ppGpp that it has in common with GDP. This would predict the similar binding affinities to GDP and ppGpp that were observed for ObgE in this study and as suggested by Buglino *et al*. (2002). In this study, ppGpp showed purely inhibitory action on GTPase activity, in contrast to previous data, suggesting that it could contain both inhibitory and stimulatory actions on GTPase activity of Obg (Buglino *et al*., 2002). The *in vivo* concentrations of all GDP, GTP and ppGpp during the *E. coli* life cycle well exceed the K_d and K_i for ObgE binding (Buckstein *et al*., 2008) (ppGpp can reach concentrations in the hundreds of micromolar). The fact that ObgE was shown to bind to ppGpp with affinity in the physiological range argues that ppGpp can be a ligand of ObgE *in vivo*.

The similar affinities of ObgE for ppGpp and GDP reinforce the fact that ObgE has a fairly open active site, allowing for the possibility that ObgE may share the binding of a nucleotide such as ppGpp with another protein, such as SpoT, RelA or Gpp. As these are all effectors of pppGpp and ppGpp levels in the cell, it may be possible that ObgE works together with these other proteins as well to produce the effects on ppGpp/pppGpp ratios observed here. ObgE's ability to physically interact with SpoT (Wout *et al*., 2004; Raskin *et al*., 2007) makes it a likely candidate; however, SpoT's pyrophosphohydrolase activity (release of the 3′ pyrophosphate) is a different chemistry than the pppGppase activity hypothesized to be responsible for the change in ppGpp/pppGpp ratio (5′-gamma-phosphate release). If SpoT is involved in the observed ObgE phenotypes, then it must play a more complicated role than originally predicted.

The similar binding affinity of ObgE to ppGpp and GDP also reenforces the idea that enzymes generally labelled as GTPases could potentially have nucleotide sensing capacities within the cell with less commonly tested nucleotides, such as pppGpp and ppGpp. In fact, to date, a small handful of GTPases in *E. coli* have also been shown to interact with ppGpp. Interestingly, aside from Gpp, translation factors IF2, EF-G and EF-Tu all have the ability to directly hydrolyse pppGpp to ppGpp *in vitro* (Cashel *et al*., 1996). IF2 binds ppGpp with similar affinity as it binds GTP, and has been proposed to be a metabolic sensor within the cell (Milon *et al*., 2006), a role often suggested for Obg. EF-Tu slows translation with a rise in ppGpp levels, causing an increase in translation fidelity during the stringent response (Dix and Thompson, 1986). Mutants in EF-G in *Salmonella typhimurium* also alter basal levels of ppGpp *in vivo* (Macvanin *et al*., 2000), and in *E. coli* EF-G controls ppGpp levels under heat shock conditions (Pao *et al*., 1981). These proteins share several features with ObgE: they are all GTPases that associate with the ribosome and can interact with ppGpp. In addition, some of them have also been shown to have downstream roles in the stringent response. Future work exploring the connections between these GTPases may prove exciting.

Obg may directly hydrolyse pppGpp or regulate this hydrolysis

In this study we observed that ObgE changes the ratio of ppGpp to pppGpp in response to serine hydroxamate. Currently, our understanding of the importance of pppGpp is far from complete. Much of the literature describing the effects of these nucleotides *in vivo* groups them both together as (p)ppGpp, and discusses their effect as one. (Distinguishing between their effects *in vitro* has been thwarted by the fact that only ppGpp is commercially available.) The current model of the stringent response is based on the fact that RelA can synthesize ppGpp and pppGpp with a similar speed (Cochran and Byrne, 1974; Cashel, 1975). Given the immense excess of GTP in *E. coli* over GDP at any given time it has been assumed that primarily pppGpp is made, which then is quickly converted to ppGpp within the cell, leading to the observed excess of ppGpp over pppGpp during the stringent response. We show here a correlation between a change in this nucleotide ratio and a deficiency in survival to amino acid starvation and DNA replication control in ObgE mutants. Although ObgE may be acting with other proteins to produce these effects, the simplest explanation of our observations is that ObgE is a pppGpp hydrolase and that this hydrolysis accompanies its roles in promoting

fork stability and cell survival during amino acid starvation. The ability of ObgE to bind ppGpp with identical efficiency to GDP suggests that it will likely bind pppGpp and may potentially hydrolyse this molecule as it does GTP. This raises the possibility that pppGpp may itself have an active role in stringent signalling, and that the act of hydrolysis of pppGpp to ppGpp may itself be a signal in this pathway.

Does Obg/CgtA directly regulate (p)ppGpp levels?

We observed higher pppGpp levels relative to ppGpp in the *obgE* mutant and a suppression of this effect by expression of ObgE$^+$ from a plasmid, although neither condition changed the overall levels of (p)ppGpp. It has been argued previously that ObgE mutants have constitutively high (p)ppGpp levels through its ability to regulate SpoT hydrolysis (Jiang *et al*., 2007; Raskin *et al*., 2007). This provided an attractive explanation for the essential nature of Obg/CgtA, to remove accumulated (p)ppGpp, and its ability to interact with SpoT. However, recent work has shown that ObgE remains essential for cell proliferation in even in *relA* or *relA spoT* strains that cannot synthesize (p)ppGpp (Jiang *et al*., 2007; Shah *et al*., 2008). Moreover, Obg/CgtA has not been shown to affect SpoT activity, *in vivo* or *in vitro*. It may be worthwhile to consider other explanations. Since (p)ppGpp levels rise naturally as cells begin to enter stationary phase and as a result of stress, the high basal levels of (p)ppGpp reported previously for ObgE/CgtA mutants could be an indirect effect of growth problems in these mutants. That is, the higher levels of ppGpp in these mutants could result from increased RelA-dependent synthesis rather than decreased SpoT hydrolysis of ppGpp.

We do not see an elevation of constitutive levels of (p)ppGpp in the *obgE*::Tn*5* mutant, as shown here, nor in ObgE-depleted cells (data not shown) in conflict with previous reports (Jiang *et al*., 2007; Raskin *et al*., 2007). A number of experimental differences, including plasmid versus chromosomal alleles, temperature and growth medium, make comparison difficult. It is possible that the differences between the two experiments with *E. coli* protein are due to the site of ObgE mutation used in these two studies [i.e. the N-terminal (Jiang *et al*., 2007) or the C-terminal of ObgE (this work)]. Recent work in *B. subtilis* suggests that the N-terminal domain is involved in Obg's growth phenotype whereas its C-terminal domain is involved with stress responses (Kuo *et al*., 2008). Although the C-terminal Tn*5* mutant could either increase or decrease overall (p)ppGpp levels through hypothetical misregulation of SpoT, we note that the observed phenotypes of the mutant with respect to replication – slow to arrest and slow to resume – is consistent with neither scenario. This suggests a role of Obg in the output of the response, over and above any effects on (p)ppGpp levels.

In addition, other observations raise doubts whether depletion of Obg/CgtA elicits a full-blown stringent response. Obg-deficient cells in a variety of conditions and in a range of organisms are significantly longer with higher DNA content than wild-type cells (Czyz *et al*., 2001; Kobayashi *et al*., 2001; Morimoto *et al*., 2002; Sikora-Borgula *et al*., 2002; Slominska *et al*., 2002; Foti *et al*., 2005; Datta *et al*., 2004). In our hands, depletion of ObgE does not affect cell growth and ability to initiate replication; cells form long polyploid filaments almost immediately upon depletion. This contrasts to stringent cells, induced either by amino acid starvation or by excess pppGpp production, which arrest replication and cease cell growth, producing short cells with a reduced, integer number of chromosomes. We conclude that if a rise in constitutive (p)ppGpp levels occurs in Obg/CgtA mutants, it does not lead to a canonical, full-blown stringent response. If ObgE depletion does cause accumulation of ppGpp, then ObgE must also be required for arrest of replication and cell growth seen during the stringent response, since this fails to occur in ObgE-depleted cells. Therefore, if Obg does indeed regulate (p)ppGpp levels, its involvement must be more complicated than previously appreciated. Future experiments to address these issues should prove informative.

A role on the output of the stringent response

The effect of ObgE on DNA replication during the stringent response fits well with the known role of ObgE to promote survival following exposure to DNA replication inhibitors (Foti *et al*., 2005). Replication in the presence of low levels of replication inhibitors and replication during amino acid starvation may both produce replication pauses or gaps. Our previous studies showed that ObgE had strongly synergistic effects with double strand break repair factors (RecA, RecBCD), both for normal viability and in the survival of mild replication inhibition, leading us to conclude that replication forks are more vulnerable to breakage in the absence of ObgE (Foti *et al*., 2005). This could provide an explanation for replication defects of *obgE*::Tn*5* seen during the stringent response: ObgE mutants have more difficulty completing replication during starvation and experience damage in the process. Interestingly, *E. coli* temperature-sensitive cells mutated in the N-terminal domain of ObgE have been reported to replicate DNA less efficiently than wild-type cells as measured by thymidine incorporation (Sikora *et al*., 2006). This may be due to problems in initiation of replication since the temperature sensitivity of this strain can be reduced by expression of the replication initiator protein, DnaA, and DnaA levels are reduced in this mutant at non-permissive

Table 2. Strain and plasmids used in this study.

	Relevant genotype	Source or derivation
Strains		
CAG12072	*sfsB203*::Tn*10*	Singer *et al.* (1989)
MG1655	K-12 wild-type *rph-1*	Blattner *et al.* (1997)
STL7679	[pSTL 346]	Cm^r transformation into MG1655
STL7742	*obgE*::Tn*5*	Foti *et al.* (2005)
STL8248	F– *ompT hsdS*($r_{B-}m_{B-}$) *dcm*– Tet^r *gal* λ (DE3) [pJT130]	Tan *et al.* (2002)
STL8783	*relA251*::*kan*	Metzger *et al.* (1989)
STL8789	*obgE*::Tn*5 sfsB203*::Tn*10*	Km^r tranductant P1 STL7742 X STL519
STL8813	*obgE*::Tn*5 relA251*::*kan sfsB203*::Tn*10*	Tc^r transductant P1 STL8789 X STL8783
STL12512	[pMK8]	Ap^r transformation into MG1655
STL12758	*pstA*::*parS*	Ferullo and Lovett (2008)
STL12760	*gadB*::*parS*	Ferullo and Lovett (2008)
STL13897	*obgE*::Tn*5* [pSTL 346]	Cm^r transformation into STL 7742
STL14013	*obgE*::Tn*5* [pMK8]	Ap^r transformation into STL7742
STL14030	*obgE*::Tn*5 pstA*::*parS sfsB203*::Tn*10*	Tc^r transductant P1 STL8789 X STL12758
STL14033	*obgE*::Tn*5 gadB*::*parS sfsB203*::Tn10	Tc^r transductant P1 STL8789 X STL12760
Plasmids		
pBAD33	*cat araC*	Schreiber *et al.* (1991)
pJT130	pET11a *obgE*	Tan *et al.* (2002)
pMK8	*sulA*::mCherry in a TGV light plasmid (from strain SS2514)	S. Sandler
pSTL346	*cat araC obgE*$^+$ in pBAD33	Foti *et al.* (2005)

Strains isogenic with MG1655, except STL8248, used for ObgE protein purfication.

temperature. However, in other studies, neither the temperature-sensitive allele nor depletion of *obgE* led to defects in replication initiation as detected by flow cytometry (Kobayashi *et al.*, 2001; Foti *et al.*, 2007) or by visualization of *oriC* foci (Foti *et al.*, 2007). ObgE-depleted cells continue to replicate to reach a median ploidy of 15–20 N after 3 h of depletion; it is possible that this high DNA content may downregulate DnaA. The hypothesis that ObgE mutants have difficulty completing replication is also consistent with our observation of an elevated constitutive SOS response in the *obgE*::Tn*5* strain and may explain the enhanced lethality during starvation in *relA* strains that cannot induce a full stringent response.

In summary, we have shown here that ObgE alters the ppGpp/pppGpp ratio in *E. coli* cells starved for amino acids. These data, together with an altered biological stringent response, place ObgE protein as an effector of the response to amino acid starvation. Correlation of the ppGpp/pppGpp ratio to a delayed change in DNA replication also further supports a connection between two functions of ObgE often considered to be disparate, namely, its involvement in DNA replication control and its connection to ppGpp.

Experimental procedures

Strains and growth conditions

Except for protein purification, the strains used in this study are derived from wild-type *E. coli* K-12 MG1655 (Table 2). All cells were grown at 37°C on LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% sodium chloride, and for plates, 1.5% agar). Antibiotics were used with the following concentrations: kanamycin (Km), 30 μg ml^{-1}; amplicillin (Ap), 100 μg ml^{-1}; tetracycline (Tc), 15 μg ml^{-1} and chloramphenicol (Cm) 30 μg ml^{-1}. Arabinose and glucose, when applicable, were used at a concentration of 0.2%. Strains isogenic with MG1655 were constructed by P1 virA transduction. Plasmids were isolated and purified from storage strains via miniprep (Qiagen and Sigma-Aldrich) and transformed by electroporation (Dower *et al.*, 1988).

Purification of ObgE

ObgE was purified as described (Kobayashi *et al.*, 2001) from STL 8248 with the following modifications: cells were grown shaking at 37°C to OD_{600} ~0.6 at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells continued to be incubated shaking at 37°C for 2.5–3.5 h. Cells were then spun down, resuspended in 1/100 the volume of Tris-sucrose buffer (50 mM Tris-HCl pH 7.5, 10% sucrose), and frozen at –80°C. To lyse the cells, DTT was added to 1 mM, NaCl was added to 100 mM and the cells were incubated on ice for 15 min. Lysozyme in Tris-sucrose was added to a final concentration of 0.2 mg ml^{-1} and the mixture was incubated on ice for 45 min, heat shocked by placing into a 37°C water bath for 2 min, then left on ice for 2 min. This process was then repeated 4–5 times and the lysed cells were then spun for 35 min at 67 100 *g*. To every 5 ml of cleared lysate 3.198 ml of saturated ammonium sulphate was added slowly with stirring. The precipitate was recovered by centrifugation at 13 250 *g* for 15 min and resuspended in TEGED100 buffer (20 mM Tris 7.5, 100 μM EDTA, 1 mM DTT, 10% glycerol and 100 mM NaCl) and dialysed overnight into 1 l of TEGED100 buffer. Insoluble particles were removed by centrifugation and the solution was loaded onto a 5 ml HiTrap Blue HP column (GE Healthcare), and

step eluted from TEGED100 buffer to TEGED1400 buffer (20 mM Tris 7.5, 100 μM EDTA, 1 mM DTT, 10% glycerol and 1400 mM NaCl). Fractions containing ObgE were collected and diluted with three volumes of TEGED100 buffer. The entire mixture was loaded onto a 5 ml Q-column (GE Healthcare) and ramp-eluted from TEGED100 buffer-TEGED1000 buffer (20 mM Tris 7.5, 100 μM EDTA, 1 mM DTT, 10% glycerol and 1000 mM NaCl). Fractions containing ObgE were pooled, dialysed into 1× Reaction Buffer (50 mM Tris 7.5, 50 mM NaCl, 1 mM DTT), concentrated in a 10 000 MWCO PES spin column (Sartorius, Vivaspin 6). Protein was either used immediately (for $K_{1/2}$ curves), or an equal volume of glycerol was added and the protein was stored at –20°C (for inhibition curves).

ObgE GTPase assays and inhibition experiments

Inhibition experiments were performed similar to experiments performed by Peluso *et al*. in 2001 (Peluso *et al*., 2001). Samples consisted of: 9.5 μl of 35.8 mM Tris, 35.8 mM NaCl, 1.8 mM DTT, 5.3 mM $MgCl_2$, 2%glycerol, 3.5 μM ObgE and 0.0032 μM of [γ-^{32}P]-GTP (EasyTides Perkin Elmer) and GDP (Sigma) or ppGpp (Trilink Technologies) with concentrations varying from 0 to 56 μM. Experiments were initiated by the addition of [γ-^{32}P]-GTP and incubated at 37°C. One-microlitre aliquots were removed at various time points, quenched with an equal volume of 40 μM cold EDTA and stored on ice. [GDP] and [ppGpp] stock solutions were diluted with water and neutralized with 50 mM Tris Base. One microlitre of each reaction was spotted on a polyethyleneimine (PEI) plate (Sigma-Aldrich). Plates were developed in 1 l beakers covered with parafilm containing a mobile phase of 1 M formic acid and 0.5 M LiCl. The plates were subsequently dried, exposed to a phosphoimager screen, and quantified using Quantity One software (Bio-Rad). None of the images used included saturated pixels (see supporting data for $K_{1/2}$ calculations and for curve-fitting information).

In vivo *pppGpp and ppGpp measurements*

In vivo measurements of ppGpp and pppGpp were performed as described previously in Metzger *et al*. (1989) with the following modifications. Cultures were grown overnight in LB medium at 37°C and supplemented with the appropriate antibiotics and sugars (Sigma). In the morning, cells were diluted to reach early log phase following 2.25 h of growth in LB and appropriate sugar, 0.2% glucose or 0.2%, for those experiments involving pBAD33 ObgE expression. Cells were grown for 15 min, at which point ^{32}P-orthophosphate (Perkin Elmer) was added to a final concentration of 120 μCi ml^{-1}, incubated for 2 h, followed by treatment with serine hydroxamate (Sigma-Aldrich) at 1.2 mg ml^{-1}. Sixty-microlitre samples prior to addition of the drug or after 20 min treatment were added to 50 μl of 13N formic acid (Sigma), frozen and thawed twice on dry ice with ethanol and the samples were subjected to centrifugation to remove debris. Six microlitres of sample was loaded onto polyethyleneimine (PEI) plates (Sigma-Aldrich). Chromatography was performed in 1 l beakers covered with parafilm containing a mobile phase of 1.5 M KH_2PO_4 (Fisher Scientific). The plates were subsequently dried, exposed to a phosphoimager screen (most exposures took two or more days), and quantified using Quantity One software (Bio-Rad), none of the images used included saturated pixels. For some experiments samples were stored in formic acid at –20°C overnight before loading onto PEI plates. Unlabelled GTP and ppGpp were spotted on the plates as markers and visualized by UV light-induced fluorescence.

ObgE protein levels following serine hydroxamate treatment

Culture of wild-type (MG1655) were grown to early log phase, split and 1 mg ml^{-1} serine hydroxamate (Sigma) was added to one culture. Samples were taken for 20 min following the addition of serine hydroxamate and the density of the culture was determined by OD_{600}. Cells were collected from 1 ml of cells by microcentrifugation and the cell pellet was resuspended in $OD_{600} \times 100$ μl of 2× FSB [4% sodium dodecyl sulphate (SDS), 200 mM DTT, 120 mM Tris pH 6.8, 0.002% bromphenol blue, 10% glycerol]. Boiled samples were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a PVDF membrane and blotted for ObgE by using polyclonal anti-ObgE primary antibody (Foti *et al*., 2007). Horseradish peroxidase-linked anti-rabbit Ig, from donkey (GE Healthcare/Amersham Biosciences), was used as a secondary antibody. Western blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). For quantitative Western blots, the protocol was slightly altered: following cell collection, cell pellets were resuspended in 100 μl of Tris-Sucrose Buffer and lysed as indicated under the ObgE purification procedures. The lysates were cleared by centrifugation and normalized to total protein level as measured by the Bradford assay (Bradford, 1976) with the addition of Blank Lysis buffer (50 mM Tris-HCl pH 7.5, 10% sucrose, 1 mM DTT, 100 mM NaCl) to relevant samples. Samples were then combined with an equal volume of 2× FSB and Western blots were performed as indicated above, except using IRDye 800CW Goat Anti-Rabbit IgG secondary antibodies (LI-COR Biosciences). Experiments were performed in triplicate and quantified using an Odyssey machine (LI-COR Biosciences). Reported are the mean and standard deviation of fluorescent intensity normalized to untreated cells originating from the same isolate.

Live/dead analysis following serine hydroxamate treatment

After 2 h of growth in fresh medium, cells were treated with and without 1 mg ml^{-1} serine hydroxamate (Sigma-Aldrich) for an additional 90 min. One millilitre of cells was resuspended in 50 μl phosphate-buffered saline (PBS) and stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) according to the manufacturer's instructions. Data were acquired for 30 000 cells by using a Becton Dickinson FACSCalibur flow cytometer and analysis was done using the FlowJo 6.4.1 software (Tree Star).

Growth curves

Two isolates each of MG1655 and STL 7742 were grown overnight at 37°C in LB and diluted to an OD_{600} of 0.05. OD_{600}

was then measured at various time points following dilution. When each culture reached an OD_{600} of 0.2–0.3, it was split with half of the cultures treatment with 1 mg ml^{-1} serine hydroxamate (Sigma-Aldrich). Shown are the average and standard deviation of the OD_{600} between the two isolates of each strain for each time point.

EdU-Click labelling of replicated DNA

Newly replicated DNA was detected using l EdU-Click labelling (Ferullo *et al.*, 2009) using a commercially available Alexa fluor 488 kit 'Click-IT Edu' (Invitrogen). All washes performed in PBS were performed twice, except the wash immediately following incubation in 0.5% Triton-X based buffer in PBS, using 1.5 ml of PBS for each wash. The final wash utilized only 1 ml of PBS for each sample. A GFP filter with exposure times of 2.0 s was used to measure cell fluorescence. All data were collected on an Olympus BX51 microscope equipped with a 100× objective and a Retiga Exi (Qimaging) camera with image analysis using Volocity imaging software (Perkin-Elmer Improvision). Figures of fluorescence shown all have a black point of 456 and a white point of 1500 intensity so that the same contrast may be shown. To quantify the average fluorescence per cell per field of view, cells were identified as objects in phase by using −1 standard deviation of the mean intensity as the upper limit of intensity allowed. Any objects identified that were touching the edge of the field of view or smaller than 1 μm^2 were not considered. Fluorescent objects were detected using a threshold pixel intensity minimum of 675 and maximum of 3000. Fluorescence was summed over the field of view and divided by the number of cells manually counted in that field of view for at least 100 cells and three fields of view for each sample; the standard deviation between fields is reported. DNA replication was measured using this assay for wild-type cells (MG1655) and *obgE*::Tn*5* cells (STL7742) at various time points for cultures at OD_{600} 0.1–0.2 followed by 1 mg ml^{-1} serine hydroxamate for 90 min. Serine hydroxamate was removed by microcentrifugation of cells, removal of the supernatant and fivefold dilution in fresh medium to resume growth.

Septation measurements

Visualization of septating cells was aided by staining inner cell membranes with FM 4–64 [*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl] pyridinium dibromide (Fishov and Woldringh, 1999). Cells were stained and analysed as described (Ferullo *et al.*, 2009). Septating cells were counted and lengths were measured using Volocity imaging software (Perkin-Elmer Improvision).

SOS induction in obgE *mutant cells using SulA::mCherry plasmid reporter*

MG1655 cells and STL7742 cells were transformed with a SulA::mCherry reporter plasmid, derived from a SulA::GFP plasmid (McCool *et al.*, 2004) by replacement with mCherry (Steven Sandler, unpubl. results). The cells were grown in LB supplemented with 100 μg ml^{-1} of ampicillin to log phase (OD_{600} = 0.3–0.4). Cells were visualized on 2% agarose-padded slides and a coverslip was added for analysis under the microscope. All data were collected on an Olympus BX51 microscope equipped with a 100× objective and Retiga Exi (Qimaging) camera. All analysis was performed using Volocity imaging software (Perkin-Elmer Improvision). To quantify the average fluorescence per cell, cells were identified as objects as described above. The average pixel intensity for mCherry fluorescence was calculated for 100 cells and averages and standard deviations are reporter.

oriC analysis

STL 12758 and STL 14030 were grown in LB Ap medium to OD_{600} = 0.3–0.4. GFP-labelled origins of replication within the living cells were immediately visualized using 2% agarose pads on an Olympus BX51 microscope equipped with a RGB liquid crystal colour filter and a Qimaging Retiga EXi camera. Foci were counted visually using Volocity imaging software (Perkin-Elmer Improvision) with a blackpoint intensity of 456 and a white-point intensity of 900. A total of 114 cells were counted for two individual isolates of STL 12758 and 79 cells were counted for two individual isolates of STL14030.

DNA content analysis using flow cytometry

DNA content per cell was determined as previously reported (Ferullo and Lovett, 2008), with the following modifications. Data were acquired for at 30 000 cells using a FACS Aria Flow Cytometer using DIVA 6.1.1 as its operating system (BD Biosciences). Data were further analysed using FloJo 6.4.7 software (Tree Star). For clarity, a lower fluorescence gate was added in the analysis of 10^1 fluorescence intensity as unlabelled cell controls ran below this intensity.

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