Molecular Microbiology (2017) 103(4), 698–712 ■
doi:10.1111/mmi.13582
First published online 16 December 2016

The stringent response plays a key role in *Bacillus subtilis* survival of fatty acid starvation

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Summary

The stringent response is a universal adaptive mechanism to protect bacteria from nutritional and environmental stresses. The role of the stringent response during lipid starvation has been studied only in Gram-negative bacteria. Here, we report that the stringent response also plays a crucial role in the adaptation of the model Gram-positive *Bacillus subtilis* to fatty acid starvation. *B. subtilis* lacking all three (p)ppGpp-synthetases (Rel_{Bs}, RelP and RelQ) or bearing a Rel_{Bs} variant that no longer synthesizes (p)ppGpp suffer extreme loss of viability on lipid starvation. Loss of viability is paralleled by perturbation of membrane integrity and function, with collapse of membrane potential as the likely cause of death. Although no increment of (p)ppGpp could be detected in lipid starved *B. subtilis*, we observed a substantial increase in the GTP/ATP ratio of strains incapable of synthesizing (p)ppGpp. Artificially lowering GTP with decoyinine rescued viability of such strains, confirming observations that low intracellular GTP is important for survival of nutritional stresses. Altogether, our results show that activation of the stringent response by lipid starvation is a broadly conserved response of bacteria and that a key role of (p)ppGpp is to couple biosynthetic processes that become detrimental if uncoordinated.

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Introduction

Bacteria in nature often face adverse growth conditions, such as nutritional limitation, physicochemical stresses and exposure to antimicrobial compounds. The capability to adapt to such changes in their surrounding environment is crucial for survival. A universal pathway for adaptation to adverse growth conditions is the stringent response, mediated by the alarmones guanosine-5′-triphosphate-3′-diphosphate and guanosine-5′-diphosphate-3′-diphosphate [ppGpp and pppGpp, henceforth collectively referred to as (p)ppGpp]. Intracellular accumulation of these signaling nucleotides produces a broad reprogramming of transcription and metabolism, with a sharp decrease in protein synthesis and other biosynthetic activities (DNA replication, cell wall and lipid synthesis) associated with growth and the activation of general and specific stress responses that favor survival (Potrykus and Cashel, 2008; Hauryliuk *et al.*, 2015; Liu *et al.*, 2015). A substantial part of (p)ppGpp actions result from the effect of this nucleotide on gene expression. Either directly or indirectly, (p)ppGpp shuts down stable RNA (rRNA and tRNA) transcription while stimulating transcription of amino acid biosynthesis operons and sets of genes associated with stationary phase and stress survival (Geiger and Wolz, 2014; Hauryliuk *et al.*, 2015; Liu *et al.*, 2015). (p)ppGpp also function by directly affecting protein targets, such as translation factors (Arai *et al.*, 1972; Milon *et al.*, 2006), small GTPases involved in ribosome maturation (Feng *et al.*, 2014; Corrigan *et al.*, 2016), DNA primase (Wang *et al.*, 2007), GTP biosynthetic enzymes (Lopez *et al.*, 1981; Kriel *et al.*, 2012), among others.

The intracellular levels of (p)ppGpp are controlled by RelA/SpoT Homolog (RSH) enzymes, which are usually bifunctional, displaying the ability to both synthesize and degrade (p)ppGpp. Synthesis and hydrolysis are mutually exclusive activities that occur at independent sites in the N-terminal catalytic half of RSH proteins (Hogg *et al.*, 2004). The balance between these activities is controlled by the C-terminal regulatory domain, which presumably senses starvation and other stress cues and relay this information to the N-terminal catalytic domain (Avarbock *et al.*, 2000; Mechold *et al.*, 2002).

Escherichia coli posseses two RSH enzymes: RelA, which has a degenerate hydrolysis domain and, thus, only produces (p)ppGpp, and SpoT, which is capable of both (p)ppGpp synthesis and hydrolysis (Sarubbi *et al*., 1988). This is also the situation for several other Proteobacteria of the gamma and beta groups. In contrast, Gram positive bacteria generally have a single bifunctional RSH enzyme and one or more small alarmome synthetases (SAS), which are truncated proteins that possess only a synthetase domain and, thus, can only produce (p)ppGpp (Mittenhuber, 2001; Atkinson *et al*., 2011). In *Bacillus subtilis*, the paradigm of Gram-positive bacteria, the RSH enzyme was named RelA and there are two SAS whose original names are YjbM and YwaC (Nanamiya *et al*., 2008). In an attempt to follow the nomenclature suggested by Atkinson and collaborators (Atkinson *et al*., 2011) we will use Rel_{Bs} to refer to the *B. subtilis* long RSH enzyme and RelP (YjbM) and RelQ (YwaC) for the SAS proteins.

Most of what is known about the circuitry that controls (p)ppGpp, how it responds to nutritional and environmental stimuli and how (p)ppGpp exerts its effects was first established in *E. coli*. The role of (p)ppGpp as a signaling molecule was initially recognized when this bacterium was subjected to amino acid deprivation (Cashel and Gallant, 1969; Cashel and Kalbacher, 1970). Subsequently, similar (p)ppGpp mediated responses were reported to carbon (Lazzarini *et al*., 1971), phosphate (Spira *et al*., 1995), iron (Vinella *et al*., 2005) and fatty acid starvation (Seyfzadeh *et al*., 1993). RelA is the protein that produces (p)ppGpp in response to amino acid deprivation, by sensing the presence of uncharged tRNAs in the A site of ribosomes (Wendrich *et al*., 2002). All the other nutritional signals act through SpoT, the second RSH protein of *E. coli*, but, with the exception of fatty acid starvation (Battesti and Bouveret, 2006), how these signal activate SpoT is still unclear. More recently, the study of (p)ppGpp signaling and the stringent response has been extended to a wide variety of bacteria, belonging to different evolutionary groups and exhibiting diverse lifestyles (Dalebroux and Swanson, 2012; Boutte and Crosson, 2013; Liu *et al*., 2015). This showed that there is substantial variation in how the stringent response becomes activated, the players involved in sensing and responding to stimuli and the downstream effects imposed by (p)ppGpp. For example, amino acid starvation, which was once thought to define the stringent response, does not seem sufficient to trigger (p)ppGpp accumulation in *Caulobacter crescentus* and perhaps other bacteria (Boutte and Crosson, 2011). Other noteworthy departure from the initial paradigm is the mechanism of transcriptional modulation by (p)ppGpp: whereas in *E. coli* it involves a direct binding of (p)ppGpp to RNA polymerase, in Gram-positive bacteria it was shown to be an indirect effect of (p)ppGpp lowering the intracellular levels of GTP (Krásný and Gourse, 2004; Srivatsan and Wang, 2008; Wolz *et al*., 2010).

Activation of the stringent response by lack of fatty acids has been demonstrated for several Gram-negative bacteria, like *E. coli* (Seyfzadeh *et al*., 1993), *C. crescentus* (Stott *et al*., 2015), *Vibrio cholera* (Das *et al*., 2009) and *Legionella pneumophila* (Dalebroux *et al*., 2009). In the case of bacteria that have two RSH enzymes, such as *E. coli* and *L. pneumophila*, the protein that responds to fatty acid starvation is SpoT, which senses lack of fatty acids due to an interaction with unacylated ACP (Battesti and Bouveret, 2006). This is likely a common mechanism for bacteria having two RSH proteins, which includes only Gram-negatives (Battesti and Bouveret, 2009; Atkinson *et al*., 2011). In comparison, much less is known about how Gram-positive bacteria cope with fatty acid starvation. There is one report that starving *Staphylococcus aureus* of fatty acids by cerulenin treatment does not induce (p)ppGpp production (Greenwood and Gentry, 2002). This, together with the observation that Gram-positive RSH enzymes seem incapable of interacting with ACP (Battesti and Bouveret, 2009), raised the possibility that this group of bacteria may not rely on the stringent response to withstand the effects of lipid starvation. Here, we tested this possibility by investigating the role of (p)ppGpp in the response of *B. subtilis* to fatty acid starvation. It is already known that *B. subtilis* activates the stringent response when faced with amino acid (Swanton and Edlin, 1972) and carbon starvation (Fortnagel and Bergmann, 1974). In addition, the effects of fatty acid starvation on general metabolism (Wille *et al*., 1975), gene expression (Schujman *et al*., 2003) and membrane composition (Porrini *et al*., 2014) of *B. subtilis* have already been reported. Interestingly, fatty acid starvation leads to an abrupt cessation of RNA and protein synthesis, a hallmark of the stringent response (Wille *et al*., 1975). However, no evidence for (p)ppGpp increases or of a role of RSH or SAS enzymes during fatty acid starvation is available in the literature.

Here, we demonstrate that the inability to activate the stringent response during fatty acid starvation causes extreme loss of viability in *B. subtilis*. Similarly to other bacteria, the long RSH enzyme of *B. subtilis*, Rel_{Bs}, is the synthetase responsible for activating the stringent response on fatty acid starvation. Surprisingly, however, we failed to detect a significant (p)ppGpp increment in *B. subtilis* cells starved of fatty acids. Instead, we observed a drop in the intracellular GTP/ATP ratio in response to starvation that does not occur in cells incapable of activating the stringent response. Loss of viability on fatty acid starvation was associated with

perturbation of membrane potential and integrity, as one would expect if cells were attempting to grow in the absence of new membrane synthesis. *In toto*, our results show that activation of the stringent response by fatty acid starvation is a broadly conserved response of bacteria. In addition, the dramatic loss of viability of cells incapable of triggering the stringent response argues that a key evolutionary drive behind the stringent response must have been the necessity to couple biosynthetic processes that are not otherwise coupled via the chemical interdependencies of metabolism.

Results

Inhibition of fatty acid biosynthesis is lethal in the absence of (p)ppGpp production

To study the role of the stringent response during fatty acid starvation in *B. subtilis*, we compared the effect of cerulenin, a well characterized inhibitor of the fatty acid synthesis enzyme FabF (Schujman *et al*., 2001; Trajtenberg *et al*., 2014) on the growth and viability of a wild-type strain (PY79) and a strain incapable of producing (p)ppGpp [ppGpp(0)]. The ppGpp(0) strain lacks the three genes that encode (p)ppGpp synthetases in *B. subtilis* (*rel*$_{Bs}$, *relP* and *relQ*) and its growth rate and viable cell count are similar to those of a wild-type strain under nonstressed conditions in rich medium (Fig. 1A and C). As reported previously (Wille *et al*., 1975), treatment of wild-type *B. subtilis* with cerulenin led to a quick growth arrest but the cells remained viable, as assessed by colony formation (Fig. 1A and B). In contrast, the viability of the ppGpp(0) strain was dramatically reduced, with close to 99.9% of the cells incapable of producing (p)ppGpp dying within 2 h of treatment with cerulenin in rich medium (LB) at 37°C (Fig. 1B). Death of the ppGpp(0) strain in response to cerulenin also occurred if cells were grown under conditions that attained slower growth rates, such as in LB at 25°C or in minimal glucose medium, although in these cases the loss of viability was significantly reduced (Supporting Information Fig. S1). These results indicate that (p)ppGpp plays a central role in the adaptation and survival to the cessation of lipid synthesis, in particular when cells are growing fast.

Rel$_{Bs}$ *is the (p)ppGpp synthetase necessary for viability on lipid starvation*

We determined which of the three (p)ppGpp synthetases of *B. subtilis* was responsible for maintaining viability when cells were treated with cerulenin by testing strains carrying mutations in individual synthetase genes, or a strain carrying simultaneous deletions of the two SAS synthetases. To inactivate Rel$_{Bs}$-dependent (p)ppGpp synthesis we employed a point mutation (Rel$_{Bs}^{D264G}$) which specifically abrogates synthetase activity without affecting (p)ppGpp hydrolase activity (Nanamiya *et al*., 2008). This is necessary because deletion of *rel*$_{Bs}$ eliminates the only source of (p)ppGpp hydrolysis in the cell, thus causing (p)ppGpp accumulation from the basal activity of the SASs (Nanamiya *et al*., 2008). As observed in Fig. 1B, the viability of the Rel$_{Bs}^{D264G}$ mutant was as reduced as that of the ppGpp(0) strain when it was challenged with cerulenin. In contrast, deletion of the SASs, either individually (data not shown) or in combination (Fig. 1B), had no effect on viability. Notably, there was also no loss in viability when a strain bearing a complete deletion of *rel*$_{Bs}$ was grown in the presence of cerulenin (Fig. 1B). This observation

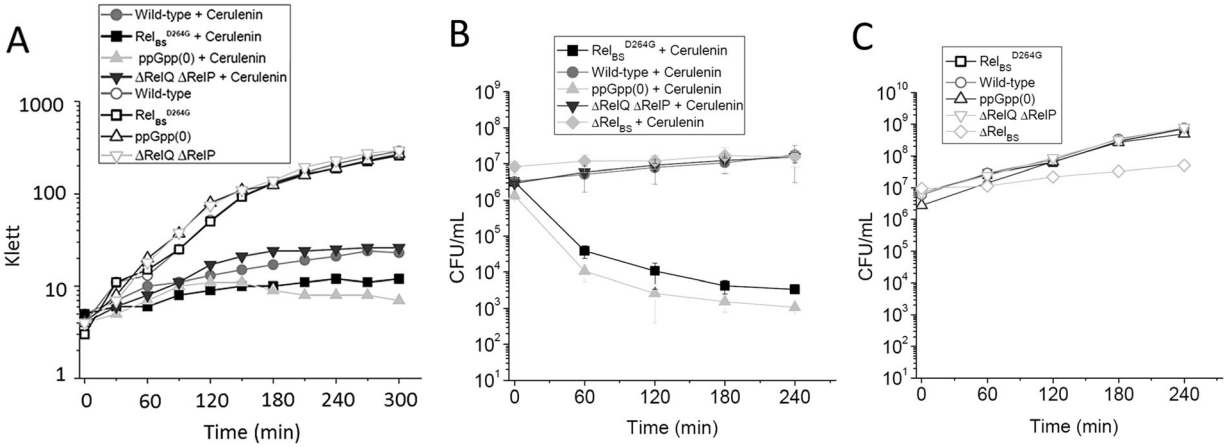

Fig. 1. (p)ppGpp is necessary for *B. subtilis* survival during fatty acid starvation.
A. Time course of viability loss in response to treatment with 10 μg.ml^{-1} of cerulenin. Strains Δ*relQ* Δ*relP* (AP10), Rel$_{Bs}^{D264G}$ (FG1316), ppGpp(0) (AP6), Wild-type (FG1) and Δ*rel*$_{Bs}$ (AP23). All assays were performed in triplicate.

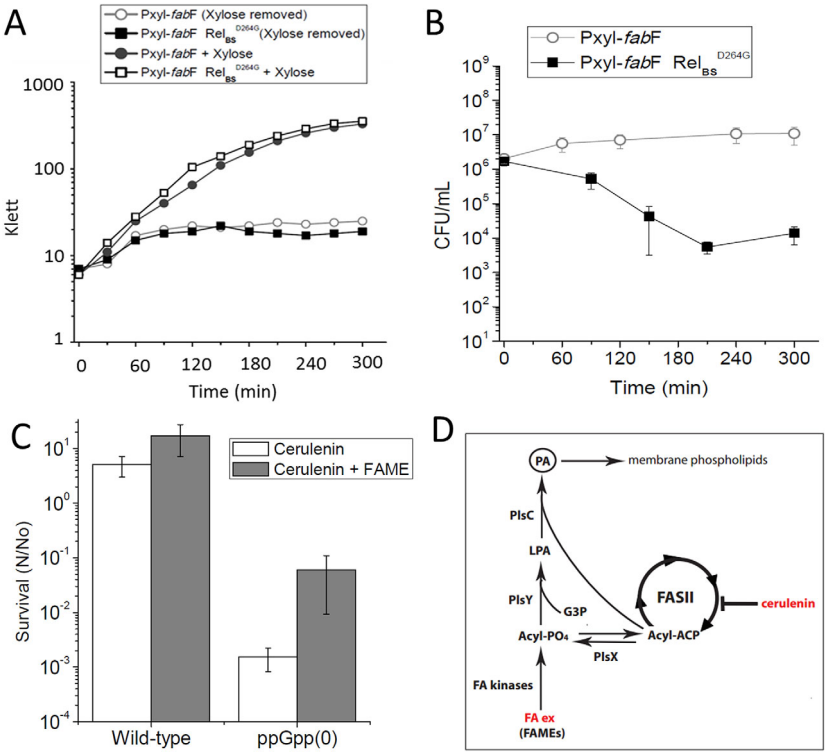

Fig. 2. A. Growth curves of P$_{xyl}$-*fabF* (AP1) and P$_{xyl}$-*fabF rel*$_{Bs}^{D264G}$ (AP2) *B. subtilis* strains starved for fatty acids by FabF depletion
(no xylose added to the media).
B. Viability of the strains after xylose removal from the media.
C. Viability rescue of the ppGpp(0) strain (AP6) by exogenous fatty acids (fatty acid methyl-esters - FAMEs). All assays were performed in triplicates. Error bars indicates the standard deviation between the triplicates.
D. Scheme of how FAMEs bypass cerulenin inhibition of FASII.

appears paradoxical, but as discussed above, a *rel*$_{Bs}$ null mutant accumulates (p)ppGpp from the unopposed activity of the SAS proteins. This suggests that an increase of (p)ppGpp above basal levels, irrespective of the synthetases that produced it, suffices to keep cells viable when facing lipid starvation. However, the fact that a strain having Rel$_{Bs}$ as the sole synthetase (the *relP relQ* double mutant) behaves like the wild-type and that the Rel$_{Bs}^{D264G}$ mutant behaves similarly to a ppGpp(0) strain, in their response to cerulenin demonstrates that Rel$_{Bs}$ is the (p)ppGpp synthetase that responds to lipid starvation in *B. subtilis* under physiological conditions.

To confirm that the lethal effect of cerulenin in strains impaired in the stringent response (SR$^-$ strains) was due to inhibition of lipid synthesis, and not to an unforeseen secondary effect of the drug, we carried out two control experiments. The first was to block lipid synthesis genetically, by placing the *fabF* gene under the control of the xylose-inducible P*xyl* promoter. As shown in Fig. 2A and B, depletion of FabF caused similar loss of viability as cerulenin treatment in a strain bearing the Rel$_{Bs}^{D264G}$ mutation. The second control was to test whether exogenous fatty acids could attenuate the lethality of cerulenin to SR$^-$ strains. As shown in Fig. 2C and D, supplementing the medium with fatty acid methyl esthers (FAMEs) rescued the viability of a ppGpp(0) strain by almost 100 fold. The effect of FAME supplementation on viability was only partial, as previously observed in a *B. subtilis plsX* mutant (Parsons *et al.*, 2014; Sastre *et al.*, 2016), probably because the salvage pathway that allows the use of exogenous fatty acids by *Bacillus* is inefficient. Nevertheless, taken together, the FabF depletion and the FAME supplementation results argue that cerulenin is acting as expected and that SR$^-$ strains die because of lipid starvation.

Fatty acid starvation causes morphological changes and membrane damage in cells compromised in (p)ppGpp synthesis

Microscopy observations revealed several morphological alterations in *B. subtilis* starved for fatty acids. Wild-type cells treated with cerulenin for 240 min became significantly shorter than nontreated cells (Fig. 3A and D). Reduction in cell size has already been reported for bacteria in which the stringent response is activated (Schreiber *et al.*, 1995) or that are starved for fatty acids (Wille *et al.*, 1975; Yao *et al.*, 2012; Stott *et al.*, 2015) and likely reflects the slow down or cessation of growth under these conditions. Interestingly, fatty acid starvation had very little or no effect on the length of cells of strains Rel$_{Bs}^{D264G}$ and ppGpp(0) (Fig. 3A and D), suggesting that these strains continued to grow after cerulenin treatment. Fluorescence microscopy using the membrane dye FM1-43 and propidium iodide (PI) showed that the membranes of wild-type cells treated with

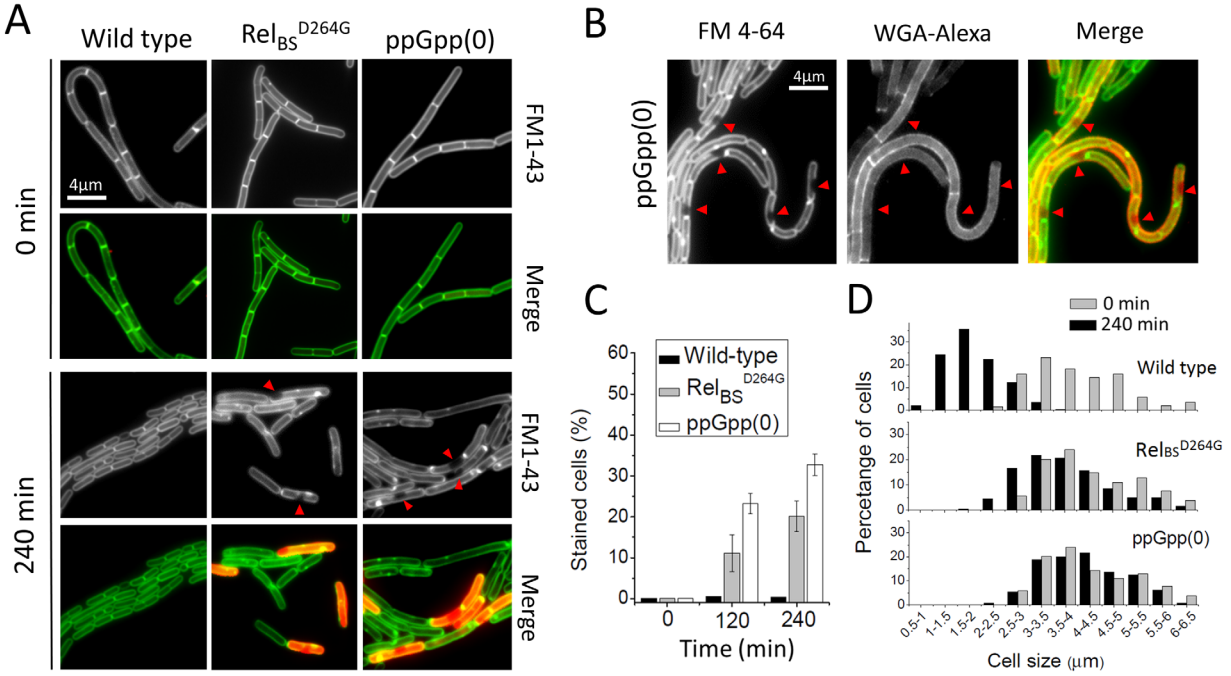

Fig. 3. Phenotype of wild-type (FG1), Rel^{D264G}_{Bs} (FG1316) and ppGpp(0) (AP6) cells starved for fatty acids by cerulenin treatment.
A. Membrane integrity and permeability assessed by treatment with the membrane dye FM1-43 (green in the merge panels) and propidium iodide (red in the merge panels). The propidium iodide only images were ommited to make the figure simpler. Membrane holes observed in strains Rel^{D264G}_{Bs} and ppGpp(0) are indicated by the red arrowheads.
B. Fluorescence microscopy of strain ppGpp(0) treated with the membrane dye FM4-64 (green in merge panels) and cell wall dye WGA-Alexa Fluor 350 (red in merge panels). Although several holes are evident in the membrane, the cell wall is intact after 240 min of fatty acid starvation.
C. Frequency of propidium iodide staining after 120 min and 240 min of cerulenin treatment.
D. Cell length measurements before and after 240 min of cerulenin treatment.

cerulenin were intact, as expected from the fact that these cells remain viable (Fig. 3A). In contrast, a significant portion of the SR^- cells treated with cerulenin [12% for Rel^{D264G}_{Bs} and 22% for ppGpp(0)] exhibited striking discontinuities in the membrane stain (Fig. 3A and B, red arrows), indicating cytoplasmic membrane rupture. This was confirmed by PI staining, which showed that the cells that have holes in their membranes have become permeable to the dye, as expected (Fig. 3A). Importantly, cells with damaged membranes still exhibited intact cell walls, as shown by staining with WGA-Alexa Fluor 350 (Fig. 3B). This indicates that lipid starvation damages membranes specifically and explains why SR^- cells treated with cerulenin do not exhibit evident lysis, detectable as a drop in OD (Fig. 1A).

One puzzling observation from the experiment in Fig. 3A was that the majority of SR^- cells (80 to 90% at 120 min) remained impermeable to PI after cerulenin treatment (Fig. 3C). Because the viability of this population (assessed by CFU in Fig. 1B) should have dropped to less than 1%, the cells that were still impermeable to PI could either represent cells that have lost viability but did not immediately became permeable to PI, or cells that entered a viable but nonculturable (VBNC) state, as recently suggested for *C. crescentus* (Stott *et al.*, 2015). To distinguish between these possibilities, we treated cells with cerulenin for 120 min, washed out the drug and followed growth on agarose pads. We observed that after a long period (300 min) following cerulenin removal all the Rel^{D264G}_{Bs} and ppGpp(0) cells became stained with PI, whereas wild-type cells resumed growth normally (Supporting Information Fig. S2). This indicates that strains that cannot activate the stringent response suffer from an irreversible cellular dysfunction when exposed to cerulenin, from which they cannot recover after drug removal. Consistently, a similar phenotype of membrane damage was observed with the P_{xyl}-*fab*F *rel*$_{Bs}^{D264G}$ strain under conditions of xylose depletion (data not shown).

Fatty acid starvation disrupts membrane potential of cells unable to synthesize (p)ppGpp

The fraction of cells that become stained with PI after 120 min of cerulenin treatment (Fig. 3A and C) is much lower than the measured 99.9% drop in viability of the same strains under identical experimental

conditions (Fig. 1). This difference suggests that most of the SR$^-$ cells lose their viability by a mechanism that does not cause immediate membrane disruption or permeabilization. Thus, we decided to investigate subtler membrane alterations that could account for the observed loss of viability. We focused on membrane potential, as perturbations in this property can have drastic effects in solute transport and ATP synthesis, both activities being crucial for cell survival (Lamsa *et al.*, 2012). Strahl and Hamoen (2010) demonstrated that loss of membrane potential causes delocalization of some cell division proteins, one of them being MinD. Using an YFP-MinD fusion, we observed that cerulenin treatment did not affect MinD localization in wild-type cells but produced strong delocalization of MinD in SR$^-$ cells (Fig. 4). Remarkably, MinD delocalization, unlike the PI staining, followed similar kinetics as the loss of viability (Fig. 1B). After 60 min of cerulenin treatment, virtually 100% of the Rel$^{D264G}_{Bs}$ and ppGpp(0) cells already showed altered MinD localization, indicating that membrane function becomes compromised by fatty acid starvation well before cells become permeable to PI.

Because MinD delocalization is an indirect readout of membrane potential we also used the dye DiSC3(5) and fluorescence microscopy (Strahl and Hamoen, 2010) to directly measure membrane potential. Using this method, we observed that the wild-type strain can maintain membrane potential after cerulenin treatment (Fig. 5A), although quantitation of the fluorescence indicated that membrane potential is slightly reduced (to about 60%) when compared to nontreated cells. In contrast, cells compromised in (p)ppGpp synthesis completely lost membrane potential when exposed to cerulenin, exhibiting a fluorescence drop similar to that of wild-type cells treated with the ionophore CCCP (Fig. 5B). Importantly, as for MinD delocalization, the loss of membrane potential followed the same kinetics as the loss of viability under identical experimental conditions: 60 min after the addition of cerulenin virtually all cells showed loss of membrane potential (Supporting Information Fig. S3), which was further compromised after 120 min. Thus, an abrupt loss of membrane potential is likely the main cause of death of cells that cannot produce (p)ppGpp in response to lipid starvation.

Alterations in the GTP/ATP ratio are associated with adaptation to fatty acid starvation in B. subtilis

Although fatty acid starvation had a dramatic effect on the viability of the Rel$^{D264G}_{Bs}$ and ppGpp(0) strains (Fig. 1), we were unable to detect a significant increment of (p)ppGpp levels in *B. subtilis* cells treated with

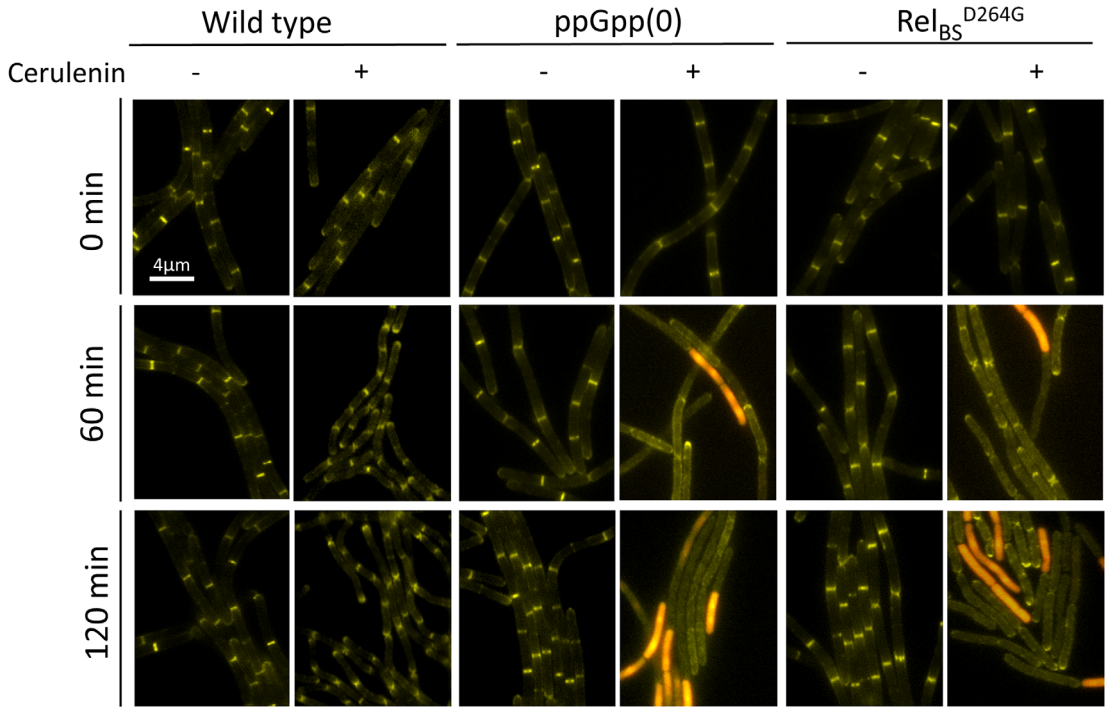

Fig. 4. Fatty acid starvation in the absence of stringent response promotes MinD delocalization. All the images are merges of MinD-YFP (yellow) and propidium iodide (red) fluorescences. Note the loss of MinD localization in essentially all ppGpp(0) and Rel$^{D264G}_{Bs}$ treated with cerulenin, most of which are still impermeable to propidium iodide. MinD was induced by adding 1mM (final concentration) of IPTG to the wild-type (FG173), Rel$^{D264G}_{Bs}$ (AP16) and ppGpp(0) (AP15) strains. Images are representative of three independent experiments.

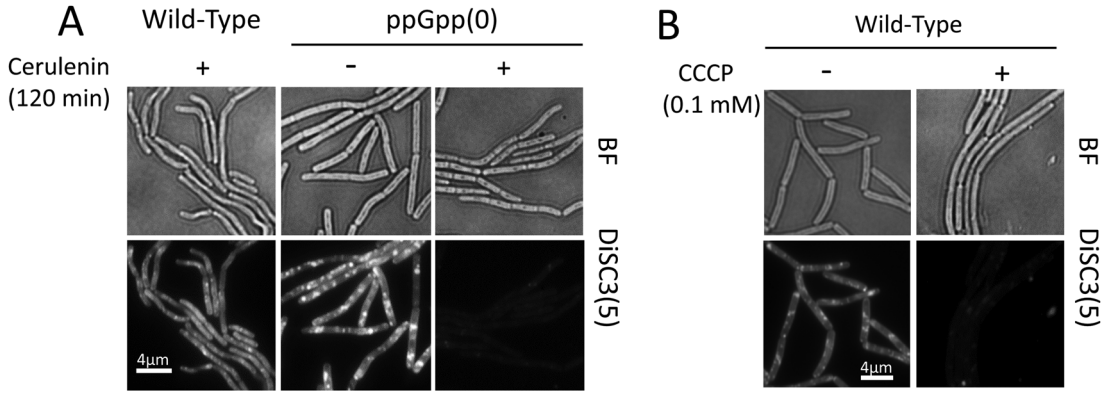

Fig. 5. Fatty acid starvation in the absence of stringent response causes collapse of membrane potential.
A. Wild-type (FG1) and ppGpp(0) (AP6) strains treated with cerulenin for 120 min and stained with the membrane potential dye DiSC3(5), whose intracellular accumulation depends on membrane potential.
B. Control treatment of the wild-type strain with the ionophore CCCP (0.1 mM, 120 min) to dissipate membrane potential. BF, bright field. Images are representative of three independent experiments. BF, bright field.

cerulenin despite exhaustive attempts. We tried different cerulenin concentrations, genetic depletion of fatty acids, different incubation times and phosphate free media (for examples of the results of some of these attempts see Supporting Information Fig. S4A and B). However, TLC experiments revealed pronounced differences in the GTP/ATP ratios of the wild-type and ppGpp(0) strains starved for fatty acids (Fig. 6A and B and Supporting Information Fig. 4C). In the presence of cerulenin, the GTP/ATP ratio of the wild-type strain decreased if compared to the levels observed in the absence of the antibiotic. In contrast, the GTP/ATP ratio of the ppGpp(0) strain increased with cerulenin treatment. In Gram-positive bacteria many of the changes promoted by the stringent response result from a reduction in intracellular GTP rather than being a direct effect of (p)ppGpp on protein targets (Krásný and Gourse, 2004; Krásný *et al*., 2008; Geiger and Wolz, 2014). The observation that the GTP/ATP ratio of the ppGpp(0) strain increased after cerulenin treatment suggested that this strain fails to reduce its intracellular GTP pool, even though our TLC experiments lacked the normalizations necessary to estimate absolute intracellular nucleotide concentrations. To verify if a drop in intracellular GTP was important for the adaptation of *B. subtilis* to lipid starvation, we treated the wild-type and ppGpp(0) strains with cerulenin in the presence of decoyinine, a drug that inhibits the synthesis of GTP by interfering with the activity of GMP synthetase. We observed that decoyinine substantially rescued survival of the ppGpp(0) strain treated with cerulenin, while it had no effect on the wild-type viability (Fig. 6C). We also employed decoyinine supplemented with FAMEs in microscopy experiments and observed that this treatment prevented the loss of membrane potential of SR^- cells exposed to cerulenin (Fig. 6D). This suggests that lowering intracellular GTP plays an important role for cell adaptation and survival of fatty acid starvation in *B. subtilis*. However, since decoyinine rescues the viability of the ppGpp(0) strain only partially (Fig. 6C), the survival of fatty acid starvation likely requires other effects of (p)ppGpp in addition to the lowering of GTP.

Lowering of intracellular GTP also inactivates the CodY repressor and turns on genes related to survival during stationary phase, such as those involved in amino acid biosynthesis, nutrient transport and differentiation (virulence, sporulation) (Geiger and Wolz, 2014). Deleting *codY* in our strains modestly delayed but did not suppress the loss of viability of SR^- cells starved of fatty acids (Supporting Information Fig. S5). This suggests that CodY target genes play little or no role in preserving viability in the absence of fatty acids.

Discussion

Activation of the stringent response by fatty acid starvation is conserved among bacteria

Despite the universality of the stringent response, recent work has shown that the inputs that activate (p)ppGpp production and the outputs produced by the accumulation of this signal may vary among bacteria, reflecting their specific metabolic needs and lifestyles (Boutte and Crosson, 2013; Gaca *et al*., 2015; Liu *et al*., 2015). Here, we showed that activation of the stringent response by fatty acid starvation, previously only reported for Gram-negative bacteria, also occurs in *B. subtilis*, a model Gram-positive bacterium. Whereas

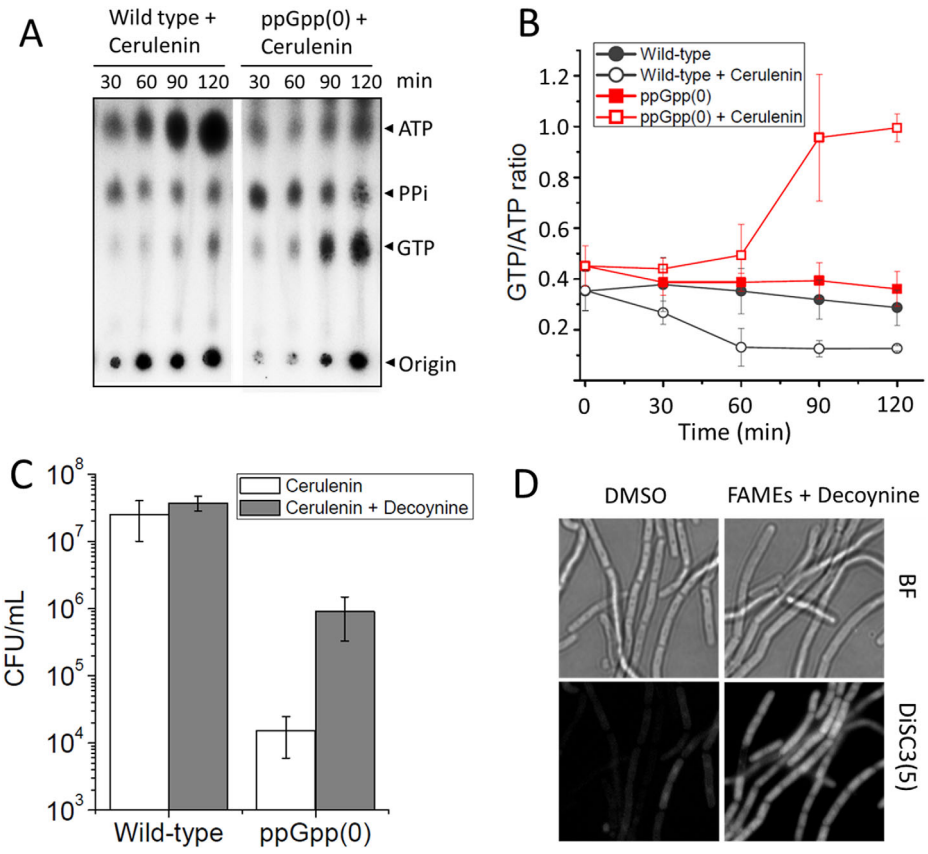

Fig. 6. Fatty acid starvation in the absence of stringent response alters the intracellular GTP/ATP ratio.
A. Thin layer chromatography showing changes of ATP and GTP levels of wild-type (FG1) and the ppGpp(0) (AP6) strains treated with 20ug.mL^{-1} of cerulenin.
B. GTP/ATP ratio of the wild-type and ppGpp(0) strains with or without cerulenin treatment. The error bars indicates the standard deviation between the triplicates.
C. Viability rescue of cerulenin-treated ppGpp(0) by decoyinine (250 μg.ml^{-1}). Decoyinine was added together with cerulenin and viability was assayed after 120 min.
D. Decoyinine and exogenous fatty acids (FAMEs) restore membrane potential to cerulenin-treated ppGpp(0) strain. Cells were treated with 10 μg.ml^{-1} cerulenin for 120 min in the absence, or presence of decoyinine (250 μg.ml^{-1}) plus FAMEs.

wild-type *B. subtilis* stops growing but stays viable when challenged with fatty acid starvation, *B. subtilis* strains incapable of activating the stringent response undergo massive death when faced with the same challenge. Even though we could not detect an increase in (p)ppGpp, survival of fatty acid starvation correlated with a decrease in the intracellular GTP/ATP ratio, a telltale sign of (p)ppGpp effects. We also employed mutant analysis to show that the bifunctional enzyme Rel_{Bs} is the (p)ppGpp synthetase that responds to fatty acid starvation. *In toto*, these data demonstrate that activation of the stringent response is a broadly conserved response of bacteria to fatty acid starvation. In addition, our results contribute to a better understanding of the functional specialization of (p)ppGpp synthetases. In all bacteria where this has been studied, responding to fatty acid starvation depends on a bifunctional RSH, usually referred to as SpoT, although in at least one case (*C. crescentus*) the bifunctional RSH is not a true ortholog of SpoT (Atkinson *et al.*, 2011). One apparent exception is *Vibrio cholerae*, where RelV, a SAS enzyme, was described as being responsive to lipid starvation. This was based on the observation that a mutant lacking $RelA_{VC}$ and $SpoT_{VC}$, where RelV is the only source of (p)ppGpp, was still able to accumulate the alarmone in response to cerulenin treatment (Das *et al.*, 2009). Nevertheless, this does not rule out that *V. cholerae* $SpoT_{VC}$ also responds to fatty acid starvation, and, in fact, the same work reported that fatty acid starvation of a strain that had $SpoT_{VC}$ as the only source of (p)ppGpp also led to accumulation of the alarmone (Das *et al.*, 2009). Taking into account previous work that investigated the response to a number of nutritional stimuli, it seems clear that long bifunctional RSHs are the enzymes that monitor endogenous signals and bring metabolism to a controlled shut down if necessary. This task is subject to some specialization in gamma and beta-proteobacteria, where RelA, a degenerate version of RSH that no longer hydrolyzes (p)ppGpp, is specifically in charge of monitoring amino acid starvation, but this is probably a circumstantial evolutionary solution, as the single long bifunctional RSH enzyme present in most bacteria is equally capable of responding to amino acid, carbon, fatty acid and other nutritional stresses (Boutte and Crosson, 2013; Haury-liuk *et al.*, 2015).

One interesting follow up question to our work is how Rel_{Bs} becomes activated by fatty acid starvation. In *E. coli*, SpoT interacts with unacylated ACP, which accumulates during fatty acid starvation (Battesti and Bouveret, 2006). However, it has been demonstrated that ACP of *B. subtilis*

does not interact with Rel_{Bs} (Battesti and Bouveret, 2009), suggesting that the signal that activates the stringent response is different in these two model organisms. The discovery of such signaling molecule would improve our knowledge of the ways in which the stringent response can be regulated. It remains a possibility that, in addition to protein–protein interactions, metabolites would be able to modulate the activity of RSH enzymes.

Stringent response without detectable accumulation of (p)ppGpp

Despite our observation that cells incapable of activating the stringent response are extremely sensitive to fatty acid starvation, we failed to detect a significant increment of intracellular (p)ppGpp under this condition (Supporting Information Fig. S4). This was surprising, but closer inspection of the literature revealed instances where stimuli expected to trigger the stringent response did not lead to (p)ppGpp accumulation, or cases in which marked phenotypic changes dependent on RSH or SAS enzymes were accompanied by marginal or no rise in (p)ppGpp. For example, *S. aureus* (Greenwood and Gentry, 2002) and *C. crescentus* (Stott *et al*., 2015) subjected to fatty acid starvation failed to generate detectable increases of (p)ppGpp, and glucose deprivation does not always lead to accumulation of (p)ppGpp in *B. subtilis* (Krásný and Gourse, 2004). Furthermore, the slow growth and increased antibiotic tolerance of RSH mutants of *B. subtilis*, *E. faecalis* and *S. aureus* was associated with little or undetectable (p)ppGpp accumulation (Lemos *et al*., 2007; Nanamiya *et al*., 2008; Abranches *et al*., 2009). The inability to detect an increase in (p)ppGpp in our experiments is in all likelihood due to technical difficulties and not because there is no increment. This conclusion is supported by the measurements of the GTP/ATP ratio of cells subjected to starvation, which exhibit the hallmark reduction expected from the effects of (p)ppGpp on GTP synthesis (Lopez *et al*., 1981; Kriel *et al*., 2012), and which does not occur in mutants compromised in the production of (p)ppGpp (Fig. 6A and B). The observation that a Rel_{Bs} null mutant stays viable when challenged with cerulenin also supports the idea that an increase in (p)ppGpp, and not some other effect of Rel_{Bs}, is the key signal to preserve viability. In the absence of Rel_{Bs}, cells should accumulate (p)ppGpp because the synthesis activity of RelP and RelQ would be unopposed. Thus, the examples from the literature and our own results highlight that a small increment of (p)ppGpp is sufficient to cause marked changes in physiology, a fact that has already been pointed out recently by Gaca and collaborators (Gaca *et al*., 2013). Given the technical difficulty associated with detecting small increases of (p)ppGpp, we would like to propose that it is insufficient to use a rise in (p)ppGpp as the sole indicator of an active stringent response. Had we relied on the detection of (p)ppGpp accumulation we may have concluded that fatty acid starvation does not trigger the stringent response in *B. subtilis*. Along the same lines, there are several published observations of bacterial species that failed to accumulate (p)ppGpp in response to starvation stimuli expected to trigger the stringent response [see (Boutte and Crosson, 2011), for a compilation of cases]. These have been interpreted as the natural variation in the way bacteria cope with starvation (Boutte and Crosson, 2011; 2013), but may instead reflect cases in which a small accumulation of (p)ppGpp, sufficient to produce stringent behavior, escaped detection. It would be advisable to revisit some of these studies using new and more sensitive techniques to detect (p)ppGpp, or looking at the GTP/ATP ratio as a surrogate marker of (p)ppGpp increase, before one can safely conclude that a bacterium does not activate the stringent response when faced with a certain stimulus.

How activation of the stringent response preserves viability?

To understand how the stringent response contributes to survival in the absence of fatty acids we carefully characterized how the SR^- cells died. We observed that *B. subtilis* compromised in (p)ppGpp synthesis quickly lost membrane potential on fatty acid starvation (Figs. 4 and 5A), indicating, as expected, perturbation of membrane function. Interestingly, however, at the same stage only a fraction of the cells exhibited evident membrane rupture and became permeable to propidium iodide, indicating that perturbation of membrane function precedes the loss of its physical integrity. A similar observation was made by Stott and collaborators (Stott *et al*., 2015) when studying *C. crescentus* response to fatty acid starvation. However, these authors interpreted the cells that remained impermeable to indicator dyes but failed to give rise to a colony in viability assays as having entered a viable but non-culturable (VBNC) state. Inasmuch as an experimental distinction between being dead or in a VBNC state could be difficult, our time course experiments of Supporting Information Fig. S4 show that essentially all cerulenin-treated cells that once were impermeable to PI go on to become stained with this dye if observed long enough. This indicates that cerulenin-treated cells had lost viability irreversibly before staining with PI. Membrane potential is a major drive for ATP production and nutrient and ion uptake (Allen *et al*., 1991; Silverman *et al*., 2003) and, thus, it

should not be surprising that loss of membrane potential would suffice to irreversibly compromise viability. Because the drop in membrane potential follows the same kinetics as the loss of viability in our experiments, we postulate that this is the primary cause of death of fatty acid starved SR$^-$ *B. subtilis* cells. Further support for the idea that loss of membrane potential suffices to make cells inviable is the study of the mode of action of SDP, the cannibalism toxin of *B. subtilis* (Lamsa *et al.*, 2012). SDP collapses membrane potential and causes a fast drop in viability similar in kinetics and extent to the one observed in our experiments. Importantly, similar to our results, most SDP treated cells remained impermeable to an indicator dye (SYTOX Green) despite having lost viability.

How does fatty acid starvation compromise membrane function in cells that cannot activate the stringent response? One simple possibility is that the absence of (p)ppGpp signaling uncouples lipid and protein synthesis in SR$^-$ cells, which try to continue growing even though they cannot make more membrane. The increment in cell volume in the absence of new membrane synthesis will eventually impose a physical stress on the membrane that should increase its permeability to small molecules and ions, thus disrupting membrane potential. The coupling between lipid and protein synthesis is supported by a previous report (Wille et al., 1975), which shows that the rate of protein synthesis drops after cerulenin treatment of *B. subtilis* cells (50% drop after 20 min of cerulenin treatment). We carried out similar experiments, but this time including a ppGpp(0) strain, and observed that, despite an initial drop in the rate of ^{35}S-labeled amino acid incorporation in both strains (20–30%), there was a tendency of the ppGpp(0) protein synthesis rate to remain higher than the wild-type after 20 min of cerulenin treatment (Supporting Information Fig. S6), as one would expect if lipid and protein synthesis were uncoupled in this strain. The hypothesis that cells die because they fail to shut down growth is also consistent with the cell size measurements of Fig. 2D, which show that SR$^-$ cells become longer than the wild-type after cerulenin treatment (we interpret the shortening of wild-type cells as a consequence of the growth rate reduction imposed by (p)ppGpp), and with the observation that slower growing cultures of SR$^-$ cells are less sensitive to lipid starvation (Supporting Information Fig. S1).

Our model for how the stringent response preserves viability in the absence of lipids is also supported by the alteration in purine nucleotide balance of SR$^-$ cells, which exhibit an increase in their GTP/ATP ratio on fatty acid starvation, instead of the decrease observed in wild-type cells (Fig. 6B). The reduction in intracellular GTP brought about by (p)ppGpp is a key effector of the stringent response in Gram-positive bacteria (Wolz *et al.*, 2010; Kriel *et al.*, 2012; Gaca *et al.*, 2013). It inhibits transcription of rRNA genes (Krásný and Gourse, 2004) and should also affect many steps of translation that rely on proteins that bind and hydrolyze GTP (Milon *et al.*, 2006; Corrigan *et al.*, 2016). Importantly, elegant work from the Wang lab recently showed that resistance to amino acid starvation in *B. subtilis* is an inverse function of the GTP/ATP ratio. They have also shown that growth rates positively correlate with the GTP/ATP ratio and that resistance to starvation is inversely proportional to growth rate (Bittner *et al.*, 2014). Thus, we propose that cells that fail to lower the GTP/ATP ratio on fatty acid starvation will continue to synthesize proteins and grow until their membranes become compromised (Fig. 7). Consistent with this possibility, treating SR$^-$ cells starved for fatty acids with an inhibitor of GTP synthesis (decoyinine) significantly rescued their viability (Fig. 6C).

Even though our results suggest that the main beneficial effect of (p)ppGpp during lipid starvation is shutting down growth, we cannot rule out other contributions of this pleotropic signaling molecule. There are reports that cells impaired in (p)ppGpp synthesis become highly sensitive to redox stress and this may be a relevant factor in lipid starved cells, in which membrane (and presumably the respiratory chain) function is compromised (Gong *et al.*, 2002; Nguyen *et al.*, 2011). (p)ppGpp is also know to regulate the acylation of membrane phospholipids (Heath *et al.*, 1994), which is downstream of fatty acid production. Thus, the possible dysregulation of the acyltransferase enzymes in the absence of their substrates may also have detrimental effects. We expect that further investigation of this system will eventually produce a more detailed picture of how (p)ppGpp preserves the viability of lipid starved cells.

(p)ppGpp as a master coordinator of biosyntheses

Creating a new bacterial cell requires tightly coordinated production of four types of macromolecules: nucleic acids, protein, lipids and polysaccharides (usually PG). This coordination is partially achieved by the intrinsic chemical interdependency of some of the biosynthesis processes. For example, because RNA is required for protein synthesis the inhibition of transcription will quickly halt protein synthesis as well. In contrast, lipids or PG are not required for protein synthesis and, thus, from a biochemical standpoint, their absence should not impact the latter process. Because lack of coordination between the biosynthetic processes can have dire consequences to the cell, as exemplified by

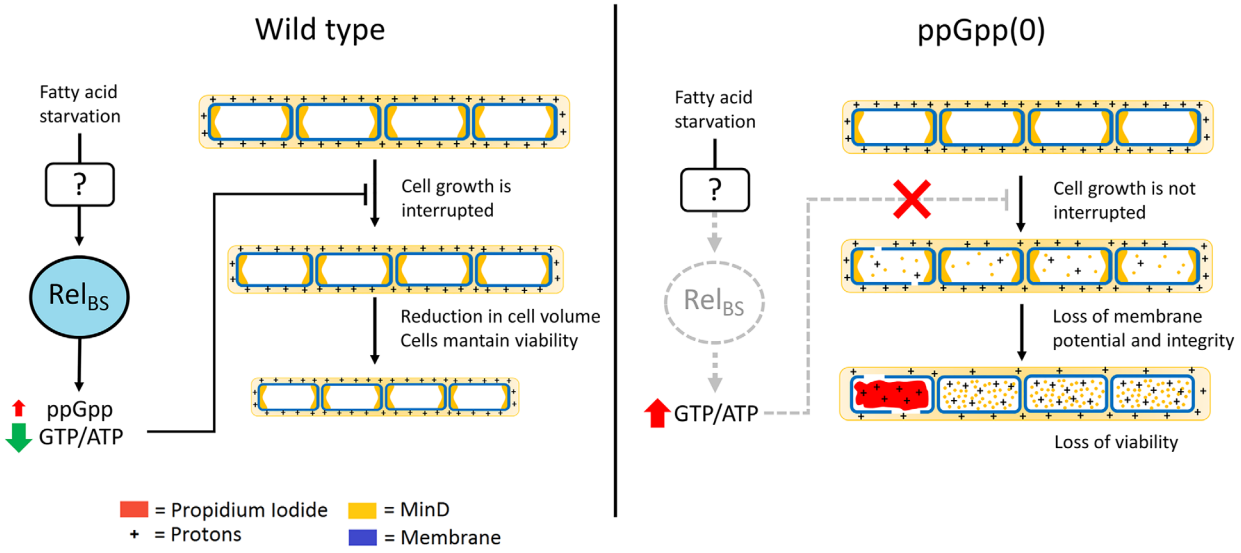

Fig. 7. Model for the protective role of the stringent response during fatty acid starvation. When fatty acid synthesis is inhibited, Rel_{Bs} activity promotes a decrease in the intracellular GTP/ATP ratio, presumably via production of (p)ppGpp. This, in turn, shuts down growth and preserves viability. In contrast, ppGpp(0) cells respond to fatty acid starvation by increasing their GTP/ATP ratio. High GTP/ATP ratios impel ppGpp(0) cells to keep growing in the absence of fatty acids. This will eventually perturb membrane function, leading to the collapse of membrane potential and death.

the dramatic loss of viability of SR$^-$ mutants starved for lipids shown here, a strong selective pressure must have been present for mechanisms to couple processes that are not normally coupled at the biochemical level. We propose that the regulatory effects of (p)ppGpp, and the stringent response itself, evolved in response to such fundamental pressure. Consistent with this idea, it is noteworthy that not every biosynthetic process triggers the stringent response if interrupted. One example is DNA replication, which when blocked still allows cells to keep making RNA, proteins, membrane and PG. Growth is not interrupted because the absence of DNA replication does not immediately impact the making of more cytoplasm, membranes or cell wall. Thus, the stringent response is only invoked by processes which can imperil cell survival if uncoordinated.

Experimental procedures

Strain construction

The strains used in this study are listed in Table 1. All the strains were derived from the wild-type strain PY79. Transformation of *B. subtilis* was performed by the one-step method as described before (Kunst *et al.*, 1994). Antibiotics, when necessary, were used at the following final concentrations: 5 $\mu g.ml^{-1}$ for chloramphenicol, 1 $\mu g.ml^{-1}$ of erythromycin plus 25 $\mu g.ml^{-1}$ of lincomycin for MLS; 100 $\mu g.ml^{-1}$ for spectinomycin; and 10 $\mu g.ml^{-1}$ for tetracycline. Isopropyl-β-D-thiogalactopyranoside (IPTG) and xylose concentrations varied from experiment to experiment and are reported in the text or figure legends. Detailed information about strains constructions can be found at the Supporting Information.

Growth conditions and viability assays

Fatty acid starvation was achieved by growing the cells in LB until a Klett measurement of 40 (O.D.600∼ 0.40). This culture was then used to inoculate a new flask to an initial cell count of 2-5 × 10^6 × ml^{-1} and cerulenin (Cayman chemical – Michigan) was added to 10 $\mu g.ml^{-1}$. Assays performed with strains bearing Rel_{Bs}^{D264G} were always performed with 1 mM of IPTG to induce its expression. For the experiments with the P_{xyl}-*fab*F strains, the pre-inoculum was grown in LB with 0.2% of xylose to the desired density, then 1 ml was taken, washed twice and inoculated in 15 ml of fresh LB without xylose. For the viable cell count, aliquots were taken, serially diluted and plated on LB agar. Plates were then incubated at 37°C overnight for the estimation of CFU/ml. For decoyinine experiments, the drug (Cayman chemical – Michigan) was added at 250 $\mu g.ml^{-1}$ together with the addition of cerulenin and viability was estimated 120 min later. For FAME complementation assays, cultures were prepared by adding BSA (10 $\mu g.ml^{-1}$, final concentration), cerulenin (10 $\mu g.ml^{-1}$ final concentration) and FAME (2.5 $\mu g.ml^{-1}$ final concentration) and viability was estimated after 120 min. All experiments were performed in triplicate.

FAME preparation

Fatty acid methyl esters (FAMEs) were extracted from *B. subtilis* cells, according to a modified procedure

Table 1. *B. subtilis* strains used in this study.

Strain code	Strain	Genotype	Strain sources and reference
FG1	PY79	Wild-type strain	Youngman *et al.* (1983)
FG173	MinD-YFP	*amyE*:: P_{xyl}-*yfp-minD cat*	Sastre *et al.* (2016)
FG1316	Rel_{Bs}^{D264G}	Δ*rel*$_{Bs}$::*erm aprE*::Pspac-}*rel*$_{Bs}^{D264G}$ *spc*	This study.
AP1	P_{xyl}-*fabF*	*fabF*ΩP_{xyl}-*fabF cat*	This study.
AP2	P_{xyl}-*fabF* Rel_{Bs}^{D264G}	Δ*rel*$_{Bs}$::*erm aprE*::Pspac-}*rel*$_{Bs}^{D264G}$ *spc fabF*ΩP_{xyl}-*fabF cat*	This study
AP6	ppGpp(0)	Δ*rel*$_{Bs}$::*erm* Δ*relQ*::*spc* Δ*relP*::*cat*	This study
AP10	Δ*relQ* Δ*relP*	Δ*relQ*::*spc* Δ*relP*::*cat*	This study
AP11	Δ*codY*	Δ*codY*	Rudner lab
AP12	Rel_{Bs}^{D264G} Δ*codY*	Δ*rel*$_{Bs}$::*erm aprE*::Pspac-}*rel*$_{Bs}^{D264G}$ *spc* Δ*codY*	This study
AP15	ppGpp(0) MinD-YFP	Δ*rel*$_{Bs}$::*erm* Δ*relQ*::*spc* Δ*relP*::*tet amyE*::P_{xyl}-*yfp-minD cat*	This study
AP16	Rel_{BS}^{D264G} MinD-YFP	Δ*rel*$_{Bs}$::*erm aprE*::Pspac-}*rel*$_{Bs}^{D264G}$ *spc amyE*::P_{xyl}-*yfp-minD cat*	This study
AP23	Δ*rel*$_{Bs}$	Δ*rel*$_{Bs}$::*erm*	This study

described by (Rogozinski, 1964). A culture of *B. subtilis* PY79 (500 ml, $OD_{600} = 1$) was harvested and free fatty acids were transesterified by heating the cells in 10% sulfuric acid in 2 ml distilled methanol for 2 h at 80°C. The FAMEs formed in the methanol/sulfuric acid solution were extracted with *n*-hexane (0.5 vol) and MilliQ water (0.5 vol) by vortexing followed by centrifugation (5 min at 3000 × *g*) to recover the upper organic phase. The sample was dried under a stream of nitrogen (N_2 gas) and resuspended in 100% ethanol.

Fluorescence microscopy

Microscopy was performed using a Nikon Eclipse TiE microscope equipped with a Plan APO VC Nikon 100X objective (NA = 1.4), a 25-mm SmartShutter and Andor EMCCD i-Xon camera. For fluorescence observations, strains were grown and starved for fatty acids as described above, concentrated 10 fold in fresh LB and treated with 50 μg.ml^{-1} of FM1-43 (Invitrogen™) and 10 μg. ml^{-1} of propidium iodide to evaluate membrane damage and permeability. At least 200 cells were examined for each experimental condition. For cell size estimation at least 200 cells of each strain were measured using ImageJ (http://rsb.info.nih.gov/ij/). For observation of the cell wall, cells were resuspended with 50 μg.ml^{-1} of FM4-64 (Invitrogen™) and 10 μg.ml^{-1} of WGA-Alexa Fluor 350 (Invitrogen™). For MinD localization, cells were grown in LB with 0.5% xylose for MinD-YFP expression until $OD_{600} \sim 0.4$ and then used as inoculum in fatty acid starvation experiments, as described above. All microscopy was performed with cells immobilized on LB 25% (LB diluted four times) agarose pads, solidified with 1.5% (w/vol) agarose.

Membrane potential assay

For the membrane potential assays, cells were grown and starved for fatty acids as described above but using LB 25% (to reduce the autofluorescence from LB) supplemented with 0.2% glucose and 100 mM KCl (Strahl and Hamoen, 2010). Similar results were obtained when growing cells in LB 100%. After 60 or 120 min of cerulenin treatment, the membrane potential indicator dye DiSC3(5) (Invitrogen®) was added at the final concentration of 1 μM, and the flasks remained under agitation at 37°C for additional 10 min, to allow the uptake of the dye by the cells. After that, cells were harvested, concentrated and applied to an LB 25% agarose pad for microscopy.

TLC measurements

A low phosphate medium (LPM), described by Vasantha and Freese (1980), was used. LPM is composed of 50 mM MOPS pH 7, 0.5% glucose, 0.5 mM K_3PO_4, 10 mM $(NH_4)SO_4$, 0.1% glutamate, 0.01% tryptophan, 0.01% phenylalanine, 0.5% casaminoacids, 2 mM $MgCl_2$ and trace elements. Strains were grown overnight in LB at 37°C with shaking and subsequently inoculated in LPM to an initial OD_{600} of 0.12. Cultures were incubated at 37°C with shaking until an $OD_{600} \sim 0.5$ was reached and then diluted to an $OD_{600} = 0.1$. ^{32}P-orthophosphoric acid (8500-9120 Ci.mmole^{-1}; 5 mCi.ml^{-1}; Perkin Elmer) was added to a final concentration of 100 μCi/ml and growth was continued for 2 h. Then, the cultures were diluted again to $OD_{600} = 0.1$ (maintaining the radionuclide concentration) and divided into fractions for the different treatments (see legend of Supporting Information Fig. S4 for details). The extraction and analysis of nucleotides were performed following previously published protocols (Schneider *et al.*, 2003) with minor modifications. Briefly, 20 μl samples of each treated culture were taken at several times, mixed with 4 μl of 12 N ice-cold formic acid and kept on ice for 30 min. Subsequently, samples were centrifuged at 4°C for 15 min at 20.000 *g*. Aliquots of the recovered supernatants, normalized by OD_{600}, were spotted on polyethyleneimine (PEI)-cellulose F thin layer chromatography (TLC, Merck) plates. For optimal separation of ppGpp and pppGpp, plates were developed in 1.5M KH_2PO_4 (pH 3.4). For optimal separation of GTP and ATP, plates were developed in 0.85M KH_2PO_4 (pH 3.4). Plates were exposed to a Storage Phosphor Screen and analyzed using a Molecular Dynamics Typhoon scanner.

Acknowledgements

Supported by grant 1169/2013 Pesquisador Visitante Especial CAPES, Brazil and Fapesp 2014/26528-4 and Fapesp 2014/13411-1 scholarships. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. We thank David Rudner, Harvard Medical School, for strains and plasmids and Jonathan Dworkin, Columbia University, for critical reading of the manuscript. F. M. is a fellow and D. d. M. and D. A. are Career Investigators of CONICET, Argentina.

Author contribution

A.A.P., D.E.S, F.M. and A.C.A. executed experiments. A.A.P., D.E.S, D.A., D.M. and F.J.G-F. designed experiments. A.A.P., D.E.S, D.A., D.M. and F.J.G-F. wrote the manuscript.

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