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ORIGINAL ARTICLE

Glyphosate induces the synthesis of ppGpp

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Abstract

Glyphosate, the most widely used herbicide in both agricultural and urban areas is toxic for plants and for many bacterial species. The mechanism of action of glyphosate is through the inhibition of the EPSP synthase, a key enzyme in the biosynthetic pathway of aromatic amino acids. Here we show that glyphosate induces the stringent response in *Escherichia coli*. Bacteria treated with glyphosate stop growing and accumulate ppGpp. Both growth arrest and ppGpp accumulation are restored to normal levels upon addition of aromatic amino acids. Glyphosate-induced ppGpp accumulation is dependent on the presence of the (p)ppGpp synthetase RelA. However, unlike other cases of amino acid starvation, pppGpp could not be discerned. In a *gppA* background both ppGpp and pppGpp accumulated when exposed to glyphosate. Conversely, the wild-type strain and *gppA* mutant treated with serine hydroxamate accumulated high levels of both ppGpp and pppGpp. Altogether, the data indicate that glyphosate induces amino acid starvation resulting in a moderate accumulation of ppGpp and a reversible stringent response.

Keywords Glyphosate · (p)ppGpp · Amino acid starvation · Stringent response

Introduction

N-(phosphonomethyl) glycine (glyphosate) is a well-known and widely used herbicide that is active against plants and bacteria. Glyphosate inhibits the enzyme enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) that catalyzes the transformation of shikimate-3-phosphate to 5-enolpyruvyl-shikimate-3-phosphate in the pathway that leads to the biosynthesis of aromatic amino acids. Glyphosate binds tightly to the active site of EPSPS preventing the binding of the enzyme substrate phosphoenolpyruvate (Funke et al. 2006). As a result, the organism stops growing due to the lack of aromatic amino acids. Bacteria algae and plants treated with glyphosate have their growth inhibited, which can be reverted by adding aromatic amino acids to the medium (Gresshoff 1979). In bacteria, EPSPS is encoded by *aroA*. There are two classes of AroA, class I, found in *Escherichia coli* that is sensitive to glyphosate, and class II AroA, present in the genus *Agrobacterium* that is glyphosate tolerant (Funke et al. 2006; Hove-Jensen et al. 2014; Sun et al. 2005; Yi et al. 2016). Some bacteria are able to metabolize glyphosate and use it as carbon, nitrogen and phosphorus sources (Hove-Jensen et al. 2014) and for that reason the herbicide is usually readily removed from contaminated soil (Borggaard and Gimsing 2008). Wild-type *E. coli* is unable to metabolize glyphosate and its EPSPS is intolerant to the herbicide (Hove-Jensen et al. 2014).

The nucleotides guanosine tetra and pentaphosphate, collectively known as (p)ppGpp, are master regulators of bacterial growth and accumulate in response to adverse environmental conditions, such as amino acid, fatty acid, carbon, nitrogen and phosphate starvation (Cashel et al. 1996; Hauryliuk et al. 2015; Steinchen and Bange 2016). The stringent response is a metabolic adjustment characterized by a dramatic reduction in the synthesis of stable RNA and ribosomes and general protein inhibition which occurs in response to the accumulation of (p)ppGpp (Traxler et al. 2008). In γ and β Proteobacteria, (p)ppGpp is synthesized by two proteins—RelA and SpoT (Atkinson et al. 2011). SpoT

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is a bi-functional enzyme that displays a strong hydrolase and a weak synthetase activity, due to the presence of two functional domains (Atkinson et al. 2011). Severe perturbations in the amino acid pool result in the accumulation of uncharged tRNAs, which upon binding to the A site of the ribosome, activates the ribosome-bound RelA protein that initiates the synthesis of (p)ppGpp. RelA synthesizes pppGpp from GTP and ppGpp from GDP, releasing AMP as a byproduct (Hauryliuk et al. 2015). Different models were proposed to explain the mechanism of RelA induction and (p)ppGpp synthesis. One of them claims that RelA hops between ribosomes monitoring for the translational state of the bacterium (Wendrich et al. 2002), while another model proposes that (p)ppGpp synthesis occurs only after RelA detaches from the deacylated tRNA-bound ribosome (English et al. 2011). (p)ppGpp influences many cellular functions, such as amino acids, carbohydrate and lipid metabolism, stable RNA synthesis, mRNA elongation, DNA replication and repair and virulence (Cashel et al. 1996; Dalebroux et al. 2010; Kamarthapu et al. 2016). The hallmark of the stringent response is the general inhibition of protein synthesis caused in most part by a strong reduction in ribosomal synthesis. (p)ppGpp also represses the expression of genes associated with cell catabolism and replication (Traxler et al. 2008). On the other hand, some genes are positively affected by (p)ppGpp, including those involved in amino acid biosynthesis (Traxler et al. 2008) and genes related to cell survival and protection, such as *rpoS* that encodes the sigma factor that coordinates the general stress response (Gentry et al. 1993).

In the present study, we investigated the effect of glyphosate on (p)ppGpp in *E. coli*. Glyphosate caused a stringent response evidenced by growth arrest and the accumulation of ppGpp in a RelA-dependent manner. Both (p)ppGpp build-up and growth inhibition were reverted by the addition of aromatic amino acids. Atypically, pppGpp which normally accumulates in wild-type cells undergoing amino acid starvation was unseen, but could be observed in a glyphosate-treated *gppA* mutant.

Material and methods

Bacterial strains and growth conditions

The bacterial strains used in this study were MG1655 (wild-type strain), MG1655 Δ*relA*::Kan (Spira et al. 2008) and MG1655 Δ*gppA*::*cat* (this study). LB/L-agar (Lysogeny broth or agar) was the standard bacterial rich medium (Miller 1992). TGP is a minimal medium containing 0.2% glucose and variable KH_2PO_4 concentrations (Spira and Yagil 1999). Different combinations of amino acids (0.1 mg/ml each) were added as specified in the text. Glyphosate (technical grade) was a kind gift from Monsanto (Brazil). Bacterial growth was followed either by measuring the culture turbidity in a spectrophotometer (OD_{600}), or by CFU counting. In the latter case, the cultures were submitted to serial dilutions in 0.9% NaCl and plated on L-agar.

Construction of the Δ*gppA*::Kan mutant

The Δ*gppA*::*cat* deletion was constructed using the λ-red recombinase system essentially as described (Datsenko and Wanner 2000; Murphy et al. 2000). Hybrid primers corresponding to the 5′-end of *gppA* (gpp-mut-f: gaatgcagccaacacagagacagattgaaggatgaagagt GTGTAGGCTGGAGCTGCTTC) and the 3′-end of *gppA* (gpp-mut-r: ccggaaaagatgcgtcagcatcgcat ccggcacttactcaCATATGAATATCCTCCTTAG) were used to amplify the chloramphenicol resistance gene (*cat*) using plasmid pKD3 (Datsenko and Wanner 2000) as a template and the GoTaq® DNA polymerase kit, as recommended by the manufacturer. The resulting amplicon (50 µl) bears a *cat* gene flanked on each side by 40 bp of *gppA* DNA sequences (lower case letters in oligo sequences). The DNA fragment was purified using the Wizard DNA purification system (Promega) and 1 µl was electroporated into strain KM44, which carries a chromosomal copy of the λ-red genes. The chloramphenicol-resistant recombinants were selected on Cm plates. Δ*gppA*::Cm was then transferred to strain MG1655 by P1 transduction (Miller 1992). The *gppA* deletion was confirmed by PCR using primers gpp-ver-f (TCAAGATGGCAAGAGAATGAATCC)/gpp-ver-r (GATGATCTGATTTGGGAACAGGAG), giving an amplicon of 1273 bp, instead of 1741 bp in the wild-type strain.

(p)ppGpp assay

(p)ppGpp was assayed essentially as described (Spira et al. 2014). Briefly, exponentially growing bacteria ($OD_{600} = 0.1$) were suspended in TGP minimal medium containing 0.2 mM KH_2PO_4 and 100 µCi/ml ^{32}P-NaH_2PO_4 (IPEN/CNEN, São Paulo). Cells were incubated with agitation for 60 min to equilibrate the ^{32}P pool. At this point an aliquot was harvested (time 0) and mixed with half of the volume of 11 M cold formic acid. Glyphosate or serine hydroxamate (SHX) were added and samples taken at different time intervals were processed as above. All samples were frozen and thawed twice at − 80 °C. Next, the cell extracts were centrifuged to precipitate debris and 5 µl of each sample were applied to polyethylenimine cellulose thin-layer chromatography (TLC) plates. The labeled nucleotides were resolved by one-dimensional TLC using 1.5 M KH_2PO_4 as solvent. The TLC plates were exposed to both X-ray films and Phosphor-Imager (molecular dynamics) screens. The amounts of (p)ppGpp on the chromatograms were estimated by measuring the radioactive content in the (p)ppGpp and GTP spots

using the Phosphor-Imager software or with the help of the ImageJ software (Schneider et al. 2012). The levels of ppGpp and pppGpp were calculated from the spots densitometries according to the formula: $(p)ppGpp = \frac{(p)ppGpp}{GTP+(p)ppGpp}$ (Svitil et al. 1993). In some cases, (p)ppGpp was normalized against time zero, by dividing the calculated (p)ppGpp values at specific times over (p)ppGpp at time zero. Unless otherwise noted, (p)ppGpp values represent the mean of at least three assays performed with three independent bacterial cultures.

Statistical analysis

The standard error of the mean was calculated according to the formula $SEM = \frac{SD}{\sqrt{n}}$, where SD is the standard deviation (Cumming et al. 2007).

Results

Glyphosate acts as a competitive inhibitor of the enzyme EPSP synthase involved in the synthesis of aromatic amino acids. We thus asked whether glyphosate-induced starvation of aromatic amino acids would cause the accumulation of (p)ppGpp. To test the effect of glyphosate on growth, bacteria growing exponentially in minimal medium were challenged with 5 or 10 mM glyphosate (Fig. 1). Growth was severely inhibited, more so in the bacterial culture treated with 10 mM glyphosate. When a mix of phenylalanine, tyrosine and tryptophan (100 µg/ml each) was added 1 h later, growth resumed in the glyphosate-treated bacterial cultures, indicating that growth inhibition was indeed caused by aromatic amino acid shortage. A very similar effect of glyphosate on growth was observed when bacteria were harvested and submitted to CFU counting (Fig. S1).

Amino acid starvation induces the stringent response that is characterized by a vigorous accumulation of (p)ppGpp (Cashel et al. 1996). To test whether glyphosate elicits a stringent response, ^{32}P-labeled bacteria were treated with 5 mM glyphosate and assayed for (p)ppGpp (Fig. 2). Following glyphosate addition, the level of ppGpp gradually increased reaching a peak at 30–60 min at which time ppGpp concentration was approximately five times above basal level. At that point, one of the following combinations of amino acids was added: 100 µg/ml of each aromatic amino acid (3 AA); 100 µg/ml of each non-aromatic amino acid (17 AA) or all twenty amino acids (100 µg/ml each) (20 AA). Addition of the three aromatic amino acids caused a strong reduction in ppGpp level, such that at 120 min ppGpp was only 1.4 times above its original level (at time 0 min). Addition of all 20 amino acids completely restored ppGpp basal level. However, when the 17 non-aromatic amino acids were added a small albeit significant reduction in ppGpp concentration was observed (ppGpp was still three times above basal level at 120 min). This suggests that although the bulk of ppGpp induction by glyphosate was caused by aromatic amino acid starvation, glyphosate may marginally affect the metabolism of other amino acids. Altogether, these results show that glyphosate induces ppGpp accumulation in

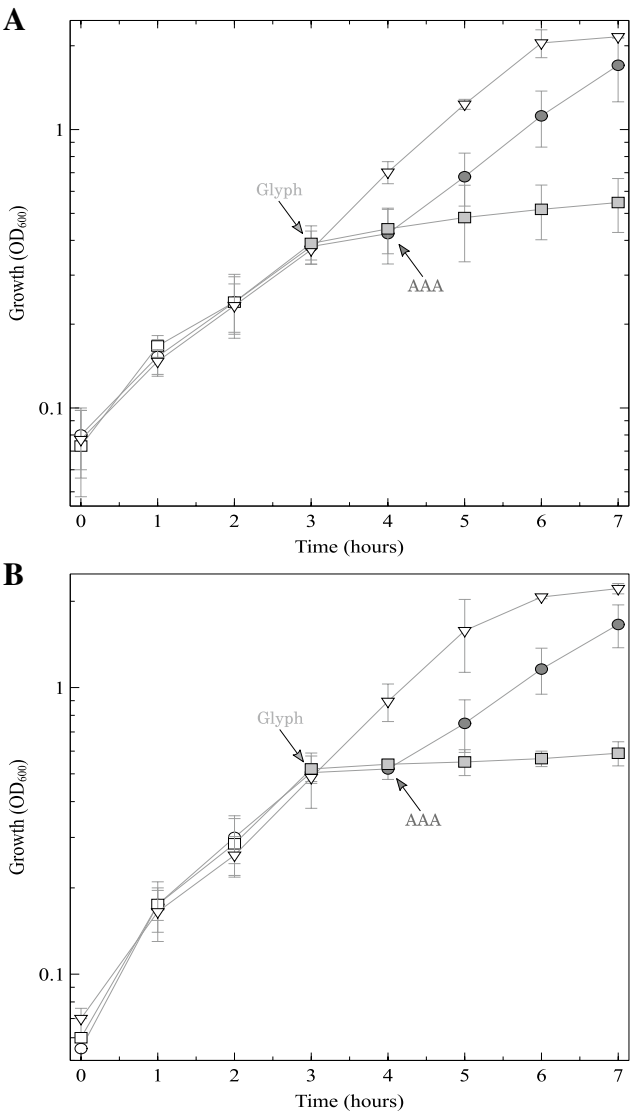


Fig. 1 Effect of glyphosate on growth. Exponentially growing bacteria in TGP minimal medium for 3 h were treated with **a** 5 mM glyphosate or **b** 10 mM glyphosate. 1 h later, one of the cultures was supplemented with a mix of 100 µg/ml of each aromatic amino acid. Unfilled squares and unfilled circles represent time points before the addition of glyphosate. Cultures treated with glyphosate only (filled squares). Cultures treated with glyphosate and aromatic amino acids (filled circles). Cultures that received neither glyphosate nor amino acids (unfilled inverted triangle). Arrows indicate the time at which glyphosate (Glyph) or aromatic amino acids (AAA) were added. Each point represents the mean ± SEM of three independent curves

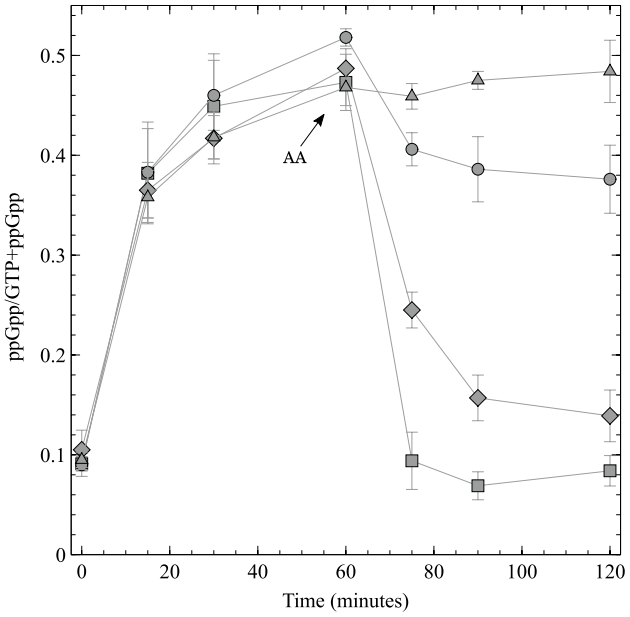
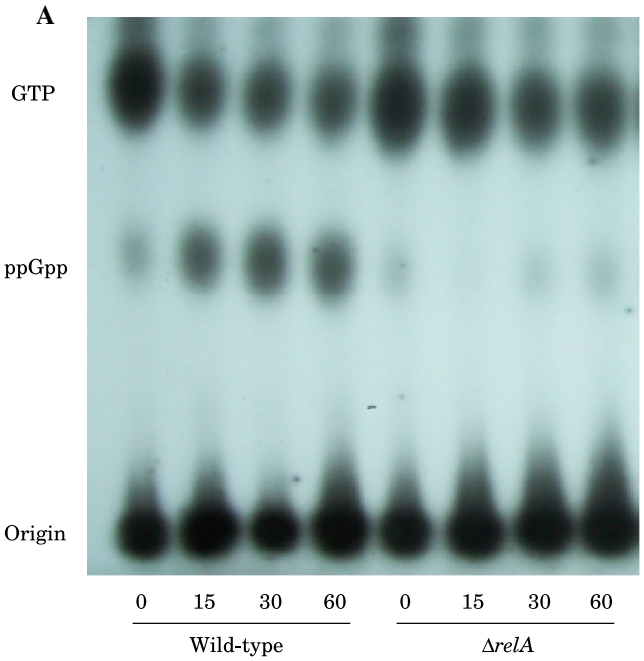


Fig. 2 Effect of glyphosate on (p)ppGpp accumulation. Four separated bacterial cultures were grown in the presence of ^{32}P-KH_2PO_4. At 0 min, 5 mM glyphosate was added to all four cultures. At 60 min, one culture received a mixture containing only aromatic amino acids (filled diamonds), another culture received a mixture containing all 20 amino acids (filled squares) and to the third one a mixture of 17 non-aromatic amino acids was added (filled circles). One culture received no combination of amino acids (filled triangles). Samples were taken for ppGpp analysis at 0, 15, 30, 60, 90 and 120 min. Each point represents the mean ± SEM of three independent curves

response to aromatic amino acid shortage. A dose-response curve showed that the highest concentration of ppGpp was attained in bacteria treated with 7 mM glyphosate (Fig. S2), while 5 mM glyphosate caused an accumulation of ppGpp that is just 30% below the maximal level. 5 mM was chosen as the working concentration of glyphosate in all subsequent experiments. It is worth noticing that the working concentration of glyphosate (Fast action ready to use Roundup) used in weed killing is around 42 mM.

The stringent response provoked by amino acid starvation depends on the *relA* gene product (Cashel et al. 1996). We thus asked whether (p)ppGpp accumulation triggered by glyphosate also depends on *relA*. Wild-type and Δ*relA* bacteria were assayed for (p)ppGpp as above. A well-defined spot corresponding to ppGpp can be observed 15 min after glyphosate addition in the wild-type strain (Fig. 3a). Conversely, the Δ*relA* mutant showed only faint spots of ppGpp, which did not increase upon glyphosate addition, confirming that glyphosate induces (p)ppGpp accumulation via RelA. Quantification of ppGpp accumulation showed that glyphosate increased ppGpp level by around fourfold in the wild-type strain. The effect of glyphosate on growth of the Δ*relA* mutant was also tested (Fig. S3). The growth of the Δ*relA* mutant was inhibited by glyphosate similarly to the wild-type strain, but its recovery upon addition of aromatic amino acids was somewhat slower.



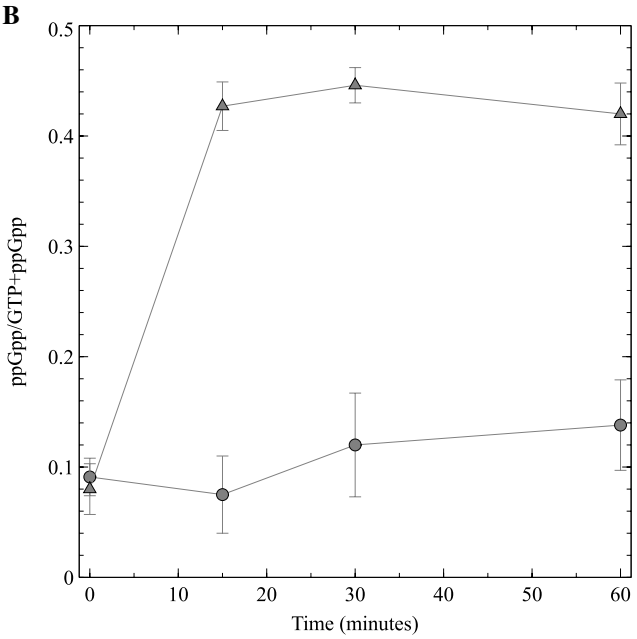


Fig. 3 RelA is essential for glyphosate-induced ppGpp accumulation. **a** Autoradiogram of a representative TLC showing the effect of 5 mM glyphosate on the levels of GTP and ppGpp in the wild-type strain MG1655 and in the Δ*relA*::Kan mutant. **b** Quantification of ppGpp in the wild-type strain (filled triangles) and in the Δ*relA*::Kan mutant (filled circles) treated with 5 mM glyphosate. Samples were taken for ppGpp analysis at 0, 15, 30 and 60 min. Each point represents the mean ± SEM of three independent experiments

Under amino acid starvation, wild-type *E. coli* cells accumulate both ppGpp and pppGpp, the former being typically 2–3 times more abundant than the latter (Gentry et al. 1993). However, under glyphosate-induced amino acid starvation pppGpp could not be observed even though (p)ppGpp accumulation under these conditions is mediated by RelA (Fig. 3). Accumulation of ppGpp in the absence of pppGpp is typically observed in bacteria starved for carbon, phosphate, fatty acids or nitrogen or submitted to other environmental stresses (Battesti and Bouveret 2006; Lazzarini et al. 1971; Mechold et al. 2013; Spira et al. 1995).

The absence of pppGpp in glyphosate-treated cells could be explained by differences in the kinetics of pppGpp synthesis (by RelA) and/or dephosphorylation (by GppA), i.e., if pppGpp dephosphorylation is considerably faster than its synthesis, it would result in far more ppGpp than pppGpp. To test this assumption, MG1655 carrying a *gppA* deletion was treated with glyphosate as above and assayed for (p)ppGpp. Figure 4 shows that in the absence of *gppA* a spot corresponding to pppGpp, less intense than that of ppGpp, was clearly observed. This result suggests that upon glyphosate treatment ppGpp is synthesized via pppGpp, which in turn is immediately hydrolyzed to ppGpp by the action of GppA. In contrast, when the *gppA* mutant was treated with serine hydroxamate (SHX), that induces amino acid starvation by inhibiting the serine tRNA synthetase (Travers 1973), the spot corresponding to pppGpp was stronger than the ppGpp spot. Quantification of the relative pools of pppGpp and ppGpp is depicted in Fig. 4b.

Overall, (p)ppGpp accumulation in the *gppA* mutant was higher in SHX than in glyphosate-treated bacteria. To emphasize the difference between the SHX and glyphosate treatments, the same data was normalized against time zero, i.e., as fold induction (Fig. S4). SHX-induced (p)ppGpp level was 3–4 times higher than when the stringent response was induced by glyphosate. Similarly, wild-type bacteria treated with SHX also displayed 3–4 times more (p)ppGpp than bacteria treated with glyphosate.

Normally, wild-type bacteria accumulate high concentrations of (p)ppGpp in response to 1 mg/ml (8 mM) SHX (Ferenci et al. 2011), while bacteria treated with an equivalent concentration of glyphosate (5 mM) accumulate considerably less ppGpp and no pppGpp at all (Fig. 3). We then asked whether RelA-dependent appearance of pppGpp depends on the severity of the stringent response, i.e., whether pppGpp accumulation requires a strong stringent response as the one induced by 1 mg/ml SHX, while a moderate stringent response such as the one induced by glyphosate does not cause the accumulation of this nucleotide. To test this assumption (p)ppGpp was followed in bacteria treated for 30 min with decreasing concentrations of SHX. Figure 5 shows that a 100-fold lower SHX concentration (0.01 mg/ml SHX) elicited a much milder induction in (p)ppGpp accumulation, but even here ppGpp was still accompanied by

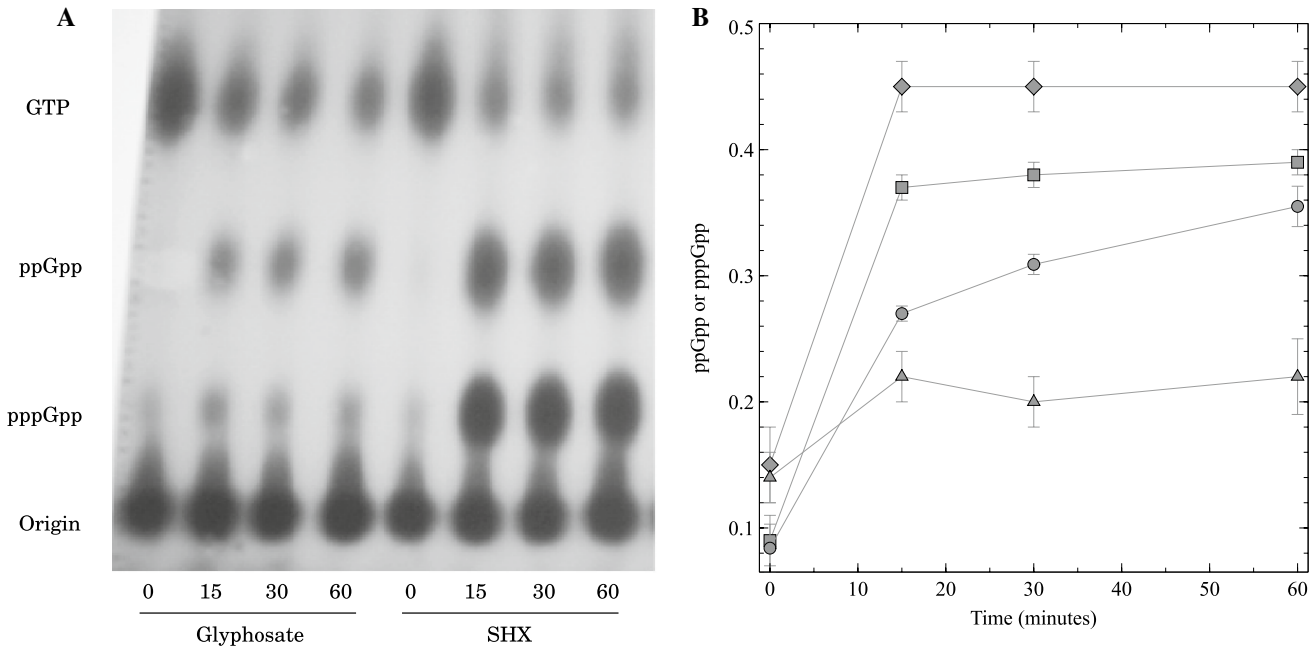


Fig. 4 ppGpp accumulation in the Δ*gppA* mutant. Bacteria were treated with either 5 mM glyphosate or 1 mM SHX. Samples were taken for ppGpp analysis at 0, 15, 30 and 60 min. **a** Autoradiogram of a representative TLC. **b** Values corresponding to pppGpp and ppGpp were normalized by dividing the intensity of each pppGpp and ppGpp spots by the respective GTP + ppGpp + pppGpp pool. Each point corresponds to the mean ± SEM of three independent experiments. (Filled squares) ppGpp in SHX-treated cells, (filled diamonds) pppGpp in SHX-treated cells, (filled circles) ppGpp in glyphosate-treated cells, (filled triangle) pppGpp in glyphosate-treated cells

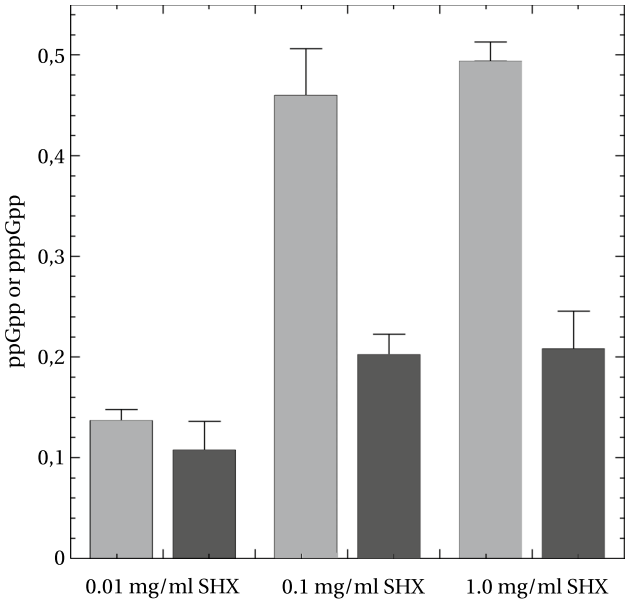


Fig. 5 (p)ppGpp accumulation in wild-type bacteria treated with increasing concentrations of SHX. Bacteria were treated for 30 min with 1.0, 0.1 or 0.01 mg/ml SHX and assayed for pppGpp (dark gray bars) and ppGpp (light gray bars). Each bar corresponds to the mean ± SEM of at least three independent cultures

pppGpp. Similar results were observed at 15 and 60 min following SHX addition and when bacteria were treated with 0.05 or 0.5 mg/ml SHX (Fig. S5). These results indicate that SHX induces the accumulation of both ppGpp and pppGpp, irrespective of the severity of the stringent response. Therefore, the absence of pppGpp in glyphosate-treated bacteria cannot be attributed to the low severity of the stringent response.

Discussion

In most plants and in many bacterial species, glyphosate blocks the biosynthesis of aromatic amino acids by inhibiting the activity of the EPSPS, which ultimately results in cell starvation and death. Depletion of amino acids in bacteria induces the stringent response, which main purpose is to stall rRNA and protein synthesis maintaining thus the bacteria in a state of latency (Cashel et al. 1996). Here we showed that glyphosate evokes the stringent response and the accumulation of ppGpp by inducing aromatic amino acid starvation.

Glyphosate-induced (p)ppGpp accumulation depends on *relA*, as expected in bacteria starved for amino acids. However, induction of amino acid starvation by other means, such as SHX treatment (Fig. 5) or amino acid imbalance (Uzan and Danchin 1978), results in the accumulation of both ppGpp and pppGpp. To the best of our knowledge, there is not a single instance of amino acid starvation in that pppGpp has not been observed. There are several possible explanations for the absence of pppGpp in glyphosate-treated cells. One possibility is that glyphosate induces the synthesis of (p)ppGpp directly from GDP resulting in mostly or exclusively ppGpp. However, the relatively low concentration of GDP in the cell [around 12%, (Varik et al. 2017)] could not account for the rapid increase in (p)ppGpp concentration during the stringent response (Fiil et al. 1977; Kudrin et al. 2018). RelA normally catalyzes the synthesis of pppGpp from GTP, a Pi moiety is then removed by GppA, resulting in higher levels of ppGpp than pppGpp (Cashel et al. 1996; Mechold et al. 2013). Our results showed that in the absence of *gppA*, there was a non-negligible accumulation of pppGpp, which indicates that (p)ppGpp synthesis occurs via pppGpp in glyphosate-treated cells. Thus, the most likely scenario is the one in which glyphosate causes a mild aromatic amino acid starvation, which in turn induces RelA activity. pppGpp is then slowly synthesized from GTP, and very rapidly hydrolyzed to ppGpp (through the activity of GppA), resulting in the accumulation of moderate levels of ppGpp and no pppGpp at all. The residual ppGpp observed in the *gppA* mutant treated with either SHX or glyphosate suggests that the conversion of pppGpp to ppGpp is processed not only by GppA, but also by other pppGpp hydrolytic enzyme(s). In fact, the GTPases IF2, EF-G and EF-Tu and the polyphosphatase PPX have been shown to catalyze the hydrolysis of pppGpp to ppGpp in vitro (Cashel et al. 1996; Keasling et al. 1993).

The accumulation of ppGpp in glyphosate-treated bacteria may have several consequences beyond maintenance of an intracellular balanced amino acids pool and adjustments in bacterial physiology and survival. For instance, (p)ppGpp is implicated in both bacterial resistance and persistence to antibiotics (Greenway and England 1999; Harms et al. 2017; Liu et al. 2017; Wu et al. 2010) and it has been claimed that glyphosate increases bacterial resistance to some antibiotics (Kurenbach et al. 2015). It is thus possible that the uplift in antibiotic resistance observed in glyphosate-treated bacteria is due to the accumulation of (p)ppGpp in these cells.

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Compliance with ethical standards

Human participants or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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