KATIA ALEXANDRA OSPINO BEJARANO

Efeito do glifosato sobre a susceptibilidade a antibióticos em *Escherichia coli*

São Paulo, Brasil

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I dedicate this work to my parents and siblings who have trusted me and supported me in the most difficult moments.

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"Science is not only a discipline of reason, but also of romance and passion."

Stephen Hawking

Resumo

Resistência e tolerância a antibióticos são mecanismos fisiológicos e moleculares desenvolvidos por bactérias para contrapor a ação de antibióticos. Alguns destes mecanismos estão relacionados apenas à resistência, outros à tolerância, e outros, ainda, a ambos os fenômenos. A resposta severa é um processo de controle gênico e fisiológico ativado por diferentes estresses ambientais e tem sido amplamente relacionado à resistência e tolerância a antibióticos, especialmente com esta última. O nucleotídeo (p)ppGpp atua como molécula efetora da resposta severa. Seu acúmulo na célula resulta na interrupção ou diminuição do crescimento bacteriano, principal razão pela qual (p)ppGpp se vincula com o desenvolvimento da tolerância a antibióticos. Glifosato é um herbicida amplamente utilizado na indústria agrícola, que induz o acúmulo de (p)ppGpp sob condições de carência de aminoácidos. Isto ocorre porque o glifosato bloqueia a enzima EPSP sintase, impedindo a síntese de aminoácidos aromáticos na via do shiquimato, presente em plantas e em microorganismos. Com a privação de aminoácidos aromáticos, a resposta severa é ativada, servindo como mecanismo de defesa para garantir a sobrevivência da bactéria. Há relatos na literatura de que o glifosato altera a suscetibilidade a antibióticos em *Escherichia coli* e *Salmonella*. Considerando a relação entre (p)ppGpp e a suscetibilidade a antibióticos, e o fato de que glifosato induz o acúmulo de (p)ppGpp, neste trabalho avaliei o efeito do glifosato sobre a resistência, tolerância e persistência a antibióticos e o possível papel do (p)ppGpp nesta resposta em *E. coli*. Foi constatado que o glifosato não afetou o perfil de resistência aos antibióticos ciprofloxacino, canamicina e ampicilina, uma vez que a concentração inibitória mínima destes antibióticos não foi alterada. Por outro lado, a adição de glifosato intensificou fortemente a tolerância ou persistência a estes três antibióticos na cepa selvagem e, com menor intensidade, no mutante $\Delta relA$ tratado com ciprofloxacino e canamicina, mas não na cepa (p)ppGpp^0 ($\Delta relA\ \Delta spoT$). Sendo assim, concluímos que o aumento da tolerância/persistência causado pelo glifosato é parcialmente dependente de *relA* e completamente dependente de (p)ppGpp.

Palavras-chave: glifosato, resistência, tolerância, persistência, antibióticos, MIC, MDK, *Escherichia coli*, (p)ppGpp

Abstract

Antibiotic resistance and tolerance are physiological and molecular mechanisms developed by bacteria to counteract the action of antibiotics. Several of these mechanisms relate only to resistance, others to tolerance, and still others to both resistance and tolerance. The stringent response is a genetic and physiological control system activated by environmental stresses, and it has been widely linked to antibiotic resistance and tolerance, especially the latter. The nucleotide (p)ppGpp acts as an effector molecule of the stringent response. By accumulating in the cell, it stops or reduces bacterial growth, which is why (p)ppGpp is linked to the development of antibiotic resistance. Glyphosate is a herbicide widely used in the agricultural industry, that induces the accumulation of (p)ppGpp under amino acid starvation conditions. This occurs because glyphosate blocks the enzyme EPSP synthase, preventing the synthesis of aromatic amino acids in the shikimate pathway, present in plants and in some microorganisms. As a result of the deprivation of aromatic amino acids, the stringent response is triggered, ensuring bacteria survival. There are reports in the literature that glyphosate alters antibiotic susceptibility in *Escherichia coli* and in *Salmonella*. Based on the relationship between (p)ppGpp and antibiotic susceptibility and the fact that glyphosate induces the accumulation of (p)ppGpp, in this work I evaluated the effect of glyphosate on antibiotic resistance, tolerance, and persistence and the possible role of (p)ppGpp in this response. Glyphosate did not to affect the minimum inhibitory concentration of the antibiotics ciprofloxacin, kanamycin, and ampicillin. On the other hand, the presence of glyphosate strongly enhanced the tolerance or persistence to these three antibiotics in the wild-type strain and somewhat less so in the $\Delta relA$ mutant treated with ciprofloxacin or kanamycin, but not in the (p)ppGpp^0 strain ($\Delta relA\ \Delta spoT$). Therefore, we conclude that the increase in tolerance/persistence caused by glyphosate is partially dependent on *relA* and completely dependent on (p)ppGpp.

Keywords: glyphosate, resistance, tolerance, persistence, antibiotics, MIC, MDK, *Escherichia coli*, (p)ppGpp

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List of abbreviations and acronyms

(p)ppGpp	guanosine tetraphosphate + guanosine pentaphosphate
ACP	acyl carrier protein
Amp	ampicillin
AMPA	aminomethylphosphonic acid
ANOVA	analysis of variance
bp	base pair
CFU	colony-forming unit
Cip	ciprofloxacin
CLSI	clinical and laboratory standards institute
DNA	deoxyribonucleic acid
ED	effective doses
EHEC	enterohemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GTP	guanosine triphosphate
GDP	guanosine diphosphate
FIC	fractional inhibitory concentration
gly	glyphosate
HGP	Hepes-glucose-phosphate
Kan	kanamycin
LB	lysogeny broth
MDK	minimum duration for killing

MIC	minimum inhibitory concentration
MOPS	3-(N-morpholino)propanesulfonic acid
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
TGP	Tris-glucose-phosphate
TLC	thin layer chromatography
tRNA	transfer RNA
UPEC	uropathogenic *E. coli*

Contents

1 INTRODUCTION

1.1 Antibiotic Resistance

In the pre-antibiotic era, humans commonly succumbed to bacterial diseases. The discovery of penicillin in 1928 and other antibiotics that followed was a game-changer, for the first time a reliable therapy enabled the successful treatment of infectious diseases, leading to the golden era of antibiotics (CLARDY *et al.*, 2010; NICOLAOU; RIGOL, 2018). However, the widespread use of antibiotics in medical and veterinary applications had a side effect, namely the selection and proliferation of resistant bacteria in the community and, more importantly, in clinical settings (ALANIS, 2005), endangering the treatment of infectious diseases and demanding the development of new and/or more potent drugs. Antibiotic-resistant bacteria evade the toxicity caused by antibiotics through several molecular mechanisms, such as target modification, efflux pump, enzyme inactivation, and membrane impermeability, which allow bacterial survival even under high concentrations of antibiotics (BLAIR *et al.*, 2015).

There are two types of antibiotic resistance: intrinsic and acquired. Intrinsic resistance can be defined as particular characteristics that are inherent to the microorganism and that reduce its susceptibility to antibiotics (BLAIR *et al.*, 2015). An example of intrinsic resistance is the outer membrane of Gram-negative bacteria whose low permeability reduces the entry of antibiotics (NIKAIDO, 1994). On the other hand, acquired resistance to antibiotics is caused by mutations in specific genes or by the acquisition of resistance genes via horizontal transfer. For instance, some mutations in *gyrA, gyrB, parC*, or *parE* change the molecular targets of fluoroquinolones, namely DNA gyrase and topoisomerase IV, resulting in resistance against this class of antibiotics (ALANIS, 2005; Van Hoek *et al.*, 2011). In addition, mobile genetic elements, e.g. plasmids, can transfer genes encoding antibiotic-inactivating enzymes, such as acetyltransferases and β-lactamases that inactivate antibiotics as diverse as chloramphenicol and penicillin (Van Hoek *et al.*, 2011).

1.2 Antibiotic Tolerance

In addition to antibiotic resistance, it has been shown that non-resistant bacteria can temporarily evade the action of high concentrations of antibiotics through physiological processes known as tolerance and persistence. Unlike resistance, tolerance and persistence are transient phenomena in which the level of antibiotic lethality depends on the length of exposure and not on the concentration of the antimicrobial drug (BRAUNER *et al.*, 2016).

This phenomenon was described for the first time in 1942 when (HOBBY, 1942) in-

vestigated the mechanism of action of penicillin in Gram-positive bacteria. They showed that only 99% of the population died at a constant rate following the addition of penicillin. The other 1% was eventually killed as well, but at a much slower rate. The biological processes that govern the acquisition of tolerance and/or persistence are influenced by different factors such as genetic background, nutrient availability and other environmental conditions (TUOMANEN *et al.*, 1986a). A physiological factor, such as nutritional deprivation, activates a variety of regulatory processes that elicit growth arrest, resulting in a much slower antibiotic killing rate (TUOMANEN *et al.*, 1986a). On the other hand, several genetic factors, such as mutations in *hipA*, *hipB* and *metG* trigger the stringent response (see subsection 1.4), which inhibits bacterial growth and result in increased tolerance to β-lactams and fluoroquinolones (GIRGIS *et al.*, 2012; BOKINSKY *et al.*, 2013; KASPY *et al.*, 2013a; GERMAIN *et al.*, 2013).

While tolerance is defined as a status through which the entire population is capable of circumventing immediate killing by antibiotics, "persistence" is a condition in which only a small fraction of the population retains this ability (WINDELS *et al.*, 2019a). Therefore, populations bearing persistent bacteria are heterogeneous (KESTER; FORTUNE, 2014) and present a bimodal time-kill curve (BALABAN *et al.*, 2019). To achieve the death of tolerant and persistent bacteria the duration of antibiotic treatment must be longer than usual for sensitive bacteria (HANDWERGER; TOMASZ, 1985).

Recently, it has been proposed that tolerance and/or persistence acquired through mutations in key genes could accelerate the evolution of antibiotic resistance (LEVIN-REISMAN *et al.*, 2017; WINDELS *et al.*, 2019b). This is mainly due to two factors, the first one is based on the ability of tolerant and/or persistent cells to survive in the presence of antibiotics for longer periods, generating a reservoir of cells that may eventually acquire mutations that confer resistance. The other factor is the abnormal high mutation rate of some persistent cells which also increases the probability of acquiring mutations that lead to resistance (LEVIN-REISMAN *et al.*, 2017; WINDELS *et al.*, 2019b). Hence, by developing strategies to combat antibiotic tolerance/persistence we may also contribute to mitigating the problem of antibiotic resistance.

1.3 Methods used to evaluate antibiotic resistance and tolerance/persistence

The golden standard method used to evaluate bacterial susceptibility to antibiotics is the minimum inhibitory concentration (MIC) assay. MIC is defined as the lowest concentration of a drug that is required to prevent the growth of a bacterial culture (ANDREWS,

2001) and provides the information needed to determine whether the tested bacterial strain is sensitive, intermediate, or resistant to a specific antibiotic (RODLOFF *et al.*, 2008). A significant limitation of the MIC assay is that it does not measure tolerance or persistence, once tolerant/persistent bacteria are in a non-growing state (TUOMANEN *et al.*, 1986a). Thus, the MIC assay can be applied to assess antibiotic resistance only (LEVIN-REISMAN *et al.*, 2017).

A suitable method for gauging tolerance is the time-kill curve assay (HANDWERGER; TOMASZ, 1985). One problem with this method is that the interpretation of the time-kill curves is not standardized, making the comparison between different assays a difficult task. Recently, the minimum duration for killing (MDK) was proposed as a standard metric for measuring tolerance (BRAUNER *et al.*, 2016; BALABAN *et al.*, 2019). This method assesses the time it takes to kill a certain proportion of a bacterial population (90%, 99%, 99.99%, and so on) in response to antibiotic treatment and does not depend on the drug concentration. The MDK is normally obtained by calculating the regression of a time-kill curve (Figure 1) (BRAUNER *et al.*, 2017).

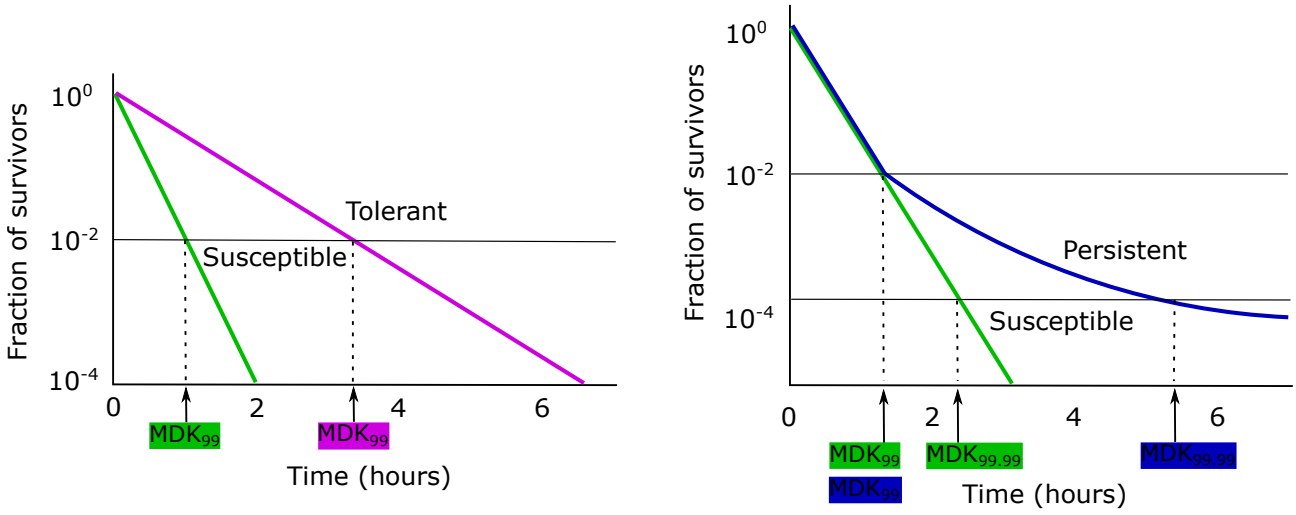

Figure 1 – Time-kill curves and minimum duration for killing. A. Determination of MDK_{99} from time kill-curves. The green and purple lines represent the regression model of hypothetical susceptible (green) and tolerant (magenta) strains, respectively. B. Determination of the $MDK_{99.99}$ from time kill-curves. The green and blue lines represent the regression model of hypothetical susceptible and persistent strains. Note that the MDK_{99} of the susceptible and the persistent strains are identical. Adapted from (BRAUNER *et al.*, 2016)

1.4 The stringent Response and (p)ppGpp

Bacteria inhabit widely diverse and changing environments. They are frequently exposed to a plethora of environmental stresses that end up triggering a variety of cellular signals. These signals activate regulatory processes that allow bacterial survival under such conditions. For example, nutrient starvation activates a regulatory mechanism known as the stringent response, in which bacteria undergo a series of physiological adaptations aiming at alleviating the effects of some nutrient deficiency. Two small molecules modulate these changes: guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) collectively known as (p)ppGpp. Under normal growth conditions, (p)ppGpp concentration in the cell is very low. However, once the cell enters a state of amino acid, carbon, nitrogen, or phosphate shortage (p)ppGpp concentration increases, and the stringent response is activated causing a drastic reduction in cell growth, (CHATTERJI; Kumar Ojha, 2001; POTRYKUS; CASHEL, 2008; MAGNUSSON *et al.*, 2005; SPIRA *et al.*, 1995; HAURYLIUK *et al.*, 2015) avoiding unnecessary energy spending, and increasing bacterial survival (HAURYLIUK *et al.*, 2015; NOWICKI *et al.*, 2016). (p)ppGpp induction and consequently the bacterial response to (p)ppGpp is considerably stronger under amino acid starvation than under any other nutritional stress. Under these conditions, bacterial growth is completely arrested in order to avoid amino acid imbalance that might result in the production of defective proteins and cell death (NOWICKI *et al.*, 2016).

In *E.coli* (p)ppGpp is synthesized by RelA and SpoT. RelA has a strong (p)ppGpp-synthetase activity that is activated under amino acid starvation when deacylated tRNAs accumulate at the ribosomal A-site, whereas SpoT is a bifunctional enzyme displaying a weak synthetase and a strong hydrolase function. SpoT responds to a variety of signals as carbon (XIAO *et al.*, 1991), phosphate (SPIRA *et al.*, 1995), iron (VINELLA *et al.*, 1992) and fatty acids (SEYFZADEH *et al.*, 1993) deprivation by inhibiting its hydrolase activity resulting in the accumulation of ppGpp, but not pppGpp (Figure 2) (SPIRA; OSPINO, 2020; HAURYLIUK *et al.*, 2015). Under unstressed growth, the synthetic activity of SpoT is repressed by the GTPase Obg, while in fatty acid-starved bacteria SpoT is activated by the acyl carrier protein ACP (HAURYLIUK *et al.*, 2015).

(p)ppGpp affects a variety of cellular processes such as transcription of stable RNA, DNA replication, bacterial pathogenicity, metabolism of nucleotides and bacterial persistence (MAGNUSSON *et al.*, 2005). Ultimately, (p)ppGpp controls growth rate by inhibiting the synthesis of stable RNA (rRNA and tRNA) and by blocking the interaction between RNA polymerase and the promoters of many genes resulting in a general inhibition of protein

synthesis. In contrast, (p)ppGpp activates the promoters of genes involved in amino acids biosynthesis (MAGNUSSON *et al.*, 2005; HAURYLIUK *et al.*, 2015; POTRYKUS *et al.*, 2011). (p)ppGpp also downregulates the initiation of DNA replication through several mechanisms: by inhibiting the transcription of *dnaA*, consequently disturbing the initiation of DNA replication at *oriC* (WEGRZYN, 1999), and by altering the expression of DNA gyrase or the binding to the DnaG primase (FERNÁNDEZ-COLL; CASHEL, 2020).

Some virulence factors of pathogenic bacteria are also regulated by (p)ppGpp. For example, in *Salmonella typhimurium* high levels of (p)ppGpp induce the expression of *hilA* that encodes a regulator of genes involved in the bacterial invasion of host cells (POTRYKUS; CASHEL, 2008; NUTT *et al.*, 2003). In enterohemorrhagic *E. coli* (EHEC), (p)ppGpp enhances the ability of this pathogen to colonize the host intestine by stimulating the transcription of the LEE operons. Similarly, (p)ppGpp is required to promote the adhesion of enteropathogenic *E. coli* (EPEC) to epithelial cells (SPIRA *et al.*, 2014). (p)ppGpp also affects the virulence of uropathogenic *E. coli* (UPEC) that invades bladder cells by upregulating the expression of the fimbrial genes *fimB* and *fimA* (SPIRA; OSPINO, 2020). It has been proposed that the pleiotropy of (p)ppGpp is due to the conformational structure of its phosphate moieties. This structure provides (p)ppGpp the elasticity required to allow the

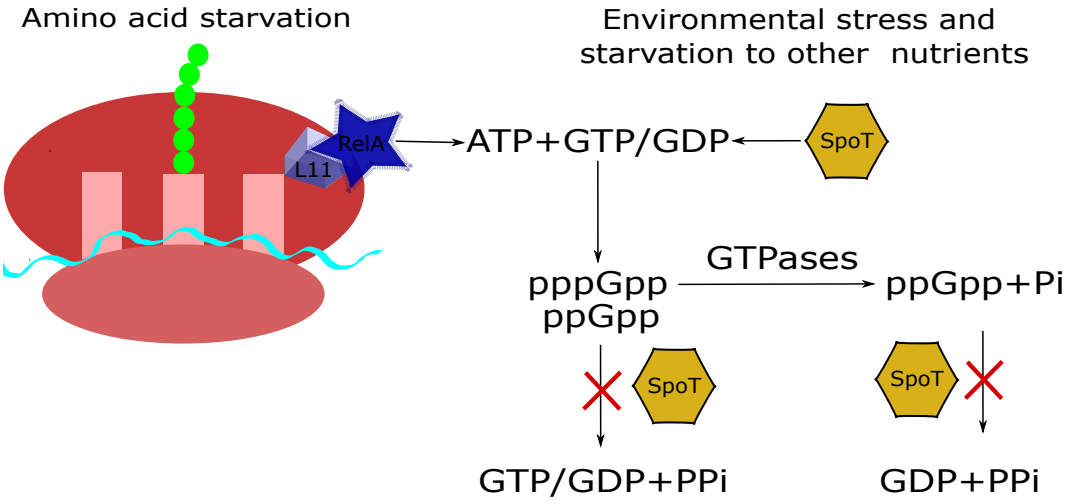

Figure 2 – Metabolism of (p)ppGpp. RelA and SpoT catalyze the synthesis and hydrolysis of (p)ppGpp. RelA forms a complex with the ribosomal protein L11. This complex binds to the A site at the ribosome where it senses the presence of deacylated tRNA. Once RelA is activated the synthesis of (p)ppGpp is initiated. pppGpp is hydrolyzed to ppGpp by the action of GTPases. In contrast, SpoT senses the shortage of several nutrients, other than amino acids, and strongly reduces (p)ppGpp hydrolysis in response. SpoT hydrolyses pppGpp and ppGpp into GTP and GDP, respectively. Adapted from (POTRYKUS; CASHEL, 2008; HAURYLIUK *et al.*, 2015).

interaction of this nucleotide with its many cellular targets (STEINCHEN; BANGE, 2016).

As mentioned above (p)ppGpp acts as a regulator of bacterial growth rate, and that effect is at the core of (p)ppGpp association with antibiotic tolerance and persistence. This strong relationship has been well documented for β-lactams and fluoroquinolones (KUSSER; ISHIGURO, 1985; JOSELEAU-PETIT *et al.*, 1994; KORCH *et al.*, 2003; BOKINSKY *et al.*, 2013; GERMAIN *et al.*, 2013; KASPY *et al.*, 2013b; AMATO *et al.*, 2013; AMATO; BRYNILDSEN, 2015; BROWN, 2019). β-lactam antibiotics act by blocking the assembly of the bacterial wall while fluoroquinolones inhibit DNA gyrase and topoisomerase IV, enzymes required for DNA replication (HOOPER, 2001). Therefore, for an antibiotic to be effective, bacteria must be actively growing. If the cell is at a non-growing state due to high levels of (p)ppGpp the β-lactam or fluoroquinolone is unable to interact with their respective targets and bacteria become tolerant to the antibiotic. Thus, the higher the growth rate the higher the killing rate (BRAUNER *et al.*, 2016). A special case of persistence association with (p)ppGpp concerns the high-persistent gene A - *hipA*, whose expression displays a positive correlation with (p)ppGpp production and formation of persisters cells (KORCH *et al.*, 2003; BOKINSKY *et al.*, 2013; GERMAIN *et al.*, 2013; KASPY *et al.*, 2013b). Some specific mutations in *hipA* confer high persistence in cells exposed to ampicillin. In the absence of (p)ppGpp ($\Delta relA\ \Delta spoT$ double mutant) the *hipA* mutant loses its ability to confer antibiotic persistence, suggesting that the high-persistence phenotype is (p)ppGpp-dependent (KORCH *et al.*, 2003).

1.5 Glyphosate

Glyphosate or N-(phosphonomethyl) glycine is one of the most widely used herbicides in the world. It was discovered in 1950, but its herbicidal properties were recognized only in the 1970s. Glyphosate is used for the control of all types of weeds due to its broad spectrum of action. The development of genetically modified crops capable of tolerating this herbicide (BENBROOK, 2016) brought great economic benefits to the industry and as a consequence, its use increased dramatically resulting in the selection of resistant plants (DUKE, 2015). Glyphosate is a weak organic acid that inhibits the synthesis of aromatic amino acids, by blocking the enzyme 5-enolpyruvylshikimic acid- 3-phosphate synthase (EPSPS). This enzyme catalyzes the production of chorismate, required for the biosynthesis of phenylalanine, tyrosine, and tryptophan in the biosynthetic shikimate pathway (Figure 3), which is present only in plants and some microorganisms (DUKE; POWLES, 2008; ANNETT *et al.*, 2014). Glyphosate is readily degraded by soil bacteria through two different pathways, in one of them glyphosate is hydrolyzed to aminomethylphosphonic acid (AMPA) and glyoxylic acid,

and in the other, it is hydrolyzed to inorganic phosphate and sarcosine (ANNETT *et al.*, 2014).

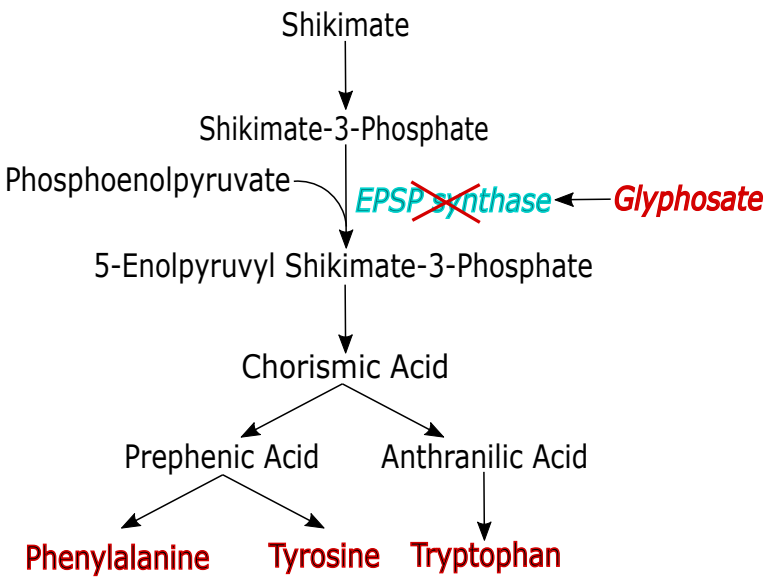

Figure 3 – Glyphosate mode of action. Glyphosate inhibits the activity of the EPSP synthase, thereby affecting the biosynthesis of aromatic amino acids. Adapted from (HAN; FOUNDATION, 2016).

By leaching, run-off, or overspray, residual levels of glyphosate and its degradation products reach aquatic and terrestrial ecosystems potentially affecting the communities that inhabit these ecosystems (SOARES *et al.*, 2019). It has been shown that glyphosate can alter the physiology and behavior of a variety of aquatic organisms depending on the dose and formulation used (ANNETT *et al.*, 2014). In addition, the toxic effects of glyphosate can be enhanced by other components, such as surfactants, present in commercial formulations of this herbicide (ANNETT *et al.*, 2014). Several studies have explored the impact of glyphosate on different microbial communities, sometimes with conflicting results (KEPLER *et al.*, 2020; NIELSEN *et al.*, 2018; ALLEGRINI *et al.*, 2015; MOTTA *et al.*, 2018; AITBALI *et al.*, 2018). These differences can be attributed to several factors, among which are the type of formulation or dose used, time of exposure, environmental conditions of the microbiome (amino acid availability for instance), and microbial composition (KEPLER *et al.*, 2020; NIELSEN *et al.*, 2018). It is known that some microbial species are able to tolerate glyphosate, by carrying an EPSPS version that is intrinsically insensitive to glyphosate (KEPLER *et al.*, 2020; MOTTA *et al.*, 2018). Therefore, it is not surprising that some studies have reported adverse effects of glyphosate on microorganisms (ALLEGRINI *et al.*, 2015; MOTTA *et al.*, 2018; AITBALI *et al.*, 2018) while others have found small or no effect at all (KEPLER *et al.*, 2020; NIELSEN *et al.*, 2018).

The effect of glyphosate on microbial susceptibility to antibiotics has also been reported (KURENBACH *et al.*, 2015). In this study, exposure to Roundup, a commercial formulation of glyphosate, elevated the minimal inhibitory concentration (MIC) of the antibiotics kanamycin and ciprofloxacin in both *E.coli* and *Salmonella Typhimurium* (KURENBACH *et al.*, 2015). In addition, (LIAO *et al.*, 2021) showed that exposure to glyphosate increases the prevalence of antibiotic resistance genes and mobile genetic elements by enriching the presence of these elements in the soil microbiome. Altogether, these studies suggest that glyphosate may indirectly promote the dissemination of antibiotic resistance. Experiments performed in our laboratory showed that glyphosate stimulates the accumulation of (p)ppGpp in *E.coli* in a *relA*-dependent fashion, as expected in bacteria starved for amino acids (CRUVINEL *et al.*, 2019a). (p)ppGpp accumulation occurs because glyphosate inhibits the synthesis of aromatic amino acids (DUKE; POWLES, 2008; ANNETT *et al.*, 2014) which in turn induce the production of high amounts of (p)ppGpp (SCHREIBER *et al.*, 1991). Given the proliferation of antibiotic-resistant bacteria in clinical settings and in the community and the widespread use of glyphosate in Brazil and elsewhere, a thorough assessment of the relationship between glyphosate, and bacterial susceptibility to antimicrobial agents would be of utmost importance. The present study aimed at investigating whether (p)ppGpp plays a role in glyphosate-induced bacterial resistance to antibiotics.

2 OBJECTIVES

2.1 General objective

Investigate whether (p)ppGpp plays a role in glyphosate-induced bacterial resistance/tolerance to antibiotics.

2.2 Specific objectives

- Evaluate the effect of glyphosate on cell viability.
- Determine the MIC of the wild-type strain, the Δ*relA* mutant and the Δ*relA* Δ*spoT* double mutant towards ampicillin, ciprofloxacin, kanamycin and glyphosate.
- Test whether glyphosate affects the MIC of these antibiotics.
- Test whether glyphosate affects bacterial tolerance towards these antibiotics.
- Assess the role of (p)ppGpp on antibiotic susceptibility in *E.coli* under glyphosate exposure.

3 MATERIALS AND METHODS

3.1 Bacterial strains and plasmids

Strains and plasmids used in this work are described in (Table 1.)

Table 1 – Bacterial strains of *E.coli* and plasmids used in this work.

Bacterial strain	**Relevant genotype**	**Reference**
MG1655	*E.coli* wild-type	Laboratory collection
CF1652	MG1655 Δ*relA*::Kan	(XIAO *et al.*, 1991)
NP52	MG1655 Δ*relA*::Kan Δ*spoT*::*cat*	Laboratory collection
BC1260	BW25113 Δ*relA*::*cat*	Laboratory collection
KO1	MG1655 Δ*relA*	This study
KO2	MG1655 Δ*relA* Δ*spoT*::*cat*	This study
Plasmids	**Utilization**	**Reference**
pCP20	pCP20 carries the yeast Flp recombinase gene, *cat*, *bla* and a temperature sensitive replicon	Laboratory collection

3.2 Growth conditions and chemicals

The composition of culture media used in this study are described in (Table 2.)

Table 2 – Composition of culture media.

Culture media	**Composition**
Lysogeny broth (LB)/ Lysogeny Agar (L-agar)	1% (m/v) tryptone extract, 0.5% (m/v) yeast extract, 1% (m/v) NaCl, 1.5% (m/v) agar (only in L-agar) (MILLER *et al.*, 1992)
TGP medium	80 mM NaCl, 20 mM KCl, 20 mM NH_4Cl, 1 mM $MgCl_2 \cdot 6\,H_2O$, 2.5 mM Na_2SO_4, 10 mM $CaCl_2$, 10 mM $FeCl_3$, 10 mM $ZnCl_2$, 0.02% (m/v) Tris, 1 mM KH_2PO_4 and 0.2% glucose; pH 7.5 (TORRIANI *et al.*, 1966)
MOPS medium	10× MOPS mixture, 0.132 M K_2HPO_4 and 0.1% glucose; pH 7.2 (ARBOR; ARBOR, 1974) 10× MOPS mixture is composed by 0.4 M MOPS, 0.04 M tricine, 10 ml of 0.01 M $FeSO_4 \cdot 7\,H_2O$, 1.9 M NH_4Cl, 0.276 M K_2SO_4, 0.02 M $CaCl_2 \cdot 2\,H_2O$, 2.5 M $MgCl_2$, 5 M NaCl, and 0.2 ml of micronutrient stock. Micronutrient stock is composed by 0.14 mM $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 20 mM H_3BO_3, 1.5 mM $CoCl_2$, 0.48 mM $CuSO_4$, 4 mM $MnCl_2$, 0.48 mM $ZnSO_4$

Bacteria stored at -80°C were streaked on L-agar and incubated overnight at 37°C. The plates were kept at 4°C until use, but no longer than one week. Plates streaked with the Δ*relA* Δ*spoT* strain were never stored at 4°C but were always collected freshly. Liquid overnight cultures were grown at 37°C in a rotator shaker at 180 rpm in TGP medium or MOPS minimum medium.

Glyphosate technical grade (94.5%) was a gift by Monsanto, Brazil. The solution was kept at a stock concentration of 80 mM and stored at room temperature. The antibiotics used in this study were the fluoroquinolone ciprofloxacin (2 mg/ml), the aminoglycoside kanamycin (1 mg/ml), and the β-lactam ampicillin (1 mg/ml). Each represents a family of antibiotics that act as bactericidal agents, a required feature for studying antibiotic tolerance (BRAUNER *et al.*, 2016). Stock solutions were stored at -20°C, except for ampicillin that was prepared freshly. All compounds mentioned above were dissolved in milli-Q water and filtered through a 0.2 µm filter.

In experiments that involved the use of ampicillin, bacteria were grown in MOPS minimal medium. In all other cases, TGP was used. The Δ*relA* Δ*spoT* double mutant was grown in minimal medium supplemented with a pool of all 20 amino acids (40 µg/ml each, with the exception of serine whose working concentration was 400 µg/ml) (POTRYKUS *et al.*, 2011).

3.3 Construction of MG1655 Δ*relA*

3.3.1 Transduction of the Δ*relA*::*cat* allele

The Δ*relA*::*cat* allele was transduced to strain MG1655 from the BW25113 Δ*relA*::*cat* strain as detailed below. The donor strain was grown overnight in LB medium. On the next day, the culture was diluted in 10 ml LB medium containing 5 mM $CaCl_2$ and 0.2% glucose (v/v) and cultivated until an OD_{600} = 0.3-0.5. P1 phages were added to the bacterial culture which was further cultivated for 2 hours or until cell lysis was observed. The lysate was centrifuged at 10,000 rpm for 2 minutes and 100 µl chloroform was added to the supernatant. In parallel, an overnight culture of the recipient strain was centrifuged at 5,000 rpm for 10 minutes and the bacteria were resuspended in 3 ml of MC buffer (0.1 M $MgSO_4$; 5 mM $CaCl_2$). 0.1 ml of the culture was mixed with 0.1 ml of the P1 phage lysate and incubated for 20 minutes at 37°C. The transduction product was centrifuged at 10,000 rpm for 3 minutes, resuspended in 100 µL LB medium, and seeded on L-agar medium containing chloramphenicol. The double mutant Δ*relA* Δ*spoT* was also constructed by P1 transduction, as described above. For this purpose, strain NP52 (Δ*spoT*::*cat*) was used as a donor strain and strain MG1655 Δ*relA*

was the recipient. The transduction product was selected on chloramphenicol.

3.3.2 Elimination of the *cat* marker from the *relA*::*cat* allele

The MG1655 Δ*relA*::*cat* transductant was transformed with plasmid pCP20. The plasmid DNA was obtained using a protocol based on the alkaline lysis method (BIMBOIM; DOLY, 1979). Electrocompetent bacteria were prepared as follows: MG1655 Δ*relA*::*cat* bacteria were grown overnight in LB medium at 37°C. On the next day, 1 ml of the culture was transferred to a microtube and incubated on ice for 5 minutes. Then, it was centrifuged for 2 minutes at 10,000 rpm at 4°C and the pellet was resuspended in 1 ml of cold water. The procedure was repeated 2 more times. In the last centrifugation, the pellet was resuspended in 200 µl of cold water. 1-2 µl of plasmid DNA was added to 200 µl of electrocompetent bacteria and the mixture was subjected to electroporation. Then, the bacterial suspension was incubated at 37°C for at least 1 hour and seeded on L-agar plates containing ampicillin. The plates were incubated overnight at 30°C. Transformants were isolated on the next day and grown at 43°C to eliminate the chloramphenicol resistance gene and pCP20, which is temperature-sensitive (DATSENKO; WANNER, 2000). The loss of the resistance gene was confirmed by PCR, using primers relA6F (ATCCACCAGGTCAATCTTCAC) and relA2633R (AGGATATACCATTGCGCGAC). PCR reactions were performed using the GoTaq Kit (Promega) according to the manufacturer's instructions. The PCR product was examined on a 1.5% agarose gel.

3.4 Cell viability assay

The effect of glyphosate on bacterial viability was tested as follows: bacteria were grown overnight in LB medium. On the next day, they were washed twice in saline and the optical density of the culture (DO_{600}) was adjusted to 3.0 – approximately 3×10^9 bacteria/ml. Then, the bacteria were diluted to a final concentration of $3 \cdot 10^3$ cells/ml in one of the following solutions: 10 ml saline (control), 10 ml saline containing 5 mM glyphosate, 10 ml water containing 5 mM glyphosate, or 10 ml saline containing 30 mM Tris and 5 mM glyphosate. The bacterial suspensions were incubated at 37°C under agitation, samples were taken at 0, 5, 15, 30, 60, 120, 180, and 240 minutes and spread on L-agar. The plates were incubated overnight at 37°C. On the next day, the number of CFU was counted and the proportion of bacterial survival was calculated. The assay was performed with 3 biological replicates.

3.5 Determining the effect of glyphosate on antibiotic susceptibility

3.5.1 Minimum Inhibitory Concentration

The MIC was established for the antibiotics ciprofloxacin, kanamycin, and ampicillin and for glyphosate according to the Clinical and Laboratory Standards Institute (CLSI) with some modifications. Serial dilutions of antibiotic and/or glyphosate were added to 96-well plates containing minimum medium. Overnight cultures were diluted 100-fold in TGP or MOPS minimal medium to an approximate OD_{600} of 0.6 ($\sim 6 \cdot 10^8$ bacteria/ml). Bacteria were then diluted 6-fold in minimal medium and 10 µL of this suspension were inoculated onto 96-well plates containing 90µl of minimal medium supplemented with one of the antibiotics or glyphosate to obtain a final concentration of 10^7 bacteria/ml. The plates were incubated at 37°C for 16-18 hours. The MIC was estimated by measuring the absorbance at 600 nm using an EPOCH™ spectrophotometer. The MIC was defined as the minimum concentration of antibiotic needed to inhibit 90% of bacterial growth. Each experiment was performed with 3 biological replicates.

3.5.2 Checkerboard assay

To determine the simultaneous effect of two compounds on bacterial growth a checkerboard assay was conducted (ALANIS *et al.*, 2015). Serial dilutions of antibiotics and glyphosate were applied to a 96-well plate as shown in (Figure 4). Wells were filled with increasing glyphosate dilutions from columns 1 to 11 and increasing antibiotic dilutions from rows A to G. Column 12 and row H were reserved to measure the MIC of each compound alone (RAND *et al.*, 1993). The bacterial inoculum and plate reading were done as described above for the determination of the MIC. The checkerboard was performed with 3 biological replicates.

3.5.3 Time-kill curves

Bacteria were grown overnight and diluted 100-fold in 2 ml of minimal medium. When cultures attained an OD_{600} of 0.5 they were diluted 50-fold in 12-well plates containing one of the following: medium (positive control), medium containing antibiotic (kanamycin, ciprofloxacin, and ampicillin) at a 20X MIC concentration, and medium containing 5 mM glyphosate plus antibiotic at a 20X MIC. Bacteria were pre-exposed for 30 minutes to 5 mM glyphosate before adding the antibiotic. The experiment was carried out in a rotator shaker at 200 rpm and 37°C. Samples were taken at different intervals of time depending on the antibiotic tested and seeded directly on L-agar plates or diluted in 0.9% NaCl and

then plated. CFU were counted on the next day. The assay was performed with at least 3 biological replicates.

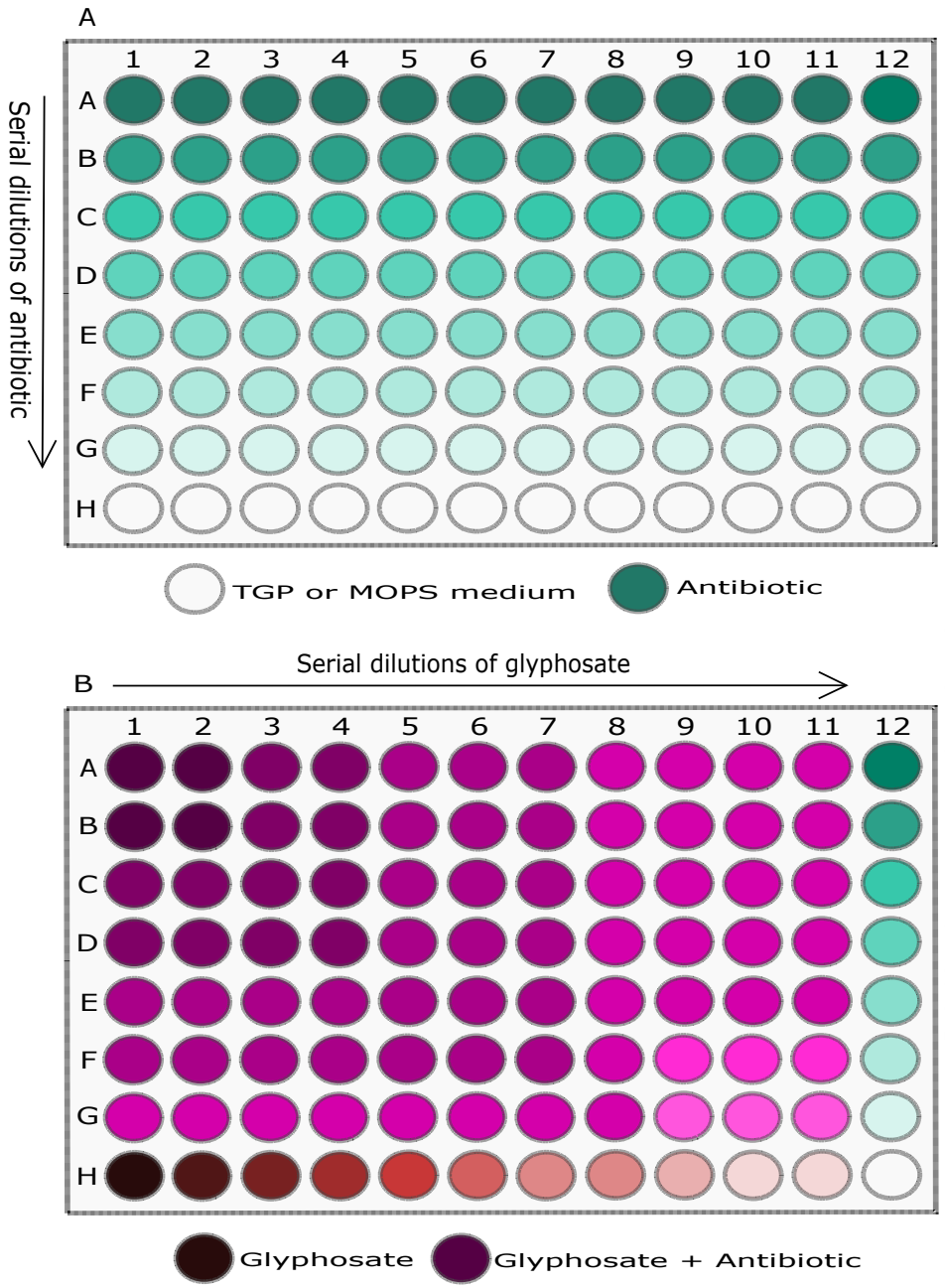

Figure 4 – Checkerboard of antibiotic + glyphosate. The dilutions of glyphosate and antibiotics were performed on the same plate, but are shown here in separate plates for the sake of clarity. Figure A shows serial dilutions of an antibiotic from row A to G as indicated by the arrow. Row H is empty. Figure B shows serial dilutions of glyphosate from columns 1 to 11. Glyphosate dilutions are made on the 96-well plate in which antibiotics dilutions have been previously added. The result is a microplate containing combinations of different concentrations of glyphosate and antibiotics. Row H contains only glyphosate and column 12 contains only antibiotic.

3.6 (p)ppGpp assay

Bacteria were grown overnight in TGP medium. On the next day, the culture was diluted in the same medium to an $OD_{600} = 0.1$ and grown at 37°C until an $OD_{600} = 0.5$. Then, bacteria were washed with 0.9% NaCl, and resuspended in TGP medium containing 0.2 mM KH_2PO_4 and 100 µCi/ml ^{32}P (control), and supplemented with an antibiotic (kanamycin, ciprofloxacin, or ampicillin) at a 20X MIC concentration, 5 mM glyphosate, or 5 mM glyphosate plus antibiotic at a 20X MIC. Cells were incubated at 37°C with agitation for 60 minutes. Aliquots of 20 µl were harvested at times 0' and 30' and mixed with 10 µl 11 M cold formic acid. All samples were frozen at -80°C. On the next day, cell extracts were centrifuged at 13,000 rpm for 10 minutes to precipitate debris and 5 µl of the supernatants 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 pH 3.4 as solvent. The radioactivity emitted by the samples was assessed by laser scanning in a Phosphor-imager (Cyclone-Perkin Elmer). The amounts of (p)ppGpp were estimated by measuring the densities of pppGpp, ppGpp, and GTP spots, using the ImageJ software. The levels of ppGpp and pppGpp were calculated from the spots densities according to the formula: $(p)ppGpp = \frac{(p)ppGpp}{GTP+(p)ppGpp}$

3.7 Statistical analyses

Time-kill curves were analyzed using R. Pairwise t-test was done to evaluate statistically significant differences between treatments in each time interval. The MDK_{99} and $MDK_{99.99}$ were determined from the time-kill curves using the R *drc* package. This package allows the fitting of non-linear regression models for dose-response analysis. Most of the models in the package assume that the response variable is a decreasing function of dose (KNEZEVIC *et al.*, 2007). Parameter estimation is performed with either nonlinear least squares or maximum likelihood estimation based on the assumption that the data have a normal distribution for the former or the assumption that the data have a binomial distribution for the latter (RITZ *et al.*, 2015). The models used in this study were the log-logistic, the Weibull, the fractional polynomial-logistic dose-response, and the exponential decay model. p-values <0.05 were considered significant.

4 RESULTS

4.1 Construction of the Δ*relA* mutant.

The *relA* knockout present in the laboratory's collection carried a kanamycin resistance gene. To test the effect of glyphosate on the resistance/tolerance to this antibiotic in the *relA* mutant, I proceeded to construct a Δ*relA* mutant without a kanamycin resistance gene. The MG1655 Δ*relA*::*cat* transductant was transformed with the pCP20 plasmid, which carries the flippase gene that catalyzes the excision of the resistance marker, leaving a clean *relA* deletion. Figure 5 shows the amplicons obtained by PCR of the *relA* region in the wild-type strain MG1655, in the Δ*relA*::*cat* mutant and in the Δ*relA* clean mutant

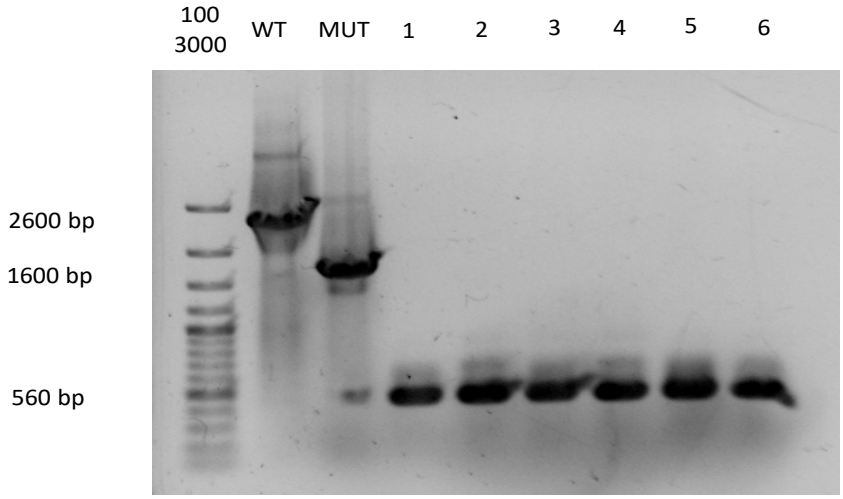

Figure 5 – Confirmation of the excision of the *cat* cassette from *relA*::*cat*. The first column shows the molecular-weight size marker 100-3000 bp; the second column labelled WT shows the amplification of *relA* gene of the wild-type strain (approximately 2600 bp). The third column labeled MUT shows the amplification product of the Δ*relA*::*cat* mutant (approximately 1600 bp). Lanes 1-6 show the amplification product of the clones obtained by curing the *cat* cassette with plasmid pCP20. The resulting fragment contains 560 bp.

To obtain a ppGpp^0 strain, the Δ*spoT*::*cat* allele from strain NP52 was transferred to the MG1655 Δ*relA* strain by P1 transduction, resulting in the double mutant strain MG1655 Δ*relA* Δ*spoT*::*cat*.

4.2 MIC determination of glyphosate, ciprofloxacin, kanamycin, and ampicillin

The MIC of glyphosate, ciprofloxacin, kanamycin, and ampicillin were assessed in the wild-type strain MG1655, in the Δ*relA* mutant and in the Δ*relA* Δ*spoT* double mutant. For the antibiotics ciprofloxacin and kanamycin, assays were performed in TGP medium, while

for ampicillin, MOPS medium was chosen. Although TGP is the standard medium in our laboratory, ampicillin had to be tested in MOPS medium because ampicillin's MIC in bacteria grown in TGP was exceptionally high and displayed great variability (data not shown). This unexpected result may have been due to the presence of Tris in medium TGP. Tris forms a complex with Zinc and catalyses penicillin degradation, possibly through a nucleophilic attack by one of the hydroxyl groups of Tris on the beta-lactam ring (COMPANY *et al.*, 1991). I also tested the MIC of ampicillin in bacteria grown in HGP medium, which contains HEPES instead of Tris, but it also resulted in a high MIC, although not as higher as the one observed in TGP. It turned out that the rate of ampicillin hydrolysis is also high in HEPES-based media (SITE, 2012).

The MICs of the antibiotics and glyphosate are summarized in (Table 3). The MICs of ciprofloxacin and kanamycin are similar in all 3 strains. Conversely, the MIC of ampicillin is four-fold higher in the $\Delta relA\Delta spoT$ mutant. A plausible explanation for this result is that the action of ampicillin is highly dependent on growth rate and since the ppGpp^0 strain grows slower than the other strains it is less affected by this antibiotic (TUOMANEN *et al.*, 1986b).

The MIC of glyphosate in the wild-type strain and in the *relA* mutant were identical (5 µg/ml). However, the $\Delta relA\Delta spoT$::*cat* double mutant showed an 8-fold increase in the MIC. The $\Delta relA\Delta spoT$::*cat* double mutant is unable to grow in the absence of amino acids, thus the higher MIC of glyphosate is probably due to the fact that the medium was supplemented with a pool of amino acids. Once glyphosate inhibits growth by preventing the synthesis of aromatic amino acids, the addition of those amino acids (at a 40 µg/ml) might partially overcome the effect of glyphosate.

Table 3 – MIC of ciprofloxacin, kanamycin, ampicillin, and glyphosate in the wild-type, $\Delta relA$ and $\Delta relA\Delta spoT$ strains

	MIC (µg/mL)		
Antibiotics/Herbicide	MG1655	$\Delta relA$	$\Delta relA\Delta spoT$
Ciprofloxacin	0.04	0.04	0.04
Kanamycin	1-2	1-2	2
Ampicillin	2-4	2-4	8-16
Glyphosate	5	5	40

4.3 Effect of glyphosate on cell viability

The principal aim of this experiment was to find out whether glyphosate has a bactericidal or bacteriostatic effect on cell viability. This has been done because, in previous experiments performed in our laboratory, it has been shown that bacteria suspended in saline rapidly lost viability when exposed to glyphosate, while in culture media the effect of glyphosate seemed to be bacteriostatic (CRUVINEL, 2019). We hypothesized that this could be related to differences in pH in each situation. Furthermore, it is known that in the absence of (p)ppGpp, stresses or compounds that induce the stringent response are bactericidal, while in wild-type bacteria they are only bacteriostatic (GENET, 1994; KRIEL *et al.*, 2012; PULSCHEN *et al.*, 2017). We, therefore, asked whether, in the Δ*relA* mutant, that does not accumulate (p)ppGpp in response to glyphosate (CRUVINEL *et al.*, 2019b), the herbicide might have a bactericidal effect. To test this possibility, the wild-type strain and the Δ*relA* mutant were suspended in buffered or not-buffered solutions with different pHs and assayed for cell viability.

Figure 6 shows that exposure of wild-type bacteria to 5 mM glyphosate diluted in either 0.9% NaCl or water resulted in the death of 99.9% of cells at 30 and 180 minutes, respectively. The Δ*relA* mutant was slightly less sensitive than the wild-type strain, as it took 60 and 240 minutes to kill 99.9% of the cells. In contrast, when glyphosate was diluted in Tris buffer (pH 7), both wild-type and Δ*relA* cells remained viable throughout the entire experiment. These results indicate that bacteria death in saline and water in combination with glyphosate was due to the acidic pH of glyphosate, which when in pure solution is around 2.0 (the measured pH of both bacterial suspensions was about 3.0). We can thus conclude that the growth arrest elicited by glyphosate reported by (CRUVINEL, 2019) was caused by a bacteriostatic effect of glyphosate and that the strong bactericidal effect of unbuffered glyphosate was caused by acid stress. Additionally, bacteria death in unbuffered glyphosate was unrelated to the presence of an intact *relA* gene.

4.4 Interaction of ciprofloxacin, kanamycin, and ampicillin with glyphosate and its effect on antibiotic resistance

Kurenbach *et al.* (2015) reported that glyphosate increases *E. coli* susceptibility to ciprofloxacin and kanamycin. We therefore asked whether this effect is reproducible and if yes, whether it is associated with the induction of the stringent response and accumulation of (p)ppGpp. As a first step, a checkerboard assay with ciprofloxacin, kanamycin, and ampicillin, each one in combination with glyphosate, was conducted. This type of assay reveals

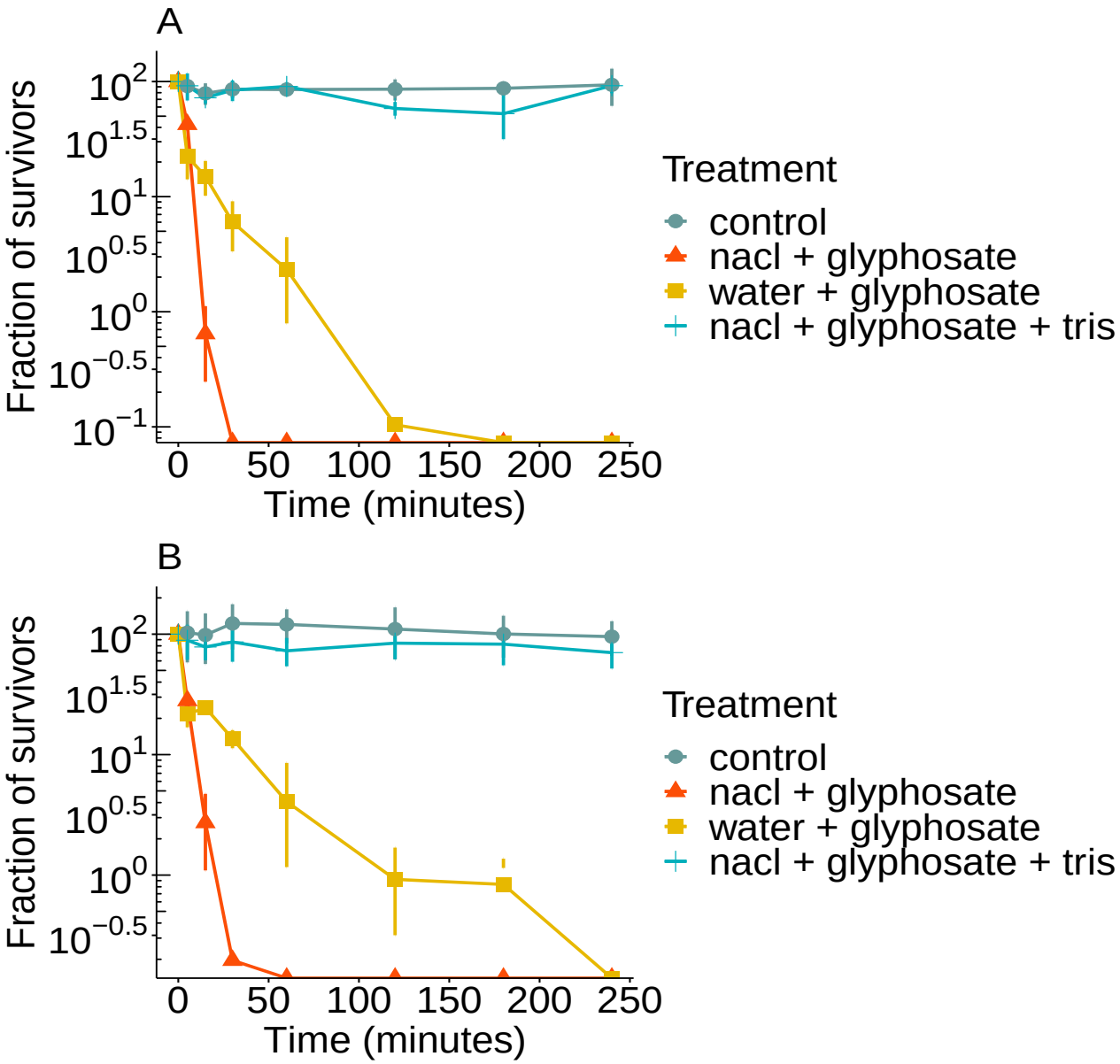

Figure 6 – Effect of glyphosate on cell viability. $3 \cdot 10^3$ cells/ml exponentially grown bacteria were exposed to 5 mM glyphosate suspended in different solutions: 0.9% NaCl, water or 0.9% NaCl + 30 mM Tris (pH 7) up to 4 hours at 37°C. Bacteria suspended in 0.9% NaCl were used as a control. The fraction of survivors was determined by L-agar plating and colony counting. (A) *E.coli* MG1655 wild-type strain; (B) MG1655 $\Delta relA$.

whether glyphosate interacts with the antibiotics and if the interaction is synergistic or antagonistic. The results were calculated using the Fractional Inhibitory Concentration (FIC) index: $FIC_{index} = FIC_A + FIC_B$ where $FIC_A = \frac{MIC_{A+Gly}}{MIC_A}$ and $FIC_B = \frac{MIC_{B+Gly}}{MIC_B}$ (SOPIRALA *et al.*, 2010; BONAPACE *et al.*, 2000) where MIC_{A+Gly} and MIC_{B+Gly} are the MIC of each antibiotic in combination with glyphosate, while MIC_A and MIC_B correspond to the MIC of each compound individually. The FIC_{index} value determines whether the interaction between glyphosate and the antibiotics is synergistic ($FIC \leq 0.5$), antagonistic ($FIC \geq 4$), or indifferent ($FIC > 0.5$ and < 4) (Figure 7).

The FIC_{index} was determined only for the wild-type strain and resulted in each combination (ciprofloxacin+glyphosate; kanamycin+glyphosate; and ampicillin+glyphosate) in $FIC_{index} = 2$, which is interpreted as an indifferent interaction as shown in (Table 4). These results indicate that, contrary to what has been reported by Kurenbach *et al.* (2015), glyphosate did not have any effect on the MIC of these antibiotics, and therefore did not affect the resistance profile of *E. coli*.

Table 4 – Checkerboard results

Combinations	FIC_{index}	Interaction
Ciprofloxacin + Glyphosate	2	Indifferent
Kanamycin + Glyphosate	2	Indifferent
Ampicillin + Glyphosate	2	Indifferent

4.5 The effect of (p)ppGpp on the viability of bacteria treated with antibiotics in the presence of glyphosate

The MDK (Minimal duration for killing) is a metric that enables the quantification of bacterial tolerance or persistence from time-kill curves (FRIDMAN *et al.*, 2014). Once the results of the checkerboard showed no alteration in bacterial resistance to antibiotics, we proceeded to determine whether the combination of antibiotics with glyphosate could alter the pattern of tolerance and/or persistence in *E. coli* cells.

I conducted time-kill curves for the wild-type strain, the Δ*relA* mutant, and the Δ*relA*Δ*spoT* double mutant. Each strain was evaluated independently for each of these antibiotics: ciprofloxacin, kanamycin, and ampicillin in the presence or absence of glyphosate. The MDK_{99} and the $MDK_{99.99}$ (minimum duration of time necessary to kill 99% and 99.99% of the population, respectively) were calculated from the data obtained in the time-kill curves. The MDK of the antibiotic+glyphosate treatment was compared to that of the antibiotic

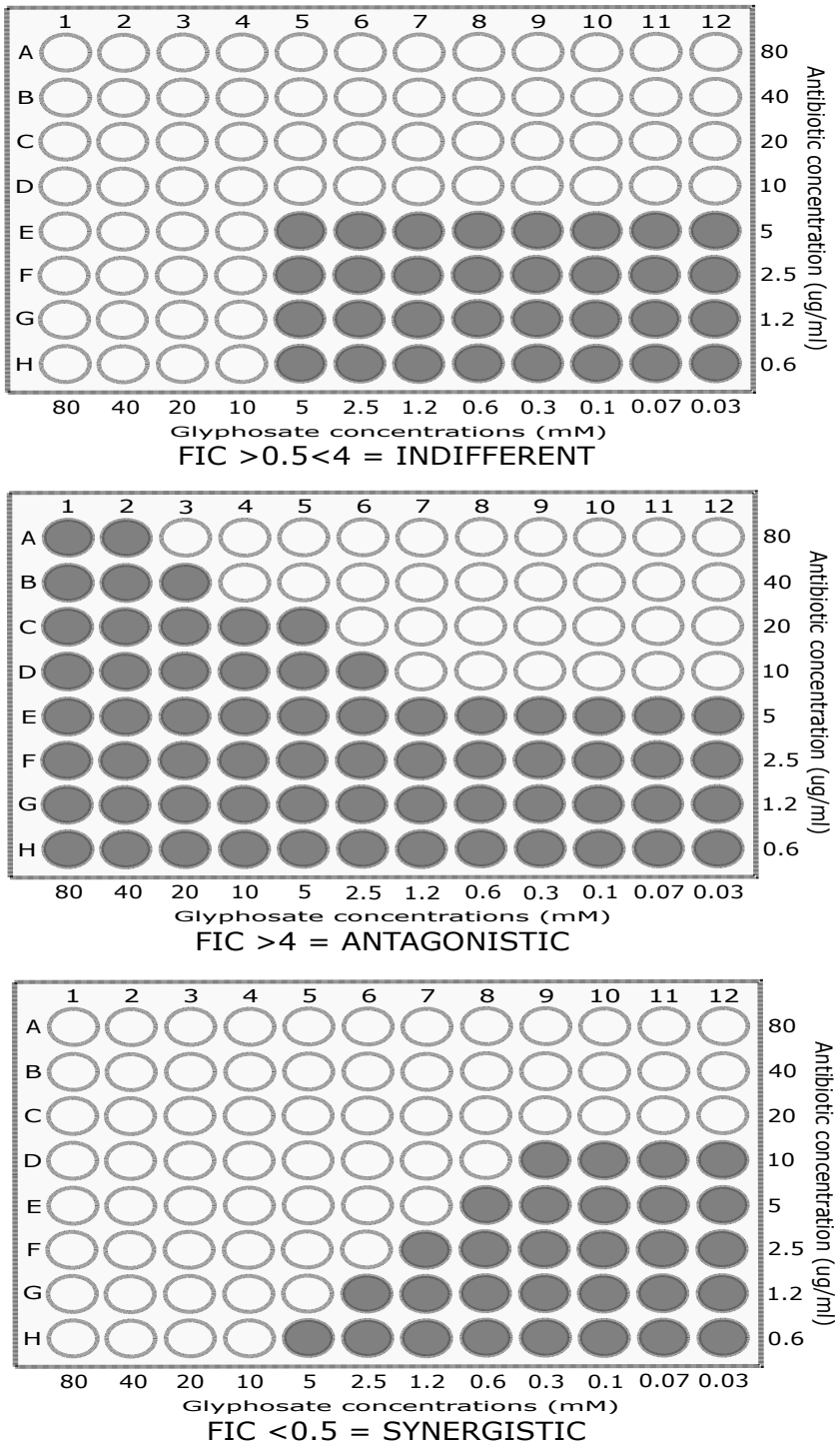

Figure 7 – Interpretation of the checkerboard assay. A. $FIC_{index} \geq 0.5$ and < 4 is interpreted as indifferent (the two different compounds do not interact). Visually, the formation of a staircase is not observed. B. $FIC_{index} \geq 4$ is interpreted as antagonistic (the combination has a negative interaction), the formation of a growing staircase towards the left part of the plate is observed. C. $FIC_{index} < 0.5$ is interpreted as synergistic (the combination has a positive interaction), the formation of a decreasing staircase is observed. Adapted from (WAMBAUGH; BROWN, 2018).

alone. An increase in the MDK indicates that the treatment (glyphosate) positively affected bacteria tolerance and/or persistence.

The time-kill curves corresponding to the antibiotic ciprofloxacin are shown in Figure 8. Because the Δ*relA* Δ*spoT* double mutant displays a considerably higher susceptibility to these antibiotics the duration of the curve in this strain was shorten to 24 hours instead of the 72 hours normally used for the wild-type strain and for the Δ*relA* mutant. Indeed, it is known that the Δ*relA* Δ*spoT* mutant is more sensitive to antibiotics and stresses, which suggests that (p)ppGpp is needed to uphold bacterial intrinsic resistance to environmental adversities (NGUYEN *et al.*, 2011; GREENWAY; ENGLAND, 1999).

Figure 8A and Figure 8B show that glyphosate reduces and delays cell death caused by the antibiotic both in the wild-type and Δ*relA* mutant strains with statistically significant differences at all measured time intervals. The reduction in the killing rate of these strains after 5 hours generates a bimodal curve, which is typical of bacterial cultures that carry persistent cells. Thus, the curves indicate that glyphosate increased both tolerance (as evidenced by the slower killing rate in the first 5 hours) and persistence (bimodal and slower cell death from 5 hours onward). Accordingly, both MDK_{99} and $MDK_{99,99}$ were higher in glyphosate-treated bacteria (Table 5). In the wild-type strain, the addition of glyphosate raised the MDK_{99} and $MDK_{99,99}$ by 4.15 and 11.4 times, respectively. Similarly, in the Δ*relA* strain, glyphosate increased the MDK_{99} by 3.9 times and the $MDK_{99,99}$ by 6.70 times. Altogether these results suggest that: (1) glyphosate antagonizes the killing caused by ciprofloxacin in both strains by increasing tolerance and persistence, and (2) the fact that the protection conferred by glyphosate was slightly higher in the wild-type strain than in the Δ*relA* mutant, suggests that the effect of glyphosate is partially dependent on *relA*.

In the Δ*relA* Δ*spoT* double mutant, a similar killing rate in both treatments (ciprofloxacin and ciprofloxacin+glyphosate) with no statistically significant differences can be observed. As a consequence, there was no alteration in the MDKs of this strain upon the addition of glyphosate (Table 5). This result suggests that the protective effect of glyphosate is thoroughly dependent on the presence of (p)ppGpp.

The time-kill curves with kanamycin are shown in (Figure 9). In the wild-type and Δ*relA* strains the experiment was conducted for 5 hours, while in the Δ*relA*Δ*spoT* double mutant one hour was sufficient to cause the death of almost all cells. Glyphosate delayed the death of all three strains, however, this protective effect was more pronounced in the wild-type strain than in the two (p)ppGpp mutants. The time-kill curves in the kanamycin+glyphosate treatment display a bimodal shape in all strains, but the biphasic behavior was only achieved

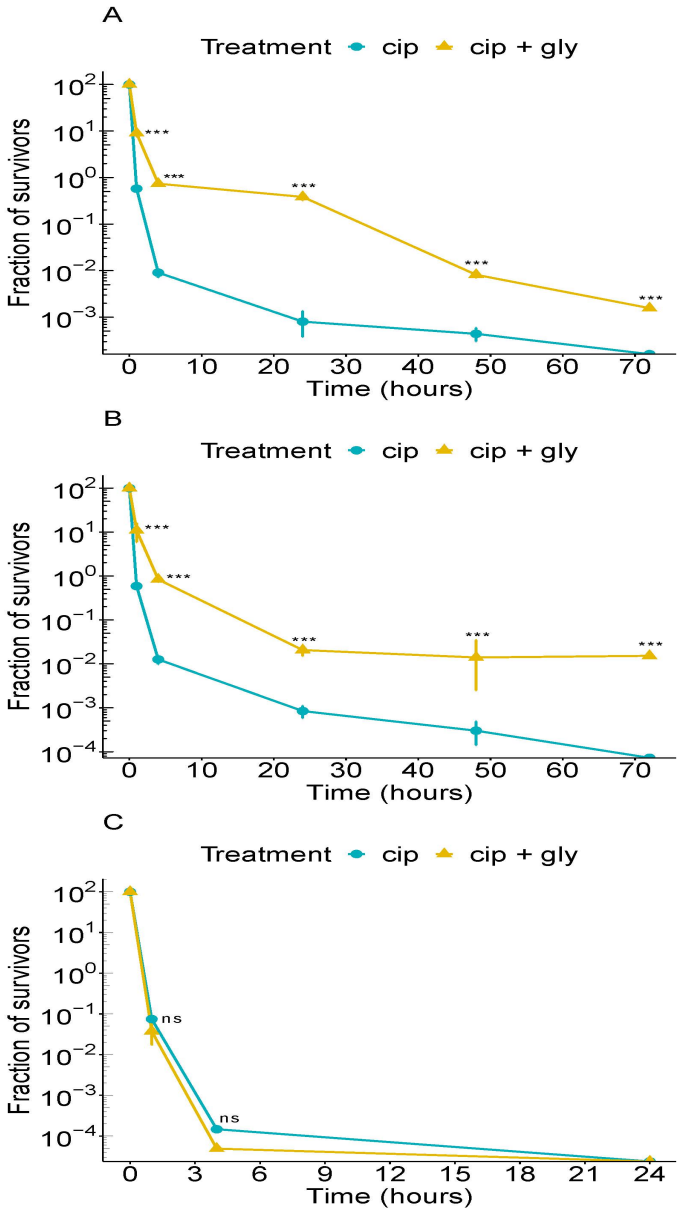

Figure 8 – Time-kill curves of bacteria growing in the presence of ciprofloxacin and ciprofloxacin + glyphosate. The wild-type strain (A) and the Δ*relA* mutant (B) were grown overnight in TGP minimal medium, while the Δ*relA* Δ*spoT* double mutant (C) was grown in TGP supplemented with amino acids (as detailed in section 3.5.3). On the next day, the overnight cultures were diluted and grown in the same medium to an OD_{600} of 0.5 and then diluted to approximately 10^7 bacteria/ml. The test group was exposed to 5 mM glyphosate for thirty minutes and immediately afterwards ciprofloxacin at a 20X MIC was added to the cip (ciprofloxacin only) and cip+gly (ciprofloxacin + glyphosate) groups. CFUs were measured at time zero (just prior to treatment) and at the times indicated in the plots. Data points correspond to the mean of at least three independent experiments with error bars representing the standard deviation of the mean. The fraction of survivors was determined by colony counting on L-agar plates. *p*-values were calculated using a pairwise *t*-test between the antibiotic and antibiotic+glyphosate treatment at each time interval. *** indicates $p \leq 0.001$; ** indicates $p \leq 0.01$; * indicates $p \leq 0.05$; *ns*, not significant.

Table 5 – MDK_{99} and $MDK_{99.99}$ of the antibiotic ciprofloxacin (Cip) in the presence of glyphosate (Gly) in the wild-type strain, $\Delta relA$, and $\Delta relA\Delta spoT$ mutants. The MDK was measured in hours.

	Wild-Type		$\Delta relA$		$\Delta relA\Delta spoT$	
Treatment	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$
Cip	0.83	3.76	0.83	4.29	0.64	1.28
Cip+Gly	3.46	42.83	3.20	28.77	0.58	1.17
Cip+Gly/Cip	4.15	11.38	3.87	6.70	0.91	0.91

after a 4-log reduction in viability. Therefore, we considered that glyphosate conferred an increase in tolerance (but not persistence) towards kanamycin, as per (BALABAN *et al.*, 2019) who defined tolerance and persistence as the time required to achieve a 2-log and 4-log reduction in viability, respectively. The fraction of the population causing the bimodal curve corresponds to a very low proportion of cells, namely less than 0.01% of the population.

The MDK_{99} increased 4.2 times in the wild-type strain and 2.6 times in the $\Delta relA$ strain treated with glyphosate (Table 6), while the $MDK_{99.99}$ went 2.9 and 2.1 times up in the presence of glyphosate. Similar to what has been observed for ciprofloxacin-treated bacteria, the protective effect of glyphosate against kanamycin was slightly higher in the wild-type strain than in the $\Delta relA$ mutant, suggesting that this effect is partially dependent on *relA*. Addition of glyphosate to the kanamycin-treated $\Delta relA\Delta spoT$ double mutant elevated the MDKs by a statistically significant rate, but the magnitude of the effect ($<$ 2-fold) was too small to be regarded as meaningful. These results suggest that the effect of glyphosate on bacterial tolerance towards kanamycin is strongly dependent on (p)ppGpp.

Table 6 – MDK_{99} and $MDK_{99.99}$ of the antibiotic kanamycin (Kan) in the presence of glyphosate (Gly) in the wild-type strain, $\Delta relA$, and $\Delta relA\Delta spoT$ mutants. The MDK was measured in hours.

	Wild-Type		$\Delta relA$		$\Delta relA\Delta spoT$	
Treatment	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$
Kan	0.57	1.13	0.56	1.13	0.35	0.70
Kan+Gly	2.40	3.23	1.48	2.33	0.40	0.80
Kan+Gly/Kan	4.21	2.86	2.61	2.06	1.14	1.14

Lastly, the time-kill curves corresponding to the antibiotic ampicillin are shown in (Figure 10). In the wild-type and $\Delta relA$ strains the curves were conducted for 24 hours, while in the $\Delta relA\Delta spoT$ double mutant the duration of the kill curve was 8 hours. The wild-type strain treated with ampicillin and glyphosate presented a unimodal curve with a 30

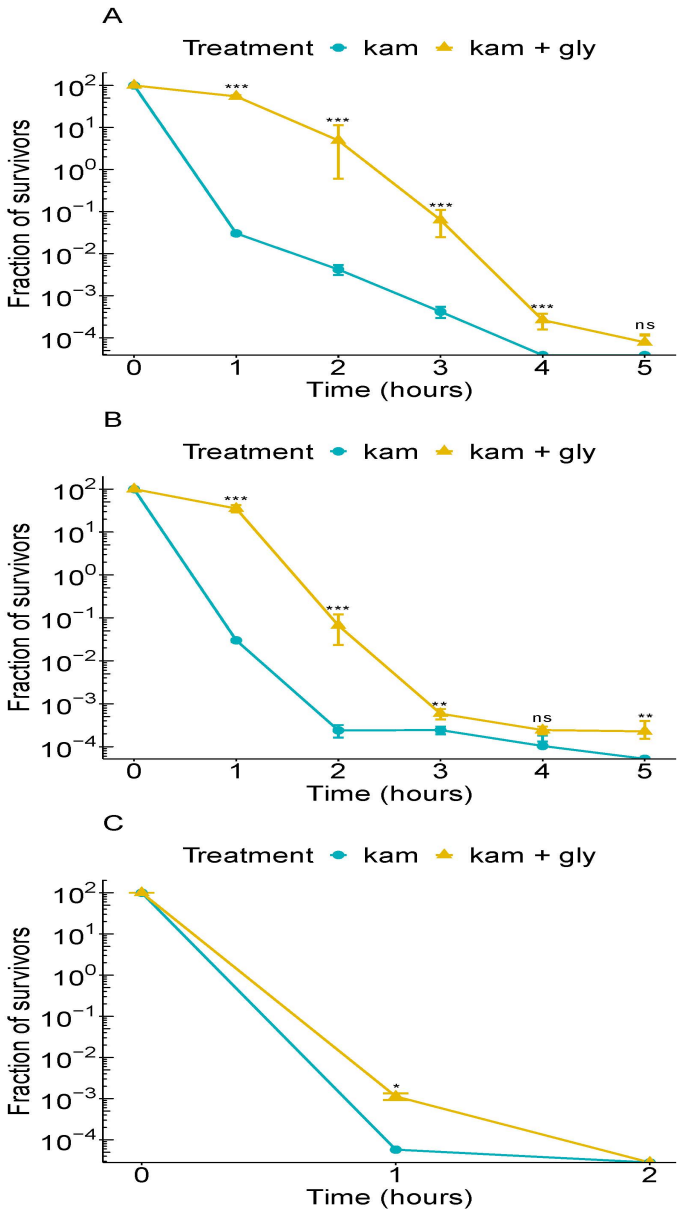

Figure 9 – Time-kill curves of bacteria growing in the presence of kanamycin and kanamycin + glyphosate. The wild-type strain (A), Δ*relA* mutant (B), and Δ*relA*Δ*spoT* double mutant (C) were grown as described in the legend to Figure 8. Approximately 10^7 bacteria/ml were then exposed to the different treatments. CFUs were measured at time zero (just prior to treatment) and at the times indicated in the plots. Data points correspond to the mean of at least three independent experiments with error bars representing standard deviation of the mean. The fraction of survivors was determined by colony counting on L-agar plates. *p*-values were calculated using a pairwise *t*-test between the antibiotic and antibiotic+glyphosate treatment at each time interval. *** indicates $p \leq 0.001$; ** indicates $p \leq 0.01$; * indicates $p \leq 0.05$; *ns*, not significant.

times increase in the MDK_{99} compared to the culture treated with ampicillin alone (Table 7), indicating a massive augment in tolerance. Similarly, the ampicillin+glyphosate $\Delta relA$ cells presented a unimodal curve with an even higher increase in the MDK_{99} (54-fold!). In any case, it is clear that both ampicillin-treated wild-type strain and $\Delta relA$ mutant greatly benefited from the presence of glyphosate (Figure 10A) and (Figure 10B).

Unlike the wild-type and $\Delta relA$ strains the glyphosate-treated $\Delta relA$ $\Delta spoT$ double mutant performed worse than the non-treated bacteria with a faster death rate than that of ampicillin treatment without glyphosate, indicating a synergistic effect with ampicillin in this strain (Figure 10C). The MDK_{99} decreased 0.53-fold and the $MDK_{99.99}$ 0.34-fold in the presence of glyphosate. These results suggest that as with ciprofloxacin and kanamycin the effect of glyphosate on tolerance/persistence towards ampicillin is totally dependent on (p)ppGpp.

Table 7 – MDK_{99} and $MDK_{99.99}$ of ampicillin (Amp) in the presence of glyphosate (Gly) in the wild-type strain, $\Delta relA$, and $\Delta relA\Delta spoT$ mutants. The MDK was measured in hours.

	Wild-Type		$\Delta relA$		$\Delta relA\Delta spoT$	
Treatment	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$	MDK_{99}	$MDK_{99.99}$
Amp	1.05	4.07	1.40	4.24	1.14	3.60
Amp+Gly	31.46	59.69	75.08	1789.46	0.61	1.21
Amp+Gly/Amp	29.96	14.65	53.63	422.04	0.53	0.34

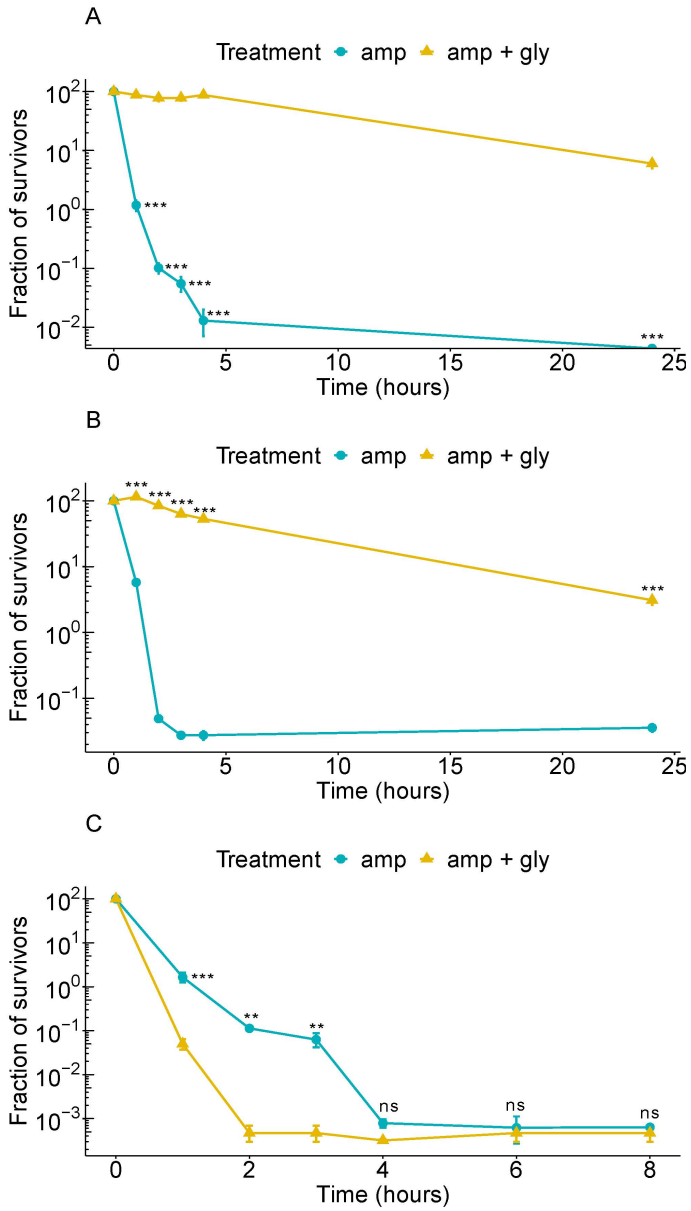

Figure 10 – Time-kill curves of bacteria growing in the presence of ampicillin and ampicillin + glyphosate. The wild-type strain (A), Δ*relA* mutant (B), and Δ*relA*Δ*spoT* double mutant (C) were grown as described in the legend to Figure 8. Approximately 10^7 bacteria/ml were then exposed to the different treatments. CFUs were measured at time zero (just prior to treatment) and at the times indicated in the plots. Data points correspond to the mean of at least three independent experiments with error bars representing standard deviation of the mean. The fraction of survivors was determined by colony counting on L-agar plates. *p*-values were calculated using a pairwise *t*-test between the antibiotic and antibiotic+glyphosate treatment at each time interval. *** indicates $p \leq 0.001$; ** indicates $p \leq 0.01$; * indicates $p \leq 0.05$; *ns*, not significant.

4.6 (p)ppGpp synthesis in the presence of glyphosate and antibiotics

It has been shown that glyphosate induces the synthesis of (p)ppGpp in a RelA-dependent manner (CRUVINEL *et al.*, 2019b). In contrast, (p)ppGpp accumulation is inhibited by several antibiotics (CASHEL *et al.*, 1996). To find out the effect of the combination of glyphosate with the antibiotics tested in this study – ampicillin, ciprofloxacin, and kanamycin, I conducted (p)ppGpp assays.

(p)ppGpp was assayed under conditions similar to those carried out in the time-kill curves in the following testing groups: control, antibiotic, glyphosate, and antibiotic+glyphosate. I managed to successfully assess (p)ppGpp in the wild-type strain and in the Δ*relA* mutant only for bacteria treated with ampicillin. For technical reasons – too high background in some of the ^{32}P-exposed screens, (p)ppGpp levels in ciprofloxacin and kanamycin-treated bacteria were only assessed in the wild-type strain.

Figure 11A and B respectively show the (p)ppGpp levels in bacteria treated for 30 minutes with ampicillin and ampicillin +glyphosate in the wild-type strain and in the Δ*relA* mutant. In the wild-type strain, (p)ppGpp increased upon exposure to glyphosate and ampicillin+glyphosate. However, ampicillin alone caused a small reduction in (p)ppGpp levels (Figure 11A). These results show that though ampicillin slightly inhibited the synthesis of (p)ppGpp, it did not affect (p)ppGpp accumulation induced by glyphosate. Conversely, in the Δ*relA* background the level of (p)ppGpp decreased in all three treatments (ampicillin, glyphosate, ampicillin+glyphosate) (Figure 11B). This was expected as glyphosate-induced (p)ppGpp accumulation is dependent on *relA* (CRUVINEL *et al.*, 2019b). These results also confirm that the protective effect of glyphosate on ampicillin-treated bacteria (Table 7) does not require RelA-dependent (p)ppGpp accumulation, since the increase in the MDK in the presence of glyphosate was higher in the Δ*relA* mutant than in the wild-type strain.

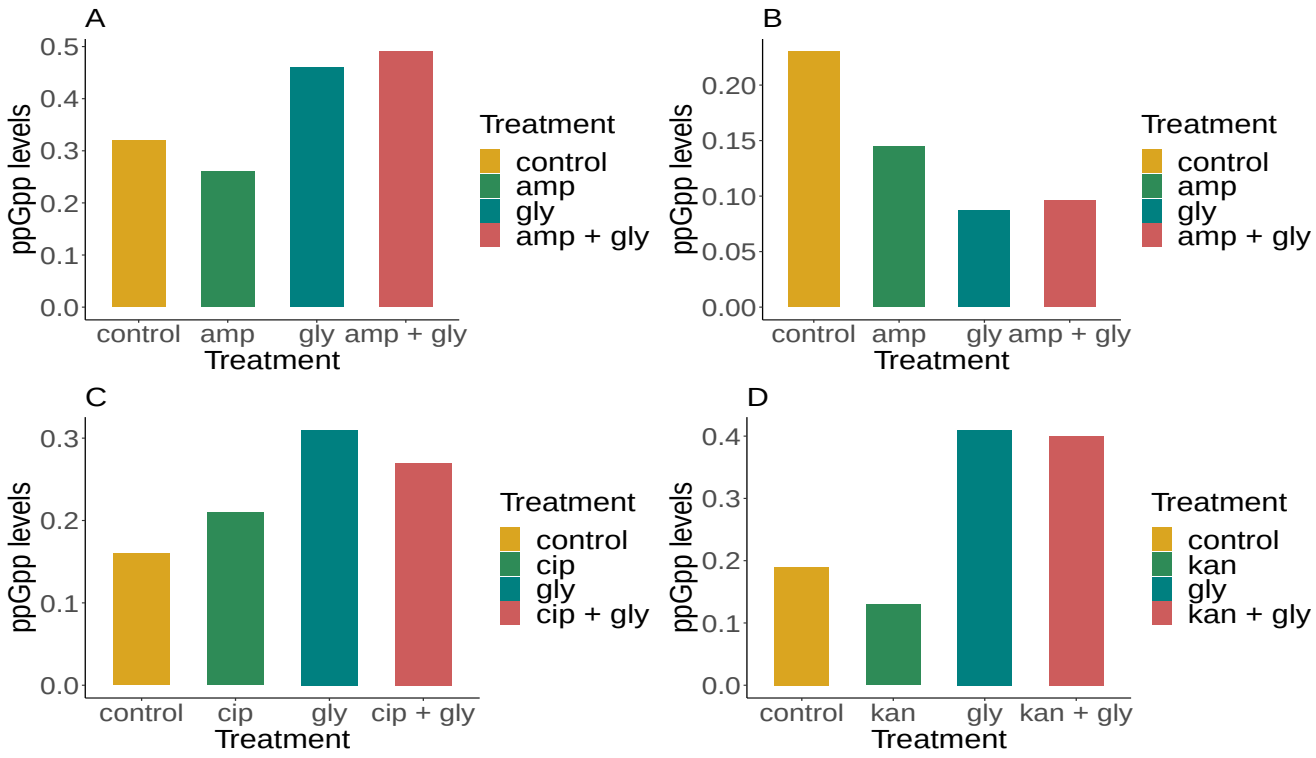

Figure 11 – (p)ppGpp accumulation in bacteria challenged with antibiotics (ampicillin, ciprofloxacin, and kanamycin) and glyphosate. Bacteria were grown in TGP medium to an OD_{600} of 0.5. Then, bacteria were resuspended in TGP medium containing 0.2 mM KH_2PO_4 and 100 µCi/ml ^{32}P supplemented with ampicillin (20X MIC) and/or 5 mM glyphosate . Aliquots were taken at 0 and 30 minutes and on the next day, the labeled nucleotides were resolved by one-dimensional TLC and read in a phosphorimager. The amounts of (p)ppGpp were estimated by measuring the densities corresponding to the spots of GTP, ppGpp, and pppGpp using the ImageJ software. Ampicillin + glyphosate assay in wild-type strain (A) and in Δ*relA* mutant (B); Ciprofloxacin + glyphosate assay in wild-type strain (C); and Kanamycin + glyphosate assay in wild-type strain (C). The values correspond to a single measurement.

Ciprofloxacin treatment slightly increased the level of (p)ppGpp, but not as much as glyphosate (Figure 11C). However, there was no synergy between ciprofloxacin and glyphosate as in the culture treated with ciprofloxacin + glyphosate the level of (p)ppGpp was somewhat lower than with glyphosate alone. In kanamycin-treated cells the results are similar to those observed for ampicillin, i.e., kanamycin treatment alone slightly reduced the level of (p)ppGpp, but kanamycin+glyphosate caused the same induction in (p)ppGpp as in glyphosate alone (Figure 11D).

It can be concluded from these results that the antibiotics tested in this study did not inhibit the accumulation of (p)ppGpp in response to glyphosate.

5 DISCUSSION

The possible harmful effects of glyphosate on the environment, human and animal health, and on the diversity and physiology of microorganisms are still subject of an intense debate. A search in the Google Scholar database revealed almost 18,000 scientific documents with the keywords "glyphosate toxicity" published in the last 10 years. In mammals, several adverse effects, like cancer and/or neurological disorders have been attributed to glyphosate exposure (BRUGGEN *et al.*, 2018). Glyphosate has also been shown to affect the microbiota in a dose and exposure time-dependent manner (NIELSEN *et al.*, 2018; ALLEGRINI *et al.*, 2015; AITBALI *et al.*, 2018; MOTTA *et al.*, 2018). In addition, it has been claimed that glyphosate influences the emergence and/or proliferation of bacterial resistance to some antibiotics (KURENBACH *et al.*, 2015; RAOULT *et al.*, 2021). However, the regulatory mechanisms behind glyphosate involvement in bacterial resistance have not been fully elucidated. The initial aim of the present study was to test the hypothesis that glyphosate-associated increase in antibiotic resistance is related to the accumulation of (p)ppGpp that occurs in the presence of glyphosate. However, very soon we found out that glyphosate does not actually increase the level of antibiotic resistance (subsection 4.4). Instead, we hypothesized that glyphosate might affect the tolerance/persistence to antibiotics via (p)ppGpp accumulation.

To begin with, I confirmed that glyphosate at a concentration of 5 mM exerts a bacteriostatic effect on *E.coli* viability in the wild-type strain and in the Δ*relA* mutant (Figure 6). This result facilitated the design of ideal conditions to test the main hypothesis and also solved a riddle raised by some experiments previously carried out in the laboratory that showed that glyphosate kills bacteria suspended in saline. It was eventually found out that the bactericidal activity of glyphosate was due to the low pH of the formulation used in our lab - technical grade glyphosate with a pH of 2. It should be noted that commercial formulations of glyphosate have a pH of 5-6, due to the addition of surfactants and other ingredients. *E.coli* has several mechanisms to cope with acid stress (DU *et al.*, 2020). Most of these mechanisms are dependent on RpoS and are therefore chiefly activated in the stationary phase (ARCARI *et al.*, 2020). An effective response to moderately low pH (5.5 to 6) may occur in exponentially-growing bacteria (SMALL *et al.*, 1994). However, in our experiments, exponential phase bacteria were subjected to strong acid stress (pH 2), which may explain the bactericidal effect caused by a relatively small concentration of glyphosate (5 mM). On the other hand, glyphosate main mechanism of action is via inhibition of the EPSP synthase, which results in aromatic amino acid starvation (HAN; FOUNDATION, 2016). The absence of aromatic amino acids induces the synthesis of *relA*-dependent (p)ppGpp, which, in turn,

inhibits stable RNA synthesis and contributes to growth inhibition (MAGNUSSON *et al.*, 2005). Therefore, one would expect a stronger inhibitory effect of glyphosate on the wild-type strain than in the Δ*relA* mutant, which does not accumulate (p)ppGpp in response to glyphosate. The fact that both strains were equally affected by glyphosate suggests that glyphosate bacteriostatic effect occurs via a regulatory mechanism unrelated to the stringent response.

By performing time-kill curves and assessing the MDK I showed that glyphosate increases *E. coli* tolerance or persistence towards ampicillin, ciprofloxacin, and kanamycin and that this effect is dependent on the presence of (p)ppGpp, but only partially dependent on *relA*. More specifically, glyphosate increased ciprofloxacin tolerance and persistence, and the tolerance toward kanamycin and ampicillin. The conclusion that the effect of glyphosate on tolerance/persistence depends entirely on (p)ppGpp stems from the observation that the glyphosate-treated (p)ppGpp^0 strain was as sensitive to the antibiotic as the control without glyphosate. On the other hand, in the relaxed strain (Δ*relA*) the protective effect of glyphosate against ciprofloxacin and kanamycin was only slightly diminished and not at all in the case of ampicillin. These results suggest that even though glyphosate induces (p)ppGpp accumulation via RelA and not via SpoT (CRUVINEL *et al.*, 2019b), active induction of (p)ppGpp synthesis is not required to promote bacterial tolerance/persistence, as long as a basal level of (p)ppGpp is present in the cell.

Unlike (KURENBACH *et al.*, 2015) that reported that glyphosate increases the concentration of ciprofloxacin and kanamycin required to reduce bacterial survival, here I showed that glyphosate did not affect the MIC of ampicillin, ciprofloxacin, or kanamycin. However, glyphosate did increase the tolerance to these antibiotics. Tolerance is defined as the extension of time that bacteria can survive in lethal concentrations of an antibiotic (usually 10-20 times the MIC) before succumbing to its effects (BRAUNER *et al.*, 2017). Though (KURENBACH *et al.*, 2015) also used the term tolerance to express the effect of glyphosate + antibiotics on bacteria, they did not clearly define it and used it interchangeably with "resistance". This misuse of terms can lead to confusion when comparing results from different laboratories. Indeed, the methodology used to evaluate a specific phenomenon will depend on the proper definition of this phenomenon, i.e. tolerance cannot be evaluated using the MIC because its definition makes it incompatible with what the MIC evaluates. Here, we relied on the definitions given in the Consensus Statement paper "Definitions and guidelines for research on antibiotic persistence" (BALABAN *et al.*, 2019) regarding the concepts of resistance, tolerance, and persistence, and therefore adopted the MDK as the metric to measure tolerance.

It has been demonstrated that (p)ppGpp accumulation induced by amino acid, nitrogen, or glucose starvation plays a fundamental role in the formation of tolerance to certain antibiotics (FUNG *et al.*, 2010) and that this effect can be *relA*-dependent or not (KUDRIN *et al.*, 2017)(BROWN, 2019) (AMATO; BRYNILDSEN, 2015). For example, (KUDRIN *et al.*, 2017) showed that exposure to mupirocin (an inhibitor of isoleucyl-tRNA synthetase) induces ampicillin tolerance in a *relA*-dependent fashion. However, when mupirocin was combined with trimethoprim the increase in tolerance occurred both in the wild-type and in the relaxed strain. In addition, they showed that mupirocin-induced (p)ppGpp accumulation was abolished in the presence of chloramphenicol and tetracycline (antibiotics that inhibit translation), without affecting tolerance to ampicillin. On the other hand, the effect of mupirocin on tolerance to norfloxacin was *relA*-independent, and for that reason, they concluded that the effect of (p)ppGpp is drug-specific. The formation of persisters toward ampicillin and ofloxacin was shown to be dependent on (p)ppGpp (AMATO; BRYNILDSEN, 2015). Interestingly, deletion of *relA* eliminated persistence towards ampicillin, but ofloxacin persistence was only eliminated when *spoT* was also removed. These authors concluded that persistence to ampicillin requires higher levels of (p)ppGpp than persistence to ofloxacin, and that, in general, tolerance to different antibiotics requires different levels of (p)ppGpp. Similarly, the results presented here show that the increase in tolerance triggered by glyphosate was largely *relA*-independent for all three antibiotics tested, but strongly dependent on (p)ppGpp, which indicates that a minimal concentration of this alarmone is required to enable glyphosate protection. It is interesting noticing that the MIC of glyphosate in the (p)ppGpp^0 strain was 8 times higher than in the wild-type or $\Delta relA$ strains. This suggests that the concentration of glyphosate used in our experiments – 5 mM, may have not been high enough to curb bacterial growth and dodge thus death by the antibiotics.

The bacteriostatic effect of glyphosate was observed in both wild-type and $\Delta relA$ strains Figure 6 which suggests that glyphosate-induced growth inhibition is mostly caused by aromatic amino acid starvation (CRUVINEL *et al.*, 2019b), which is sufficient to halt bacterial growth, even in the absence of (p)ppGpp accumulation. Accordingly, Traxler *et al.* (2013) noted that despite nutritional shortage induces (p)ppGpp synthesis, the effects of this stress are global and go beyond the increase in (p)ppGpp levels. Likewise, Fung *et al.* (2010) and Hobbs e Boraston (2019) claimed that although (p)ppGpp plays a key role in tolerance formation, it is not the only relevant factor. Other players are the SOS response, RpoS, toxin-antitoxin systems, quorum sensing, efflux pumps and reactive oxygen species (TRASTOY *et al.*, 2018), as well as some individual genes such as *hipA*, *phoU*, and *glpD* (FUNG *et al.*, 2010). Chowdhury *et al.* (2016) demonstrated that the formation of persister

cells occurred in the absence of (p)ppGpp and that this process depended on the production of stress-induced toxins. These toxins reduce cell growth and as a result increase persistence in a (p)ppGpp-independent manner. (KALDALU; TENSON, 2019) concluded that there is no specific molecular mechanism involved in persister formation and that any environmental stress that reduces bacterial growth would contribute to the formation of persisters.

The relationship between dormant cells and persistence is well known, bacteria that enter into a state of dormancy protect themselves and ensure their survival during upcoming adverse environmental conditions through growth arrest or decreased metabolism (JUNG *et al.*, 2019). Thus, it is not surprising that a bacterial stressor like glyphosate leads bacteria to a dormant state and consequently boosts tolerance/persistence to antibiotics.

The concentration of glyphosate used in this work was the same as that used by (CRUVINEL, 2019). She determined that a concentration of 5 mM is adequate to evaluate the effect of glyphosate on bacteria without causing alterations in their biology. In agricultural or non-agricultural environments where glyphosate is the active ingredient of commercial formulations, its concentration varies from 1% to 41% (BRADBERRY *et al.*, 2004). The minimum concentration used for gardening (6 mM) is similar to that used in our experiments (5 mM). However, the most commonly used concentration of glyphosate is 21 mM. It is thus highly probable that glyphosate applied to agricultural fields would affect the level of tolerance/persistence to antibiotics, at least transiently. On the other hand, the maximum residue limit or concentration of glyphosate allowed legally in food according to the Code of Federal Regulations of the US government varies between 0.6 µM and 2.36 mM depending on the crop (Federal Government of U.S., 2002). In addition, the residual levels of glyphosate found in agricultural soil range from 29 to 47 µM, and in water the maximal residual concentration was 88 µM (SOARES *et al.*, 2019). These levels are well below the glyphosate concentration used in this study, and should not, therefore, cause a significant change in microbial tolerance/persistence. Indeed, concentrations below 5 mM do not have any effect on growth rate (CRUVINEL, 2019) and will not drive the bacteria into a state of dormancy with the consequent emergence of tolerance. Whether the concentration of glyphosate in natural environments is high enough to increase tolerance or not, our results clearly showed that glyphosate does not increase the MIC to antibiotics and that the effect of glyphosate on bacterial tolerance/persistence is indirect. Once any environmental stress that slows down growth rate may potentially lead to tolerance, it is not surprising that glyphosate, which causes amino acid starvation, increases bacterial tolerance. In this sense, the use of glyphosate does not generate any more risk to human or animal health than any other environmental stress that bacteria encounter during their lifetime.

6 CONCLUSIONS

- Glyphosate acts as a bacteriostatic agent in both wild-type and $\Delta relA$ strains.
- Glyphosate does not affect bacterial resistance to ampicillin, ciprofloxacin, and kanamycin.
- Glyphosate increases bacterial tolerance or persistence to ampicillin, ciprofloxacin, and kanamycin in a mechanism that completely depends on (p)ppGpp, but only partially on *relA*.

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Annex

frontiers in Microbiology

REVIEW
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Diversity in *E. coli* (p)ppGpp Levels and Its Consequences

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(p)ppGpp is at the core of global bacterial regulation as it controls growth, the most important aspect of life. It would therefore be expected that at least across a species the intrinsic (basal) levels of (p)ppGpp would be reasonably constant. On the other hand, the historical contingency driven by the selective pressures on bacterial populations vary widely resulting in broad genetic polymorphism. Given that (p)ppGpp controls the expression of many genes including those involved in the bacterial response to environmental challenges, it is not surprising that the intrinsic levels of (p)ppGpp would also vary considerably. In fact, null mutations or less severe genetic polymorphisms in genes associated with (p)ppGpp synthesis and hydrolysis are common. Such variation can be observed in laboratory strains, in natural isolates as well as in evolution experiments. High (p)ppGpp levels result in low growth rate and high tolerance to environmental stresses. Other aspects such as virulence and antimicrobial resistance are also influenced by the intrinsic levels of (p)ppGpp. A case in point is the production of Shiga toxin by certain *E. coli* strains which is inversely correlated to (p)ppGpp basal level. Conversely, (p)ppGpp concentration is positively correlated to increased tolerance to different antibiotics such as β-lactams, vancomycin, and others. Here we review the variations in intrinsic (p)ppGpp levels and its consequences across the *E. coli* species.

Keywords: (p)ppGpp, polymorphism, growth rate, evolution, stress resistance, antibiotic resistance, virulence

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DIVERSITY OF (p)ppGpp CONCENTRATIONS–IMPACT ON GROWTH RATE AND BEYOND

"The study of bacterial growth is the essence of microbiology" (Jacques Monod).

The success of an organism in evolutionary terms resides in its ability to reproduce and perpetuate its genes. It would thus be expected that bacterial resources would be devoted most of the time to maximizing growth rate. This may be true under some circumstances, especially, under optimal laboratory growth conditions. However, bacteria actually keep growth rate under very tight control. At the core of growth regulation is a small nucleotide that appears in two different forms—guanosine tetra- and penta-phosphate—ppGpp and pppGpp, collectively known as (p)ppGpp. The grip of (p)ppGpp on growth rate is achieved mainly through an effective inhibition of stable RNA (rRNA and tRNA) synthesis during amino acid starvation and other nutritional stresses in a process that became known as the stringent control (Cashel and Gallant, 1968; Potrykus and Cashel, 2008; Potrykus et al., 2011). Nevertheless, the impact of (p)ppGpp on cell physiology goes far beyond stable RNA control. (p)ppGpp also inhibits DNA replication, lipid and protein synthesis and ultimately cell division (Potrykus and Cashel, 2008; Traxler et al., 2008). Whenever the growth conditions deteriorate, (p)ppGpp concentration increases, severely repressing the expression of

growth-related genes. This repression is necessary in order to promote the reallocation of resources, which are then shifted from growth promotion to the maintenance of amino acid as well as energy pools and to cell protection and survival. In fact, (p)ppGpp concentration increases stepwise according to the severity of nutrient depletion (Traxler et al., 2011).

In *E. coli* and related bacterial species, (p)ppGpp is synthesized by two different proteins—RelA and SpoT. These proteins evolved by duplication from a bifunctional ancestral RelA/SpoT Homolog (RSH) possessing both (p)ppGpp synthetic and hydrolytic capabilities, resulting in two proteins with overlapping functionalities (Mittenhuber, 2001; Atkinson et al., 2011). The RelA and SpoT proteins contain 744 and 702 amino acids, respectively. Both proteins can be divided in two parts of similar size (**Figure 1**). The NTD half of the protein harbors the catalytic HD (hydrolytic) and Synth (Synthetic) domains. In RelA, the HD domain is not active. The CTD portion of the protein contains four regulatory domains: TGS (ThrRS, GTPase, SpoT/RelA domain), AH (α-helical domain), RIS (Ribosome-InterSubunit domain) and ACT (Aspartate kinase-Chorismate mutase-TyrA domain) (Atkinson et al., 2011; Loveland et al., 2016). RelA responds to intracellular amino acid imbalancies, such as amino acid starvation, by synthesizing large amounts of (p)ppGpp (Cashel, 1969). RelA carries an inactive (p)ppGpp-hydrolytic domain and does not hydrolyze the alarmone under any conditions. SpoT is a bifunctional enzyme that contains functional (p)ppGpp-synthetic and hydrolytic domains, but displays weak (p)ppGpp-synthetic activity and strong ppGpp hydrolytic activity. The *relA* knockout accumulates ppGpp in response to several environmental stresses, such as carbon and nitrogen (Edlin and Donini, 1971), phosphate (Spira et al., 1995), iron (Vinella et al., 2005), and fatty acid (Battesti and Bouveret, 2006) starvation.

Early in (p)ppGpp research different spontaneous alleles of *relA* and *spoT* have been isolated. For instance, the *spoT*1 allele (Laffler and Gallant, 1974), that confers a spotless phenotype (absence of pppGpp under amino acid starvation), was isolated from the old 58-161 strain and is now common in many K-12 derivatives (Alföldi et al., 1962). Bacteria that carry the *spoT*1 allele overproduce (p)ppGpp both under nutrient starvation and under normal growth conditions. The *spoT*1 allele contains two different mutations - a H255Y substitution in the synthetase domain (Synth) and a two-amino acid insertion between residues 82 and 83 (+QD) in the hydrolytic domain (HD), both at the NTD portion of SpoT (**Figure 1**). The two amino acid insertions in the HD domain are likely to negatively affect the ppGpp-hydrolytic activity of SpoT resulting in high (p)ppGpp basal levels, while the H255Y substitution hits a conserved residue (Atkinson et al., 2011), but its effect on the (p)ppGpp-synthetic activity of SpoT is hard to predict. Interestingly, *spoT*1 is usually accompanied in many strains by the defective *relA*1 allele, consisting of an IS2 insertion in the HD domain that is likely to disrupt RelA (p)ppGpp-synthetic activity (Metzger et al., 1989). The *relA*1 mutant displays lower ppGpp basal level than the *relA*$^+$ strain (Lagosky and Chang, 1980) and does not accumulate (p)ppGpp in response to amino acid starvation. Apparently, the high ppGpp basal level caused by the *spoT*1 allele is compensated by the defect in (p)ppGpp synthesis caused by the presence of *relA*1. It is therefore no wonder that both alleles often appear together in the same genome.

Later on, other *spoT* alleles, such as *spoT*201, *spoT*202 an *spoT*203 were isolated by selection on amino-triazole plates (Sarubbi et al., 1988). Amino-triazole is a herbicide that inhibits the synthesis of histidine. Bacteria that synthesize high levels of (p)ppGpp overcome histidine starvation by inducing the expression of the *his* operon (Rudd et al., 1985). A critical difference between *spoT*201 and the other three alleles was that the former confers an almost normal growth rate. The other alleles (*spoT*202-203) considerably reduced growth rate and for that reason could be transferred only to a *relA*1 background, but not to a bacterium that carries a wild-type *relA* allele. The *spoT* alleles *spoT*202 and *spoT*203 consist, respectively, of T78I and R140C substitutions, both in the HD domain (Potrykus et al., 2011). The molecular nature of the *spoT*201 mutation has not been published. Given the high (p)ppGpp level in strains bearing these alleles, the *spoT*201-203 mutations have probably compromised the ppGppase activity of SpoT.

It became evident that an inverse linear correlation exists between the intrinsic level of (p)ppGpp in a bacterium (basal level under unrestricted growth conditions) and the bacterial growth rate (Sarubbi et al., 1988). This negative correlation was confirmed when *spoT* mutant alleles were transferred to other genetic backgrounds (Spira et al., 2008). The recombinant strains displayed all the hallmarks of the previously analyzed *spoT* mutations, namely slower growth rate, high levels of the sigma factor RpoS (coordinator of the general stress response) and high resistance to environmental stresses (see below).

The above mentioned *relA* and *spoT* alleles and most data on (p)ppGpp physiology and homeostasis were obtained by studying laboratory strains derived from the ancestral K-12 strain. To date very few attempts have been made to analyze (p)ppGpp homeostasis in natural isolates of *E. coli*. In two of these studies, the basal level and starvation-induced levels of (p)ppGpp were assessed in a set of strains derived from the ECOR collection (Ferenci et al., 2011) and in a collection of Shiga toxin-producing *E. coli* (STEC) strains (Stella et al., 2017). The ECOR collection contains 72 strains from various locations and environments and from five phylogenetic groups (A, B1, B2, D, and E) that supposedly represents the variability in the *E. coli* species (Ochman and Selander, 1984). Most ECOR isolates are commensal, but some are pathogenic. The levels of (p)ppGpp in non-limited minimal medium, in response to amino acid starvation or carbon starvation were reported for 33 strains of the ECOR collection. ppGpp concentrations in the ECOR strains treated with serine hydroxamate, an inhibitor of seryl-tRNA synthetase that induces amino acid starvation, were quite similar in all tested strains. However, (p)ppGpp response to carbon starvation was less homogeneous, consistent with the variation in SpoT observed in those strains. A T13N amino acid substitution was common in strains that showed low (p)ppGpp accumulation in response to carbon starvation and was absent in strains presenting high levels of ppGpp (Ferenci et al., 2011). These data suggested that *spoT* is being subjected to microevolutionary pressures.

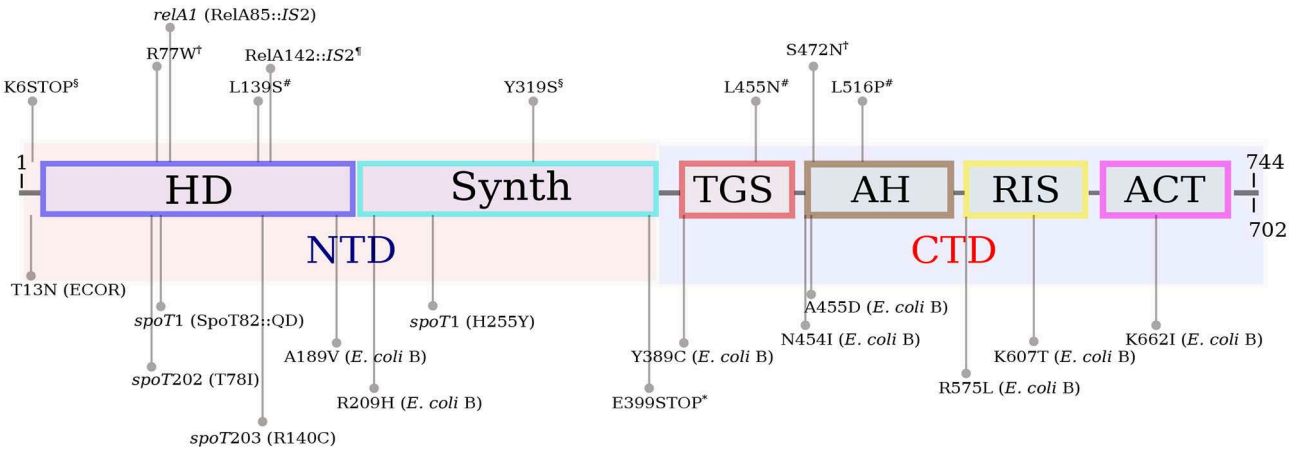

FIGURE 1 | Schematic representation of the long RSH (RelA/SpoT) architecture, as per Atkinson et al. (2011) and Loveland et al. (2016). The RelA and SpoT proteins contain 744 and 702 amino acids, respectively. The NTD half of RSH proteins harbors the catalytic HD (hydrolytic) and Synth (Synthetic) domains. In RelA, the HD domain is not active. The CTD portion of the protein contains four regulatory domains: TGS (ThrRS, GTPase, SpoT/RelA domain), AH (α-helical domain), RIS (Ribosome-InterSubunit domain) and ACT (Aspartate kinase-Chorismate mutase-TyrA domain). The mutations in RelA and SpoT mentioned in the main text are shown above and below the protein diagram, respectively. *relA*1, *spoT*1, *spoT*202 and *spoT*203 are known mutations present in many *E. coli* K-12 derivatives. The *spoT*1 allele consists of two mutations. All mutations, with the exception of those that are followed by *E. coli* B or ECOR (in parentheses) were found in K-12 strains. The T13N substitution is common in strains of the ECOR collection (Ferenci et al., 2011). The SpoT mutations in *E. coli* B were selected in an evolution experiment in glucose-limited minimal medium (Cooper et al., 2003). Mutations ending with "*" were selected during adaptation to high temperature (Kishimoto et al., 2010); "¶" labels indicate RelA mutations selected for n-butanol tolerance (Reyes et al., 2012); "#" labels point to mutations in RelA selected under high ethanol concentration (Horinouchi et al., 2015); RelA mutations ending with "†" were selected for isopropanol tolerance (Horinouchi et al., 2017) and "§" labels indicate RelA mutations selected under growth with lactate (Conrad et al., 2009).

It is well-established that the intrinsic concentration of (p)ppGpp is inversely correlated with growth rate (Ryals et al., 1982; Sarubbi et al., 1988; Potrykus et al., 2011; Jin et al., 2012). In fact, gratuitous induction of (p)ppGpp synthesis mediated by *relA* overexpression causes an almost instantaneous growth arrest (Schreiber et al., 1991; Svitil et al., 1993; Cruvinel et al., 2019). However, the vast majority of studies that analyzed this correlation used isogenic *E. coli* laboratory strains harboring different *relA* or *spoT* alleles. If growth rate is mainly regulated by (p)ppGpp a good correlation between (p)ppGpp levels and growth rate, even in a set of non-isogenic strains, would be expected. Indeed, when the intrinsic ppGpp concentrations of the ECOR isolates growing under non-limited growth conditions are plotted against their respective growth rates, an inverse correlation is observed (**Figure 2A**), with a Pearson's correlation coefficient= −0.58. Though not perfect, the inverse correlation between ppGpp concentration and growth rate in these strains validates the central role of (p)ppGpp in governing growth rates across the *E. coli* species, even in strains that come from very different genetic backgrounds as is the case of the ECOR collection. It is worth mentioning that in this as well as in other studies that analyzed (p)ppGpp in exponentially growing bacteria or in response to stresses other than amino acid starvation, pppGpp was below the detection level (Varik et al., 2017; Cruvinel et al., 2019).

In another analysis of (p)ppGpp fluctuation in natural isolates, ppGpp concentration was measured in 50 STEC strains growing under two different culture conditions—non-limited growth medium and phosphate starvation (Stella et al., 2017). A significant variability in ppGpp levels was observed among the STEC isolates (**Figure 2B**). On average, ppGpp values were twice as high in bacteria submitted to Pi starvation than in the same bacteria growing exponentially in minimal medium. ppGpp values in this set of strains went from 0.08 to 0.30 units for bacteria growing exponentially and from 0.17 to 0.42 units for phosphate-starved bacteria (units correspond to the ratio of ppGpp over GTP+ppGpp). Though this study did not evaluate the variability of ppGpp with growth rate, it correlated the levels of this alarmone with STEC cytotoxicity, as described below.

Altogether, the data presented here highlight the existence of variability in intrinsic ppGpp concentrations across the *E. coli* species and that this variability has a substantial impact on growth rate. However, in addition to growth rate control (p)ppGpp directly and indirectly affects many important bacterial characteristics, such as stress responses, virulence, antibiotic resistance and persistence, biofilm formation, genome stability, and more (Potrykus and Cashel, 2008; Dalebroux et al., 2010; Martin-Rodriguez and Romling, 2017; Rasouly et al., 2017; Hobbs and Boraston, 2019). Variability in (p)ppGpp basal levels is thus likely to affect these traits as well.

It is important to notice that in the studies mentioned above that compared (p)ppGpp values in isogenic and non-isogenic strains, (p)ppGpp was assessed using the classical method of formic acid extraction of ^{32}P-labeled bacterial nucleotide pools. These studies did not provide absolute values of (p)ppGpp concentration, but instead presented the level of ppGpp relative to that of GTP+ppGpp as detailed in Cashel (1994). The most relevant limitations of this method is the lack of absolute

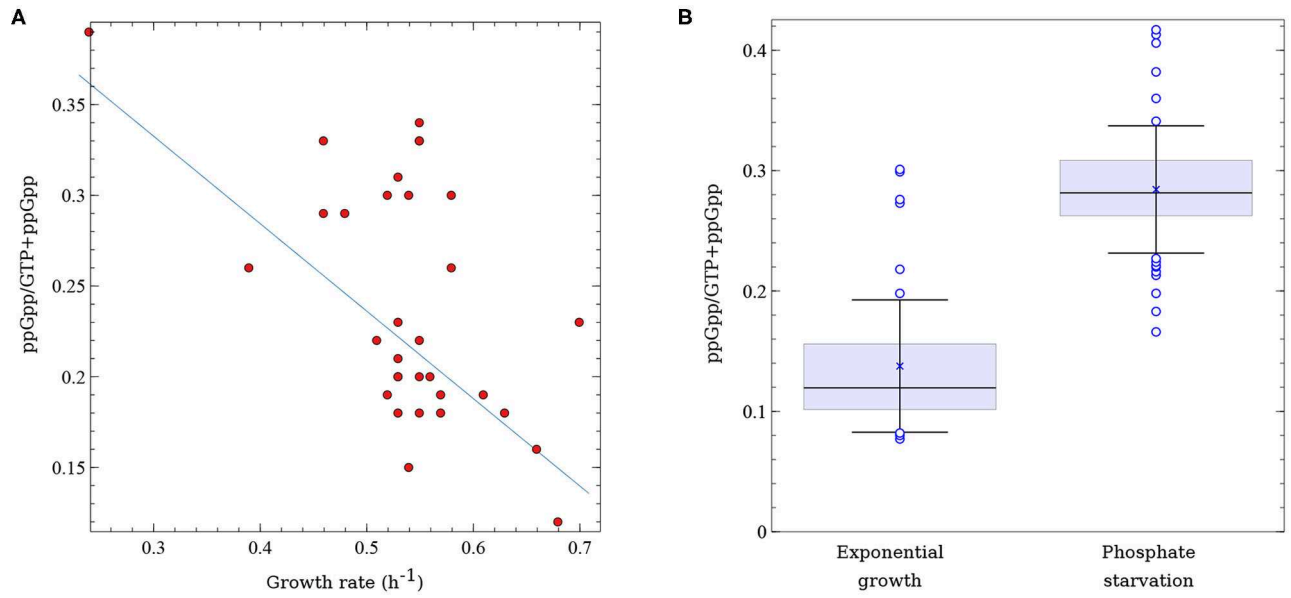

FIGURE 2 | Variability in (p)ppGpp levels across *E. coli* natural isolates. **(A)** Correlation between (p)ppGpp basal levels and growth rate (h^{-1}) in 33 strains of the ECOR collection growing exponentially in glucose minimal medium. ppGpp levels correspond to the ratio between ppGpp and GTP + ppGpp. Data was extracted from Ferenci et al. (2011). **(B)** Distribution of ppGpp levels (ppGpp/GTP+ppGpp) in 50 STEC isolates growing exponentially in non-limited (glucose) minimal medium or under phosphate starvation. x corresponds to the mean values, horizontal lines represent the median and the error bars correspond to 1 standard deviation; ○ represent outliers. Data was extracted from Stella et al. (2017).

numerical estimates of (p)ppGpp concentrations and that it leaves out GDP, which constitutes 7.7–15% of the total pool of guanosine nucleotides (Varik et al., 2017), as the resolution of the ^{32}P-labeled nucleotides on the TLC plate is not usually good enough to identify GDP spots on the autoradiogram. Because of these limitations, the ppGpp values obtained in those studies cannot be easily compared to the ones found in other reports. However, the relative values of (p)ppGpp obtained by the classical method are reproducible and give a reasonable estimate of (p)ppGpp status in a particular set of strains. More recent techniques for evaluating (p)ppGpp, based on Ion Chromatography-High-Resolution MS (Patacq et al., 2018), HPLC (Varik et al., 2017), or UPLC (Ihara et al., 2015) largely overcome the disadvantages of the ^{32}P-classical method.

ROLE OF (p)ppGpp IN STRESS RESISTANCE AND NUTRITIONAL COMPETENCE

(p)ppGpp supports survival by either directly or indirectly stimulating the expression of genes involved in stress protection. The cell response to environmental stresses such as extreme pH and osmolarity, dehydration or oxidative stress is coordinated by the sigma factor RpoS (Landini et al., 2014; Schellhorn, 2014), whose synthesis and stability is enhanced by (p)ppGpp (Gentry et al., 1993; Battesti et al., 2011). The culture history of a bacterial population determines its overall physiology, and more specifically, the strength of its response to environmental challenges (Ryall et al., 2012). The specific hurdles that a bacterial lineage experiences throughout its existence would eventually leave their imprints in its genome. For instance, alleles that maintain high levels of RpoS and other stress-related genes would be selected in a population that is being often exposed to environmental stresses Conversely, bacteria growing in a stress-free environment accumulates mutations in genes that downregulate RpoS synthesis, promotes its proteolysis or even acquire null mutations in the *rpoS* gene itself (King et al., 2004; Spira and Ferenci, 2008; Wang et al., 2010). Likewise, genes involved in (p)ppGpp metabolism are under selective pressures driven by culture conditions (Spira et al., 2008; Ferenci et al., 2011). ppGpp pleiotropy indicates that variations in intrinsic (p)ppGpp levels might have broad consequences on bacterial physiology and genotypic characteristics of bacterial populations. Bacteria that display intrinsic high levels of (p)ppGpp are more resistant to environmental stresses either because they express high levels of RpoS or because (p)ppGpp directly stimulates the transcription of other genes related to stress protection. However, the correlation between (p)ppGpp and RpoS is not as straightforward as would be expected from extrapolating data on K-12 strains (Gentry et al., 1993; Spira et al., 2008; Battesti et al., 2011). Analysis of *E. coli* natural isolates does not give a simple relationship in which RpoS concentration is proportional to (p)ppGpp concentration. While some strains exhibit a proportionality between the two measured entities, others display mediocre levels of RpoS but high (p)ppGpp levels (Ferenci et al., 2011). Surely, there are other inputs, other than (p)ppGpp that modulate the levels of RpoS.

Both (p)ppGpp and RpoS directly affect the transcription of dozens of genes and indirectly the transcription of many others (Peano et al., 2015; Wong et al., 2017). RpoS competes with other sigma factors, particularly with σ^{70} for binding to the core RNA polymerase. The outcome of this competition is that under nutrient limitation or in the stationary phase (circumstances that cause the accumulation of RpoS), the transcription of σ^{70}-dependent genes, i.e., the majority of bacterial genes, is considerably diminished. Hence, the stimulatory effect of (p)ppGpp on RpoS adds another layer of growth control in addition to the already discussed inhibition of stable RNA. Bacterial strains that accumulate high levels of (p)ppGpp or RpoS are less fit for growing on poor carbon sources or under nutrient limitation (King et al., 2004). A trade-off is thus characterized in which a certain bacterial strain cannot simultaneously be nutritionally competent and highly stress resistant (Ferenci, 2016). **Figure 3** shows how bacteria with high or low intrinsic (p)ppGpp concentrations deal with environmental challenges.

INTRINSIC (p)ppGpp CONCENTRATION AS A TARGET IN EVOLUTION EXPERIMENTS

Given that (p)ppGpp is the most important source of growth rate control (Potrykus et al., 2011), polymorphism in *relA* and *spoT* are likely to occur throughout the course of bacterial evolution and adaptation to different environments, especially in those limited in one or more nutrients, a situation that suppresses normal growth. Several evolution experiments, which resulted in the emergence of mutants related to (p)ppGpp both in batch and in continuous cultures, have been conducted to date. The mutations observed in these studies are summarized in **Figure 1**.

In one of them, 12 *E. coli* populations have been daily diluted in glucose limited minimal medium and grown for 20,000 generations. Different non-synonymous mutations in *spoT* have been observed in 8 out of 12 evolved populations (Cooper et al., 2003). The first one, A189V is located at the very end of the HD domain; R209H is at the ∼45-residues region between the HD and Synth domains; Y389C is at the regulatory TGS domain; N454I and A455D are located at the beginning of the α-helical domain; the mutations R575L and R607L are in the RIS domain and K662I is at the ACT domain. Although (p)ppGpp levels were not measured in this study, the expression of aminoacyl-tRNA synthetases and ribosomal proteins were shown to be upregulated in one of these *spoT* mutants (K662I), suggesting that the mutation caused a reduction in (p)ppGpp intrinsic concentration that led to an increase in growth rate. The ACT domain interacts with the ribosome A site in order to activate the (p)ppGpp synthetic activity (Loveland et al., 2016), thus the K662I substitution is likely to interfere with Synth activation resulting in low (p)ppGpp. A non-sense mutation in the TGS domain of *spoT* (E399*) was observed in another case of adaptive evolution of *E. coli* growing at 43.2° (Kishimoto et al., 2010). This mutant displayed high growth rate at the high temperature, possibly due to a reduction in intrinsic (p)ppGpp levels. This finding is puzzling, once it has been shown that the truncation of the CTD leads to an upshift in (p)ppGpp synthesis (Mechold et al., 2002; Battesti and Bouveret, 2006). However, this particular evolved strain carried additional mutations in *lrp* and *rho* that might have strengthened the observed phenotype.

In another experiment, *E. coli* subjected to adaptive evolution under high ethanol concentrations acquired different mutations in *relA* (L139S, L455N, and L519P) that contributed to an increased tolerance in the presence of 5% ethanol (Horinouchi et al., 2015). According to these authors, the *relA* mutations enabled a relaxed response to ethanol, by diminishing (p)ppGpp concentration, thereby increasing growth rate. The L139S mutation occurred in the pseudo-hydrolytic domain of RelA and is therefore unlikely to affect (p)ppGpp synthesis. The other two mutations—L455N and L516P, were in the TGS and AH domains, respectively. These mutations might have affected the regulation of (p)ppGpp synthesis by RelA as both TGS and AH subunits form the elbow of the boomerang-shaped RelA that interacts with the 30S ribosome and with the deacyl-tRNA (Loveland et al., 2016). A similar study with bacteria growing with increasing concentrations of isopropanol (up to 450 mM) for 210 generations showed that the evolved isolates acquired mutations in *relA* (Horinouchi et al., 2017). Again, the suggested mechanism was that the *relA* mutants expressed RelA proteins that synthesized reduced levels of (p)ppGpp in response to isopropanol, resulting in higher growth rates. The mutations—R77W and S472N, were, as before, in the pseudo-HD and AH domains, respectively.

In another experiment of guided evolution, bacteria grown in a chemostat with increasing butanol concentrations (up to 1.3%) for 144 generations acquired mutations in several genes (Reyes et al., 2012). One of the evolved isolates presented an IS2 insertion at the end of the HD domain of RelA, which has probably compromised the integrity of the entire protein, resulting in a RelA-negative phenotype. Mutations in *relA* also appeared in 2 out of 11 populations growing in lactate minimal medium (Conrad et al., 2009). One mutation—K6*, caused a frameshift at the very beginning of the gene, while the other mutation, Y319S, occurred in the Synth domain of RelA.

In addition to the direct effect of (p)ppGpp on growth, low concentrations of this alarmone also results in reduced levels of RpoS (Gentry et al., 1993; Battesti et al., 2011). Due to the competition between σ^S and σ^{70}, the former negatively affects the expression of growth-related genes, especially those involved in the uptake and assimilation of alternative carbon sources with a consequent reduction in growth rate (Gentry et al., 1993; King et al., 2004; Magnusson et al., 2005; Spira et al., 2008; Ferenci et al., 2011). Thus, mutations in *relA* would also improve growth by diminishing RpoS concentration in the cell. **Figure 3** summarizes the outcomes of bacterial evolution experiments in which mutations in (p)ppGpp-related genes have been observed.

In conclusion, selection of different *relA* and *spoT* alleles in evolution experiments is not uncommon. In fact, in most of these experiments regulatory genes are the primary targets of adaptive selection (Maharjan et al., 2006; Wang et al., 2010). Given the central role that (p)ppGpp plays in the regulation of gene transcription, protein synthesis and growth, it is not

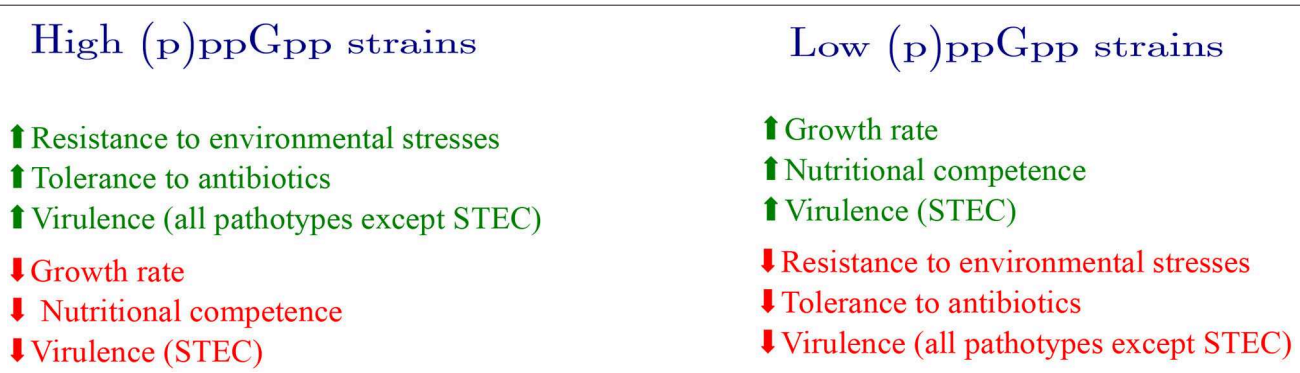

FIGURE 3 | Diversity in (p)ppGpp levels and its consequences. (p)ppGpp intrinsic concentrations are not constant across *E. coli* strains. *E. coli* isolates displaying high (p)ppGpp are benefited under environmental challenges. For instance, these bacteria tolerate higher concentrations of antibiotics and display a higher survival rate when challenged with environmental stresses. Most high-(p)ppGpp pathotypes also show increased ability to colonize host tissues (higher virulence). On the other hand, these strains display lower growth rates and less nutritional competence (ability to utilize alternative carbon sources) than other strains with intrinsically lower (p)ppGpp values. Strains displaying high (p)ppGpp usually carry mutations in the HD domain of SpoT. Mutations in the Synth domain of both RelA or SpoT or in the regulatory domains often cause a reduction in (p)ppGpp levels. The resulting strains display an increased growth rate and higher nutritional competence, but are more sensitive to environmental stresses and antibiotics. STEC is the only known *E. coli* pathotype whose virulence capability benefits from low (p)ppGpp levels.

surprising that modulation of (p)ppGpp is a primary target for evolution.

VARIABILITY IN (p)ppGpp LEVELS AND ITS INFLUENCE ON ANTIBIOTIC SUSCEPTIBILITY

The stringent response has been linked to bacterial tolerance to β-lactam antibiotics in *E. coli*. Tolerance to antibiotics is defined as the ability of microorganisms to survive transient exposure to high concentrations of an antibiotic without a change in the minimum inhibitory concentration (MIC) (Brauner et al., 2016). When both the wild-type strain and *relA* null mutants were exposed to penicillin under amino acid starvation, only the former was able to avoid cell lysis triggered by the presence of the antibiotic (Goodell and Tomasz, 1980; Kusser and Ishiguro, 1985). Moreover, the protective effect of the stringent response against β-lactam antibiotics was reverted by the addition of chloramphenicol (Kusser and Ishiguro, 1985), a well-known inhibitor of the stringent response (Cortay and Cozzone, 1983). In the aforementioned studies (p)ppGpp levels were not directly measured, however, it has been subsequently shown that mecillinam-tolerant mutants accumulated more (p)ppGpp than mecillinam-sensitive strains (Vinella et al., 1992). It became thus evident that high concentrations of (p)ppGpp increase the level of mecillinam tolerance (Joseleau-Petit et al., 1994). The mechanism by which (p)ppGpp confers tolerance to β-lactams was not entirely elucidated. One possibility is that (p)ppGpp acts by inhibiting the biosynthesis of phospholipids. In fact, treatment with cerulenin, an inhibitor of fatty acid biosynthesis, induced β-lactam resistance in the Δ*relA* mutant (Rodionov et al., 1995). In addition, the gratuitous induction of (p)ppGpp accumulation by overexpression of *relA* resulted in the inhibition of phospholipid and peptidoglycan synthesis and in penicillin tolerance (Rodionov and Ishiguro, 1995) supporting the idea that (p)ppGpp mediates penicillin tolerance through the inhibition of phospholipid synthesis (Rodionov and Ishiguro, 1996). However, a more recent study has demonstrated that antibiotic tolerance to β-lactams occurs even in the absence of RelA (Kudrin et al., 2017). *E. coli* cells treated with mupirocin, an isoleucyl-tRNA syntethase inhibitor, displayed increased ampicillin tolerance in the wild-type but not in the relaxed strain. In contrast, the combination of trimethoprim with mupirocin, tetracycline or chloramphenicol significantly increased tolerance to ampicillin in both strains. These data indicate that growth arrest/protein synthesis inhibition can, at least in some cases, increase bacterial tolerance to antibiotics in a (p)ppGpp-independent fashion.

The positive relation between antibiotic tolerance and intrinsic (p)ppGpp concentrations is not restricted to β-lactam antibiotics. The wild-type strain of *E. coli* displayed higher MIC values for trimethoprim, gentamicin and polymixin when compared to the Δ*relA* or Δ*relA* Δ*spoT* mutants (Greenway and England, 1999). The increase in MIC values characterizes an augment in bacterial resistance to these antibiotics (Brauner et al., 2016). Likewise, it has been shown that mutations in the aminoacyl-tRNA synthetase genes *leuS* and *aspS* reduced susceptibility to ciprofloxacin, chloramphenicol, rifampicin, mecillinam, ampicillin, and trimethoprim. Deletion of the *relA* gene in these mutants restored the original MIC values of these antibiotics (Garoff et al., 2018). In another instance bacteria expressing high levels of (p)ppGpp displayed resistance to microcin J25, while strains unable to produce (p)ppGpp were completely sensitive to this antibiotic. In addition, overexpression of *relA* in a strain naturally susceptible to microcin J25 resulted in high MIC values and higher survival rates in killing curves (Pomares et al., 2008).

Several studies have shown a positive correlation between the expression of *hipA*, that encodes a serine/threonine-protein kinase that belongs to a type-II toxin/anti-toxin module,

(p)ppGpp production and the formation of persisters (Korch et al., 2003; Bokinsky et al., 2013; Germain et al., 2013; Kaspy et al., 2013). Persistence is the ability of a subpopulation of an antibiotic-sensitive strain to survive for longer periods of time in the presence of high concentrations of an antibiotic than the majority of the population (Brauner et al., 2016). Some strains are able to form a higher percentage of persisters than others. For instance, strains carrying the *hipA*7 allele formed 100-fold more persistent cells than the wild-type strain when exposed to ampicillin. In the absence of (p)ppGpp (Δ*relA* Δ*spoT* double mutant) the *hipA*7 allele did not confer any advantage regarding antibiotic persistence, suggesting that the high-persistence phenotype elicited by *hipA*7 is (p)ppGpp-dependent. Accordingly, overexpression of *relA* in the *hipA*7 strain increased the frequency of persisters (Korch et al., 2003). On the other hand, overexpression of *hipA* granted resistance to ampicillin, but only in *relA*$^+$ bacteria, as bacteria overexpressing *hipA* but lacking *relA* were considerably more sensitive to ampicillin. Interestingly, the level of (p)ppGpp in the *relA*$^+$ strain overexpressing *hipA* was as high as under amino acid starvation (Bokinsky et al., 2013). Two other studies confirmed the findings of Bokinsky et al. and extended their observations to fluoroquinolone antibiotics (Germain et al., 2013; Kaspy et al., 2013). In addition, these studies suggested a mechanism for *hipA* stimulation of persistence via (p)ppGpp. In their model *hipA* inactivates the glutamyl-tRNA synthetase GltX resulting in the accumulation of uncharged tRNAs which ultimately leads to the activation of RelA and (p)ppGpp synthesis.

Formation of persister cells in bacteria exposed to ofloxacin and ampicillin was also observed upon carbon source transitions, a situation that causes the accumulation of (p)ppGpp (Amato et al., 2013; Amato and Brynildsen, 2015). Deletion of *relA* abolished the formation of ampicillin, but not of ofloxacin persistence, which required the deletion of both *relA* and *spoT*. Furthermore, by controlling the level of (p)ppGpp it has been shown that formation of ampicillin persisters required higher concentrations of (p)ppGpp than formation of ofloxacin persisters. It has also been shown that under conditions of nitrogen starvation *E. coli* accumulates high levels of (p)ppGpp and forms high percentages of persisters when treated with ciprofloxacin, but only in a *relA*$^+$ strain (Brown, 2019).

Integrons are important elements in the dissemination of antibiotic resistance genes. It has been shown that (p)ppGpp plays a role in the regulation of *intI*1, which encodes an integrase protein found in class 1 integrons (Strugeon et al., 2016). Accumulation of (p)ppGpp causes the stalling of RNA-polymerase and the formation of R-loops, which in turn activates the SOS response. The autoproteolysis of the *intI*1 repressor, LexA, ensues resulting in the transcription of *intI*1. *In trans* expression of this gene in the Δ*relA* Δ*spoT* double mutant resulted in reduced *intI*1 promoter activity when compared to the parental strains. Overall, these data indicate that (p)ppGpp helps propagating antibiotic resistance genes through activation of integrase in class 1 integrons.

VARIABILITY IN (p)ppGpp–EFFECT ON BACTERIAL PATHOGENICITY

The expression of virulence-related genes in pathogenic *E. coli* is very well-integrated with (p)ppGpp homeostasis Dalebroux et al. (2010). For instance, (p)ppGpp influences the ability of enterohemorrhagic *E. coli* (EHEC) to colonize the host intestine (Nakanishi et al., 2006). This *E. coli* pathotype secretes a potent cytotoxin—Shiga toxin, that causes serious diseases in humans—bloody diarrhea and HUS (hemolytic uremic syndrome). In addition, bacteria of this pathotype harbor a 35 Kb pathogenicity island known as the Locus of Enterocyte Effacement (LEE), which carries most genes implicated in EHEC intimate adherence (Nguyen and Sperandio, 2012). The passage from the nutrient-rich higher intestine to the nutrient-limited lower intestine triggers the accumulation of (p)ppGpp, which in turn stimulates the transcription of the LEE operons. The EHEC Δ*relA* mutant was unable to induce bacterial adherence or expression of the LEE (Nakanishi et al., 2006). Overexpression of *relA* greatly stimulated the expression of EspB and Tir, two proteins encoded by the LEE and increased the transcription of several LEE genes, implying a positive correlation between (p)ppGpp concentration and EHEC virulence. EPEC (Enteropathogenic *E. coli*) is another diarrheogenic pathotype that carries the LEE, but unlike EHEC it does not produce Shiga toxin. EPEC strains harbor a plasmid (EAF) that encodes both the BFP fimbria associated with bacterial adherence to the intestine cells and the *perABC* operon whose products control the transcription of the chromosomal LEE region (Pearson et al., 2016; Serapio-Palacios and Finlay, 2020). Deletion of *relA* partially impaired EPEC adherence to epithelial cells by diminishing the transcription of the *perABC* operon that controls the expression of the adhesins BFP and intimin (Spira et al., 2014). However, gratuitous overproduction of (p)ppGpp slightly inhibited the expression of *perABC*. The antagonistic effects of (p)ppGpp on *perABC* expression suggests that a fine-tuned concentration of (p)ppGpp is required to maximize EPEC adherence. Even though (p)ppGpp concentrations were not assessed in different EHEC and EPEC isolates the data presented in these studies suggest that the expression of virulence genes and virulence traits are modulated by this alarmone.

Shiga toxin-producing *E. coli* (STEC) is another diarrheogenic pathotype that secretes Shiga toxin, but unlike EHEC, does not harbors a LEE and, consequently, does not display intimate adherence to intestinal cells (Bryan et al., 2015; Joseph et al., 2020). The role of (p)ppGpp in STEC virulence and particularly in toxin production and secretion has been examined in detail. The *stx* genes that encode Shiga toxin were introduced in the STEC genome by means of lambdoid bacteriophages, a phenomenon known as phage lysogenic conversion (Harrison and Brockhurst, 2017). The synthesis and release of Shiga toxin is preceded by the induction of the bacteriophage, a development that eventually results in cell lysis (Waldor and Friedman, 2005; Nowicki et al., 2013). Therefore, the level of Shiga-toxin production and release is directly related to the number of STEC bacteria in a population undergoing phage induction. On the other hand, (p)ppGpp has been shown to inhibit stx phage replication, as the Δ*relA* Δ*spoT* double

mutant displayed a higher degree of phage DNA replication and formed larger plaques on Δ*relA* Δ*spoT* lawns (Nowicki et al., 2013). A subsequent report has shown that intrinsic (p)ppGpp concentration is indeed inversely correlated with Stx toxin production, as STEC strains showing higher cytotoxicity toward Vero cells (the golden standard method for measuring toxin production and STEC virulence) usually contained lower levels of (p)ppGpp (Stella et al., 2017).

The extraintestinal uropathogenic *E. coli* (UPEC) causes recurrent infections in the urinary tract. A critical mechanism of UPEC infection is the ability to invade the bladder cells by means of Type-I fimbriae. The expression of fimbrial genes is controlled by (p)ppGpp and DksA (Aberg et al., 2008). DksA is a transcription factor that binds to RNA polymerase and greatly enhances the effect of (p)ppGpp on transcription regulation (Gourse et al., 2018). (p)ppGpp activates the promoter of *fimB* that encodes a recombinase that specifically inverts the promoter of the *fimAICDFGH* operon. This operon codes for the structural components of the type-I fimbria. By inverting the promoter orientation FimB allows the transcription of the *fimAICDFGH* operon switching the promoter from "off" to "on" state (Eisenstein, 1981). Amino acid starvation or growth arrest caused by bacteria entering the stationary phase increase (p)ppGpp which activates the *fimB* and *fimA* promoters (Aberg et al., 2006). Likewise, *relA* overexpression also induces the transcription from these promoters resulting in the synthesis of Type-I fimbria and the invasion and colonization of bladder cells. Altogether, the data suggest that UPEC strains with high (p)ppGpp intrinsic levels present higher levels of virulence toward the host.

Lastly, (p)ppGpp is directly associated with the pathogenicity of many bacterial species and is required for the full expression of virulence genes (Dalebroux et al., 2010; Kalia et al., 2013). Interestingly, STEC, the only *E. coli* pathotype in which a populational study correlating (p)ppGpp and pathogenicity has been performed stands out as an outlier. STEC toxin production is coupled to phage induction, which is inhibited by (p)ppGpp. By inhibiting phage replication (p)ppGpp acts as a legitimate promoter of bacterial survival.

CONCLUSIONS

The intrinsic concentration of (p)ppGpp in strains of the species *Escherichia coli* is not constant. Rather, the level of (p)ppGpp is been shaped by the historical contingency of bacterial populations. There are two types of evidence that support this assertion: direct assessment of (p)ppGpp in *E. coli* natural isolates and the selection of *relA* and *spoT* mutant alleles in evolution experiments. These data indicate that the genes that govern (p)ppGpp synthesis and degradation are subjected to frequent microevolutionary pressures that will eventually determine the optimal concentration of (p)ppGpp in a population. Given the pleiotropic effects of (p)ppGpp in the cell, adjustments of (p)ppGpp intrinsic concentration should have broad implications on bacterial physiology (**Figure 3**). In fact, intrinsic variations in (p)ppGpp levels differentially affect growth, stress response, virulence and antibiotic resistance. However, the intrinsic levels of (p)ppGpp in *E. coli* natural isolates do not perfectly correlate with the expected phenotypes. For instance, growth rate and (p)ppGpp inverse correlation across the ECOR strains was significant but not perfect, which suggests that the role of this alarmone in growth is intertwined with other regulatory circuits and that bacterial physiology is always more complex than firstly assumed.

AUTHOR CONTRIBUTIONS

BS and KO drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

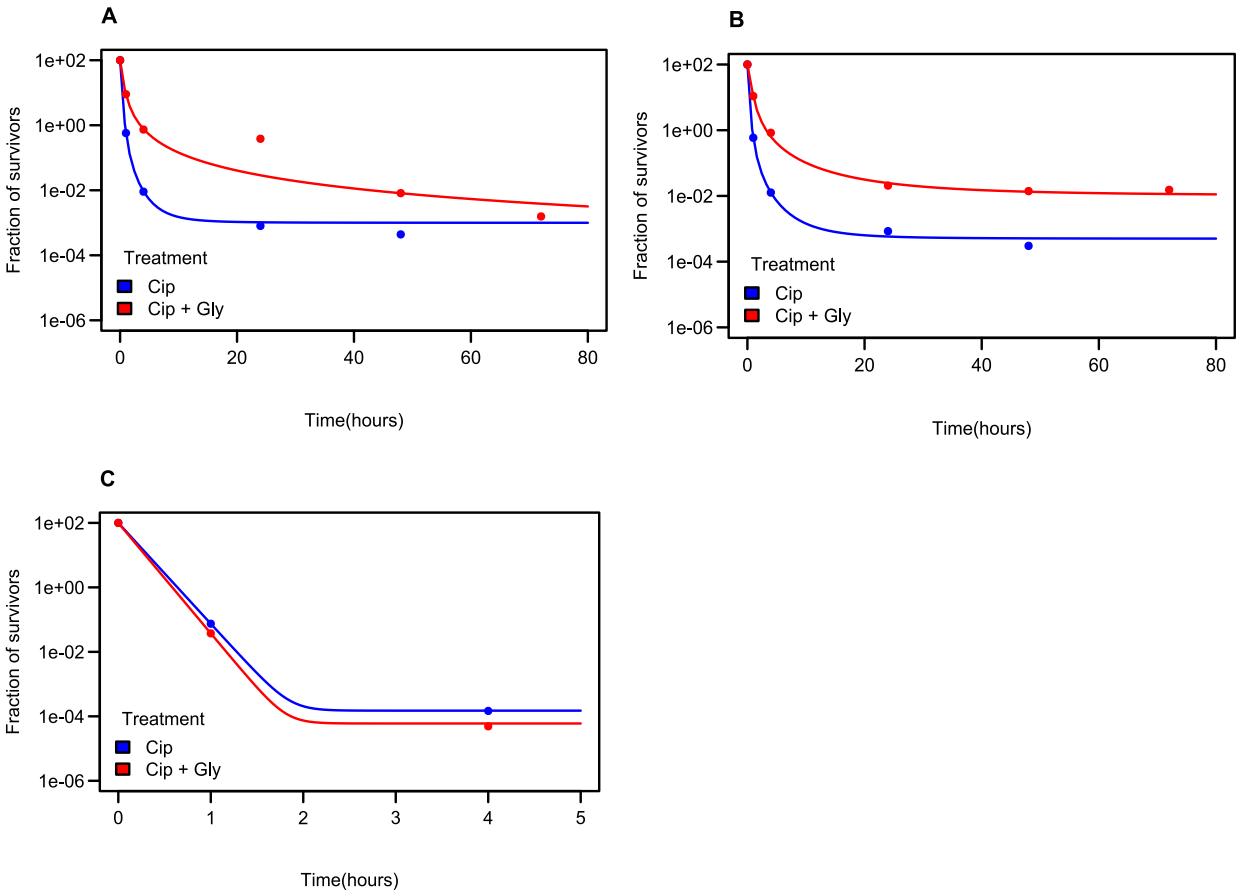

Figure 12 – Non-linear regression of ciprofloxacin. Wild-type strain (A), Δ*relA* mutant (B), and Δ*relA*Δ*spoT* double mutant (C) are shown. MDK_{99} and $MDK_{99.99}$ were extracted from the kill curves. The R *drc* package was used to fit the available models for each curve. The corresponding MDK values were calculated with the Effective Doses (ED) function.

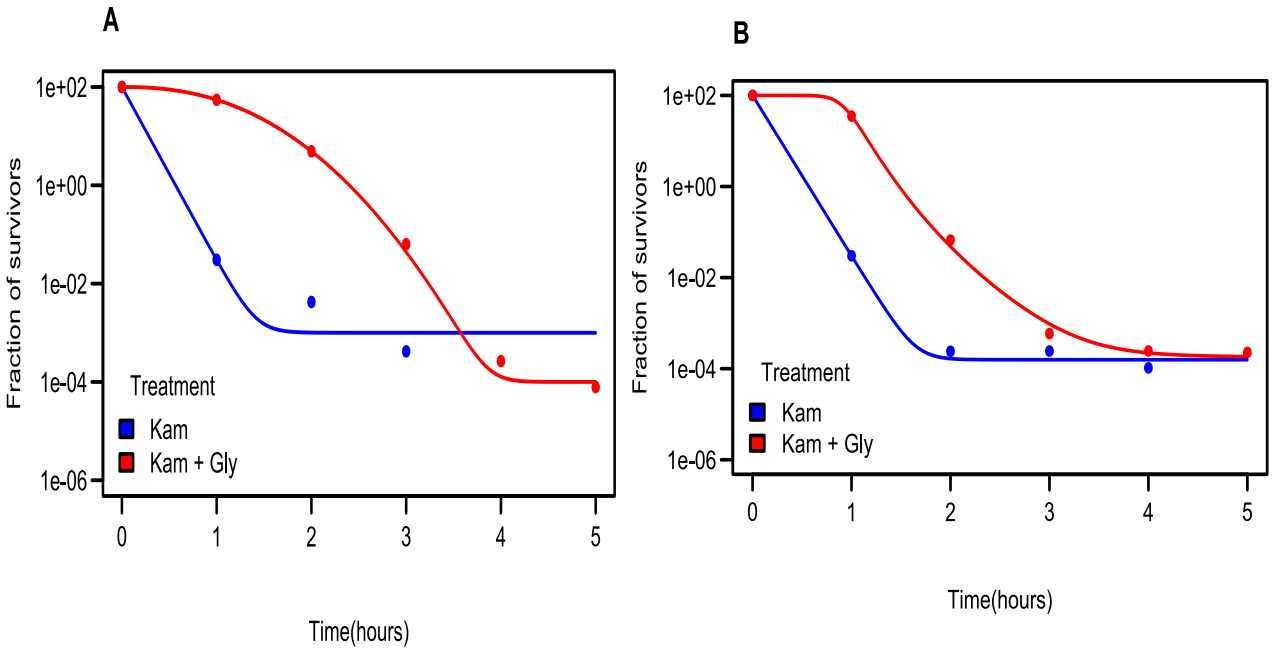

Figure 13 – Non-linear regression of kanamycin. Wild-type strain (A), Δ*relA* mutant (B), and Δ*relA*Δ*spoT* double mutant (C) are shown. MDK_{99} and $MDK_{99.99}$ were extracted from the kill curves. The R *drc* package was used to fit the available models for each curve. The corresponding MDK values were calculated with the ED function.

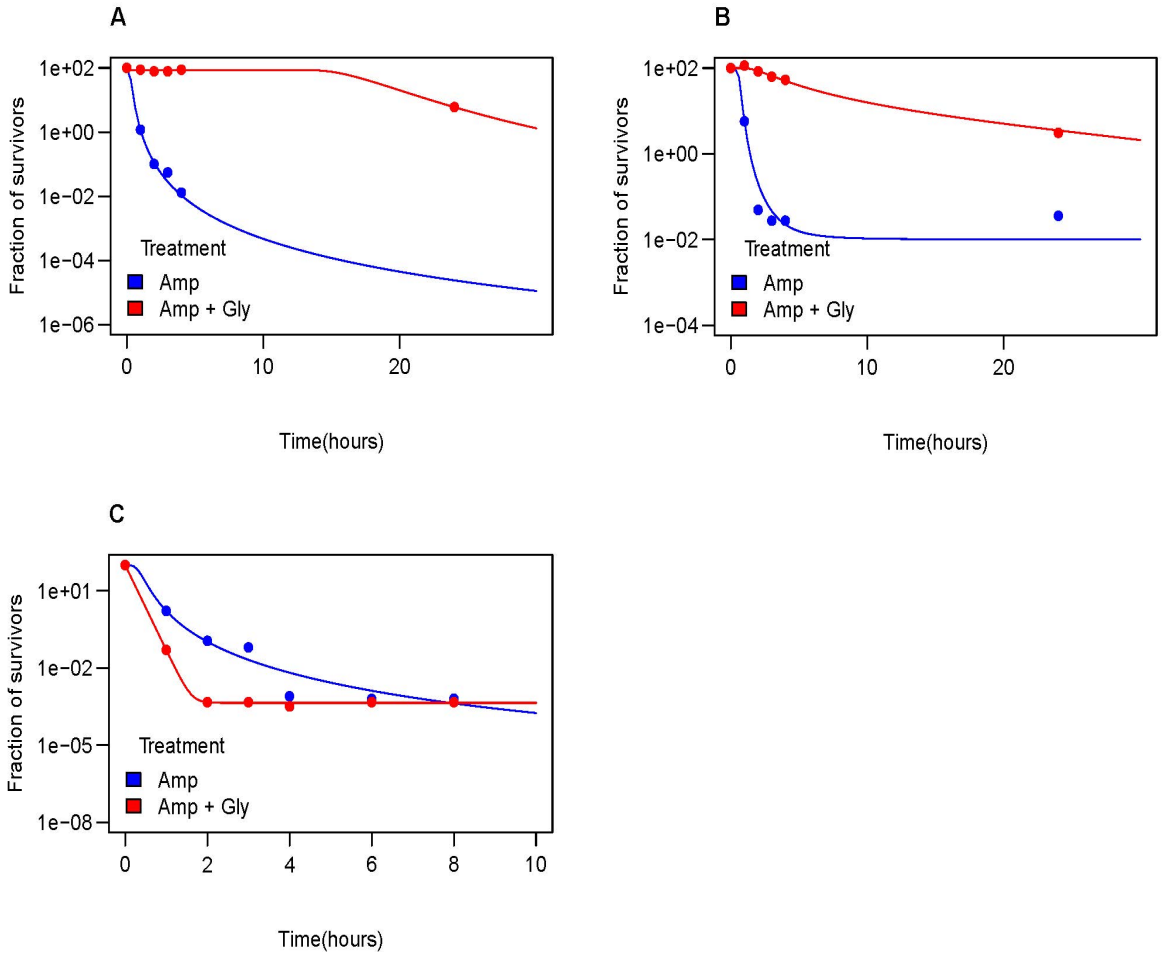

Figure 14 – Non-linear regression of ampicillin. Wild-type strain (A), Δ*relA* mutant (B), and Δ*relA*Δ*spoT* double mutant (C) are shown. MDK_{99} and $MDK_{99.99}$ were extracted from the kill curves. The R *drc* package was used to fit the available models for each curve. The corresponding MDK values were calculated with the ED function.