A paradox of bacterial persistence and antibiotic resistance: chloramphenicol acetyl transferase as a double barrel shot gun

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Abstract

The problematic microbial resistance to antibiotics has led to an increasing interest in bacterial persistence and its impact on infection. Nonetheless, these two mechanisms are often assessed in independent studies, and there is a lack of knowledge about their relation or possible interactions, both at cellular and population levels. This work shows evidence that the insertion of the resistance gene Chloramphenicol Acetyl Transferase (*cat*) together with its cognate antibiotic chloramphenicol (CAM), is capable to modulate *Salmonella* Typhimurium persistence to several antibiotics and decrease its survival. This effect is independent of the antibiotics' mechanisms of action or the locus of *cat*. RelA [p(ppGpp) syntetase] has been shown to be involved in persistence. It was recently proposed that RelA [(p)ppGpp synthetase], binds to uncharged tRNAs, forming RelA.tRNA complexes. These complexes bind to vacant A-sites in the ribosome, and this mechanism is essential for the activation of RelA. In this study, we propose that the antibiotic chloramphenicol blocks the A-site of the ribosome, hindering the binding of RelA.tRNA complexes to the ribosome thus preventing the activation of RelA and (p)ppGpp synthesis, with a consequent decrease in the level of persistence of the population. Our discovery that the concomitant use of chloramphenicol and other antibiotics in chloramphenicol resistant bacteria can decrease the persister levels can be the basis of novel therapeutics aiming to decrease the persisters and recalcitrant infections.

Keywords: persistence, antibiotic resistance, chloramphenicol, chloramphenicol acetyl transferase

Introduction

Persister cells have been gaining increasing attention since they were discovered (Bigger 1944). The phenomenon of bacterial persistence can be defined as the ability of a bacterial subpopulation to survive in the presence of antimicrobial concentrations far above the minimal inhibitory concentration (MIC). Persister cells do not employ a mechanism of resistance; instead, they are metabolically inactive and escape antibiotic action. When these cells are no longer exposed to the antibiotic, they are able to regrow (Gerdes and Maisonneuve 2012).

Persister cells are considered one of the major causes of antibiotic failure and relapsing infections, leading to the overuse of antibiotics and contributing to the emergence of new antibiotic resistances (Fisher et al. 2017). It is still under discussion whether persisters are preexistent in the population or they are induced by exposure to stress. However, most of the scientific community accepts that they are stochastically formed (Balaban et al. 2019). In the last years, several efforts have been made to understand the mechanisms that lie behind the onset of persistent bacteria. Toxin–antitoxin modules have been shown to play a major role in the formation of these cells. These modules are composed by a toxin that inhibits cell growth by interfering with some essential processes in the cell, and an antitoxin that counteracts the effect of the toxin until its own degradation by alternative signaling mechanisms (Gerdes and Maisonneuve 2012).

Antibiotic persistence during infection is gaining particular relevance in *Salmonella* infection research (Hill and Helaine 2019). *Salmonella enterica* serovar Typhimurium is a facultative intracellular pathogen commonly found in the intestinal lumen that is able to infect and survive inside host cells (Lhocine et al. 2015). This successful pathogen is one of the major causes of non-typhoidal salmonellosis in humans (Canals et al. 2019). According to the CDC, *Salmonella* infections are responsible for ∼1.35 million illnesses, 26 500 hospitalizations, and 420 deaths in the United States every year (CDC 2019). In the EU, over 91 000 salmonellosis cases are reported each year. For these reasons, *Salmonella* diseases are life-threatening, and most of them require antibiotic therapy (Fabrega and Vila 2013). Chloramphenicol binds to the ribosomes, inhibiting protein synthesis, and therefore was commonly used for the treatment of severe *Salmonella* infections. However, the emergence of multidrug-resistant strains together with the discovery of novel antibiotics led to the discontinuation of chloramphenicol usage (Rowe et al. 1997). Fluoroquinolones, a class of antibiotics that inhibit DNA gyrase, turn out to be the first-line antibiotics for salmonellosis treatment, among which ciprofloxacin has become one of the most used (Drescher et al. 2019). In the last years, the decrease in ciprofloxacin susceptibility as well as an increase in the number of resistances have been reported, and this was mostly attributed to the indiscriminate usage of fluoroquinolones (Crump et al. 2015). This fact, coupled with the

decrease in *Salmonella* resistance to chloramphenicol caused by its discontinuation (Shrestha et al. 2016, Bhatia et al. 2007), is paving the way to the reintroduction of chloramphenicol in the treatment of salmonellosis.

The genetic background of chloramphenicol resistance was accessed in a collection of *S.* Typhimurium strains, revealing that 37.5% of these cells harbored the gene chloramphenicol acetyl transferase (*cat*) (Nógrády et al. 2005). This gene encodes for a bacterial enzyme that detoxifies chloramphenicol conferring resistance to it. More specifically, the mechanism of action of this enzyme is based on the covalent attachment of an acetyl group from acetyl-CoA to chloramphenicol. The acetylation prevents the antibiotic from binding to the A-site of the ribosome, allowing protein synthesis. Chloramphenicol acetyl transferase is also often used as a selection marker in current genetic transformations (Shaw 1983).

The present report reveals an unknown connection between the expression of *cat*, the antibiotic chloramphenicol, and persistence of *S.* Typhimurium populations exposed to different antibiotics, including fluoroquinolones.

Materials and methods

Bacterial strains and oligonucleotides

All the strains used in this study are listed in Supplementary Table S1 and are isogenic of *S.* Typhimurium SL1344. Strains were stored at $-80°C$ in Luria Bertani broth (LB) supplemented with 10% (v/v) of glycerol and DMSO. Strain CMA667 was obtained by excising the *cat* gene from strain CMA820 using recombination with plasmid pCP20 (Cherepanov and Wackernagel 1995). The oligonucleotides used in this work were synthesized by STAB Vida and are listed in Supplementary Table S1.

Bacterial growth conditions

Salmonella Typhimurium was cultivated in Luria Bertani broth at 37°C and 220 rpm unless otherwise stated. When appropriate, antibiotics were used in the following concentrations: 25 µg ml^{-1} chloramphenicol, 0.3125 µg ml^{-1} ciprofloxacin, 1.25 µg ml^{-1} ofloxacin, and 3.75 µg ml^{-1} cefotaxime.

Minimum inhibitory concentration determination

Determination of MIC was performed by the microdilution method using 96-well round-bottom microtiter plates and bacterial suspensions prepared in LB broth adjusted to a final 1×10^6 CFU ml^{-1}. In each well, 100 µl of fresh medium with increasing antibiotic concentrations were introduced and 100 µl of bacterial suspension were added, reaching a final concentration of 5×10^5 CFU ml^{-1}. Microtiter plates were incubated at 37°C for 18 h, and the OD_{600} was measured in a Spectra-Max Plus 384 microplate reader (Molecular Devices). MIC was recorded as the lowest concentration, where no growth was detected.

Persistence determination

Overnight cultures were diluted to an OD_{600} of 0.1 in fresh medium and grown until an OD_{600} of 0.4. At this time, a sample was taken to determine the number of CFUs prior to exposure to antibiotics. In the next step, the selected antimicrobial was added. Samples were taken after 2, 4, and 6 h of exposure for determination of CFUs. The experimental design is represented in Fig. 7.

RNA extraction

Overnight cultures were diluted to an OD_{600} of 0.1 in fresh LB and grown until the indicated cell densities (growth conditions are detailed in the respective figure legends). Culture samples were collected, mixed with stop solution (10 mM Tris pH 7.2, 25 mM $NaNO_3$, 5 mM $MgCl_2$, 500 µg ml^{-1} chloramphenicol) in a proportion of 1:1, and harvested by centrifugation (10 min, 6 000 g, 4°C). RNA was isolated using the phenol/chloroform extraction method, precipitated with isopropanol, and resuspended in RNase-free water (Viegas et al. 2007). RNA was quantified in a Nanodrop 1000 machine (NanoDrop Technologies), and its integrity was confirmed by running the samples in a 1.5% Agarose gel.

Northern blot

For northern blot analysis, total RNA was separated under denaturing conditions by 8.3 M urea/6% polyacrylamide gel in TBE (Tris/Borate/EDTA) buffer. Transfer of RNA onto Hybond-N$^+$ membranes (GE Healthcare) was performed by electroblotting in TAE (Tris/acetic acid/EDTA) buffer. RNA was UV cross-linked to the membranes immediately after transfer. Membranes were then hybridized with PerfectHyb buffer (Sigma) at 68°C. Signals were visualized by PhosphorImaging (Fujifilm FLA-5100, FUJIFILM Life Science) and analyzed and quantified using ImageQuant software (Molecular Dynamics).

Hybridization probe

Labeling of the riboprobe was performed according to the method previously described (Viegas et al. 2007). The riboprobe was obtained using the primer pair CATFORW/CATREVT7 that can be found in Supplementary Table S2.

Statistical analysis

A statistical analysis was conducted recurring to a model of "Kruskal-Wallis test" of GraphPad Prism 6 software. Differences between means were considered statistically significant for *P*-values ≤ 0.05.

Results

Chloramphenicol affects the level of persistence to fluoroquinolones

The existence of persister cells in a population can be easily detected by the typical biphasic curve observed when bacterial cultures are exposed to antibiotics. The first part of the curve represents the majority of the population that is killed by the antibiotic, while the second part represents the small fraction of the population that remains viable in time (Harms et al. 2016). Due to the extensive use of fluoroquinolones in salmonellosis therapy (Cuypers et al. 2018), we have tested *Salmonella* persistence in the presence of ciprofloxacin and ofloxacin. Minimum inhibitory concentrations (MIC) for these antibiotics were determined. According to the minimum concentration necessary to reach a plateau in killing curves, we have used a dosage between 10× and 30× the MIC for these assays (Table 1). For this, we used two isogenic *Salmonella* Typhimurium SL1344 strains: in the first one, a chloramphenicol-resistance cassette (*cat*$^+$) was inserted in the locus of the previously deleted *bolA* gene (STM0446). The second strain was used as a control, to make sure that the absence of *bolA* was not influencing our results. For that, we used a *bolA* mutant strain in which the resistance cassette for chloramphenicol was removed (*cat*$^-$).
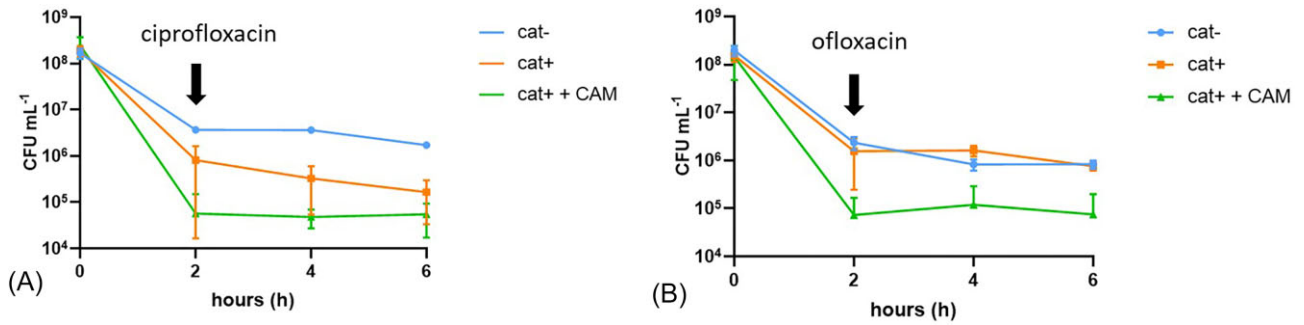
Figure 1. S. Typhimurium killing curve in the presence of ciprofloxacin **(A)** and ofloxacin **(B)** measured in a chloramphenicol-susceptible (cat^-) and in a chloramphenicol-resistant (cat^+) strain in the presence or absence of chloramphenicol. The arrows indicate the time of antibiotic addition. These results were obtained from at least three independent assays.
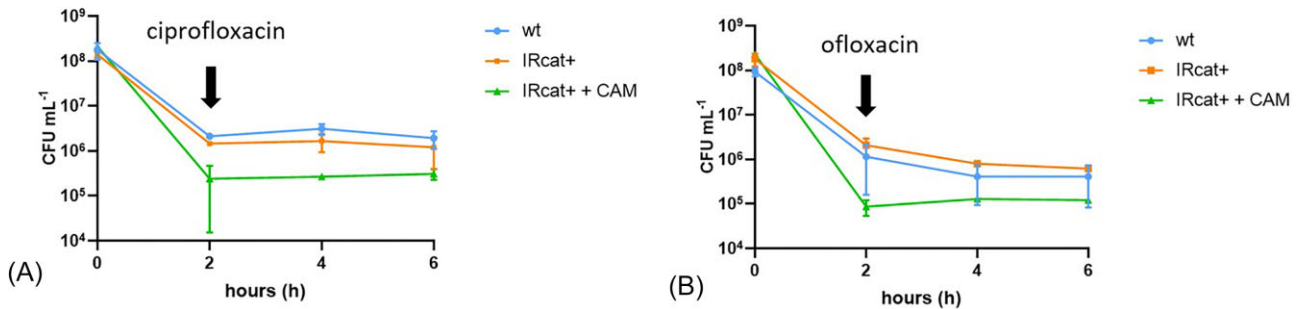

Figure 2. S. Typhimurium killing curve in the presence of ciprofloxacin **(A)** and ofloxacin **(B)** measured in chloramphenicol-susceptible (IRcat^-) and chloramphenicol-resistant (IRcat^+) strains in the presence or absence of chloramphenicol. The arrows indicate the time of antibiotic addition. Results obtained from at least three independent assays.
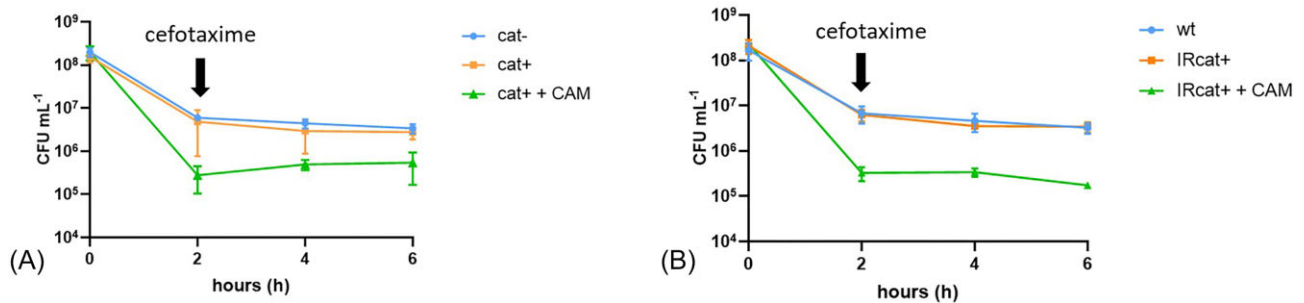

Figure 3. (A)—Survival to cefotaxime measured in chloramphenicol-susceptible (cat^-) and chloramphenicol-resistant (cat^+) strain in the presence or absence of chloramphenicol. **(B)**—Same experiment done in A using strains IRcat^- and IRcat^+. The arrows indicate the time of antibiotic addition. Results obtained from at least three independent assays.

Table 1. MIC of the antibiotics used in this study determined for S. Typhimurium SL1344 and the respective concentration used for persistence assays.

Antibiotic	MIC (μg ml^{-1})	Concentration used for persistence determination (μg ml^{-1})
Ciprofloxacin	0.031	0.312
Ofloxacin	0.125	1.25
Cefotaxime	0.125	3.75
Chloramphenicol	4	25

The S. Typhimurium population was reduced in at least 2 log of viability upon addition of both ciprofloxacin and ofloxacin. Accordingly, the antibiotics exhibited good activity against this pathogen. In case of ciprofloxacin, the presence of cat gene (cat^+) was associated with a lower level of persisters when comparing with the cat^- strain. In the case of ofloxacin the level of persister cells was similar in both strains. We also evaluated the response of the strain cat^+ in the presence of chloramphenicol (CAM) added from the beginning of the growth. Interestingly, for the strain harboring the cat, a higher reduction in viability (3 log) could be observed with both antibiotics when chloramphenicol was also present in the medium. The results obtained can be found in Fig. 1A and B.

Persistence is not affected by the locus of cat

In order to test if the differences observed in persistence were related with the insertion locus of chloramphenicol-resistance cassette, we have used a strain in which the cat gene was inserted in an intergenic region (IR) (Silva et al. 2013) (Fig. 2A and B).
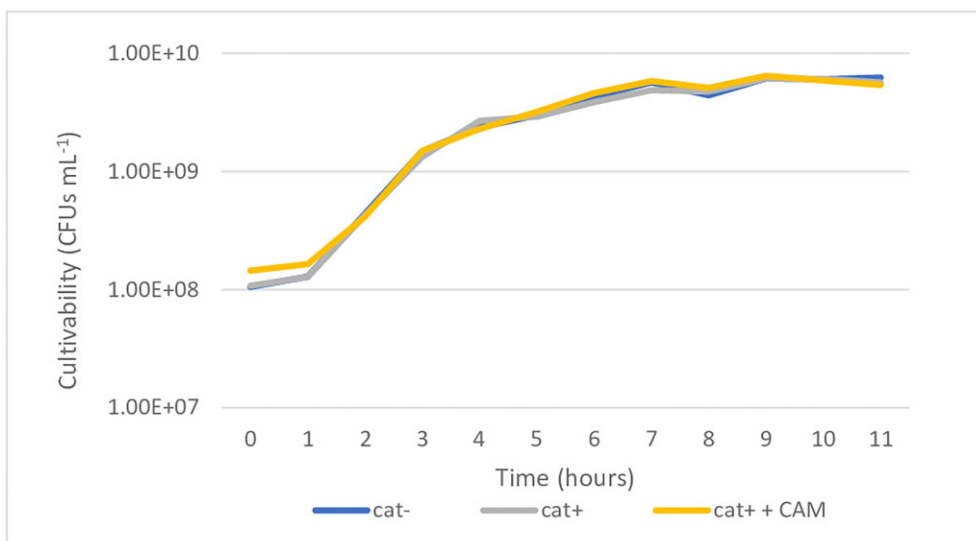
Figure 4. Growth curves determined by colony former units for cat^-, cat^+, and cat^+ + chloramphenicol. The results were obtained from two independent assays.
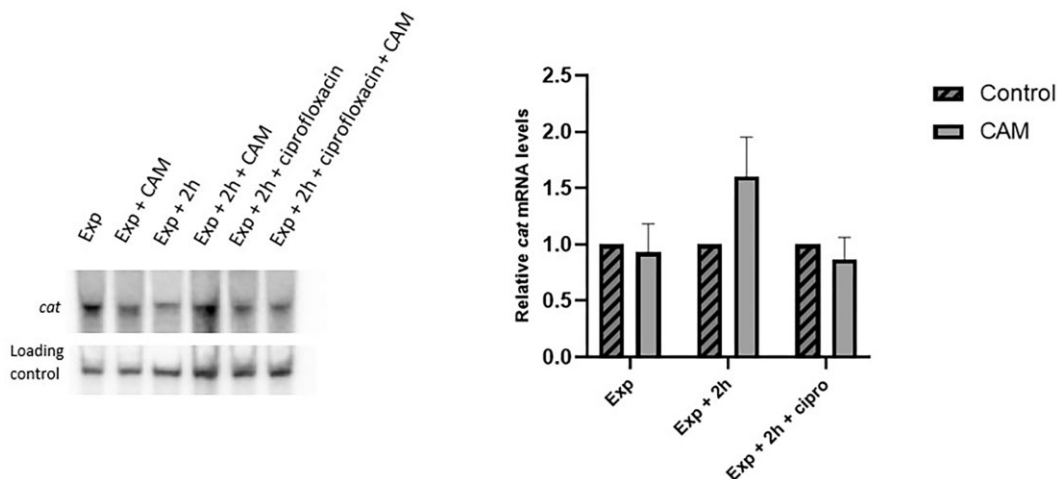

Figure 5. Effect of chloramphenicol on the expression of *cat* transcript. Total cellular RNA was extracted from the cat^+ strain grown in LB at 37°C until OD_{600} of 0.5 (Exp) and 2 h after OD_{600} of 0.5 in the absence (Exp + 2 h) and presence (Exp + 2 h + ciprofloxacin) of ciprofloxacin. The expression levels of *cat* mRNA were determined by Northern blot using 15 µg of total RNA separated in a 6% PAA gel. Membranes were probed for tmRNA as a loading control. The amount of RNA in the strains grown without chloramphenicol is represented in a bar with oblique lines and was set as one (control). The ratio between the RNA amount of each strain and control is represented in a gray bar (relative levels). A representative membrane is shown, and values indicated correspond to the average of five northern blot experiments using RNAs from five independent extractions *(P-value $\leq$ 0.05).
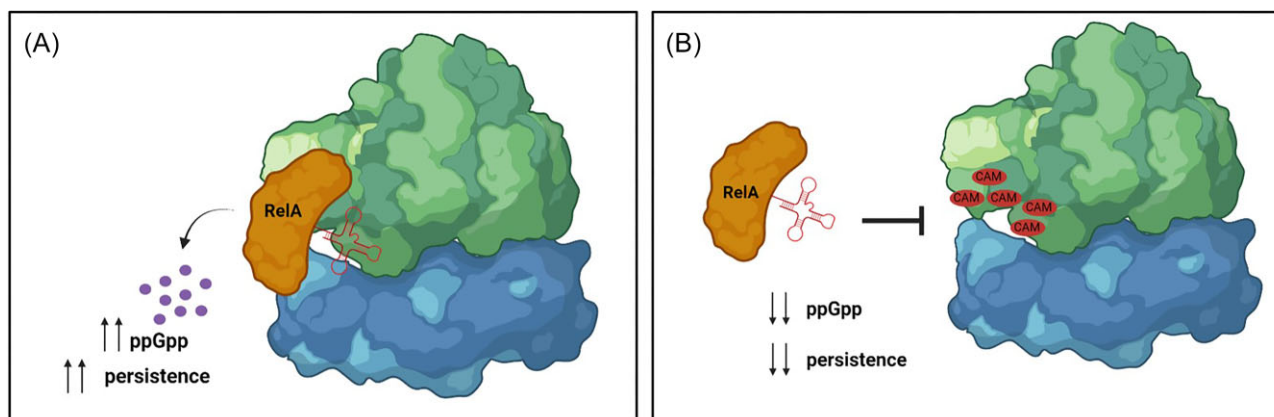

Figure 6. (A)—RelA.tRNA complex binds to the A-site of the ribosome activating RelA's (p)ppGpp synthetic activity leading to persisters formation. **(B)**—Chloramphenicol binds to the A-site of the ribosome preventing the activation of RelA and decreasing the formation of persisters.
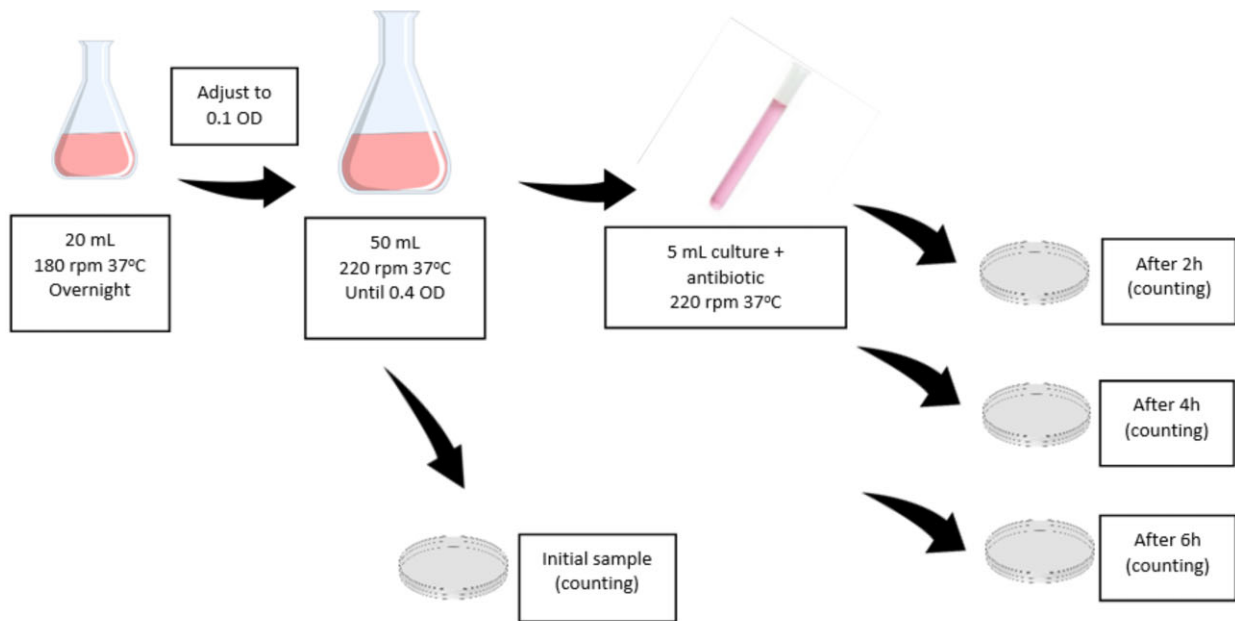
Figure 7. Schematic representation of bacterial persistence determination.

As presented in Fig. 2, changing the location of *cat* in the genome did not cause significant differences in the levels of *Salmonella* persistence. Moreover, there was also a reduction of persister cells when chloramphenicol was present in the medium with both ciprofloxacin and ofloxacin.

Chloramphenicol influences bacterial persistence to different classes of antibiotics

To investigate whether chloramphenicol also influences bacterial persistence upon exposure to antibiotics with different mechanisms of action, we have measured the survival of the strains in response to cefotaxime, an antibiotic that acts on the cell wall. As observed for fluoroquinolones, cefotaxime exhibited good activity against S. Typhimurium. The population was reduced in at least 2 logs of viability. Again, a higher reduction in viability (3 logs) was observed when chloramphenicol was also present in the medium. The same effect was obtained independently of *cat* insertion locus (Fig. 3A and B).

Bacterial fitness is not affected by *cat*

It was previously highlighted the existence of a strict relation between persistence and fitness of bacterial populations (Hong et al. 2012). The authors presented evidence supporting that persistence increases as environmental fitness decreases. For this reason, we hypothesized that the fitness of the strains carrying the *cat* gene could be different from the others, thus explaining the differences previously observed (Fig. 1). To access this, we conducted an experiment in which the colony former units of each strain were measured during its growth in Luria Bertani (LB) broth. The *cat⁻* exhibited a growth rate very similar to that of *cat⁺* both in the presence or absence of chloramphenicol (Table 2). Moreover, the strains revealed a very similar growth profile reaching the several stages of growth at the same time (Fig. 4). Therefore, the fitness obtained was similar for all the strains and conditions used.

Table 2. Growth rates determined by colony former units for cat^-, cat^+, and cat^+ + chloramphenicol.

Allele	Growth rate (μ h^{-1})
cat^-	1.17
cat^+	1.22
cat^+ + chloramphenicol	1.10

The results were obtained from two independent assays.

The expression of *cat* is affected by chloramphenicol and ciprofloxacin and it is dependent on the growth phase

The expression of genes that confer resistance to antibiotics are known to be tightly regulated, being in some cases, dependent on the presence of the cognate antibiotic (Mak et al. 2014). In order to investigate the influence of chloramphenicol in the expression of the *cat* gene, we have quantified the transcription levels of *cat* in the *cat⁺* strain in the presence or absence of chloramphenicol in exponential phase of growth. The results showed that there were no significative differences in *cat* mRNA expression. However, following 2 h of growth in both conditions, the expression of the *cat* transcript was higher when the bacteria were exposed to chloramphenicol. Interestingly, when ciprofloxacin was also present in the media the transcription levels of *cat* mRNA were not significantly affected (Fig. 5).

Discussion

In the last years, bacterial persisters have been gaining recognition, since many recalcitrant infections are associated with bacterial persistence (Fisher et al. 2017, Helaine and Kugelberg 2014). However, not much is known regarding the relation between persistence and resistance, both at cellular and population levels.

Our data indicate that the insertion of the *cat* gene in the S. Typhimurium genome together with its cognate antibiotic, chloramphenicol, decreases the persistence levels of the popu-

lation to different antibiotics. At the beginning of this study, we intended to test persistence to fluoroquinolones since they are extensively used for *Salmonella* treatment (Cuypers et al. 2018). This class of antibiotics act based on the inhibition of DNA gyrase, a bacterial enzyme that belongs to a class known as topoisomerases. It is involved in the control of topological transitions of DNA, introducing negative supercoils into closed-circle double-stranded DNA (Reece and Maxwell 1991). Later, we have also investigated the effect of cefotaxime, an antibiotic with a different mechanism of action, in *Salmonella* persistence. However, the mechanism of action of cefotaxime affects the cell wall. Interestingly, the results consistently showed a decrease of *Salmonella* persistence with both classes of antibiotics, meaning that the results obtained with chloramphenicol are not dependent on the mechanism of action of the antibiotics.

When working with insertions of foreign genes in an organism, it is always a concern that the construction itself may cause an effect in the cell rather than the gene. Since *cat* was inserted in the locus of the *bolA* gene, the location of *cat* was moved to an intergenic region. The results showed the same effect in persistence was observed independently of the locus (Fig. 2A and B).

Hong and colleagues reported that bacterial persistence increases as environmental fitness decreases (Hong et al. 2012). Therefore, it would be expected that the strain carrying the *cat* gene would have a higher fitness both in the presence and absence of chloramphenicol in the medium. Contrarily to what was expected, the strain *cat*[+] presented an environmental fitness very similar to the *cat*[−]. In fact, the mechanisms involved in the control of bacterial persistence are diverse (Harms et al. 2016), and other factors than fitness could be causing the decrease in persister cells.

In order to gain additional insights about what could be possibly happening during the persistent state, we have quantified the expression of *cat* transcript using *cat*[+] strain. The quantification revealed that 2 h after exponential phase (early stationary), *cat* transcript is significantly more expressed when chloramphenicol is present in the medium. However, when cells were also exposed to ciprofloxacin (persistence state), a decreased *cat* mRNA expression is observed, when compared to the control without ciprofloxacin (P-value ≤ 0.05). It could be that the decreased *cat* expression with ciprofloxacin could be causing a loss of chloramphenicol resistance and somehow lead to a decrease in the persister levels. However, even with a lower *cat* expression, the bacteria could still survive to chloramphenicol.

Toxin–antitoxin (TA) modules have been extensively described as mechanisms that explain the formation of bacterial persister cells. Guanosine tetraphosphate (ppGpp) is a stress-response alarmone, whose levels are controlled by the RelA–SpoT Homologs (RSH), a highly conserved family of proteins (Ronneau and Hallez 2019). ppGpp has a crucial role in the regulation of several activities in the cell (Kundra et al. 2020), and it is synthesized in stationary phase of growth, in response to stress or nutrient depletion. It is not essential for persistence (Chowdhury et al. 2016), but the most accepted models that currently explain the formation of bacterial persisters point to ppGpp as the responsible alarmone that triggers the formation of persisters (Song and Wood 2020, Winther et al. 2018).

Despite the divergence of results in studies that address the chloramphenicol interaction with the ribosome, they are unanimous that chloramphenicol binds to the A-site of the ribosome (Bulkley et al. 2010). Winther et al. (2018) proposed a model by which RelA, the (p)ppGpp synthetase, interacts with uncharged tRNAs and that this RelA.tRNA complex binds to the A-site of the ribosome. This process is essential for the activation of RelA's (p)ppGpp synthetic activity.

The well-known heterogeneity of bacterial populations implies that in a susceptible population, there is always a small fraction of bacteria that can survive to an antibiotic due to persistence, tolerance, or even spontaneous mutations. The opposite must also be true. When considering a chloramphenicol-resistant population being treated with chloramphenicol, it would not be surprising that some of their ribosomes would still be affected by the antibiotic. In fact, in order to every ribosome escape chloramphenicol action, it would be required that 100% of chloramphenicol molecules were acetylated, which is a very unlikely scenario. A fraction of bacteria that survive the antibiotic can undergo some residual cell division during the initial period after the start of the treatment. Some chloramphenicol molecules can bind to their ribosomes, resulting in an overall reduction of CFUs. Despite this, we believe that the population has a significant part of its ribosome A-sites blocked by chloramphenicol, thus hampering the activation of RelA and reducing the persistence levels. Taking into account our results, we propose a model, in which in presence of chloramphenicol, the tRNA.RelA complexes compete with chloramphenicol for the A-site of the ribosome leading to a decreased ppGpp synthesis and therefore lower persistence (Fig. 6). Our discovery that the concomitant use of chloramphenicol and other antibiotics in chloramphenicol-resistant bacteria can decrease the persister levels can be the basis of novel therapeutics aiming to decrease the persisters and recalcitrant infections.

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Supplementary data

Supplementary data is available at *FEMSML Journal* online.

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