



## Research paper

# An alternative penicillin-binding protein involved in *Salmonella* relapses following ceftriaxone therapy

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## ABSTRACT

**Background:** *Salmonella* causes intracellular infections in humans. Besides quinolones, third generation cephalosporins are first line drugs used for salmonellosis therapy. An unresolved anomaly of this practice involves high relapse rates associated to quinolone- or cephalosporin-susceptible *Salmonella* isolates in patients that are discharged clinically following initial recovery. Reduced drug accessibility to intracellular locations has been hypothesized to impair pathogen eradication although supporting evidence is lacking *in vivo*. Here, we uncover a novel penicillin-binding protein as the first *Salmonella* factor likely contributing to relapse following beta-lactam, mainly ceftriaxone, therapy.

**Methods:** We used *Salmonella enterica* serovar Typhimurium mutants lacking the alternative penicillin-binding proteins PBP2<sub>SAL</sub> or PBP3<sub>SAL</sub>. Affinity of PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> for beta-lactam antibiotics was tested. Relapse after ceftriaxone therapy was analysed in the murine typhoid model.

**Findings:** *S. Typhimurium* does not express PBP2<sub>SAL</sub> or PBP3<sub>SAL</sub> in the Mueller-Hinton medium used for susceptibility testing. The pathogen produces these PBPs in response to acidic pH and nutrient limitation, conditions found in phagosomes of mammalian cells. PBP3<sub>SAL</sub> has low affinity for beta-lactams, even at acidic pH. *In vitro* susceptibility to ceftriaxone at low pH is strongly reduced. *S. Typhimurium* lacking PBP3<sub>SAL</sub> was unable to cause relapse in mice following ceftriaxone therapy.

**Interpretation:** The reduced capacity of ceftriaxone to clear *S. Typhimurium in vivo* is favoured by a switch in beta-lactam targets. This switch, involving production of the less-susceptible PBP3<sub>SAL</sub>, remains invisible for standard procedures used in clinical therapy. We conclude that eradication of salmonellosis will be possible only upon targeting of PBP3<sub>SAL</sub> with novel drugs.

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## 1. Introduction

*Salmonella enterica* has an enormous impact in human health causing high morbidity and mortality [1,2]. The evidence accumulated in the last decades has demonstrated that *S. enterica* infections progress intracellularly with bacteria located inside epithelial cells and phagocytes [2]. Intracellularity has marked the evolution of *S. enterica* as pathogen following the acquisition of novel genes that enabled the bacterium to invade mammalian cells and to survive and proliferate inside host cells [3,4].

Most intracellular infections impose a barrier for effective antimicrobial chemotherapy due to the poor penetration of commonly used

antibiotics [5]. *In vitro* assays show that beta-lactams do not accumulate enough in intracellular locations to reach bactericidal effects [6]. Treatment failure of intracellular infections might also relate to other factors like: adaptive variations in the physiology of intracellular bacteria [2]; exposure of the pathogen to heterogeneous host stresses in distinct cell types leading to emergence of persistence phenotypes [7]; and, inhomogeneous distribution of the drug in intracellular locations [8]. These factors are thought to contribute the lack of effectiveness following antibiotic therapy in relapses associated to drug-susceptible isolates.

The epidemiology of *S. enterica* in recent years shows an alarming increase in antibiotic resistance in both typhoid and non-typhoid serovars [9,10]. Resistance is frequently reported to first choice antibiotics used to treat human salmonellosis such as quinolones and third generation cephalosporins. These drugs replaced chloramphenicol, trimethoprim-sulphamethoxazole and ampicillin due to the

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## Research in context

### Evidence before this study

In an extensive search of literature about relapses and antibiotic resistance published up to March 2020, the available information refers to widespread resistance to third generation cephalosporins in typhoid and non-typhoid *S. enterica* serovars, and randomized controlled trials in which failure of the antibiotic therapy and relapse rates with the original isolate were estimated. The most representative original and review articles are cited in this study. Importantly, in early studies of 2002 involving randomized controlled trials using third generation cephalosporins in typhoid fever patients, principally ceftriaxone and cefixime, the rates of treatment failure were estimated 5 to 10 per cent, with relapse rates that were 3 to 6 per cent. In another study of 2016 performed in Nepal and involving uncomplicated enteric fever, third generation cephalosporins were associated with slow clinical improvement and high relapse burden. In a 2016 study in Japan, the relapse rate with intravenous ceftriaxone exceeded 10%. In a review published in 2011, relapse rates reaching up to 17% are reported in diverse randomized clinical trials involving typhoid fever patients. Relapses caused by drug-susceptible isolates have also been reported in cases of combined therapies involving quinolones and cephalosporins. Insufficient effect of beta-lactam antibiotics *in vivo* was attributed to low intracellular concentrations, although these observations are restricted to *in vitro* infection assays based on tissue cultured mammalian cells.

### Added value of this study

*Salmonella* is recognized as one of the main bacterial pathogens causing asymptomatic and chronic infections in humans and livestock. Clinical evidence also associates drug-susceptible *Salmonella* isolates to high relapse rates in human patients that were clinically discharged after an antibiotic treatment that resulted in remission of symptoms. These relapses are attributed to a reduced antibiotic penetration into the intracellular locations where the pathogen resides, although no evidence has yet formally obtained *in vivo* for such potential cause. To our knowledge, our study provides the first clue explaining this clinical anomaly for a *Salmonella* enzyme that is involved in cell wall metabolism -the penicillin binding protein PBP3<sub>SAL</sub>- promoting pathogen persistence in host tissues after antibiotic chemotherapy. Targeting of this alternative PBP3<sub>SAL</sub> is therefore needed to successfully treat salmonellosis patients and eradicate this important pathogen.

### Implications of all the available evidence

The data obtained in the assays involving binding of beta-lactams to PBP3<sub>SAL</sub> indicate that this enzyme, which replaces PBP3 in intracellular *Salmonella*, has low affinity for ceftriaxone, resulting in reduced *in vitro* susceptibility and a significant increase in relapse after therapy in an animal model. These findings suggest that modifications of current cephalosporins to better inhibit PBP3<sub>SAL</sub> might have consequences in the success in treatment of human infections.

symptoms with third generation cephalosporin therapies [13]. Thus, in randomized controlled trials of third generation cephalosporins in typhoid fever, principally ceftriaxone and cefixime, the rates of treatment failure were 5 to 10 percent, with relapse rates that were 3 to 6 percent [14]. In other studies of uncomplicated enteric fever, third generation cephalosporins are generally associated with slow clinical improvement and high relapse burden [15]. In some series, the relapse rate with intravenous ceftriaxone, a first-choice cephalosporin, exceeded 10 percent [16]. The causes sustaining these high relapse rates remain unknown.

In this study, we characterize in serovar Typhimurium the production in intracellular bacteria of two pathogen-specific penicillin binding proteins (PBPs), natural targets of beta-lactam antibiotics. These alternative PBPs are expressed by the pathogen inside the mammalian cell and bind current beta-lactams with low affinity. Here, we demonstrate that these two factors, involving niche-dependant expression and reduced antibiotic binding, contribute to the failure of ceftriaxone treatment in terms of pathogen eradication.

## 2. Materials and methods

### 2.1. Bacterial strains, media and growth conditions

Bacterial strains and plasmids used are listed in Table S1. All *S. enterica* serovar Typhimurium (*S. Typhimurium*) strains used are derivatives of virulent wild-type strain SV5015, an His<sup>+</sup> prototroph of strain SL1344 [17]. Strains were grown in Luria-Bertani (LB) broth, composed of 1% (w/v) casein peptone, 0.5% (w/v) yeast extract and, 0.5% (w/v) sodium chloride; or, phosphate-carbon-nitrogen (PCN) minimal medium [18]. When necessary, pH was buffered with 80 mM MES [2-(N-morpholino) ethanesulfonic acid]. The composition of PCN medium is: 4 mM Tricine [N-[Tris (hydroxymethyl) methyl]glycine], 0.1 mM FeCl<sub>3</sub>, 376 μM K<sub>2</sub>SO<sub>4</sub>, 50 mM NaCl, 15 mM NH<sub>4</sub>Cl, 1 mM MgSO<sub>4</sub>, 1 μM CaCl<sub>2</sub>, 0.4% (w/v) glucose, 0.4 mM inorganic phosphate (P<sub>i</sub>), and micronutrients [18]. The 80 mM MES solution was adjusted to the desired pH value with NaOH and used to buffer the medium. To grow strains harbouring plasmids, the media were supplemented with 100 μg/mL ampicillin or, 10 μg/mL chloramphenicol. For kanamycin, the antibiotic was used at 30 μg/mL or 60 μg/mL in neutral (7.0) or acidic (4.6) pH, respectively.

### 2.2. Construction of *S. Typhimurium* mutants lacking PBPs

To delete the native copy of *mrdA* encoding PBP2, a Kan<sup>R</sup> cassette was used. This cassette contained upstream and downstream regions (50 bp) of *mrdA* including some codons of the gene due to short distance between *mrdA* and the flanking gene *mrdB*. The Kan<sup>R</sup> cassette was amplified from pKD13 template plasmid [19] using primers KO STBP2 FW/ KO STBP2 RV (Table S2). Gene replacement was done using Lambda Red-mediated recombination [20]. After electroporation, Super Optimal Broth with Catabolite repression (SOC) medium buffered at pH 4.6 with MES was added and cells were grown for 3 h. Recombinants were selected on LB agar pH 4.6 supplemented with 60 μg/mL kanamycin. Primers pbp2-flanking FW and pbp2-flanking RV (Table S2) were used to confirm the gene deletion. The construction was verified by sequencing. Deletion of the SL1344\_1845 gene, encoding PBP2<sub>SAL</sub>, was done with the primers KO PBP2\* FW/ KO PBP2\* RV (Table S2) using essentially similar inactivation procedure as for *mrdA* but selecting recombinants on LB agar pH 7.4 with 60 μg/mL kanamycin. Primers FL-2\* Fw and FL-2\* Rv (Table S2) were used to confirm gene deletion. *S. Typhimurium* mutants lacking PBP3 or PBP3<sub>SAL</sub>, have been published elsewhere [21].

emergence of resistance in mobile genetic elements and, in the case of chloramphenicol, to undesirable haematologic side effects [11, 12]. In addition, worrisome relapse rates are also repeatedly reported in salmonellosis patients discharged clinically after remission of

### 2.3. Plasmid constructions involving PBP expression for Bollicin-650 binding assays

*mrda* (PBP2) and *SL1344\_1845* (PBP2<sub>SAL</sub>) genes were cloned in the expression vector pFUS-P<sub>ara</sub> [22]. The *mrda* and *SL1344\_1845* genes were amplified using primers fwSpeI-PBP2/revSpe-PBP2 and primers fwSpeI-PBP2\*/revSpeI-PBP2\* (Table S2), respectively, digested with SpeI and ligated into pFUS-P<sub>ara</sub> [21]. The final constructs were sequenced to ensure no undesired mutation. To construct pAC::PBP2<sub>SAL</sub> and pAC-HIS::PBP2<sub>SAL</sub>, SpeI-SpeI fragments containing their respective coding sequences were ligated into pAC-P<sub>lac</sub> and pAC-6xHIS-P<sub>lac</sub>, respectively. To construct pAC-HIS::PBP2, the coding sequence of *mrda* (SpeI-SpeI fragment) was ligated also into pAC-6xHIS-P<sub>lac</sub>. Generation of plasmids expressing PBP3-6xHIS and PBP3<sub>SAL</sub>-6xHIS variants has been previously described [21].

### 2.4. Bocillin-650 binding assays to detect PBPs

Subcellular fractions containing bacterial membranes were prepared from 100 mL cultures of *E. coli* strains SP4500 [*mrda* (ts)] [23] or RP41 [*ftsI*(ts)] [23] expressing PBP2/PBP2<sub>SAL</sub> or PBP3/PBP3<sub>SAL</sub>, respectively, from a *lac* inducible promoter. At OD<sub>600</sub> = 0.3, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression of the PBP of interest. After 2 h induction, bacteria were pelleted by centrifugation (4400 x g, 10 min, 4 °C), washed in 0.1 M phosphate buffer adjusted to pH 7.4, 5.8 or 4.6 and frozen at -80 °C. Preparation of lysates and binding assays with Bocillin-650 were done as previously described [21].

### 2.5. DNA techniques

All primers used in this study are listed in Table S2. PCR was performed using Q5 polymerase (New England Biolabs) according to manufacture instructions. PCR fragments were purified using the Speedtools PCR Clean-Up kit (Biotools).

### 2.6. Immunoblot analyses

The following antibodies were used as primary antibodies: mouse monoclonal anti-Flag (1:5000; Sigma), mouse monoclonal anti-6xHis (1:2500), rabbit polyclonal anti-PBP2 (1:1000; lab collection), rabbit polyclonal anti-PBP3 (1:1000, lab collection) [21]. Goat polyclonal anti-mouse (1:20,000) or anti-rabbit IgG (1:20,000) conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies. Proteins were separated by 4-20% precasted polyacrylamide gels, 7% tricine gels, or 6% acrylamide gels.

### 2.7. Synthesis of PBPs during a shift from pH 7.4 to 4.6

*S. Typhimurium* strain MD5064 (PBP2<sub>SAL</sub>::3xFlag PBP3<sub>SAL</sub>::3xFlag) (Table S1) was grown overnight at 37 °C in PCN pH 7.4 medium. Cultures were prepared to a starting optical density at 600 nm (OD<sub>600</sub>) of 0.05 in PCN pH 4.6 medium. Bacteria were collected at different times for protein analysis by western blot, as described [21].

### 2.8. Synthesis of PBPs in macrophage and mouse infections

The murine macrophage line RAW 264.7 (Cell Lines Service) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% (v/v) foetal bovine serum (FBS) and 4 mM glutamine at 37 °C in a 5% CO<sub>2</sub> atmosphere. RAW 264.7 macrophages were seeded in p150 plates and pre-cultured for 24 h before infection in the presence of 2.5 ng/mL interferon-γ. For infection, culture medium was replaced by fresh medium without interferon-γ and macrophages exposed to *S. Typhimurium* strain MD5064 (PBP2<sub>SAL</sub>::3xFlag PBP3<sub>SAL</sub>::3xFlag) (Table S1) at a multiplicity of infection (MOI) of 10:1 during 10 min.

Macrophages were washed with phosphate-buffered saline (PBS) supplemented with 0.5 mM MgCl<sub>2</sub> and 0.9 mM CaCl<sub>2</sub>. Extracellular non-phagocytosed bacteria were killed by incubation in medium containing gentamicin (100 μg/mL for 1 h, followed by 10 μg/mL for the remaining incubation). To determine levels of PBPs produced by intracellular bacteria the infected macrophages were lysed at 2 and 8 hpi, as described [21]. Production of PBPs in bacteria colonizing spleen of BALB/c mice was monitored with *S. Typhimurium* strain MD5064 (PBP2<sub>SAL</sub>::3xFlag PBP3<sub>SAL</sub>::3xFlag), as described [21].

### 2.9. Spot dilution and antibiotic susceptibility assays

Bacterial viability was monitored after spotting 5 μL of 10-fold serially dilutions of the cultures onto agar plates containing PCN medium at pH 7.4 or 4.6, with or without antibiotic added. Antibiotic susceptibility was also tested using MIC strips (MIC Test Strip; Liofilchem) in PCN agar plates containing 0.01% yeast extract (w/v) at neutral or acid pH.

### 2.10. Relapse experiments

BALB/c mice were challenged intraperitoneally with a dose of ~5 × 10<sup>3</sup> cfu of *S. Typhimurium* wild-type strain SV5015 or mutant derivatives lacking PBPs of interest (PBP2, PBP3, PBP2<sub>SAL</sub> or PBP3<sub>SAL</sub>) (Table S1). One group of infected mice was left non-treated. In the second infected group, ceftriaxone was administrated orally at a dose of 400 mg/kg per 24 h for three days. This antibiotic therapy controlled infection (no symptoms after 3 days) although all mice of this group showed relapse after 7 days of therapy discontinuation. Mice were sacrificed at 3 days (group with no antibiotic therapy) and 10 days (group showing relapse after antibiotic therapy) for bacterial counts in spleen and liver, as previously described [21].

### 2.11. Ethical regulations

Animal experiments were performed in accordance with the guidelines of the European Commission for the handling of laboratory animals (directive 2010/63/EU) and approved by the Environment Council (Consejería de Medio Ambiente) of the Regional Government of Madrid, under license PROEX 110/19.

### 2.12. Statistical analysis

We analysed data with GraphPad Prism, version 8.0, software (GraphPad Inc. San Diego, CA). In the mice experiments, total bacterial numbers per organ were normalized to the median of the group infected with wild-type bacteria. Significant differences amongst these normalized values for each of the groups challenged with the different bacterial mutants (WT, ΔPBP2, ΔPBP3, ΔPBP2<sub>SAL</sub>, ΔPBP3<sub>SAL</sub>) were determined by Kruskal-Wallis multiple comparison tests, using the group of mice challenged with wild-type bacteria as control. Significance was established at *P* values ≤ 0.05.

## 3. Results

### 3.1. *S. Typhimurium* replaces PBP2 and PBP3 by the alternative PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> in infection conditions

The clinical bases accounting for therapy failure in cases of salmonellosis associated to drug-susceptible isolates remain unknown [11, 14]. Third generation cephalosporins, commonly used to treat salmonellosis, target penicillin-binding proteins (PBPs) involved in cell wall metabolism. We reasoned that *S. enterica*, as intracellular pathogen, could persist during the initial antibiotic therapy by modifying expression of alternative PBPs in the intracellular niche of mammalian cells. The first insight supporting this hypothesis came

with the comparison of genome sequence from *S. enterica* serovar Typhimurium reference strain SL1344 (genome sequence NC\_016810.1) and that of the non-pathogenic *Escherichia coli* K-12 strain MG1655 (genome sequence NC\_000913.3). This comparative analysis showed the presence in the pathogen of several genes encoding enzymes involved in peptidoglycan metabolism that are absent in *E. coli*. amongst these are PBP2<sub>SAL</sub>, which *S. Typhimurium* uses to replicate inside mammalian cells [21,24], and a second pathogen-specific PBP that we named PBP2<sub>SAL</sub> due to its high homology to the shape-maintaining PBP2 (63% identity at the amino acid level) (Fig. S1). BLAST analyses demonstrated that the genes of *S. Typhimurium* strain SL1344 encoding PBP2<sub>SAL</sub> (SL1344\_1845) and PBP3<sub>SAL</sub> (SL1344\_1765) are conserved in all species, subspecies, and serovars of the *Salmonella* genus. Importantly, in addition to PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub>, all members of the *Salmonella* genus have functional the "classic" PBP2 and PBP3, which are targets of beta-lactams and are responsible for peptidoglycan synthesis during cell elongation and cell division, respectively [25].

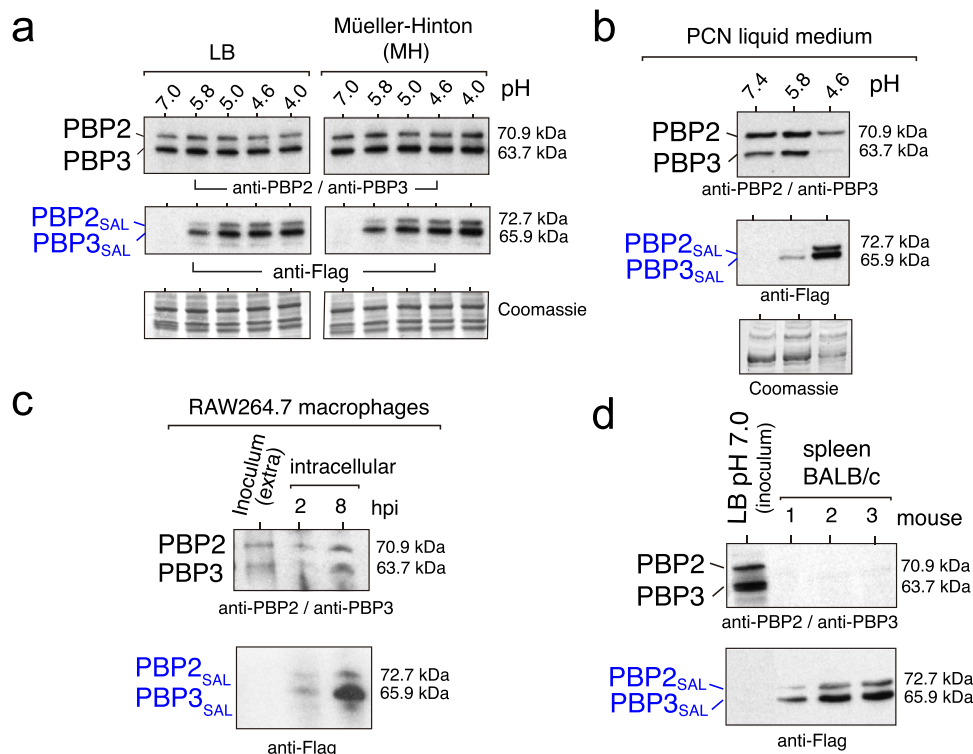
Similar to PBP3<sub>SAL</sub>, whose production is stimulated in intracellular *S. Typhimurium* [21], we reasoned that PBP2<sub>SAL</sub> could be expressed selectively by bacteria located in the acidic phagosome of mammalian cells. *S. Typhimurium* produces PBP2<sub>SAL</sub> exclusively in acidic pH (Fig. 1a), a feature reminiscent of what it is observed for PBP3<sub>SAL</sub> [21]. We also noted that as the nutrient rich media (Mueller-Hinton, LB) was prepared progressively more acidic, the bacteria produced the complete repertoires of PBPs involved in cell elongation and division: PBP2 vs PBP2<sub>SAL</sub> and PBP3 vs PBP3<sub>SAL</sub> (Fig. 1a). However, in contrast to such behaviour in nutrient rich medium, we noticed that bacteria growing in a minimal medium with limited amount of nutrients expressed preferentially PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub>, compared to PBP2 and PBP3 (Fig. 1b). PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> also prevailed over PBP2 and PBP3 in infection conditions, as denoted in intracellular bacteria

obtained from infected macrophages or, in bacteria isolated from spleen of infected mice (Fig. 1c-d). Altogether, these data demonstrated that PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub>, which are not produced by *S. Typhimurium* in Mueller-Hinton medium at neutral pH (Fig. 1a), are however the main PBPs that sustain pathogen growth in the host.

### 3.2. PBP3<sub>SAL</sub> has low affinity for beta-lactams, even in acidic pH

Our initial premise considered that *S. Typhimurium* could modify PBP expression inside the mammalian cells. Our findings indeed unveiled major changes consistent with the idea of a "replacement" of functional PBPs involving main beta-lactam targets (Fig. 1). Although it remains unknown the exact location of surviving bacteria in the period that follows antimicrobial therapy and that precedes relapses, it has been shown for *S. Typhimurium* in the murine model that bacteria surviving antibiotic therapy are in a non-growing physiological state that is induced "intracellularly" and, that allow them to regrow in the absence of antibiotic pressure [26-28]. How PBPs contribute to reach this intracellular persistence state is unknown. We hypothesized that the novel PBP2<sub>SAL</sub> and/or PBP3<sub>SAL</sub> produced by intracellular bacteria could have less affinity for known beta-lactams and, as consequence, contribute to survival inside the infected host cell despite exposure to the drug along the antibiotic therapy.

To test this hypothesis involving low affinity for current drugs, we performed antibiotic binding assays using a fluorescent-labelled beta-lactam, Bocillin-650, commonly used to detect PBPs [29]. We expressed *S. Typhimurium* PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> in *E. coli* strains bearing endogenous PBP2 and PBP3 labile variants that do not bind Bocillin-650. In this manner, we were confident that the Bocillin-650 signal detected at the expected molecular weight corresponded to PBP2<sub>SAL</sub> or, PBP3<sub>SAL</sub>. Antibiotic binding was performed at three different pH values (7.4, 5.8, and 4.6) with membrane material prepared



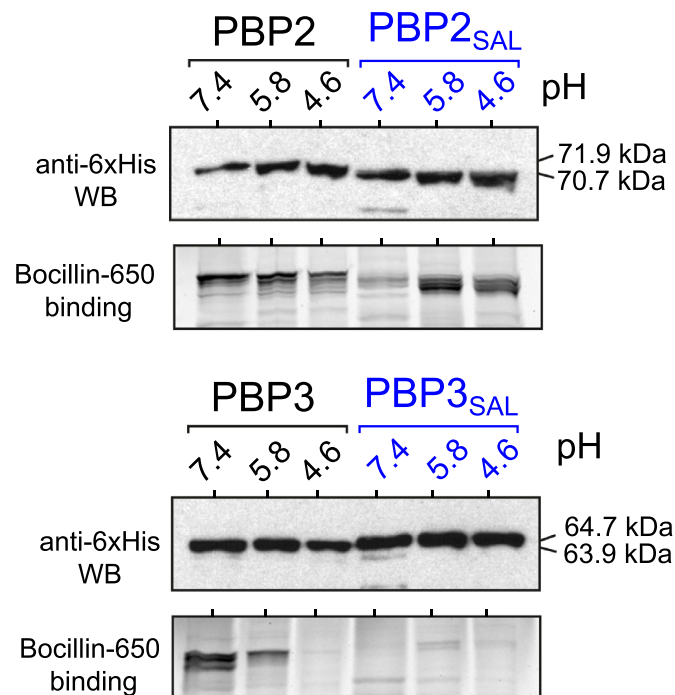
**Fig. 1.** *S. Typhimurium* produces the pathogen-specific PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> in acidic pH and during infection. (a) Levels of PBP2, PBP2<sub>SAL</sub>, PBP3, and PBP3<sub>SAL</sub> determined by western blot in bacteria grown in the indicated media and pH values. For these assays, bacteria expressing functional 3x-flag-tagged PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub>, were used; (b) replacement of PBP2/PBP3 by PBP2<sub>SAL</sub>/PBP3<sub>SAL</sub> occurring in minimal medium PCN adjusted to pH 4.6. Note that such replacement is favoured at the lowest pH value tested; (c) production of PBP2<sub>SAL</sub>/PBP3<sub>SAL</sub> prevail over that of PBP2/PBP3 in intracellular bacteria; (d) PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> replace PBP2 and PBP3 in bacteria colonizing the spleen of susceptible mice used in a murine typhoid model. The assays were repeated in at least two independent biological replicates.



from the corresponding recombinant strains overproducing PBP2<sub>SAL</sub> or, PBP3<sub>SAL</sub>. For comparison, we also produced in *E. coli* the fully functional PBP2 or PBP3 from *S. Typhimurium*. These Bocillin-650 binding assays showed that PBP2<sub>SAL</sub> binds efficiently the beta-lactam at acidic pH (5.8 and 4.6), although at neutral pH the affinity was lower than that displayed by PBP2 (Fig. 2). Remarkably, PBP3<sub>SAL</sub> did not bind Bocillin-650 at neutral pH with poor binding capacity even at the acidic pH (Fig. 2), at which this enzyme is functional *in vivo* [21]. This observation prompted us to classify PBP3<sub>SAL</sub> as a specialized enzyme that plays an essential role in intracellular *S. Typhimurium* persistence and has low affinity to known beta-lactams currently used in clinics.

### 3.3. PBP3<sub>SAL</sub> expression decreases beta-lactam susceptibility in *S. Typhimurium*

To further determine whether PBP2<sub>SAL</sub> or PBP3<sub>SAL</sub> could alter beta-lactam susceptibility in *S. Typhimurium*, we generated mutants lacking each of the four PBPs under study: PBP2, PBP2<sub>SAL</sub>, PBP3, or, PBP3<sub>SAL</sub> (see Materials and methods). As we previously reported, the *S. Typhimurium* mutant lacking PBP3 is unable to grow at neutral pH since PBP3<sub>SAL</sub> is only active at acid pH [21]. These four mutants were then tested for antimicrobial susceptibility to ceftriaxone, a drug of choice to treat salmonellosis that binds with high affinity to PBP3 [30]. The production of PBP3<sub>SAL</sub> at pH 4.6 resulted in a pronounced decrease in ceftriaxone susceptibility (Fig. 3a). Thus, viability assays revealed that the mutant lacking PBP3<sub>SAL</sub> was four-log more sensitive to ceftriaxone when plated on low-pH plates containing 5 µg/mL of the drug (Fig. 3a). E-test assays further confirmed that the presence of PBP3<sub>SAL</sub> in acid pH also reduces cephalothin, cefuroxime, ceftazidime, and aztreonam susceptibility (Fig. 3b-c). Certainly, the effect of reduction in ceftriaxone susceptibility in conditions mimicking the intra-phagosomal environment, might be enhanced by the relatively low intracellular active concentrations of the drug, facilitating pathogen persistence in the host, and, as consequence, the relapse.



**Fig. 2.** PBP3<sub>SAL</sub> has low affinity for binding of Bocillin-650, a fluorescent reagent that detects PBPs. The binding was performed at the indicated pH values. The upper panels refer to levels of the respective PBPs, the lower panels to the signal obtained with the fluorescent Bocillin-650. Note that PBP3<sub>SAL</sub> binds Bocillin-650 with low efficiency at all pH values tested, despite acidic pH the optimal for its function. The assays were repeated in three independent biological replicates.

### 3.4. Production of PBP3<sub>SAL</sub> in the host contributes to *S. Typhimurium* relapse in the typhoid murine model

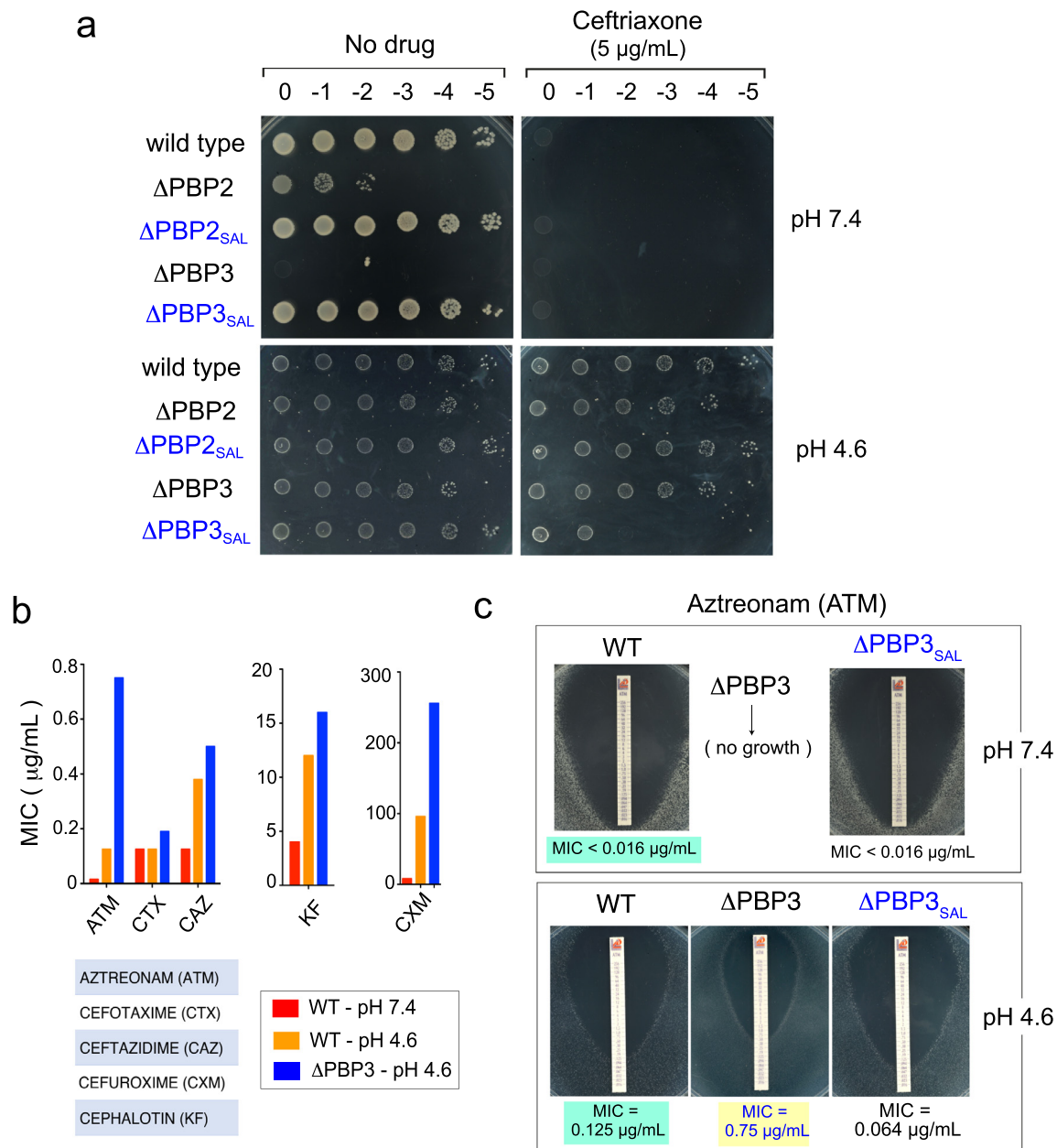
The possible contribution of alternative PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> to relapses after ceftriaxone therapy was further tested by using the murine typhoid model, in which serovar Typhimurium infection progresses as an invasive disease similar to the typhoid fever caused by serovar Typhi in humans [31]. Mice were challenged with the isogenic mutants lacking each of the PBPs of interest. Of the two groups of infected mice, one received ceftriaxone therapy for 3 days starting at 24 h after inoculation, which prevented appearance of symptoms. However, once the antibiotic therapy was discontinued, this group of mice showed relapse after seven days, with a significant bacterial burden in target organs such as liver and spleen (Fig. 4). The only exception was the mutant lacking PBP3<sub>SAL</sub>, which reached up to 3-logs less bacterial loads in the liver during relapse ( $P = 0.0021$ , Kruskal-Wallis multiple comparison test) (Fig. 4). These observations unveiled an unsuspected contribution of PBP3<sub>SAL</sub> to relapses associated to *Salmonella* invasive infections.

## 4. Discussion

Many human trials involving patients infected with *Salmonella* have reported unusual high rate of relapses caused by isolates that show *in vitro* susceptibility to the drug used in the initial therapy. These relapses occur weeks to months after remission of first symptoms, with recurrence of clinical disease that is usually milder than the initial illness. In the case of typhoid in humans, with an estimated annual global burden greater than 27 million, clinical relapse rate accounts for 5% to 20% of patients treated with antimicrobials, especially third generation cephalosporins [32]. This clinical anomaly manifests inability of the drug to effectively eradicate the pathogen. In many instances such treatment failure has been linked to inhomogeneous distribution of the drug in host tissues [8], or poor accessibility to intracellular locations where the pathogen resides [32, 33]. Reactivation and dissemination of dormant bacteria have been reported in some cases to occur from mesenteric and caecum draining lymph nodes [28, 34]. Intracellularly might also impair an effective immune response facilitating relapses and decreasing the effectivity of vaccination [35, 36]. However, pathogen factors that contribute to relapse have not yet been reported to date.

We show here that *Salmonella* replaces the main PBPs involved in cell elongation and division concomitantly to the adaptation to an intracellular lifestyle inside an acidic phagosome. This event definitively influences antibiotic susceptibility in a way that is undetectable by standard susceptibility testing. In these tests, *Salmonella* only expresses the known repertoire of PBPs for elongation and division used in extracellular conditions, PBP2 and PBP3, the main beta-lactams targets that are non-expressed by intracellular bacteria (Fig. 1). Noteworthy, the experiments performed in mice showed that the  $\Delta$ PBP3<sub>SAL</sub> mutant causes disease (Fig. 4), despite the replacement of PBP3 by this PBP3<sub>SAL</sub> that was observed in wild-type bacteria (see Fig. 1d). A possibility explaining this phenotype is that the lack of PBP3<sub>SAL</sub> due to a genetic inactivation may alter the regulation that normally down-regulates PBP3 production in response to acid pH and limited nutrients (Fig. 1b). These PBPs are essential for cell division, so the lack of one of them may render constitutive the expression of the other, an idea supported by the growth exhibited by the  $\Delta$ PBP3<sub>SAL</sub> mutant on minimal PCN medium plates at pH 4.6 without antibiotic (Fig. 2a). Note, however, that the reverse does not apply for PBP3<sub>SAL</sub> since the  $\Delta$ PBP3 mutant is unable to growth at neutral pH (Fig. 2a).

The advantage for *S. Typhimurium* for acquiring PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> remains at present unknown, although we could speculate

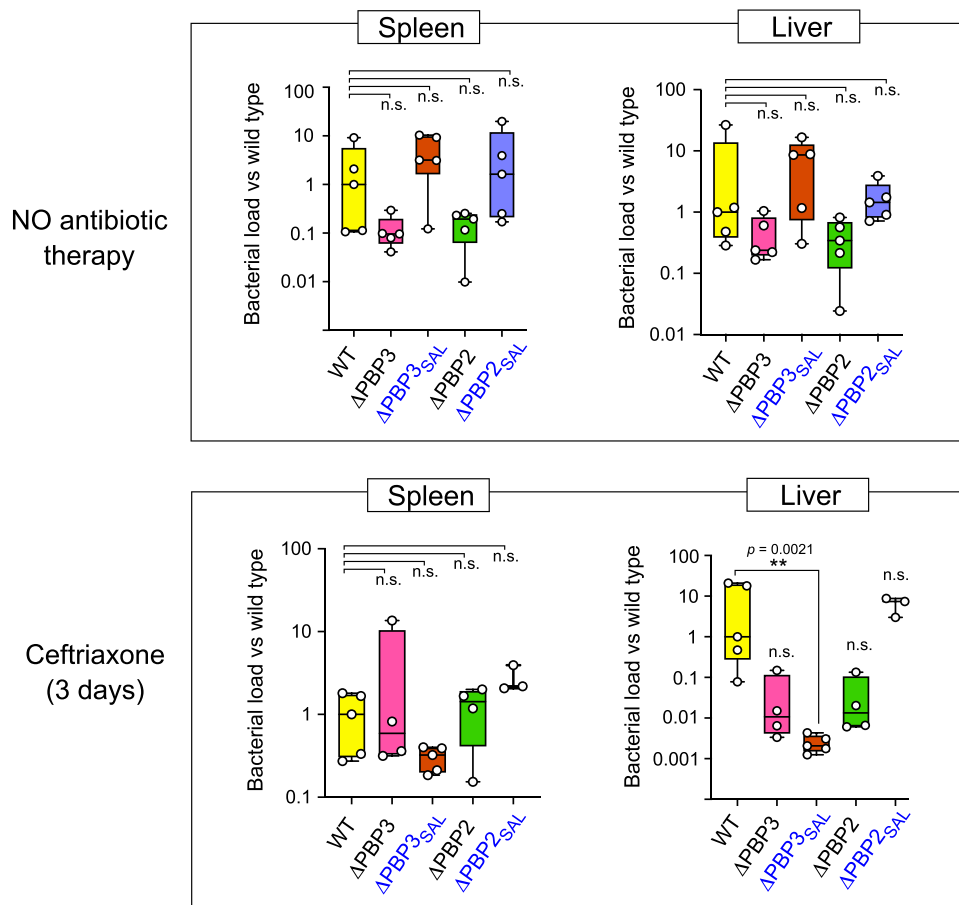


**Fig. 3.** PBP3<sub>SAL</sub> decreases ceftriaxone susceptibility at acidic pH. (a) Bacterial viability was tested in the indicated strains in PCN minimal medium at pH values 7.4 and 4.6 in the presence/absence of ceftriaxone 5  $\mu\text{g/mL}$ . While the antibiotic drastically reduces viability at neutral pH (condition of PBP3 production), it only affects bacterial viability in acidic pH in the presence of PBP3<sub>SAL</sub>. (b) MIC values obtained by the E-test assay for different beta-lactams that bind with high affinity to PBP3: aztreonam (ATM), cefotaxime (CTX), ceftazidime (CAZ), cefuroxime (CXM) and cephalotin (KF). (c) Representative E-test assays showing a marked increase in the MIC value to aztreonam (ATM) in response to acid pH (4.6) that is further enhanced in the  $\Delta\text{PBP3}$  mutant, expressing only PBP3<sub>SAL</sub>. The assays were repeated in three independent biological replicates.

about a probable correlation between the production of these alternative PBPs and a reduction in the growth rate, required to successfully persist inside the mammalian cell. Growth control is essential to co-exist in the infected host and, the fact that PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> are exquisitely regulated when the growth conditions are not optimal like acidic pH and nutrient limitation, led us to support such hypothesis. In this context, it is also tempting to postulate a putative relation between the origin of these PBPs and competition with other microbiota producing antimicrobial compounds affecting mainly actively growing cells, which is the beta-lactams mechanism of action. Together with the acquisition of other functions allowing invasion and survival inside mammalian cells, like the specialized type III secretion systems encoded by pathogenicity islands, the growth control linked to the production of PBP2<sub>SAL</sub> and PBP3<sub>SAL</sub> might have

culminated in the successful pathogen that *Salmonella* is, prone to cause persistent intracellular infections.

Our study therefore indicates that, to improve current antimicrobial therapy aimed to control salmonellosis, we need novel drugs with increased affinity for PBP3<sub>SAL</sub>. Wild type bacteria exposed to such drugs would be unable to progress in the infection since the intracellular signals -acid pH and nutrient limitation- would trigger the substitution of PBP3 by PBP3<sub>SAL</sub> during the course of the adaptive program to the intracellular lifestyle. We also envision such novel drugs will not only reduce relapse rates but also chronic and asymptomatic infections involving dormant intracellular bacteria, major contributors of pathogen dissemination, both in humans and livestock.<sup>25</sup> The activity of this novel PBP3<sub>SAL</sub>, which might evolved to ensure a long lasting residence of *Salmonella* inside mammalian cells,



**Fig. 4.** PBP3<sub>SAL</sub> allows *S. Typhimurium* to resist antibiotic (ceftriaxone) therapy and facilitate relapses. One group of susceptible mice (lower panel) was treated with ceftriaxone preventing appearance of symptoms. All members of this group showed relapse seven days after discontinuing ceftriaxone treatment. Note that colonization of the liver in the group of mice with relapse was much lower, 3-log difference and statistically significant, \*\*,  $P = 0.0021$ , (Kruskal-Wallis multiple comparison test), in bacteria lacking PBP3<sub>SAL</sub> compared to the rest of strains used. No statistically significant differences were found in untreated mice (upper panels), n.s., not significant.

will certainly illuminate medical research to improve therapies to control this highly successful human pathogen.

#### Declarations of Competing Interest

The authors declare no conflict of interest. Dr. García-del Portillo, Dr. Pucciarelli, Dr. Castanheira, Dr. Cestero and, David López-Escarpa have a patent EP20382036 pending to Spanish Office of Patents, which is relevant to this work.

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#### Author Contributions

SC, FB and FG-P contributed to the study design. SC, DL-E, MGP and JJC collected and analysed the data that were further discussed with FB and FG-P. SC, FB, MGP and FG-P interpreted the data. The first draft was written by FG-P, which was critically commented by FB and SC. All authors revised and approved the final version of the manuscript.

#### Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2020.102771](https://doi.org/10.1016/j.ebiom.2020.102771).

#### References

- [1] Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004;82(5):346–53.
- [2] Pucciarelli MG, Garcia-Del Portillo F. Salmonella intracellular lifestyles and their impact on host-to-host transmission. *Microbiol Spectr* 2017;5(4).
- [3] Ilyas B, Tsai CN, Coombes BK. Evolution of salmonella-host cell interactions through a dynamic bacterial genome. *Front Cell Infect Microbiol* 2017;7:428.
- [4] Hiyoshi H, Tiffany CR, Bronner DN, Baumler AJ. Typhoidal Salmonella serovars: ecological opportunity and the evolution of a new pathovar. *FEMS Microbiol Rev* 2018;42(4):527–41.
- [5] Abed N, Couvreur P. Nanocarriers for antibiotics: a promising solution to treat intracellular bacterial infections. *Int J Antimicrob Agents* 2014;43(6):485–96.
- [6] Bongers S, Hellebrekers P, Leenen LPH, Koenderman L, Hietbrink F. Intracellular penetration and effects of antibiotics on *Staphylococcus aureus* inside human neutrophils: a comprehensive review. *Antibiotics (Basel)* 2019;8(2).

- [7] Bumann D, Cunrath O. Heterogeneity of Salmonella-Host interactions in infected host tissues. *Curr Opin Microbiol* 2017;39:57–63.
- [8] Bumann D, Fanous J, Li J, Goormaghtigh F. Antibiotic chemotherapy against heterogeneous pathogen populations in complex host tissues. *F1000Res*. 2019;8.
- [9] McDermott PF, Zhao S, Tate H. Antimicrobial resistance in nontyphoidal Salmonella. *Microbiol Spectr* 2018;6(4).
- [10] Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in Salmonella enterica serovar typhi, the etiological agent of typhoid. *PLoS Negl Trop Dis* 2018;12(10):e0006779.
- [11] Butler T. Treatment of typhoid fever in the 21st century: promises and shortcomings. *Clin Microbiol Infect* 2011;17(7):959–63.
- [12] Soe GB, Overturf GD. Treatment of typhoid fever and other systemic salmonellosis with cefotaxime, ceftriaxone, cefoperazone, and other newer cephalosporins. *Rev Infect Dis* 1987;9(4):719–36.
- [13] Dragsted UB, Pedersen P. Relapse of multiresistant Salmonella typhi after combined therapy with ciprofloxacin and ceftriaxone. *Clin Microbiol Infect* 2000;6(3):167–8.
- [14] Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. *N Engl J Med* 2002;347(22):1770–82.
- [15] Arjyal A, Basnyat B, Nhan HT, Koirala S, Giri A, Joshi N, et al. Gatifloxacin versus ceftriaxone for uncomplicated enteric fever in Nepal: an open-label, two-centre, randomised controlled trial. *Lancet Infect Dis* 2016;16(5):535–45.
- [16] Matono T, Kato Y, Morita M, Izumiya H, Yamamoto K, Kutsuna S, et al. Case series of imported enteric fever at a referral center in Tokyo, Japan: antibiotic susceptibility and risk factors for relapse. *Am J Trop Med Hyg*. 2016;95(1):19–25.
- [17] Vivero A, Banos RC, Mariscotti JF, Oliveros JC, Garcia-del Portillo F, Juarez A, et al. Modulation of horizontally acquired genes by the HHA-YDGT proteins in Salmonella enterica serovar Typhimurium. *J Bacteriol* 2008;190(3):1152–6.
- [18] Deiwick J, Hensel M. Regulation of virulence genes by environmental signals in Salmonella typhimurium. *Electrophoresis* 1999;20(4–5):813–7.
- [19] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* 2000;97(12):6640–5.
- [20] Ellermeier CD, Janakiraman A, Slauch JM. Construction of targeted single copy lac fusions using lambda red and FLP-mediated site-specific recombination in bacteria. *Gene* 2002;290(1–2):153–61.
- [21] Castanheira S, Cestero JJ, Rico-Perez G, Garcia P, Cava F, Ayala JA, et al. A specialized peptidoglycan synthase promotes Salmonella cell division inside host cells. *MBio* 2017;8(6).
- [22] Lobato-Marquez D, Moreno-Cordoba I, Figueroa V, Diaz-Orejas R, Garcia-del Portillo F. Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic cells. *Sci Rep* 2015;5:9374.
- [23] Garcia del Portillo F, de Pedro MA. Differential effect of mutational impairment of penicillin-binding proteins 1A and 1B on Escherichia coli strains harboring thermosensitive mutations in the cell division genes ftsA, ftsQ, ftsZ, and pbpB. *J Bacteriol* 1990;172(10):5863–70.
- [24] Castanheira S, Cestero JJ, Garcia-del Portillo F, Pucciarelli MG. Two distinct penicillin binding proteins promote cell division in different Salmonella lifestyles. *Microb Cell* 2018;5(3):165–8.
- [25] Spratt BG. Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12. *Proc Natl Acad Sci U S A* 1975;72(8):2999–3003.
- [26] Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. *Science* 2014;343(6167):204–8.
- [27] Claudi B, Sprote P, Chirkova A, Personnic N, Zankl J, Schurmann N, et al. Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. *Cell* 2014;158(4):722–33.
- [28] Kaiser P, Regoes RR, Dolowschiak T, Wotzka SY, Lengefeld J, Slack E, et al. Cecum lymph node dendritic cells harbor slow-growing bacteria phenotypically tolerant to antibiotic treatment. *PLoS Biol* 2014;12(2):e1001793.
- [29] Zhao G, Meier TI, Kahl SD, Gee KR, Blaszcak LC. BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. *Antimicrob Agents Chemother* 1999;43(5):1124–8.
- [30] Kocaoglu O, Carlson EE. Profiling of beta-lactam selectivity for penicillin-binding proteins in Escherichia coli strain DC2. *Antimicrob Agents Chemother* 2015;59(5):2785–90.
- [31] Tsolis RM, Kingsley RA, Townsend SM, Ficht TA, Adams LG, Baumler AJ. Of mice, calves, and men Comparison of the mouse typhoid model with other Salmonella infections. *Adv Exp Med Biol*. 1999;473:261–74.
- [32] Ahmad KA, Khan LH, Roshan B, Bhutta ZA. Factors associated with typhoid relapse in the era of multiple drug resistant strains. *J Infect Dev Ctries* 2011;5(10):727–31.
- [33] Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 2016;14(5):320–30.
- [34] Griffin AJ, Li LX, Voedisch S, Pabst O, McSorley SJ. Dissemination of persistent intestinal bacteria via the mesenteric lymph nodes causes typhoid relapse. *Infect Immun* 2011;79(4):1479–88.
- [35] Griffin AJ, McSorley SJ. Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* 2011;4(4):371–82.
- [36] Abrahams GL, Hensel M. Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells. *Cell Microbiol* 2006;8(5):728–37.