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Affinity of cefotiam for the alternative penicillin binding protein $PBP3_{SAL}$ used by *Salmonella* inside host eukaryotic cells

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Background: Following the invasion of eukaryotic cells, *Salmonella enterica* serovar Typhimurium replaces PBP2/PBP3, main targets of β-lactam antibiotics, with $PBP2_{SAL}$/$PBP3_{SAL}$, two homologue peptidoglycan synthases absent in *Escherichia coli*. $PBP3_{SAL}$ promotes pathogen cell division in acidic environments independently of PBP3 and shows low affinity for β-lactams that bind to PBP3 such as aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefalotin.

Objectives: To find compounds with high affinity for $PBP3_{SAL}$ to control *Salmonella* intracellular infections.

Methods: An *S.* Typhimurium ΔPBP3 mutant that divides using $PBP3_{SAL}$ and its parental wild-type strain, were exposed to a library of 1520 approved drugs in acidified (pH 4.6) nutrient-rich LB medium. Changes in optical density associated with cell filamentation, a read-out of blockage in cell division, were monitored. Compounds causing filamentation in the ΔPBP3 mutant but not in wild-type strain—the latter strain expressing both PBP3 and $PBP3_{SAL}$ in LB pH 4.6—were selected for further study. The bactericidal effect due to $PBP3_{SAL}$ inhibition was evaluated *in vitro* using a bacterial infection model of cultured fibroblasts.

Results: The cephalosporin cefotiam exhibited higher affinity for $PBP3_{SAL}$ than for PBP3 in bacteria growing in acidified LB pH 4.6 medium. Cefotiam also proved to be effective against intracellular *Salmonella* in a $PBP3_{SAL}$-dependent manner. Conversely, cefuroxime, which has higher affinity for PBP3, showed decreased effectiveness in killing intracellular *Salmonella*.

Conclusions: Antibiotics with affinity for $PBP3_{SAL}$, like the cephalosporin cefotiam, have therapeutic value for treating *Salmonella* intracellular infections.

Introduction

Salmonella enterica infections progress in intracellular locations of phagocytic and non-phagocytic host cells.[1] The avidity of this pathogen to be rapidly internalized by host cells imposes a barrier for commonly used antibiotics, which are more effective in extracellular locations.[2,3] Many studies have shown limited accessibility of drugs to the intracellular niche occupied by the pathogen, a factor decreasing their inhibitory activity.[3] In intraphagosomal intracellular pathogens, other factors such the acidity of the compartment colonized by the pathogen can also affect antibiotic activity. To counterbalance these negative effects, new approaches based on highly penetrating nanoparticles carrying the drug as cargo are currently under intense investigation and development.[4]

The clinical evidence accumulated in *S. enterica* infections both in livestock and humans indicates that this pathogen is prone to cause persistent infections.[5,6] The reduced proliferation rate inside the infected cell, associated in many instances with latency, imposes an additional drawback for chemotherapy, which normally requires the pathogen to be undergoing active metabolism. The standard antimicrobial therapy based on cephalosporins and quinolones seems in some instances ineffective to eradicate *S. enterica* infections. In this scenario, it is alarming that relapse caused by drug-susceptible isolates is reported in the order of 5%–15% by many studies.[7,8] Intracellularity as a lifestyle therefore impacts the antimicrobial chemotherapy of *S. enterica* infections.

Our recent studies show an additional factor hampering effective treatment of *S. enterica* infections. Besides PBP2 and PBP3, the two peptidoglycan synthases conserved in enteric bacteria that are responsible for cell elongation and division, respectively,[9] *S. enterica* encodes two homologous enzymes named

$PBP2_{SAL}$ and $PBP3_{SAL}$, which replace PBP2 and PBP3 when located inside host cells.[10–12] Intracellular *S. enterica* therefore upregulates certain functions compared with bacteria growing extracellularly, and among these are enzymes related to the metabolism of peptidoglycan, one of the main targets in antimicrobial therapy. Importantly, it has been shown in a mouse animal model that an *S.* Typhimurium mutant lacking $PBP3_{SAL}$ does not cause relapse following ceftriaxone therapy,[12] highlighting how $PBP3_{SAL}$ contributes to prevent effective eradication of *Salmonella* infections. These observations indicate that new antimicrobial therapies directed to selectively inhibit $PBP2_{SAL}$ and/or $PBP3_{SAL}$ are imperative to control intracellular infections caused by this pathogen.

In this study, we identified the second-generation β-lactam cephalosporin cefotiam (IUPAC name: (6*R*,7*R*)-7-[[2-(2-amino-1,3-thiazol-4-yl)-acetyl]amino]-3-[[1-[2-(dimethylamino)ethyl]tetrazol-5-yl]sulfanylmethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid) as an antibiotic showing higher affinity for $PBP3_{SAL}$ than for PBP3. Consistently with the replacement of these two PBPs by intracellular *S.* Typhimurium, cefotiam proved to be effective in controlling the infection inside host cells in a $PBP3_{SAL}$-dependent manner. Conversely, cefuroxime, a cephalosporin with higher affinity for PBP3 than for $PBP3_{SAL}$,[10] showed reduced effectiveness in the killing of intracellular bacteria.

Methods

Prestwick Chemical Library and selected compounds

The Prestwick Chemical Library (Prestwick Chemical Libraries, GreenPharma S.A.S., Orléans, France) was used to screen compounds that induce filamentation in the *S.* Typhimurium ΔPBP3 mutant having $PBP3_{SAL}$ as the only PBP promoting cell division.[10] The library has 1520 off-patent compounds approved by agencies such as the FDA and the EMA. The compounds were supplied at 10 mM in 100% DMSO.

Bacterial strains, eukaryotic cell lines and growth conditions

S. Typhimurium strains used were isogenic to the parental wild-type strain SV5015,[13] a His^+ derivative of the virulent strain SL1344 isolated from calf.[14] The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in LB broth buffered with 80 mM MES [2-(*N*-morpholino) ethanesulfonic acid] and adjusted to pH 4.6. When necessary, ampicillin and kanamycin were added at 0.1 mg/mL and 0.03 mg/mL, respectively. The *S.* Typhimurium double mutants MD5063 [Δ*mrdA* (Δ*PBP2*) Δ*ftsI* (Δ*PBP3*)] and MD2588 [Δ*STM1910* (Δ$PBP2_{SAL}$) Δ*STM1836* (Δ$PBP3_{SAL}$)] were constructed by bacteriophage P22-mediated transduction using donor strains MD2502 [Δ*STM1836*::Km (Δ$PBP3_{SAL}$::Km)] and MD5049 [Δ*mrdA*::Km (Δ*PBP2*::Km)]. The P22 lysates obtained in MD2502 and MD5409 strains, were used to transduce recipient strains MD2576 [Δ*STM1910* (Δ$PBP2_{SAL}$)] and MD4356 [Δ*ftsI* (Δ*PBP3*)], respectively. Subsequent removal of the kanamycin cassette from strains MD2580 [Δ*STM1910* (Δ$PBP2_{SAL}$) Δ*STM1836*::Km (Δ$PBP3_{SAL}$::Km)] and MD5061 [Δ*mrdA*::Km (Δ*PBP2*::Km) Δ*ftsI* (Δ*PBP3*)] (see Table 1), was performed as described.[15]

Identification of drugs blocking cell division based on monitoring of OD_{600} values

As a previous control, the growth of *S.* Typhimurium wild-type and Δ*PBP3* isogenic strains was monitored in LB medium at pH 4.6 in the absence/presence of 0.001 mg/mL aztreonam using a Spark Automatic Microplate Reader (Tecan Trading AG, Switzerland). Differences in final OD_{600} values were correlated with the presence of filamented bacteria in which cell division was blocked by the antibiotic. Response to compounds of the Prestwick Library was directed to the monitoring of growth curves and final OD_{600} values. Drugs having a bacteriostatic effect were discarded from further analyses. Those compounds causing bacteriolytic effect at the initial dose used (1:100 dilution of the 10 mM stock) were re-screened at lower concentrations and reanalysed for increased values in the final OD_{600} measurements. Growth curves were monitored in the automatic reader for a minimum period of 8 h. Control samples consisted of bacteria grown in LB medium pH 4.6 containing 1% DMSO.

Antibiotic susceptibility tests

MIC values were determined using MIC strips (MIC Test Strip, Liofilchem, Roseto degli Abruzzi, Italy) in LB plates at pH 4.6. Because no cefotiam MIC strips are commercially available, MIC values for this antibiotic were determined in 96-well microplates by serial dilutions of the antibiotic in liquid LB pH 4.6 medium and overnight incubation at 37°C. The starting inoculum was 6×10^5 cfu per well.

Preparation of membrane extracts for BOCILLIN-FL (Boc-FL) labelling

S. Typhimurium Δ*ftsI* (Δ*PBP3*) and Δ*STM1836* (Δ$PBP3_{SAL}$) isogenic strains were grown overnight at 37°C in LB pH 4.6. Cultures were diluted 1:100 in 200 mL fresh media and bacteria grown up to exponential phase (OD_{600} ~0.2–0.3). Bacteria were harvested by centrifugation (6000×g, 10 min, 4°C) and washed in 50 mM sodium phosphate buffer pH 4.6. After subsequent centrifugation (12 000×g, 15 min, 4°C), bacteria were suspended in 20 mL of 50 mM sodium phosphate buffer. Cells were disrupted by passing through a French press and lysates centrifuged at low speed (4000×g, 10 min, 4°C) to remove unbroken cells. The supernatant was further centrifuged (150 000×g, 35 min, 4°C) and pellets containing membrane material were suspended in 150 μL of 50 mM sodium phosphate buffer pH 4.6. The protein concentration was measured using Pierce 660 nm Protein Assay reagent (Thermo Scientific).

Cefotiam competition in Boc-FL binding assays

Cefotiam hydrochloride (ref. 66309-69-1, Molekula GmbH, Munich, Germany) was added to 0.02 mg membrane extracts prepared in 50 mM sodium phosphate buffer pH 4.6 to reach final concentrations of the antibiotic in the 0.00005–0.005 mg/mL range. Sample volume was 10 μL in all cases. Binding conditions were pH 4.6 for 10 min at 30°C, as described.[12] Subsequently, 20 μM Boc-FL (Molecular Probes) was added and the sample incubated for 20 min at 30°C. Samples were finally processed by adding Laemmli buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE in 8% (w/v) acrylamide gels. The gel was washed with 30% (v/v) methanol/10% (v/v) acetic acid, and fluorescence was detected on a Typhoon 8410 variable-mode imager (General Electric) with an excitation wavelength of 588 nm and a 520BP40 emission filter. Quantification of gel bands was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Bands with no signal changes in the presence of cefotiam were used as loading controls.

Microscopy analyses

Overnight bacterial cultures were diluted at OD_{600} ~0.01 in LB pH 4.6 and grown in 96-well plates for 8 h in the presence of aztreonam (ref. 78110-38-0, Molekula GmbH, Munich, Germany) or cefotiam hydrochloride at varied concentrations. A volume of 120 μL of each culture was harvested (4300×g, 5 min, room temperature), washed in PBS pH 7.4 and fixed with 3% (w/v) paraformaldehyde. Images were acquired on an inverted Leica

Table 1. Bacterial strains and plasmids used in this study

Strain	Genotype	Reference/ source
SV5015	wild type, SL1344 *hisG+*	13
MD2502	SV5015, Δ*STM1836*::Km (Δ$PBP3_{SAL}$::Km)	10
MD2576	SV5015, Δ*STM1910* (Δ$PBP2_{SAL}$)	12
MD2577	SV5015, Δ*STM1836* (Δ$PBP3_{SAL}$)	10
MD2580	SV5015, Δ*STM1910* (Δ$PBP2_{SAL}$) Δ*STM1836*:: Km (Δ$PBP3_{SAL}$::Km)	This study
MD2588	SV5015, Δ*STM1910* (Δ$PBP2_{SAL}$) Δ*STM1836* (Δ$PBP3_{SAL}$)	This study
MD4356	SV5015, Δ*ftsI* (Δ*PBP3*)	10
MD5052	SV5015 Δ*mrdA* (Δ*PBP2*)	12
MD5049	SV5015 Δ*mrdA*::Km (Δ*PBP2*::Km)	This study
MD5061	SV5015 Δ*mrdA*::Km (Δ*PBP2*::Km) Δ*ftsI* (Δ*PBP3*)	This study
MD5063	SV5015 Δ*mrdA* (Δ*PBP2*) Δ*ftsI* (Δ*PBP3*)	This study
Plasmid		
pCP20	FLP^+, Amp^R, Cm^R	15

DMI 6000B microscope with an automated CTR/7000 HS controller (Leica Microsystems) and an Orca-R2 CCD camera (Hamamatsu Photonics).

Bacterial infection of fibroblasts

NRK-49F fibroblasts (ATCC CRL-1570) were propagated in DMEM containing 10% (v/v) FBS at 37°C in a 5% CO_2 atmosphere as previously described.[16] Fibroblasts were infected for 20 min at a multiplicity of infection of 10:1 with wild-type or mutant strains, all of them previously grown overnight in LB pH 4.6 without shaking. After this time, fibroblasts were washed three times with PBS pH 7.4, and fresh tissue culture medium containing 0.1 mg/mL gentamicin to kill remaining extracellular bacteria was added. At 2 hours post-infection (hpi), tissue culture medium was further replaced with fresh medium containing 0.01 mg/mL gentamicin and 0.001 mg/mL cefotiam hydrochloride. Cefuroxime at 0.02 mg/mL was also used as an alternative cephalosporin. Unlike cefotiam, cefuroxime has higher affinity for binding to PBP3 compared with $PBP3_{SAL}$.[10] At 2 and 24 hpi, fibroblasts were lysed in a 0.1% Triton X-100 solution and the number of viable intracellular bacteria was calculated by plating of 10-fold serial dilutions using PBS pH 7.4. Volumes plated were 100 µL onto LB pH 4.6 plates to calculate number of viable intracellular bacteria or 5 µL for the drop assay.

Statistical analyses

Data were analysed with GraphPad Prism, version 8.0, software (GraphPad Inc.). A *t*-test was used for data analysis. Significance was established at *P* values ≤0.05.

Results

Design of a screening method to identify drugs selectively inhibiting $PBP3_{SAL}$ versus PBP3

Unlike in *E. coli*, it is possible to generate *S.* Typhimurium mutants defective in PBP3 due to the presence of an alternative PBP, named $PBP3_{SAL}$, which accomplishes cell division in acid pH.[10] To select drugs targeting $PBP3_{SAL}$ with higher affinity than PBP3, we designed a screening method to search for compounds inducing filamentation, a manifestation of cell division blockage. The candidate compound was expected to cause filamentation in an *S.* Typhimurium ΔPBP3 mutant expressing only $PBP3_{SAL}$ for division but not in wild-type bacteria that express both PBP3 and $PBP3_{SAL}$ when growing in acidified (pH 4.6) LB medium.[12] To detect differences in filamentation using high-throughput screening, we monitored the growth curve of wild-type and ΔPBP3 bacteria in the presence of aztreonam, a β-lactam that binds PBP3 with high affinity and blocks cell division at very low concentrations.[17] The growth curves in the presence of a low dosage of aztreonam (0.001 mg/mL, equivalent to 2.3 µM) reached higher final optical density values (Figure 1a). This change was consistent with the presence of long filaments (Figure 1a). A dose–response assay for aztreonam confirmed the higher affinity for PBP3 compared with other PBPs. Thus, aztreonam caused *S.* Typhimurium filamentation starting at 0.0000078 mg/mL (0.018 µM) up to 0.05 mg/mL (115 µM), the highest concentration used (Figure S1, available as Supplementary data at *JAC* Online). No massive cell lysis was observed even at 115 µM of this antibiotic.

Based on these preliminary controls, we screened the Prestwick Chemical Library consisting of 1520 off-patent compounds. This library contains diverse anti-infective (antiviral, antiprotozoal, antifungal and antibacterial) compounds as well as drugs used in clinics to treat a number of pathologies, all supplied at 10 mM in DMSO. *S.* Typhimurium wild type and its isogenic ΔPBP3 mutant were exposed to these compounds at 1:100 dilution of the stock (100 µM). A few compounds increased final optical density values compared with control cultures not exposed to drug. These compounds were further discarded because no differential effect on wild-type and ΔPBP3 strains was observed. Compounds of this first group included β-lactam antibiotics like aztreonam, which validated our previous control assays (see Figures 1a and S1), cloxacillin, amoxicillin, piperacillin and cefazolin. A second group of β-lactams included in the Prestwick Library, comprising cefixime, cefotetan, ceforanide and cefotiam, caused lysis of wild-type and ΔPBP3 bacteria at 100 µM. Aiming to find different responses in these two strains, these β-lactams were subsequently tested at more diluted concentrations starting from 100 µM up to 3.125 µM. We also included an isogenic Δ$PBP3_{SAL}$ to discern whether the differences, if found, were related to the presence/absence of $PBP3_{SAL}$.

Within this second group of β-lactams, cefotiam hydrochloride (MW = 598.56) was the only antibiotic causing a differential effect. In LB pH 4.6 medium, a condition in which *S.* Typhimurium wild type expresses PBP3 and $PBP3_{SAL}$,[12] cefotiam hydrochloride at 0.00375 mg/mL (6.25 µM) increased final optical density values in wild-type and ΔPBP3 strains, but not in the Δ$PBP3_{SAL}$ mutant (Figure 1b). We next acquired cefotiam hydrochloride powder at ≥98% purity from commercial sources. The response of bacteria exposed to cefotiam was then examined by microscopy in wild-type, ΔPBP3 and Δ$PBP3_{SAL}$ strains. Only those strains expressing $PBP3_{SAL}$ (wild-type and ΔPBP3) filamented in LB pH 4.6 medium containing 0.00032 mg/mL cefotiam hydrochloride whereas such an effect was not seen in the Δ$PBP3_{SAL}$ strain expressing only PBP3 (Figure 1c). The data obtained in liquid culture were confirmed in LB pH 4.6 agar plates containing distinct concentrations of cefotiam hydrochloride (Figure 1d). In the assays involving solid media we also tested

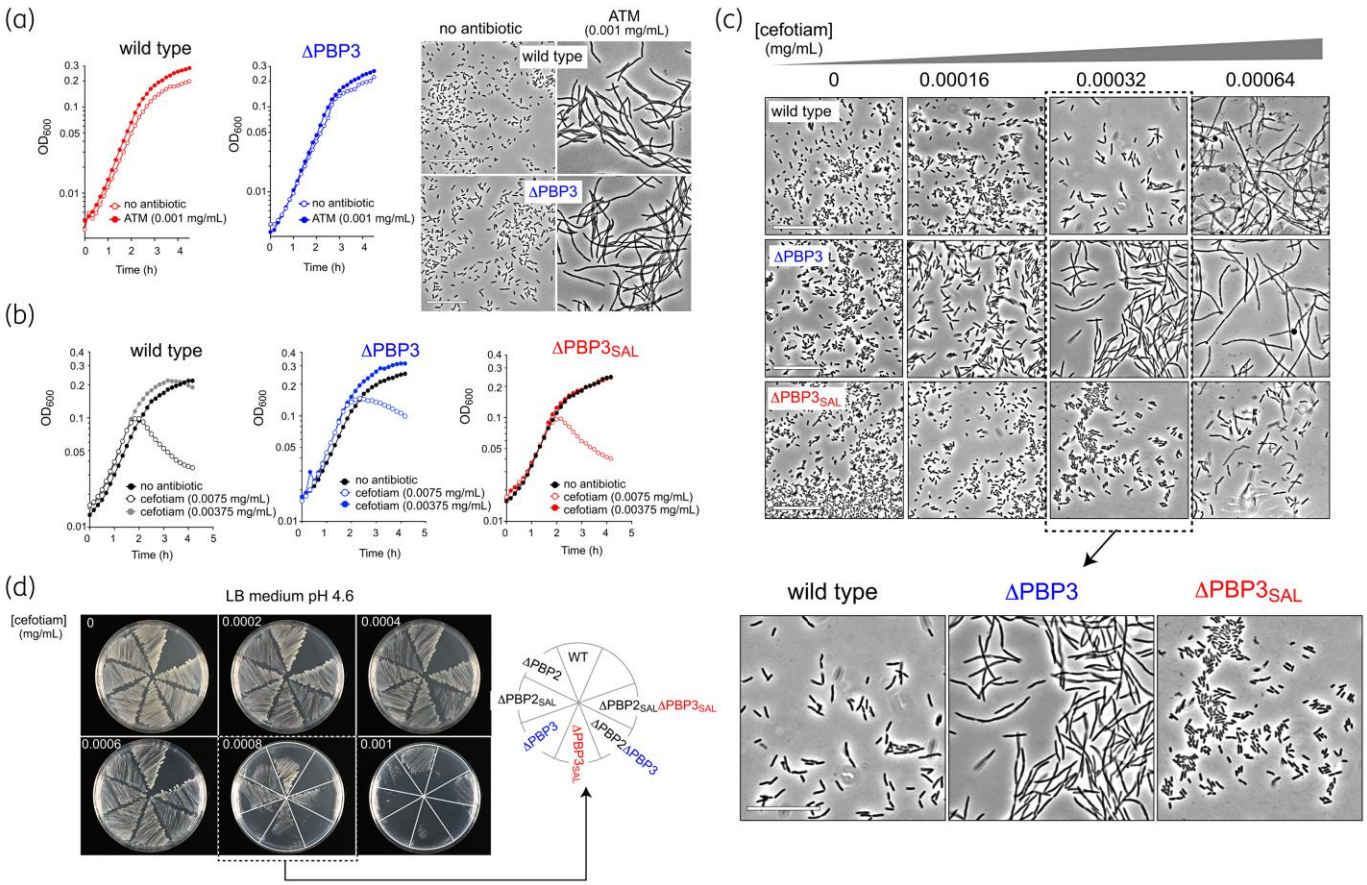

Figure 1. Identification of cefotiam in a screening designed to identify compounds that inhibit cell division by targeting $PBP3_{SAL}$. (a) Higher final OD_{600} values detected in *S.* Typhimurium wild-type and ΔPBP3 isogenic strains incubated in the presence of 0.001 mg/mL (2.3 μM) aztreonam in LB medium pH 4.6. Microscope images were taken at 6 h after the onset of growth. Bar: 25 μm. (b) Effect on growth of *S.* Typhimurium wild-type, ΔPBP3 and $\Delta PBP3_{SAL}$ isogenic strains incubated in LB medium pH 4.6 with the indicated concentrations of cefotiam hydrochloride. For these assays, the antibiotic was taken from the aliquot supplied at 10 mM in the Prestwick Chemical Library. (c) Effect of cefotiam on cell division visualized through the microscope in the indicated *S.* Typhimurium strains and antibiotic concentrations using LB medium pH 4.6. Note the differential effect when used at 0.00032 mg/mL (0.51 μM) causing blockage of cell division only in the strains producing $PBP3_{SAL}$. The cefotiam used in these assays was acquired commercially with a purity of ≥98%. Bar: 25 μm. (d) Phenotype of the indicated isogenic *S.* Typhimurium single or double mutants lacking PBPs in response to commercially acquired cefotiam. Note the lack of growth at 0.0008 mg/mL (1.28 μM) cefotiam of the strains producing $PBP3_{SAL}$ as the only enzyme promoting cell division (ΔPBP3 genetic background). Assays were performed for a minimum of two independent biological replicates. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

isogenic single and double mutants lacking distinct PBPs involved in morphogenesis: PBP2, PBP3, $PBP2_{SAL}$ and $PBP3_{SAL}$. Those strains having $PBP3_{SAL}$ as the single enzyme for division (ΔPBP3 genetic background) exhibited higher susceptibility to cefotiam (Figure 1d). Taken together, these observations suggested that the second-generation cephalosporin cefotiam could bind more efficiently to $PBP3_{SAL}$ than to PBP3.

Boc-FL bindings assays reveal higher affinity of cefotiam for $PBP3_{SAL}$

To further demonstrate the distinct behaviour of cefotiam for binding to PBP3 and $PBP3_{SAL}$, a competition binding assay with the fluorescent derivative Boc-FL antibiotic was performed in membrane extracts obtained from ΔPBP3 and $\Delta PBP3_{SAL}$ strains (Figure 2a). The assay revealed IC_{50} values for cefotiam of 0.0004 mg/mL in the case of $PBP3_{SAL}$ and 0.00375 mg/mL for PBP3 (Figure 2b). This result contrasted with our previous Boc-FL competition assays with other cephalosporins like cefuroxime, which exhibited higher affinity for PBP3.[10] Cefotiam also showed avidity for binding to the high molecular weight bifunctional PBP1A and PBP1B, although with relatively high IC_{50} values of 0.004 and 0.0028 mg/mL for the ΔPBP3 and $\Delta PBP3_{SAL}$ strains, respectively (Figure 2b). MIC values obtained for single and double mutants with deficiencies in PBP2, PBP3, $PBP2_{SAL}$ or $PBP3_{SAL}$ corroborated the higher susceptibility to cefotiam of those strains expressing $PBP3_{SAL}$ as the only PBP involved in cell division (Table 2). Conversely, the determination in *S.* Typhimurium by the Etest assay of MIC values for other cephalosporins known to have high affinity for PBP3, like aztreonam, ceftriaxone, cefuroxime, cefotaxime and cefepime, revealed an opposite phenotype compared with the response to cefotiam. Thus, the

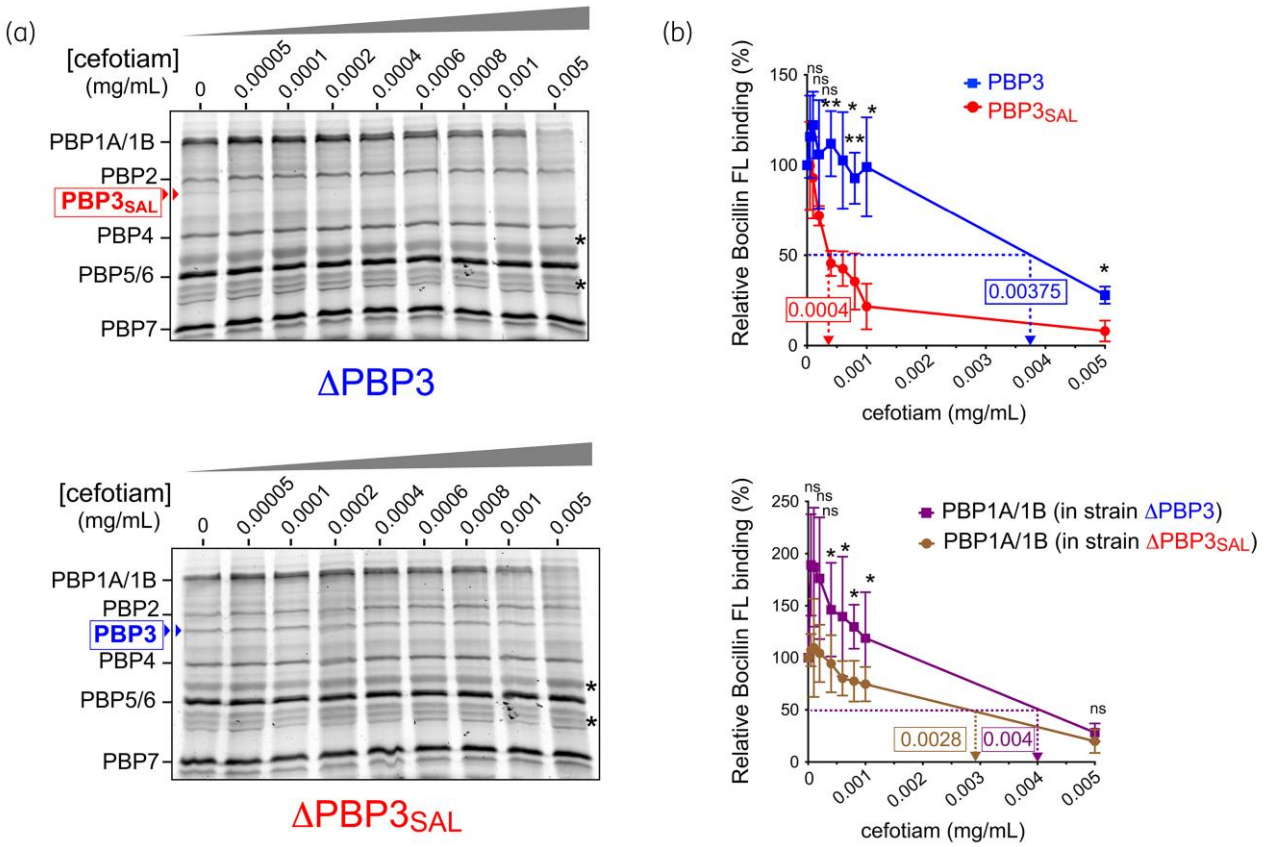

Figure 2. Cefotiam binds to $PBP3_{SAL}$ with higher affinity than to PBP3. (a) Representative BOCILLIN-FL competition binding assays performed in membrane extracts obtained from ΔPBP3 and $\Delta PBP3_{SAL}$ isogenic mutants grown in LB medium pH 4.6. Binding conditions were also pH 4.6. Asterisks point to protein used in the densitometry analyses to adjust values for protein content. (b) Determination of the IC_{50} of cefotiam for competing BOCILLIN-FL binding to PBP1A/1B, PBP3 and $PBP3_{SAL}$. For PBP1A/1B, data are shown separately corresponding to the data of ΔPBP3 and $\Delta PBP3_{SAL}$, respectively. Data are from three independent experiments and were analysed by Student's *t*-test. *, $P \leq 0.05$; **, $P \leq 0.005$. ns, not significant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

susceptibility to these other β-lactams increased notoriously in strains lacking $PBP3_{SAL}$, i.e. having PBP3 as the only PBP for division (Table 2). The lack of $PBP3_{SAL}$ associated with increases in MIC values in the order of more than 8-fold (cefuroxime, cefepime), 6-fold (aztreonam) or 2.65-fold (ceftriaxone, cefotaxime) (Table 2). The MIC to mecillinam (amdinocillin), a β-lactam that

Table 2. MIC values obtained for distinct β-lactam antibiotics in *S.* Typhimurium isogenic mutant strains lacking morphogenetic PBPs involved in cell elongation and division (PBP2, PBP3, $PBP2_{SAL}$ and $PBP3_{SAL}$)

	MIC value (mg/mL)[a]							
Strain	CTM	ATM	MEC	CRO	CXM	CTX	FEP	CS[b]
wild type	**0.00256**	0.00025	0.00019	0.000094	0.032	0.000094	0.000047	0.001
ΔPBP2	**0.00256**	0.000094	0.00019	0.000047	0.008	0.000094	0.000094	0.003
ΔPBP3	**0.00128**	0.0015	0.00019	0.00025	>0.256	0.00025	0.00038	0.001
$\Delta PBP2_{SAL}$	**0.00256**	0.000125	0.000125	0.000094	0.032	0.000125	0.000064	0.001
$\Delta PBP3_{SAL}$	**0.00512**	0.000094	0.000125	0.000094	0.004	0.000094	0.000094	0.00075
ΔPBP2 ΔPBP3	**0.00128**	0.00038	0.000125	0.00025	>0.256	0.00019	0.00025	0.002
$\Delta PBP2_{SAL}$ $\Delta PBP3_{SAL}$	**0.00512**	0.000125	0.000125	0.000094	0.006	0.000064	0.000047	0.001

ATM, aztreonam; CRO, ceftriaxone; CTM, cefotiam; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; MEC, mecillinam.
[a]MIC values were calculated with the Etest assay in LB pH 4.6 plates except for cefotiam, which was calculated in liquid LB pH 4.6 using serial dilutions of the antibiotic. MIC values for cefotiam (CTM) are highlighted in bold.
[b]CS, colistin: non-β-lactam antibiotic used as control.

binds specifically to PBP2 in *E. coli*,[18] showed less than 2-fold difference in strains expressing either PBP3 or $PBP3_{SAL}$ (Table 2). The MIC to mecillinam was also very similar in strains expressing either PBP2 or $PBP2_{SAL}$ (Table 2). Unlike PBP3 and $PBP3_{SAL}$, the pair PBP2/$PBP2_{SAL}$ might therefore bind mecillinam with similar affinity. Altogether, these data indicated that, unlike the other cephalosporins tested, cefotiam shows higher affinity for $PBP3_{SAL}$ compared with PBP3 and with no binding to other PBPs at concentrations in the 0.0001–0.001 mg/mL (0.16–1.6 μM) range.

Unlike cefuroxime, cefotiam is highly bactericidal against intracellular S. Typhimurium in a $PBP3_{SAL}$-dependent manner

$PBP3_{SAL}$ is not produced by *S.* Typhimurium when growing extracellularly in neutral pH but its expression is up-regulated in acidic compartments of eukaryotic cells.[10,12] Because cefotiam showed increased affinity for $PBP3_{SAL}$, we reasoned that it could inhibit bacterial growth by blocking the activity of this PBP. To differentiate such predicted selectivity, we used the isogenic series of single and double *S.* Typhimurium mutants lacking PBP2, PBP3, $PBP2_{SAL}$ or $PBP3_{SAL}$ to infect the rat fibroblast NRK-49F cell line, in which we have extensively characterized mechanisms used by the pathogen to survive and persist intracellularly.[16,19,20] Non-phagocytic cells consistent with fibroblasts are also targeted *in vivo* by *S.* Typhimurium in the intestinal lamina propria.[16] The addition of 0.001 mg/mL (1.6 μM) cefotiam to the tissue culture medium at 2 hpi led to a drastic drop in the viability at 24 hpi of those strains dividing only with $PBP3_{SAL}$ (ΔPBP3 genetic background). This was demonstrated by drop assays performed on agar plates containing the antibiotic (Figure 3a) as well as by plating and cfu counting corresponding to viable intracellular bacteria at 2 hpi and 24 hpi (Figure 3b).

To further demonstrate the selective action of cefotiam against intracellular bacteria due to inhibition of $PBP3_{SAL}$, we also tested cefuroxime (Figure 3c), a cephalosporin that has higher affinity for PBP3 than $PBP3_{SAL}$.[10] The bactericidal effect of cefuroxime in intracellular bacteria was the reverse to that of cefotiam, i.e. more pronounced for the Δ$PBP3_{SAL}$ mutant expressing PBP3 (Figure 3d,e). Overall, these data demonstrated that cefotiam is an antibiotic that reaches the intracellular compartment colonized by *S.* Typhimurium inside host cells and that it inhibits selectively $PBP3_{SAL}$ at the concentrations used.

Discussion

In enteric bacteria like *E. coli* and *S.* Typhimurium, PBP2 and PBP3 are morphogenetic peptidoglycan synthases directing the cell elongation and cell division phases, respectively.[9,21] Due to these essential roles, PBP2 and PBP3 have been traditionally selected as targets for developing new drugs within the group of β-lactam antibiotics. Cephalosporins are widely used in clinics; however, some factors decrease their effectiveness. Besides the emergence of MDR and extensive-resistant isolates in *S. enterica* serovars with high incidence in humans such as Typhi, Typhimurium and Enteritidis,[22–24] there are also reports of relapses in infections caused by antibiotic-susceptible isolates. This phenomenon is repeatedly documented in salmonellosis, with rates that can reach up to 15% of treated patients.[8,25–27] Our previous work showed that *S.* Typhimurium mutants defective in $PBP3_{SAL}$ and, therefore, more susceptible to β-lactams, were less capable of causing relapse in a mouse typhoid model following ceftriaxone therapy.[12] $PBP3_{SAL}$ is envisioned as a novel target of extreme importance in the biology of intracellular *S.* Typhimurium and consequently should be considered in future research directed to develop new effective antimicrobial drugs.

Unlike PBP3, $PBP3_{SAL}$ is expressed by the pathogen exclusively in acid environments,[12] which precludes its analysis by the standard methods used in hospitals to determine antimicrobial susceptibility. In view of our findings, this practice, based on nutritional media at neutral pH like the Mueller–Hinton broth, should be revisited when analysing *Salmonella* isolates. Despite the unique feature of $PBP3_{SAL}$ of not binding antibiotics when expressed artificially at neutral pH and showing in acid pH reduced affinity for Boc-FL and other β-lactams like cefuroxime,[10] this study provides evidence for the contrasting view of an antibiotic, cefotiam, with higher affinity to $PBP3_{SAL}$ than to PBP3. The encouraging data obtained in the *in vitro* infection model with cultured fibroblasts indicate that, when *S.* Typhimurium enters into a susceptible human or animal host, it should be possible to kill this pathogen 'only' in intracellular locations. The ∼10-fold difference in IC_{50} values for PBP3 and $PBP3_{SAL}$ obtained in the Boc-FL competition assays with cefotiam supports this possibility when using appropriate doses of the antibiotic. This innovative therapy should be theoretically innocuous to the endogenous microbiota given the absence of genes encoding $PBP2_{SAL}$/$PBP3_{SAL}$ orthologues in the beneficial bacteria.

It is also worth recalling that cefotiam can be now be exploited to analyse computationally at the atomic level how it behaves as a ligand when entering the catalytic pocket of modelled PBP3 and $PBP3_{SAL}$ atomic structures. These analyses can provide insights into the different affinity found for this antibiotic to bind to these PBPs and facilitate the identification of new drugs acting with even higher selectivity. The structures of related cephalosporins that behave in an opposite manner to cefotiam regarding the bactericidal effect in intracellular bacteria, such as cefuroxime, are certainly of much value for the design of new antimicrobial drugs selectively controlling the intracellular infection.

Cefotiam was first described in 1979 as a second-generation cephalosporin more potent than others like cefazolin and being active against Gram-positive and Gram-negative bacteria, including Enterobacteriaceae genera such as *Enterobacter* spp., *Klebsiella* spp., *E. coli*, *Salmonella* spp. and *Proteus*.[28–30] Some of these bacteria like *E. coli* and *Proteus* spp. do not have an alternative $PBP3_{SAL}$-like homologue to PBP3. Furthermore, the antimicrobial susceptibility assays against these bacteria were performed in standard neutral pH (Mueller–Hinton medium), a condition in which at least in *Salmonella* spp., $PBP3_{SAL}$ is not expressed.[10] These observations unequivocally demonstrate that cefotiam targets PBP3 at neutral pH, although probably at higher concentrations than those required to target $PBP3_{SAL}$ in acid pH. This difference regarding the effective inhibitory concentration in extra- and intracellular niches should be considered in future therapies based on cefotiam. Surprisingly, despite the optimal pharmacokinetics exhibited by cefotiam,[31] to our knowledge no study has investigated the therapeutic potential of this cephalosporin to eradicate *Salmonella* infections. The data presented here, sustained with isogenic *S.* Typhimurium mutant strains lacking specific PBPs involved in cell elongation and division and alternative

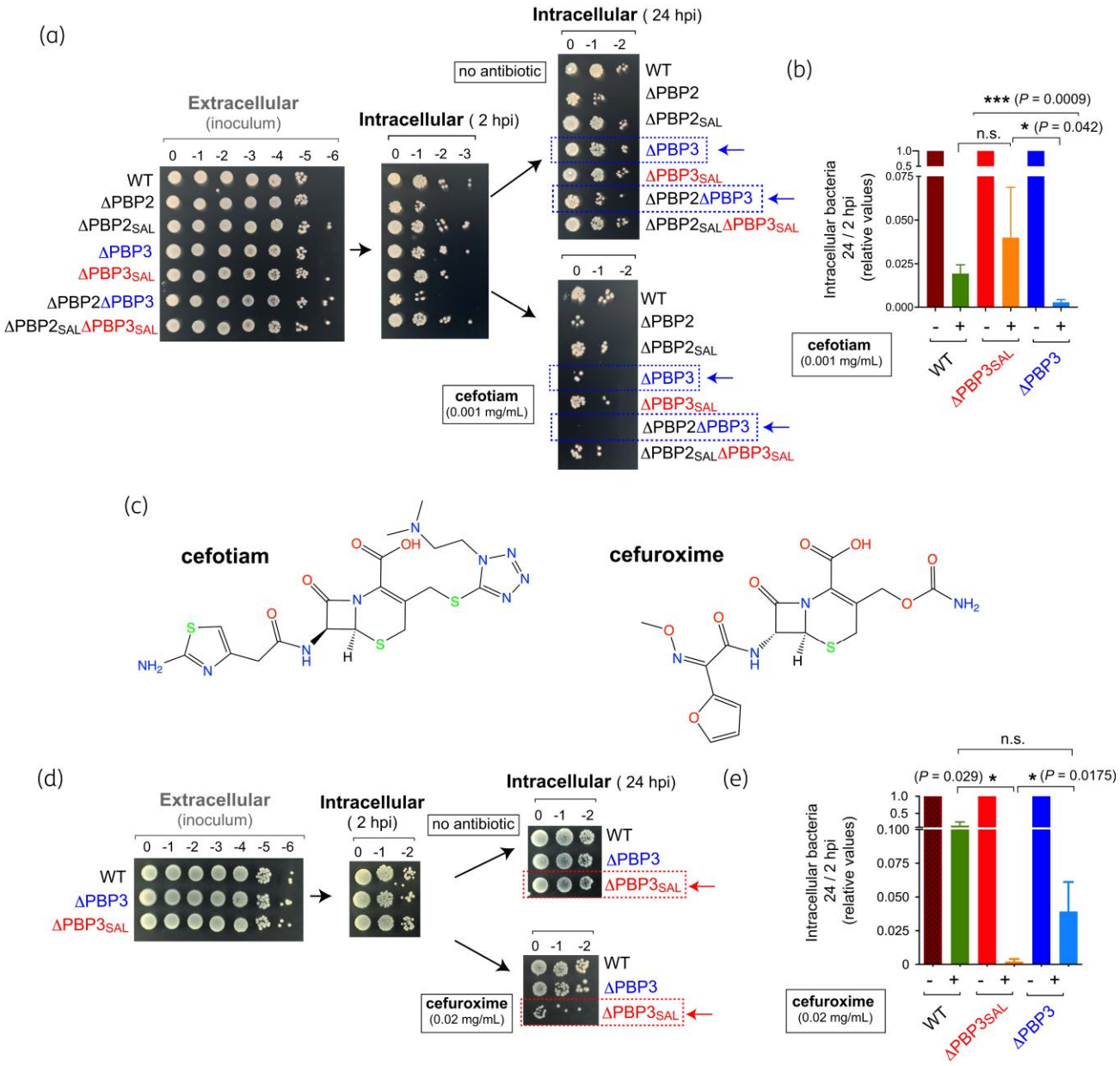

Figure 3. Cefotiam shows stronger viability inhibition in intracellular *S.* Typhimurium compared with other cephalosporins like cefuroxime. (a) Viability of the series of *S.* Typhimurium isogenic single and double mutants defective in PBPs inside NRK-49F rat fibroblasts at distinct post-infection times (2 hpi, 24 hpi) determined by the drop assay. Shown are serial dilutions of the overnight culture used for infection (inoculum) and the extracts obtained from the infected NRK-49F fibroblast culture. Cefotiam (0.001 mg/mL, 1.6 μM) was added to the tissue culture medium at 2 hpi. (b) Ratios of viable intracellular bacteria at 24 hpi versus 2 hpi determined for the indicated strains in the absence/presence of cefotiam. The values are shown relative to the samples not treated with the antibiotic and are the mean and SD of a total of the four independent biological replicates. Experimental mean of the 24 hpi/2 hpi ratios corresponding to four independent assays for samples without antibiotic were: 0.675 (wild type), 0.675 (ΔPBP3) and 0.738 ($\Delta PBP3_{SAL}$). (c) Structures of the cephalosporins cefotiam and cefuroxime, which show high affinity for $PBP3_{SAL}$ and PBP3, respectively. (d) Drop assay depicting the viability of *S.* Typhimurium wild-type, ΔPBP3 and $\Delta PBP3_{SAL}$ strains inside NRK-49F fibroblasts at 2 hpi and 24 hpi. Shown are serial dilutions corresponding to inoculum (extracellular bacteria) and extracts containing intracellular bacteria. Cefuroxime (0.02 mg/mL, 47 μM) was added to the tissue culture medium at 2 hpi. (e) Ratios of viable intracellular bacteria at 24 hpi versus 2 hpi obtained for the indicated strains in the absence/presence of cefuroxime. The values are shown relative to the samples with no β-lactam added and are the mean and SD of four independent biological replicates. Data were analysed by Student's *t*-test. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$. n.s., not significant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

cephalosporins, clearly support the usage of cefotiam for treating salmonellosis.

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Transparency declarations

The authors declare no conflict of interest. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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