Evolutionary adaptability of β-lactamase: a study of inhibitor susceptibility in various model systems

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Mycobacterium tuberculosis β-lactamase variant reduces sensitivity to ampicillin/avibactam in a zebrafish model for tuberculosis

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Mycobacterium marinum blaC frameshift mutant was created by J. J. Maaskant and C. P. Kuijl. Microinjections were performed in cooperation with M. A. Aguirre García.

Abstract

The β-lactamase of *Mycobacterium tuberculosis*, BlaC, hydrolyzes β-lactam antibiotics, hindering the use of these antibiotics for the treatment of tuberculosis. Inhibitors, such as avibactam, can reversibly inhibit the enzyme, allowing for the development of combination therapies using both antibiotic and inhibitor. However, laboratory evolution studies using *Escherichia coli* resulted in the discovery of single amino acid variants of BlaC that reduce the sensitivity for inhibitors or show higher catalytic efficiency against antibiotics. Here, we tested these BlaC variants under more physiological conditions using the *Mycobacterium marinum* infection model of zebrafish, which recapitulates hallmark features of tuberculosis, including the intracellular persistence of mycobacteria in macrophages and the induction of granuloma formation. To this end, the *M. tuberculosis blaC* gene was integrated into the chromosome of a *blaC* frameshift mutant of *M. marinum.* Subsequently, the resulting strains were used to infect zebrafish embryos in order to test the combinatorial effect of ampicillin and avibactam. The results show that embryos infected with an *M. marinum* strain producing BlaC show lower infection levels after treatment than untreated embryos. Additionally, embryos infected with the strain producing BlaC K234R showed higher infection levels after treatment than those infected with bacteria producing the wild-type enzyme, demonstrating that the zebrafish host is less sensitive to the combinatorial therapy of β-lactam antibiotic and inhibitor. These findings are of interest for future development of combination therapies to treat tuberculosis.

Introduction

One-quarter of the world's human population is estimated to be latently infected with tuberculosis (TB) and TB is the leading cause of death by bacterial infection in 2020.[189] *Mycobacterium tuberculosis* (Mtb), the pathogen that causes TB, can be transmitted between hosts in aerosol particles and parasitizes macrophages in the lungs of the patient.[190] Mtb expresses the *blaC* gene, coding for a class A extended-spectrum β-lactamase, BlaC, that can hydrolyze β-lactam antibiotics, such as ampicillin and early generations of cephalosporins.[27,53] The presence of this protein prohibits the use of β-lactam antibiotics for the treatment of TB. However, the discovery and development of β-lactamase inhibitors opens up possibilities for combination therapies. Clavulanic acid is a naturally occurring β-lactam that is produced by *Streptomyces clavuligerus* and has FDA approval to be used in combination with several antibiotics for the treatment of bacterial infections in both humans and domestic animals.[88,89] Sulbactam and avibactam are both synthetic inhibitors, the former structurally similar to clavulanic acid, while the latter is a diazabicyclooctane. Sulbactam is in clinical use in combination with ampicillin or cefoperazone, and avibactam is combined with ceftazidime.[94,105,137] *In vitro* data show that these inhibitors inhibit BlaC by covalently binding to the catalytic residue Ser70.[104]

Previous experiments using *Escherichia coli* have shown that BlaC can be inhibited by β-lactamase inhibitors.[54,119,172] Single amino acid mutations have been identified that improve catalytic efficiency against ampicillin or reduce sensitivity for inhibitors (chapters 2-4).[54,116,118,125,172] It is currently unknown whether the phenotypes of mutations in BlaC in *E. coli* translate to Mtb. Therefore, we aimed to test the effect of amino acid mutations under more physiological conditions using a zebrafish infection model.

Zebrafish (*Danio rerio*) are widely used as a model system of tuberculosis for both active and latent disease.[191–195] *Mycobacterium marinum* (Mmar) is a natural pathogen of zebrafish that shares 3,000 orthologs with Mtb, including crucial virulence factors, with an average sequence similarity of 85%.[196] After infection of zebrafish by Mmar, the bacteria are phagocytosed by macrophages, which subsequently invade tissues and initiate the formation of granulomas.[197] These are collections of infected and uninfected macrophages surrounded by epithelial cells and other immune cells and are similar to granulomas found in the lungs of human patients infected with Mtb.[198–202] The early stages of granuloma formation can be studied in zebrafish embryos, which are optically transparent, allowing for easy and non-invasive visualization of cell tissues or bacteria using fluorescent labels. Mmar infection studies in zebrafish embryos have contributed key insights into the function of granulomas and into the role of different immune response genes in host resistance.[194,202,203] Furthermore, the zebrafish embryo model has successfully been used to screen for novel anti-infectious treatments.[204–206]

Here, we aimed to establish whether the properties of BlaC variants result in changed infection patterns in the zebrafish TB model. A *blaC* knockout strain of Mmar was created and Mtb *blaC*

variants were introduced for chromosomal expression. Growth on plates shows the same trends as the data obtained previously with an expression system in *E. coli*, indicating that this organism yields representative data. Furthermore, the zebrafish model system shows that inhibition effects of wild-type BlaC and BlaC K234R observed in Mmar and *E. coli* lead to the predicted changes in infection levels, demonstrating that ampicillin/avibactam is effective as a treatment of Mmar infection in zebrafish.

Results

Mmar expressing BlaC variants show reduced sensitively to β-lactam inhibitors

We first aimed to test the effect of single amino acid mutations and compare them with other studies on Mtb BlaC. Mmar BlaC and Mtb BlaC share only 70% of their amino acid sequence, necessitating the replacement of Mmar *blaC* by the homologous Mtb gene. *Streptococcus thermophilus* CRISPR1-Cas9 was used to produce a frameshift in the Mmar *blaC* gene,[207] resulting in a stop codon after Ser31 (Ambler numbering[65]). A mycobacterial integration vector was used to introduce the Mtb *blaC* sequence coding for the mature protein, preceded by the promotor region and signal sequence of Mmar *blaC*, into the chromosome of Mmar (Figure S5.1). The resulting strains were tested for their ability to grow in the presence of either ampicillin, carbenicillin, or a combination of ampicillin and inhibitor (Figures 5.1, S5.2, and S5.3). The strain carrying the wild type Mtb *blaC* can grow on a plate with these antibiotics. Mutation S70A results in a catalytically inactive BlaC, as it prohibits the formation of the acyl-enzyme. Mmar expressing this variant does not grow in the presence of ampicillin or carbenicillin, serving as a negative control. Ile105 has been named the gatekeeper residue,[125] and is present in the loop that restricts access to the active site. A Phe in position 105 improves the catalytic efficiency of the enzyme and allows for the hydrolysis of ampicillin in the presence of clavulanic acid.[125] Production of Mtb BlaC I105F allows Mmar to grow on 80 μg mL^{-1} ampicillin, whereas Mmar expressing the wild type Mtb *blaC* or other variants grow up to 40 μg mL^{-1} ampicillin. Position 132 is generally occupied by Asn in class A β-lactamases, yet BlaC has Gly in this position. Mutation G132N restores the canonical motif, which was reported to result in reduced sensitivity for both clavulanic acid and avibactam,[116–118] while G132S was found in laboratory evolution experiments to confer reduced sensitivity for sulbactam and increased sensitivity for avibactam.[172] When expressed in Mmar, the mutation of residue 132 to either Asn or Ser was observed to confer reduced sensitivity for sulbactam. BlaC K234R was previously found to reduce sensitivity for clavulanic acid[54,118] and the K234R mutation is known to confer reduced sensitivity for avibactam to the β-lactamase KPC-2.[208] In Mmar, Mtb BlaC K234R also confers reduced sensitivity for avibactam. The other mutations tested, D172N and D179N, decreased sensitivity for sulbactam and avibactam, respectively, when produced in *E. coli* (chapter 3).[127,172] This is likely due to increased protein stability, and no such decrease was observed in Mmar. The addition of clavulanic acid showed no effect on bacterial growth for the concentrations tested (Figure S5.4), probably caused by the instability of clavulanic acid at 30 °C in combination with the long growth time in these experiments (8 days).[209,210] In conclusion, most of the mutation effects previously observed in *E. coli* translate to Mmar.

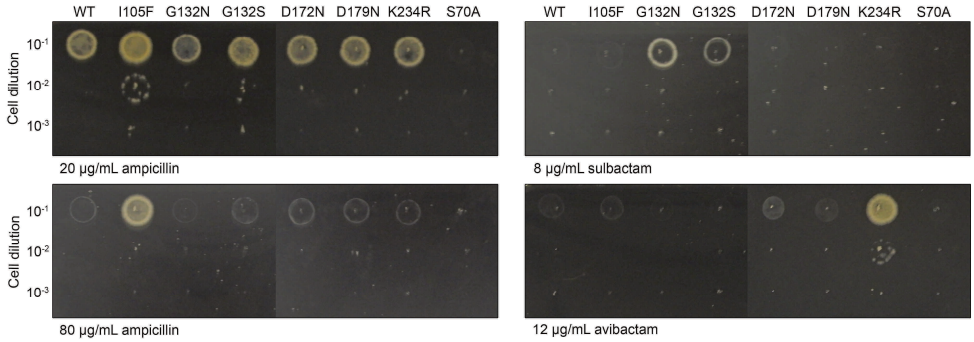

Figure 5.1. Activity of Mtb BlaC mutants produced in Mmar. Cultures of Mmar chromosomally expressing wild-type BlaC or variants S70A (negative control), I105F, G132N, G132S, D172N, D179N, and K234R were incubated for 8 days at 30 °C on plates containing 20 or 80 µg mL^{-1} ampicillin, or 8 µg mL^{-1} sulbactam or 12 µg mL^{-1} avibactam in the presence of 15 µg mL^{-1} ampicillin. The complete plates are shown in Figures S5.2 and S5.3.

Inhibition of BlaC in zebrafish embryos infected with Mmar

To test whether ampicillin, clavulanic acid, sulbactam, and avibactam could be used to treat zebrafish for mycobacterial infections, embryotoxicity tests were performed by injecting high concentrations of the compounds into the blood island of the embryos. After 4 days, no developmental abnormalities were observed for any groups and more than 80% of the injected larvae survived (Figures S5a-d), similar to the non-injected controls.

To test the effect of β-lactam antibiotics and inhibitors on Mtb BlaC activity in zebrafish embryos, ampicillin, and avibactam were selected as treatment as they result in a clear difference in phenotype for BlaC variants on plate (Figure 5.1). Embryos were injected in the blood island with Mmar expressing Mtb *blaC* and producing the fluorescent protein mWasabi around 30 hours post-fertilization. Embryos showing systemic infections were treated 24 hours after infection by injection in the Duct of Cuvier with ampicillin, avibactam, or both, and the bacterial load was determined 4 days after infection. Embryos injected with PBS showed clear signs of infection, with the formation of the typical granuloma-like aggregates of infected cells, as did those treated with ampicillin or avibactam (Figures 5.2 and S5.6a). Zebrafish embryos treated with both ampicillin and avibactam showed lower fluorescence intensity than untreated larvae, indicating a reduced bacterial load. Interestingly, the same effect was observed for larvae infected with Mmar harboring native BlaC (Figures S5.5e and S5.6c). Injection of ampicillin or avibactam alone had no significant effect on infection. These results show that ampicillin/avibactam combination therapy is effective in the zebrafish TB model.

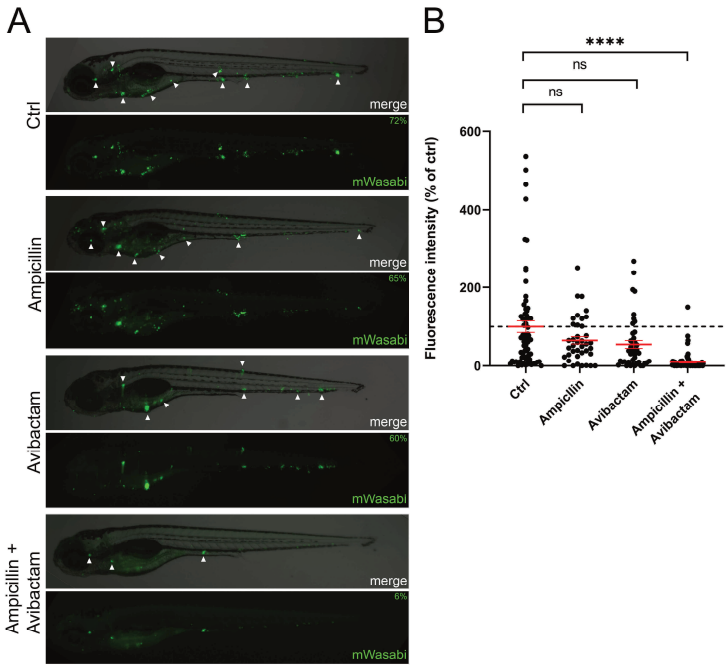

Figure 5.2. Effect of combination treatment on zebrafish embryos infected with mWasabi-labelled Mmar producing Mtb BlaC at 4 dpi. (A) Representative larvae showing systemic infection from the control group and groups treated with ampicillin, avibactam, or both. Arrows indicate collections of bacteria indicative of granuloma formation, and percentages indicate the percentage of the mean of the control. (B) Bacterial load of Mmar producing wild type Mtb BlaC as represented by fluorescence intensity after being given the indicated treatment 1 dpi. Each dot represents a larva. Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin in PBS (estimated concentration 100 µg mL^{-1} in the embryo, n=39), 65 mg mL^{-1} avibactam in PBS (225 µg mL^{-1}, n=43), ampicillin and avibactam in PBS (same concentrations, n=67) or PBS only (ctrl, n=69). Data for the groups treated with both ampicillin and avibactam or the control were accumulated in three, and the groups treated with only ampicillin or avibactam in two independent experiments. Data were normalized by setting the mean of the control to 100%. Error bars represent the mean and standard error. Mood's median test with Holm-Bonferroni post hoc test for multiple comparisons was used to compare groups with the control: ns, not significant; ****, $p < 0.0001$.

Mutation K234R reduces sensitivity to ampicillin/avibactam in zebrafish

To check whether the properties of BlaC variants could influence Mmar pathogenesis in zebrafish, we used the K234R mutation that moderately reduces the sensitivity for avibactam, by 8-fold and 10-fold in growth assays for KPC-2 in *E. coli* and BlaC in Mmar, respectively.[208] Zebrafish embryos were infected with Mmar strains producing either wild-type BlaC or BlaC K234R and those with systemic infections were injected with a combination of ampicillin and avibactam 24 hours after infection. Infection levels in the control group were not influenced by the mutation, but a clear difference was observed for the treatment group (Figures 5.3 and S5.6b). In the presence of ampicillin/avibactam combination therapy, infection levels in larvae infected with Mmar producing BlaC K234R were significantly higher than the infection levels for wild-type BlaC. Therefore, we conclude that the K234R mutation reduces sensitivity to ampicillin/avibactam in the zebrafish TB model.

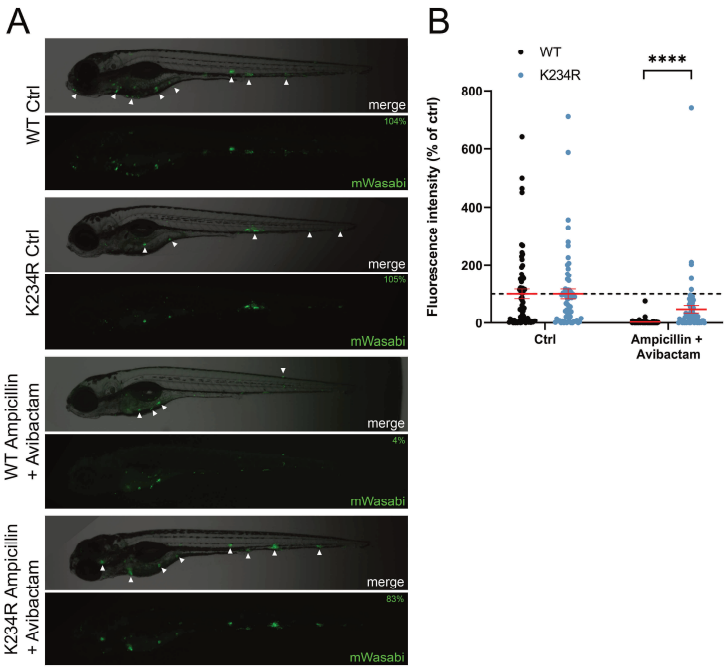

Figure 5.3. Effect of combination treatment on zebrafish embryos infected with mWasabi-labelled Mmar producing Mtb BlaC variants at 4 dpi. (A) Representative larvae showing systemic infection from the control group and groups treated with ampicillin, avibactam, or both. Arrows indicate collections of bacteria indicative of granuloma formation, and percentages indicate the percentage of the mean of the control. (B) Bacterial load of Mmar producing either wild type (black dots) or K234R Mtb BlaC (blue dots) as represented by fluorescence intensity after being given the indicated treatment 1 dpi. Each dot represents a larva. Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 22 mg mL^{-1} avibactam in PBS (estimated concentration 100 µg mL^{-1} ampicillin and 75 µg mL^{-1} avibactam in the embryo, n=69 for WT and n=62 for K234R) or PBS only (ctrl, n=66 for WT and n=63 for K234R). Data were accumulated in three independent experiments and normalized by setting the mean of the control to 100%. Error bars represent the mean and standard error. Mood's median test was used to compare treated groups: ****, $p < 0.0001$.

Discussion

In vivo experiments testing inhibition of Mtb BlaC are generally performed using *E. coli*. While this method is efficient, *E. coli* bacteria differ substantially from mycobacteria in terms of accessory proteins, cell physiology, and cell wall composition, posing the question of whether results obtained in *E. coli* translate to Mtb. Furthermore, it is not clear whether the observed phenotypes affect the pathogenic behavior of mycobacteria *in vivo*. Here, we aimed to test the inhibition of wild-type BlaC and the effect of previously documented mutations in Mmar, using both a growth assay on plate and an infection assay in zebrafish embryos that is used as a model for TB in humans. Six mutants were compared to both wild-type BlaC and the catalytically inactive mutant S70A. It was found that the effects on enzyme activity and inhibition were similar to the effects previously found using *E. coli*. Only the marginal increases in antibiotic resistance of cells that was ascribed to enhanced enzyme stability of the variants could not be observed in Mmar.

Interestingly, the concentrations for antibiotics and inhibitors at which Mmar growth is inhibited are comparable to concentrations used in growth assays using *E. coli*,[125,127,172] while the permeability of the mycobacterial outer membrane has been reported to be 1000 times lower than for *E. coli*.[211,212] Nevertheless, it was reported before that the concentrations of both β-lactam and other antibiotics used for the selection of bacteria during cloning are similar for *E. coli* and Mmar.[213] Addition of detergent, such as Tween 80, which is added to 7H9 medium to prevent aggregation, probably disorganizes the cell wall, making it more permeable.[214,215] While Tween 80 was present in the liquid cultures used to grow the Mmar bacteria, and therefore present in the drops placed on the plates, it was absent from the plates themselves during the growth assays.

Zebrafish embryos and larvae are often used for high-throughput screens in drug research.[216–218] Potential drugs are added to the egg water rather than injected into the embryo, and the drugs should diffuse through the embryonic skin. However, not all small molecules are readily taken up and this mechanism differs from mammalian uptake.[218–221] Here, we injected the drugs into the Duct of Cuvier to precisely control concentrations ensuring full bioavailability for all individual larvae. To distinguish between the avibactam sensitivity of Mmar producing the wild type or BlaC K234R of Mtb, the concentration of avibactam is critical. At 75 μg mL^{-1}, a clearly higher infection level was seen for the mutant BlaC. At three times more avibactam, also BlaC K234R becomes fully inhibited (Figures S5f and S6d), in line with the modest difference in avibactam sensitivity observed in an *E. coli* growth assay for KPC-2.[208] Thus, our results demonstrate that introduction of the Mtb BlaC K234R variant into Mmar reduces sensitivity to ampicillin/avibactam in the zebrafish TB model. It will be of interest to establish whether the results are also of relevance to lowering the bacterial burden of Mtb in human cells.

Previous studies tested the inhibition of β-lactamases in zebrafish using either *Mycobacterium abscessus* or *Staphylococcus aureus* expressing their native β-lactamases.[222–224] Treatment with amoxicillin and avibactam decreased both the development of abscesses and the overall mortality when compared to fish treated with only amoxicillin 13 days post-infection with *M. abscessus* expressing Bla_{Mab}.[222] Combining avibactam with imipenem had a similar effect.[223]

In conclusion, the study shows that results obtained with mutants in the rather reductionistic and simple one gene - one phenotype system of *blaC* expression in *E. coli* are representative of the phenotype of these mutants in a mycobacterium and the zebrafish TB model. The model offers the possibility to test for the effects of antibiotic/inhibitor combinations, also together with other therapies, in an efficient way and to determine the evolvability of resistance due to mutations in BlaC.

Materials and methods

Bacterial strains, media, and zebrafish handling

All mycobacterium strains were derived from Mmar M and cultured in Middlebrook 7H9 medium, supplemented with 10% ADC and 0.05% Tween 80 or plated on Middlebrook 7H10 agar containing 10% ADC/OADC and 0.5% glycerine.[225] Antibiotics were added when appropriate at concentrations of 50 µg mL^{-1} kanamycin, 30 µg mL^{-1} streptomycin, or 50 µg mL^{-1} hygromycin. Cultures and plates were incubated at 30 °C. *E. coli* KA797 cells were used to clone and generate plasmids, and incubated in LB medium or on agar plates with appropriate antibiotics at 37 °C.[147] Zebrafish (*D. rerio*) of wild type line AB/TL were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (http://zfin.org) and in compliance with the directives of the local animal welfare committee of Leiden University. They were exposed to a cycle of 14 h light and 10 h dark to maintain circadian rhythmicity. Fertilization was performed by natural spawning at the beginning of the light period and eggs were raised at 28.5 °C in egg water (60 µg mL^{-1} Instant Ocean sea salts and 0.0025% methylene blue).

Construction of Mtb *blaC* in Mmar

Mmar Δ*blaC* strain was created as described before using *S. thermophilus* CRISPR1-Cas9.[207] sgRNAs were designed to target the *blaC* gene and pTdTomato-L5 was electroporated into the knock-out strain to replace pCRISPRx-Sth1Cas9-L5.[207] To introduce Mtb *blaC* variants on the chromosome of Mmar, the Mtb *blaC* gene (Uniprot P9WKD3) was cloned into the integration vector pML1337, replacing *psmyc-gfpm2+*.[226] Mtb *blaC* is preceded by the 140 bp of the upstream flanking region and signal peptide of Mmar *blaC* (Figure S5.1). Mutations S70A, I105F, G132N, G132S, D172N, D179N, or K234R were introduced into the Mtb *blaC* gene, and plasmids were electroporated into the Mmar Δ*blaC* pTdTomato-L5 strain, replacing pTdTomato-L5 in the MMAR_5512 locus (attB recombination site, position 4733000..4733042 of sequence NC_010612.1). Colonies were screened for resistance against kanamycin and sensitivity to streptomycin, and the constructs were confirmed by sequencing.

Antibiotic resistance and inhibitor sensitivity

Antibiotic resistance and inhibitor susceptibility were tested by adding 10 µL of Mmar Δ*blaC* pML1337-Mtb *blaC* liquid cultures with optical densities of 0.3, 0.03, 0.003, and 0.0003 on plates with various concentrations of ampicillin and inhibitors. Plates were incubated for 8 days at 30 °C before imaging.

Zebrafish embryo toxicity test

One-day-old zebrafish embryos (30 hpf) were manually dechorionated using surgical forceps (Dumont #5), anesthetized using 0.02% aminobenzoic acid ethyl ester (tricaine, Sigma Aldrich), and injected with 1 nL of either PBS, ampicillin, clavulanate, sulbactam, or avibactam

in PBS. The solutions contained 10% phenol red (Sigma-Aldrich) to aid the visualization of injections. The solutions were injected into the blood island of the embryos using glass microcapillary needles and survival rates were tracked for 4 days.

Zebrafish embryo infection

To allow for imaging of the mycobacteria in zebrafish larvae, the pTEC15 plasmid (Addgene plasmid #30174)[227] was electroporated into the Mmar Δ*blaC* pML1337-Mtb *blaC* strains and colonies were selected for both kanamycin and hygromycin resistance. One-day-old zebrafish embryos (30 hpf) were dechorionated, anesthetized, and injected in the blood island with 1 nL of Mmar pTEC15, Mmar Δ*blaC* pML1337_Mtb *blaC* pTEC15, or Mmar Δ*blaC* pML1337_Mtb *blaC*_K234R pTEC15 (300 cfu in PBS). At ~24 hours post-infection, embryos showing systemic infection were injected in the Duct of Cuvier with 1 nL of either PBS, ampicillin, avibactam or a combination of ampicillin and avibactam.

Image quantification and statistical analysis

Zebrafish larvae were anesthetized and imaged at 4 dpi using a Leica M205FA fluorescence stereomicroscope equipped with a Leica DFC 345FX camera and Leica Las X software. Fluorescence intensity was quantified using QuantiFish version 2.1.1.[228] Intensities were normalized by using the mean of the control group as 100%. Many of the distributions are highly skewed and consequently, non-parametric statistical analysis was required to analyze the data (Figure S5.6). In addition, there is unequal variance between groups. Mood's median test with Holm-Bonferroni post hoc test for multiple comparisons was employed to compare the treated groups to the control (Figures 5.2b, S5.5c, and S5.5d) or treated groups for BlaC WT and K234R (Figure 5.3b).[229]

Supporting information

A

GTCATGGGCAAATACCTTAACCGGGCAGGTGCGGCGCCCTACGACCGGCCGTCGTAGAGCGCCGCGGCGGGGGC
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CGTGAAGCACTGCTGGCGGAAGCAGCAACCTGTGTTGCCGGTGTTCTGGCACTCGAGTA

B

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                                                      30         40
MRPSNPRSAV NRRQLLAAMA ALLPLSACAK AASDQHMAST MAVPSPDLAD RFAELERRYD

        50         60         70         80         90         100
ARLGVYVPAT GTTAAIEYRA DERFAFCSTF KAPLVAAVLH QNPLTHLDKL ITYTSDDIRS

     110        120        130        140             150        160
ISPVAQQHVQ TGMTIGQLCD AAIRYSDGTA ANLLLADLGG PGGGTAAFTG YLRSLGDTVS

          170        180        190        200        210        220
RLDAEEPELN RDPPGDERDT TTPHAIALVL QQLVLGNALP PDKRALLTDW MARNTTGAKR

          230        240        250        260           270        280
IRAGFPADWK VIDKTGTGDY GRANDIAVVW SPTGVPYVVA VMSDRAGGGY DAEPREALLA

          290
EAATCVAGVL ALE
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Figure S5.1. DNA and amino acid sequences of Mtb BlaC in Mmar. (A) The Mtb *blaC* gene is preceded by 140 bp of the upstream flanking region of Mmar *blaC* (shaded) and the sequence coding for the Mmar *blaC* signal peptide (underlined, locus MMAR_3050). (B) Residues 28-291 are numbered according to the Ambler notation[65] (BlaC misses residue 58, 84, 85, 239, and 253 and has 6 additional residues: 145A, B, C, D, 269A, and B; this corresponds to residue numbers 43-307 of Mtb BlaC Uniprot entry P9WKD3-1. The last two residues (L and E) are remnants of cloning. Residues of the Mmar signal peptide are underlined (residues 0-46 of Uniprot entry B2HFS3).

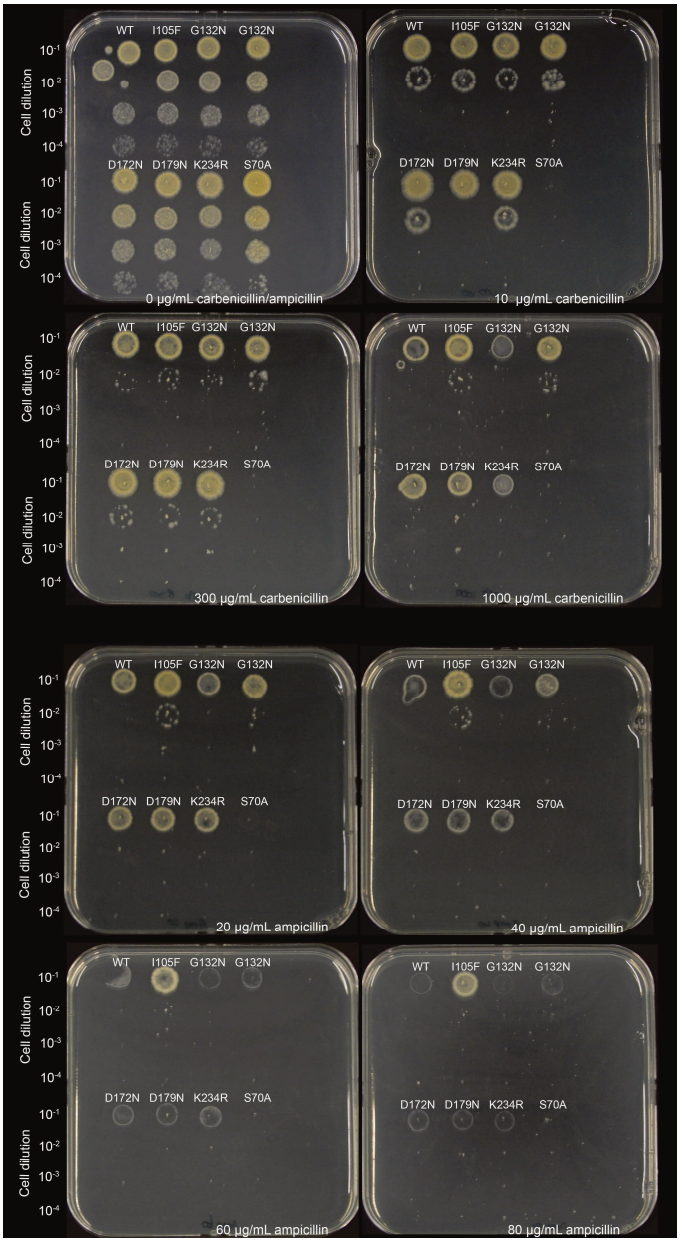

Figure S5.2. Activity of Mtb BlaC variants produced in Mmar. Cultures of Mmar chromosomally expressing wild-type BlaC or variants S70A (negative control), I105F, G132N, G132S, D172N, D179N, and K234R were incubated for 8 days at 30 °C on plates containing indicated concentrations of carbenicillin and ampicillin.

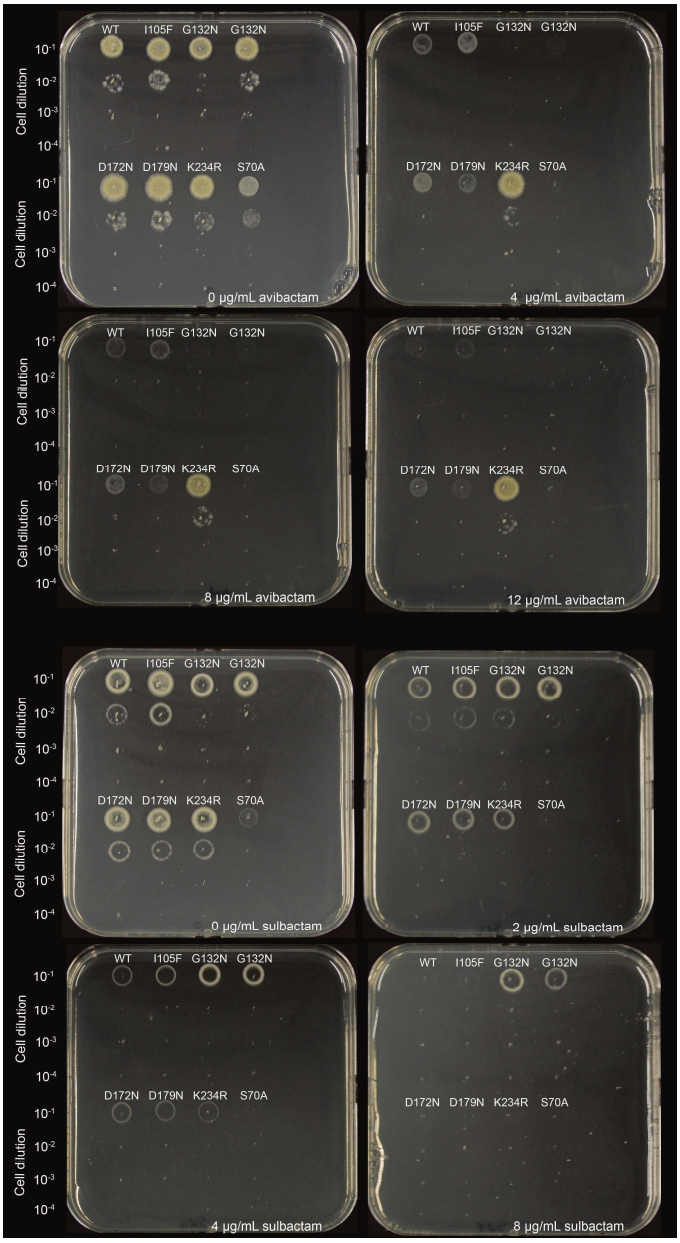

Figure S5.3. Activity of Mtb BlaC variants produced in Mmar. Cultures of Mmar chromosomally expressing wild-type BlaC or variants S70A (negative control), I105F, G132N, G132S, D172N, D179N, and K234R were incubated for 8 days at 30 °C on plates containing indicated concentrations of avibactam and sulbactam in the presence of 15 µg mL^{-1} ampicillin.

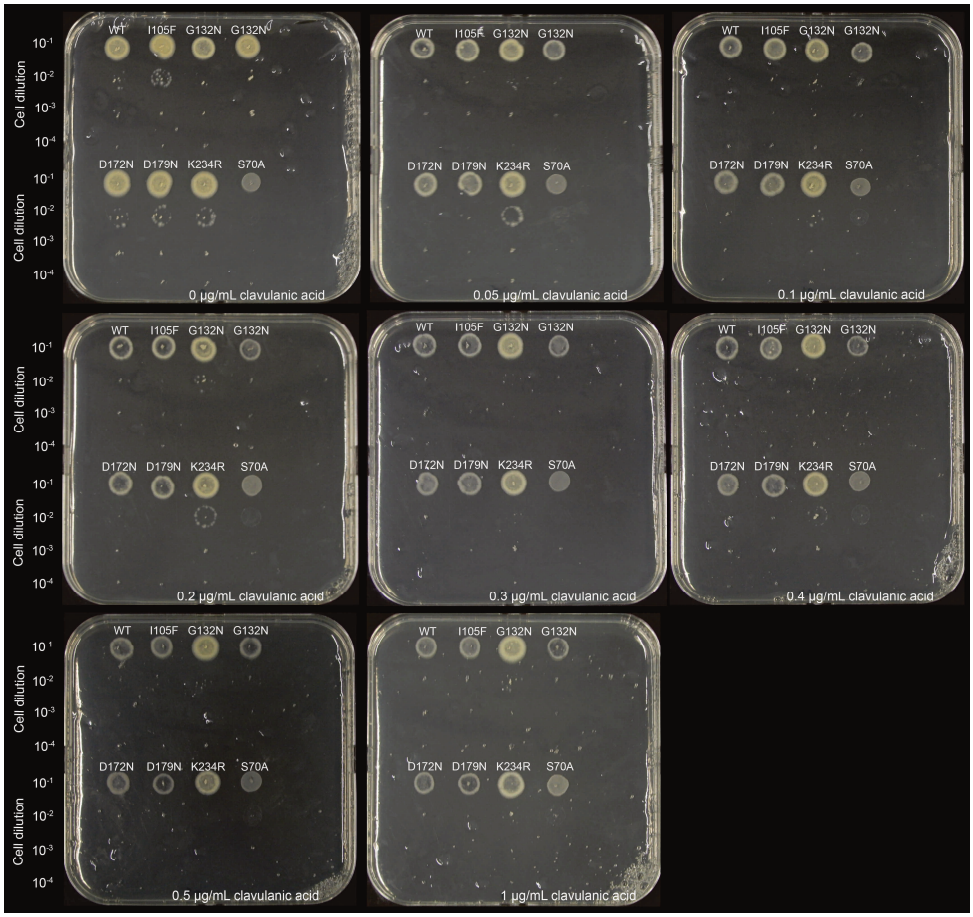

Figure S5.4. Activity of Mtb BlaC variants produced in Mmar. Cultures of Mmar chromosomally expressing wild-type BlaC or variants S70A (negative control), I105F, G132N, G132S, D172N, D179N, and K234R were incubated for 8 days at 30 °C on plates containing indicated concentrations of clavulanic acid in the presence of 15 µg mL^{-1} ampicillin.

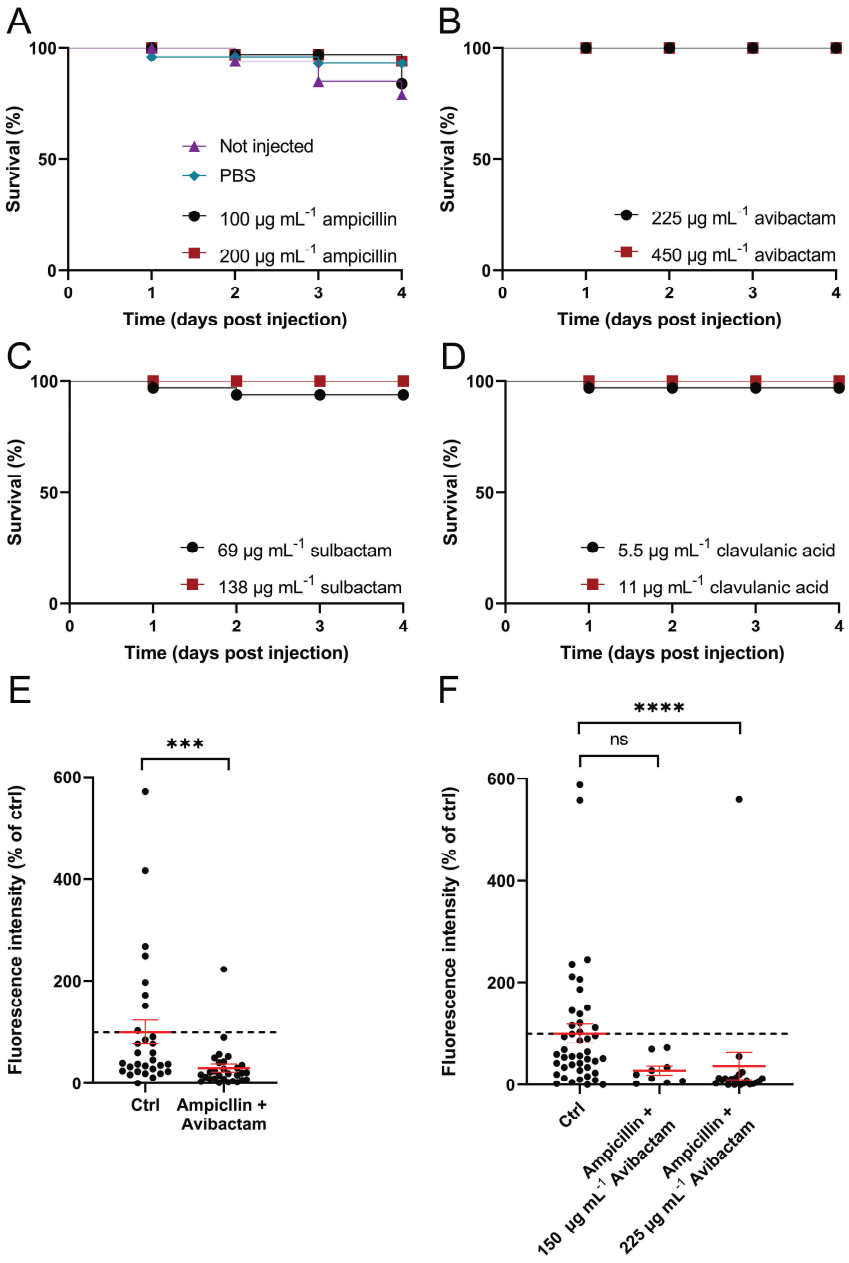

Figure S5.5. Effect of treatment by injection with ampicillin and/or inhibitors on zebrafish embryos. (A-D) Survival rates of embryos (A) not injected (n=34) or injected at 1dpf with 1 nL of either PBS (n=28) or ampicillin in PBS (29 mg mL^{-1}, n=32 or 58 mg mL^{-1} n=33), (B) avibactam in PBS (65 mg mL^{-1}, n=31 or 131 mg mL^{-1}, n=29), (C) sulbactam in PBS (20 mg mL^{-1}, n=35 or 40 mg mL^{-1}, n=23), (D) clavulanic acid in PBS (1.6 mg mL^{-1} or 3.2 mg mL^{-1}, both n=31). The injected concentrations were calculated to reach the desired concentrations of 100 or 200 µg mL^{-1} ampicillin, 5.5 or 11 µg mL^{-1} clavulanic acid, 69 or 138 µg mL^{-1} sulbactam, or 225 or 450 µg mL^{-1} avibactam in the embryo. (E) Bacterial load of wild type Mmar

represented by fluorescence intensity after being given the indicated treatment 1dpi. Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 22 mg mL^{-1} avibactam in PBS (estimated concentration 100 µg mL^{-1} ampicillin and 75 µg mL^{-1} avibactam in the embryo, n=29) or PBS only (ctrl, n=30) at 4 dpi. (F) Bacterial load of Mmar producing Mtb BlaC K234R as represented by fluorescence intensity after been given the indicated treatment 1dpi. Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 44 or 65 mg mL^{-1} avibactam in PBS (100 µg mL^{-1} ampicillin and 150 µg mL^{-1} or 225 µg mL^{-1} avibactam in the embryo, n=9 or n=21) or PBS only (ctrl, n=44) at 4dpi. Each dot represents a single larva and data were normalized by setting the mean of the control to 100%. Error bars represent the mean and standard error. Mood's median test with Holm-Bonferroni post hoc test for multiple comparisons was used to compare groups with their control: ns, not significant; ***, p <0.001; ****, p < 0.0001.

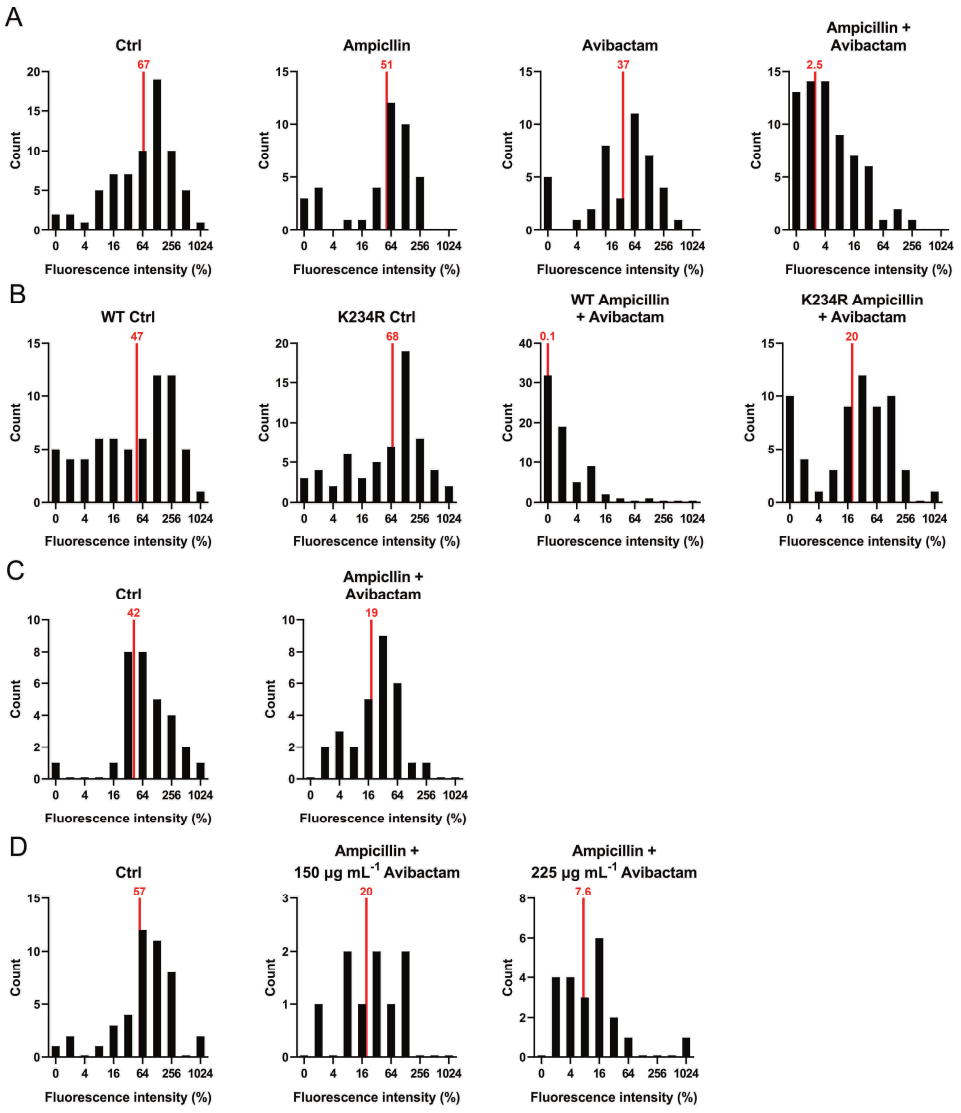

Figure S5.6. Distribution of fluorescence intensity for the Mmar infection datasets. (A) Number of larvae infected with Mmar producing wild type Mtb BlaC after being given the indicated treatment 1dpi (Figure 2b). Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin in PBS (estimated concentration 100 µg mL-

[1] in the embryo, n=39), 65 mg mL^{-1} avibactam in PBS (225 µg mL^{-1}, n=43), ampicillin and avibactam in PBS (same concentrations, n=67) or PBS only (ctrl, n=69). Data for the groups treated with both ampicillin and avibactam or the control were accumulated in three, and the groups treated with only ampicillin or avibactam in two independent experiments. (B) Number of larvae infected with Mmar producing either wild type or K234R Mtb BlaC after being given the indicated treatment 1dpi (Figure 2c). Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 22 mg mL^{-1} avibactam in PBS (estimated concentration 100 µg mL^{-1} ampicillin and 75 µg mL^{-1} avibactam in the embryo, n=69 for WT and n=62 for K234R) or PBS only (ctrl, n=66 for WT and n=63 for K234R). Data were accumulated in three independent experiments. (C) Number of larvae infected with wild type Mmar after been given the indicated treatment 1dpi (Figure S5e). Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 22 mg mL^{-1} avibactam in PBS (estimated concentration 100 µg mL^{-1} ampicillin and 75 µg mL^{-1} avibactam in the embryo, n=29) or PBS only (ctrl, n=30) at 4 dpi. (F) Number of larvae infected with Mmar producing Mtb BlaC K234R after been given the indicated treatment 1dpi (Figure S5f). Larvae were injected with 1 nL of 29 mg mL^{-1} ampicillin and 44 or 65 mg mL^{-1} avibactam in PBS (100 µg mL^{-1} ampicillin and 150 µg mL^{-1} or 225 µg mL^{-1} avibactam in the embryo, n=9 or n=21) or PBS only (ctrl, n=44) at 4dpi. Data were normalized by setting the mean of the control to 100%. The bin ranges are 0%, 0.01% - 2%, 2.01% - 4%, 4.01% - 8%, 8.01% - 16%, etc., with the maximum values as labels below the x-axis. Medians are shown in red.