Supplementary material to

Mutation G132S enhances resistance of *Mycobacterium tuberculosis* β-lactamase against sulbactam

Ilona van Alen[1,†], *Aleksandra Chikunova*[1,†], *Adil A. Safeer*[1,3,†], *Misbha Ud Din Ahmad*[2], *Anastassis Perrakis*[2], *Marcellus Ubbink*[1,*]

[1] Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333CC Leiden, The Netherlands

[2] Division of Biochemistry, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

[3] Current address: NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University Padualaan 8, 3584 CH Utrecht, The Netherlands

Table S1. List of primers used to introduce mutations in *blaC.*

Mutation	Primers
A55E	CAACCGGCACCACCGAAGCAATTGAATATCGTG
	CACGATATTCAATTGCTTCGGTGGTGCCG GTTG
G132S	GCAATTCGTTATAGTGATAGCACCGCAGCCAATC
	GATTGGCTGCGGTGCTATCACTATAACGAATTGC
D172N	GCCTGGTGATGAACGTAATACCACCACACCGCATGC
	GCATGCGGTGTGGTGGTATTACGTTCATCACCAGGC
G269S	GAGCGATCGTGCCAGTGGTGGCTATGATGCC
	GGCATCATAGCCACCACTGGCACGATCGCTC

Table S2. Data collection and refinement statistics of crystal structures

	A55E	**G132S**	**G132S + sulbactam**	**D172N**
PDB	7A5T	7A71	7A72	7A5W
Wavelength (Å)	1.000	1.000	0.912	1.000
Resolution (Å)	38.2-1.40 (1.42-1.40)	44.7-1.40 (1.42-1.40)	78.2-1.30 (1.32-1.30)	44.8-1.40 (1.42-1.40)
Space group	P 1 21 1	P 21 21 21	P 21 21 21	P 21 21 21
Unit cell a, b, c (Å)	39.00, 54.36, 53.69	53.30, 54.32, 78.85	53.49, 54.66, 78.20	54.03, 54.47, 78.78
β	91.94	90.00	90.00	90.00
$CC_{1/2}$	98.5 (75.2)	98.9 (65.4)	99.9 (65.5)	99.7 (77.7)
R_{meas} (%)	13.4 (56.8)	16.3 (130.6)	9.3 (140.7)	7.5 (94.7)
<I/σ(I)>	5.2 (1.7)	8.3 (2.6)	11.1 (1.3)	9.3 (1.8)
Completeness (%)	96.9 (94.2)	98.9 (97.4)	98.2 (99.8)	98.0 (98.0)
Multiplicity	2.5	3.7	5.7	3.0
Unique reflections	42718	45293	56098	45377
Atoms protein/ligands/water	2013/59/207	2047/61/201	2034/37/221	2008/29/156
Bfactors protein/ligands/water ($Å^2$)	10/22/19	8/25/20	15/24/26	16/28/28
R_{work}/R_{free} (%)	14.8/17.7	13.0/16.1	15.2/18.9	13.5/17.2
Bond lengths RMSZ/RMSD (Å)	1.056/0.014	1.270/0.016	1.249/0.016	1.236/0.016
Bond angles RMSZ/RMSD (°)	1.085/1.781	1.120/1.837	1.168/1.940	1.155/1.919
Ramachandran plot preferred/outliers	248/2	248/2	259/2	245/2
RamaZ score	-1.177	-0.595	-0.889	-1.019
Clash score	2.42	4.52	1.94	1.48
MolProbity score	1.02	1.23	0.96	0.89

Table S3. Overview of non-synonymous and synonymous mutations found during screenings for resistance against sulbactam.

Clones	Non-synonymous mutations		Synonymous mutations	
	Amino acid	**Codon**	**Amino acid**	**Codon**
1	D172N	GAT > AAT	S130S	AGT > AGC
	G269S	GGT > AGT		
2	A55E	GCA > GAA	R61R	CGT > CGC
	D100E	GAT > GAA	F66F	TTT > TTC
	G132S	GGC > AGC	V194V	GTT > GTA
	D179N	GAT > AAT	D209D	GAT > GAC
3	G132S	GGC > AGC	A51A	GCA > GCT

Table S4. Melting temperatures of BlaC mutants. Thermal shift measurements were performed with SYPRO orange fluorescent dye and melting curves were fitted to determine the melting temperature. Values represent the average and standard deviation of six measurements.

Protein	T_m (SD) (°C)
WT	52.5 (0.3)
A55E	52.4 (0.2)
G132S	52.5 (0.2)
D172N	55.5 (0.1)
G269S	53.1 (0.4)

Table S5. Kinetic parameters for the inactivation of BlaC and BlaC G132S by sulbactam as determined with model 3. K_i is the ratio k_6/k_5. Measurements were performed in duplicate. Errors in brackets represent one standard deviation.

BlaC	K_i (10^1 μM)	k_7 (10^{-2} s^{-1})	k_8 (10^{-3} s^{-1})
WT	3.4 (0.3) [a]	10 (1)	11.7 (0.3)
G132S	17 (1) [a]	5.95 (0.02)	15.2 (0.4)

[a] Errors represent propagated standard deviation.

```
MANNDLFQAS RRRFLAQLGG LTVAGMLGPS LLTPRRATAA QADLADRFAE LERRYDARLG
                                                  30        40

VYVPATGTTA AIEYRADERF AFCSTFKAPL VAAVLHQNPLT HLDKLITYT SDDIRSISPV
    50        60         70         80        90          100

AQQHVQTGMT IGQLCDAAIR YSDGTAANLL LADLGGPGGGT AAFTGYLRS LGDTVSRLDA
 110        120        130        140             150        160

EEPELNRDPP GDERDTTTPH AIALVLQQLV LGNALPPDKRA LLTDWMARN TTGAKRIRAG
     170        180        190        200        210        220

FPADWKVIDK TGTGDYGRAN DIAVVWSPTG VPYVVAVMSDR AGGGYDAEP REALLAEAAT
     230        240        250        260        270        280

CVAGVLALEH HHHHH
   290        300
```

Figure S1. Amino acid residues of BlaC as used for *in vivo* experiments. Residues 27-293 are numbered according to Ambler notation,[S1] this corresponds to residue numbers 43-307 of BlaC Uniprot entry P9WKD3-1. Residues of the TAT-signal sequence are underlined and the His-tag residues are highlighted in grey.

```
MGSSHHHHHH SSGLVPRGSH MENLYFQGDL ADRFAELERR YDARLGVYVP ATGTTAAIEY
                                  30         40         50        60

RADERFAFCS TFKAPLVAAV LHQNPLTHLD KLITYTSDDI RSISPVAQQH VQTGMTIGQL
         70         80        90         100        110        120

CDAAIRYSDG TAANLLLADL GGPGGGTAAF TGYLRSLGDT VSRLDAEEPE LNRDPPGDER
       130        140              150        160        170

DTTTPHAIAL VLQQLVLGNA LPPDKRALLT DWMARNTTGA KRIRAGFPAD WKVIDKTGTG
 180        190        200        210        220        230

DYGRANDIAV VWSPTGVPYV VAVMSDRAGG GYDAEPREAL LAEAATCVAG VLA
240        250       260        270        280        290
```

Figure S2. Amino acid residues of BlaC as used for *in vitro* experiments. Residues 27-293 are numbered according to Ambler notation,[S1] this corresponds to residue numbers 43-307 of BlaC Uniprot entry P9WKD3-1. Residues of the TEV-cleavable His-tag are highlighted in grey.

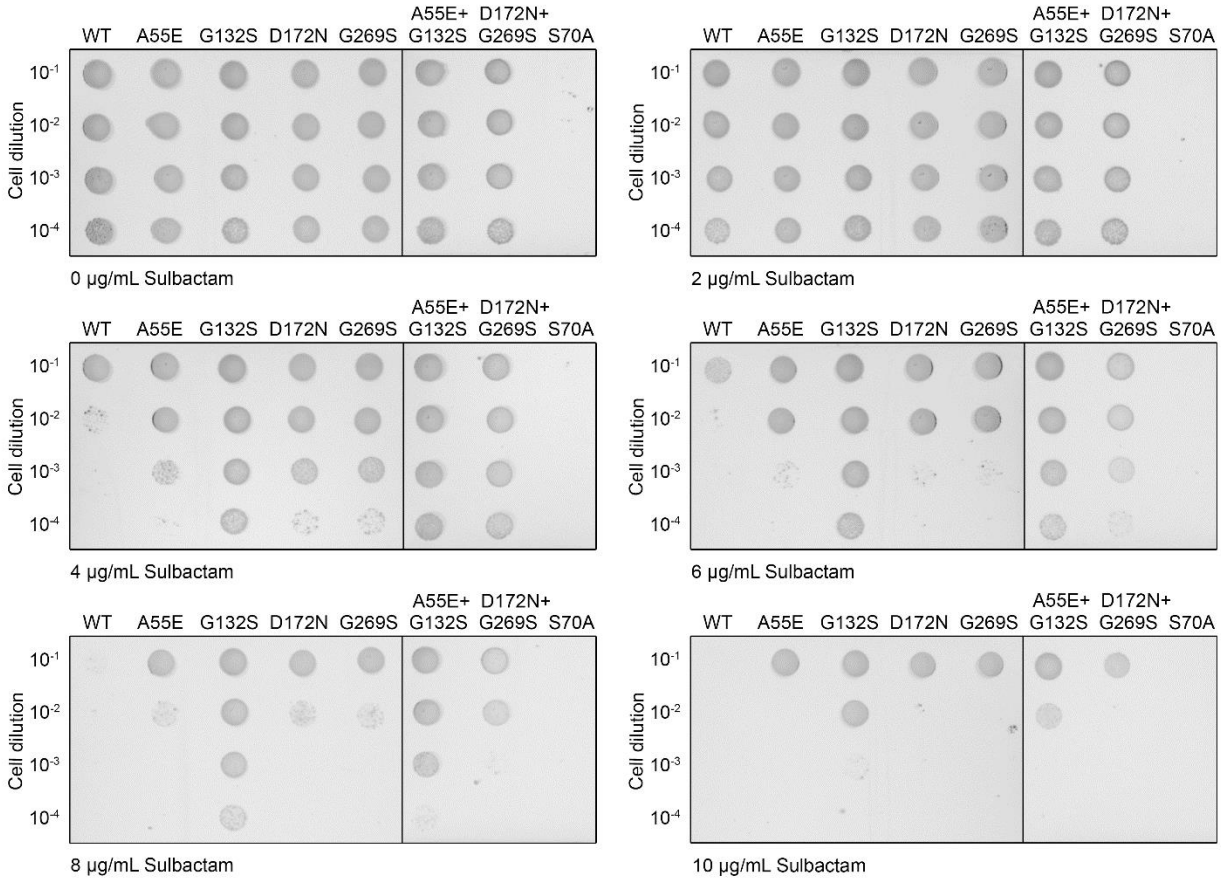


Figure S3. *In vivo* activity of Mtb BlaC mutants in *E. coli*. *E. coli* cells expressing the genes of wild-type BlaC, and BlaC variants A55E, G132S, D172N, G269S, A55E/G132S, or D172N/G269S were spotted on a plate containing 10 µg/mL ampicillin and sulbactam. BlaC S70A cannot hydrolyze ampicillin and is used as a negative control. The two panels at each sulbactam concentration originate from the same LB-agar plate.

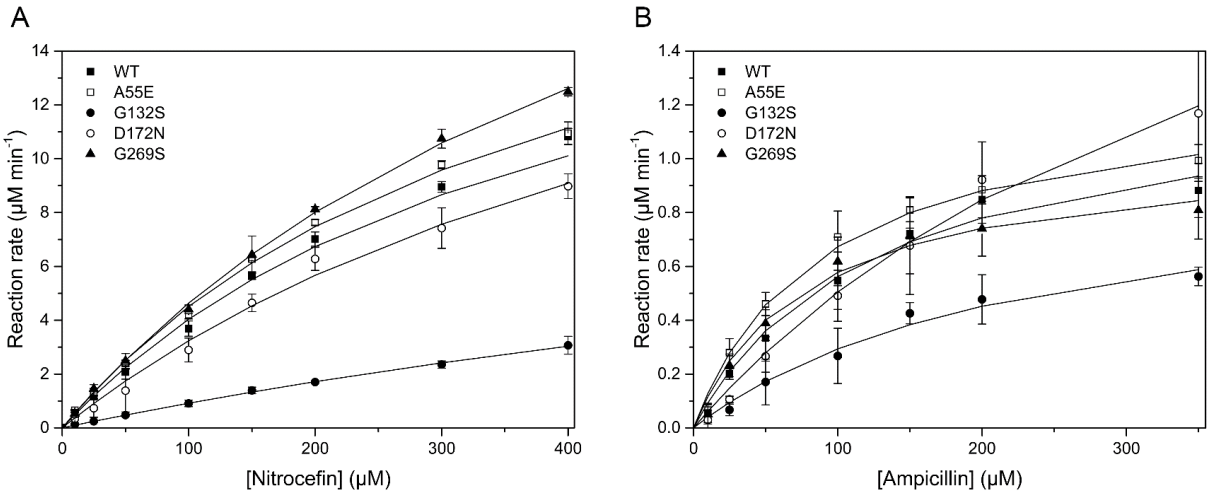


Figure S4. Michaelis-Menten curves of BlaC mutants for (A) nitrocefin and (B) ampicillin. Experiments were performed at 25 °C in 100 mM sodium phosphate at pH 6.4. Error bars represent the standard deviation of three measurements.

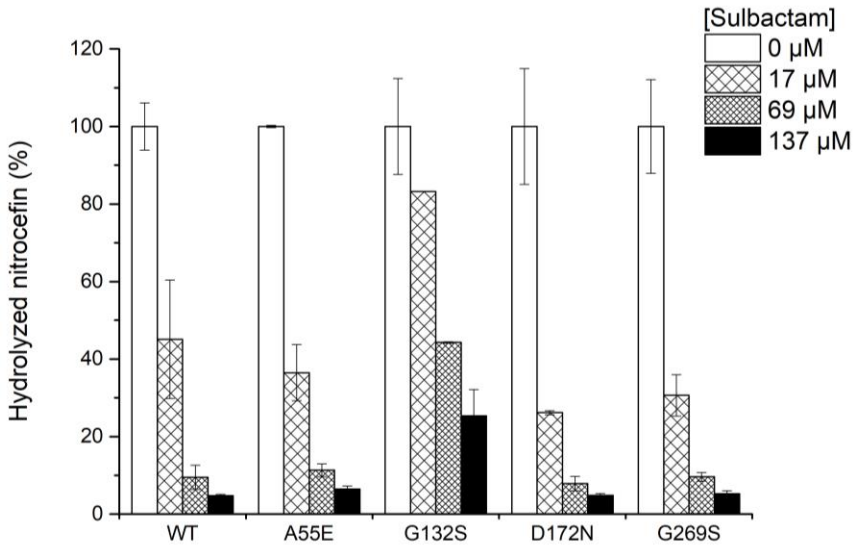
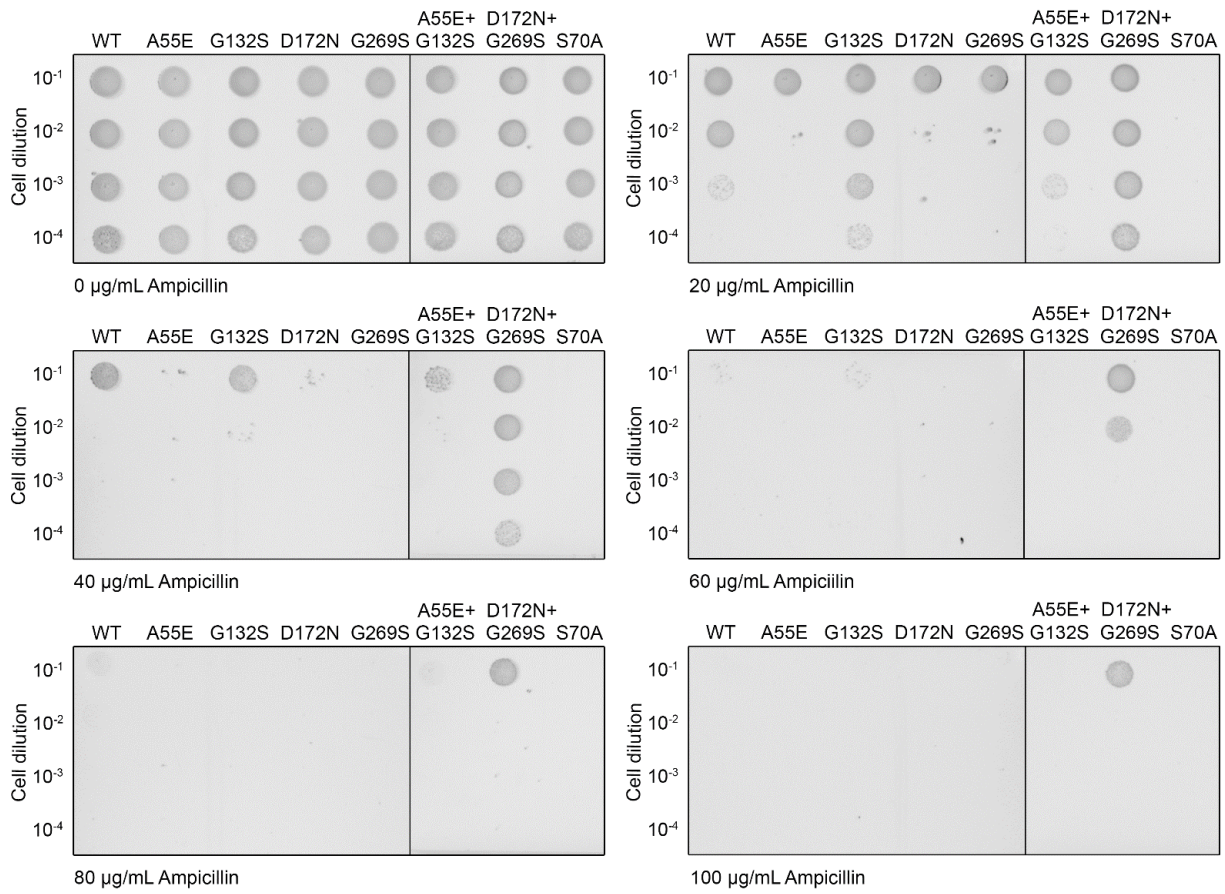


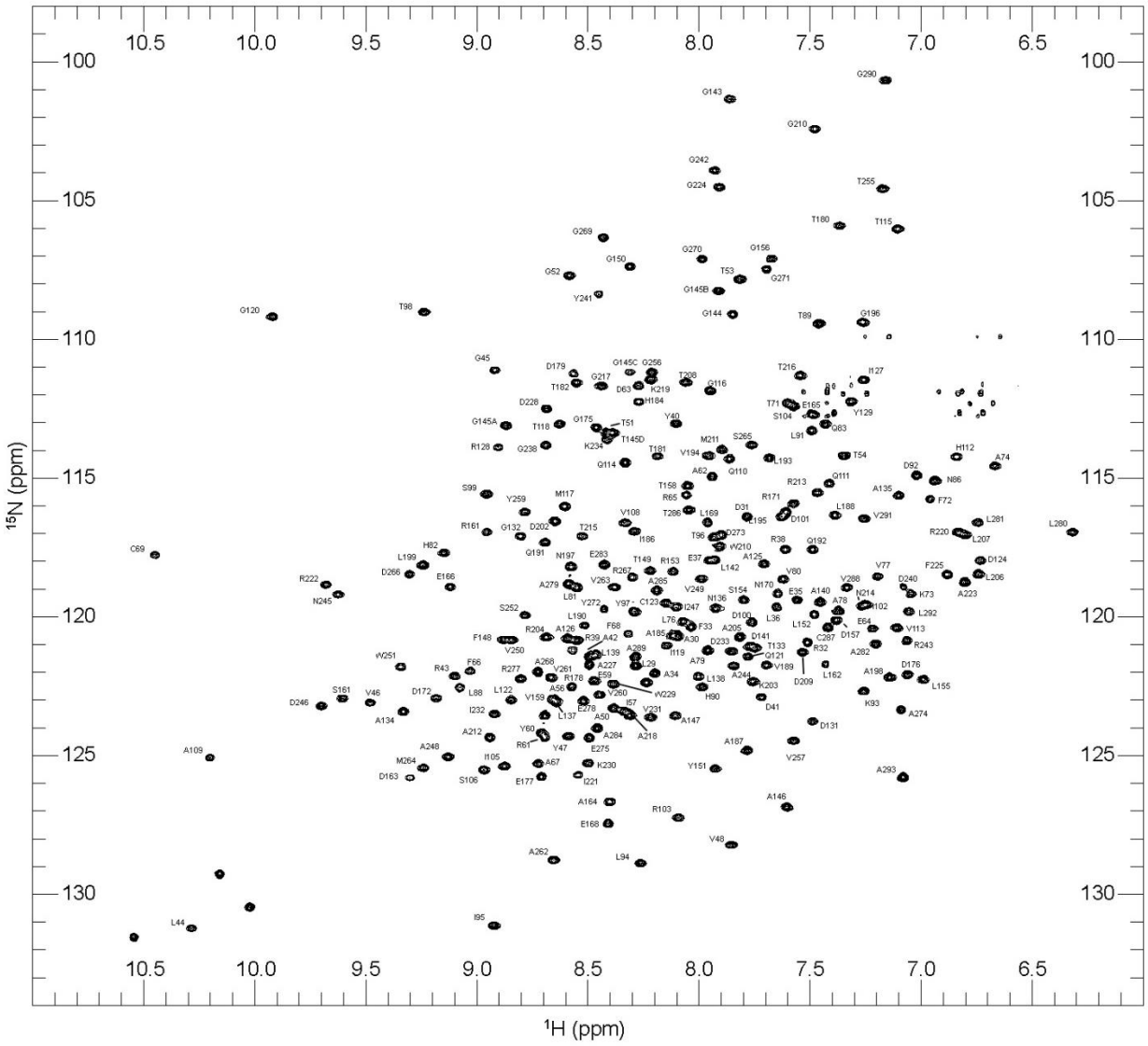
Figure S5. Hydrolysis of nitrocefin after incubation with sulbactam and BlaC for 15 minutes at 37 °C. Measurements were performed in duplicate. The error bars represent one standard deviation.



64

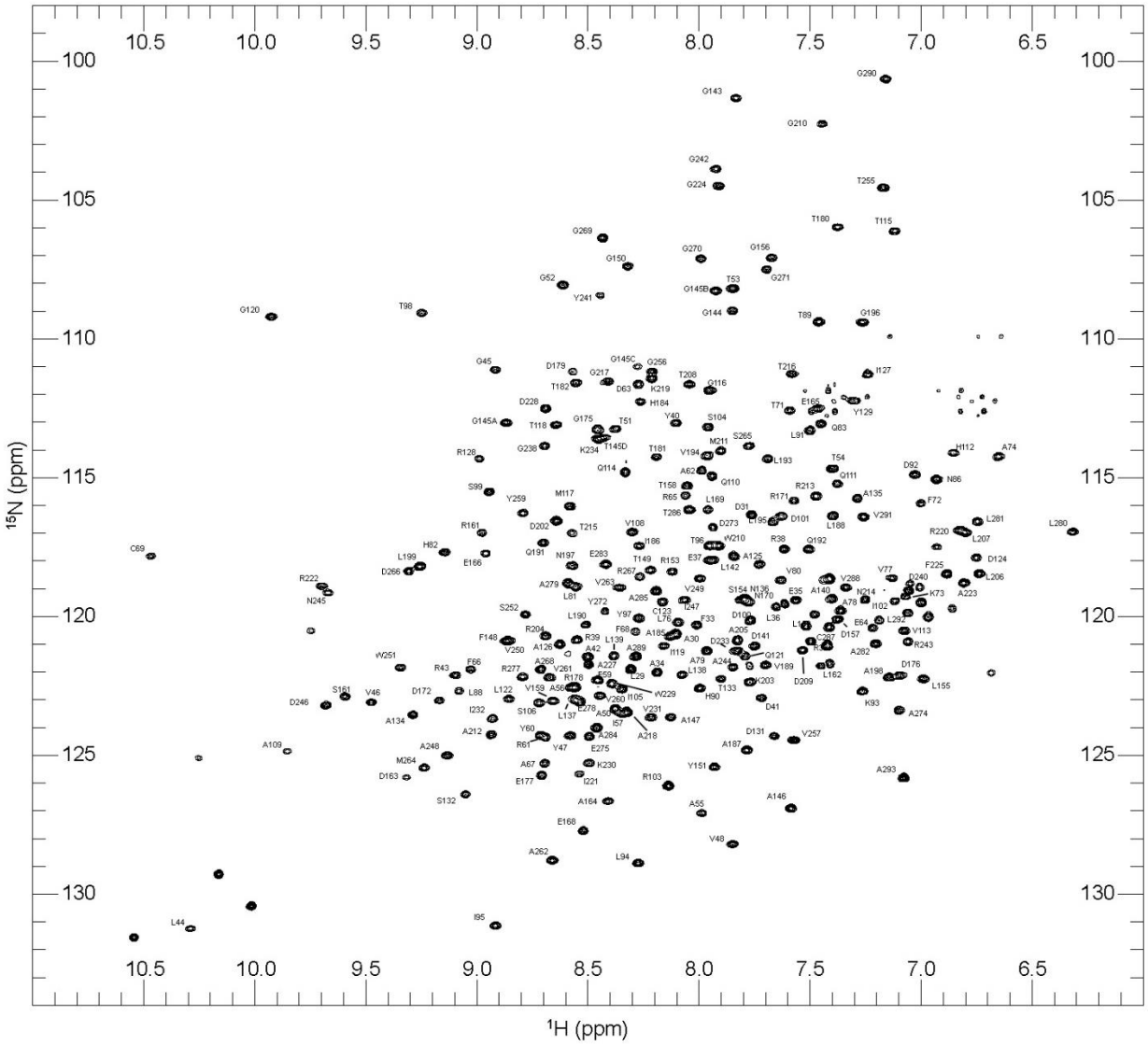
65 **Figure S6**. *In vivo* ampicillin conversion activity of MtB BlaC mutants in *E. coli*. *E. coli* cells
66 expressing the genes of wild-type BlaC, or BlaC variants A55E, G132S, D172N, G269S,
67 A55E/G132S, or D172N/G269S were spotted on a plate containing ampicillin. BlaC S70A cannot
68 hydrolyze ampicillin and is used as a negative control. The two panels at each ampicillin
69 concentration originate from the same LB-agar plate. Note that the combination mutant
70 D172N/G269S has enhanced activity against ampicillin.

71



72

73 **Figure S7.** TROSY-HSQC spectrum of BlaC A55E with residue assignments. Spectrum was
74 obtained at 25 °C using a Bruker AVIII HD 850 MHz spectrometer equipped with a TCI cryoprobe.
75 Sample contained ca. 0.15 mM ^{15}N BlaC in 100 mM sodium phosphate pH 6.4 and 6% D_2O.
76 Numbering refers to Ambler numbering.[S1] The assignments of the backbone amide resonances
77 have been deposited in the BMRB under entry 50565.



78

79 **Figure S8.** TROSY-HSQC spectrum of BlaC G132S with residue assignments. Spectrum was
80 obtained at 25 °C using a Bruker AVIII HD 850 MHz spectrometer equipped with a TCI cryoprobe.
81 Sample contained ca. 0.15 mM ^{15}N BlaC in 100 mM sodium phosphate pH 6.4 and 6% D_2O.
82 Numbering refers to Ambler numbering.[S1] The assignments of the backbone amide resonances
83 have been deposited in the BMRB under entry 50563.

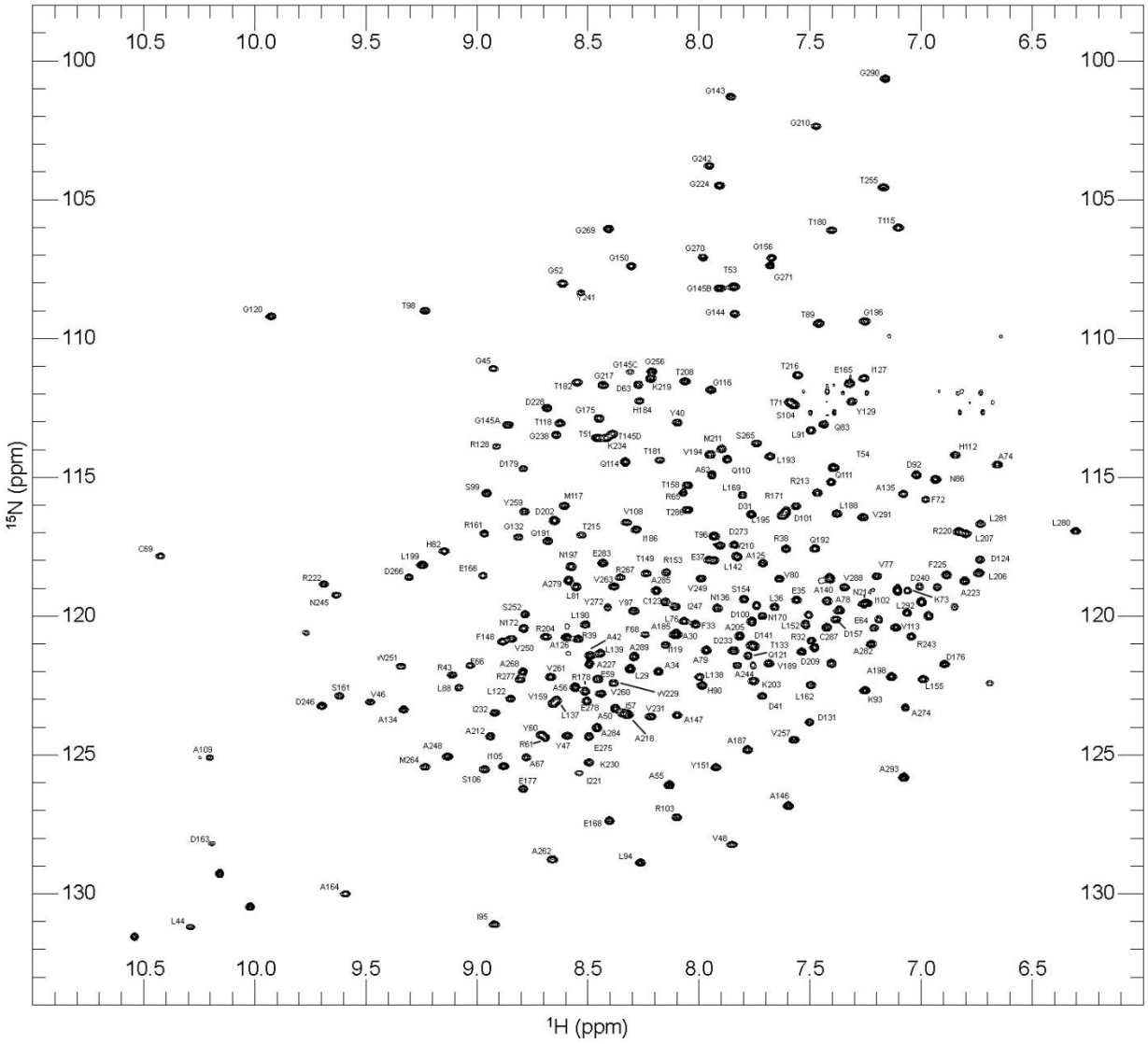
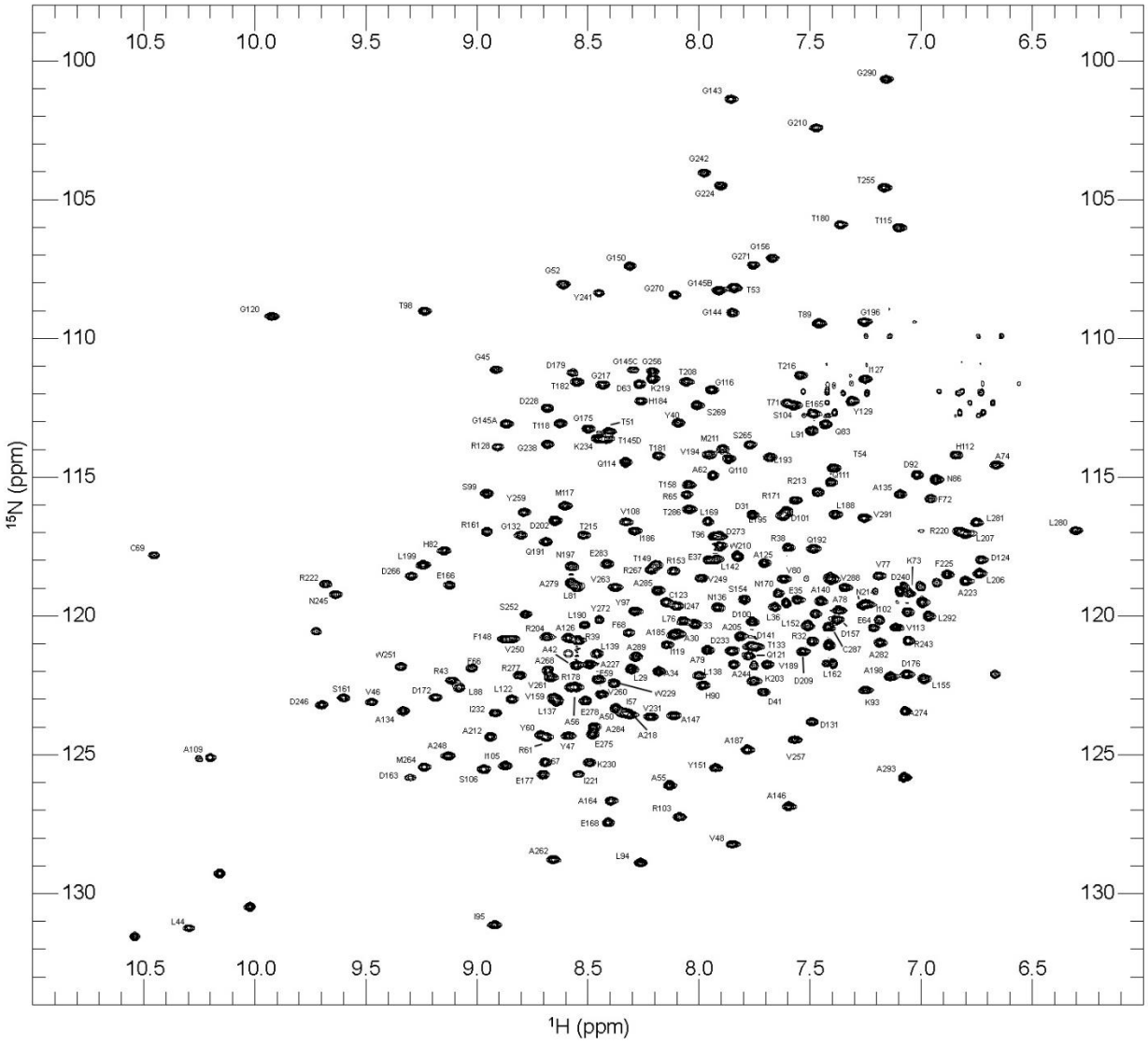


Figure S9. TROSY-HSQC spectrum of BlaC D172N with residue assignments. Spectrum was obtained at 25 °C using a Bruker AVIII HD 850 MHz spectrometer equipped with a TCI cryoprobe. Sample contained ca. 0.15 mM ^{15}N BlaC in 100 mM sodium phosphate pH 6.4 and 6% D_2O. Numbering refers to Ambler numbering.[S1] The assignments of the backbone amide resonances have been deposited in the BMRB under entry 50566.



90

91 **Figure S10.** TROSY-HSQC spectrum of BlaC G269S with residue assignments. Spectrum was
92 obtained at 25 °C using a Bruker AVIII HD 850 MHz spectrometer equipped with a TCI cryoprobe.
93 Sample contained ca. 0.15 mM ^{15}N BlaC in 100 mM sodium phosphate pH 6.4 and 6% D_2O.
94 Numbering refers to Ambler numbering.[S1] The assignments of the backbone amide resonances
95 have been deposited in the BMRB under entry 50564.

96

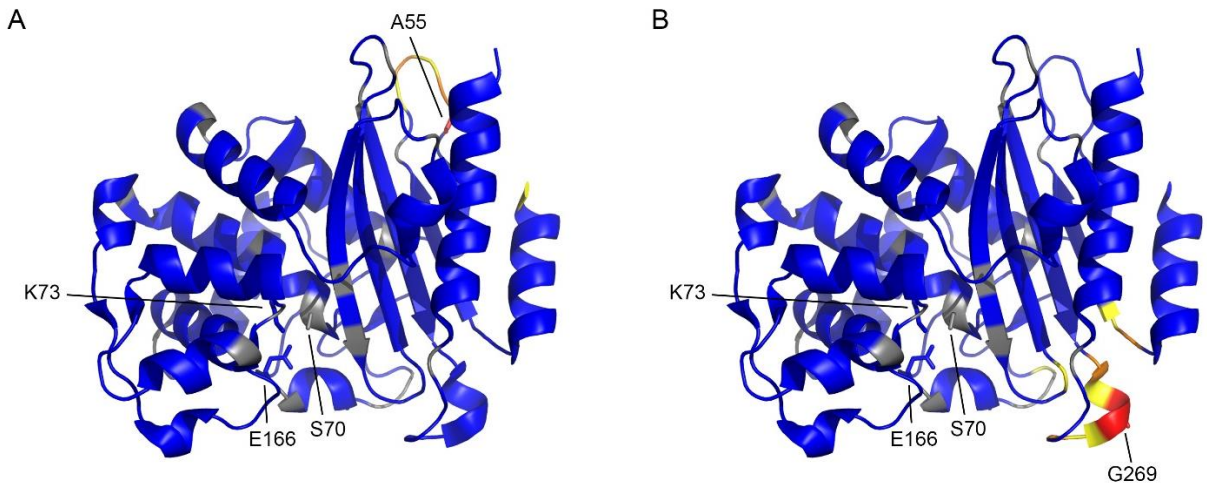


Figure S11. Average CSP of BlaC A55E (A) and BlaC G269S (B) plotted on the crystal structure of wild-type BlaC (PDB 5NJ2).[S2] Residues with CSP over 0.025, 0.05, and 0.1 ppm are displayed in yellow, orange, and red, respectively, those with no or small CSPs are in blue, and the ones for which no data were available are displayed in grey. Mutation sides are indicated and the side chains of active site residues S70, K73 and E166 are represented as sticks. For amides that were detected in the wild-type spectrum, but undetectable in the mutant spectrum, we determined the minimal chemical shift with reference to unassigned peaks and used these values to determine the CSP.

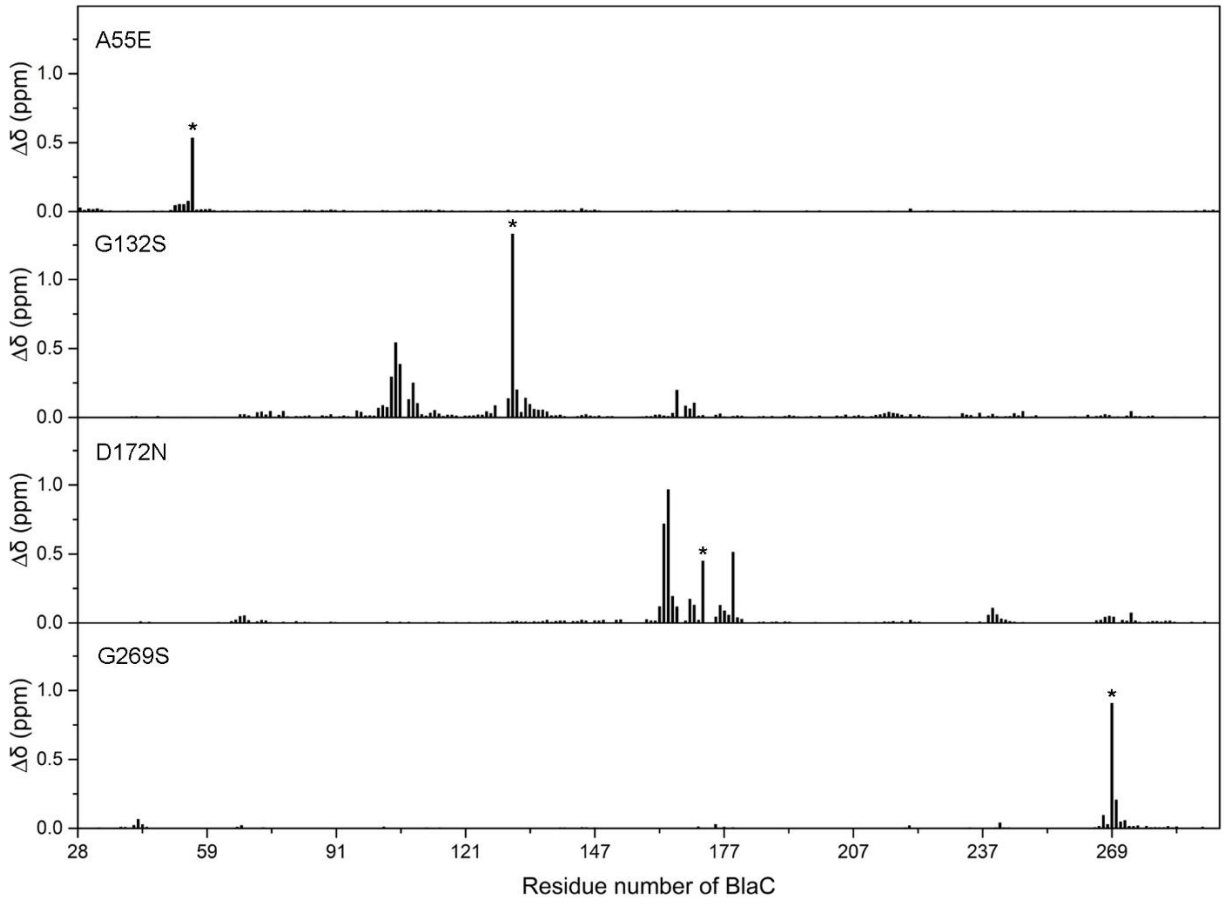


Figure S12. Average CSP for the backbone amides of the BlaC mutants. Residue numbers refer to Ambler numbering.[S1] Mutated residues are indicated with an asterisk.

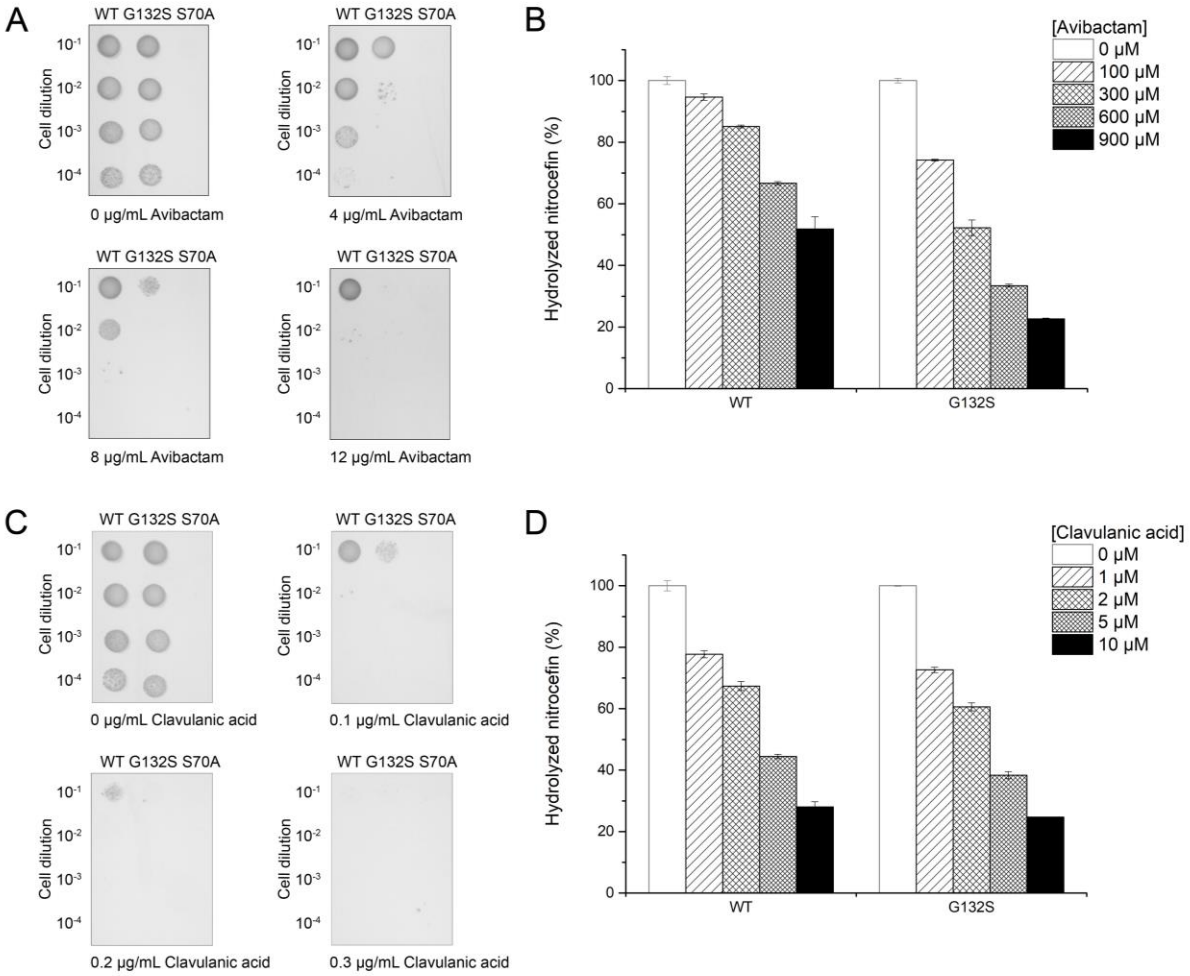


Figure S13. Activity of Mtb BlaC G132S. (A, C) *E. coli* cells expressing the genes of wild-type BlaC or BlaC G132S were spotted on a plate containing 10 μg/mL ampicillin and (A) avibactam or (C) clavulanic acid. BlaC S70A cannot hydrolyze ampicillin and is used as a negative control. (B,D) Hydrolysis of nitrocefin by BlaC wild-type or G132S in the presence of avibactam (B) or clavulanic acid (D) for 15 minutes at 25 °C. Measurements were performed in duplicate. The error bars represent one standard deviation.

Supplementary references

(S1) Ambler, R. P., Coulson, A. F. W., Frère, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., and Waley, S. G. (1991) A standard numbering scheme for the class A β-lactamases. *Biochem. J. 276*, 269–270.

(S2) Elings, W., Tassoni, R., Van Der Schoot, S. A., Luu, W., Kynast, J. P., Dai, L., Blok, A. J., Timmer, M., Florea, B. I., Pannu, N. S., and Ubbink, M. (2017) Phosphate promotes the recovery of *Mycobacterium tuberculosis* β-Lactamase from clavulanic acid inhibition. *Biochemistry 56*, 6257–6267.