Supplementary Information

Materials and Methods

β-lactamase induction assays

To assess induction of β-lactamases, 10 µl of overnight cultures of selected isolates were diluted into 490 µl of fresh Mueller-Hilton Broth (MHB) and grown aerobically at 37±1°C to an optical density of 0.4 (600 nm). For imipenem experiments, the isolates were then incubated in the presence or absence of imipenem (10 µg/ml) for two hours. For aztreonam (ATM) and avibactam (AVI) induction assays, isolates were grown in MHB supplemented with 10 µg/ml imipenem, 4 µg/ml AVI, 16 µg/ml ATM, or 4 µg/ml AVI + 0.5 µg/ml ATM. Aliquots were removed at 30 min, and 1, 1.5 and 2 hours post-induction. For each culture, 1 ml of cells was pelleted, the supernatant was removed, and the pellet was frozen at -20°C overnight. Crude lysates were then prepared using 50 µl of lysis buffer (50 mM Tris-HCl pH 7.4, 0.04 mg/ml lysozyme, 1 mM $MgSO_4$, and 5 U/ml benzonuclease (Novagen; Darmstadt, Germany)). After 30 min of incubation at 25°C, the lysate was centrifuged at 10,000 x g and 10 µl of each supernatant was loaded on an analytical isoelectric focusing (aIEF) gel (Novex® Isoelectric Focusing Electrophoresis System; Thermo Fisher Scientific, Waltham, MA). Purified L1 and L2 proteins (100 µg; kindly provided by Dr. James Spencer, at the University of Bristol, UK) were included as controls, along with the IEF protein standard (Bio-Rad, Hercules, CA). β-lactamase activity was detected by a 2 mM nitrocefin overlay.

Enzyme specific activity measurement

Enzyme specific activity was monitored using an Agilent 8453 Diode Array

spectrophotometer, following previously described protocols with some modifications (1). Briefly, crude lysates were obtained after isolate A1 was grown aerobically at 37±1°C for 2 hours in MHB alone (as growth control) or supplemented with 10 μg/ml imipenem, 4 μg/ml AVI, 16 μg/ml ATM, or 4 μg/ml AVI + 0.5 μg/ml ATM. To measure the activity of the L2 β-lactamase, nitrocefin hydrolysis was monitored in 10 mM HEPES (pH 7.5) supplemented with 100 μM EDTA (to inhibit the L1) and 20 μg/mL bovine serum albumin (BSA). Specific activity of the L1 was assessed by following imipenem hydrolysis in 10 mM HEPES (pH 7.5) supplemented with 20 μM Zn_2SO_4, and 20 μg/mL bovine serum albumin (BSA). Rates of hydrolysis for each reaction were recorded for 3 min. The following extinction coefficients were used: nitrocefin, $\Delta_{\varepsilon 482}$ = 17400 M^{-1} cm^{-1}; imipenem, $\Delta_{\varepsilon 300}$ = −9000 M^{-1} cm^{-1}.

Steady-state kinetics

Steady-state kinetic parameters were determined using an Agilent 8453 Diode Array spectrophotometer. Briefly, each assay was performed in 10 mM phosphate-buffered saline (PBS) at pH 7.4 at room temperature. For determination of V_{max} and K_m, L2 was maintained at 2.6 nM with nitrocefin in excess molar concentration to establish pseudo-first order kinetics, as previously described (2). Kinetic values $K_{i\ apparent\ (app)}$, k_2/K, k_{off}, and K_D were determined using previously described methodology and equations (2, 3). Briefly, $K_{i\ apparent\ (app)}$ for AVI was determined by direct competition assays under steady-state conditions. The initial velocity was measured in the presence of a constant concentration of enzyme (2 nM) with increasing concentrations of inhibitor (0.75-6 μM) against a fixed concentration (120 μM) of the indicator substrate, nitrocefin. For the k_2/K assessment, progress curves were obtained by mixing 2 nM of L2 with increasing concentrations of AVI (0.75 - 6 μM), using nitrocefin (120 μM) as a reporter

substrate. Finally, the k_{off} value was determined by incubating 1 μM of L2 with 4 μM of AVI for 5 min. Samples were serially diluted to a final enzyme concentration of 2.6 nM and hydrolysis of 120 μM nitrocefin was measured.

Electrospray Ionization Mass Spectrometry (ESI-MS)

ESI-MS was performed on a Waters SynaptG2-Si quadrupole-time-of-flight mass spectrometer electrospray ion source and Waters Acquity UPLC BEH C18 1.7 um column (2.1 x 50 mm), as previously described (2). MassLynx V4.1 was used to deconvolute protein peaks. The tune settings were as follows: capillary voltage at 3.5 kV, sampling cone at 35, source offset at 35, source temperature of 100 °C, desolvation temperature of 500 °C, cone gas at 100 liters/h, desolvation gas at 800 liters/h, and nebulizer bar at 6.0. Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. Mass accuracy for this system is ±5 Da. For the experiments, 1:1 molar ratio of L1 or L2 and AVI (170 nM) was incubated for 15 sec, 1 min, 15 min, 1 hr and 24 hr at room temperature in 10 mM PBS, pH 7.4. Reactions were quenched with 1% acetonitrile, and added to 1 mL of 0.1% formic acid.

Molecular Modeling

The crystal structure (PDB:1N40) of L2 β-lactamase was used for molecular modeling. The structure was prepared using Discovery Studio 4.1 software (multiple conformers removed and minimized), and the AVI was manually docked into the active site. The AVI-L2 complex was created, solvated and minimized using CHARMm force field with Conjugate Gradient method and SHAKE algorithm.

Table S1. Susceptibility results obtained by agar dilution for clinical isolates of *S. maltophilia* [a]

Strains	TMX		CIP		MIN		COL		TCV		CAZ		ATM		ATM/AVI	
A1	1/19	S	4	I	0.5	S	4	I	16	S	32	**R**	>128	**R**	4	S
A2	1/19	S	16	**R**	1	S	2	S	>256	**R**	256	**R**	>128	**R**	8	S
A3	1/19	S	2	S	0.25	S	32	**R**	32	I	128	**R**	>128	**R**	8	S
A4	1/19	S	32	**R**	1	S	4	I	>256	**R**	>256	**R**	>128	**R**	8	S
A6	1/19	S	4	I	1	S	32	**R**	>256	**R**	>256	**R**	>128	**R**	32	**R**
A8	1/19	S	2	S	0.12	S	8	**R**	64	I	256	**R**	>128	**R**	8	S
A9	1/19	S	32	**R**	4	S	4	I	64	I	256	**R**	>128	**R**	8	S
B1	1/19	S	2	S	0.25	S	4	I	256	**R**	256	**R**	>128	**R**	8	S
B2	1/19	S	8	**R**	0.25	S	4	I	32	I	128	**R**	>128	**R**	8	S
B4	1/19	S	2	S	0.5	S	2	S	64	I	256	**R**	>128	**R**	16	I
B5	1/19	S	4	I	0.25	S	16	**R**	64	I	256	**R**	>128	**R**	8	S
B6	>4/76	**R**	2	S	1	S	16	**R**	128	**R**	256	**R**	>128	**R**	8	S
B7	>4/76	**R**	2	S	1	S	64	**R**	128	**R**	256	**R**	>128	**R**	8	S
B9	>4/76	**R**	2	S	0.5	S	0.5	S	256	**R**	256	**R**	>128	**R**	4	S
C1	1/19	S	2	S	0.12	S	2	S	256	**R**	256	**R**	>128	**R**	16	I
C3	1/19	S	2	S	0.25	S	8	**R**	4	S	8	S	64	**R**	4	S
E1	1/19	S	16	**R**	2	S	8	**R**	>256	**R**	256	**R**	>128	**R**	8	S
E2	1/19	S	2	S	0.25	S	16	**R**	128	**R**	256	**R**	>128	**R**	8	S
E3	2/38	S	4	I	0.25	S	2	S	128	**R**	256	**R**	>128	**R**	8	S
E4	>4/76	**R**	8	**R**	0.5	S	32	**R**	256	**R**	>256	**R**	>128	**R**	16	I
E5	1/19	S	0.5	S	0.12	S	4	I	32	I	128	**R**	>128	**R**	4	S
E6	1/19	S	0.5	S	0.12	S	4	I	4	S	4	S	64	**R**	2	S
F1	1/19	S	4	I	0.5	S	32	**R**	8	S	16	I	>128	**R**	4	S
F2	2/38	S	4	I	0.25	S	4	I	32	I	32	**R**	>128	**R**	8	S
F3	1/19	S	8	**R**	1	S	16	**R**	32	I	4	S	>128	**R**	4	S
F4	1/19	S	2	S	0.25	S	16	**R**	8	S	64	**R**	>128	**R**	8	S
F5	1/19	S	0.5	S	16	**R**	32	**R**	64	I	>256	**R**	>128	**R**	>128	**R**
F6	1/19	S	4	I	0.25	S	32	**R**	64	I	256	**R**	>128	**R**	8	S
F9	2/38	S	2	S	0.25	S	32	**R**	64	I	256	**R**	>128	**R**	8	S

[a] Values correspond to μg/mLconcentration of the following antibiotics: TMX: trimethoprim-sulfamethoxazole; CIP: ciprofloxacin; MIN: minocycline; COL: colistin; TCV: Tircacillin-clavulanate; ATM: aztreonam; CAZ: ceftazidime; ATM-AVI: aztreonam avibactam. The letter (S = susceptible, R = resistant) next to each value is the interpretation according to CLSI. For ATM and ATM-AVI, interpretation was made according to the CLSI breakpoint of ATM for *P. aeruginosa.*

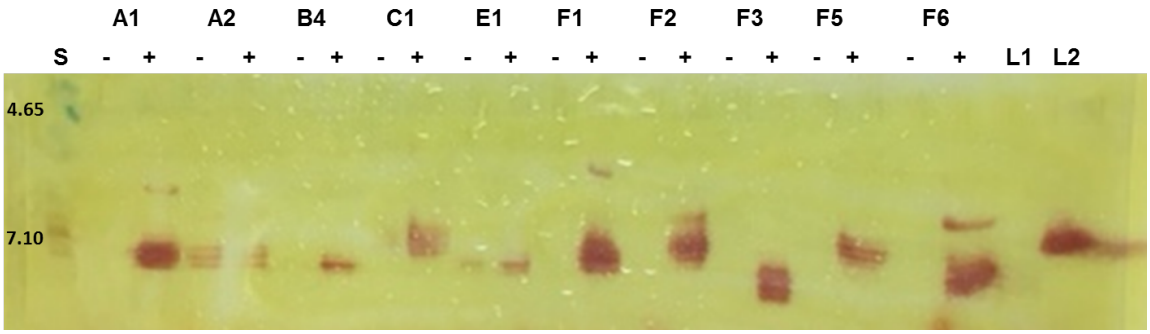

Figure S-1. aIEF demonstrates diversity of β-lactamases.

Selected *S. maltophilia* isolates were grown with (+) or without (-) imipenem (10 μg/ml) for two hours and crude lysates were obtained as described in *Supplemental Materials and Methods*. The crude lysates were run on an aIEF gel and nitrocefin hydrolysis was used to detect β-lactamases. S: IEF protein standard; L1: 100 μg of L1; L2: 100 μg of L2. Numbers next to the standard bands are the approximate isoelectric points (p*I*) of phycocyanin (three blue bands pIs 4.75, 4.65, and 4.45), equine myoglobin (pI 7.0), human hemoglobin A (pI 7.1), and human hemoglobin C (pI 7.5), as listed from the top to the bottom of the gel. Different bands in the clinical isolates represent diversity of β-lactamases.

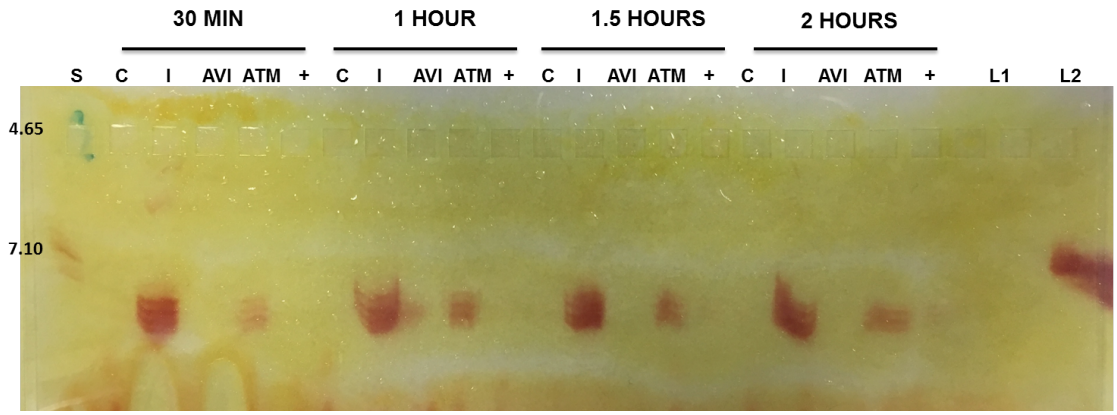

Figure S-2. ATM-AVI combination does not induce β-lactamase expression.

S. maltophilia isolate A1 was challenged to grow in MHB alone (C), or in presence of 10 μg/ml imipenem (I), 4 μg/ml AVI, 16 μg/ml ATM, or 4 μg/ml AVI + 0.5 μg/ml ATM (+). Samples were taken at the specified times, and crude lysates were prepared as described in *Supplemental Materials and Methods*. β-lactamase activity was detected in the aIEF gel based on nitrocefin hydrolysis. S: IEF protein standard; L1: 100 μg of L1; L2: 100 μg of L2. Numbers next to the standard bands are the approximate isoelectric points (p*I*) of phycocyanin (three blue bands pIs 4.75, 4.65, and 4.45), equine myoglobin (pI 7.0), human hemoglobin A (pI 7.1), and human hemoglobin C (pI 7.5), as listed from the top to the bottom of the gel.

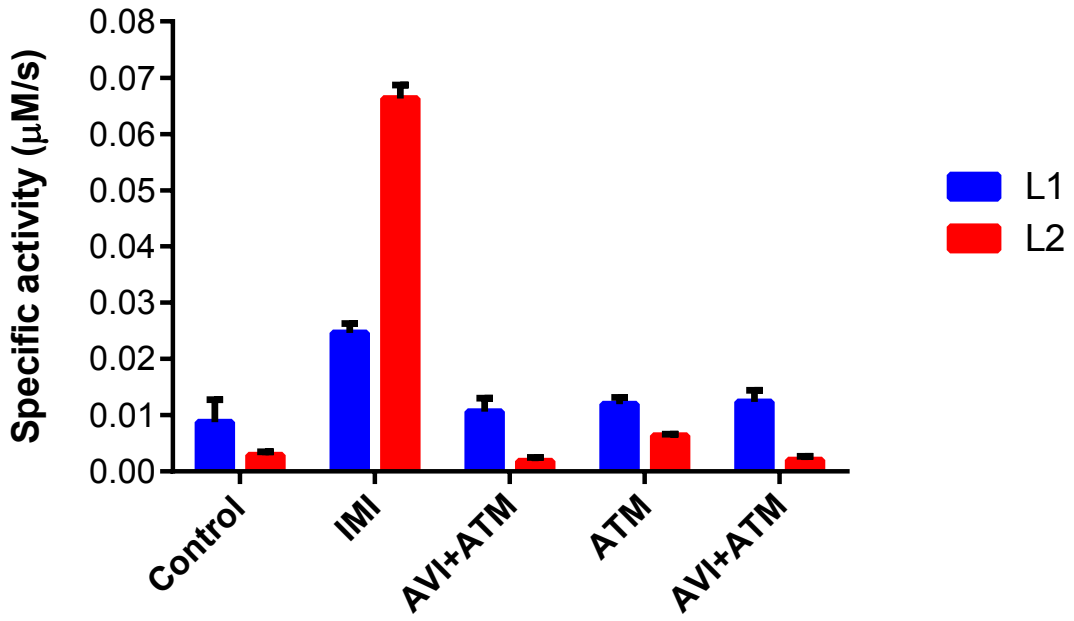

Figure S-3. ATM-AVI combination does not induce β-lactamase expression.

S. maltophilia isolate A1 was challenged to grow in MHB alone (control), or in MHB supplemented with 10 μg/ml imipenem, 4 μg/ml AVI, 16 μg/ml ATM, or 4 μg/ml AVI + 0.5 μg/ml ATM. Samples were after 2 hours of incubation, and crude lysates were prepared as described in *Supplemental Materials and Methods*. Recording the initial velocity of hydrolysis of imipenem and nitrocefin assessed specific enzymatic activity of L1 and L2, respectively.

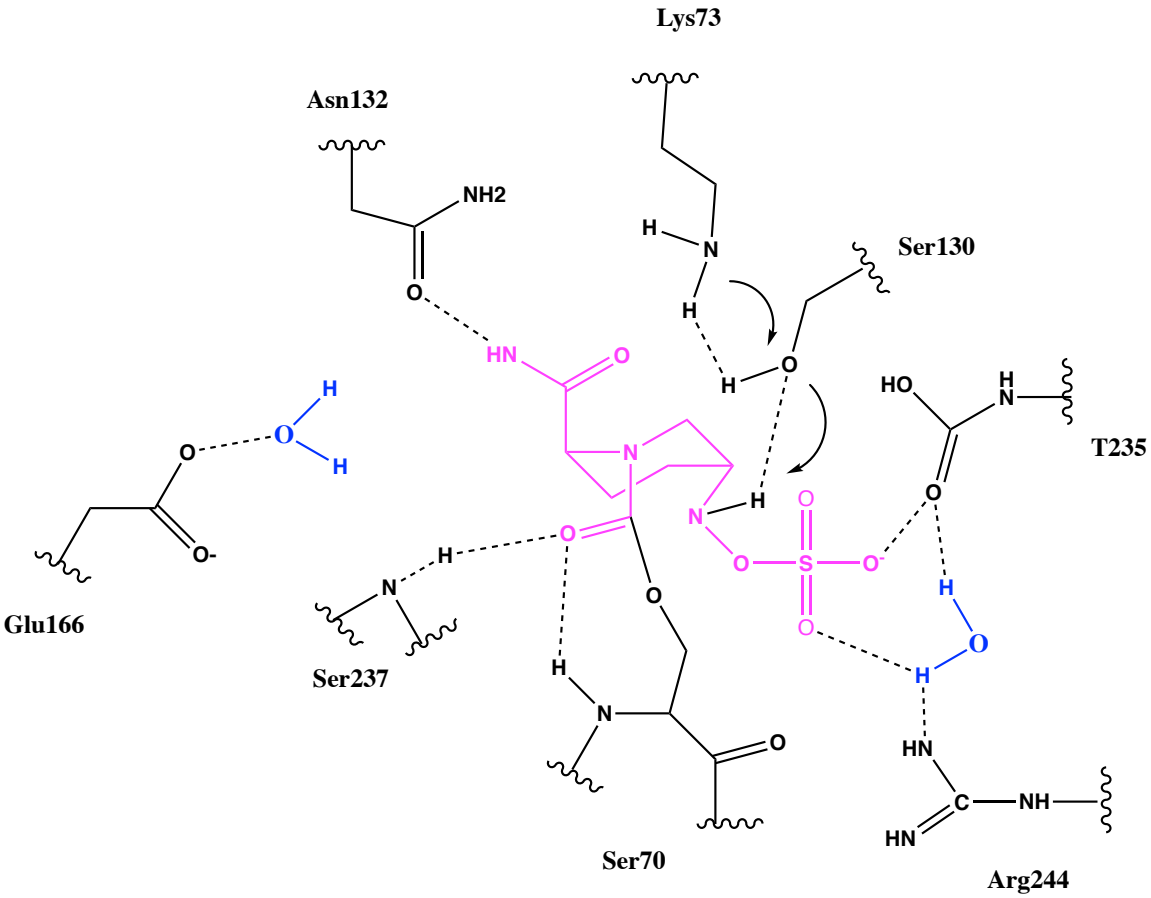

Figure S-4. Proposed mechanism of avibactam recyclization and decarbamylation. in L2 (based on crystal structure PDB: 1N40).

Supplementary References

1. **Avison MB, Niumsup P, Walsh TR, Bennett PM.** 2000. *Aeromonas hydrophila* AmpH and CepH β-lactamases: derepressed expression in mutants of *Escherichia coli* lacking *creB*. J Antimicrob Chemother **46:**695-702.
2. **Mojica MF, Mahler SG, Bethel CR, Taracila MA, Kosmopoulou M, Papp-Wallace KM, Llarrull LI, Wilson BM, Marshall SH, Wallace CJ.** 2015. Exploring the role of residue 228 in substrate and inhibitor recognition by VIM metallo-β-lactamases. Biochemistry **54:**3183-3196.
3. **Ehmann DE, Jahić H, Ross PL, Gu R-F, Hu J, Kern G, Walkup GK, Fisher SL.** 2012. Avibactam is a covalent, reversible, non-β-lactam β-lactamase inhibitor. Proc Natl Acad Sci USA **109:**5.