

# **Monitoring Drug-Protein Interactions in the Bacterial Periplasm by Solution Nuclear Magnetic Resonance Spectroscopy**

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## METHODS

### *Bacterial cultures*

All the constructs and bacterial strains are listed in **Supplementary Table 1**. *E. coli* BW25113 $\Delta ampC$  was obtained by excising the Km<sup>R</sup> resistance cassette from the *ampC* locus, as previously described<sup>2</sup>.

For protein purification, cells were cultured at 37 °C in LB or M9 medium supplemented with 50 µg/ml kanamycin for KPC-2 and MltG or 30 µg/ml chloramphenicol for PBP2 with vigorous shaking (200 rpm). For <sup>13</sup>C/<sup>15</sup>N protein labelling, the M9 medium was supplemented with <sup>15</sup>N-labelled ammonium chloride and uniformly <sup>13</sup>C-labelled glucose (Cambridge Isotope Laboratories, USA). Protein production was induced when the cultures reached OD<sub>600</sub> of 0.6 to 0.8 by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM for KPC-2 and PBP2 or 0.5 mM for MltG. The cultures were further incubated 16 hours at 20 °C (KPC-2 and PBP2) or at 16 °C (MltG). Cells were collected by centrifugation (10 min, 4,000 rpm, 20 °C) and the pellets were used for protein purification.

For in-cell experiments, M9 minimal medium supplemented with MgSO<sub>4</sub>, CaCl<sub>2</sub> and thiamine was used. IPTG was added once the culture reached an OD<sub>600</sub> of 1.0 to 1.2. incubation was continued for three hours at 37 °C with agitation at 200 rpm. Cells were collected by centrifugation (10 min, 4,000 rpm, 20 °C) and washed once with PBS. For NMR data collection, pellets were supplemented with D<sub>2</sub>O (10% final) and resuspended in 600 µl of warm PBS solution supplemented with agar (0.5% final). Samples were transferred to 5-mm NMR tubes for extemporaneous data collection. For KPC-2 inhibition, the bacterial pellet was resuspended in PBS and incubated for one hour at 30 °C in the presence of avibactam at 700 µM with agitation (200 rpm). Cells were collected by centrifugation. In-cell mecillinam hydrolysis was monitored in bacterial pellets corresponding to 700 µl of bacterial cultures. KPC-2 and mucopeptide 2D NMR spectra were collected from bacterial pellets corresponding to 150 ml of cultures.

### *Colony forming unit counts*

Cell cultures grown in LB were prepared as described above for in-cell experiments. 500 µl of pTRC99K $\Omega kpc-2$  culture was collected and washed twice in PBS. Pepsin (4 mg/ml final) was mixed with the cells in PBS and the samples were incubated for 1 hour at room temperature. To determine the number of colony forming units (CFUs), serial ten-fold dilutions were plated on LB agar plates supplemented with kanamycin (50 µg/ml). CFUs were enumerated after overnight incubation at 37 °C. Experiments were performed in duplicate.

### *Protein purification*

For KPC-2, cells were resuspended in buffer A (50 mM MES pH 5.9, 500 mM NaCl, 25 mM imidazole, 3% glycerol) and disrupted by sonication. The lysate was centrifuged (46,000 × g, 4 °C, 45 min) and soluble proteins were loaded onto a Ni-NTA agarose column, washed with 5 column volumes of buffer A and eluted in buffer B (50 mM MES pH 5.9, 500 mM NaCl, 500 mM imidazole, 3% glycerol). KPC-2 was further purified by size-exclusion chromatography (Superdex 75 16/600, GE, USA) in 50 mM MES pH 5.9, 100 mM NaCl, 3% glycerol and concentrated by ultrafiltration (Amicon centrifugal filters 10-kDa cutoff, Merck, Germany).

For MltG, bacteria were lysed by sonication in buffer C (25 mM Tris pH 7.5, 300 mM NaCl) and the enzyme was purified from a clarified lysate by Ni-NTA and size exclusion chromatography in buffer C.

For PBP2, the cell pellet was resuspended in lysis buffer (25 mM CHES pH 9.0, 500 mM NaCl, 10 mM MgCl<sub>2</sub>, 5% glycerol, 0.5% CHAPS, lysozyme at 10 mg/ml, 1x protease inhibitor and

2  $\mu\text{L}$  of benzonase at 250 U/ $\mu\text{L}$ ) and cells were disrupted by cell disruptor (Microfluidizer LM20) at 18,000 psi. The sample was centrifuged at  $20,000 \times g$ , and the soluble fraction containing the His-tagged PBP2 was incubated and purified with Protino Ni-NTA Agarose using buffer A (25 mM CHES pH 9.0, 500 mM NaCl, 10 mM  $\text{MgCl}_2$ , 5% glycerol), following elution of PBP2 in buffer B (Buffer A supplemented with 300 mM imidazole). The eluted sample was injected onto a Superdex 200 16/600 (GE, USA) column, and peak fractions were collected. A third purification step using a Mono Q<sup>TM</sup> anion exchange chromatography 5/50 GL column was performed to remove contaminants and to concentrate purified PBP2.

All protein aliquots were flash frozen in liquid nitrogen and kept at  $-80\text{ }^\circ\text{C}$  until experiments were performed.

#### *Periplasm extraction*

The protocol was previously described<sup>3</sup>. Briefly, bacterial cells were cultured in 20 ml of LB or M9 media in the same conditions as used for in-cell experiments. Cells were collected by centrifugation (10 min, 4,000 rpm,  $4\text{ }^\circ\text{C}$ ), washed once in PBS, resuspended in 160  $\mu\text{L}$  of 0.5 M sucrose, 0.2 M Tris pH 8.0, 0.5 mM EDTA, and incubated on ice for 15 min. Then, 640  $\mu\text{L}$  of 10 mM Tris pH 8.0, 1 mM EDTA were added, the samples were further incubated on ice for 30 min, and the supernatant (10 min, 4,000 rpm,  $4\text{ }^\circ\text{C}$ ) corresponding to the periplasmic fraction was collected.

#### *KPC-2 release evaluation*

To estimate KPC-2 release from bacterial cells due to cell lysis, *E. coli* pTRC99K (negative control) and pTRC99K $\Omega$ kpc-2 cultures were prepared in LB medium, induced with 1 mM IPTG at  $\text{OD}_{600}$  of 1.0 to 1.2, and cultured for three hours at  $37\text{ }^\circ\text{C}$  with agitation at 200 rpm. Pellets corresponding to 700  $\mu\text{L}$  of bacterial cultures were collected by centrifugation (10 min, 4,000 rpm,  $20\text{ }^\circ\text{C}$ ) and resuspended in an equivalent volume of PBS. At indicated timepoints (time "0", 30 min, 60 min and 90 min), each sample was centrifugated (5 min, 4,000 rpm,  $20\text{ }^\circ\text{C}$ ) and transferred to new tube. Collected supernatants were subjected to nitrocefin activity assay.

#### *Nitrocefin activity assay*

Hydrolysis of the chromogenic  $\beta$ -lactam nitrocefin (cephalosporin) was evaluated using a micro plate reader (FLUOstar<sup>®</sup> Omega, BMG LABTECH). The reaction was performed in a volume of 150  $\mu\text{L}$  containing 20 nM KPC-2 or 4  $\mu\text{L}$  of periplasmic extracts or 120  $\mu\text{L}$  of supernatants collected from bacterial cells due to cell lysis, 200  $\mu\text{M}$  nitrocefin, and 50 mM phosphate buffer pH 7.0 for 1 hour at  $25\text{ }^\circ\text{C}$ . The absorbance at 486 nm was recorded every min. The assay was performed in technical triplicate. For KPC-2 quantification in the fraction released due to cell lysis, calibration using recombinant KPC-2 (0-50 nM) and 200  $\mu\text{M}$  nitrocefin was performed in the same experimental conditions.

#### *Peptidoglycan digestion*

<sup>13</sup>C,<sup>15</sup>N-labelled PG of *E. coli* K-12 (10 mg/mL) was mixed with MltG (1.6 mg/mL) or mutanolysin (1 kU commercial enzyme; Merck, Germany), and the reaction was topped up to 500  $\mu\text{L}$  with PBS. After overnight incubation at  $37\text{ }^\circ\text{C}$ , digestion products were evaluated by collecting 2D <sup>1</sup>H,<sup>15</sup>N-BEST-TROSY NMR spectra.

#### *NMR spectroscopy*

All NMR experiments reported in this study were collected on Avance III HD or NEO Bruker spectrometers equipped with helium-cooled cryogenic solution-state <sup>1</sup>H, <sup>2</sup>H, <sup>13</sup>C, <sup>15</sup>N-

resonance probes operating at 700/850, or 950 MHz  $^1\text{H}$  NMR frequency, respectively. All NMR spectra were processed with Topspin (Bruker) and analysed with CcpNmr software (version 3.0.4.)<sup>4</sup>.

2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST experiments were recorded to gain signal sensitivity and retain relatively good resolution of NH signals. These were collected over 12 hours (with 1024 scans and a 46.2 Hz/pts resolution in the indirect dimension) using cell cultures overexpressing KPC-2 prepared as described above, and reference experiment for the interaction studies with avibactam, collected on 50  $\mu\text{M}$   $^{15}\text{N}$ -labelled purified KPC-2 in PBS at pH 7.2 with 5%  $\text{D}_2\text{O}$ , before and after addition of avibactam in 1:1 molar ratio. CSPs for *in vitro* and in-cell experiments were calculated as a weighted average distance between the inhibitor-free protein sample and the protein sample at the 1:1 inhibitor-protein ratio. Default weighting parameters from the CcpNmr software were used<sup>4</sup>.

2D  $^1\text{H}$ - $^{15}\text{N}$  BEST-TROSY experiments were collected to provide optimal resolution on: 50  $\mu\text{M}$   $^{15}\text{N}$ -labelled purified KPC-2 enzyme diluted in PBS pH 7.2; periplasm extract in its extraction buffer; the PG sample processed by mutanolysin or lytic transglycosylase MltG used as a reference to identify PG turnover products in-cell, recorded on soluble  $^{13}\text{C}$ , $^{15}\text{N}$ -labelled PG fragment samples in PBS pH 7.2. In all cases samples were recorded at 298 K and supplemented with 5%  $\text{D}_2\text{O}$ <sup>5</sup>.

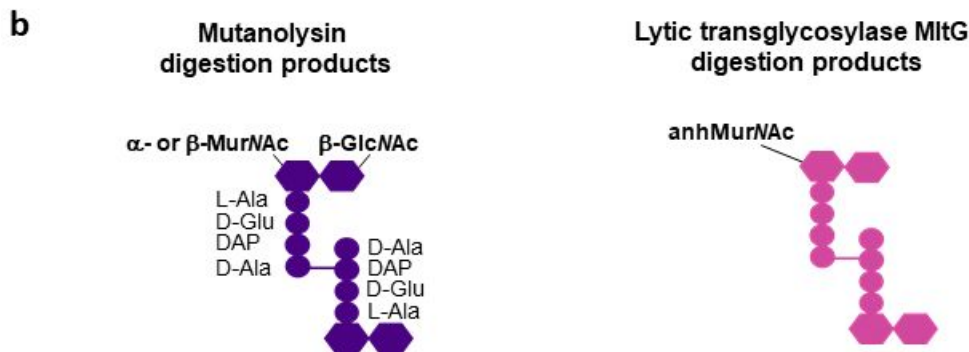
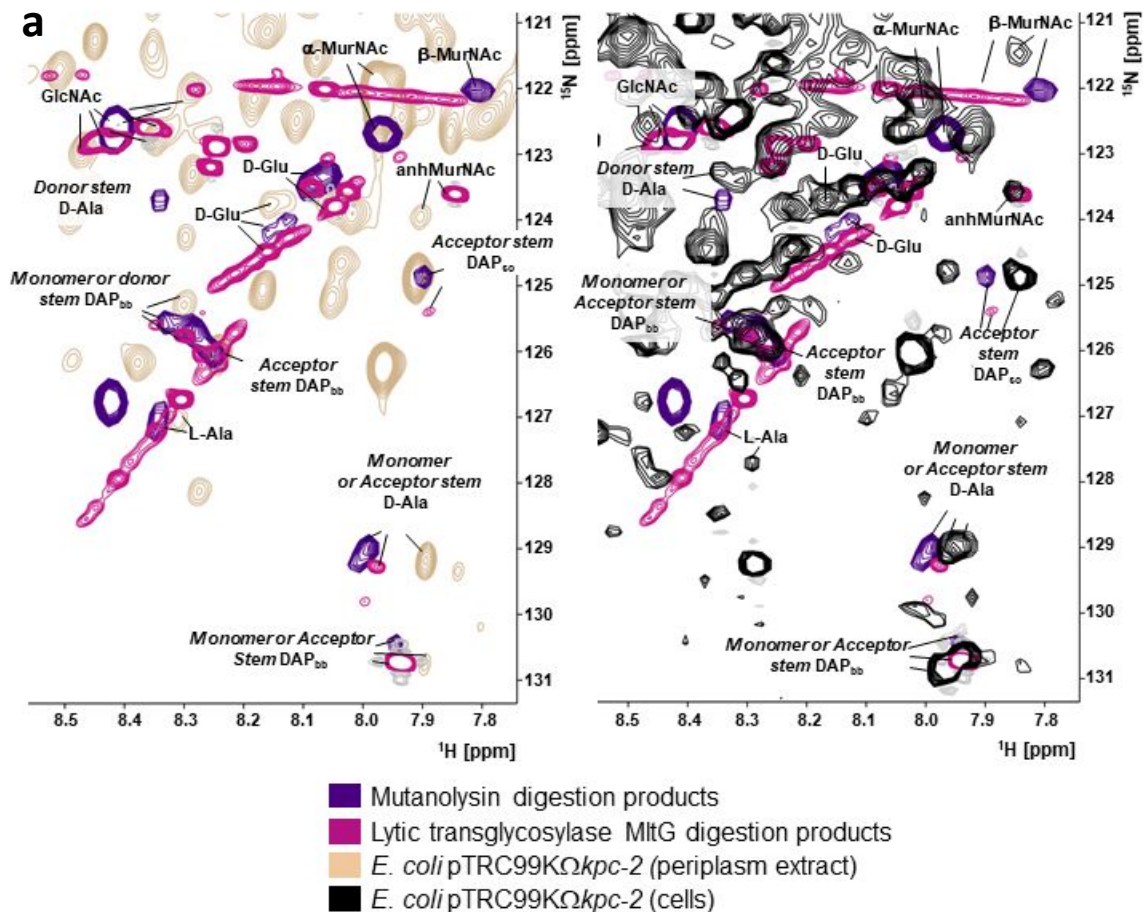
Sequential  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$ -NMR resonance assignments of mutanolysin digested products was performed using sets of 3D experiments. Assignment of backbone resonances from the amino acids of the peptide stems were performed using 3D BEST-TROSY implementations of HNC0, HNcaCO, HNCACB, and HNcoCACB experiments with experimental times of 2.5, 2.5, 4.9, and 5.2 hours, respectively. Missing amino acid side-chain resonances were identified through HcoNH- and CcoNH-TOCSY experiments collected over 11 hours each with acquisition times of 15 ms over a 10-ppm spectral width and 8 ms over a 70-ppm spectral width in the  $^1\text{H}$  and  $^{13}\text{C}$  dimensions, respectively. Finally, glycan, D-Glu (2) and DAP (3) side-chain resonances were assigned using an hCCH-TOCSY experiment acquired over 19 hours with an acquisition time of 6 ms over a 100-ppm window in the  $^{13}\text{C}$ -dimension. 3D data were processed using squared sine-bell window functions within TopSpin version 3.5pl6. All datasets were analyzed with CcpNmr version 2.5.2 for assignment and 2D spectra were superimposed within this software for sample comparison and spectral edition. Chemical shifts were referenced to an external DSS sample at the same temperature.

To follow mecillinam hydrolysis, 1D  $^1\text{H}$  NMR spectra using  $T_{1\rho}$  filtering method were recorded with a spinlock of 50-ms duration and a 1.7-kHz amplitude. 200  $\mu\text{M}$  mecillinam was mixed with (1) purified KPC-2 (20 nM or 100 nM) or/and PBP2 (20 nM) and topped up with PBS, (2) periplasm in its extraction buffer, or (3) bacterial pellets prepared as described above.



**Supplementary Figure 1** Sample containing cells solidified in agar used in the study for in-cell experiments.

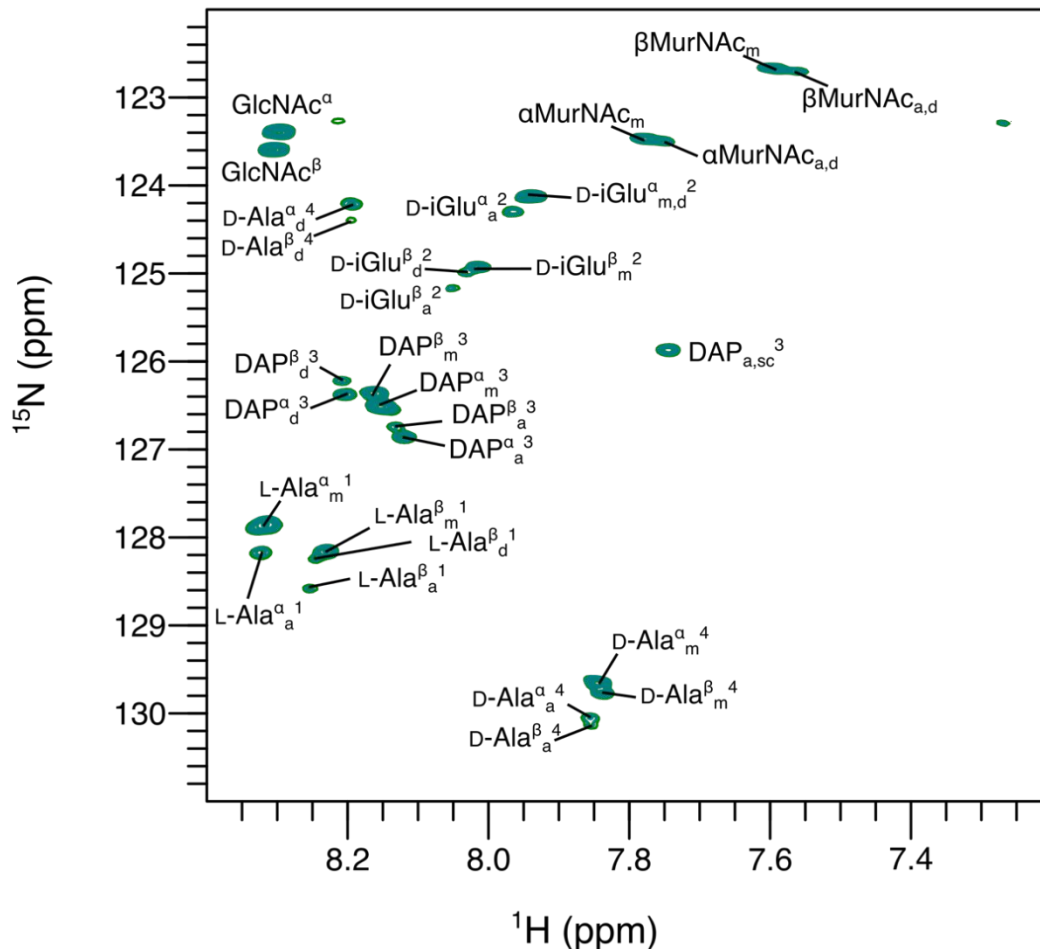
*E. coli* pTRC99K $\Omega$ *kpc-2* was cultured in 150 ml of minimal  $^{15}\text{N}$ -M9 medium. Culture was induced with 1 mM IPTG in minimal medium for 3 hours. Cells were collected by centrifugation (10 min, 4000 rpm). Pellets were washed once in PBS, resuspended in  $\text{D}_2\text{O}$  (10% final) and 600  $\mu\text{l}$  of warm PBS solution supplemented with agar (0.5% final). Samples were immediately transferred to 5-mm NMR tubes. The optimized agar concentration prevents cell lysis and aggregation during long-term NMR experiments. These conditions maintain sample homogeneity and, consequently, positively impacts the shimming process.



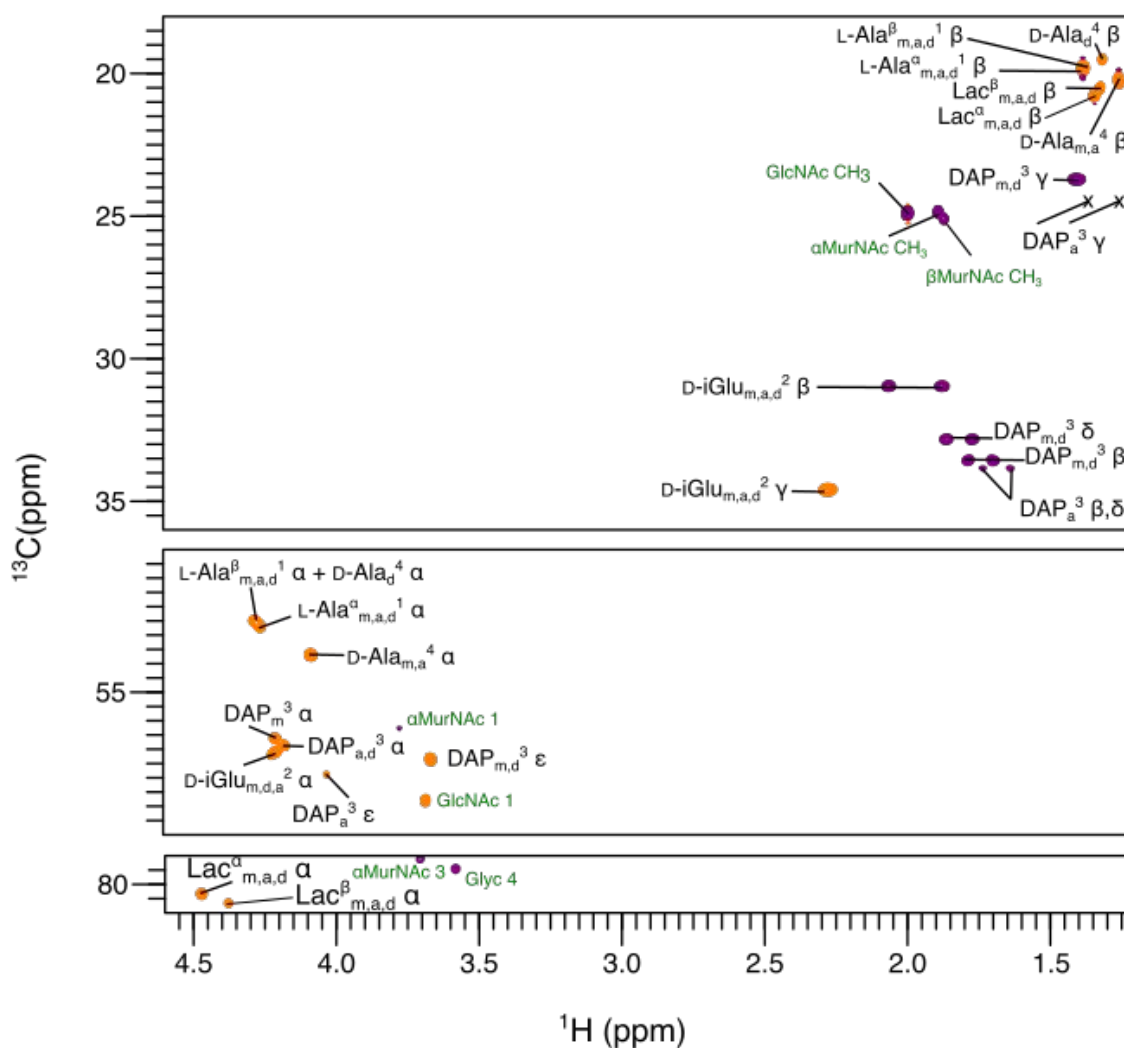
**Supplementary Figure 2** Identification of peptidoglycan (PG) turnover products.

**a.** PG turnover products identified in 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST experiment recorded using extracted periplasm (left part of the panel) and cells (right part of the panel) from *E. coli* pTRC99K $\Omega$ kpc-2 induced with 1 mM IPTG. Mutanolysin and lytic transglycosylase MltG digested samples served as references to identify muuropeptides. Saccharide and amino acid components are named as in **Fig. 1b**.

**b.** Schematic representation of PG digestion products by mutanolysin (pink) and MltG (violet).

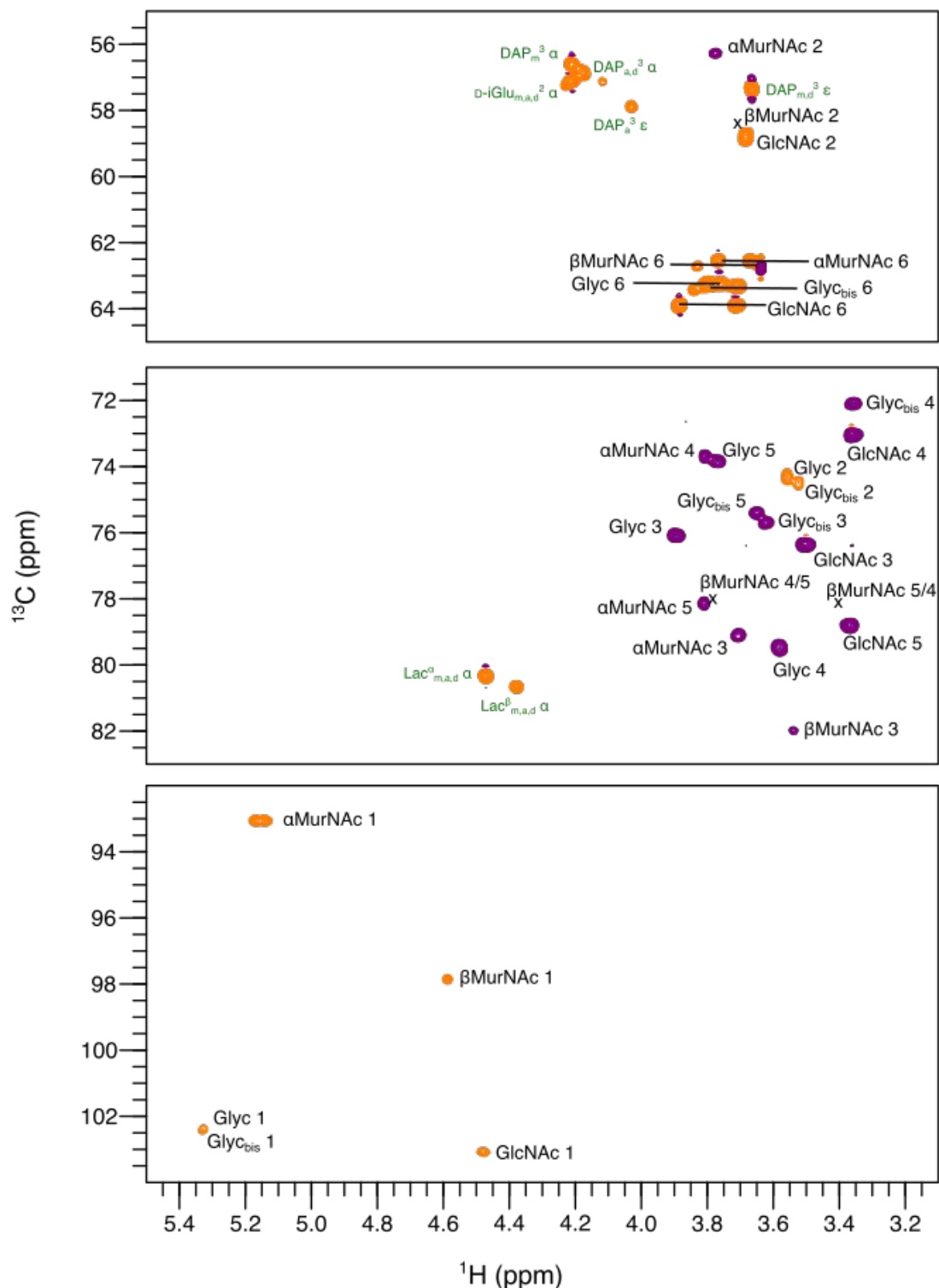


**Supplementary Figure 3**  $^{15}$ N-BEST-TROSY NMR spectrum of *E. coli* peptidoglycan soluble fragments obtained after mutanolysin digestion. Muropeptide samples were dialyzed against water and lyophilized. 2.35 mg of sample were solubilized in 200  $\mu$ L of 100 mM ammonium hydrogenocarbonate buffer at pH 6.7 with 10%  $D_2O$  and NMR spectra were collected at 27  $^{\circ}C$  on a Bruker Avance III HD spectrometer operating at 850 MHz proton-frequency and equipped with a Cryo TCI ( $^1H$ ,  $^{13}C$ ,  $^{15}N$ )-triple resonance probe with z-gradients. Each amide resonance of amino acid is labeled with (i) the name of the amino acid (e.g. L-Ala), (ii) its occurrence in a motif containing a MurNac in configuration  $\alpha$  or  $\beta$  (e.g.  $\alpha$ ), (iii) its occurrence in a monomer (m), acyl donor (d), or acyl acceptor (a) peptide stem (e.g. a), and (iv) the position of the amino acid in the peptide stem (e.g. 1) yielding in the present example to L-Ala $^{\alpha}$ <sub>a</sub><sup>1</sup>. Resonances labelled with sugar names correspond to the N-acetyl group of the corresponding GlcNac or MurNac. The superscript on the GlcNac specifies the  $\alpha$  or  $\beta$  configuration of the covalently linked MurNac. Since the  $\alpha$  configuration of MurNac is in excess in comparison to the  $\beta$  configuration relative intensities were used to decipher specific assignment of resonances from the corresponding peptide stems. 3D HNCOC, HNCaCO, HNCACB, HNCoCACB, HCoNH- and CCoNH-TOCSY experiments were instrumental in the discrimination of peptide stems belonging to monomers vs dimers, and to donor vs acceptor stems. The occurrence of D-Ala<sup>4</sup> as the terminus of any stem peptide is evidenced from the chemical shift of its carbonyl group in the HNCaCO experiment. These resonance assignments were transferred to the spectra shown in Figure S2a that were collected in the same conditions than the periplasmic extracts and cell samples. For the later spectra details of the monomeric, acceptor or donor stems when uncertain are omitted due to the lower resolution of the 2D  $^1H$ - $^{15}N$  SOFAST experiment and changes in the sample conditions.

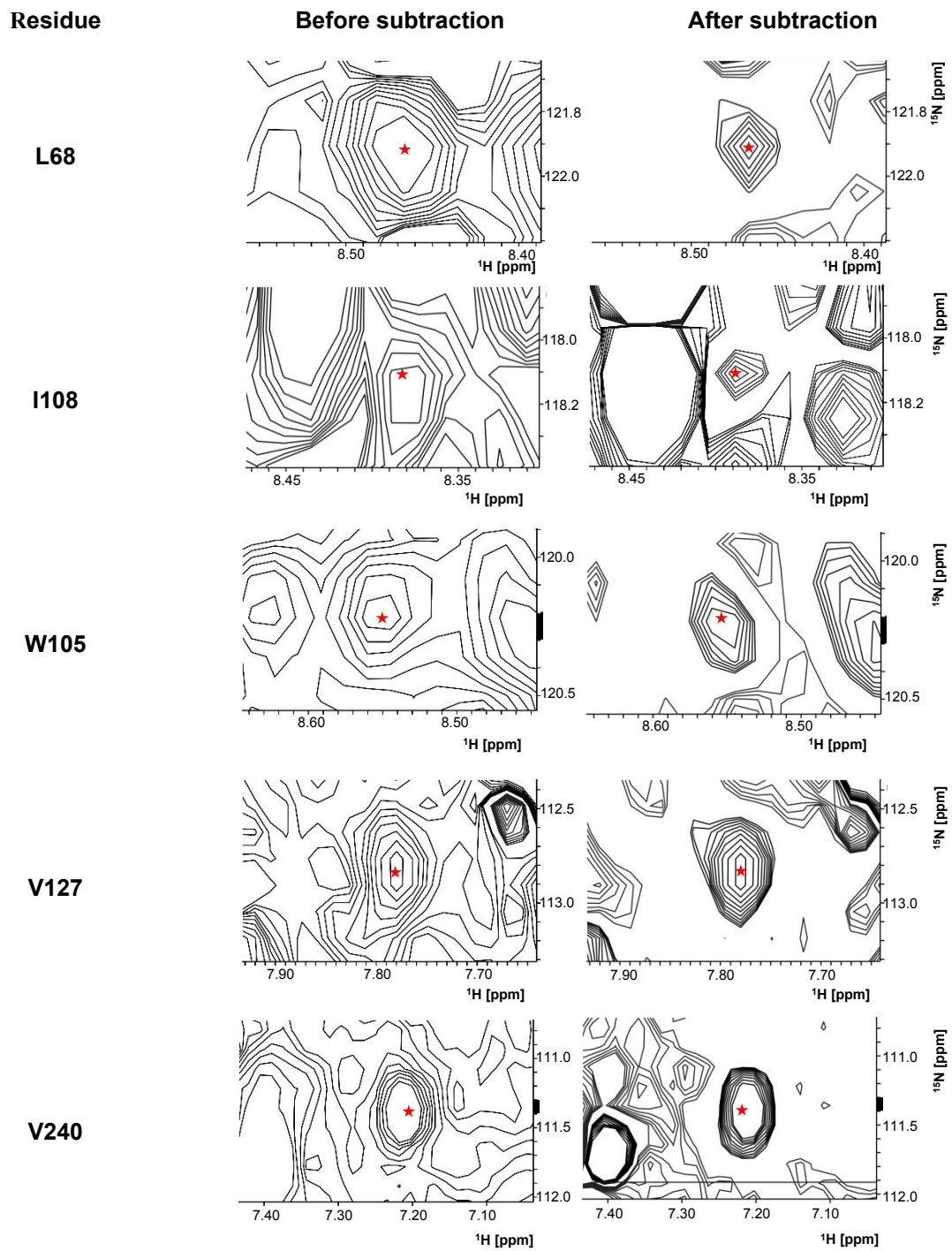


**Supplementary Figure 4** Focus on the amino acid portion of the  $^{13}\text{C}$ -SE-CT-HSQC spectrum of *E. coli* peptidoglycan soluble fragments obtained after mutanolysin digestion. Data were collected in the same conditions and amino acid resonances are labeled according to the same nomenclature as in Figure S3. The impact of the  $\alpha$  or  $\beta$  configuration of MurNAc extends only to the D-Lac and L-Ala<sup>1</sup> C-H correlations. C-H correlations of D-iGlu<sup>2</sup>, DAP<sup>3</sup> and D-Ala<sup>4</sup> residues are degenerate for the  $\alpha$  and  $\beta$  configuration of MurNAc. For this reason, this precision is omitted for the corresponding residues. The position of the C-H group in the amino acid side-chain is specified last for each resonance with the corresponding Greek symbol  $\alpha$  through  $\epsilon$ . Resonances in green originate from sugars. Resonances in orange and purple are positive and negative, respectively, resulting from a constant time delay of 26 ms in the  $^{13}\text{C}$ -HSQC sequence used.

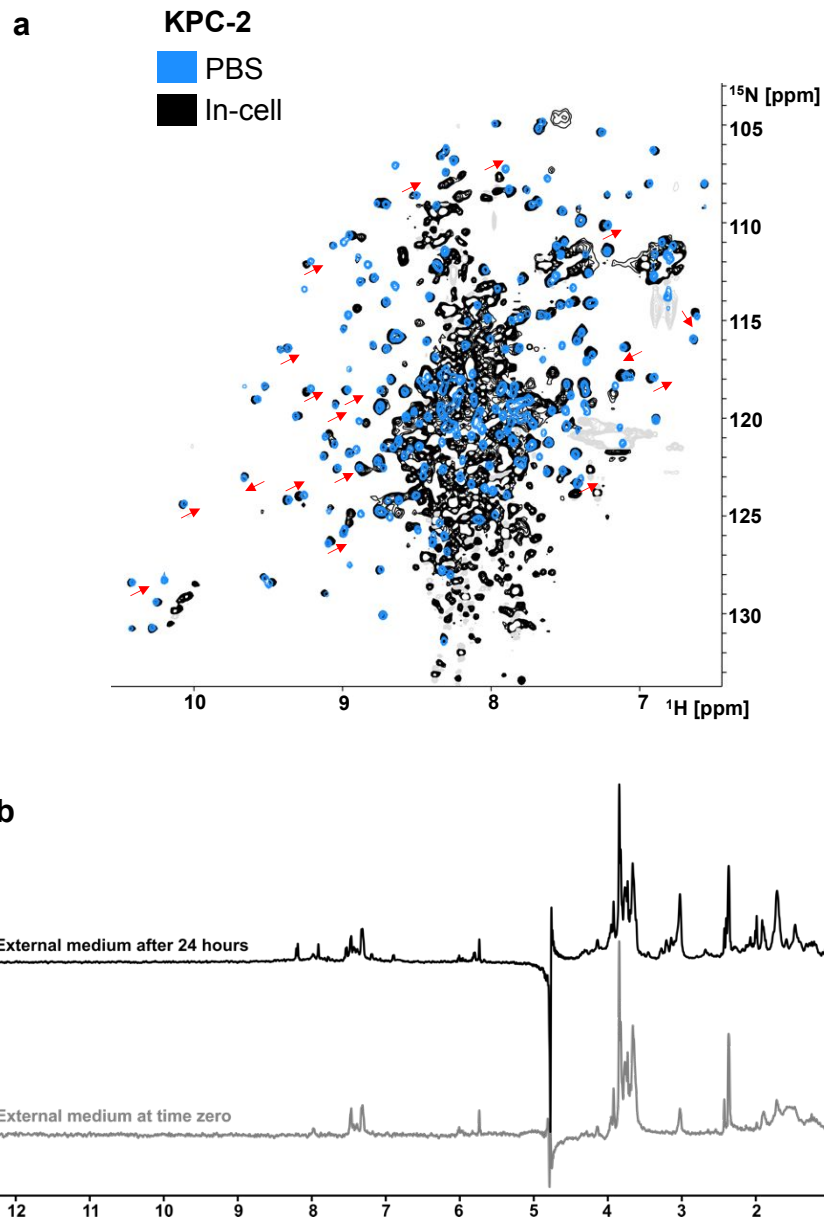




**Supplementary Figure 5** Focus on the sugar region of the  $^{13}\text{C}$ -SE-CT-HSQC spectrum of *E. coli* peptidoglycan soluble fragments obtained after mutanolysin digestion. This portion is part of the same spectrum as in Fig. S4. Resonances from the disaccharide are labelled with the sugar type assorted with its configuration when necessary ( $\alpha$  or  $\beta$  for MurNAc) and position of the C-H group in the ring starting from the cyclic oxygen (anomeric carbon is C1). Crosses indicate resonances at lower contour level due to the choice of the constant time 26 ms delay for the collection of this experiment. Resonances labelled in green originate from amino acid resonances of the peptide stem. Resonances from glycogen (polymer of  $\alpha$ -glucose) contaminants copurified with peptidoglycan are labelled with Glyc and have been identified by comparison with the literature<sup>1</sup>.

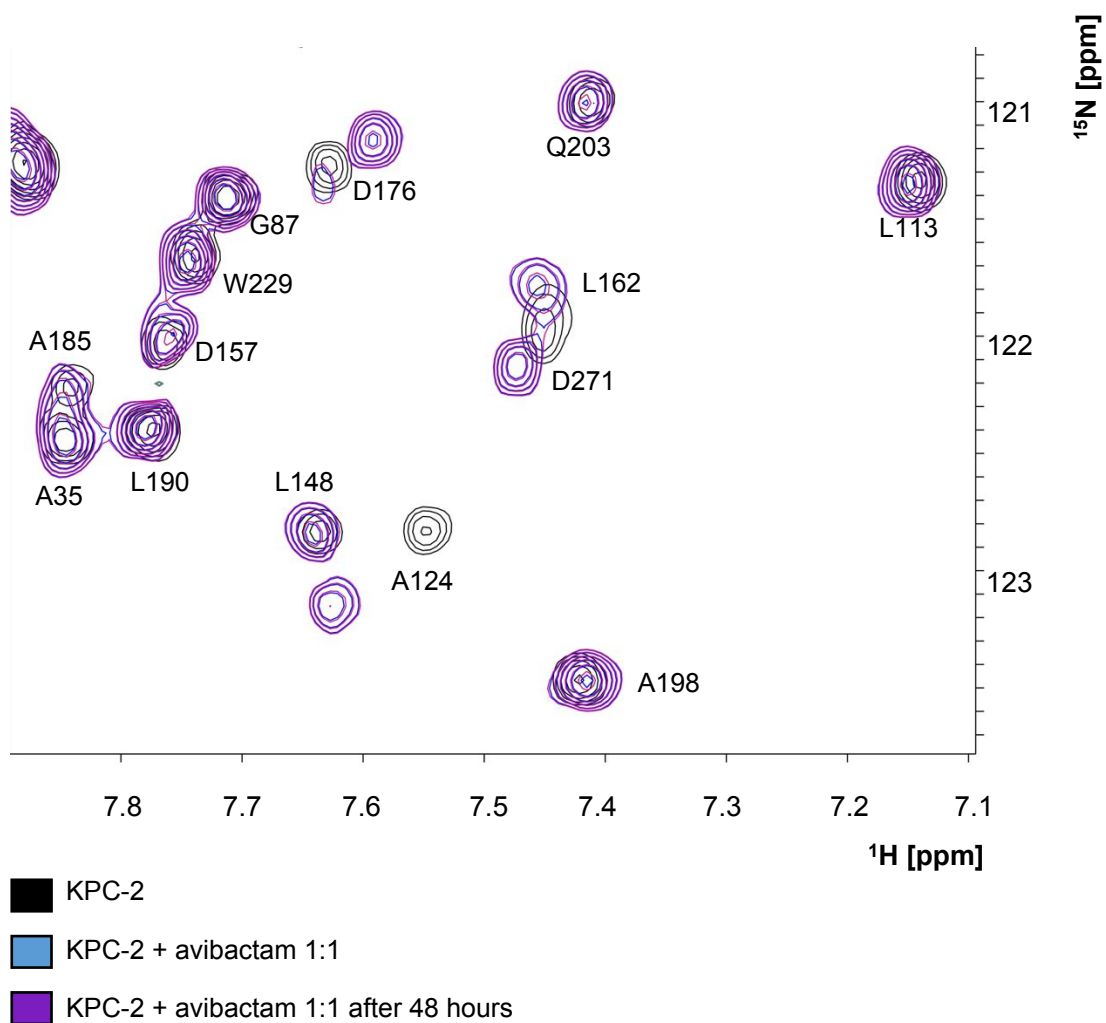


**Supplementary Figure 6** Excerpts of 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST spectrum recorded on *E. coli* pTRC99K $\Omega$ kpc-2 induced with 1 mM IPTG before and after subtraction of the reference dataset recorded on non-induced *E. coli* pTRC99K $\Omega$ kpc-2. Red asterisks mark the KPC-2 amide resonances.

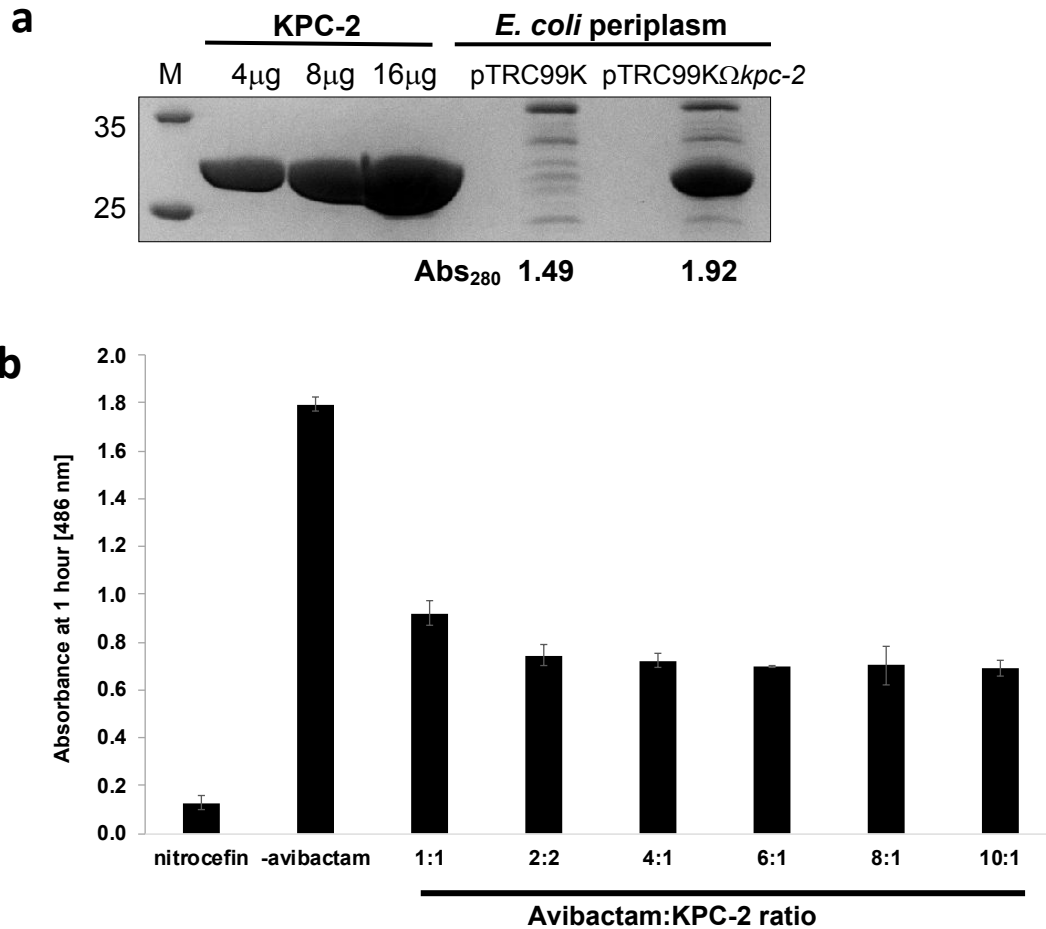


**Supplementary Figure 7** Validation of internal localization of KPC-2

- Comparison of 2D  $^1\text{H}$ - $^{15}\text{N}$  BEST-TROSY spectrum recorded on purified KPC-2 in PBS (blue) and 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST spectrum of *E. coli* pTRC99K $\Omega$ *kpc-2* induced with 1 mM IPTG (black). Red arrows mark NH chemical shift perturbations (CSPs) of KPC-2, due to different physicochemical conditions in each sample.
- Comparison of 1D  $^1\text{H}$  spectrum recorded on external medium (PBS) of *E. coli* pTRC99K $\Omega$ *kpc-2* induced with 1 mM IPTG, collected by centrifugation immediately (“time zero” in grey) or after prolonged incubation (“after 24 hours” in black).

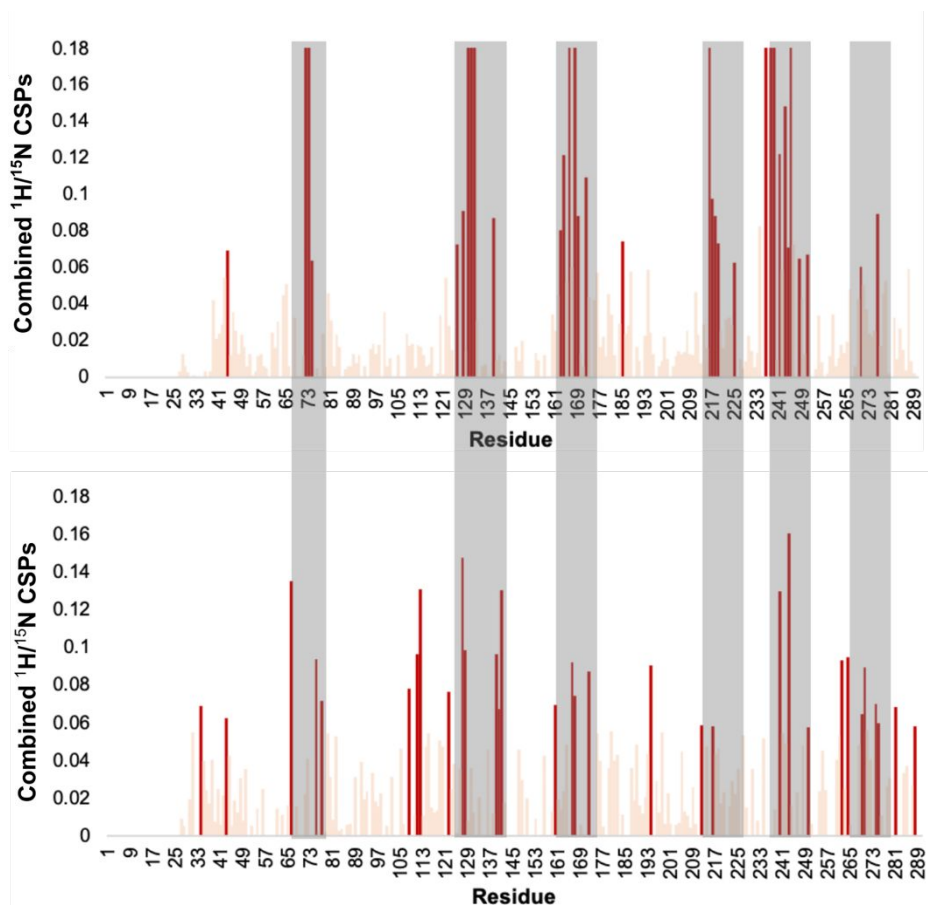


**Supplementary Figure 8** Region of the  $^1\text{H}, ^{15}\text{N}$ -BEST-TROSY spectra of  $^{15}\text{N}$ -labelled KPC-2 recorded at 25 °C and pH 7.4. The experiment was conducted before (black) and after (blue) addition of avibactam to the NMR tube. The same experiment was recorded 48 h after addition of avibactam (violet) to evaluate the stability of the complex.

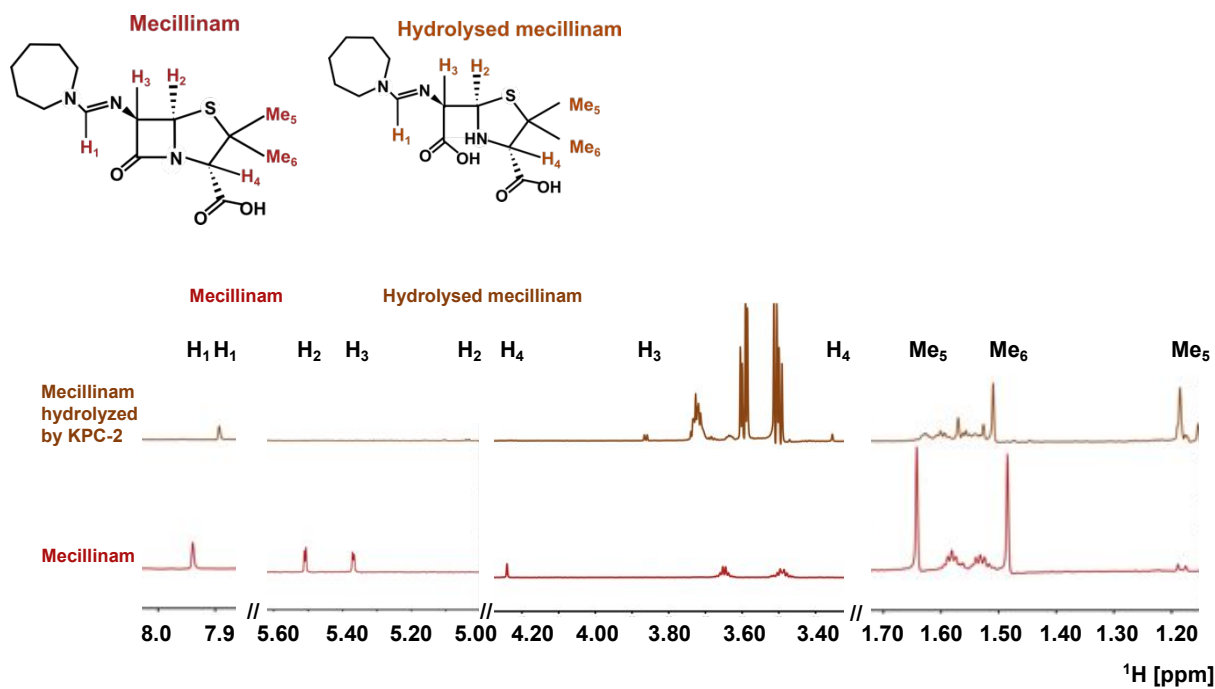


**Supplementary Figure 9** Evaluation of KPC-2 inhibition by avibactam in cells.

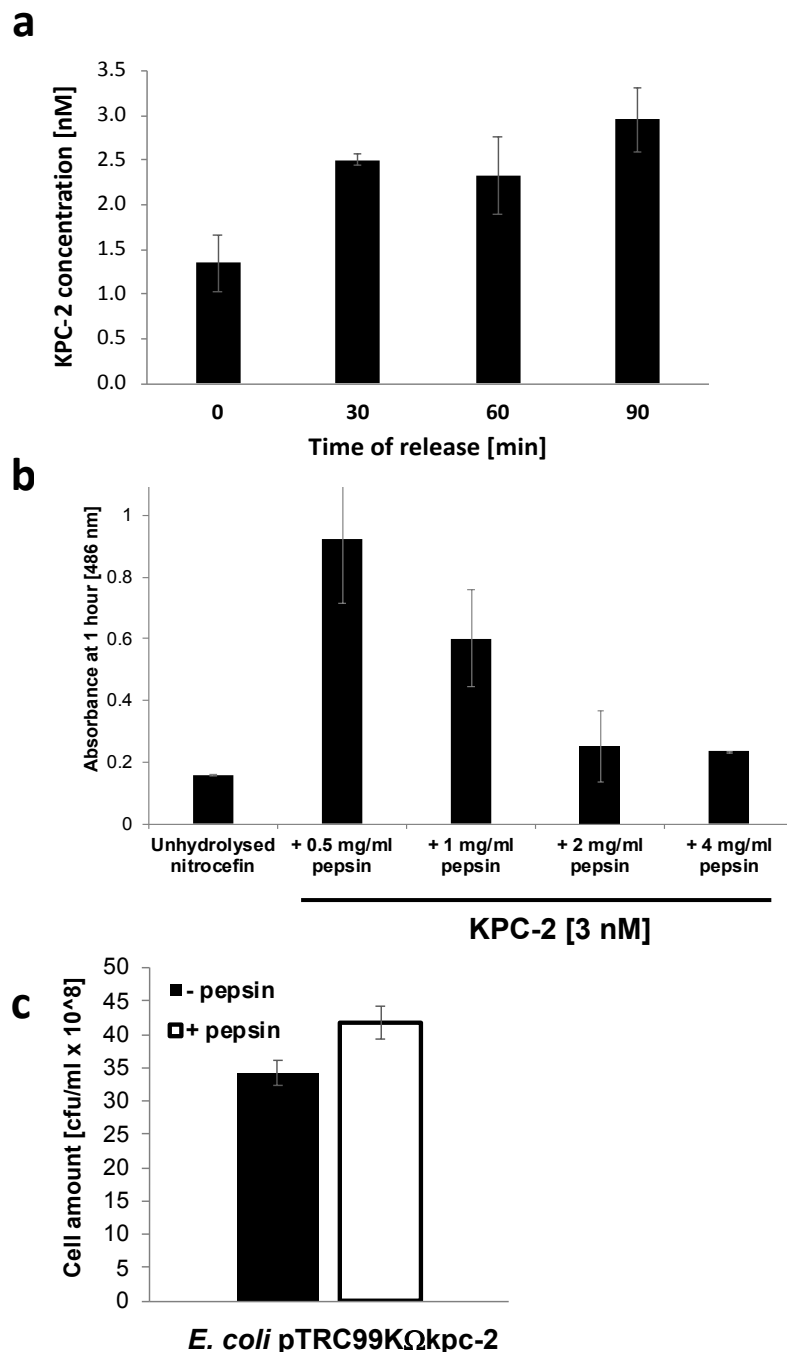
- a.** SDS-PAGE gel (15%) comparing the intensity of protein bands corresponding to purified KPC-2 and to KPC-2 in periplasmic extracts from cultures of *E. coli* pTRC99K (negative control) and *E. coli* pTRC99KΩ*kpc-2* induced with 1 mM IPTG. The absorbance at 280 nm of the periplasm extracts was evaluated by Nanodrop to refer to comparable total protein concentration. KPC-2 concentration in periplasmic extracts was estimated to be 70 μM.
- b.** Hydrolysis of nitrocefin by *E. coli* pTRC99KΩ*kpc-2* cells induced with 1 mM IPTG in the presence of increasing avibactam concentrations. The amount of avibactam was adjusted to the amount of KPC-2 in cell samples as estimated in panel A. Experiment was performed in PBS at 25 °C in triplicate.



**Supplementary Figure 10** Combined <sup>1</sup>H- and <sup>15</sup>N-chemical shift perturbations calculated for each KPC-2 residue from the data displayed in **Figure 2a** and **b** as the weighted-average distance between the resonance position of the free form of KPC-2 and its equivalent position in the complex with avibactam. Top and bottom panels correspond to CSP measured *in vitro* and in-cell, respectively. CSPs superior to 1 standard deviation (0.032 and 0.031 ppm for *in vitro* and in-cell data, respectively) are colored in crimson. Unaffected and unassigned protein regions are colored in wheat and pale-grey, respectively. KPC-2 sequence regions affected upon avibactam binding in both *in vitro* and in-cell experiments are highlighted in grey.



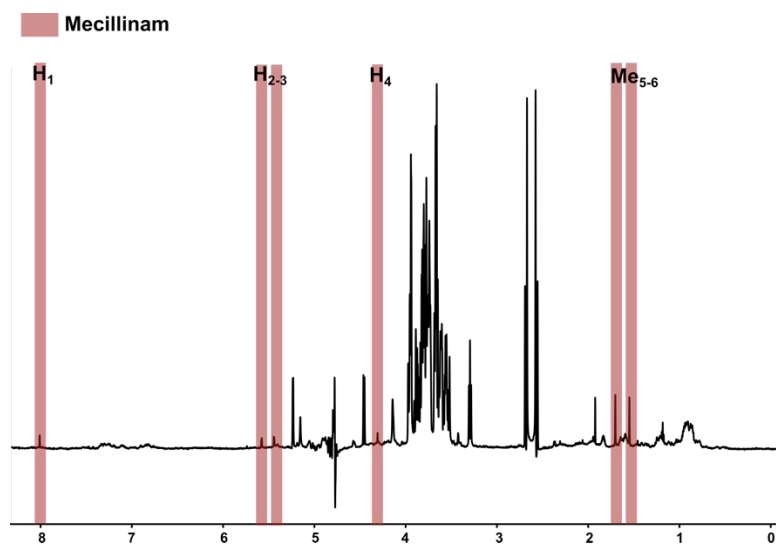
**Supplementary Figure 11** 1D <sup>1</sup>H spectra recorded at pH 7.4 and 25 °C on 200 μM of mecillinam and its KPC-2 hydrolysis product are shown in top and bottom panels, respectively. <sup>1</sup>H NMR peaks unique to mecillinam and its hydrolysis product have been highlighted in red and brown, respectively.



**Supplementary Figure 12** Determination of pepsin concentration to inactivate KPC-2 released due to cell lysis during mecillinam hydrolysis NMR experiments.

- Hydrolysis of nitrocefin by culture supernatants sampled at different time points from cultures of *E. coli* pTRC99KΩkpc-2 induced with 1 mM IPTG. Reaction readouts recorded for *E. coli* pTRC99K collected at the corresponding timepoints were subtracted from the presented results. Experiment was done in PBS for 2 hours at 25 °C in duplicate. Error bars correspond to the standard deviation. Amount of KPC-2 for a given sample was estimated using calibration curve based on the activity of recombinant KPC-2 probed in the nitrocefin activity assay performed in the same experimental conditions.
- Hydrolysis of nitrocefin by purified KPC-2 (3 nM) treated for 30 minutes with increasing concentrations of pepsin. Experiment was done in PBS for 1 hour at 25 °C in duplicate.
- Number of cell forming units of *E. coli* pTRC99KΩkpc-2 induced with 1 mM IPTG upon treatment with 4 mg/ml pepsin for 1 hour at room temperature.

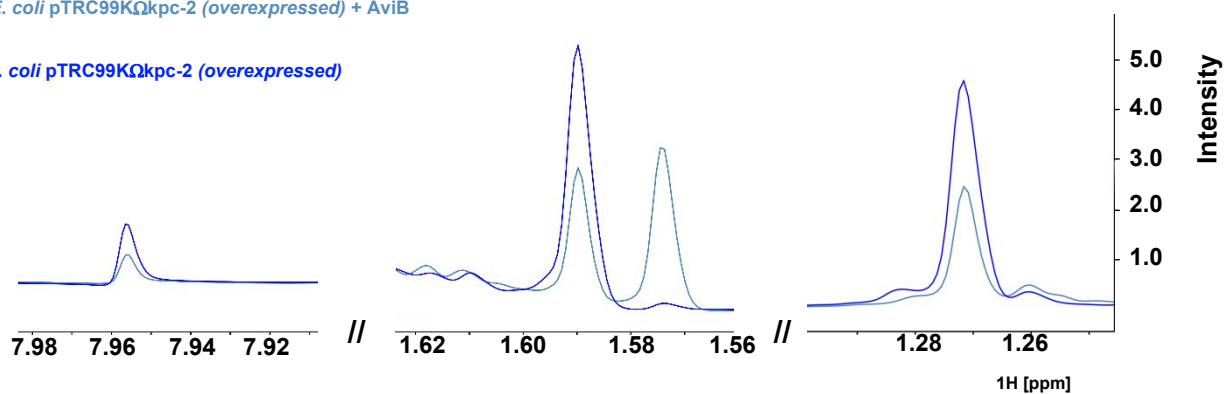




**Supplementary Figure 13** 1D <sup>1</sup>H spectra recorded at pH 7.4 and 25 °C on 200 μM of mecillinam in the presence of 4 mg/ml pepsin. Mecillinam <sup>1</sup>H NMR peaks have been highlighted in red.

*E. coli* pTRC99KΩkpc-2 (overexpressed) + AviB

*E. coli* pTRC99KΩkpc-2 (overexpressed)



Integral value

$4.76 \times 10^8$	$2.07 \times 10^9$	$1.89 \times 10^9$
$9.11 \times 10^8$	$4.14 \times 10^9$	$3.79 \times 10^9$

**Supplementary Figure 14** Three different regions of the 1D <sup>1</sup>H T<sub>1ρ</sub>-filtered spectra recorded at pH 8.0 and 25 °C on *E. coli* pTRC99KΩkpc-2 induced by 1mM IPTG and corresponding cell culture pre-incubated with avibactam in 10-fold molar excess above KPC-2 determined based on **Figure S9a**. Each variant of cell culture was mixed with mecillinam (200 μM). Each peak was integrated and the integral value corresponding to the area under the peak for a given signal has been determined using the CcpNmr software. 1D spectra as well as integral values are colored in pale blue and blue for data recorded in presence and in absence of avibactam, respectively.

**Table S1.** <sup>1</sup>H-, <sup>13</sup>C- and <sup>15</sup>N-resonance chemical shifts (in ppm) of *E. coli* monomers and dimers in 100 mM NH<sub>4</sub>HCO<sub>3</sub> buffer at pH 6.7 and 27 °C obtained from the NMR spectra analysis<sup>a,b</sup>

<b>Disaccharide tetrapeptide monomer 1 (most abundant)</b>								
	<b>GlcNAc sugar</b>	<b>αMurNAc sugar</b>		<b>Lac</b>	<b>L-Ala<sup>1</sup></b>	<b>D-iGlu<sup>2</sup></b>	<b>DAP<sup>3</sup></b>	<b>D-Ala<sup>4</sup></b>
<b>H1</b>	4.48	5.15	<b>H<sub>N</sub></b>		8.32	7.94	8.15	7.84
<b>C1</b>	103.08	93.06	<b>N</b>		127.85	124.12	126.51	129.65
<b>H2</b>	3.68	3.77	<b>CO</b>	178.11	176.78	180.38	175.78	182.47
<b>C2</b>	58.80	56.27				(COOH)		(COOH)
<b>H3</b>	3.50	3.71	<b>Hα</b>	4.47	4.26	4.21	4.21	4.08
<b>C3</b>	76.37	79.12	<b>Cα</b>	80.34	52.69	57.10	56.62	53.70
<b>H4</b>	3.36	3.81	<b>Hβ</b>	1.34	1.38	1.87/2.06	1.69/1.78	1.25
<b>C4</b>	73.05	73.69	<b>Cβ</b>	20.76	19.80	30.96	33.57	20.22
<b>H5</b>	3.37	3.81	<b>Hγ</b>			2.28	1.40	
<b>C5</b>	78.81	78.13	<b>Cγ</b>			34.60	23.71	
			<b>COγ</b>			178.35		
<b>H6</b>	3.71/3.88	3.67/3.76	<b>Hδ</b>				1.77/1.86	
<b>C6</b>	63.89	62.55	<b>Cδ</b>				32.83	
<b>NHAc</b>			<b>Hε</b>				3.66	
			<b>Cε</b>				57.35	
<b>H<sub>N</sub></b>	8.30	7.79						
<b>N</b>	123.40	123.47						
<b>CO</b>	177.41	176.87						
<b>CH<sub>3</sub></b>	1.99	1.89						
<b>CH<sub>3</sub></b>	24.89	24.86						
<b>Disaccharide tetrapeptide monomer 2 (less abundant)</b>								
	<b>GlcNAc sugar</b>	<b>βMurNAc sugar</b>		<b>Lac</b>	<b>L-Ala<sup>1</sup></b>	<b>D-iGlu<sup>2</sup></b>	<b>DAP<sup>3</sup></b>	<b>D-Ala<sup>4</sup></b>
<b>H1</b>	4.48	4.59	<b>H<sub>N</sub></b>		8.23	8.02	8.16	7.84
<b>C1</b>	103.08	97.85	<b>N</b>		128.16	124.93	126.36	129.76
<b>H2</b>	3.68	3.66	<b>CO</b>	177.47	176.95	180.29	175.78	182.47
<b>C2</b>	58.80	58.29				(COOH)		(COOH)
<b>H3</b>	3.50	3.54	<b>Hα</b>	4.38	4.28	4.21	4.21	4.08
<b>C3</b>	76.37	81.99	<b>Cα</b>	80.67	52.51	57.10	56.62	53.70
<b>H4</b>	3.36	3.77	<b>Hβ</b>	1.32	1.38	1.87/2.06	1.69/1.78	1.25
<b>C4</b>	73.05	78.07	<b>Cβ</b>	20.55	19.81	30.96	33.57	20.22
<b>H5</b>	3.37	3.42	<b>Hγ</b>			2.28	1.40	
<b>C5</b>	78.81	78.53	<b>Cγ</b>			34.60	23.71	
			<b>COγ</b>			178.35		
<b>H6</b>	3.71/3.88	3.64/3.82	<b>Hδ</b>				1.77/1.86	
<b>C6</b>	63.89	62.71	<b>Cδ</b>				32.83	
<b>NHAc</b>			<b>Hε</b>				3.66	
			<b>Cε</b>				57.35	
<b>H<sub>N</sub></b>	8.31	7.60						
<b>N</b>	123.60	122.67						
<b>CO</b>	177.41	177.22						
<b>CH<sub>3</sub></b>	1.99	1.87						
<b>CH<sub>3</sub></b>	24.89	25.10						

Table S1 (continued).

Disaccharide tetrapeptide dimer 1								
Donor stem								
	GlcNAc sugar	$\alpha$ MurNAc sugar		Lac	L-Ala <sup>1</sup>	D-iGlu <sup>2</sup>	DAP <sup>3</sup>	D-Ala <sup>4</sup>
H1 C1	4.48 103.08	5.15 93.06	H <sub>N</sub> N		8.32 127.85	7.94 124.12	8.21 126.37	8.20 124.21
H2 C2	3.68 58.80	3.77 56.27	CO	178.11	176.78	180.38 (COOH)	176.74	176.68
H3 C3	3.50 76.37	3.71 79.12	H $\alpha$ C $\alpha$	4.47 80.34	4.26 52.69	4.21 57.10	4.17 56.86	4.28 52.51
H4 C4	3.36 73.05	3.81 73.69	H $\beta$ C $\beta$	1.34 20.76	1.38 19.80	1.87/2.06 30.96	1.70/1.77 33.47	1.31 19.50
H5 C5	3.37 78.81	3.81 78.13	H $\gamma$ C $\gamma$ CO $\gamma$			2.28 34.60 178.43	1.40 23.71	
H6 C6	3.71/3.88 63.89	3.67/3.76 62.55	H $\delta$ C $\delta$				1.77/1.86 32.83	
NHAc			H $\epsilon$ C $\epsilon$				3.66 57.35	
H <sub>N</sub> N	8.30 123.40	7.79 123.47	HN $\epsilon$ N $\epsilon$ COOH $\epsilon$					
CO	177.41	176.87						
<u>CH</u> <sub>3</sub> <u>CH</u> <sub>3</sub>	1.99 24.89	1.89 24.86						
Acceptor stem								
	GlcNAc sugar	$\alpha$ MurNAc sugar		Lac	L-Ala <sup>1</sup>	D-iGlu <sup>2</sup>	DAP <sup>3</sup>	D-Ala <sup>4</sup>
H1 C1	4.48 103.08	5.15 93.06	H <sub>N</sub> N		8.32 128.17	7.96 124.30	8.12 126.86	7.86 130.05
H2 C2	3.68 58.80	3.77 56.27	CO	177.94	176.98	180.44 (COOH)	175.87	182.42 (COOH)
H3 C3	3.50 76.37	3.71 79.12	H $\alpha$ C $\alpha$	4.47 80.34	4.26 52.69	4.21 57.10	4.17 56.86	4.08 53.70
H4 C4	3.36 73.05	3.81 73.69	H $\beta$ C $\beta$	1.34 20.76	1.38 19.80	1.87/2.06 30.96	1.63/1.73 33.84	1.25 20.32
H5 C5	3.37 78.81	3.81 78.13	H $\gamma$ C $\gamma$ CO $\gamma$			2.25 34.58 178.25	1.29/1.37 24.47	
H6 C6	3.71/3.88 63.89	3.67/3.76 62.55	H $\delta$ C $\delta$				1.63/1.73 33.84	
NHAc			H $\epsilon$ C $\epsilon$				4.03 57.88	
H <sub>N</sub> N	8.30 123.40	7.79 123.47	HN $\epsilon$ N $\epsilon$ COOH $\epsilon$				7.74 125.87 181.26	
CO	177.41	176.87						
<u>CH</u> <sub>3</sub> <u>CH</u> <sub>3</sub>	1.99 24.89	1.89 24.86						

Table S1 (continued).

Disaccharide tetrapeptide dimer 2								
Donor stem								
	GlcNAc sugar	$\beta$ MurNAc sugar		Lac	L-Ala <sup>1</sup>	D-iGlu <sup>2</sup>	DAP <sup>3</sup>	D-Ala <sup>4</sup>
H1 C1	4.48 103.08	4.59 97.85	H <sub>N</sub> N		8.25 128.25	8.03 124.99	8.21 126.22	8.20 124.39
H2 C2	3.68 58.80	3.66 58.29	CO	177.46	176.95	180.29 (COOH)	176.74	176.68
H3 C3	3.50 76.37	3.54 81.99	H $\alpha$ C $\alpha$	4.38 80.67	4.28 52.51	4.21 57.16	4.17 56.86	4.28 52.51
H4 C4	3.36 73.05	3.77 78.07	H $\beta$ C $\beta$	1.32 20.55	1.38 19.81	1.87/2.06 30.96	1.70/1.77 33.47	1.31 19.50
H5 C5	3.37 78.81	3.42 78.53	H $\gamma$ C $\gamma$ CO $\gamma$			2.28 34.60 178.42	1.40 23.71	
H6 C6	3.71/3.88 63.89	3.64/3.82 62.71	H $\delta$ C $\delta$				1.77/1.86 32.83	
NHAc			H $\epsilon$ C $\epsilon$				3.66 57.35	
H <sub>N</sub> N	8.31 123.60	7.56 122.70						
CO	177.41	177.22						
CH <sub>3</sub> CH <sub>3</sub>	1.99 24.89	1.87 25.10						
Acceptor stem								
	GlcNAc sugar	$\beta$ MurNAc sugar		Lac	L-Ala <sup>1</sup>	D-iGlu <sup>2</sup>	DAP <sup>3</sup>	D-Ala <sup>4</sup>
H1 C1	4.48 103.08	4.59 97.85	H <sub>N</sub> N		8.25 128.58	8.05 125.17	8.13 126.74	7.86 130.13
H2 C2	3.68 58.80	3.66 58.29	CO	177.33	176.95	180.34 (COOH)	175.87	182.42 (COOH)
H3 C3	3.50 76.37	3.54 81.99	H $\alpha$ C $\alpha$	4.38 80.67	4.28 52.51	4.21 57.10	4.17 56.86	4.08 53.70
H4 C4	3.36 73.05	3.77 78.07	H $\beta$ C $\beta$	1.32 20.55	1.38 19.81	1.87/2.06 30.96	1.63/1.73 33.84	1.25 20.32
H5 C5	3.37 78.81	3.42 78.53	H $\gamma$ C $\gamma$ CO $\gamma$			2.25 34.58 178.25	1.29/1.37 24.47	
H6 C6	3.71/3.88 63.89	3.64/3.82 62.71	H $\delta$ C $\delta$				1.63/1.73 33.84	
NHAc			H $\epsilon$ C $\epsilon$				4.03 57.88	
H <sub>N</sub> N	8.31 123.60	7.56 122.70	H $\epsilon$ N $\epsilon$ COOH $\epsilon$				7.74 125.87 181.26	
CO	177.41	177.22						
CH <sub>3</sub> CH <sub>3</sub>	1.99 24.89	1.87 25.10						

- a) <sup>1</sup>H and <sup>15</sup>N chemical shift of amide resonances are measured on the TROSY line and are therefore shifted by approximately 48 Hz in both dimensions with respect to the actual resonance position.
- b) We cannot exclude the presence of a mixture of  $\alpha$ - and  $\beta$ -MurNAc isomers in the dimers as D-iGlu resonances are insensitive to the presence of the 4 $\rightarrow$ 3 cross-links or the MurNAc isomerization. The MurNAc isomerization is due to the presence of the reducing end of MurNAc in disaccharides after mutanolysin cleavage.

**Table S2.** Constructs and bacterial strains used in this study.

	<b>Name</b>	<b>Expressed protein</b>	<b>Reference</b>
<b>Constructs</b>	KPC-2-pET41a	<i>K. pneumoniae</i> KPC-2 <sub>L26-G290</sub> , C-terminal His-tag	This study
	PBP2-pACYC-Duet 1	<i>P. aeruginosa</i> PAO1 PBP2 <sub>L53-Q646</sub> , N-terminal His-tag	
	MltG-pET-TEV (modified pET28a)	<i>E. coli</i> MltG <sub>E76-Q340</sub> , N-terminal His-tag	
	pTRC99k	Empty vector	
	pTRC99kΩkpc2	<i>K. pneumoniae</i> KPC-2 <sub>S1-Q294</sub>	

	<b>Name</b>	<b>Application</b>	<b>Reference</b>
<b>Bacterial strains</b>	<i>E. coli</i> BW25113Δ <i>ampC</i>	In-cell NMR experiments, periplasm purification	This study
	<i>E. coli</i> BL21(DE3)	KPC-2 and MltG expression	Laboratory strain
	<i>E. coli</i> BL21(DE3) Gold	PBP2 expression	
	<i>E. coli</i> K12	PG digestion	

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