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Supplementary Materials for

Peptidoglycan editing provides immunity to *Acinetobacter baumannii* during bacterial warfare

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Published 22 July 2020, *Sci. Adv.* **6**, eabb5614 (2020)
DOI: 10.1126/sciadv.abb5614

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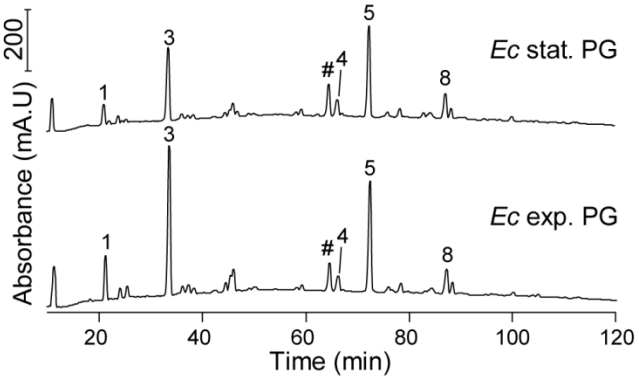

Fig. S1. Stationary *E. coli* cells do not modify their PG. Muropeptide profiles of exponential (exp.) and stationary (stat.) phase *E. coli* BW25113 (*Ec*) cells. Main muropeptides are labelled and their structures are shown in Fig. S2.

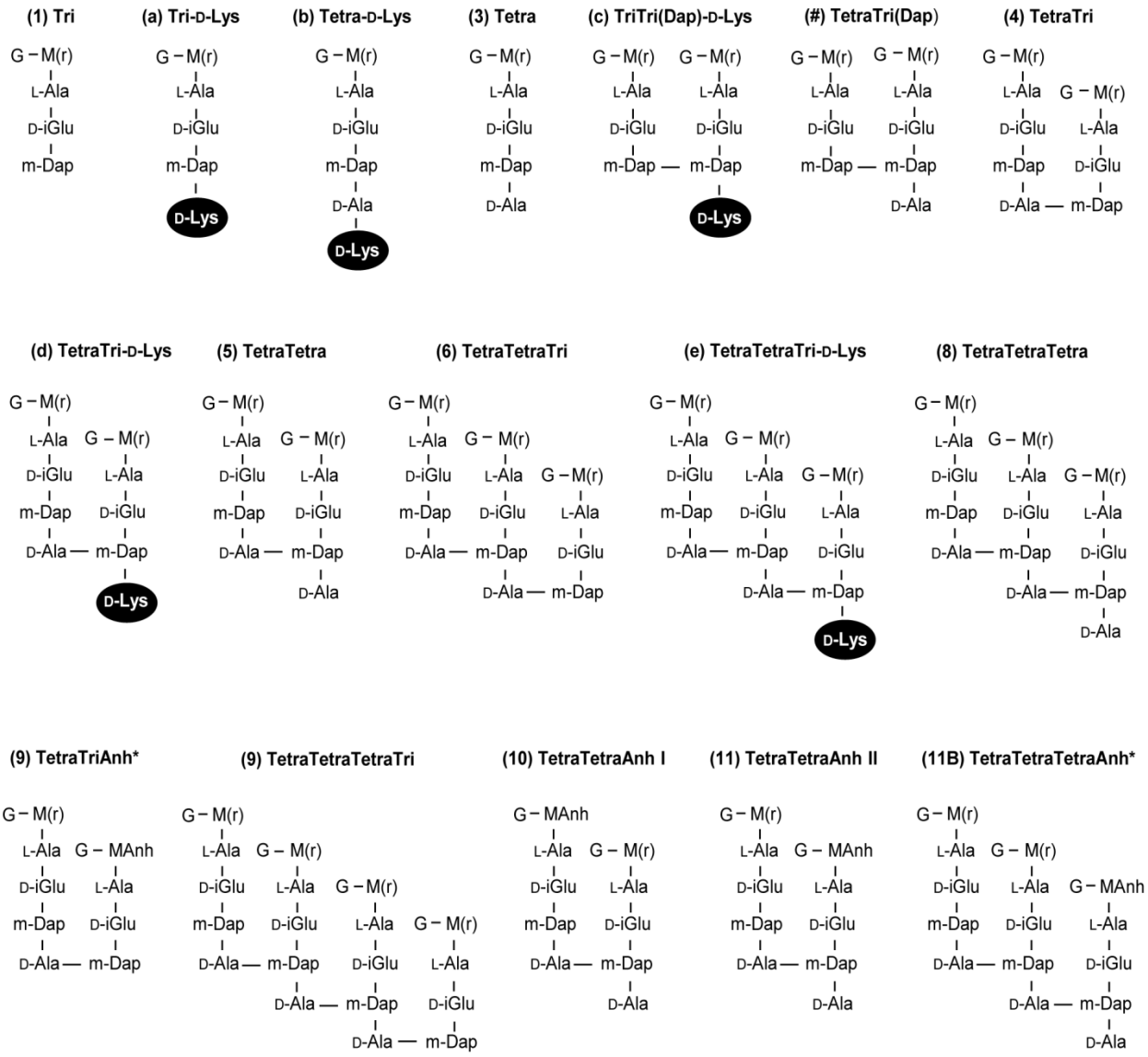

Fig. S2. Proposed muropeptide structures of the chromatograms shown in Figs. 1, 2C, 3B, S1 and S4. M(r), *N*-acetylmuramitol; G, *N*-acetylglucosamine. MS analysis of peak 9 reavealed a composition of two muropeptides (~70% d43Anh and ~30% p4443). * position of the anhydro-group is unknown. Peak 0 is generated by acid hydrolysis of unused lipid II or glycan chains ends carrying the C55-PP moiety. m = monomer, d = dimer, t = trimer, p = polymer. 3,4,5 = number of amino acids in the peptide stem. Anh = anhydro-group.

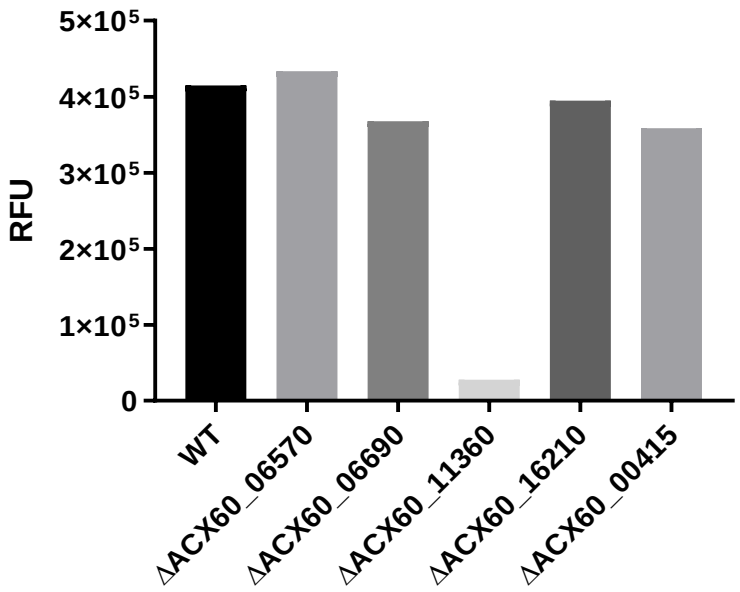

Fig. S3. Identification of racemase responsible for D-Lysine production in Ab17978. AmaD assay described in the Methods section was used to quantify D-Lys concentrations of stationary phase culture supernatants of *A. baumannii* 17978 strains individually lacking possible racemases.

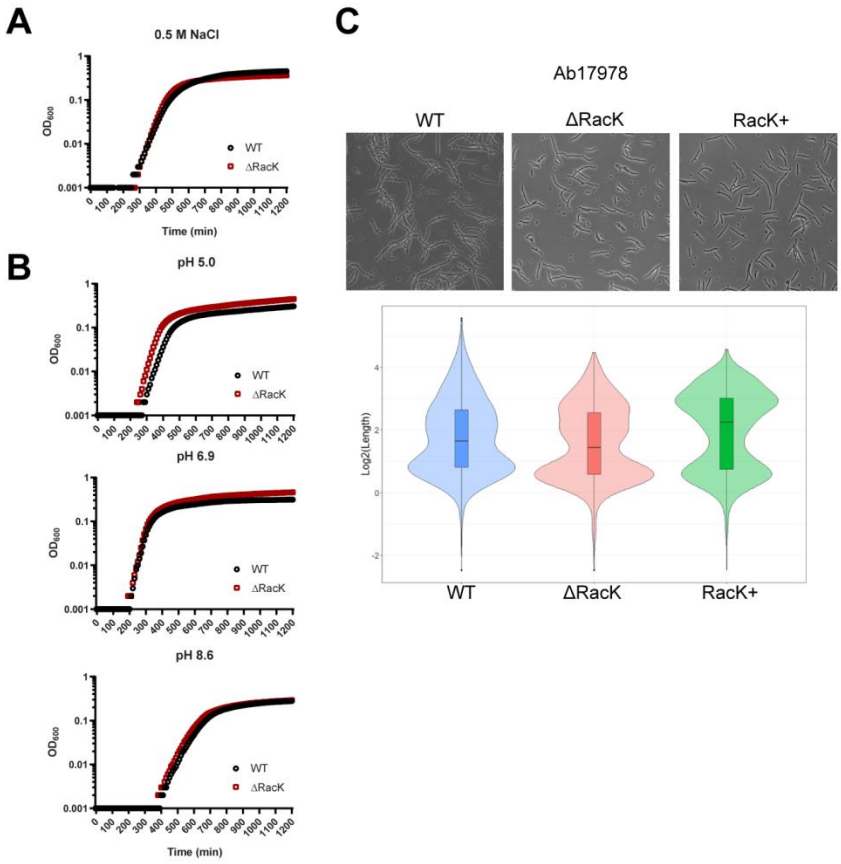

Fig. S4. PG modification in Ab17978 does not have notable impact on its response to osmotic (A) and pH stress (B) or bacteria morphology (C). (A) Growth curve of Ab17978 WT or ΔRacK grown in LB supplemented with 0.5M NaCl. **(B)** Growth curve of Ab17978 WT or ΔRacK growth in LB buffered at pH 5.0, 6.9 or 8.6. **(C)** Ab17978 WT, ΔRacK or RacK+ bacteria cells were observed under phase contrast microscopy. Cell length of each strain was obtained by using cell segmentation SuperSegger algorithm (Stylianidou et al. 2016) on 10 microscopy images. Violin and box plots represent length distribution of 800+ bacteria cells in log2 scale.

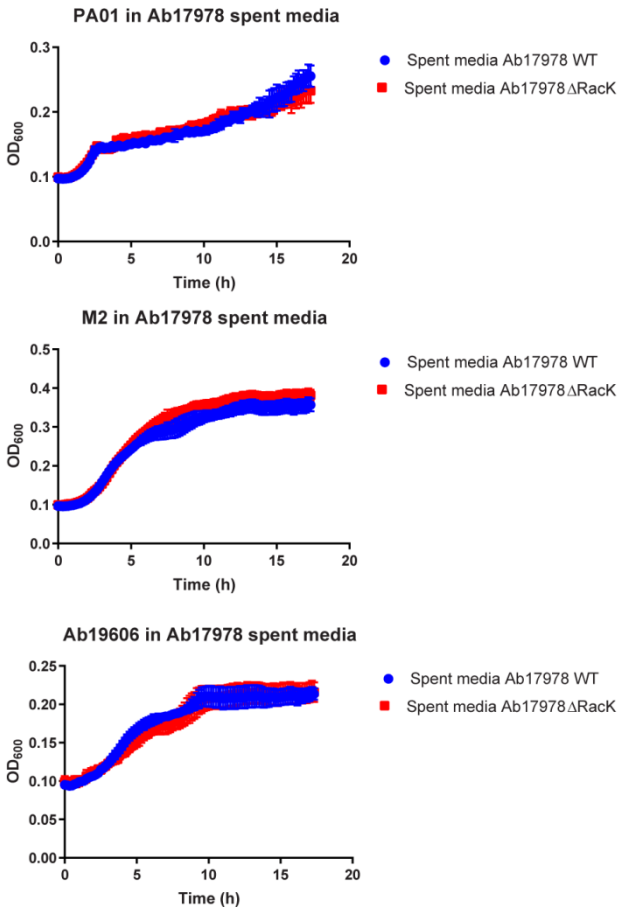

Fig. S5. Concentration of secreted D-Lys by Ab17978 is not enough to induce inter-bacterial growth inhibition in liquid culture. Stationary culture supernatants of Ab17978 WT and ΔRacK were use as growth media of *P. aeruginosa* PAO1, *A. nosocomialis* M2 and *A. baumannii* Ab19606. Data are representative of 3 biological replicates.

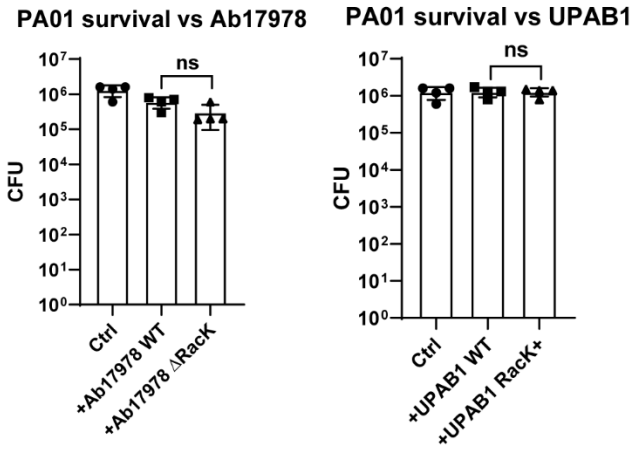

Fig. S6. *P. aeruginosa* PAO1 (PAO1) growth is unaffected by co-incubation with Ab17978 or UPAB1 during T6SS killing assays shown in Fig. 2. Bar graphs represent the mean ± SD of 4 biological replicates. Statistical analyses were performed using the unpaired Student's t test, ns: non significative.

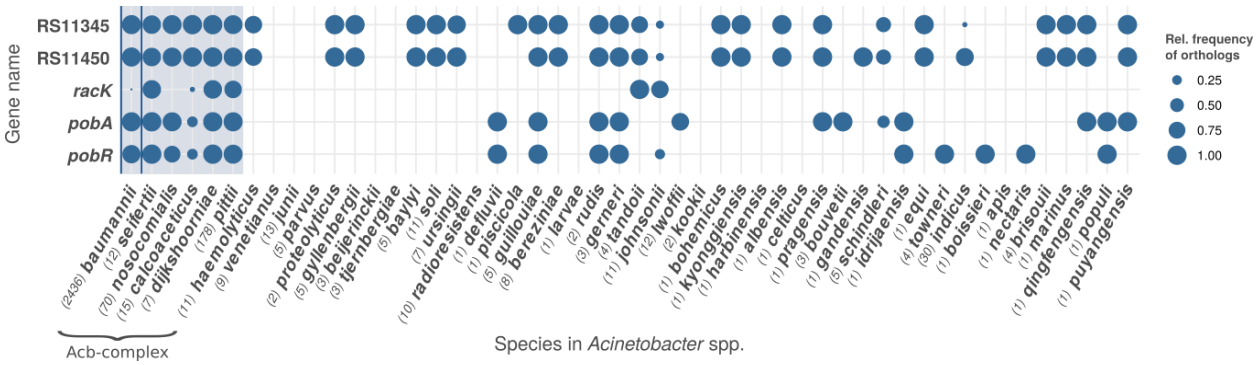

Fig. S7A. Presence/absence pattern of *racK* and its flanking genes in the genus *Acinetobacter*. The phylogenetic profile reveals the occurrence of orthologs to *racK* and of its two flanking genes up- and downstream, respectively, across 3,052 *Acinetobacter spp.* genomes. The information was summarized on the species level with the number of genomes per species given in parentheses. A blue dot indicates the presence of an ortholog in the respective species. The dot diameter is proportional to the relative frequency of genomes that harbour an ortholog. In total, only 7 species of *Acinetobacter* harbour an ortholog including *A. tandoii* and *A. johnsonii*. Within the Acb-complex *racK* orthologs were found in only 7% of all *A. baumannii* strains (cf Figure 7), 10% of *A. calcoaceticus* and none of *A. nosocomialis*. In contrast, it is almost ubiquitously present in *A. seiffertii*, *A. pittii*, and *A. dijkshoorniae*. The presence of orthologs to all five genes is restricted to the clade constituting the Acb-complex. The profile was visualized using PhyloProfile v. 1.1.2 [10.1093/bioinformatics/bty225].

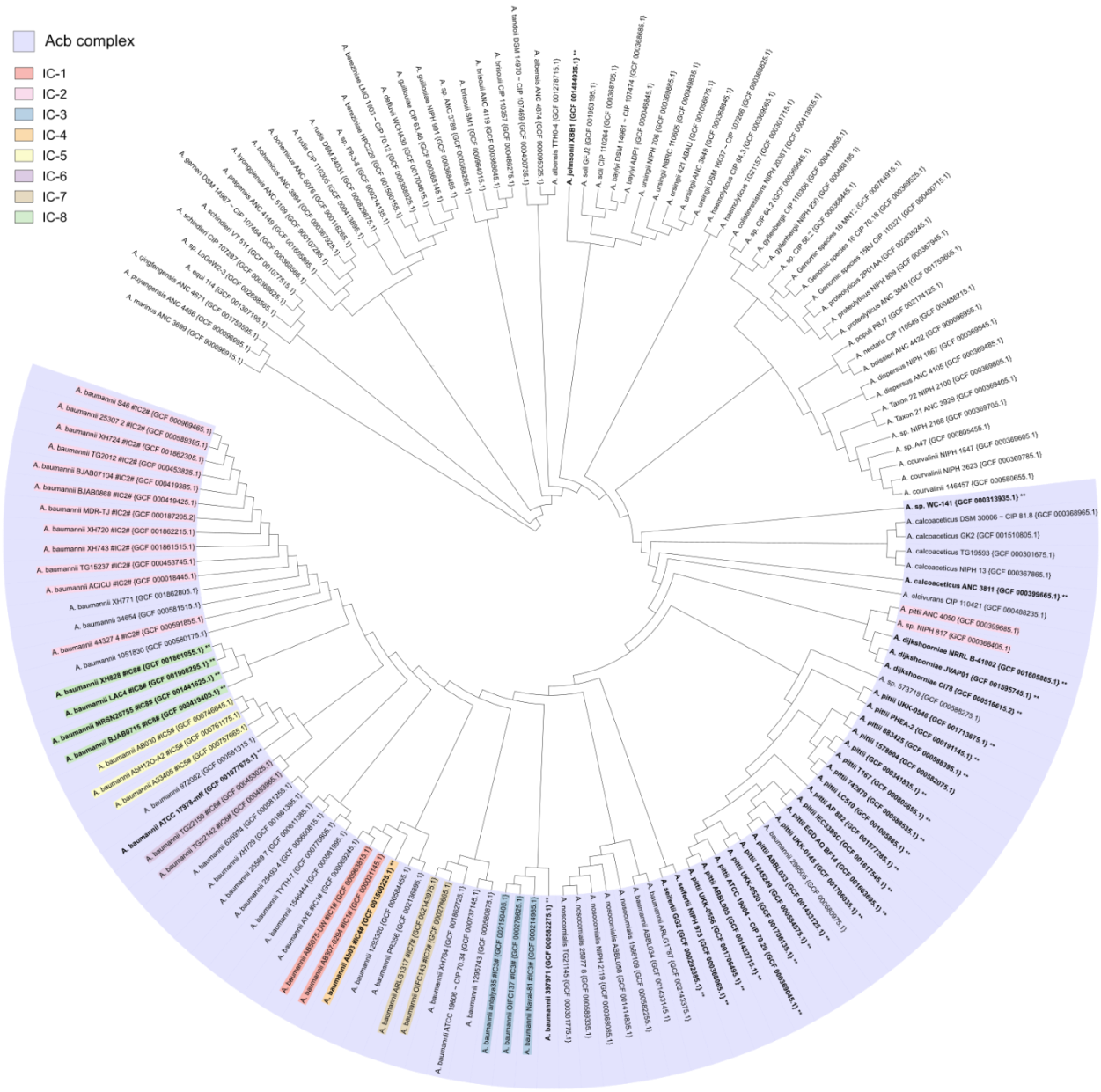

Fig. S7B. Maximum-Likelihood phylogeny of *racK*'s genomic neighborhood across Acinetobacter spp. The tree was reconstructed under the best fitting model TIM+F+I+G4 and was inferred from concatenated codon alignments of *racK*'s 4 flanking genes (ACX60_RS11345, ACX60_RS11350, ACX60_RS11360, and ACX60_RS11365) that we traced across 232 genomes. Leaf labels comprise species names, strain names and identifiers, as well as RefSeq assembly accessions (in curly brackets). Clone Type (IC) affiliation of an *A. baumannii* strain is indicated by label background colors (cf. figure legend). For visualization, the branch lengths have

universally been set to 1. Branch labels specify bootstrap supports. Strains harboring a *racK* ortholog (bold faced tip labels) are distributed within the phylogeny of the *Acinetobacter calcoaceticus/baumannii* (Acb-) complex (blue background).

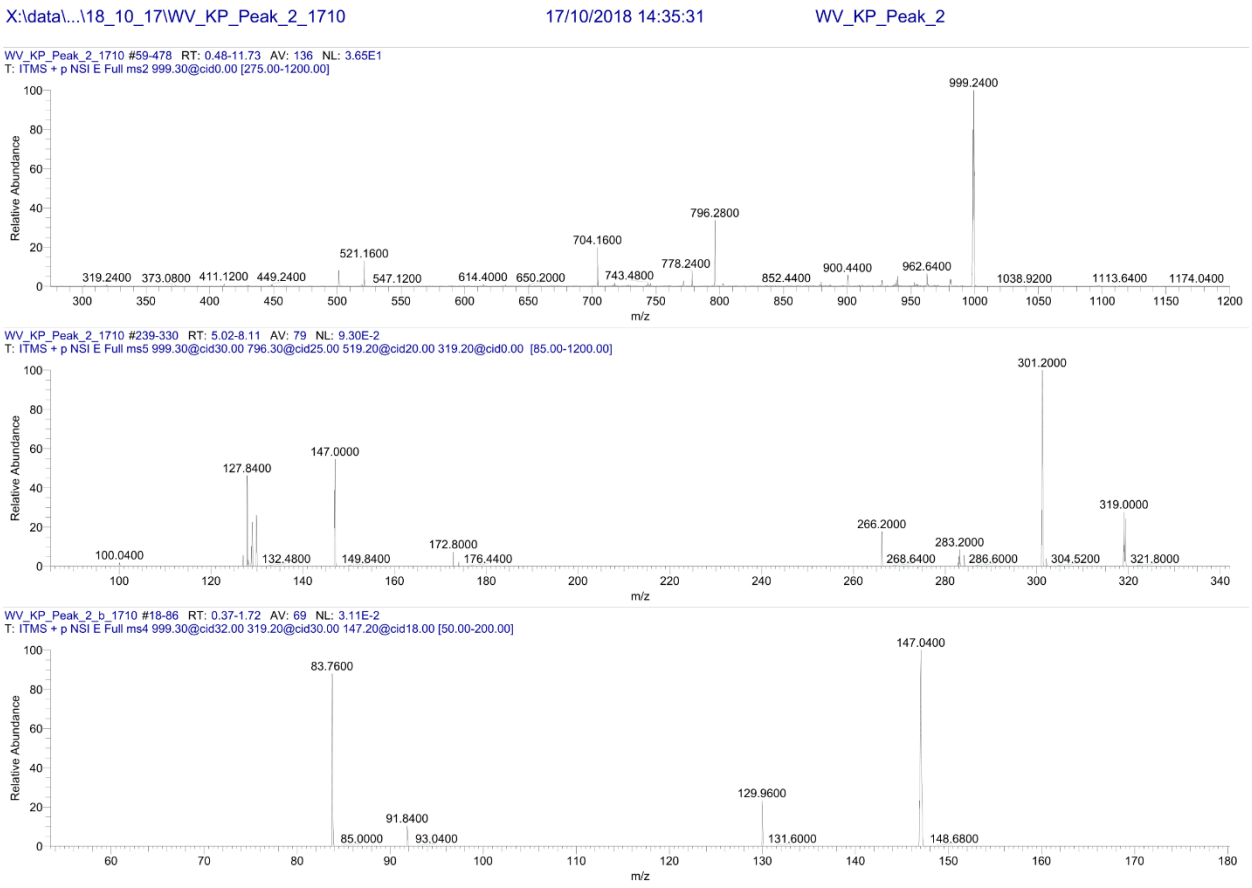

Fig. S8A. Mass spectrometry analysis of peak a (Tri-D-Lys) as presented in Fig. 1

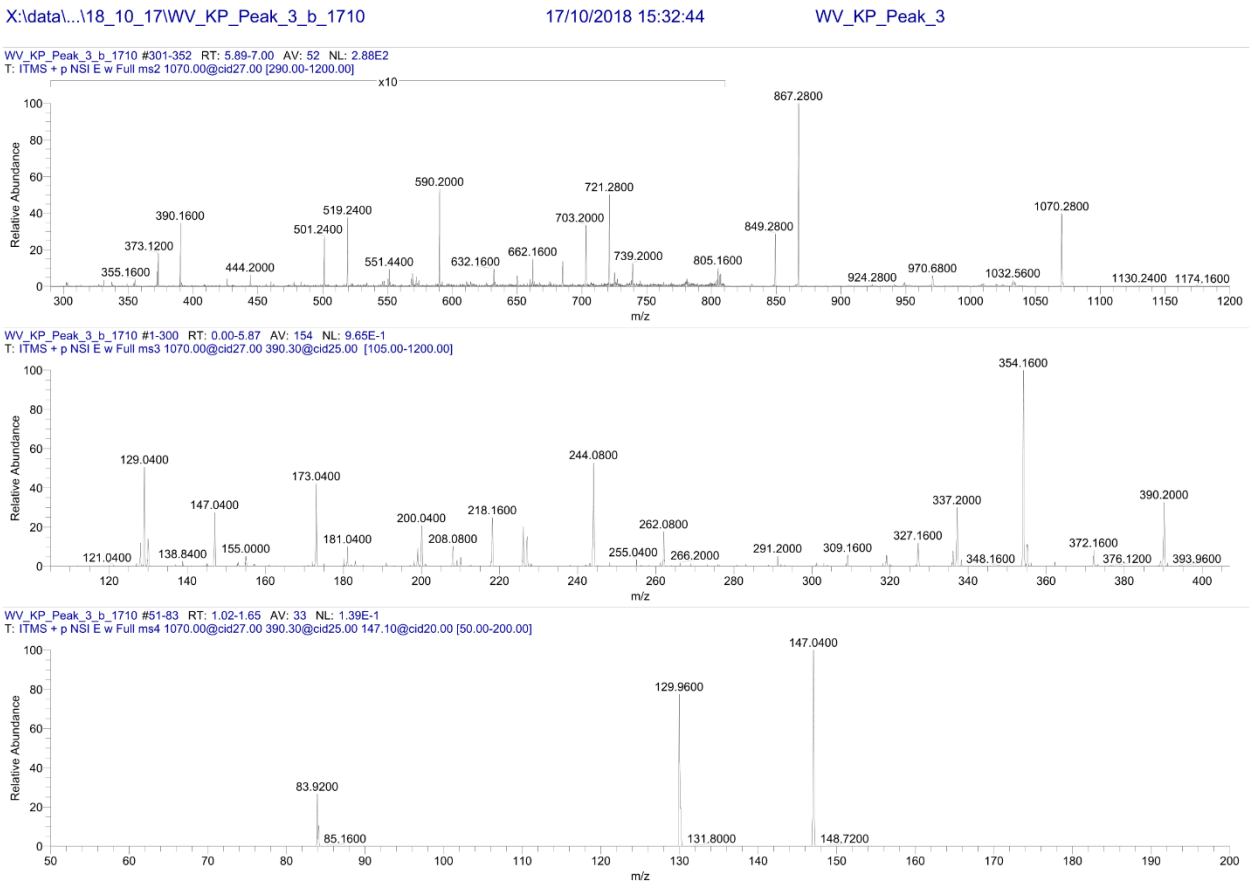

Fig. S8B. Mass spectrometry analysis of peak b (Tetra-D-Lys) as presented in Fig. 1

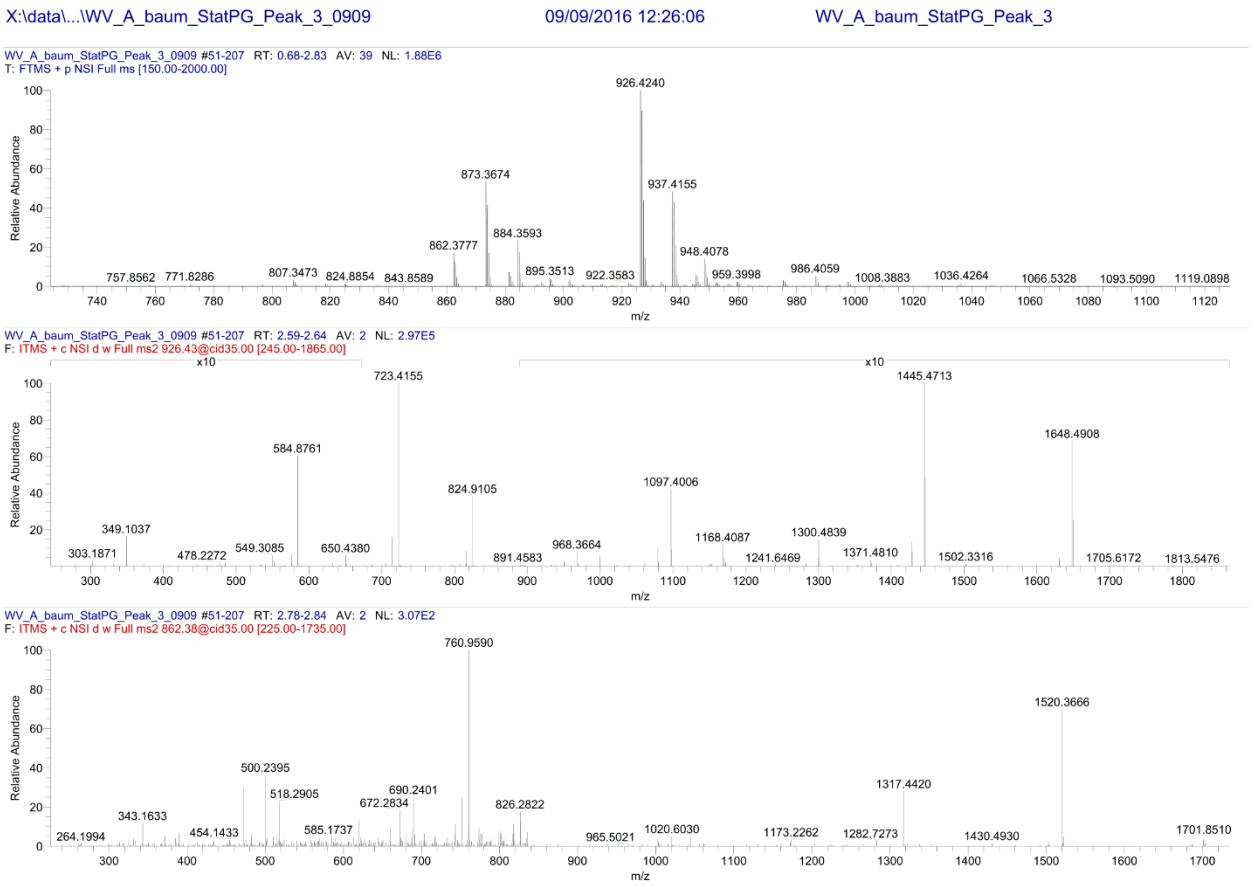

Fig. S8C. Mass spectrometry analysis of peak c (TriTri(Dap)-D-Lys) as presented in Fig. 1

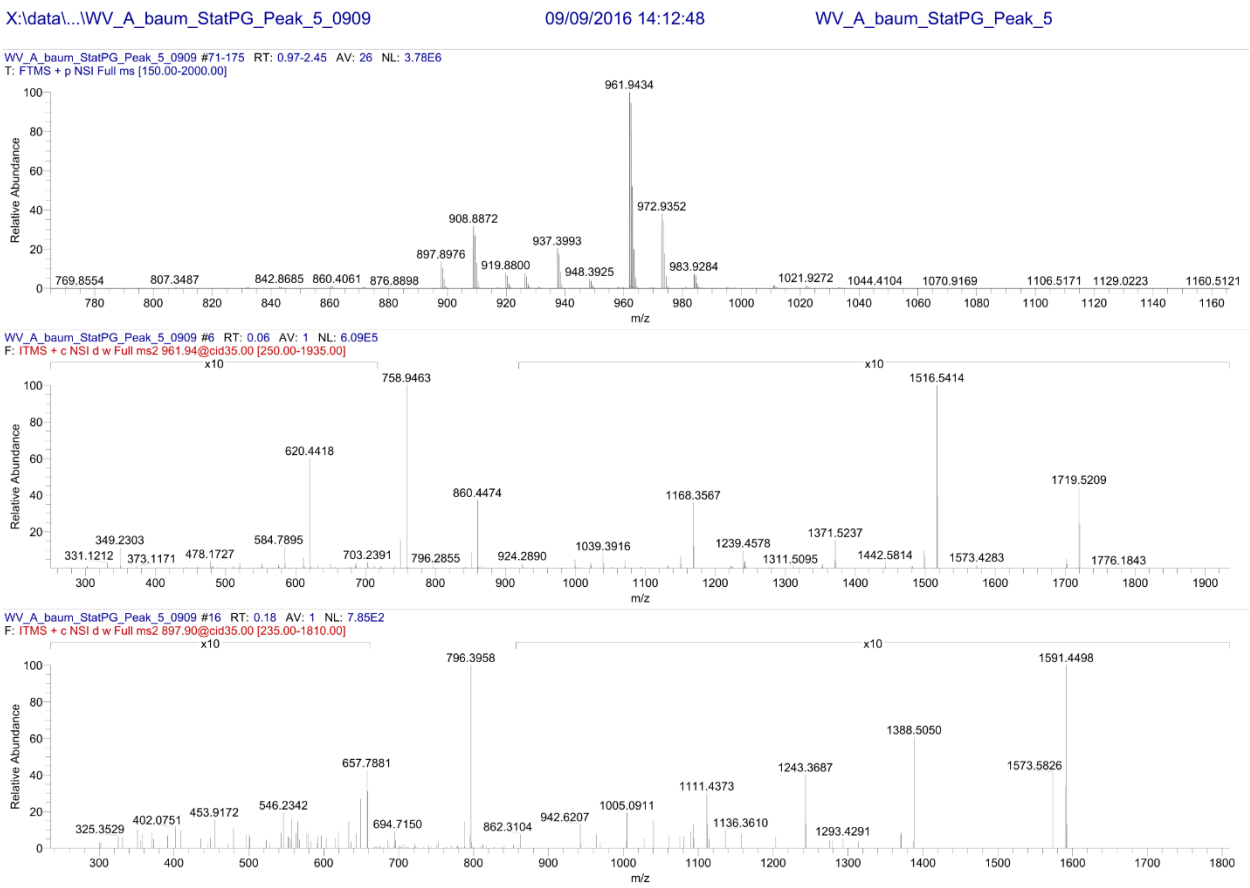

Fig. S8D. Mass spectrometry analysis of peak d (TetraTri-D-Lys) as presented in Fig. 1

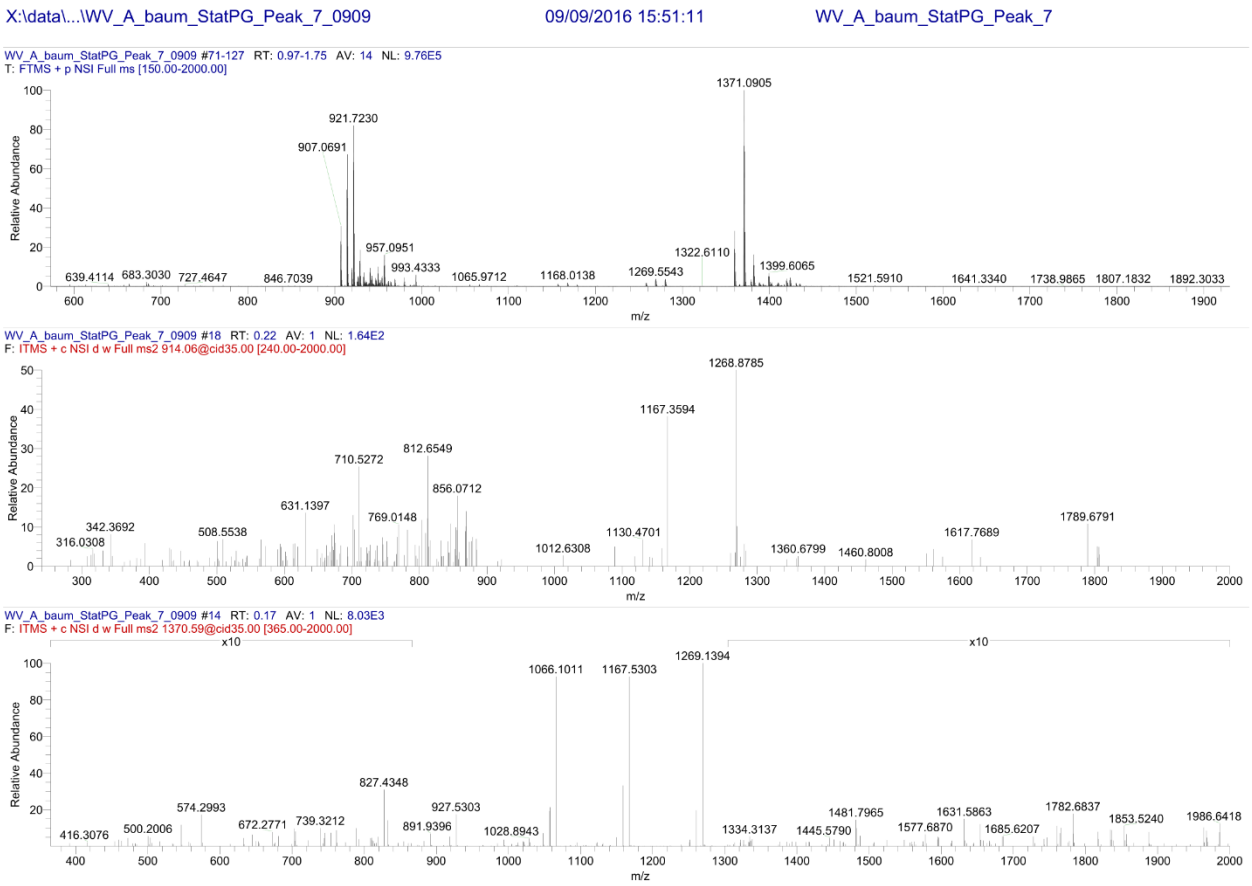

Fig. S8E. Mass spectrometry analysis of peak e (TetraTetraTri-D-Lys) as presented in Fig. 1

Table S1. Reduced D-Lys containing muropeptides from stationary *A. baumannii* 17978 cells in HPLC fractions detected by LTQ- FT MS. (1) Nomenclature of muropeptides according to Glauner (1988) is used. Muropeptides were assigned according to their retention times, which were identical to known, unmodified muropeptides from *E. coli*. Muropeptides were numbered according to Boll et al., 2016. D-Lys containing muropeptides (a-e) were confirmed by MS/MS-analysis. (2) MS analysis of peak 9 revealed a mixture of two muropeptides (~70% TetraTriAnh and ~30% TetraTetraTetraTri).

Label	Proposed structure(s)[1]	Retention time (min)	Theoretical neutral mass (Da)	Measured neutral mass (Da)
1	**Tri**	21.2		
a	**Tri-D-Lys**	24.4	998.4656	998.3662
b	**Tetra-D-Lys**	32.5	1069.5027	1069.4276
3	**Tetra**	33.3		
c	**TriTri(Dap)-D-Lys**	59.0	1850.8256	1850.8324
4	**TetraTri**	64.1		
d	**TetraTri-D-Lys**	66.4	1921.8627	1921.8712
5	**TetraTetra**	72.0		
6	**TetraTetraTri**	82.4		
e	**TetraTetraTri-D-Lys**	84.2	2845.2598	2845.7044
8	**TetraTetraTetra**	88.1		
9	**TetraTetraTetraTri/ TetraTriAnh[2]**	96.4		
10	**TetraTetraAnh I**	100.6		
11	**TetraTetraAnh II**	102.1		
11B	**TetraTetraTetraAnh**	112.2		

Table S2. Exponential and Stationary peptidoglycan composition of *A. baumannii* strains (Quantification of the chromatograms shown in Fig. 1A). (1) Muropeptides 1-11B are numbered according to Boll et al., 2016 and D-Lys containing muropeptides are labelled a-e. (2) The relative peak areas were estimated as percentage of all known peaks. (3) MS analysis of peak 9 revealed a composition of two muropeptides (~70% TetraTriAnh and ~30% TetraTetraTetraTri). (4) The chain length is given in disaccharide unit calculated from the % of anhydro groups in the compound.

		Relative peak area (%)[2]	
Label	**Muropeptide[1]**	**17978 expo.**	**17978 stat.**
1	**Tri**	3.0	2.1
a	**Tri-D-Lys**	2.1	10.5
b	**Tetra-D-Lys**	0.0	10.0
3	**Tetra**	19.6	10.7
c	**TriTri(Dap)-D-Lys**	1.0	3.3
4	**TetraTri**	6.1	4.3
d	**TetraTri-D-Lys**	0.9	8.1
5	**TetraTetra**	37.5	28.2
6	**TetraTetraTri**	1.6	1.5
e	**TetraTetraTri-D-Lys**	2.6	2.5
8	**TetraTetraTetra**	17.1	12.9
9	**TetraTriAnh (~70%)[3]**	3.2	2.3
9	**TetraTetraTetraTri (~30%)[3]**	1.4	1.0
10	**TetraTetraAnh I**	0.5	0.2
11	**TetraTetraAnh II**	0.7	0.5
11B	**TetraTetraTetraAnh**	2.8	1.9
Monomers		24.7	33.3
Dimers		49.8	47.0
Trimers		24.1	18.7
Tetramers		1.4	1.0
% peptides in crosslinkage		75.3	66.7
Average chain length[4]		31.9	46.1
D-Lys containing muropeptides		6.5	34.4

Table S3. Exponential and Stationary peptidoglycan composition of *E. coli* (Quantification of the chromatograms shown in Fig. S1). (1) Muropeptides 1-11B are numbered according to Boll et al., 2016 and D-Lys containing muropeptides are labelled a-e. (2) The relative peak areas were estimated as percentage of all known peaks.

		Relative peak area (%)[2]	
Label	**Muropeptide[1]**	**E. coli expo.**	**E. coli stat.**
1	**Tri**	9.15	6.72
3	**Tetra**	39.09	29.34
*****	**TetraTri(Dap)**	7.80	12.01
4	**TetraTri**	4.86	7.43
d	**TetraTri-D-Lys**	0.9	8.1
5	**TetraTetra**	31.17	34.15
6	**TetraTetraTri**	0.00	0.00
8	**TetraTetraTetra**	7.92	10.35
Monomers		48.24	36.06
Dimers		43.83	53.59
Trimers		7.92	10.35
% peptides in crosslinkage		51.76	63.94

Table S4. Stationary peptidoglycan composition of *A. baumannii* strains (Quantification of the chromatograms shown in Fig. 2C). (1) Muropeptides 1-11B are numbered according to Boll et al., 2016 and D-Lys containing muropeptides are labelled a-e. (2) The relative peak areas were estimated as percentage of all known peaks. (3) MS analysis of peak 9 revealed a composition of two muropeptides (~70% TetraTriAnh and ~30% TetraTetraTetraTri). (4) The chain length is given in disaccharide unit calculated from the % of anhydro groups in the compound.

		Relative peak area (%)[2]		
Label	**Muropeptide[1]**	**17978**	**17978 ΔRacK**	**17978 RacK+**
1	**Tri**	2.2	4.1	3.9
a	**Tri-D-Lys**	11.5	0.6	9.9
b	**Tetra-D-Lys**	10.4	0.3	5.4
3	**Tetra**	10.6	12.5	12.6
c	**TriTri(Dap)-D-Lys**	3.5	0.8	2.3
4	**TetraTri**	4.7	4.0	6.0
d	**TetraTri-D-Lys**	9.2	2.6	7.5
5	**TetraTetra**	21.3	30.7	26.4
6	**TetraTetraTri**	1.4	2.1	1.9
e	**TetraTetraTri-D-Lys**	4.8	2.1	3.5
8	**TetraTetraTetra**	11.1	23.0	12.1
9	**TetraTriAnh (~70%)[3]**	3.1	6.7	2.0
9	**TetraTetraTetraTri (~30%)[3]**	1.3	2.9	0.9
10	**TetraTetraAnh I**	0.8	3.0	1.6
11	**TetraTetraAnh II**	1.2	1.2	1.4
11B	**TetraTetraTetraAnh**	2.9	3.2	2.7
Monomers		34.7	17.6	31.8
Dimers		43.8	49.1	47.2
Trimers		20.2	30.5	20.1
Tetramers		1.3	2.9	0.9
% peptides in crosslinkage		65.3	82.4	68.2
Average chain length[4]		28.5	15.4	29.5
D-Lys containing muropeptides		39.4	6.5	28.5

Table S5. Bacterial strains and plasmids used in this study

Bacterial strains	**Description**
Ab17978	*Acinetobacter baumannii* strain ATCC17978 devoid of pAB3 plasmid. Spontaneous Rifampin resistant. (Weber et al.)
Ab17978 ΔRacK	Ab17978 *racK* (*acx60_11360*) deletion mutant. Spontaneous Rifampin resistant
Ab17978 ΔACX60_06570	Ab17978 *acx60_06570* deletion mutant
Ab17978 ΔACX60_06690	Ab17978 *acx60_06690* deletion mutant
Ab17978 ΔACX60_16210	Ab17978 *acx60_11210* deletion mutant
Ab17978 ΔACX60_00415	Ab17978 *acx60_00415* deletion mutant
Ab17978 RacK+	Ab17978 ΔRacK with pSH_RacK plasmid
UPAB1	*Acinetobacter baumannii* strain UPAB1 (Di venanzio et al., 2019)
UPAB1 RacK+	*Acinetobacter baumannii* strain UPAB1 with chromosomal insertion of racK and its 300 bp upstream region
Ab19606	*Acinetobacter baumannii* strain ATCC19606
M2	*Acinetobacter nocosomialis* strain M2 with pVRL2 plasmid. Gentamycin resistant
PAO1	*Pseudomonas aeruginosa* strain PAO1
BW25113	*Escherichia coli* strain BW25113
Serratia	*Serratia marcescens* strain 66262

Plasmids	**Description**
pET22b_AmaD	*amaD* gene from *Pseudomonas putida* cloned into pET22b+ plasmid
pBAD_RacK	*racK* gene from Ab17978 cloned into an in-house modifier version of the arabinose inducible vector pBAD
pUC-miniTn7-racK	*racK* with its 300 bp upstream region from Ab17978 cloned into a pUC18 vector
pVRL2	As described in Lucidi et al. 2018, Gentamycin resistant

Table S6. Primers used in this study

Primers	Sequence
AB RacK Fwd	ATAAAACAAAGTTTCGGATG
AB RacK Rev	TCCAGCCTACACAATCGCGAGTTTTTTAATCTTTCCTGG
CD RacK Fwd	TAAGGAGGATATTCATATGTAAACTTTAGGTGAATTGATAAG
CD RacK Rev	ATGCTGCGCATATTGTTCC
AB 06570 Fwd	ATTAGCTTAAGTGAGGCTTCG
AB 06570 Rev	TCCAGCCTACACAATCGCTATTTAAACCTTTTACTGGAC
CD 06570 Fwd	TAAGGAGGATATTCATATGTAAATACTCATCAACTTTTAATTTATTCTG
CD 06570 Rev	TTAGTTTAGGTCGTGTTGTCG
AB 06690 Fwd	TACTGAAGTTCGTGCCCGAGC
AB 06690 Rev	TCCAGCCTACACAATCGCCCTCATTTACTCCTTTGGGC
CD 06690 Fwd	TAAGGAGGATATTCATATGTAAAGTTTACTAAATAAAAAATCC
CD 06690 Rev	ATATCAGTTCAGATACCCAAG
AB 16210 Fwd	ATGGCATCCAAAGCATTGCGG
AB 16210 Rev	TCCAGCCTACACAATCGCGGCAGTACTAGAGAGTGTCGG
CD 16210 Fwd	TAAGGAGGATATTCATATGTAAGCTCTGTTGTAGGCATTCAG
CD 16210 Rev	AAGTTCTAAATCACGTGGGC
AB 00415 Fwd	ACAAACAACTTACGCCTAGAG
AB 00415 Rev	TCCAGCCTACACAATCGCGCTTTATCCTGTATTTCATAT
CD 00415 Fwd	TAAGGAGGATATTCATATGTAAAGCCAAGAAAAACGCCTAGCC
CD 00415 Rev	AAAATCTGGTCTAATGGCAGC
P1_kanR	AGCGATTGTGTAGGCTGGAGCTG
P2_kanR	CATATGAATATCCTCCTTAGTTCCTATTCCG
amaD Fwd	TAAGAAGGAGATATACATATGCATTGCCAGACCCTTGTC
amaD Rev	GTGGTGGTGGTGCTCGAGGTCGAAACGGGTCGCGCTGTA
pSH_racK Fwd	TAGCGGCCGCTGCAGGCCTATGAAACTTAAAACAAATTTTTC
pSH_racK Rev	TTAAGCGGCGGCATCGATCGTCAGTGGTGGTGGTGGTGGTGC
Prom_racK Fwd	ATGAGCTCACTAGTGGATCCAAAGTTTCGGATGTTTACCTC
Tn7_racK Rev	GAGGTACCGGGCCCAAGCTTTTACTCAGCGGTTGCTGCAAC
UPAB1_check Fwd	TAGAGCTATAAAAAGCCC
UPAB1_check Rev	TTGGCGAAGTCAGTAACTG