

1 **Multifactorial Chromosomal Variants Regulate Polymyxin Resistance in Extensively**
2 **Drug-Resistant *Klebsiella pneumoniae***

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5 **SUPPLEMENTARY FIGURES AND TABLES**

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9 **Table S1.** Oligonucleotides used in this study

Assay	Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Reference
PCR				
	<i>mgrB</i>	ATT CTG CCG CTT TTG CTG	CGT TTT GAA ACA AGT CGA TGA	24
Complementation				
	<i>mgrB</i>	TTA AGA AGG CCG TGC TAT CC	AAG GCG TTC ATT CTA CCA CC	48
	<i>phoP</i>	CAC CAG GGG CCC TTT TTA T	GCT AAC GCT ATA GCC CAC CA	This study
	<i>phoQ</i>	ATA CCC ACA GGA CGT CAT CA	CAG GTG TCT GAC AGG GAT TA	48
	<i>pmrB</i>	ACC TAC GCG AAA AGA TTG GC	GAT GAG GAT AGC GCC CAT GC	48

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11 **Table S2.** Broth microdilution values acquired for the 24 clinical isolates

Strain*	Resistance Profile [†]																							
	1		2	3	4		5	6		7	8	9	10	11	12	13	14	15	16		17			
	AMK	GEN	TOB	CPT	TZP	IPM	MEM	CFZ	FEP	CTX	CAZ	FOX	CIP	SXT	TGC	ATM	AMP	SAM	CHL	FOF	CST	PMB	MIN	TET
ATCC 25922	≤2	≤0.5	≤0.5	≤0.25	≤1/4	≤0.5	≤0.25	≤2	≤0.5	≤0.25	≤0.5	≤2	≤0.125	≤0.5/ ≤16	≤0.25	≤0.5	8	8/4	≤8	≥27	0.25	0.25	≤0.5	≤0.5
ATCC 700603	≤1	≤8	≤8	≤4	128/ 64	≤0.5	≤0.25	≥64	≤0.5	2	64	≤16	≤0.125	≤2/ 128	≥4	≥32	>64	>64/ ≥32	≥32	≤12	0.5	0.5	≥16	8
ATCC 13883	≤0.5	≤0.5	≤0.5	≤0.25	16/4	≤0.5	≤0.25	≤2	≤0.5	≤0.25	≤0.5	≤2	≤0.125	≤0.5/ ≤32	≤1	≤0.5	>64	>64/ ≥8	≤4	≤18	≤4	≤4	≤1	≤0.5
ATCC BAA-2146	>64	>64	>64	>32	>128/ >64	>32	>32	>64	>64	>32	>64	>64	>16	>64/ >128	≥4	>64	>64	>64/ ≥32	>64	≤20	≤0.25	≤0.25	≥64	>64
1_GR_13	>64	>64	>64	>32	>128/ >64	≥64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	≥2	>64	>64	>64/ >64	64	≤19	16	≥8	≥16	>64
2_GR_12	>64	>64	>64	>32	>128/ >64	≥64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤20	16	8	16	>64
3_GR_13	>64	>64	>64	>32	>128/ >64	≥16	≥8	>64	≥16	>32	>64	64	>16	>64/ 128	2	>64	>64	>64/ >64	≥32	≤22	16	8	≥8	>64
4_GR_12	>64	>64	>64	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	20	32	≥16	≥8	>64
5_GR_13	≤16	≤4	≥8	>32	>128/ >64	≥32	≥4	>64	>64	>32	>64	>64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤21	≥16	16	16	>64
6_GR_12	≤8	2	8	>32	>128/ >64	32	≥8	>64	≥64	>32	>64	>64	>16	>64/ 128	≥2	>64	>64	>64/ >64	>64	20	≤0.5	≤1	≤8	>64
7_GR_13	64	≤2	64	>32	>128/ >64	≥64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	≥2	>64	>64	>64/ >64	>64	19	>64	≥64	4	≤4
8_GR_13	>64	>64	>64	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	≥2	>64	>64	>64/ >64	>64	≤19	64	≥32	≥16	>64
9_GR_12	≥32	≤2	≥32	>32	>128/ >64	16	≥4	>64	>64	>32	>64	64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤8	≥16	≥8	≤8	≤4
10_GR_13	16	≤1	≥16	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤20	64	≥32	≤4	≤4
11_BR_13	≤16	≤0.5	≥16	>32	>128/ >64	>64	>32	>64	≥64	>32	≥64	>64	>16	>64/ 128	≤2	>64	>64	>64/ >64	8	≤15	≥64	64	≤4	≤2
12_BR_13	≤1	>64	≥8	>32	>128/ >64	≥64	>32	>64	>64	>32	≥64	>64	>16	>64/ 128	≤2	>64	>64	>64/ >64	>64	23	>64	>64	≤8	≤2
13_GR_14	≤32	≤0.5	≥16	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	≤1	>64	>64	>64/ >64	>64	20	16	16	≤1	≤0.5
14_GR_14	≤32	≤0.5	≥16	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	≤1	>64	>64	>64/ >64	>64	20	≥32	≥16	≤2	≤1
15_GR_13	≤32	≤1	≥16	>32	>128/ >64	32	≥8	>64	≥64	>32	>64	64	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤22	64	≥32	≤4	1

16_GR_13	>64	>64	>64	>32	>128/ >64	≥8	≥16	>64	>64	>32	>64	64	>16	>64/ 128	≥2	>64	>64	>64/ >64	32	≤19	64	64	≤8	>64
17_GR_14	>64	>64	>64	>32	>128/ >64	≥4	≥4	>64	>64	>32	>64	32	>16	>64/ 128	2	>64	>64	>64/ >64	≥16	≤20	64	32	4	>64
18_GR_14	≤32	≤0.5	≥16	>32	>128/ >64	>64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	2	>64	>64	>64/ >64	≥32	≤20	64	64	2	≤2
19_GR_14	≤32	≤0.5	≥16	>32	>128/ >64	≥16	≥8	>64	>64	>32	>64	>64	>16	>64/ 128	≥4	>64	>64	>64/ >64	64	21	64	64	≥8	8
20_GR_12	64	1	64	>32	>128/ >64	≥8	≥8	>64	≥16	>32	>64	64	>16	>64/ 128	≥2	>64	>64	>64/ >64	≥32	≤22	0.125	≤0.25	16	>64
21_GR_13	≤4	≤4	≥8	>32	>128/ >64	8	≤2	>64	≥16	>32	>64	>64	0.25	>64/ 128	1	≤4	>64	>64/ >64	≤4	≥24	≤0.25	0.125	≤1	≤0.5
22_GR_12	≤32	≤1	≥16	>32	>128/ >64	≥16	≥8	>64	≥16	>32	>64	64	>16	>64/ 128	≥1	>64	>64	>64/ >64	32	20	≤0.25	≤0.25	≤4	1
23_GR_12	>64	>64	>64	>32	>128/ >64	≥64	>32	>64	>64	>32	>64	>64	>16	>64/ 128	8	>64	>64	>64/ >64	>64	≤20	≥8	≥4	>64	>64
24_GR_13	≤32	≤1	≥16	>32	>128/ >64	≥8	≥4	>64	≥16	>32	>64	≥32	>16	>64/ 128	2	>64	>64	>64/ >64	>64	≤23	≤0.25	≤0.25	≤8	2

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14 *Strain identification, numerical order catalogued at IMB_Country (GR:Greece, BR:Brazil)_last two digits of isolation year.

15 †Antibiotic resistance as determined by broth microdilution according to CLSI guidelines except fosfomycin (disk diffusion) and tigecycline which

16 followed EUCAST breakpoints. Antibiotic classes tested include **1**, Aminoglycosides (Amikacin, AMK; Gentamicin, GEN; Tobramycin, TOB);

17 **2**, Anti-MRSA cephalosporins (Ceftaroline, CPT); **3**, Antipseudomonal penicillins + β-lactamase inhibitors (Piperacillin-tazobactam, TZP); **4**,

18 Carbapenems (Imipenem, IPM; Meropenem, MEM); **5**, Non-extended spectrum cephalosporins (1st and 2nd generation) (Cefazolin, CFZ); **6**,

19 Extended-spectrum cephalosporins (3rd and 4th generation) (Cefepime, FEP; Cefotaxime, CTX, Ceftazidime, CAZ); **7**, Cephamycins (Cefoxitin,

20 FOX); **8**, Fluoroquinolones (Ciprofloxacin, CIP); **9**, Folate pathway inhibitors (Trimethoprim-sulfamethoxazole, SXT); **10**, Glycylcyclines

21 (Tigecycline, TGC); **11**, Monobactams (Aztreonam, ATM); **12**, Penicillins (Ampicillin, AMP); **13**, Penicillins + β-lactamase inhibitors

22 (Amipicillin-sulbactam, SAM); **14**, Phenicol (Chloramphenicol, CHL); **15**, Phosphonic acids (Fosfomycin, FOF); **16**, Polymyxins (Colistin, CST);

23 Polymyxin B, PMB); 17, Tetracyclines (Minocycline, MIN; Tetracycline, TET). The two antibiotics for TZP, SXT and SAM were assayed
24 separately and both MICs separated with a /. Shading indicates R, Resistant; I, Intermediate; S, Susceptible.

25 **Table S3.** Non-synonomous changes relative to 20_GR_12 and PROVEAN prediction

Strain *	Polymyxin Profile [†]	Gene	Variant [‡]	PROVEAN score [§]
ATCC 700603	S	<i>pmrA</i>	S64A, N131D, L140Q, E199D, N219H	-
		<i>pmrB</i>	T8N, N105S, A228T, Q232E, I242V, N244S, G256R, E272Q, Q356R	-
		<i>phoP</i>	R34K	-
		<i>phoQ</i>	Q92K, A106T, E112D, I139V, L163F, V196I, T372S, Q424P, Q482L, Q487E	-
1_GR_13 ^Δ	R	<i>pmrB</i>	T140P	-4.39
2_GR_12 ^Δ	R	<i>phoP</i>	A95S	-2.97
		<i>phoQ</i>	N253T	-3.86
3_GR_13	R	<i>mgrB</i>	D29E	-3.97
4_GR_12 ^Δ	R	<i>phoP</i>	P74L	-9.98
		<i>phoQ</i>	N253T	-3.86
5_GR_13	R	<i>mgrB</i>	Q30STOP	-14.86
		<i>pmrA</i>	N131D, G144D, D149E, N219H	-
		<i>pmrB</i>	A5V, N105S, M175V, A228T, G256R	-
6_GR_12	S	<i>pmrA</i>	N131D, G144D, D149E, N219H	-
		<i>pmrB</i>	A5V, N105S, M175V, A228T, G256R	-
8 GR_13	R	<i>phoQ</i>	G385C	-8.06
9 GR_12	R	<i>phoQ</i>	T281M	-5.36
10 GR_13 ^Δ	R	<i>phoQ</i>	A225T, N253T	-, -3.86
13_GR_14 ^Δ	R	<i>pmrB</i>	P158R (66)	-8.68
		<i>phoQ</i>	V446G (65)	-5.46
14_GR_14 ^Δ	R	<i>pmrB</i>	P158R (57)	-8.68
		<i>phoQ</i>	V446G (52)	-5.46
19 GR_14	R	<i>mgrB</i>	C28STOP	-19.00
21_GR_13	S	<i>pmrA</i>	S64A, N131D, L140Q, E199D, N219H, I220N, D221E	-
		<i>pmrB</i>	T8N, N105S, A228T, Q232E, I242V, N244S, G256R, E272Q, Q356R, G358A	-
		<i>phoP</i>	R34K	-
		<i>phoQ</i>	Q92K, A106T, E112D, I139V, L163F, V196I, T372S, Q424P, Q482L, Q487E	-
23_GR_12 ^Δ	R	<i>phoP</i>	A95S (57)	-2.97
		<i>phoQ</i>	N253T (57)	-3.86

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27 *Strains also harboring an IS element disruption in *mgrB* shown as, ^Δ.

28 [†]S, Susceptible (MIC: ≤2 μg/ml); R, Resistant (MIC: >2 μg/ml).

29 [‡]Variant represented as initial amino acid, position and new amino acid; () corresponds to
30 percentage of reads mapping to alteration if ≤98%.

31 [§]PROVEAN predicted deleterious score which is set to ≤-2.5; -, value not predicted deleterious.

32 **Table S4.** Non-synonymous mutations relative to ATCC 700603

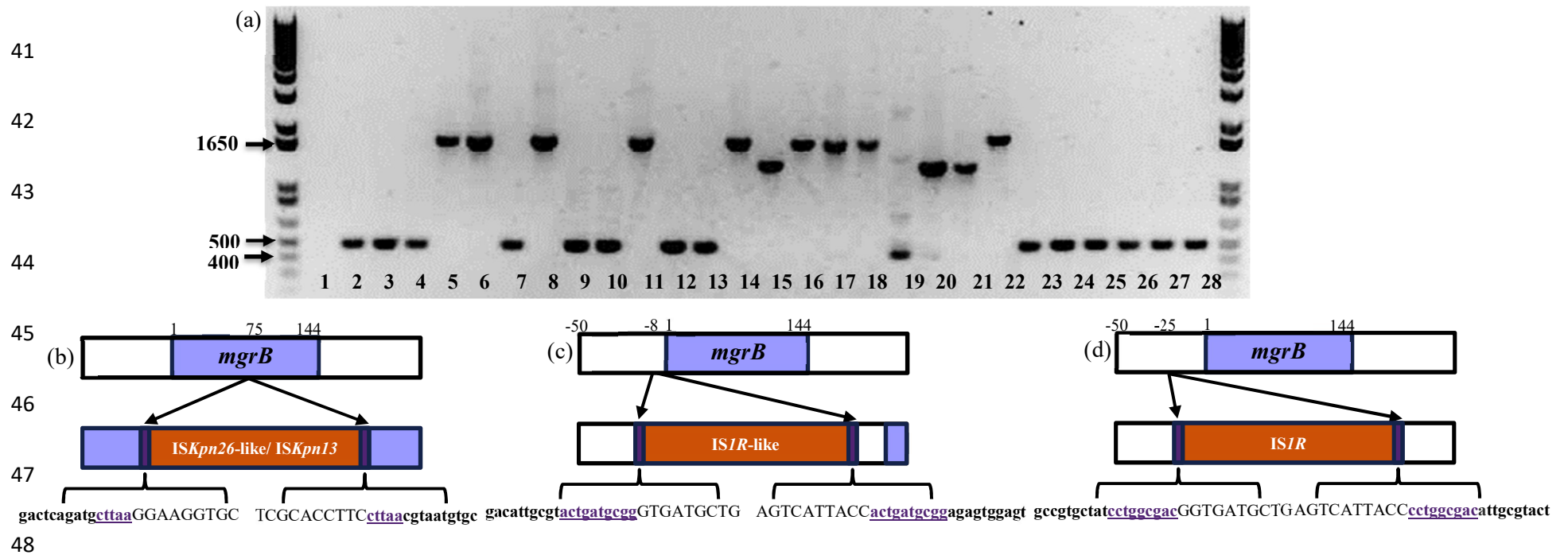
33

MLST*	Gene [†]				
	<i>mgrB</i>	<i>phoP</i>	<i>phoQ</i>	<i>pmrA</i>	<i>pmrB</i>
11 (3)	-	K34R	K64R, K92R, T106A, D112E, V139E, F163L, I196V, S372T, P424Q, L482Q, E487Q	A64S, D131N, Q140L , D199E, H219N	N8T, S105N, T228A, E232Q, V242I, S244N, R256G , Q272E, R356Q,
147 (1)	-	K34R	K64R, K92R, T106A, D112E, V139E, F163L, I196V, S372T, P424Q, L482Q, E487Q	A64S, D131N, Q140L , D199E, H219N	N8T, S105N, T140P , T228A, E232Q, V242I, S244N, R256G , Q272E, R356Q,
258 (16)	C28STOP , D29E	K34R, P74L , A95S	K64R, K92R, T106A, D112E, V139E, F163L, I196V, A225T , N253T , T281M , G385C S372T, P424Q, V446G , L482Q, E487Q	A64S, D131N, Q140L , D199E, H219N	N8T, S105N, P158R , T228A, E232Q, V242I, S244N, R256G , Q272E, R356Q
383 (2)	Q30STOP	K34R	K64R, K92R, T106A, D112E, V139E, F163L, I196V, S372T, P424Q, L482Q, E487Q	A64S, Q140L , G144D, D149E, D199E	A5V, N8T, M175V, E232Q, V242I, S244N, Q272E, R356Q
437 (1)	-	K34R	K64R, K92R, T106A, D112E, V139E, F163L, I196V, S372T, P424Q, L482Q, E487Q	A64S, D131N, Q140L , D199E, H219N	N8T, S105N, T228A, E232Q, V242I, S244N, R256G , Q272E, R356Q
2401 (1)	-	-	-	I220N, D221E	G358A

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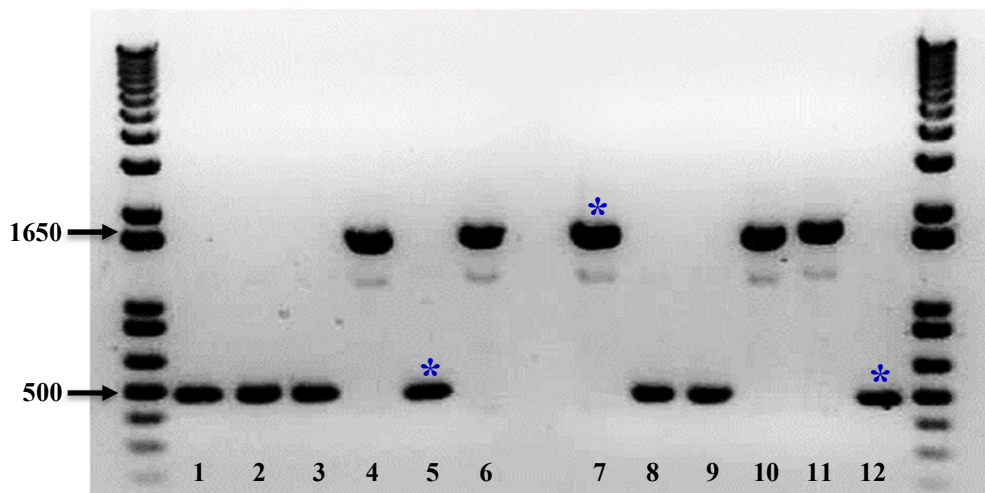
35 *Multi-locus sequence type and in brackets, the number of isolates in this group.

36 †Genes *mgrB*, *pmrAB* and *phoPQ* were aligned to ATCC 700603 and represented as initial
37 amino acid, position and new amino acid. Bold indicates a predicted deleterious variant in
38 polymyxin-resistant strains according to PROVEAN analysis and an underline identifies this
39 change also being present in polymyxin-susceptible isolates. Strains impacted by an IS element
40 disruption in *mgrB* only had flanking regions interrogated to detect variants.

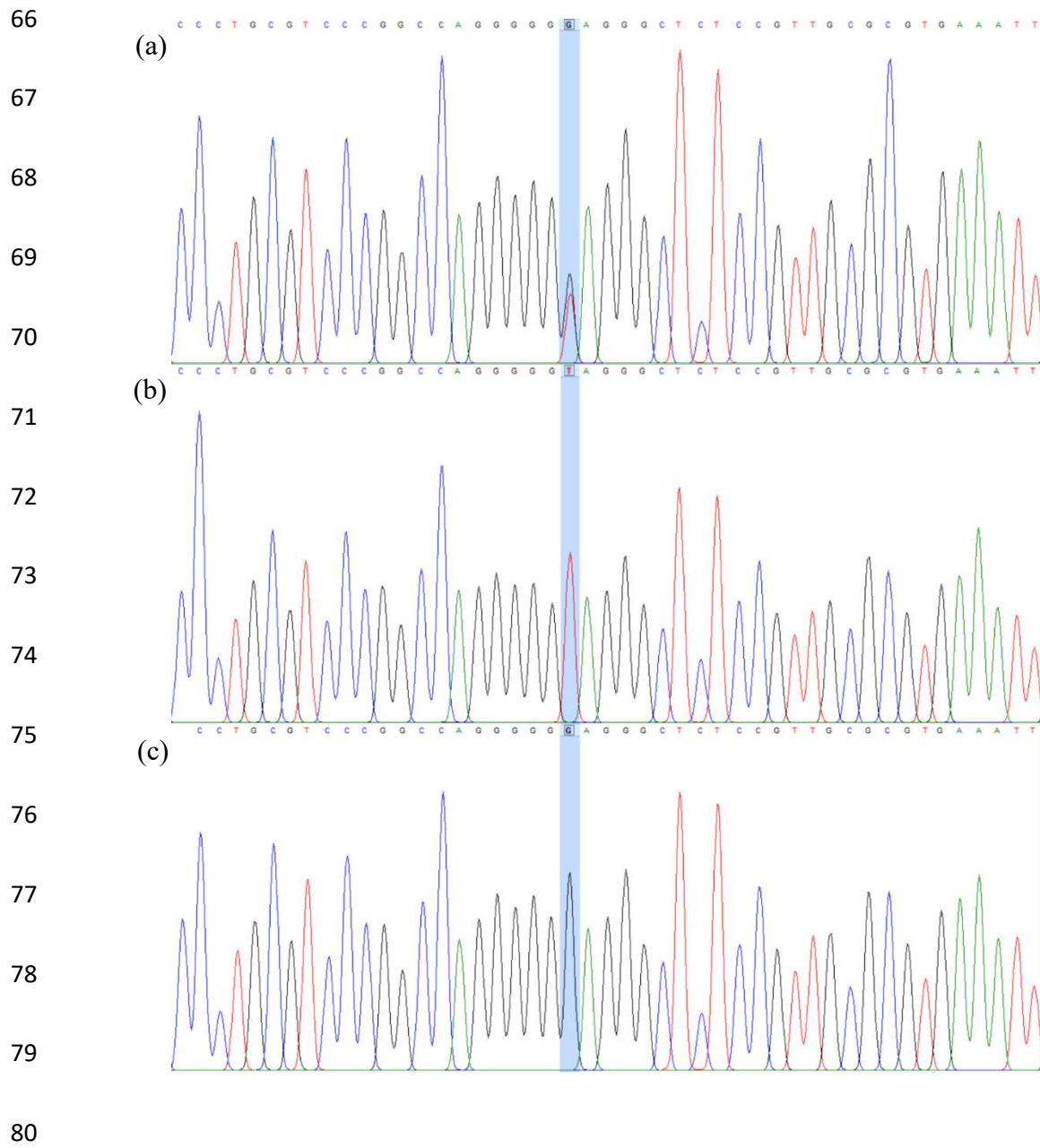


49 **Fig. S1.** Amplification of *mgrB*. (a) PCR products of 1, ATCC 25922 (*E. coli* negative control); 2, ATCC 700603; 3, ATCC 13883; 4, ATCC BAA-2146; 5,
 50 1_GR_13; 6, 2_GR_12; 7, 3_GR_13; 8, 4_GR_12; 9, 5_GR_13; 10, 6_GR_12; 11, 7_GR_13; 12, 8_GR_13; 13, 9_GR_12; 14, 10_GR_13; 15, 11_BR_13; 16,
 51 12_BR_13; 17, 13_GR_14; 18, 14_GR_14; 19, 15_GR_13; 20, 16_GR_13; 21, 17_GR_14; 22, 18_GR_14; 23, 19_GR_14; 24, 20_GR_12; 25, 21_GR_13; 26,
 52 22_GR_12; 27, 23_GR_12; 28, 24_GR_13. (b) IS5-like (*ISKpn26*-like or *ISKpn13* for 12_BR_13) element producing 1700 bp amplicon. (c) *ISIR*-like element
 53 resulting in 1268 bp amplicon in 16_GR_13 and 17_GR_14 (d) *ISIR* element in 11_BR_13 resulting in 1268 bp amplicon.

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61 **Fig. S2.** Amplification of chromosomal *mcrB* for complemented 13_GR_14 (lanes 1 to 6) and
62 14_GR_14 (lanes 7 to 12). Complementation via pTOPO-*mcrB* is shown in lanes 1 to 3 and 7 to 9 and
63 for pTOPO-*pmrB*, lanes 4 to 6 and 10 to 12. Three colonies were tested per treatment group and
64 reversion to a polymyxin-susceptible phenotype is indicated by *. A 500 bp amplicon represents an
65 intact *mcrB* whilst a 1700 bp amplicon is the result of the IS*Kpn26*-like insertion in *mcrB*.



81 **Fig. S3.** Sanger sequencing traces of amplified *phoQ* from (a) DNA extraction of 14_GR_14 used for
 82 whole genome sequencing. (b) complementation of 14_GR_14 where susceptibility was recovered by
 83 pTOPO-*mgrB*. (c) complementation of 14_GR_14 where susceptibility was recovered by pTOPO-
 84 *pmrB*. WT sequence contains **T** at nucleotide position 1337 (shaded) and mutation of **G** causing V446G
 85 was isolated in condition (c).