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Supplementary Materials for

Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants

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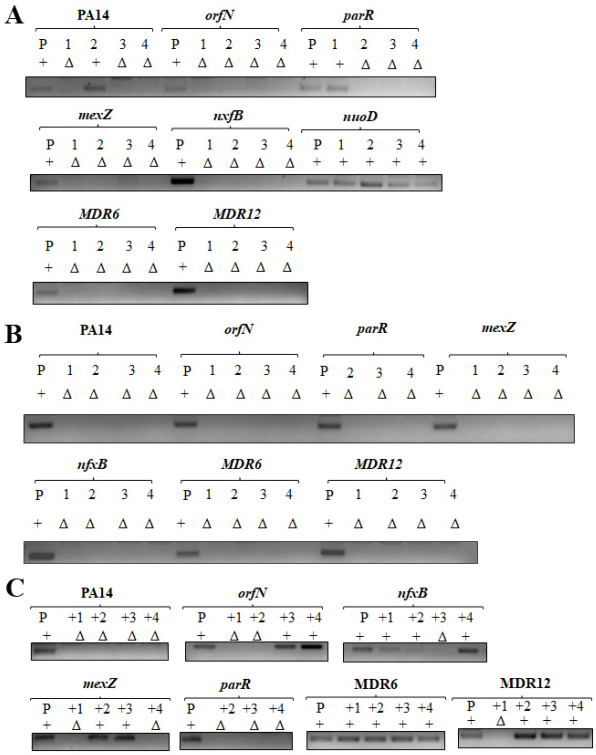
Supplementary information



Supplementary Figure S1. *mexXY* genotype of *P. aeruginosa* populations or individual clones analysed during this work. Presence or absence of chromosomal deletions was determined by absence (Δ) or presence (+) of a 163 bp PCR fragment corresponding to *mexXY* in 2% agarose gel. Primers used for *mexXY* genotyping are included in Supplementary Table S6. **(A)** Chromosomal deletions (including *mexXY*) are selected at first stages of ceftazidime evolution in most of the *P. aeruginosa* genetic backgrounds analysed. **(B)** *mexXY* genotype of pyomelanogenic clones isolated from different genetic backgrounds of *P. aeruginosa* after ceftazidime short-term evolution. **(C)** *mexXY* genotype of heterogeneous pyomelanogenic populations from different genetic backgrounds of *P. aeruginosa* after tobramycin/ceftazidime sequential evolution.

Supplementary Table S1 | *P. aeruginosa* PA14 mutants used in this work.

Type of mutant	Mutant	Protein (aa change)	Antibiotic selection	Reference
Single	*nfxB177*	NfxB (Phe177Ser)	TGC	(*16*)
	parR87	ParR (Glu87Lys)	TGC	(*36*)
	mexZ43	MexZ (Val43Gly)	TOB	(*16*)
	orfN50	OrfN (Val50fs)	TGC TOB	(*16, 36*)
	nuoD184	NuoD (Gln184*)	TOB	(*16*)
Multiple	MDR6	NfxB (Leu10fs), PhoQ (Val260Gly), Frr (Ile98Ser), PmrB (Leu87Gln)	TGC	(*16*)
	MDR12	FusA (Tyr552Cys; Tyr683Cys), OrfN (Val50fs), PmrB (Met46Ile), MexZ (Val43Gly), Gabp (Ile263Ser266del), PtsP (Leu537Pro), NuoD (Gln184*)	TOB	(*16*)

Amino acid (aa) location of the mutations. Fs: frameshift. Del: deletion. *: stop. TGC: tigecycline. TOB: tobramycin.

Supplementary Table S2 | MICs (µg/ml) of ceftazidime and tobramycin for *P. aeruginosa* populations after short-term evolution in the presence or absence of ceftazidime.

	Parental strain	Replicate 1		Replicate 2		Replicate 3		Replicate 4	
		CAZ	TOB	CAZ	TOB	CAZ	TOB	CAZ	TOB
CAZ (+)	**PA14** [a, b] *	8	0.5	12	0.25	8	0.5	24	0.5
	nfxB177 [a, b] *	6	0.38	8	0.38	6	0.5	8	0.38
	parR87 [a, b]	6	4	>256	0.5	>256	0.5	>256	0.5
	orfN50 [a, b] **	24	0.38	24	0.75	24	0.38	12	0.5
	nuoD184 [a, b]	12	1.5	8	2	8	3	8	1.5
	mexZ43 [a, b] **	6	0.25	48	0.5	6	0.38	3	0.5
	MDR6 [a, b] **	12	0.38	8	0.5	8	0.5	12	0.38
	MDR12 [a, b] **	48	3	48	3	6	3	48	3
CAZ (-)	**PA14** [a, b]	1	1.5	1	1.5	0.75	1.5	1	1.5
	nfxB177 [a, b]	1	1	1.5	1	1	1	1	1
	parR87 [a, b]	1	1.5	1	1.5	1	1.5	1	1.5
	orfN50 [a, b]	3	3	3	3	3	3	3	2
	nuoD184 [a, b]	1.5	2	1.5	2	1	2	1.5	1.5
	mexZ43 [a, b]	1	1.5	1	1.5	1	1.5	1	1.5
	MDR6 [a, b]	0.75	1.5	1	1.5	0.75	2	0.75	1.5
	MDR12 [a, b]	0.75	48	1	32	1	32	1	32

CAZ: ceftazidime. TOB: tobramycin.

Four different replicates of each genetic background were submitted to short-term evolution in the presence (+) or absence (-) of ceftazidime and the tobramycin MIC values measured. Asterisks indicate genetic backgrounds showing a significant change

of tobramycin MIC after ceftazidime treatment according to a bilateral Dunnet's *post hoc* test using the $\log_2$ of fold change with respect to each parental strain (* $P < 0.01$, ** $P < 0.001$).

[a] CAZ MIC (μg/ml) of parental strains PA14, 1; *nfxB177*, 1.5; *parR87*, 1; *orfN50*, 3; *nuoD184*, 1; *mexZ43*, 1; MDR6, 1.5; MDR12, 1.

[b] TOB MIC (μg/ml) of parental strains PA14, 1; *nfxB177*, 1; *parR87*, 1.5; *orfN50*, 3; *nuoD184*, 2; *mexZ43*, 1.5; MDR6, 2; MDR12, 32.

Supplementary Table S3 | MICs (μg/ml) of ceftazidime and tobramycin of pyomelanogenic clones isolated from *P. aeruginosa* populations after ceftazidime short-term evolution.

	Clone 1		Clone 2		Clone 3		Clone 4	
	CAZ	**TOB**	**CAZ**	**TOB**	**CAZ**	**TOB**	**CAZ**	**TOB**
PA14 [a, b] **	8	0.38	8	0.38	8	0.25	8	0.38
nfxB177 [a, b] **	8	0.5	8	0.5	8	0.5	8	0.5
parR87 [a, b] **	-	-	>256	0.5	>256	0.38	>256	0.38
orfN50 [a, b] **	24	0.5	24	0.5	12	0.38	16	0.5
mexZ43 [a, b] **	8	0.38	8	0.38	6	0.38	4	0.38
MDR6 [a, b] **	12	0.5	12	0.5	8	0.75	12	0.5
MDR12 [a, b] **	8	1.5	6	2	4	3	6	2

CAZ: ceftazidime. TOB: tobramycin.

Asterisks indicate pyomelanogenic genetic backgrounds showing a significant change in tobramycin MIC with respect to their parental strain according to Dunnet's bilateral *post hoc* test (** $P < 0.001$).

[a] CAZ MIC (μg/ml) of parental strains PA14, 1; *nfxB177*, 1.5; *parR87*, 1; *orfN50*, 3; *mexZ43*, 1; MDR6, 1.5; MDR12, 1.

[b] TOB MIC (μg/ml) of parental strains PA14, 1; *nfxB177*, 1; *parR87*, 1.5; *orfN50*, 3; *mexZ43*, 1.5; MDR6, 2; MDR12, 32.

Supplementary Table S4 | MICs (µg/ml) of ceftazidime and tobramycin for heterogeneous pyomelanogenic populations of *P. aeruginosa* after tobramycin and ceftazidime sequential evolution.

		+1 [b]		+2 [b]		+3 [b]		+4 [b]	
		CAZ	**TOB**	**CAZ**	**TOB**	**CAZ**	**TOB**	**CAZ**	**TOB**
First step (TOB)	**PA14** [a, c]	1	32	0.75	6	2	24	1.5	16
	nfxB177 [a, c]	0.5	4	0.75	48	1	4	1	16
	parR87 [a, c]	-	-	1	16	1	16	1	32
	orfN50 [a, c]	3	48	2	64	2	64	1.5	8
	mexZ43 [a, c]	1	32	1	16	1	24	0.75	24
	MDR6 [a , c]	0.75	8	2	8	2	4	1.5	8
	MDR12 [a, c]	0.75	32	2	64	2	96	1.5	96
Second step (CAZ)	**PA14 ****	6	0.25	4	1	16	0.38	8	0.75
	nfxB177 *	3	0.75	4	1	6	0.75	8	1
	parR87 *	-	-	8	2	4	0.75	6	2
	orfN50 *	6	0.5	6	1	4	12	48	1.5
	mexZ43*	8	0.75	4	1.5	4	4	6	0.75
	MDR6	6	6	16	2	8	2	4	2
	MDR12	6	3	16	48	32	96	12	64

TOB: tobramycin. CAZ: ceftazidime.

Heterogeneous pyomelanogenic populations (dubbed +1, +2, +3 and +4) were constructed by mixing each of the 27 pyomelanogenic clones with its parental strain, in a 1:1 ratio. Extinction of pyomelanogenic populations after first step on tobramycin was determined by comparing the CAZ MIC value of each heterogeneous population with the ones of parental strains and pyomelanogenic clones. Asterisks indicate populations

showing a significant difference in tobramycin MIC between the first and second step of sequential evolution according to Dunnett's *post hoc* test (**P* < 0.01; ***P* < 0.001).

[a] CAZ MIC (µg/ml) of parental strains: PA14, 1; *nfxB177*, 1.5; *parR87*, 1; *orfN50*, 3; *mexZ43*, 1; MDR6, 1.5; MDR12, 1.

[b] CAZ MIC (µg/ml) of pyomelanogenic clones: PA14 (from 1 to 4), 8; *nfxB177* (from 1 to 4), 8; *parR87* (from 2 to 4), >256; *orfN50* (1, 2, 3 and 4), 24, 24, 12 and 16, respectively; *mexZ43* (1, 2, 3 and 4), 8, 8, 6 and 4, respectively; MDR6 (1, 2, 3 and 4), 12, 12, 8 and 12, respectively; MDR12 (1, 2, 3 and 4), 8, 6, 4 and 6, respectively.

[c] TOB MIC (µg/ml) of parental strains PA14, 1; *nfxB177*, 1; *parR87*, 1.5; *orfN50*, 3; *mexZ43*, 1.5; MDR6, 2; MDR12, 32.

Supplementary Table S5 | MICs (µg/ml) of antibiotics belonging to different structural families in the final populations of *P. aeruginosa* PA14 after tobramycin/ceftazidime sequential evolution.

	TGC	TET	CIP	LEV	CAZ	ATM	IPM	FOF	ERY	TOB	AMK	CHL
PA14	6	64	0.094	0.25	1	2	0.75	48	64	1	4	64
PA14+1	8	64	0.5	1.5	6	96	1.5	6	>256	0.25	0.75	>256
PA14+2	2	12	0.125	0.25	4	8	4	6	>256	1	4	>256
PA14+3	8	64	0.25	1	16	32	0.75	8	>256	0.38	1	>256
PA14+4	2	12	0.125	0.25	8	12	12	6	>256	0.75	2	>256
parR87	8	24	0.125	0.5	1	3	2	32	>256	1.5	8	64
***parR87*+2**	3	12	0.19	0.38	8	12	16	4	>256	2	12	192
***parR87*+3**	12	12	0.19	0.5	4	48	16	12	>256	0.75	2	>256
***parR87*+4**	2	12	0.125	0.5	6	12	24	4	>256	2	8	>256
orfN50	32	48	0.19	0.38	3	8	2	8	>256	3	12	>256
***orfN50*+1**	3	12	0.094	0.38	6	12	3	8	>256	0.5	6	>256
***orfN50*+2**	3	8	0.094	0.25	6	16	6	3	>256	1	4	>256
***orfN50*+3**	48	48	0.25	0.38	4	8	1	8	>256	12	192	>256

***orfN50*+4**	32	48	0.19	0.75	48	48	1.5	8	>256	1.5	16	>256
nfxB177	32	48	3	4	1.5	2	1	32	>256	1	2	>256
***nfxB177*+1**	32	48	3	4	3	12	1	48	>256	0.75	3	>256
***nfxB177*+2**	16	24	0.125	0.38	4	16	8	32	>256	1	8	>256
***nfxB177*+3**	16	24	2	4	6	16	16	12	>256	0.75	4	>256
***nfxB177*+4**	48	48	0.25	0.5	8	24	12	6	>256	1	32	>256
mexZ43	8	24	0.38	0.5	1	3	1.5	32	>256	1.5	8	48
***mexZ43*+1**	4	24	0.38	1	8	32	8	8	>256	0.75	1.5	>256
***mexZ43*+2**	8	24	0.38	0.75	4	2	8	8	>256	1.5	>256	>256
***mexZ43*+3**	96	48	0.75	2	4	16	2	24	>256	4	16	>256
***mexZ43*+4**	4	24	0.25	1	6	32	24	8	>256	0.75	2	>256
MDR6	48	32	0.19	0.38	1.5	1.5	1.5	24	>256	2	8	48
MDR6+1	32	32	0.125	0.38	6	8	1	8	>256	6	48	>256
MDR6+2	96	48	0.125	0.38	16	8	4	6	>256	2	16	>256
MDR6+3	48	32	0.25	0.5	8	12	16	4	>256	2	16	>256
MDR6+4	128	64	0.125	0.38	4	6	2	12	>256	2	16	>256

MDR12	64	24	0.5	1	1	2	1.5	32	>256	32	>256	48
MDR12+1	2	6	0.094	0.38	6	12	4	16	>256	3	8	>256
MDR12+2	>256	48	0.38	1.5	16	12	3	12	>256	48	>256	>256
MDR12+3	>256	48	0.75	1.5	32	32	3	12	>256	96	>256	>256
MDR12+4	>256	48	0.5	1.5	12	12	3	12	>256	64	>256	>256

Post-hoc tests using Fisher's exact test with Hochberg correction for multiple comparisons showed that the observed trends towards sensitivity or resistance were significant in all cases ($P < 0.0001$), except for erythromycin (P=0.8841). Among the significance levels for antibiotics showing a predominant MIC reduction, fosfomycin presented the higher significance (P=0.0000000023), followed by tobramycin (P =0.0000003482) and tetracycline (P= 0.0000630420).

TGC: tigecycline, TET: tetracycline, CIP: ciprofloxacin, LEV: levofloxacin, CAZ: ceftazidime, ATM: aztreonam, IPM: imipenem, FOF: fosfomycin, ERY; erythromycin, TOB: tobramycin, AMK: amikacin, CHL: chloramphenicol.

Supplementary Table S6 | Primers used in this study.

Primer	Sequence (5'-3')	Description
nfxB177.Fw	AAGCTTATGACCCTGATTTCCCATGA	To amplify mutant allele of *nfxB* by PCR
nfxB177.Rv	AAGCTTTCGTTGAGACGATCGAGCTG	
parR87.Fw	AAGCTTCGCTCAAGCGCGGAAGTGCTTT	To amplify mutant allele of *parR* by PCR
parR87.Rv	AAGCTTCAGGTAGAGGCGCAGGAACA	
mexZ43.Fw	AAGCTTACGTCCTGGCCTTCCTCGTA	To amplify mutant allele of *mexZ* by PCR
mexZ43.Rv	AAGCTTAACTGCGCAGGCTATCGAGG	
nuoD184.Fw	AAGCTTATGACTGCAGACTCCGCTCT	To amplify mutant allele of *nuoD* by PCR
nuoD184.Rv	AAGCTTTCGGTGAAGGTGAAGAACAC	
lasR.Fw	AAGCTTAGCGCCATCCTGCAGAAGAT	To amplify wild type allele of *lasR* by PCR
lasR.Rv	AAGCTTGCCGACCAATTTGTACGATC	
mexXYgntyp.Fw	GTACGAGGAAGGCCAGGAC	To genotype *mexXY* by PCR
mexXYgntyp.Rv	CTTGATCAGGTCGGCGTAG	