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Quantitative contribution of target alteration and decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone resistance

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ABSTRACT

20 Quinolone antibiotics constitute a clinically successful and widely used class of broad spectrum antibiotics, however, the emergence and spread of resistance increasingly limits the use of fluoroquinolones in the treatment and management of microbial disease. In this study we evaluated the quantitative contribution of quinolone target alteration as well as the expression of efflux pumps to fluoroquinolone resistance in 25 *Pseudomonas aeruginosa*. We generated isogenic mutations in hot spots of the quinolone resistance determining regions (QRDR) of *gyrA, gyrB* and *parC,* and inactivated the efflux regulator-genes to overexpress the corresponding MDR efflux pump. We then introduced the respective mutations in the PA14 reference strain singularly and in various combinations. Whereas the combined inactivation of two efflux-30 regulator-encoding genes did not lead to higher resistance levels as compared to the inactivation of only one efflux-regulator-encoding gene; the combination of mutations leading to an increased efflux and target alteration clearly exhibited an additive effect. This combination of target alteration and overexpression of efflux pumps was commonly observed in clinical *P. aeruginosa* isolates; however, these two mechanisms were 35 frequently found not to be sufficient to explain the level of fluoroquinolone resistance. Our results implicate that there are additional mechanisms to increase ciprofloxacin resistance in isolates with mutations in the QRDRs, which are independent of the expression of the MexAB-OprM, MexCD-OprJ, MexEF-OprN and/or MexXY-OprM efflux pumps.

40 **INTRODUCTION**

Fluoroquinolones are very potent antimicrobial agents with an excellent oral bioavailability reaching serum drug concentrations equivalent to intravenous administrations. They are broad-spectrum antibiotics with an antibacterial activity against Gram-positive as well as Gram-negative bacteria (1, 2). As a consequence
45 fluoroquinolones are widely and increasingly used for the treatment of bacterial infections not only in the hospital setting but also for the treatment of outpatients. The broad, frequent and world-wide use of the fluoroquinolones and also the frequently inappropriate application of the antibiotics are important factors that drive resistance, which has reached clinically relevant levels in the last decade (3, 4, 5).

50 The fluoroquinolones act by directly inhibiting DNA replication via an interaction of the drug with complexes composed of DNA and either of the two target enzymes, DNA-gyrase and topoisomerase IV (1, 6). The molecular mechanisms of fluoroquinolones resistance include two dominant mechanistic categories for all bacterial species studied so far (7, 8). The activity of multidrug resistance (MDR) efflux pumps act to decrease
55 intracellular fluoroquinolone concentrations (9) and alterations of the drug target by mutations at key sites in the so called quinolone resistance determining regions (QRDR) in genes encoding the DNA-gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*) lead to a decreased binding affinity of the quinolones to their respective drug target (10, 11). More recently mobile genetic elements have also been described,
60 carrying the *qnr* (12), *qepA* (13) or the *aac(6')-Ib-cr* gene (14), which confer reduced susceptibility to quinolones in members of the Enterobacteriaceae family.

In this study we analyzed a panel of 100 clinical *Pseudomonas aeruginosa* isolates in respect to the presence of mutations in the QRDRs and the expression of four major efflux pumps in a subset of these. We furthermore introduced the most dominant
65 mutations in the QRDR into the susceptible *P. aeruginosa* reference strain PA14 and inactivated the efflux regulator-encoding genes *nfxB, mexR* and *mexZ* as well as the oxidoreductase *mexS* with the aim to generate mutants that overexpress the MexCD-OprJ, MexAB-OprM, MexXY and MexEF-OprN efflux pump, respectively. The results of this study implicate that there are additional yet unknown mechanisms that contribute to
70 increased ciprofloxacin MIC levels in clinical *P. aeruginosa* isolates that go beyond mutations in the QRDRs and overexpression of the MexAB-OprM, MexCD-OprJ, MexEF-OprN and/or MexXY-OprM efflux pumps.

MATERIALS AND METHODS

Bacterial isolates and antibiotic resistance profile. A collection of 100 clinical *P.*
75 *aeruginosa* isolates collected at Hannover Medical School (MHH) between 2005 and
2007 was used in this study. These isolates were obtained from clinical infections at
various sites (Table S1), from overall 90 individuals, 24 of whom were cystic fibrosis
patients. A maximum of two isolates per patient were analyzed in this study only when
both isolates clearly differed in their antibiotic resistance profile. Antibiotic resistance
80 profiles (Table S1) were determined using a VITEK 2 system (bioMérieux).

Pyrosequencing. To extract DNA for pyrosequencing, 500 µl of an overnight culture
were harvested and lysed for 15 min at 95 °C in 100 µl lysis buffer (0,25 % (m/v) SDS,
50 mM NaOH). After addition of 900 µl dH_2O, 2 µl was used as a PCR template.
85 To identify mutations at amino acid position 83 and 87 in *gyrA* and position 87 in *parC* a
pyrosequencing assay was established. Amplification was performed as described by
Doostzadeh *et al*. (15) using a 24-mer universal biotinylated primer (UBP) adopted from
Royo *et al*. (16). All primers used in this study are shown in Table S2. Sequencing
primers were designed with Primer3 (17) to anneal 3 bp upstream (*gyrA*) or 5 bp
90 upstream (*parC*) from the SNP. Pyrosequencing was performed on a PSQ 96MA
pyrosequencer (Pyrosequencing AB) with PyroMark gold chemistry (Qiagen) as
described in Royo *et al*. (16).

Sanger sequencing. To identify mutations in *gyrB* and *parE* the QRDRs of both genes
95 were amplified with primers gyrBfp4, gyrBrp4, parEfp3 and parErp3 (Table S2). PCR

products were sequenced by the Sanger method using the same sets of primers on a 3730xl DNA Analyzer (Applied Biosystems).

Quantitative real time RT-PCR. The expression levels of, *mexA*, *mexC*, *mexE, mexX* and the housekeeping gene *rpoD* were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). RNA was isolated at late logarithmic growth (OD600 1.5 – 2.0) from 3 ml Mueller-Hinton liquid culture using the RNeasy kit (Qiagen), as described by the manufacturer. RNA was eluted from the RNeasy columns in a volume of 50 µl water and treated with DNA-*free* kit (Ambion). cDNA was synthesized using random hexamer primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) according to the manufactures instructions. qRT-PCRs were performed in duplicates in a 20 µl volume with 25 ng cDNA and a primer concentration of 500 nmol/l on a LightCycler 480 (Roche) using SYBR Green I Mastermix (Roche). The primers were adopted from Tomás *et al.* (18), sequences are listed in Table S2. Gene expression was calculated using the $\Delta\Delta$ Ct method and a standard curve to measure the PCR efficiency. All results were normalized to the expression of the house-keeping gene *rpoD* of the same clinical isolate and calibrated relative to *P. aeruginosa* PA14. According to Cabot *et al*. (19) isolates with a *mexA* expression of ≥3-fold were regarded as positive, whereas values between 2- and 3-fold were regarded as borderline expression. An overexpression of *mexC*, *mexE* and *mexX* of ≥10-fold was regarded as positive, an overexpression of 5- to 10-fold as borderline expression.

Genetic manipulations. To generate mutations of *gyrA*, *gyrB* and *parC* as well as gene knockouts of *nfxB*, *mexR*, *mexS* and *mexZ* in an isogenic background, the *P. aeruginosa*
120 strain PA14 was used. Mutagenesis was carried out by homologous recombination using the pEX18Ap plasmid (20) and approximately 1000 bp long mutagenic fragments created by overlap extension PCR as described previously (21). To generate knockout mutants the 500 bp upstream and downstream regions of the gene of interest were amplified using overlapping primers. All primers are listed in Table S2. Mutant
125 candidates were identified by replica plating on LB agar plates with appropriate antibiotic concentrations and further analyzed by PCR and Sanger sequencing to verify the mutation. The antibiotic resistance profile of all mutants was determined in Mueller-Hinton broth as described previously (22).

130 **Cloning of *gyrA* and complementation of clinical isolates.** To complement *gyrA* mutations in clinical isolates with the wildtype gene, *gyrA* was amplified from the PA14 chromosome with primers gyrAFPSacI and gyrARPSacI and cloned into the *Sac*I restriction site of plasmid pME6032 (23) yielding plasmid pME::gyrA. The correct insertion and sequence was verified by Sanger sequencing using primers gyrAseqF,
135 gyrAseqR, pMEseqF and pMEseqR. Complementation was performed as follows: Each isolate was grown over night at 37 °C and 180 rpm in 4 ml LB broth. 1.5 ml of these cultures were centrifuged, washed three times with 1 ml 0.3 M sucrose and resuspended in 100 µl 0.3 M sucrose. 500 ng of the plasmid pME::gyrA and 50 µl of the cell suspension were used for electroporation. The cells were plated on LB agar plates
140 supplemented with 100 µg/ml tetracycline. Ciprofloxacin minimal inhibitory concentration

(MIC) of isolates containing pME::gyrA with and without the addition of 1mM IPTG was determined using Etest stripes (bioMérieux) on LB agar plates supplemented with 100 μg/ml tetracycline. Results were recorded after 24 h of growth at 37 °C.

RESULTS AND DISCUSSION

145 **Frequency and nature of mutations in the quinolone resistance determining regions (QRDR) of *gyrA, gyrB, parC* and *parE* in clinical *P. aeruginosa* isolates.** In this study we used Sanger sequencing and pyrosequencing to determine the nature and frequency of hot spot SNP mutations in the QRDRs of the genes *gyrA* and *gyrB* encoding the DNA-gyrase as well as *parC* and *parE* encoding the topoisomerase IV. A 150 panel of 100 clinical *P. aeruginosa* isolates isolated from patients of the Hannover Medical School over a time period of two years (2005 - 2007) was analyzed in this study. The isolates were recovered from various clinical sites and most of them exhibited resistance not only towards fluoroquinolones but also towards various other antimicrobial compounds (Table S1). The pyrosequencing technology has been proven 155 to be time and cost competitive and to allow efficient detection of SNPs in localized regions where the nucleotide variants are known (24). We have designed two different pyrosequencing assays for sequencing analysis of the most prominent mutation hotspots in the QRDR of the A-subunit of the DNA-gyrase encoded by *gyrA* that spans amino acid position 83 to 87 and of the A–subunit of the topoisomerase IV encoded by 160 *parC* that spans amino acid position 82 to 84. The QRDR of the B-subunits of the DNA-Gyrase encoded by *gyrB* and the topoisomerase IV (*parE*) are larger as they span amino acid position 429 to 585 in *gyrB* and 357 to 503 in *parE.* Therefore, Sanger sequencing was performed for the identification of relevant mutations in the QRDRs of *gyrB* and *parE*.

165 Sequencing confirmed the presence of mutations in the QRDR in most of the clinical isolates. The relative frequency of the specific mutations is depicted in Fig. 1A. In

accordance with several previous studies (25, 26, 27, 28, 29, 30, 31), the most frequently observed mutation was within the QRDR of *gyrA*, with the T83I being the dominant mutation, whereas mutations in *gyrB* were less frequent (30, 32, 33). Here, the

170 majority of mutations were found at amino acid positions 466 to 468, however we also found two isolates with an I529V mutation which to our knowledge has not been described previously. Two mutations within the QRDR of *parC* were detected in our panel of 100 clinical isolates (S87W and S87L) and overall only three mutations were present in *parE* (one M437I and two A473V). The majority of clinical isolates harbored

175 either a singular mutation in *gyrA* or *gyrB* or the combination of mutations in *gyrA* and *parC* (Fig. 1B). Fewer isolates exhibited mutations in *gyrB* in combination with *parE* and in *gyrA* in combination with *gyrB* with and without additional mutations in the QRDR of *parC.* Single *parC* mutations were not found in our panel of clinical *P. aeruginosa* isolates - as also observed in previous studies (28, 33), two of the isolates harbored a

180 single mutation in *parE* and in 14 isolates no mutation in the QRDRs were detected.

It has been observed before that highly resistant *P. aeruginosa* isolates harboring a double *gyrA* and *parC* mutation are almost exclusively isolated from non-cystic fibrosis (CF) patients, whereas in isolates from CF patients single mutations within the QRDRs dominate (34, 35, 36). Interestingly, in accordance with the previous reports 27 of 29

185 *gyrA/parC* mutants of this study were isolated from non-CF patients. It has been suggested that higher drug levels of ciprofloxacin in non-CF patients might account for this phenomenon, since drug levels in CF-sputum were found to be significantly lower than in blood (37). Although the lower drug concentration levels might select for intermediate resistant strains in distinct niches, it might also indicate that there is a co-

190 selection of single mutations in QRDRs with other phenotypic traits that provide the strains with a selective advantage. Thereby, the unique environment of the CF lung might play a significant role and therefore influences the process of mutation and selection (35).

195 **Correlation of the presence of SNPs within the QRDR of *gyrA, gyrB, parC* and *parE* with the ciprofloxacin resistance phenotype in clinical *P. aeruginosa* isolates.** The presence of SNPs within the QRDR of *gyrA, gyrB, parC* and *parE* was correlated with phenotypic resistance to fluoroquinolones in the clinical *P. aeruginosa* isolates. In Fig. 2 the relationship of ciprofloxacin MIC values of all 100 clinical *P. aeruginosa*
200 isolates and the presence of mutations in the QRDR is depicted. The majority of clinical isolates harbored single mutations in *parE*, *gyrA* or *gyrB*, however, those mutations did not necessarily lead to a ciprofloxacin MIC values exceeding 2µg/ml (noteworthy, resistance according to the Clinical Laboratory and Standards Institute (CLSI) breakpoints is categorized by MIC values exceeding 2 µg/ml). In contrast, combinations
205 of mutations in the QRDR of *gyrA* and *parC* always resulted in a ciprofloxacin resistant phenotype exhibiting MIC values of ≥8 µg/ml. The two isolates that harbored a singular *parE* mutation and the 14 isolates without mutations within the QRDRs exhibited MIC values that did not exceed 2 µg/ml and thus were categorized as susceptible or intermediate according to CLSI breakpoints.

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Introduction of dominant SNPs within the QRDR of *gyrA, gyrB* and *parC* into the susceptible *P. aeruginosa* reference strain PA14. With the aim to pin-point the contribution of the most frequent mutations within the QRDR of *gyrA, gyrB* and *parC* to fluoroquinolone resistance, we introduced the respective SNPs into the fluoroquinolone
215 susceptible reference strain PA14 and measured the resistance profile. Plasmid constructs for allelic exchange were generated for the two SNPs T83I and D87N in *gyrA*, the three SNPs S466F, S466Y and E468D in *gyrB* and two SNPs S87L and S87W in *parC*. Those SNPs were introduced into the reference strain singularly and in various combinations. As shown in Table 1, the introduction of *parC* mutations alone did not
220 have an impact on the ciprofloxacin susceptibility, whereas mutations in the QRDR of *gyrB* or *gyrA* increased the MIC of ciprofloxacin 8- to 16-fold. Similarly, the introduction of a single *parC* mutation did not alter the fluoroquinolone susceptibility in *E. coli* (38). The simultaneous introduction of two SNPs within the QRDR of *gyrA* (T83I and D87N) did not further contribute to ciprofloxacin resistance as compared to the introduction of
225 T83I alone. However, the simultaneous introduction of SNPs in *gyrA* (T83I) and *parC* (either S87L or S87W) increased the MIC of ciprofloxacin of the reference strain 256-fold. None of the mutations within the QRDR had an impact on the resistance profile of the parental strain against beta-lactam antibiotics, carbapenems and aminoglycosides (data not shown).

230

Most clinical *P. aeruginosa* isolates harboring mutations in the QRDR additionally express efflux pumps. Mutations in genes encoding the two subunits of DNA-gyrase raised the MIC of ciprofloxacin already 8- to 16-fold in the *P. aeruginosa* reference strain.

Those *gyrA* mutants as well as the majority of the clinical isolates harboring relevant
235 mutations in the QRDR of *gyrA* and/or *gyrB* exhibited MIC values of ≤2 µg/ml. However,
we identified clinical isolates with a singular mutation within *gyrA* that exhibited MIC
values reaching up to 8 µg/ml. The broad MIC range in clinical *P. aeruginosa gyrA*
mutants has been observed in several studies before (34, 39) and although it is tempting
to speculate that this can be explained by a differential expression of efflux pumps (32,
240 40), no clear association of increased MICs against the *gyrA* mutants and an increased
expression of efflux pumps could be demonstrated in previous studies (39, 41, 42). In
the same line, it has been demonstrated for some individual clinical *P. aeruginosa*
isolates that elevated MIC levels of meropenem could not be explained by decreased
levels of OprD and/or expression of the MexAB-OprM and MexEF-OprN efflux pumps
245 (43, 44) and it was speculated that other resistance mechanisms yet to be identified
might account for the resistance phenotype. We therefore tested whether a differential
expression of efflux pumps in those isolates could account for the high MIC values and
monitored the expression of four efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM in 29 selected clinical isolates. Nine of these isolates did not
250 harbor any mutation in one of the QRDRs, ten isolates harbored a singular *gyrA*
mutation and ten isolates had mutations in *gyrA* and *parC*. The quantification of the
transcription of the genes encoding the membrane fusion proteins of the pumps (*mexA*,
mexC, *mexE* and *mexX*) using qRT-PCR is shown in Table 2. According to work done by
Cabot *et al* (19) overexpression of *mexA* of ≥3-fold was regarded as positive, whereas
255 values between 2- and 3-fold were regarded as borderline expression. An
overexpression of *mexC*, *mexE* and *mexX* of ≥10-fold was regarded as positive, an

overexpression of 5- to 10-fold as borderline expression. The majority of clinical isolates exhibited an increased expression of at least one efflux pump, with MexXY-OprM being the one expressed the most. This finding has been observed before (36). However, no

260 clear association between the expression of efflux pumps and increased fluoroquinolone MIC values against the isolates harboring mutations within the QRDRs could be observed (Fig. 3).

Inactivation of the efflux regulator-encoding genes *mexR, nfxB, mexS* and *mexZ* in

265 **susceptible *P. aeruginosa* reference strain.** With the aim to pin-point the contribution of overexpression of the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux pumps to fluoroquinolone resistance, we inactivated the respective efflux regulator-encoding genes in the fluoroquinolone susceptible reference strain PA14 and measured the resistance profile. Deletion of the efflux regulator-encoding genes *mexR,*

270 *nfxB* and *mexZ* as well as the oxidoreductase *mexS* lead to an overexpression of the efflux pump MexAB-OprM by 1.6-fold, the MexCD-OprJ pump by 16-fold, the MexXY-OprM pump by 6-fold and the MexEF-OprN pump by 320-fold, respectively (Table 2). We also inactivated the efflux regulator-encoding genes in a PA14 strain background in various combinations (Δ*nfxB*-*mexZ*; Δ*mexR*-*mexS*; Δ*mexR*-*mexZ* and Δ*mexS*-*mexZ*)

275 and also combined the deletion of the four efflux regulator-encoding genes with mutations in the QRDRs (*gyrA*, *gyrB* and *gyrA*/*parC*). As shown in Table 1, overexpression of the efflux pumps clearly increased the ciprofloxacin MIC values in the susceptible *P. aeruginosa* reference strain by 2- to 16-fold. Thereby, inactivation of *nfxB* and *mexS* had the most pronounced phenotype. The combined inactivation of various

280 efflux regulator-encoding genes (∆*nfxB*-*mexZ*; ∆*mexR*-*mexS*; ∆*mexR*-*mexZ* and ∆*mexS*-*mexZ*) did not lead to further increased MIC levels. This absence of an additive effectmight be explained by antagonistic interactions of efflux pumps during planctonic growth, which was found to occur in *nfxB* mutants (45). However, the inactivation of efflux regulator-encoding genes in the PA14 *gyrA*, *gyrB* and *gyrA/parC* mutant
285 background clearly further enhanced the fluoroquinolone resistance level in an additive manner. In accordance to our results the deletion of efflux pumps in resistant *P. aeruginosa* strains with multiple target alterations has previously been demonstrated to lead to a reduced fluoroquinolone MIC (46). It might thus be surprising that we did not find a clear correlation of an increased fluoroquinolone MIC value in clinical isolates
290 harboring a particular QRDR genotype with the expression of major efflux pumps.

Mutation within the QRDR of *gyrA* adds to preexisting isolate specific resistance levels. Although overexpression of efflux pumps further enhanced fluoroquinolone resistance in a QRDR mutant background, we did not find a clear association between
295 expression of efflux pumps and increased fluoroquinolone MIC values in our set of clinical isolates harboring mutations within the QRDRs. We therefore wondered; whether the contribution of a *gyrA* mutation to the fluoroquinolone resistance level could vary between different isolates. To address this question we cloned the wild-type *gyrA* gene into the pME6032 vector resulting in vector pME::gyrA and introduced the gene into
300 various clinical isolates in *trans*. All of those clinical isolates exhibited a *gyrA* mutation but the MIC values varied from 0.125 - 2 µg/ml (Table 3). We found that the complementation with the wild-type *gyrA* gene led to a reduction in fluoroquinolone

resistance by 2- to 8-fold irrespective of the original resistance level. These results indicate that mutations within the QRDR of *gyrA* adds to preexisting isolate specific
305 resistance levels of unknown origin. Two comprehensive screenings of a *P. aeruginosa* PA14 mutant library showed that approximately 100 to 200 genes are involved in the ciprofloxacin resistome (47, 48). It thus will be an interesting task for the future to determine whether and which of the identified gene inactivations play a role for fluoroquinolone resistance in clinical settings.

310 53 of the 100 clinical *P. aeruginosa* isolates harbored a single mutation within only one of the QRDR and 40 of them expressed MIC values of 2 µg/ml or less. Although these *P. aeruginosa* isolates are categorized as susceptible it remains to be shown that it is safe to treat them with fluoroquinolones (49). The stepwise enrichment of fluoroquinolone resistant mutations has been described previously in several *in vitro* studies (50, 51, 52).
315 Mutants at each step are enriched when drug concentrations fall within a specific range called the mutant selection window (53, 54). Even antibiotic concentrations below the MIC might select for resistance conferring mutations, as in a recent study, it could be demonstrated that ciprofloxacin concentrations at 1/10 the MIC was sufficient to select *de novo* fluoroquinolone resistant mutants in *E. coli* (55).

320 Although low level resistance conferred by first-step SNPs within the QRDR does not prevent bacterial eradication in the presence of sufficient levels of a quinolone, they may substantially enhance the number of (secondary) resistant mutants that can be selected from this population. In line with this, it has been demonstrated that deletion of efflux pumps significantly reduces the frequency of emergence of fluoroquinolone resistant
325 mutant isolates (46, 52). A key to preventing fluoroquinolone resistance in *P. aeruginosa*

may therefore be to strictly avoid low dose use of fluoroquinolones and thus to prevent the raise of *P. aeruginosa* first-step mutations that confer resistance to fluoroquinolones.

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TABLE 1. MICs of *in vitro* generated PA14 mutants.

	PA14 mutant	**CIP MIC in µg/ml**
wild type	PA14	0.125
mutations in QRDR	*gyrA* T83I	2
	gyrA D87N	1
	gyrA T83I-D87N	2
	gyrB S466F	1
	gyrB S466Y	1
	gyrB E468D	1
	parC S87L	0.125
	parC S87W	0.125
efflux mutations	Δ*nfxB*	2
	Δ*mexR*	0.5
	Δ*mexS*	2
	Δ*mexZ*	0.25
	Δ(*nfxB-mexZ*)	2
	Δ(*mexR-mexS*)	2
	Δ(*mexR-mexZ*)	0.5
	Δ(*mexS-mexZ*)	2
combination of efflux and QRDR mutations	*gyrA* T83I + Δ*nfxB*	32
	gyrA T83I + Δ*mexR*	8
	gyrA T83I + Δ*mexS*	32
	gyrA T83I + Δ*mexZ*	2
	gyrB E468D + Δ*nfxB*	8
	gyrB E468D + Δ*mexR*	2
	gyrB E468D + Δ*mexS*	8
	gyrB E468D + Δ*mexZ*	1
	gyrA T83I + *parC* S87L	32
	gyrA T83I + *parC* S87W	32
	gyrA T83I + *parC* S87L + Δ*nfxB*	256
	gyrA T83I + *parC* S87L + Δ*mexR*	64
	gyrA T83I + *parC* S87L + Δ*mexS*	256
	gyrA T83I + *parC* S87L + Δ*mexZ*	32

505 CIP, ciprofloxacin

TABLE 2. Expression of *mexA*, *mexC*, *mexE* and *mexX* in 29 clinical isolates and in four *in vitro* generated PA14 knock-out mutants compared to PA14 wildtype strain.

Isolate	**fold change**				**CIP MIC**	**mutation**
	mexA	***mexC***	***mexE***	***mexX***	**in µg/ml**	
10049	0.99	**11.60**	**n.d.**	**50.57**	0.5	wt
12178	1.72	3.22	3.50	**11.87**	0.5	wt
7624	0.93	2.10	1.88	**17.25**	1	wt
9229	1.19	**18.24**	1.82	*6.45*	1	wt
9639	0.22	*8.52*	3.18	**93.65**	1	wt
10047	*2.48*	3.01	*5.63*	**21.86**	1	wt
11935	*2.01*	*5.43*	**13.05**	**21.27**	1	wt
9748	0.95	1.24	**43.69**	4.47	2	wt
9830	*2.87*	**10.60**	**33.72**	**76.38**	2	wt
7508	0.38	3.03	**10.75**	*7.16*	1	*gyrA* D87N
7807	1.18	1.42	4.94	**45.14**	1	*gyrA* D87Y
7091	0.87	0.80	1.58	**63.14**	1	*gyrA* T83I
11445	1.47	2.00	3.87	**110.02**	1	*gyrA* T83I
7252	1.16	1.93	2.39	*6.34*	2	*gyrA* T83I
14088	0.62	**14.94**	2.83	**74.33**	2	*gyrA* T83I
7313	1.31	3.04	*5.66*	**15.72**	4	*gyrA* T83I
11148	0.73	0.67	0.74	**27.32**	4	*gyrA* T83I
12274	0.85	1.25	1.92	**55.25**	4	*gyrA* T83I
7055	**4.38**	1.88	2.69	**38.43**	8	*gyrA* T83I
6964	0.87	1.13	4.02	**42.34**	8	*gyrA* T83I + *parC* S87L
6829	1.01	0.90	**13.53**	*6.71*	>8	*gyrA* T83I + *parC* S87L
6870	0.92	1.27	*5.55*	**53.41**	>8	*gyrA* T83I + *parC* S87L
7176	1.11	1.39	*5.36*	**54.14**	>8	*gyrA* T83I + *parC* S87L
7823	0.95	0.73	3.23	**37.57**	>8	*gyrA* T83I + *parC* S87L
7863	1.17	0.75	4.92	**31.39**	>8	*gyrA* T83I + *parC* S87L
8349	1.22	1.25	4.46	**45.60**	>8	*gyrA* T83I + *parC* S87L
8478	1.13	1.83	*6.00*	**56.84**	>8	*gyrA* T83I + *parC* S87L
8614	1.00	1.69	*6.13*	**49.78**	>8	*gyrA* T83I + *parC* S87L
9652	1.19	1.08	4.43	**41.37**	>8	*gyrA* T83I + *parC* S87L
∆nfxB	0.59	**16.08**	0.70	0.58	2	knockout of *nfxB* in PA14
∆mexR	*1.58*	0.64	0.81	0.73	0.5	knockout of *mexR* in PA14
∆mexS	0.65	0.33	**318.2**	0.67	2	knockout of *mexS* in PA14
∆mexZ	0.81	0.75	2.30	*6.17*	0.25	knockout of *mexZ* in PA14

CIP, ciprofloxacin; nd, not determined

510 Overexpression is shown as bold digits and borderline expression values are underlined and in italics according to Cabot *et al.* (19)

TABLE 3. Complementation of clinical isolates with plasmid pME::gyrA

Isolate	**CIP MIC in µg/ml**		**Dif**	**Mutation**
	-IPTG	**+IPTG**	**log2**	**in *gyrA***
7252	2	0.5	2	T83I
7444	4	0.5	3	T83I
7807	0.25	0.25	0	D87Y
8044	1	0.25	2	T83A
8931	1	0.25	2	D87N
9481	1	0.25	2	T83I
9674	8	2	2	D87Y
12207	1	0.5	1	T83I
13224	1	0.5	1	D87N
13428	0.5	0.125	2	D87N
14088	0.5	0.125	2	T83I
PA14 *gyrA* T83I	1	0.125	3	T83I
PA14 wt	0.125	0.125	0	wt

Dif, difference

515

FIG. 1. Mutations identified in *gyrA*, *gyrB*, *parC* and *parE* in 100 clinical isolates. (A) Frequency and nature of the mutations found in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* in 100 clinical *Pseudomonas aeruginosa* isolates. Labels indicate the mutations with wild type allele, amino acid position and mutant allele and the number of isolates
520 harboring this mutation. 'Δ' indicates a deletion at the specified position. 'wt' indicates wild type allele or a silent mutation. (B) Co-occurence of mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, *parE* in the individual clinical *P. aeruginosa* isolates. The fraction of isolates with a single mutation is depicted in light gray, with a double mutation in dark gray and with a triple mutation in black. The fraction of isolates without any identified
525 QRDR mutation is shown in white.

FIG. 2. Correlation of the minimal inhibitory concentration (MIC) values for ciprofloxacin with the presence of mutations in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* (and combinations thereof) of 100 *P. aeruginosa* clinical isolates. The
530 number written inside a circle displays the amount of isolates with the same combination of MIC and genotype. The color of the circles represent the clinical breakpoints according to CLSI standards with light gray, gray and dark gray representing sensitive, intermediate and resistant, respectively.

535 **FIG. 3. Influence of multidrug efflux (MEX) pump overexpression and QRDR mutation on ciprofloxacin MIC.** The variations of MIC values for a particular genotype

cannot be explained by the additional expression of MEX pumps. The figure shows the expression of four MEX pumps in 29 selected clinical *P. aeruginosa* isolates. Each circle represents one clinical isolate while each quarter represents one efflux pump (top left:
540 MexAB-OprM, top right: MexCD-OprJ, bottom left: MexEF-OprN and bottom right: MexXY-OprM) with black quarters representing an overexpression of a pump, gray quarters representing borderline expression and white quarter representing wild -type expression levels (according to Cabot *et al.* 2011). The isolates were arranged according to their QRDR genotype (wild type, single mutation in *gyrA* or simultaneous mutation in
545 *gyrA* and *parC*) and their ciprofloxacin MIC.