

## Resistance Mechanisms of Multiresistant *Pseudomonas aeruginosa* Strains from Germany and Correlation with Hypermutation<sup>∇†</sup>

B. Henrichfreise,<sup>1\*</sup> I. Wiegand,<sup>1</sup> W. Pfister,<sup>2</sup> and B. Wiedemann<sup>1</sup>

University of Bonn, Institute for Medical Microbiology, Immunology and Parasitology, Pharmaceutical Microbiology Unit, Meckenheimer Allee 168, 53115 Bonn, Germany,<sup>1</sup> and University of Jena, Institute for Medical Microbiology, Germany<sup>2</sup>

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**In this study, we analyzed the mechanisms of multiresistance for 22 clinical multiresistant and clonally different *Pseudomonas aeruginosa* strains from Germany. Twelve and 10 strains originated from cystic fibrosis (CF) and non-CF patients, respectively. Overproduction of the efflux systems MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM was studied. Furthermore, loss of OprD, alterations in type II topoisomerases, AmpC overproduction, and the presence of 25 acquired resistance determinants were investigated. The presence of a hypermutation phenotype was also taken into account. Besides modifications in GyrA (91%), the most frequent mechanisms of resistance were MexXY-OprM overproduction (82%), OprD loss (82%), and AmpC overproduction (73%). Clear differences between strains from CF and non-CF patients were found: numerous genes coding for aminoglycoside-modifying enzymes and located, partially in combination with  $\beta$ -lactamase genes, in class 1 integrons were found only in strains from non-CF patients. Furthermore, multiple modifications in type II topoisomerases conferring high quinolone resistance levels and overexpression of MexAB-OprM were exclusively detected in multiresistant strains from non-CF patients. Correlations of the detected phenotypes and resistance mechanisms revealed a great impact of efflux pump overproduction on multiresistance in *P. aeruginosa*. Confirming previous studies, we found that additional, unknown chromosomally mediated resistance mechanisms remain to be determined. In our study, 11 out of 12 strains and 3 out of 10 strains from CF patients and non-CF patients, respectively, were hypermutable. This extremely high proportion of mutator strains should be taken into consideration for the treatment of multiresistant *P. aeruginosa*.**

The ubiquitous environmental bacterium *Pseudomonas aeruginosa* is one of the most important nosocomial pathogens, especially in intensive care units (ICUs) (39, 42). It is also the pathogen most frequently associated with morbidity and mortality in cystic fibrosis (CF) and other underlying chronic respiratory diseases causing chronic lung infections (6, 8, 22).

Intrinsic and acquired antibiotic resistance makes *P. aeruginosa* one of the most difficult organisms to treat. The high intrinsic antibiotic resistance of *P. aeruginosa* is due to several mechanisms: a low outer membrane permeability, the production of an AmpC  $\beta$ -lactamase, and the presence of numerous genes coding for different multidrug resistance efflux pumps (20).

A high number of acquired resistance genes coding for aminoglycoside-modifying enzymes (AME) and  $\beta$ -lactamases have been noted in *P. aeruginosa* (2). Extended-spectrum  $\beta$ -lactamases have been increasingly reported (3, 7, 33, 41, 44), and metallo- $\beta$ -lactamases have also started to emerge in *P. aeruginosa* (43).

Besides horizontal transfer of resistance genes, acquired resistance also occurs via mutations in chromosomal genes. Alter-

ations in the quinolone resistance-determining regions (QRDR) in the genes coding for DNA gyrase and topoisomerase IV play an important role in quinolone resistance in *P. aeruginosa* (14). Loss of OprD is the most prevalent mechanism of resistance to carbapenems and is associated with resistance to imipenem and reduced susceptibility to meropenem (28). To date, four efflux systems of the RND (resistance nodulation division) family, MexAB-OprM, MexEF-OprN, MexCD-OprJ, and MexXY-OprM, are well characterized and known to contribute significantly to antimicrobial resistance in *P. aeruginosa* (17, 27, 34, 35). Their overproduction confers cross-resistance or reduced susceptibility to several  $\beta$ -lactams (piperacillin, ticarcillin, ceftazidime, cefepime, and meropenem), quinolones, and aminoglycosides (20). Hyperproduction of the inducible AmpC  $\beta$ -lactamase is mostly due to the inactivation of the amidase AmpD (15) and two additional AmpD homologues (16), leading to an increase of inducer molecules.

Among *P. aeruginosa* strains from CF patients, a high proportion are hypermutable (30). Hypermutation is characterized by an increased spontaneous-mutation rate and seems to be an advantage for fast adaptation to a heterogeneous and fluctuating environment, like the lung of a chronically infected CF patient (30). Recently, studies found hypermutation to be the key factor in development of mutation-mediated multiresistance in patients with chronic *P. aeruginosa* lung infections (23). Among strains from patients with acute infections, mutator strains are rare (9).

Accumulation of resistance mechanisms frequently occurs in *P. aeruginosa* strains from patients with CF, where sequential

\* Corresponding author. Mailing address: University of Bonn, Institute for Medical Microbiology, Immunology and Parasitology, Pharmaceutical Microbiology Unit, Meckenheimer Allee 168, 53115 Bonn, Germany. Phone: 49 228 732111. Fax: 49 228 735267. E-mail: bhenrich@uni-bonn.de.

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treatment of the chronic infection with various antibiotics is common. In patients without CF, multiresistant *P. aeruginosa* strains are less frequent but are associated with severe adverse clinical outcomes (1).

The aim of our study was to investigate the mechanisms of resistance of multiresistant *P. aeruginosa* strains from CF patients and non-CF patients from Germany. Furthermore, we intended to get insights into the correlation between the genetic resistance determinants detected and the respective resistance phenotypes.

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## MATERIALS AND METHODS

**Bacterial strains.** Twenty-two multiresistant, clonally different (determined by pulsed-field gel electrophoresis [PFGE]) *P. aeruginosa* strains (I to XXII) were obtained from three German hospitals participating in the German Network for Antimicrobial Resistance Surveillance (GENARS) from January through March 2004. The strains showed reduced susceptibility (intermediate or resistant according to the guidelines of the German Institute for Standardization) to each of the following class representatives: imipenem, ceftazidime, piperacillin, ciprofloxacin, and gentamicin. A reidentification was performed by verifying growth on cefrimide agar plates and confirming the presence of the *P. aeruginosa*-specific chromosomal *algD* gene by PCR (see the supplemental material).

The 22 strains originated from 11 CF and 10 non-CF patients. For three CF patients, two clonal variants (VIa and VIIa) and another clonal isolate of strain XI were also investigated. All CF patients were medicated at ambulant CF wards. Of the non-CF patients, five were inpatients from normal wards (heart vessel surgery, infection, internal medicine, and neurosurgery) and five were from ICU wards (anesthesiology, interdisciplinary ICU, internal medicine, neurology, and neurosurgery). All strains from CF patients were isolated from sputum specimens. The strains isolated from non-CF patients originated from several anatomic sites: respiratory tract (sputum, bronchoalveolar lavage, and tracheal secretion), wound, vagina, and urinary tract.

**In vitro susceptibility testing.** Susceptibility testing was performed by broth microdilution with reference to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (5).

**PFGE.** Genomic DNA was prepared using GenePath group 3 reagents as recommended by the manufacturer (Bio-Rad, Marnes-la-Coquette, France). Analysis of genomic DNA macrorestriction patterns by PFGE was carried out after digestion with SpeI using the CHEF mapper system (Bio-Rad, Hercules, CA). Clonal relationships were interpreted in accordance with the guidelines proposed by Tenover et al. (40).

**Mutation frequency determination.** Mutation frequencies conferring rifampin resistance were determined in triplicate, following a previously described method (29). According to Oliver et al., a strain was considered a mutator strain when the mutation frequency conferring rifampin resistance was at least 20-fold higher than that observed for the wild-type strain PAO1 (30).

**Efflux pump inhibition test.** Efflux pump overexpression was detected using an efflux pump inhibitor (EPI) test (21). Levofloxacin MICs (concentrations ranging from 256 mg/liter to 0.06 mg/liter) were tested either with or without 20  $\mu$ g/ml of the EPI Phe-Arg- $\beta$ -naphthylamide (Sigma-Aldrich, Taufkirchen, Germany). An at least 16-fold reduction of the levofloxacin MIC in the presence of Phe-Arg- $\beta$ -naphthylamide indicated the overproduction of efflux pumps.

**Cephalosporinase inhibition test.** Overproduction of the chromosomal cephalosporinase AmpC was evaluated by a disk diffusion test with 30- $\mu$ g ceftazidime discs (Oxoid Limited, Basingstoke, United Kingdom) on Mueller-Hinton agar with or without cloxacillin (Sigma-Aldrich, Taufkirchen, Germany) at 500 mg/liter as described elsewhere (4). The test result was considered positive when the ceftazidime inhibition zone diameter increased by at least 10 mm in the presence of cloxacillin.

**EDTA-phenanthroline-imipenem microdilution test.** To detect metallo- $\beta$ -lactamases, MICs of imipenem (concentrations in the range of 512 mg/liter to 0.25 mg/liter) were tested in the absence or presence of 0.4 mM EDTA and 0.04 mM 1,10-phenanthroline (Sigma-Aldrich, Taufkirchen, Germany) (26).

**Molecular analysis techniques.** Extraction of plasmid DNA was performed using a Fast Plasmid mini kit (Eppendorf, Hamburg, Germany). Genomic DNA was purified from *P. aeruginosa* with a QIAGEN tissue kit (QIAGEN, Hilden,

Germany). To analyze chromosomally mutation-mediated resistance mechanisms, the QRDRs of the DNA gyrase and topoisomerase IV genes *gyrA* and *parC*, in addition to the gene *oprD*, encoding the porin OprD, were amplified and sequenced. Furthermore, the efflux regulator-encoding genes *mexR*, *nalC*, *nfxB*, *mexZ*, *mexT*, and *mexS* were sequenced.

The relevance of amino acid changes not previously reported was analyzed. The positions in functional domains were investigated by localizing the changed amino acid either in the tertiary structure if available (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Structure>) or in the secondary structure by using LOOPP @ CBSU software, version 3.0 (<http://cbsuapps.tc.cornell.edu/loop.aspx>).

Class 1 and 2 integrons were detected using specific primers for the respective integrase genes *intI1* and *intI2*. To detect acquired resistance genes, an elaborate PCR screening for  $\beta$ -lactamase and AME genes and *qnrA* was carried out.

The genetic context of the detected resistance genes was analyzed by PCR mapping (19). Class 1 integron-specific primers binding to the 5'-conserved segment (*intI1*) and to the 3'-conserved segment ( $\Delta$ *qacE1*) and a set of primers binding to the resistance genes found were used. The identities of PCR screening and mapping products were partially verified by sequencing.

All primers used for PCR techniques and sequencing are listed in the supplemental material (see Table S1 in the supplemental material). Sequencing was performed by SEQLAB Sequence Laboratories Göttingen, Göttingen, Germany.

## RESULTS

**Hypermutation.** Fourteen of the 22 clonally different multiresistant strains showed at least a 20-fold-higher frequency of mutations conferring rifampin resistance than that observed for the wild-type strain PAO1. The mutation frequencies ranged from  $(5.85 \pm 0.68) \times 10^{-7}$  to  $(1.43 \pm 0.15) \times 10^{-5}$  (Table 1).

Eleven out of 12 multiresistant strains from CF patients were hypermutable. Of note, for two hypermutable strains (VI and XI) collected from CF patients, clonally related and identical strains, respectively, that did not show hypermutation phenotypes were found. The clonal variant VIa had a mutation frequency that was 2 orders of magnitude lower than that of strain VI. The same phenomenon was found for an additionally tested strain with a PFGE pattern identical to that of strain XI. All CF patients were infected by multiresistant strains with mutator phenotypes. Interestingly, three mutator strains were also found among the multiresistant strains isolated from non-CF patients. Two of those hypermutable strains originated from the respiratory tract, but one was isolated from wound specimens.

**Chromosomally mediated resistance mechanisms. (i) Efflux pump overproduction.** The EPI test indicated efflux pump overexpression for 10 strains (Table 2). For strain VIII, the EPI test yielded no result because the strain showed no growth in the presence of the efflux inhibitor.

The phenotypic EPI test is useful only for indicating the collective extent of overproduction of the efflux pumps MexAB-OprM, MexCD-OprJ, and MexEF-OprN, which can all be inhibited by Phe-Arg- $\beta$ -naphthylamide. To differentiate between those efflux pumps and to investigate the overproduction of MexXY-OprM, we used the following strategy. For strains with positive EPI test results, the efflux regulator-encoding genes *mexR* (MexAB-OprM) and *nfxB* (MexCD-OprJ) were sequenced. Strains without modifications or with functionally irrelevant modifications in MexR were additionally investigated for alterations in *nalC* leading to the overexpression of *mexAB-oprM*. *mexZ* (MexXY-OprM) was sequenced for all strains. For strains showing no disruption of *oprD*, the carbapenem resistance was further investigated by sequencing *mexT* (MexEF-OprN). As the influence of the oxidoreductase MexS on the expression of MexEF-OprN had been proven

TABLE 1. Multiresistant *P. aeruginosa* strains from CF and non-CF patients<sup>a</sup>

Strain	Epidemiology					Phenotypic test result(s)			
	Patient	Material	Ward	Type of ward	Center	Mutation frequency <sup>b</sup>	Cephalosporinase inhibition test	MBL test	EPI test
From CF patients									
I	A	SPU	CF	A	1	$(3.26 \pm 0.65) \times 10^{-9}$			
II	A	SPU	CF	A	1	<b><math>(3.56 \pm 1.67) \times 10^{-6}</math></b>	NR	–	+
III	B	SPU	CF	A	1	<b><math>(1.09 \pm 0.21) \times 10^{-5}</math></b>	+	NG	–
IV	C	SPU	CF	A	1	<b><math>(2.00 \pm 0.19) \times 10^{-6}</math></b>	NR	–	–
V	D	SPU	CF	N	1	<b><math>(1.27 \pm 0.93) \times 10^{-5}</math></b>	NR	–	+
VI	E	SPU	CF	A	1	<b><math>(6.57 \pm 1.14) \times 10^{-7}</math></b> ,	+	NG	–
VIa <sup>g</sup>		SPU	CF			$3.61 \times 10^{-9}$		–	–
VII	F	SPU	CF	A	1	<b><math>(2.50 \pm 0.82) \times 10^{-6}</math></b>	+	–	+
VIIa <sup>g</sup>		SPU	CF						
VIII	G	SPU	CF	A	1	<b><math>(7.30 \pm 4.91) \times 10^{-7}</math></b>	–	NG	NG
IX	H	SPU	CF	A	1	<b><math>(1.94 \pm 0.24) \times 10^{-6}</math></b>	+	–	–
X	I	SPU	CF	A	1	<b><math>(5.85 \pm 0.68) \times 10^{-7}</math></b>	+	–	–
XI	J	SPU	CF	A	1	<b><math>(1.43 \pm 0.15) \times 10^{-5}</math></b> ,	+	–	–
XII	K	SPU	CF	A	2	<b><math>(1.20 \pm 0.14) \times 10^{-6}</math></b>	+	–	+
From non-CF patients									
XIII	L	SPU	MED	N	1	$(8.03 \pm 3.68) \times 10^{-9}$	+	–	+
XIV	M	SPU	INF	N	1	$(1.47 \pm 0.48) \times 10^{-8}$	+	–	–
XV	N	BAL	ID	I	2	<b><math>(3.06 \pm 1.26) \times 10^{-6}</math></b>	+	–	–
XVI	O	BAL	NEU	I	2	$(2.01 \pm 0.66) \times 10^{-8}$	+	–	+
XVII	P	SPU	MED	I	2	$(3.50 \pm 0.66) \times 10^{-8}$	+	–	–
XVIII	Q	BLO	MED	I	2	$(3.24 \pm 0.55) \times 10^{-8}$	+	–	+
XIX	R	URM	HGC	N	2	$(1.57 \pm 0.44) \times 10^{-8}$	+	–	–
XX	S	SPU	INF	N	3	$(5.86 \pm 1.41) \times 10^{-8}$	+	–	+
XXI	T	WUP	ANA	I	3	<b><math>(2.26 \pm 0.23) \times 10^{-6}</math></b>	–	–	+
XXII	U	TRS	NEC	N	3	<b><math>(5.40 \pm 1.65) \times 10^{-6}</math></b>	–	–	+
PAO1						$(3.07 \pm 0.58) \times 10^{-8}$	–	–	–

<sup>a</sup> MBL, metallo-β-lactamase; BAL, bronchoalveolar lavage sample; BLO, blood culture; SPU, sputum; TRS, tracheal secretion; URM, midstream urine; WUP, wound pus; ANA, anesthesiology; HGC, heart vessel surgery; INF, infection ward; ID, interdisciplinary ICU; MED, internal medicine; NEC, neurosurgery; NEU, neurology; A, ambulant; I, intensive; N, normal; +, positive test result; –, negative test result or no modification or resistance gene found; NR, no result; NG, no growth in the presence of the inhibitors.

<sup>b</sup> Mutation frequencies of mutator strains are indicated in bold. Each strain was tested in triplicate; the additionally tested variant of clone VI and another strain with a PFGE pattern identical to that of clone XI showed divergent results for hypermutability (tested once).

<sup>c</sup> Modifications that are relevant for the function of the regulator are indicated in bold.

<sup>d</sup> Relevance postulated on the basis of the localization and sort of the amino acid change.

<sup>e</sup> Relevance elsewhere experimentally proven.

<sup>f</sup> Repeated sequencing attempts revealed nondifferentiable multisignals for bp 318 to 592.

<sup>g</sup> Clones VIa and VIIa are additionally tested variants of clones VI and VII, respectively, showing differences in mutation frequencies and/or chromosomal alterations.

TABLE 1—Continued

Genotypic test result(s)									
Chromosomal alteration(s) in indicated repressor									Acquired resistance and integrase genes
MexR <sup>c</sup>	NalC <sup>c</sup>	NfxB <sup>c</sup>	MexT <sup>c</sup>	MexS <sup>c</sup>	MexZ <sup>c</sup>	OprD	GyrA	ParC	
–	Gly71Glu	–			<b>Leu138Arg<sup>d</sup>, deletion C595, frameshift<sup>d</sup></b>	Deletion bp 745–748, frameshift	Thr83Ile	–	–
–	Gly71Glu	<b>Leu62Pro<sup>d</sup></b> Arg186His	Functional	<b>Gly68Ser<sup>d</sup></b>	<b>Gln134stop<sup>d</sup></b> <b>Tyr49Cys<sup>d</sup></b>	Trp138stop Trp277stop, Asp318Asn	–	–	–
–	Gly71Glu		Functional		<b>Leu123stop<sup>d</sup></b> Val58Ala, <b>Gly89Ser<sup>d</sup>,</b> <b>Arg143Gln<sup>d</sup></b>	Tyr91stop Trp138stop	Asp87Asn Thr83Ile	–	–
Val126Glu	Gly71Glu, Arg209Ser	–	Functional	<b>Phe273Ile<sup>d</sup></b>	<b>deletion G142, frameshift<sup>d</sup></b>	–	Thr83Val	–	–
–	Gly71Glu, Arg209Ser	–	Functional Val199Ala				Thr83Ala	–	–
–	Gly71Glu, Arg209Ser	–			<b>Thr32Ile<sup>d</sup>,</b> Ala175Val <b>Ser44Phe<sup>d</sup></b> <b>Asp155Gly<sup>d</sup></b>	Deletion G1017, frameshift Trp277stop G insertion after G635, frameshift	Thr83Ile Thr83Ile Thr83Ile	–	–
Val126Glu	Gly71Glu, Arg209Ser	Arg21His <b>Asp56Gly<sup>d</sup></b> <b>Ser167Pro</b>			<b>Arg45Leu<sup>d</sup></b>	Trp277stop	Asp87Asn	–	–
–	Gly71Glu, Arg209Ser	–			<b>Leu138Arg<sup>d</sup>, deletion bp 447–630, frameshift<sup>d</sup></b>	Trp277stop	Thr83Ile	–	–
–	Gly71Glu, Arg209Ser	–			<b>bp 318–592<sup>d,f</sup></b>	Trp277stop	Thr83Ile	–	–
–	Gly71Glu, Arg209Ser	–			<b>Deletion bp 491–496, frameshift<sup>d</sup></b>	Trp339stop	Thr83Ile	–	–
Val126Glu	Gly71Glu	–			<b>Leu138Arg, deletion bp 515–532, frameshift<sup>d</sup></b>	Deletion bp 155–167, frameshift	Thr83Ile	–	–
Val126Glu	Gly71Glu	–			–	Deletion C393, frameshift	Thr83Ile	Ser80Leu	<i>aac(6')Ib', intI1</i>
Val126Glu	Gly71Glu	–			<b>Gly89Ser<sup>d</sup>,</b> <b>Asp155Gly<sup>d</sup></b>	Trp138stop	Thr83Ile	–	<i>aadA1, aadB, aphA1-IAB, intI1</i>
<b>Lys44Met<sup>c</sup></b> Val126Glu	Gly71Glu Glu153Gln	–	Functional	–	<b>Val48Ala<sup>e</sup></b>	Deletion A1007, frameshift	Thr83Ile Asp87His Thr83Ile	Ser80Leu Ser80Leu	<i>aacA7, aacA8, bla<sub>OXA-2</sub>, intI1</i>
<b>C insertion after G75, frameshift<sup>d</sup></b> <b>Asn insertion after Leu52<sup>d</sup></b>	–	–	Functional	–	<b>Val48Ala<sup>e</sup></b>	Gln327stop	Thr83Ile	–	<i>bla<sub>PSE-1</sub>, intI1</i> <i>aac(6')Ib', aadA2, intI1</i>
Val126Glu	<b>C19 deletion</b> Gly71Glu Ala145Val	Arg21His <b>Asp56Gly<sup>d</sup></b>	Functional	–	<b>Val48Ala<sup>e</sup>,</b> Asn186Ser	–	Thr83Ile Asp87Gly	Ser80Leu	–
Val126Glu	Ala145Val	–			–	C insertion after T1002, frameshift	–	–	–

previously (38), for these strains the gene *mexS* was also sequenced.

For 6 (V, XII, XVIII, XX, XXI, and XXII) of the 10 strains with positive EPI test results, functionally relevant modifications in the repressors of MexAB-OprM (MexR/NalC) and/or MexCD-OprJ (NfxB) were detected (Table 2). Functionally relevant modifications in the repressors of both efflux systems

were found for only one strain (XXII). For the VI (except the variant VIa), VII, XIX, and XXI strains, whose carbapenem resistance levels were not due to the loss of the porin OprD, MexT was found to be a functional expression activator. For two of those strains, modifications in MexS were detected. A high proportion of strains (18 out of 22) with functionally relevant modifications in MexZ were found.

TABLE 2. Correlation of resistance phenotypes and mechanisms relevant for resistance to ureidopenicillins and cephalosporins, carbapenems, aminoglycosides, and quinolones for 22 multiresistant *P. aeruginosa* strains<sup>a</sup>

Strain	MIC (mg/liter) for:										
	Ureidopenicillins and cephalosporins				Carbapenems		Aminoglycosides			Quinolones	
	Piperacillin	Piperacillin-tazobactam	Ceftazidime	Cefepime	Imipenem	Meropenem	Gentamicin	Tobramycin	Amikacin	Ciprofloxacin	Levofloxacin
From CF patients											
I	128	128	>32	>32	32	8	16	2	32	4	8
II	>128	>128	>32	>32	32	8	32	4	>32	2	4
III	>128	>128	>32	>32	32	8	32	8	>32	4	8
IV	>128	>128	>32	>32	8	16	>32	32	>32	2	4
V	>128	>128	>32	>32	16	16	>32	8	>32	4	8
VI	64	32	32	32	8	4	>32	4	>32	4	8
VIa <sup>b</sup>											
VII	>128	>128	>32	>32	16	16	16	2	32	8	8
VIIa <sup>c</sup>											
VIII	64	32	8	32	8	4	16	2	>32	8	16
IX	>128	>128	>32	>32	>32	32	16	4	32	>8	128
X	>128	>128	>32	32	16	4	4	1	8	4	4
XI	128	128	32	32	16	8	16	2	16	2	4
XII	>128	128	>32	32	16	4	4	4	32	>8	16
From non-CF patients											
XIII	>128	>128	>32	>32	32	16	32	8	>32	4	8
XIV	>128	>128	>32	>32	>32	32	16	2	>32	4	8
XV	64	32	16	>32	32	16	>32	>32	>32	>8	32
XVI	64	32	32	16	16	8	>32	16	8	>8	32
XVII	64	32	>32	>32	32	16	>32	>32	16	>8	16
XVIII	32	16	16	16	16	16	32	16	>32	>8	128
XIX	>128	>128	32	>32	32	16	16	16	32	>8	16
XX	>128	>128	>32	>32	16	8	>32	16	16	>8	32
XXI	32	32	16	8	4	2	8	2	16	>8	128
XXII	32	32	16	16	16	16	2	1	8	2	8
PAO1	4	4	2	2	2	0.5	2	0.5	4	0.13	0.5

<sup>a</sup> Variants of strains are listed only for cases in which divergent results for resistance mechanisms were obtained. NA, no analysis possible; NM, no modification; +, positive test result or detection of resistance-relevant modification(s); -, negative test result or no detection of resistance-relevant modification(s) or acquired genes.

<sup>b</sup> Variant of clone VI.

<sup>c</sup> Variant of clone VII.

(ii) **Loss of OprD.** Loss of OprD due to premature stop codons or frameshifts was found in 18 out of 22 multiresistant *P. aeruginosa* strains.

(iii) **Type II topoisomerase alterations.** Modifications in the QRDR in the genes coding for the quinolone target enzymes DNA gyrase and topoisomerase IV were found in 20 out of the 22 multiresistant *P. aeruginosa* strains investigated in this study. Sixteen strains had alterations in GyrA only. Simultaneous mutations in *gyrA* and *parC* were found four times, exclusively among strains from non-CF patients. In two strains and one clonal variant (VIa), no target modifications were detected. None of the strains showed mutations in *parC* only.

(iv) **AmpC overproduction.** Up-regulated production of the chromosomal  $\beta$ -lactamase AmpC was shown for 17 of the 22 multiresistant *P. aeruginosa* strains by using the cephalosporinase inhibition test. For three strains (II, IV, and V), the test revealed no result because, in the presence and absence of the inhibitor cloxacillin, no ceftazidime inhibition zone was detected.

**Acquired resistance determinants.** Acquired resistance genes were detected in only five (XVI, XVII, XVIII, XIX, and XX) multiresistant *P. aeruginosa* strains, all from non-CF patients. *intI1*, coding for the integrase of class 1 integrons, was

detected in these strains. *intI2* was not detected. The acquired resistance determinants found were three adenyltransferase genes (*aadA1*, *aadA2*, and *aadB*), three acetyltransferase genes [*aac(6')-Ib*, *aac(6')-Ib'*, *aacA7*, and *aacA8*], and one phosphotransferase gene (*aphA1-IAB*). Two  $\beta$ -lactamase genes, *bla*<sub>OXA-2</sub> and *bla*<sub>PSE-1</sub>, were detected. The EDTA-phenanthroline-imipenem microdilution test and the PCR screening revealed the absence of metallo- $\beta$ -lactamases. The *qnrA* gene lowering quinolone susceptibility was not found.

To characterize the genetic context of the acquired resistance genes found, PCR mapping experiments were carried out. As depicted in Fig. 1, all resistance genes detected by PCR but *aphA1-IAB*, an AME-encoding gene usually not integron associated (36), were located in class 1 integrons.

## DISCUSSION

We analyzed the contribution of eight chromosomally mediated resistance mechanisms and 25 acquired resistance determinants to multiresistance for clinical multiresistant *P. aeruginosa* strains from CF and non-CF patients, taking into account the presence of a hypermutation phenotype.



TABLE 2—Continued

EPI test	Result for indicated resistance mechanism						OprD status	Type II topoisomerase		AmpC overproduction	Acquired resistance gene(s)
	Efflux							GyrA	ParC		
	MexR, NalC (MexAB-OprM)	NfxB (MexCD-OprJ)	MexT (MexEF-OprN)	MexS (MexEF-OprN)	MexZ (MexXY-OprM)						
-						+	Loss	Thr83Ile	-	+	-
+	-	-				+	Loss	-	-	NA	-
-						+	Loss	Asp87Asn	-	+	-
-						+	Loss	Asp87Asn	-	NA	-
+	-	+				+	Loss	Thr83Ile	-	NA	-
-			Intact			-	NM	Thr83Ile	-	+	-
+	-		Intact	+		+	Loss	-	-	+	-
+	-			+		+	NM	Thr83Val Thr83Ala	-	+	-
NA	-					+	Loss	Thr83Ile	-	-	-
-	+						Loss	Thr83Ile	-	+	-
-	+						Loss	Thr83Ile	-	+	-
-	+						Loss	Thr83Ile	-	+	-
+	-	+				+	Loss	Asp87Asn	-	+	-
+	-						Loss	Thr83Ile	-	+	-
+	-					-	Loss	Thr83Ile	-	+	-
-						+	Loss	Thr83Ile	-	+	-
+	-					+	Loss	Thr83Ile	Ser80Leu	+	<i>aac(6')Ib'</i>
+	+					+	Loss	Thr83Ile	Ser80Leu	+	<i>aadA1, aadB,</i> <i>aphA1-IAB</i>
-	Intact					-	NM	Thr83Ile	Ser80Leu	+	<i>aacA7, aacA8,</i> <i>bla<sub>OXA-2</sub></i>
+	+					+	Loss	Thr83Ile	-	+	<i>aac(6')Ib,</i> <i>aadA2,</i> <i>bla<sub>PSE-1</sub></i>
+	+		Intact	-		-	NM	Thr83Ile	Ser80Leu	-	<i>aac(6')Ib',</i> <i>aadA2</i>
+	+	+				+	Loss	Thr83Ile	-	-	-
+	+						NM	Thr83Ile	Ser80Leu	-	-
+	+						Loss	Asp87Gly	-	-	-

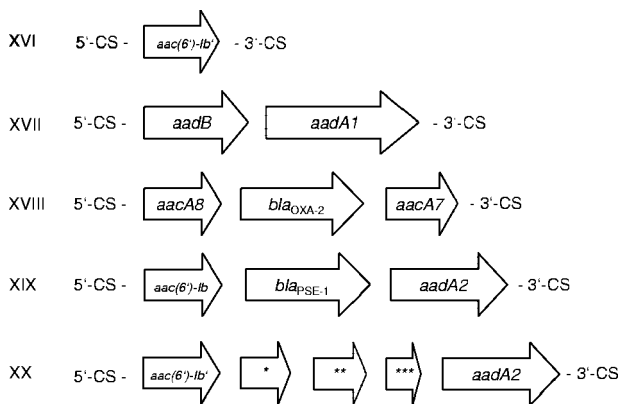


FIG. 1. Structures of class 1 integrons detected exclusively in multiresistant *P. aeruginosa* clones from non-CF patients. Sequence analysis (with the BLASTX program) revealed 79% amino acid sequence homology with QecE (NP\_044260) (\*), 45% amino acid sequence homology with hypothetical protein MED121\_04115 of *Marinomonas* sp. strain MED121 (ZP\_01077982) (\*\*), and 57% amino acid sequence homology with the RecA/RadA recombinase of *Yersinia bercovieri* (ZP\_00823172) (\*\*\*). 5'-CS, 5' conserved segment; 3'-CS, 3' conserved segment.

**Ureidopenicillin and cephalosporin resistance.** Regarding the phenotypes resistant to ureidopenicillins (piperacillin) and antipseudomonal cephalosporins (ceftazidime and cefepime), an overall higher resistance level was observed for strains from CF patients (Table 2). Consistent with former studies (20), overproduction of AmpC was, with a proportion of 73%, the predominant and, for the resistance level, the most important mechanism of resistance to ureidopenicillins and cephalosporins among the multiresistant *P. aeruginosa* strains investigated in this study. Overproduction of AmpC leads usually to MICs of cefepime lower than those of ceftazidime. However, in at least five strains (VIII, XI, XV, XVIII, and XXII) with cefepime MICs as high as or higher than the MICs of ceftazidime, this phenotype may be explained by the (additional) overproduction of MexXY-OprM, as found in a previous study (11). Of note,  $\beta$ -lactam resistance was almost exclusively due to chromosomally mediated resistance mechanisms. The acquired  $\beta$ -lactamase genes *bla<sub>PSE-1</sub>* and *bla<sub>OXA-2</sub>* were detected in only two strains, both from non-CF patients.

**Carbapenem resistance.** Metallo- $\beta$ -lactamases are rare in Germany (10, 43) and were not detected in this study. Confirming former studies (32, 20), loss of OprD was, with a proportion of 82%, the predominant and, for the resistance level, the most important mechanism of imipenem resistance

(Table 2). In so-called *nfxC* mutants, the reduced production of OprD leads to borderline resistance to imipenem (MIC, 4 mg/liter) (18). Accordingly, for two strains (VII and VI) the reduced production of OprD due to modifications in MexT and MexS (*nfxC* mutants) seems to be the reason for reduced imipenem susceptibility. *nfxB* mutants overproducing MexCD-OprJ have heterogeneous phenotypes. In this study, the hypersusceptibility to imipenem that was reported for type B *nfxB* mutants (24, 45) was not found.

For meropenem, several mechanisms reducing susceptibility are known. However, each of these mechanisms alone leads only to a slight decrease in susceptibility, not conferring full resistance. In this study, the most frequent mechanisms lowering meropenem susceptibility were loss of OprD (82%) and overproduction of AmpC (73%), which result in meropenem MICs of 4 mg/liter and up to 2 mg/liter, respectively (16). Meropenem is unlike the imipenem substrates of several efflux pumps: while overproduction of MexCD-OprJ and MexXY-OprM leads to poorly increased MICs of meropenem, overexpression of MexAB-OprM confers meropenem MICs of 4 mg/liter (25). For the two *nfxC* mutants (strains VII and VI), the interaction of reduced OprD production and AmpC overproduction seems to be the reason for reduced meropenem susceptibility, whereas additional efflux pump overproduction leads to full meropenem resistance in strain VII. For several strains (e.g., IX and XIX) resistant to meropenem and imipenem, mechanisms other than the known mechanisms of carbapenem resistance seem to contribute additionally to resistance. The high levels of resistance seen for these strains are unlikely to be caused solely by the mechanisms detected.

**Aminoglycoside resistance.** The analysis of aminoglycoside resistance mechanisms and the associated aminoglycoside susceptibility phenotypes revealed one important difference between multiresistant *P. aeruginosa* strains from CF and non-CF patients: numerous AME genes were found only in strains from non-CF patients (Table 2). These resistance genes were located in class 1 integrons, partially repeated and found in combination with  $\beta$ -lactamase genes. The finding of acquired resistance genes in half of the multiresistant strains from non-CF patients points to the decisive role of horizontal transfer of resistance mechanisms for *P. aeruginosa* strains from this group of patients. The absence of any acquired resistance genes in multiresistant strains from CF patients emphasizes the relevance of the mutator phenotype for the autarkic evolution of multiresistant *P. aeruginosa* strains during chronic infection of the lungs of CF patients. The only chromosomally mediated high-level aminoglycoside resistance mechanism in clinical *P. aeruginosa* strains known so far, overproduction of MexXY-OprM, was found in 82% of the multiresistant strains. Some studies, including this work, show that the impact of this efflux pump on resistance in *P. aeruginosa* may have been underestimated so far (11, 12, 37). The overproduction level of MexXY-OprM can vary significantly. Quantitative real-time PCR experiments revealed 3- to 727-fold-increased amounts of MexY mRNA in overproducing strains (12). Variety in *mexXY* expression levels might explain the broad range of aminoglycoside resistance phenotypes, particularly in multiresistant *P. aeruginosa* strains from CF patients found in this study. The cause may be the different impacts and numbers of modifications in the repressor MexZ. However, previous studies pro-

vided evidence of the existence of additional components involved in *mexXY* expression regulation besides MexZ, as overproducing strains without modifications in MexZ were detected. Furthermore, the contribution of other, unknown components of aminoglycoside resistance was suggested, as no clear correlation between *mexXY* expression levels and aminoglycoside MICs was found (12, 37). These components might also be involved in aminoglycoside-resistant strains found in this work that lack modifications in MexZ and acquired AME genes.

**Quinolone resistance.** The most noticeable result was the difference in quinolone resistance levels between strains from CF patient and non-CF patient strains: while 7 out of 10 strains from non-CF patients showed ciprofloxacin MICs of >16 mg/liter, only 2 of 12 strains from CF patients had this level of resistance to ciprofloxacin (Table 2). The analysis of surveillance data generated as part of the GENARS project confirmed this phenomenon with a higher number of strains, including the strains investigated in this study: of 207 *P. aeruginosa* strains from non-CF patients, 32.9% were intermediately resistant and 67.1% were fully resistant to ciprofloxacin. In contrast, of 78 strains from CF patients, 46.2% showed intermediate resistance and 51.3% showed full resistance to ciprofloxacin. Lower concentrations of quinolones in the mucus of the CF lung or biofilm formation may reduce the need for high-level resistance in CF patient strains.

The obvious differences in quinolone resistance between *P. aeruginosa* strains from CF and non-CF patients were also found on a molecular level. High-level quinolone resistance conferred by modifications in ParC in combination with (multiple) modifications in GyrA was exclusively detected in *P. aeruginosa* strains from non-CF patients. Besides target modifications, the overproduction of MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM can also contribute to quinolone resistance. A previous study of quinolone resistance in *P. aeruginosa* strains from CF patients revealed no modifications in *mexR* or *parC*, but overproduction of MexCD-OprJ was most prevalent (13). In a comparative study with quinolone-resistant *P. aeruginosa* isolates from urinary tract and wound infections, numerous modifications in *gyrA*, *parC*, and *mexR* were found, while overproduction of MexCD-OprJ played a minor role (14). This study confirms the results of both studies. However, several strains with different resistance levels showing modifications in GyrA and MexZ only were found in this study. Their quinolone resistance levels may be due to different high expression levels of *mexXY* comparable to those found for aminoglycoside resistance due to MexXY-OprM overproduction. These findings emphasize the need for further investigations about the variable contribution of MexXY-OprM to multiresistance in *P. aeruginosa*.

**Mutator strains.** A high proportion of resistance mechanisms detected in the multiresistant *P. aeruginosa* strains were mutation mediated; therefore, we were interested in the mutator phenotypes of the strains.

In our study, all but one multiresistant *P. aeruginosa* strain from CF patients were hypermutable. This extremely high proportion of mutator phenotypes is in concordance with recent studies that found hypermutation to be the key factor in development of mutation-mediated multiresistance in patients with chronic *P. aeruginosa* lung infections (23, 31). The ex-

tremely high proportion of mutator strains found in this study is expected to have important consequences for the treatment of multiresistant *P. aeruginosa* strains from CF patients. A multiresistant *P. aeruginosa* strain from a CF patient is most probably also hypermutable and very likely to acquire additional resistance properties during therapy. Three mutator strains were also detected among the multiresistant strains from non-CF patients. Two of those originated from respiratory tract specimens and might be assigned to chronic lung infections other than CF that seem to favor in the same way the selection of hypermutability (23). Interestingly, one hypermutable *P. aeruginosa* strain was isolated twice from wound specimens of a patient who was medicated in an ICU anesthesiology ward and subsequently in a normal abdominal surgery ward. To our knowledge, this is the first report of a *P. aeruginosa* mutator strain that was not isolated in the context of a chronic lung infection.

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