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Dynamics of Mutations during Development of Resistance by *Pseudomonas aeruginosa* against Five Antibiotics

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***Pseudomonas aeruginosa* is an opportunistic pathogen that causes considerable morbidity and mortality, specifically during intensive care. Antibiotic-resistant variants of this organism are more difficult to treat and cause substantial extra costs compared to susceptible strains. In the laboratory, *P. aeruginosa* rapidly developed resistance to five medically relevant antibiotics upon exposure to stepwise increasing concentrations. At several time points during the acquisition of resistance, samples were taken for whole-genome sequencing. The increase in the MIC of ciprofloxacin was linked to specific mutations in *gyrA*, *parC*, and *gyrB*, appearing sequentially. In the case of tobramycin, mutations in *fusA*, *HP02880*, *rplB*, and *capD* were induced. The MICs of the beta-lactam compounds meropenem and ceftazidime and the combination of piperacillin and tazobactam correlated linearly with beta-lactamase activity but not always with individual mutations. The genes that were mutated during the development of beta-lactam resistance differed for each antibiotic. A quantitative relationship between the frequency of mutations and the increase in resistance could not be established for any of the antibiotics. When the adapted strains are grown in the absence of the antibiotic, some mutations remained and others were reversed, but this reversal did not necessarily lower the MIC. The increased MIC came at the cost of moderately reduced cellular functions or a somewhat lower growth rate. In all cases except ciprofloxacin, the increase in resistance seems to be the result of complex interactions among several cellular systems rather than individual mutations.**

The medical consequences of antibiotic resistance, such as fewer options for and increased costs of treating infectious diseases, are well recognized. The pathway to resistance consists of sequential mutations or acquisition of resistance genes driven by the selective pressure caused by antibiotic exposure (1). Once resistance has been acquired, the cell rarely reverses to become sensitive again, compensating for the metabolic costs instead (2, 3). The increased level of resistance caused by an antibiotic treatment typically prescribed by primary care physicians is very noticeable when subsequent further treatment is necessary (4). Hence, in order to limit the development of resistance when antibiotics have to be used, treatment protocols need to be devised to prevent this side effect. Rational design of such protocols requires knowledge of the molecular mechanisms that cause resistance. One of the central questions is whether similar mechanisms are operational for all drugs or whether resistance to each drug is induced in a distinct manner. Other basic questions center on evolutionary pathways to clinically significant resistance and the persistence of molecular changes after treatment.

Molecular changes that cause the development and persistence of drug resistance can be identified by combining experimental evolution and whole-genome sequencing (WGS), provided that the proper controls are used (5, 6). This study used the pathogen *Pseudomonas aeruginosa* as a model to achieve this goal, as it is an important opportunistic pathogen, for example, in patients suffering from cystic fibrosis (7). Several antibiotics are used as the treatment of choice for intensive care patients infected with *P. aeruginosa*. The bacteria were adapted to the following five most-often-used drugs: the fluoroquinolone ciprofloxacin, the aminoglycoside tobramycin, and the beta-lactam antibiotics ceftazidime, meropenem, and piperacillin in combination with the beta-lactamase inhibitor tazobactam. This experimental design allows the comparison of three classes of antibiotics and has three members of a single class to document the variability within a class as a biological control for the interclass comparison. The outcome suggests that the *de novo* buildup of resistance cannot be attributed only to DNA mutations but rather develops as a result of intricate interactions between cellular adaptation and mutations.

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions. The antibiotic-susceptible wild-type strain *P. aeruginosa* ATCC 27853 was used as the ancestor strain in all resistance evolution experiments. Batch cultures were grown in either rich or defined minimal medium to assess the influence of the growth environment on the development of resistance. The rich medium was cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) autoclaved at 115°C for 10 min. The minimal medium was Evans medium containing 55 mM glucose at pH 6.9 (8). Evans medium was autoclaved for 20 min at 121°C, with the exception of glucose, which was autoclaved for 10 min at 110°C and added afterward. Continuous cultures were performed only with Evans medium with the concentrations of glucose and Na_2HPO_4 lowered to 5 and 10 mM, respectively.

Precultures for the inoculation of 96-well plates, batch cultures, and

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continuous cultures were grown overnight in 100-ml flasks shaken at 200 rpm at 37°C. Continuous cultures were carried out in Sixfors fermenter vessels (Infors AG, Bottmingen, Switzerland) consisting of six vessels with a working volume of 250 ml at 37°C and constant stirring at 250 rpm. The pH was maintained at 6.9 by automatically adding sterilized 2 N NaOH. Culture parameters such as pH, temperature, and the stirring rate were monitored continuously. The continuous culture was assumed to have reached a steady state when all of the parameters measured, including cell density and optical density at 600 nm (OD_{600}), remained constant after five to seven volume changes. Samples were taken at every steady state to determine the dry weight and number of cells and the glucose concentration of the culture medium.

Evolution experiments. For experiments documenting the development of resistance, cultures were initially grown at the maximum antibiotic concentration that allowed growth. Whenever normal or approximately normal growth (OD_{600} at >75% of the OD_{600} for normal growth) occurred, a small aliquot of the culture was used to start two more incubations, one at the same concentration and the other at double the concentration (9). The stepwise increasing exposure to an antibiotic was stopped when the saturation level of the antibiotic was reached or continued for 30 days at most. After the adaptation, cultures were grown in fresh medium without drugs for 15 days to observe the sustainability of the acquired resistance after treatment. Independent duplicates were performed with each antibiotic. The MIC was determined every day for both duplicates. Daily samples were preserved at −80°C for further tests, including WGS, beta-lactamase activity measurement, and fitness evaluation. These tests were done with samples revived at the concentration of antibiotic to which they were adapted.

Antibiotics and MIC measurement. Five antibiotics were tested in this study, i.e., three beta-lactam antibiotics, ceftazidime, meropenem, and piperacillin, combined with the beta-lactamase inhibitor tazobactam; the aminoglycoside tobramycin; and the fluoroquinolone ciprofloxacin. The 10-mg/ml stock solutions of ceftazidime (Fresenius Kabi), meropenem (Fresenius Kabi), piperacillin-tazobactam (Fresenius Kabi), and ciprofloxacin (Fluka) were filter sterilized (0.2 μm) and preserved in a freezer at −20°C. Each stock solution of these drugs was used only once and remade freshly every week. The tobramycin was purchased in a solution of 80 mg/2 ml (Obracin), stored according to the manufacturer's instructions, and used before the expiration date.

MICs were measured by monitoring the growth of cells exposed to antibiotic concentrations increasing by factors of 2 in 96-well plates as described previously (10). The ranges of antibiotic concentrations were adjusted according to the expected resistance level of the sample tested. All measurements were performed in duplicate. The starting OD_{600} was 0.05. The MIC was defined as the minimal concentration of antibiotic that limited growth to an OD_{600} of ≤0.2 after 23 h.

***De novo* sequencing and annotation of the reference strain.** The culture's genome was isolated with the DNeasy blood and tissue kit (Qiagen). *De novo* sequencing of the reference strain was performed by using the Illumina and PacBio platforms at BaseClear B.V. (Leiden, The Netherlands). For Illumina sequencing, high-molecular-weight genomic DNA (gDNA) was used as the input for library preparation with the Illumina Nextera XT library preparation kit (Illumina). Briefly, the gDNA was fragmented by random transposon integration, DNA adapters with sample-specific bar codes were added, and the library was amplified by PCR. The resulting Illumina library was checked on a Bioanalyzer (Agilent) and quantified. The library was multiplexed, clustered, and sequenced on an Illumina HiSeq 2500 by a paired-end 125-cycle protocol. For PacBio sequencing, high-molecular-weight gDNA was sheared to about 10-kb lengths with g-TUBES (Covaris) and further processed into a PacBio sequencing library by standard protocols (Pacific Biosciences). The resulting PacBio library was checked on a Bioanalyzer (Agilent), quantified, and sequenced on a PacBio RSII.

The quality of the Illumina FASTQ sequences was enhanced by trimming off low-quality bases with the program bbduk, which is part of the BBMap suite, version 34.46. The quality-filtered sequence reads were assembled into a number of contig sequences with ABySS version 1.5.1 (11). These contigs were then linked and placed into superscaffolds based on the alignment of the PacBio CLR reads with BLASR (12). From the alignment, the orientation, order, and distance between the contigs were estimated. This analysis was performed with the SSPACE-LongRead scaffolder, version 1.0 (13). The gapped regions within the superscaffolds were (partially) closed in an automated manner with GapFiller, version 1.10 (14). The complete sequence was reached in one scaffold with a total size of 6,827,737 bp.

Genome annotation of the assembled contig or scaffold sequences was performed with the BaseClear (BaseClear B.V., Leiden, The Netherlands) annotation pipeline, which is based on the Prokka Prokaryotic Genome Annotation System (Victorian Bioinformatics Consortium, Melbourne, Australia).

WGS and data analysis. WGS was used at those points where the MIC significantly increased in response to drug exposure at the end of the drug exposure period and after 15 days of continued growth in the absence of the drug. Strains grown in either Mueller-Hinton broth or mineral medium for 30 days served as controls for mutations occurring during growth in the absence of antibiotics. DNA was also collected with the DNeasy blood and tissue kit (Qiagen). gDNA libraries were generated according to the manufacturer's protocols with the Ion Xpress Plus gDNA Fragment Library Preparations (Life Technologies). Shearing of 100 ng of gDNA was performed with the Covaris M220 Focused-ultrasonicator in accordance with the 200-bp protocol provided by Life Technologies. Barcoded libraries were prepared with the Ion Plus fragment library kit (Life Technologies) and the Ion Xpress DNA bar-coding kit (Life Technologies) according to the instructions of the manufacturer of the 200-base-read Ion Proton libraries. The size distribution and yield of the bar-coded libraries were assessed with the 2200 Tapestation System by using Agilent High Sensitivity D1000 ScreenTapes (Agilent Technologies). Sequencing templates were prepared with the Ion PI Template OT2 200 kit v3 on an Ion OneTouch 2 system and enriched on an Ion OneTouch ES system (Life Technologies). Sequencing was performed with the Ion Proton system with the Ion PI Chip v2 and the Ion PI Sequencing 200 kit v3 (Life Technologies) according to the manufacturer's protocols.

The FASTQ files were subjected to quality control procedures. The quality of individual samples was assessed with fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In addition, several quality metrics (sequencing depths, read length distributions, read quality distributions, mean read quality along the read, base frequencies at each read position) were compared across the samples, in relation to the experimental factors, with in-house software based on samtools (15) and R statistical software (https://www.r-project.org/). Tmap (16) was used to map all accepted reads from all samples to the *P. aeruginosa* ATCC 27853 reference genome. The Ion Proton system generates sequencing reads of variable lengths, and Tmap combines a short-read algorithm (17) and long-read algorithms (18, 19) in a multistage mapping approach. The average sequencing depth was 220, with a range of 185 to 253. Deviations from the reference genome, such as single-nucleotide variants, insertions, and deletions, were identified with the standalone Torrent Variant Caller, v4.2. (Thermo Fisher Scientific, Waltham, MA). The abundance of the mutations is presented as a mutation frequency calculated as the ratio of the number of reads containing a genetic variation to the overall read number.

Possible gene duplication was searched for by aligning reads of experimental samples with the reference genome and calculating the number of reads that map for every gene. Both forward and reverse complemented reads were counted in nonoverlapping "windows" of 100 nucleotides. Counts were normalized and copy numbers were estimated with haplocn.mops, which is especially designed for haploid organisms (http://www.bioinf.jku.at/research/ehec/ehec.html) (20). Pairwise comparisons of the control and experimental samples for every gene were performed in the R

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TABLE 1 Sequences of the primers used for PCR of the areas of interest within the genes indicated

Gene	Primer sequence (5′-3′)	
	Forward	Reverse
ampD	GTAGACCACCACCAGAAG	AATACCTTTCCTCGACGC
dacB	ATCGGGCCTGGAGAAT	TTCGCGTGATGTCCGT
yerD	GACATGAAAAAGCCGGAG	CGAAGAAGGTGACTACCA
hfq	CCCTTCCAGATGCACCA	TTGTCCGTCTGTTTCCG
prkC	GAA AAC CAG GAC GC	GTC TTT CTG CCC CGT
HP06356	AGC AAT GTT GTG CCG AT	CTT TGC CGT AAC TTT CAT TC
oprD	CTGCGTGCTATAAGTTAG	CTACGCCCTTCCTTTATA
mexR	AAGCGGATACCTGAAACG	AAGCCTCGCGTGAAAACA
mexB	TCGAGGTGAAGACCGT	GTCGATCCTCAAGCATCG
NC3312	TCA CCC TGA TCG CTC	TTT CCT GGG TTG ATT GAT CG
ampC	GCT CAT GGC ACC ATC ATA G	GGG GCG GTT TCT CAT
NC3725	ACC GTG TTC GAG AAA GG	TCG ACC TCC TCC AAC
gyrA	CGT TGA TTT GTA GTG AGT TGG	ACT CTC GCT ATA GGT AGG G
gyrB	ACCCGCAACTATTGAAAG	CAAGCTACAGGCAAA
parC	GGGAGACGACATTC	GCTACTCGGCATG
CPA_2	CCATCGGTAGCGAA	CGT CTA CGC ATT GTC
fusA	CAA GAA GCG TGA AGA	AGCCACGACAGTAAA
UK02280	CCCGTCCGTTTT	GCGTTATACTCGCC
rplB	GGGCAAGCGTAA	CTTGCGGTCGTT
capD	TATTTGCCAGACCAA	AAA GCA GTC GCT TC

programming language to calculate differences as follows: diff = $\log_2$ sample count − $\log_2$ control count.

Genes were plotted in the order of the genome and assessed for individual genes or cluster of genes that gained a copy or was deleted. A gene was considered to have been duplicated if the ratio exceeded 1, representing a single duplication, and it occurred in more than one experimental sample. Applying these standards, no gene was found to have been duplicated.

To distinguish mutations caused by sequencing errors or selected for by the growth medium, the detected genetic variations were excluded in the final analysis when one of the following conditions applied: (i) the Phred quality score was <20, (ii) the depth was <100, (iii) mutations appeared only once and at a frequency of <10%, or (iv) mutations also occurred in cells growing in the absence of drugs for 30 days. Mutations with Phred quality scores between 10 and 20 and/or sequencing depths between 40 and 100 were included when they were found more than once.

Reproducibility of mutations detected. WGS of one of the duplicate strains was performed, and PCR was used to ascertain the presence of these mutations in the other replicate at the same time points. The Primers used are shown in Table 1. The PCR products of six colonies for each combination of antibiotic and time point were purified with the MSB Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe by Sanger sequencing with an ABI 3730XL DNA analyzer.

Beta-lactamase assay. Beta-lactamase activity was measured with an assay based on the chromogenic substrate nitrocefin (21) Briefly, 1 ml of culture was harvested and washed in buffer (pH 7.0). The cells were lysed in 1% Triton X-100, and the lysates were centrifuged. Beta-lactamase activity was determined as the rate of nitrocefin hydrolysis with protein as the normalization factor.

Assessment of fitness cost. To determine maximum growth rates (μ_{max}) the growth of batch cultures was monitored for 23 h by measuring OD_{600}. The μ_{max} was calculated as the average growth rate of four independent replicates during exponential growth. Dry weight was calculated as the added weight on a preweighed filter dried overnight at 110°C. Cell number was quantified by counting colonies on antibiotic-free agar plates after dilution. Glucose concentrations were determined enzymatically.

RESULTS

To explore the role of mutations in the *de novo* development of resistance by *P. aeruginosa*, cells were made resistant to medically relevant antibiotics and WGS was performed at several time points during this process. Resistance to three beta-lactam antibiotics, ceftazidime, meropenem, and piperacillin, in combination with the beta-lactamase inhibitor tazobactam; to the aminoglycoside tobramycin; and to the fluoroquinolone ciprofloxacin was induced. Identification of the mutations that accompany the acquisition of resistance allowed a comparison of the potential differences within the beta-lactam class of antibiotics and the expected differences between the representatives of separate classes. Since preliminary experiments showed that the acquisition of resistance differs when *P. aeruginosa* is grown in mineral or rich medium, both media were used.

No or barely any resistance to the beta-lactam antibiotics developed during growth and exposure in mineral medium (Fig. 1). There was no meaningful difference in the development of resis-

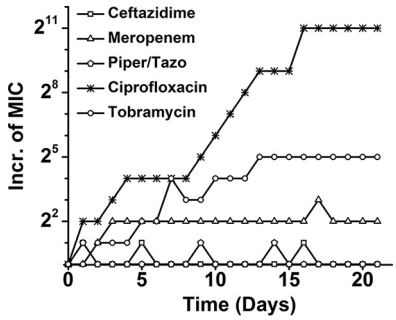

FIG 1 Antibiotic resistance development by *P. aeruginosa* expressed as 2-fold increases (Incr.) in MICs as a function of time (days) in minimal medium with stepwise increasing concentrations of the antibiotics indicated. Piper, piperacillin; Tazo, tazobactam.

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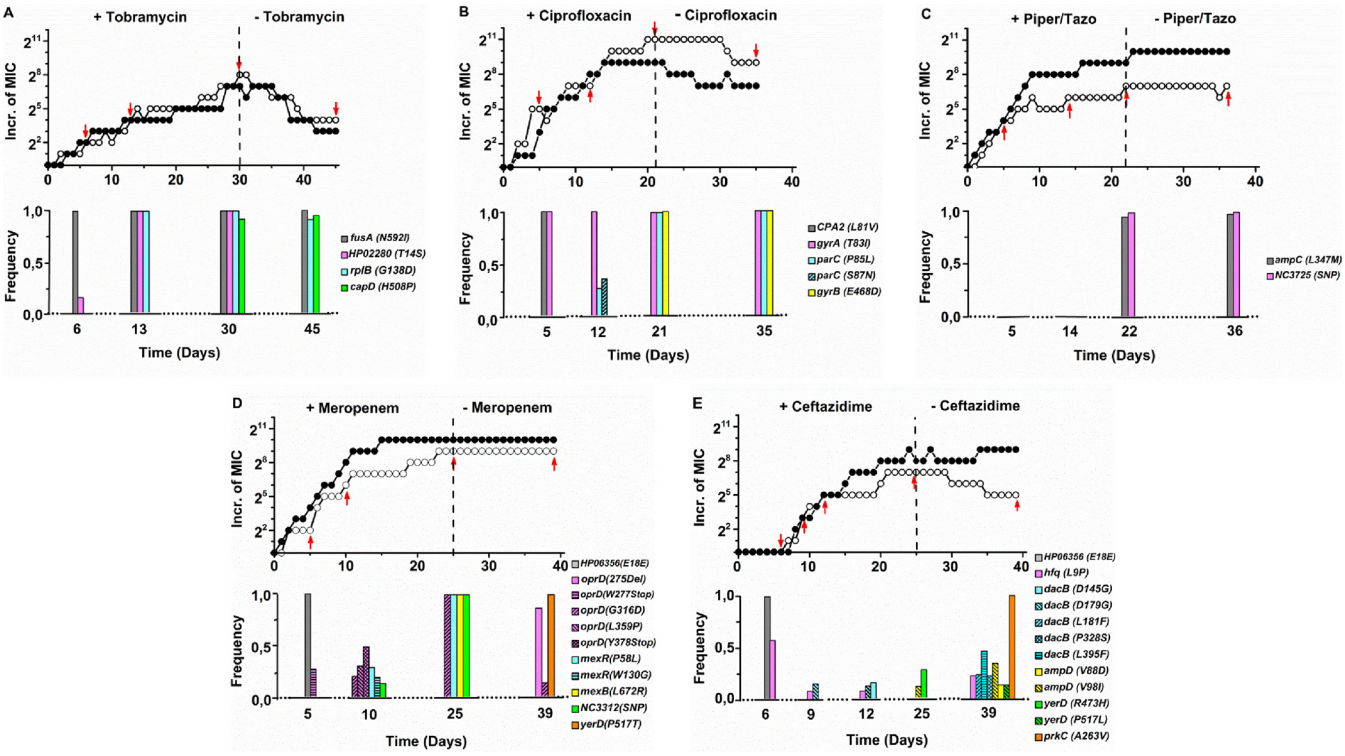

FIG 2 Increase (Incr.) in the MIC by factors of 2 and frequencies of mutations observed in the replicates on which WGS was performed as a function of time (days) in the presence of the antibiotic indicated and after its removal from the growth medium. All mutations found at frequencies exceeding 0.5 were also discovered in the second replicate by PCR, except for the *parC* mutations in response to ciprofloxacin, where P85L in the first was replaced by S87N in the second. Piper, piperacillin; Tazo, tazobactam.

tance to tobramycin and ciprofloxacin in mineral or rich medium (compare Fig. 1 and 2). In rich medium, the MIC of a drug for *P. aeruginosa* increased within 20 to 30 days by 7 to 11 2-fold steps, depending on the drug (Fig. 2). Once the drug was removed and growth continued in its absence, the MIC decreased slightly or not at all. The difference between the replicates in the acquisition of resistance was meaningful only in the case of the 8-fold lower MIC of piperacillin-tazobactam for one replicate than for the other. The MIC of ceftazidime decreased for one replicate after exposure was ended but not for the other.

At four or five time points (indicated with red arrows in Fig. 2), WGS of one of the replicates was performed with a sequencing depth of roughly 200 times. The hypothesis was that mutations would appear at a low frequency in the beginning and over time become dominant. The actual outcome differed from that expected to various degrees (Fig. 2). In the figures, mutations are omitted that appeared only once and at a frequency of <10%. The presence of the same mutations in the replicate cultures was checked by sequencing PCR products of the DNA in the region around the mutation. Unless specifically mentioned, the first and second replicates had the same mutations. The theoretical possibility that additional mutations had occurred in the replicate strain was not verified experimentally.

The accumulation of mutations was comparatively straightforward when cells were exposed to ciprofloxacin. After five transfers, the usual T83I *gyrA* mutation for fluoroquinolones (22) appeared, accompanied by a mutation in *CPA2* (Fig. 2A). After another seven transfers, the *CPA2* mutation disappeared entirely and two *parC* mutations emerged in less than half of the population. Analysis of the individual reads, averaging 200 bases, suggests that the neighboring *parC* mutations never occurred in a single chromosome. In the next stage, when the highest MIC had been reached, only one of the two *parC* mutations, P85L in the first replicate and S87N in the second, remained and a *gyrB* mutation appeared in the whole population. Even though these mutations remained, the MIC was slightly reduced during growth in the absence of ciprofloxacin. Exposure to tobramycin immediately yielded a mutation in *fusA* in the whole population and a low frequency of mutation in *HP02280* (Fig. 2B). Both were found at a frequency of one a few transfers later, accompanied by a mutation in *rpB*. When the highest MIC was reached, a new *capD* mutation attained a frequency of almost one as well. After 15 transfers in drug-free medium, the *HP02280* mutation disappeared and the MIC was considerably reduced.

The picture for the three beta-lactam antibiotics was considerably more complex. No persistent mutations were observed in the initial stages, when considerable resistance had already developed. When cells were already almost completely resistant to the combination of piperacillin and tazobactam, two mutations were observed, the usual one in *ampC* and an unknown single-nucleotide polymorphism in a noncoding region (Fig. 2C). Both remained when the antibiotic pressure was removed, while the MIC remained unchanged. Growth in the presence of meropenem initially resulted in one synonymous mutation with a frequency of one in a hypothetical protein (Fig. 2D). This mutation was no longer observed afterward. Instead, a low-frequency mutation ap-

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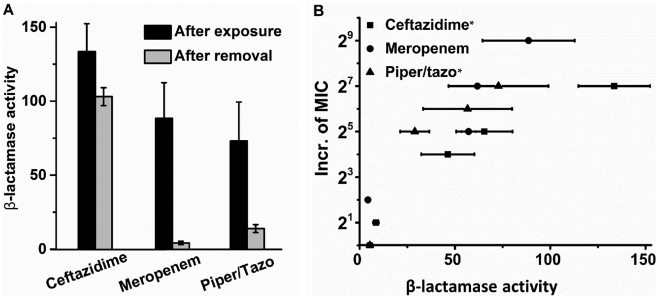

FIG 3 (A) β-Lactamase activities measured in cells adapted to the beta-lactam antibiotics indicated and after subsequent growth for 15 days in the absence of the drugs. The unit of β-lactamase activity is nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. (B) Increase (Incr.) in the MIC as a function of β-lactamase activity. The correlation between β-lactamase activity and MIC is statistically significant for the two antibiotics indicated with asterisks. Piper, piperacillin; tazo, tazobactam.

peared in *oprD*, which codes for a membrane channel known to be involved in meropenem resistance (23). In the next stage, at an intermediate MIC, the frequency of none of the six observed mutations exceeded 0.5. The mutations occurred three times in *oprD*, twice in *mexR*, and once in a noncoding region. None of the different mutations detected in a specific gene occurred together on the chromosome of a single cell. In cells with the highest MIC, mutations in *oprD*, *mexR*, *mexB*, and a noncoding region had taken over the entire population and attained a frequency of one. After removal of the drug, the MIC remained unchanged at high levels, but two new mutations were detected, while the earlier *oprD* mutation decreased to a very low frequency. The exact same synonymous mutation that was observed at the start of the meropenem experiment also appeared upon exposure to ceftazidime (Fig. 2E). While the MIC increased steadily, low-frequency mutations appeared and disappeared. Only at the very end, when the MIC had decreased because of removal of the drug, was a mutation in *prkC*, which codes for a serine/threonine-protein kinase, detected in the entire population.

The experimental samples were searched for evidence of gene duplication or deletion, but none was found, indicating that under these experimental conditions, only point mutations occurred.

Strains adapted to ceftazidime maintained their beta-lactamase activity when grown in the absence of a drug, but meropenem- and piperacillin-tazobactam-adapted cells did not (Fig. 3A). Since the MICs of the latter two antibiotics did not decrease during growth in their absence, beta-lactamase activity cannot be the sole factor determining the MICs. Still, there is a considerable correlation between the measured beta-lactamase activity and the increase in the MICs of the three beta-lactam antibiotics (ceftazidime, $R^2 = 0.926$; piperacillin-tazobactam, $R^2 = 0.921$; meropenem, $R^2 = 0.635$) (Fig. 3B). This correlation does not necessarily mean that beta-lactamase activity determines the MIC of this class of antibiotics. The positive cross-resistance of only two to four 2-fold steps (Table 2) suggests that a common mechanism is merely part of the total beta-lactam resistance system. Cross-resistance with the antibiotics of the other classes is either absent or negative. The negative cross-resistance of tobramycin-adapted cells indicates that becoming resistant to this antibiotic increases sensitivity to the others.

The costs of resistance for the cell were measured as a decrease in μ_{max} (Fig. 4A) and as maintenance energy, defined as energy metabolism devoted to purposes other than growth (Fig. 4B). Resistance to all antibiotics caused some decrease in μ_{max}. This decrease was statistically significant in cells adapted to ceftazidime, meropenem, and tobramycin. Ceftazidime-adapted cells had the lowest μ_{max} of 0.85 h^{-1}, compared to 1.15 for the wild type. Maintenance energy could only be measured in cells adapted to ciprofloxacin and tobramycin, because only these grew in mineral medium. In rich medium, too much glucose and other potential carbon and energy sources are available to use the carbon and energy source as a growth rate-limiting factor. The results indicate an increase in the maintenance energy by up to 0.3 mmol of glucose/10^{10} cells/h when cells become resistant to ciprofloxacin and roughly half of that amount in the case of tobramycin. Taken together, these observations indicate that *P. aeruginosa* pays a modest metabolic price for becoming resistant to the antibiotics tested.

Induced resistance in *Escherichia coli* came at the price of a reduced ability to grow under less-than-optimal conditions (24). Therefore, growth at nonoptimal pHs and salt concentrations was measured in *P. aeruginosa* as well. Only cells adapted to tobramycin had a reduced pH range (Fig. 4C), indicating that regulation of the internal pH interfered with counteraction of the drug. In all other cases, growth rates at different pHs and salt concentrations were similar to those of the wild type (data not shown).

DISCUSSION

The outcome of this study suggests that *P. aeruginosa* acquires resistance to the five antibiotics tested by different genetic mechanisms. WGS did not reveal mutations common to all five types of resistance, not even when limited to the three beta-lactam antibiotics. Some of the genes that were mutated were identified as resistance genes in *P. aeruginosa* before (25, 26). There was no clear correlation between the frequency of specific mutations and the increase in the MICs of any of the antibiotics tested, though for fluoroquinolone resistance, the *gyrA* mutation is always required.

TABLE 2 Cross-resistance after drug exposure/removal[a]

Drug(s)	Ceftazidime	Meropenem	Piperacillin-tazobactam	Tobramycin	Ciprofloxacin
Ceftazidime	$+2^7/2^5$	$+2^3/+2^2$	$+2^3/+2^2$	–[b]	–
Meropenem	$+2^4$/–	$+2^9/+2^9$	$+2^2$/–	–	–
Piperacillin-tazobactam	$+2^4/+2^2$	$+2^2$/–	$+2^7/+2^7$	–	–
Tobramycin	$-2^2/-2^4$	–	$-2^2/-2^3$	$+2^8/+2^4$	$-2^3/-2^4$
Ciprofloxacin	–	–	–	–	$+2^7/+2^5$

[a] Shown are the increases (+) or decreases (−) in the MICs of the antibiotics at the top upon acquisition of resistance to the antibiotics on the left. After *P. aeruginosa* became resistant to tobramycin, it was more sensitive to the other antibiotics.
[b] –, no change in the MIC.

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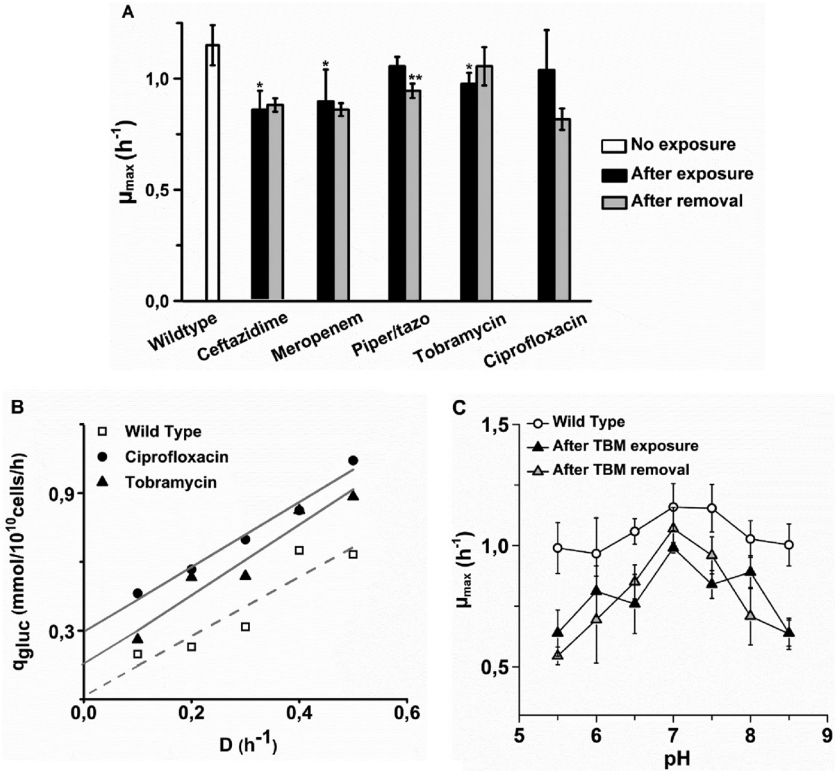

FIG 4 μ_{max} and maintenance energy of strains adapted to the antibiotics indicated immediately after growth in the presence of the drugs (after exposure) and after 14 days of subsequent growth in their absence (after removal). An asterisk indicates that the growth rate was significantly lower than that of the wild type, while double asterisks indicate that the growth rates before and after growth in the absence of the antibiotic differed significantly. Panels: A, μ_{max} in duplications (Piper, piperacillin; tazo, tazobactam); B, estimation of the maintenance energy by extrapolation to D (specific growth rate in duplication per h) = 0 of the specific glucose (gluc) consumption measured at several dilution rates; C, μ_{max} of wild-type *P. aeruginosa* and cells adapted to tobramycin (TBM) in the presence or absence of the drug as a function of pH.

Below, we will argue that all of the data combined indicate that, as shown for *E. coli* (22, 24), mutations alone cannot explain the development of resistance but that an intricate interaction between the adaptation of cellular systems and the DNA mutation level leads to higher levels of resistance.

Intrinsic resistance of *P. aeruginosa* to fluoroquinolones is due to four RND-type drug efflux pumps of the Mex-Opr families (27), which were not affected during *de novo* development of ciprofloxacin resistance in this study. In a hospital setting, fluoroquinolone use did not correlate with the occurrence of the *gyrA* and *parC* mutations found in this study (28). Therefore, it seems that several distinct mechanisms can neutralize the effects of fluoroquinolones on *P. aeruginosa*. The role of the initial *CPA2* mutation is unclear, but mutations often co-occur without an obvious functional relationship during the adaptation of *P. aeruginosa* to changing environmental conditions (29).

Cell elongation and clustering were shown to be essential steps during the initial development of resistance in *P. aeruginosa* (30). Therefore, the role of *fusA*, which codes for an elongation factor, in the initial protection against tobramycin can be understood in terms of its function in biofilm formation by *Pseudomonas chlororaphis* (31). The *rplB* gene codes for a 50S ribosome-associated L2 protein that is known to be involved in bactobolin resistance (32). The effect of the *rplB* mutation may be exerted through interaction with the 30S target protein of aminoglycosides (27). The CapD protein has a function in type 1 capsular polysaccharide biosynthesis (33) and therefore can have an indirect effect on the cellular access of aminoglycosides.

The ceftolozane-tazobactam combination caused several mutations during the development of resistance in *P. aeruginosa* (34), but the *ampC* mutation was the only functional one observed when piperacillin-tazobactam was used in this study. Since this mutation appeared at the very last MIC increase stages, its contribution may not have been very important. Possibly, the induction of increased beta-lactamase activity did not involve DNA mutations but was the result of increased expression that remained at higher levels even after many generations, as found in *E. coli* (24). In contrast, the highest levels of meropenem resistance correlated with mutations in the well-known resistance genes of the *oprD* and *mex* families (35). There are, however, several other genes involved in resistance to beta-lactam antibiotics (36, 37) that did not show mutations in this study. The *dacB* mutations observed at low frequencies during the development of resistance to ceftazidime influence the expression of AmpC beta-lactamases (38). The *ampD* mutations observed after the growth of cells made resistant to ceftazidime in antibiotic-free medium fulfill a role in peptidoglycan synthesis (39). The mechanism by which the two mutations in *yerD*, which codes for the large subunit of glutamate synthase, interact with ceftazidime resistance is unclear. It is possible that genetic hitchhiking (40), rather than a functional relationship, is the cause of these mutations.

While development of resistance to tobramycin and cipro-

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floxacin seems to be a more or less linear process with two unsuccessful mutations in the initial stages in the case of ciprofloxacin, the process is less straightforward in the case of beta-lactam antibiotics. In the latter case, the diverse-community model (41, 42) seems more applicable, in particular in the case of ceftazidime. In the early stages of development of resistance to meropenem and ceftazidime, multiple mutations in different genes are observed at frequencies of less than one and sometimes even at very low frequencies. Either the mutated cells also provide neighboring cells with protection against the antibiotic (43) or it represents evolution by trial and error. Alternatively, the well-documented regulation at the level of cellular processes (24, 44) may be the origin of the initial increase in the MIC observed in a similar manner in the case of all five antibiotics tested.

The fitness costs of resistance can come as a reduced growth rate, lower pathogenicity (2, 45), or a reduced ecological range (24). The μ_{max} of *P. aeruginosa* was affected less than its maintenance energy, indicating that the operation of, for example, efflux pumps requires additional energy (46). The increased maintenance energy was observed in the absence of antibiotics, indicating a more or less permanent change in metabolism. The moderate effect on μ_{max} is in agreement with observations on clinical strains (47). Whether the acquisition of resistance *in vitro* also influences pathogenicity, as in these clinical strains, is unclear. No mutations were observed in known pathogenicity genes, but expression levels may have been affected.

The overall conclusion from the comparison of the development of resistance to five antibiotics by *P. aeruginosa* is that no common mutations or mechanisms can be discerned. Only resistance to ciprofloxacin is built up in a straightforward manner by mutations in the genes coding for the target proteins. The genes affected are different for each of the three beta-lactam antibiotics. While this does not exclude the possibility of a common mechanism regulating the acquisition of resistance, as would be expected, assuming a common killing mechanism for bactericidal antibiotics (48, 49), it does not support that notion either. The lack of correlation between the persistence of mutations and the MIC after removal of the antibiotic from the growth medium indicates that these mutations are not the sole factor determining the MIC.

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