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Pseudomonas aeruginosa populations in the cystic fibrosis lung lose susceptibility to newly applied β-lactams within 3 days

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Background: Chronic pulmonary infections by *Pseudomonas aeruginosa* require frequent intravenous antibiotic treatment in cystic fibrosis (CF) patients. Emergence of antimicrobial resistance is common in these patients, which to date has been investigated at long-term intervals only.

Objectives: To investigate under close to real-time conditions the dynamics of the response by *P. aeruginosa* to a single course of antibiotic therapy and the potentially associated rapid spread of antimicrobial resistance, as well as the impact on the airway microbiome.

Methods: We investigated a cohort of adult CF patients that were treated with a single course of antimicrobial combination therapy. Using daily sampling during treatment, we quantified the expression of resistance by *P. aeruginosa* (median of six isolates per daily sample, 347 isolates in total), measured bacterial load by *P. aeruginosa*-specific quantitative PCR and characterized the airway microbiome with a 16S rRNA-based approach. WGS was performed to reconstruct intrapatient strain phylogenies.

Results: In two patients, we found rapid and large increases in resistance to meropenem and ceftazidime. Phylogenetic reconstruction of strain relationships revealed that resistance shifts are probably due to *de novo* evolution and/or the selection of resistant subpopulations. We observed high interindividual variation in the reduction of bacterial load, microbiome composition and antibiotic resistance.

Conclusions: We show that CF-associated *P. aeruginosa* populations can quickly respond to antibiotic therapy and that responses are patient specific. Thus, resistance evolution can be a direct consequence of treatment, and drug efficacy can be lost much faster than usually assumed. The consideration of these patient-specific rapid resistance shifts can help to improve treatment of CF-associated infections, for example by deeper sampling of bacteria for diagnostics, repeated monitoring of pathogen susceptibility and switching between drugs.

Introduction

Pseudomonas aeruginosa is one of the most problematic MDR bacteria, combining low intrinsic susceptibility to many antibiotics with a propensity to evolve further resistances.[1] Patients with a number of lung diseases are at risk of *P. aeruginosa* infection.[2] In adult cystic fibrosis (CF) patients, *P. aeruginosa* is the most prevalent cause of lung infection, contributing to loss of lung tissue and excess mortality.[3] As these infections are difficult to eradicate and usually relapse even if temporarily undetectable, patients frequently or even permanently receive antibiotic therapy. As a consequence, antibiotic resistance is widespread in CF-associated *P. aeruginosa* and is achieved through phenotypic changes (e.g. biofilm formation) and direct resistance mutations.[4,5]

Genetically mediated drug resistance can have two origins. Patients can become infected with an already-resistant strain. Alternatively, resistance evolves as a consequence of novel selective pressures induced by therapy. While long-term drug resistance evolution in chronic *P. aeruginosa* infections has been studied,[6,7]

little is known about the consequences of individual selective events. It is thus unclear how a single course of antibiotics may cause changes in resistance. Such information could reveal constraints of the involved selection dynamics, which may be exploited to improve therapy.[8,9]

Objectives

The primary objectives of this study were to: (i) assess to what extent a course of antibiotic treatment causes resistance changes in CF-associated *P. aeruginosa* infections; and (ii) investigate whether resistance shifts are due to *de novo* resistance evolution, the overgrowth of initially present resistant variants or novel infection with resistant strains. The secondary objectives were the characterization of the impact of antimicrobial chemotherapy on: (i) *P. aeruginosa* population size; and (ii) the diversity of the upper airway microbiome.

Therefore, daily sputum samples were collected from a cohort of patients undergoing standard-of-care intravenous antibiotic combination treatment. Multiple strains were isolated from daily samples, providing resistance profiles on a population level. In selected cases, we used WGS to infer intra-patient strain relationships and presence of resistance mutations.

Materials and methods

Ethics

The study was conducted in accordance with the Declaration of Helsinki and national and institutional standards. It was approved by the ethics committee of the Kiel University Medical Faculty (ref. D 479/15). All patients gave written informed consent.

Patient recruitment and sampling

A total of 12 adult CF patients were recruited between October 2015 and March 2016. Four of these had to be excluded because of absence of *P. aeruginosa* or incomplete sampling. The remaining eight patients had a history of *P. aeruginosa* bronchitis and received intravenous antibiotic combination therapy, in addition to existing permanent inhalation therapy, at the adult CF clinic of Kiel University Medical Center. Sputum was collected every morning, following inhalation with hypertonic NaCl solution (DNase in patient 5), for the duration of therapy. When sputum production was impossible, a throat swab was taken. More invasive sampling was deemed too harmful to be performed repeatedly. The collected samples were streaked on non-selective and *Pseudomonas*-selective agar plates at the microbiological diagnostics laboratory (Kiel Institute for Infection Medicine). The remaining sputum was frozen at −80°C. Up to 16 *P. aeruginosa* isolates were picked per sample, representing all observed colony morphologies. All isolates were confirmed to be *P. aeruginosa* by MALDI-TOF MS.

Isolation of metagenomic DNA

Sputum samples were homogenized by mixing with 2% *N*-acetyl-L-cysteine solution and mechanical agitation, as described.[10] Metagenomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) with recommended lysis buffer and lysozyme (20 mg/mL).

Bacterial quantification

Pseudomonas load was measured by quantitative PCR (qPCR) of a *P. aeruginosa*-specific *oprL* fragment, using primers and the TaqMan probe published previously (Table S1, available as Supplementary data at *JAC* Online),[11] including 300 nM each primer, 200 nM probe and amplification over 35 cycles of 5 s at 95°C and 30 s at 56°C. A serially diluted, cfu-counted *P. aeruginosa* UCBPP-PA14 culture served as standard, to translate the qPCR output to cell counts.

Airway microbiome analysis

For 16S rRNA amplicon sequencing, equal amounts of sputum DNA were used for amplification of the V1–V2 region (primers 27 F–338 R, Table S1). Sequencing was performed on the MiSeq platform (Illumina); the reads of two runs were combined and normalized to 5000 per sample. Taxonomic classification was carried out with USEARCH/UTAX, using the RDP training set 15 as reference.[12] Diversity and dissimilarity measures were computed with the 'vegan' package in R.[13] Microbiome diversity was quantified with Shannon's index, which incorporates both species abundance and evenness within a sample (α-diversity). Bray–Curtis dissimilarity was used to measure differences between microbiota samples (β-diversity).

Antibiotic susceptibility testing

Resistance of bacterial isolates was measured in three technical replicates using Etests (bioMérieux, Liofilchem), following EUCAST guidelines and the manufacturers' instructions. Briefly, axenic cultures were grown overnight in LB, diluted to 0.5 McFarland turbidity and inoculated on Mueller–Hinton agar. Etests were read after 20 h incubation. We additionally assessed exact resistance levels of individual colonies found within the inhibition zone, using the above Etest protocol.

Resistance genomics

Genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB) protocol.[14] WGS was performed using the MiSeq platform and Nextera XT library preparation (Illumina). After quality control and trimming in Trimmomatic,[15] sequences were aligned to the PAO1 reference using bowtie2.[16] Variants were called with samtools/bcftools and the GATK variant calling pipeline for cohort data, following recommended protocols.[17–19] Variant annotation and filtering were done with SnpEff.[20]

MLST was performed on pubmlst.org with seven core loci for *P. aeruginosa*.[21] Forty-nine antibiotic resistance genes were selected for further analysis, following previous work.[5,22] For protein analysis, gene sequences were translated *in silico*.[23] MUSCLE was used for multiple sequence alignment.[24] Maximum likelihood phylogenetic analysis was performed with RAxML, using the gamma model of rate heterogeneity coupled with a general time-reversible model for DNA and the WAG amino acid replacement matrix for proteins, and at least 1000 bootstraps.[25] Phylogenetic trees were visualized using the Interactive Tree of Life tool.[26]

The genome sequences are deposited at NCBI, BioProject PRJNA504944.

Results

Patient treatment consisted of two or three combined drugs, chosen based on the individual needs of the patients. In some patients, prior outpatient therapy with inhaled tobramycin or colistin was continued. Clinical markers (BMI and lung function) ranged from almost within normal limits to severely compromised (Table 1).

Rapid shifts in β-lactam resistance

Resistance analysis revealed large increases in meropenem and ceftazidime resistance during the first days of therapy (Figure 1a). In contrast, we did not observe an overall increase in tobramycin

Table 1. Patient overview

Patient	CFTR genotype	Age (years)	BMI (kg/m^2)	FEV_1 (%)	Treatment	Continued treatment
1	ΔF508/ΔF508	44	22.5	36	CST/MEM	–
2	ΔF508/ΔF508	29	15.9	21	CST/MEM	TOB[a]
3	ΔF508/CFTRdele2, 3	41	24.3	67	CAZ/MEM	CST[a]
4	ΔF508/c.2051_2052delAAinsG	33	17	43	CST[a]/CAZ/TOB	–
5	ΔF508/1078delT	30	20.4	43	CST/CAZ	CST[a]
8	G542X/G542X	37	20.7	64	CAZ/MEM/TOB[a]	–
9	ΔF508/ΔF508	21	22.4	49	CST[a]/MEM/TOB	–
11	ΔF508/pR347/P	29	25.7	65	CST[b]/TZP	CST[a]

FEV_1, forced expiratory volume in 1 s as a percentage of the predicted average value for the individual patients; CST, colistin; MEM, meropenem; CAZ, ceftazidime; TOB, tobramycin; TZP, piperacillin/tazobactam.
[a]Inhaled drugs.
[b]Simultaneous inhaled and intravenous application; all others, intravenous administration only.

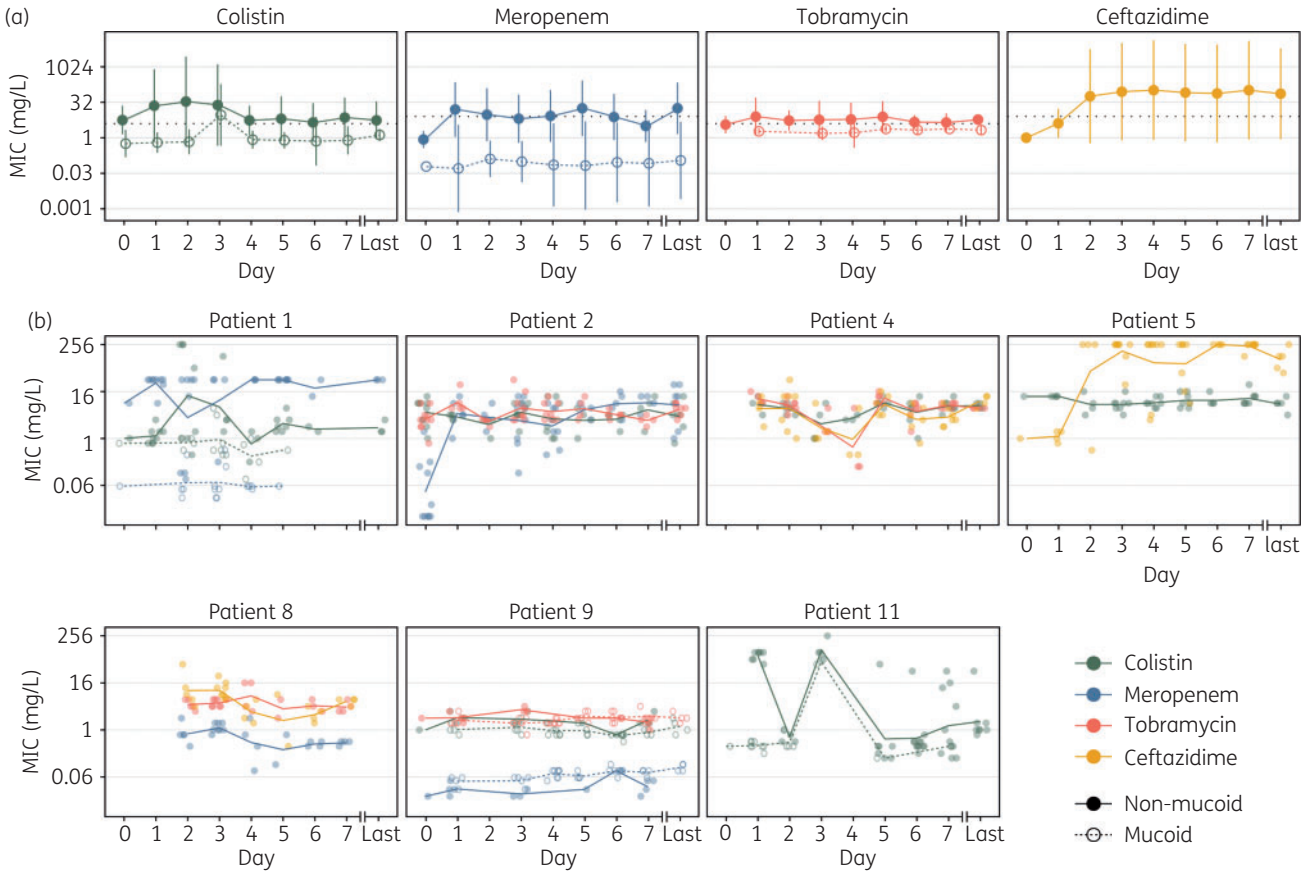

Figure 1. Antibiotic resistance profiles. (a) Mean MIC ± SD of all isolates treated with the respective antibiotics. Open circles and dashed lines denote mucoid isolates. Black dotted lines indicate EUCAST clinical resistance breakpoints. No patients with mucoid isolates were treated with ceftazidime. (b) MIC profiles by patient for those drugs used in treatment. Each dot represents one isolate–drug combination. Lines mark population means, continuous for all non-mucoid, dashed for mucoid. Day '0' indicates isolates gathered directly before the beginning of therapy, 'Last' indicates the last day of treatment between days 12 and 14.

and colistin resistance, irrespective of whether patients were previously treated with these drugs or not (Figure S1, Table 1).

Mucoid isolates were present in three patients, and were significantly more susceptible to colistin than the non-mucoid isolates (mean MICs of 2.48 mg/L and 11.48 mg/L, respectively), tobramycin (0.09 versus 9.65 mg/L) or meropenem (1.97 versus 5.66 mg/L; $P<0.001$ for all three drugs in Wilcoxon Rank Sum Tests). The mucoid isolates treated with meropenem ($n=53$) did

not display the resistance shifts observed in the non-mucoid isolates (n=134).

Splitting the resistance data by patient revealed that the observed overall resistance shifts were due to changes in individual patients (Figure 1b). For meropenem, this was observed in patient 2, where the population mean MIC increased from 0.21 mg/L to 5.8 mg/L within 1 day, crossing the EUCAST threshold to 'intermediate'.[27] The ceftazidime resistance shift occurred in patient 5, from a mean MIC of 1.19 mg/L on the first day to 192 mg/L on day 3, signifying strong resistance. In both cases, no low resistance isolates remained at the end of therapy. Thus, the resistance shifts observed in this cohort were not a general phenomenon, but were patient specific.

For some isolates from patient 11, accurate determination of the MIC for piperacillin/tazobactam was impeded by sporadic colonies within the zone of inhibition (Figure 2). The proportion of isolates with this phenotype increased during therapy. When tested individually, most of these colonies displayed stable high resistance.

Genetics of rapid resistance shifts

To gain mechanistic insight into the β-lactam resistance shifts, we sequenced the genomes of isolates from patients 2 and 5 before and after the shifts (Figure S2). MLST analysis assigned all isolates to ST 2331 (Table S2). Phylogeny reconstruction from the seven core MLST genes revealed tight clustering of all isolates, separated by a maximum of five SNPs (Figure S3), but sharing 53 common variants compared with the PAO1 reference. Even when considering full MLST gene sequences, all patient 5 isolates were completely identical. Thus, the sequenced strains from either of the two patients were very closely related and, on the MLST level, we found no evidence of strain replacement during the resistance shift.

Next, we examined 49 known *P. aeruginosa* antibiotic resistance genes (Table S3) to achieve higher phylogenetic resolution and characterize possible causative mutations underlying the resistance shifts. Like the MLST analysis, most variants at these loci were shared across isolates, regardless of patient, and included 61 non-synonymous mutations (Table S4). Individual strains had a median of five non-shared non-synonymous mutations in these genes (range 0–16, Tables S5 and S6). Nine of these were found in major β-lactam resistance genes only after the resistance shifts (Table 2).

We inferred phylogenetic trees from the DNA and protein sequences of these resistance genes. The patient 2 isolates from before the resistance shift clustered around the insertion point of PAO1 (Figure 3a). Of these, the more resistant clones formed two clusters: isolates 2-0-01 and 2-0-03 versus 2-0-02 and 2-0-04, which varied in non-silent mutations in the efflux pump gene *mexCD* and a few other genes. Several variants in important β-lactam resistance genes were unique to the later isolates (Table 2). The *emrB* frameshift mutation was shared by almost all strains and only absent in the highly susceptible strains, which formed a distinct cluster in the protein phylogeny (Figure 3b).

For patient 5, most isolates fell into one of two clusters (Figure 3c and d). The susceptible early population and the less-resistant later isolates formed one cluster, which shared a large deletion in *mexB*. The remaining, highly resistant isolates had pairs of frameshift mutations in *mexB*, which together restored the

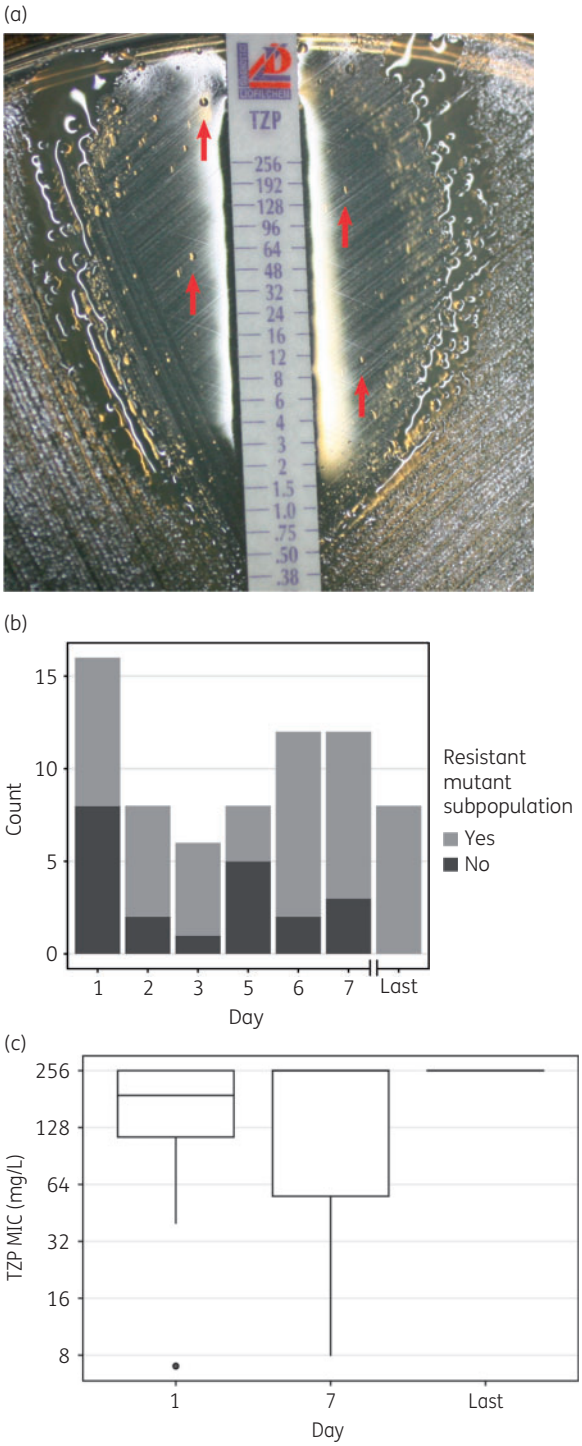

Figure 2. Individual high resistance colonies in the Etest for piperacillin/tazobactam (TZP) of isolates from patient 11. (a) Several colonies grow well inside the zone of inhibition (red arrows), indicating a resistant mutant subpopulation (RMS), thereby compromising reliable MIC determination. (b) Number of isolates with an RMS in the TZP Etest. (c) TZP resistance of the RMS colonies picked from the original Etests, producing higher resistance than most of the original isolates from patient 11, shown in Figure 1(b). Horizontal lines indicate medians; boxes indicate IQR. Dot indicates value outside of 1.5×IQR from the box. The upper limit of the TZP Etest is 256 mg/L.

Table 2. β-Lactam resistance gene variants found after resistance shifts

Variant	Isolates	Potential resistance mechanism	Reference citation
Patient 2			
AmpD D28G	5-03, 6-01	AmpR activation, derepression of *ampC*	52
AmpR R86C	5-03, 6-01	gain-of-function increases *ampC* expression	52
AmpR E88K	5-02, 5-04	gain-of-function increases *ampC* expression	52
FtsI Q208R	5-01, 5-02, 5-04	modification of PBP3	53
FtsI N242S[a]	5-01	modification of PBP3	35
FtsI P527S[a]	5-02, 5-03, 5-04, 6-01	modification of PBP3	35
Patient 5			
AmpC V239G[a]	[b]	gain-of-function of β-lactamase	54
AmpR R86C	[b]	gain-of-function increases *ampC* expression	52
FtsI A421T	6-03	modification of PBP3	53
FtsI R504H[a]	[b]	modification of PBP3	36

Variants in main β-lactam resistance genes that were not present before the resistance shifts in patients 2 and 5.
[a]Variants previously described in clinical *P. aeruginosa* isolates in the references listed.
[b]Variants present in the highly ceftazidime-resistant population as well as the first highly resistant clone isolated on day 2 (see Figure S2). Position in AmpC includes the 26 amino acid signal peptide.

reading frame. This mutation pair was different in isolate 5-6-03, causing a larger disruption. Both groups carried additional mutations, mainly in the β-lactamase *ampC* gene and the *ftsI* gene (which encodes PBP3).

Overall branch lengths and inferred nucleotide diversity were higher for patient 2, indicating a more diverse population than in patient 5 (1.98×10^{-6} versus 7.49×10^{-7} per-site nucleotide diversity, respectively). For both patients, pairwise genetic distances and resistance differences of the isolates were positively correlated (Mantel test, $r=0.43$ for patient 2 and $r=0.63$ for patient 5, $P<0.001$ for both).

To explore the presence of mutator strains (i.e. strains with increased spontaneous mutation rates and thus higher likelihood of resistance mutations), we quantified silent mutations in the resistance and MLST genes, revealing almost identical numbers for all isolates, which were, however, smaller than those for a previously characterized putative mutator (Figure S4a). We further found several common variants in genes affecting mutation rates (Table S7). As an independent indicator of mutator strains, we assessed for each isolate the presence of highly resistant colonies within the Etest inhibition zone. Even though this phenotype appeared to be more common in isolates from patients with resistance shifts, the overall variation among patients was not significant (Figure S4b).

Antibiotic effects on P. aeruginosa abundance

Five of the eight recruited patients remained *P. aeruginosa* positive on their last day of therapy. One patient had a negative final culture (patient 9), and two were consistently negative (patients 3 and 8) (Figure 4a). In three patients, sputum production ceased, even with induction, and throat swabs were taken instead (patients 1, 8 and 9). The *P. aeruginosa* load in sputum, as measured by qPCR, differed significantly between patients (Table S8). While a trend towards an overall decrease was observed, it was not statistically significant at the end of the first or second week (Figure 4b). Individual patient trajectories were variable, with either decreases of cell numbers in the early and late treatment phases or little overall change (Figure 4c). One patient showed a load increase in week 2 after an initial decrease (patient 11).

Impact on upper airway microbiome

Airway microbiome composition varied substantially between patients. *Pseudomonas* spp. were present in all samples, but their relative abundance ranged from low to >80% of reads, the latter corresponding to low diversity within samples (i.e. low α-diversity; Figure 4e, patients 2 and 8). Antimicrobial therapy caused little disturbance to these communities, as highlighted by the lack of common directionality of change in diversity between samples (i.e. weighted β-diversity; Figure 4d) or diversity within samples (i.e. community α-diversity; Figure 4e). While two patients almost exclusively harboured *Pseudomonas* spp. and *Staphylococcus* spp., other patients had more diverse communities (detailed composition in Figure S5). No statistically significant correlations were found between microbiome diversity or *P. aeruginosa* abundance and clinical parameters (Table S9).

Discussion

We here provide one of the few studies examining the impact of a single course of antibiotics on *P. aeruginosa* infection and resistance evolution.[28–30] In most patients antibiotic resistance did not change over time. In two patients, however, we observed surprisingly rapid shifts to high resistance to β-lactam antibiotics. Genomic analysis indicates that these shifts are not due to replacement with a more resistant strain, but the result of *de novo* resistance evolution and/or the spread of originally low-frequency resistant subpopulations. Our results highlight that changes in both airway microbiome and pathogen characteristics are patient specific.

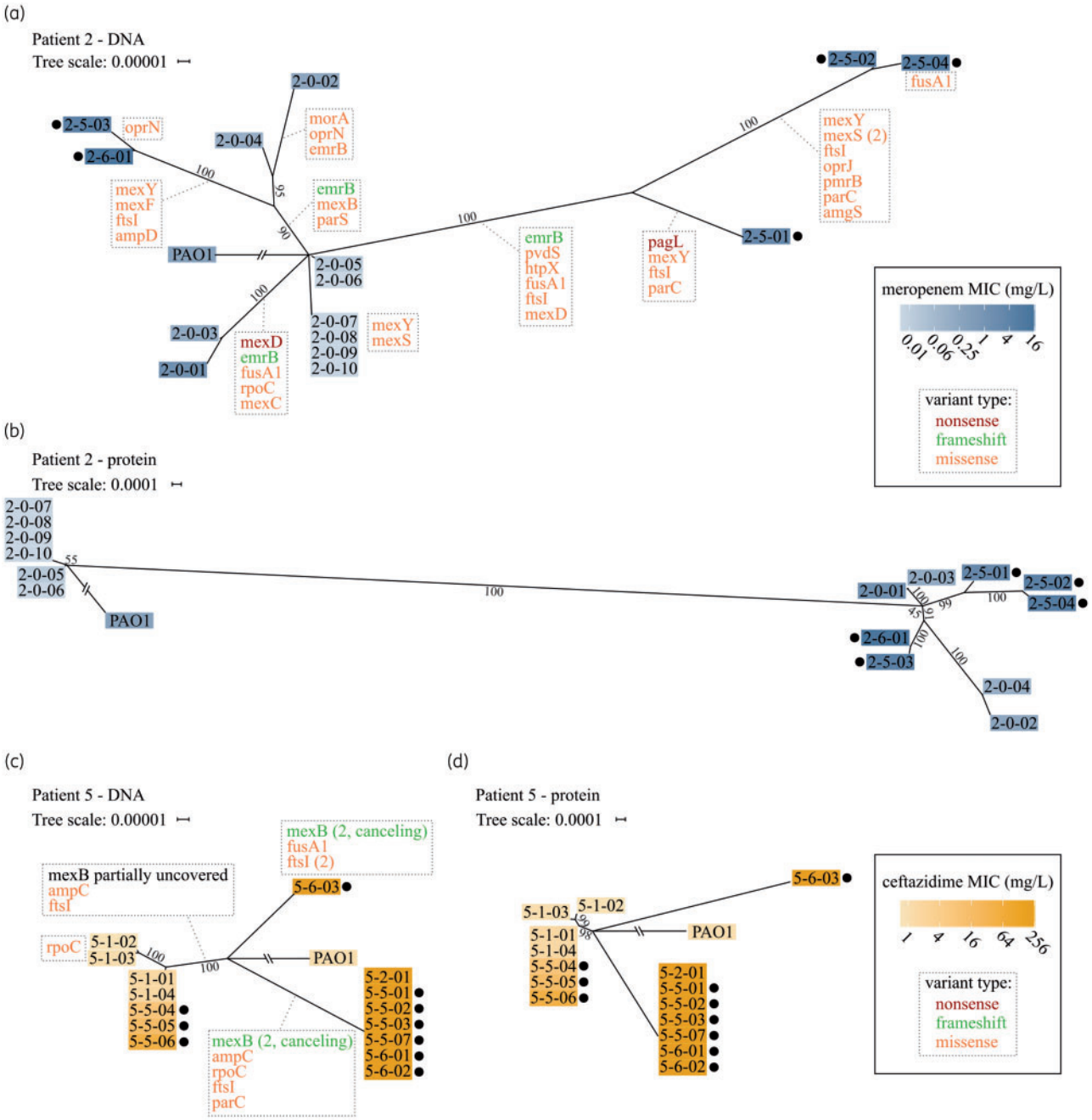

Figure 3. Phylogenetic trees of 49 resistance gene sequences in *P. aeruginosa* isolates from two patients showing resistance shifts. Leaf labels denote isolates, and background shading indicates MICs of the corresponding antibiotics. Filled circles mark isolates collected after resistance shifts. Branch labels indicate bootstrap support. The very long branch to the reference strain PAO1 was shortened for illustrative purposes, as indicated by two slashes. Dashed boxes indicate non-synonymous resistance gene variants. (a) and (c) show DNA sequence-based phylogenies. (b) and (d) show protein sequence-based phylogenies.

Resistance shifts are caused by selection of resistant subpopulations and/or de novo evolution

Previous studies have examined the adaptation of *P. aeruginosa* to the human airway over longer periods of time, showing common adaptations such as reduction of virulence and increased biofilm formation.[31] Simultaneously, phenotypic diversity increases over time, and mutations in antibiotic resistance genes are frequently observed.[6,7,32] The effect of single courses of antibiotics on bacterial resistance has mostly been explored in case reports, often after treatment failure. Pronounced resistance increases have been documented for several pathogens, including *P. aeruginosa*.[28,29,33,34] In our study, we found rapid and significant increases in *P. aeruginosa* antibiotic resistance in two patients, in both cases to newly applied β-lactam antibiotics, ceftazidime

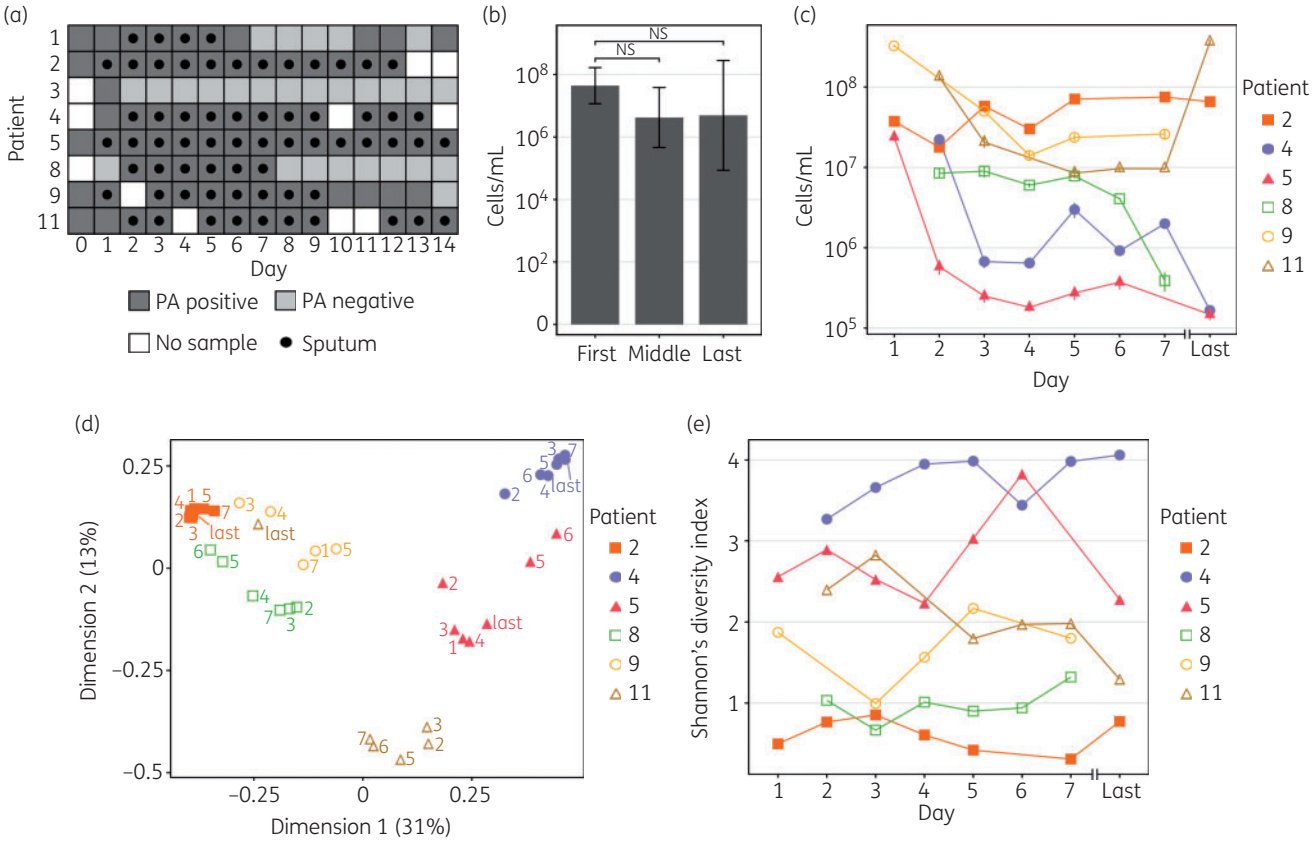

Figure 4. Overview of sampling, *P. aeruginosa* frequency dynamics and changes in microbiome characteristics during the course of antibiotic treatment. (a) Sample overview. Dark grey tiles represent samples with at least one *P. aeruginosa* (PA) isolate; light grey indicates *P. aeruginosa*-negative samples. Filled circles mark days with a sputum sample; those without mark throat swabs. Only patients with five or more sputum samples were included in the following analyses. (b) Mean ± SD of *P. aeruginosa* counts in sputum, determined by qPCR. 'First', 'Middle' and 'Last' indicate first available sample per patient, last sample of week 1 per patient and last available samples per patient, respectively. NS, not significant (Wilcoxon signed-rank test, $V=20$, $P=0.063$ for week 1 and $V=7$, $P=0.63$ for the full duration). (c) Time course of *P. aeruginosa* counts from sputum, determined by qPCR, for each patient. 'Last' indicates the last available sputum sample between days 12 and 14. Data represent the mean ± SD of three technical replicates. (d) 16S rRNA-based airway microbial community composition. Multidimensional scaling of square root-transformed Bray–Curtis dissimilarity indices. Coloured labels indicate day of therapy. (e) Time course of Shannon's diversity index of airway microbial communities, based on operational taxonomic units (OTUs). 'Last' indicates the last available sputum sample between days 12 and 14.

(MIC change from 1.19 mg/L to 192 mg/L) and meropenem (0.21 mg/L to 5.8 mg/L). This result may be explained by faster emergence and spread of resistance to the two antibiotics, compared with other drugs, or novelty of drug application in the two affected patients. However, no resistance shifts for other antibiotics such as tobramycin or colistin were observed when they were newly applied (as done in five patients, Table 1), and not all applications of meropenem and ceftazidime led to resistance shifts (as seen for five patients, Table 1).

The finding of a significant correlation between genomic and MIC variation among isolates from either patient 2 or 5 suggests that the resistance shifts have at least some genetic basis and are not exclusively due to phenotypic effects. Moreover, the identified variants in known resistance genes, found in the late resistant isolates (Table 2), are sufficient to explain the resistance shifts at the genetic level. Three alternative processes may then underlie these shifts on the population level: (i) the more-resistant isolates could have originated from a new strain that invaded and replaced the old population due to its selective advantage under antibiotic therapy; (ii) a more-resistant variant was already present at low frequency before therapy was started and then spread due to antibiotic-induced selection; or (iii) resistance mutations arose *de novo* after therapy was started and subsequently spread in response to antibiotic selection. The first alternative appears to be unlikely in the two tested patients because we found no evidence of a new strain in our analysis of MLST or resistance genes. The second and third alternatives are difficult to distinguish in infections caused by diverse populations, without very deep sampling to reliably identify the ancestors of later, more resistant isolates. Nevertheless, our current data for patient 2 are consistent with one and that for patient 5 with both alternatives.

In detail, patient 2 harboured a diverse initial population, including some strains with medium-level resistance that were closely related to the late and highly resistant isolates. All late isolates carried previously described variants in *ftsI* (encoding PBP3), which can confer resistance to meropenem.[35,36] However, the late

isolates also had several additional variants compared with all early strains, including those with mild resistance levels (Figure 3a). Therefore, a complete *de novo* differentiation from these mildly resistant strains is unlikely within the single days observed here (Figure 1b). Thus, the most parsimonious explanation is that selection of pre-existing genetic variation gave rise to the resistance shift.

In patient 5, one cluster of highly similar strains included susceptible early and also resistant late strains (left bottom cluster in Figure 3c and d). This pattern is consistent with *de novo* evolution of resistance within this particular patient, even though the resistance-causing mutation itself could not be found in the considered resistance genes (i.e. all strains from this cluster had identical sequences in the resistance genes). Patient 5 also harboured a different cluster with an early and several late isolates, which were all highly resistant (right bottom cluster in Figure 3c and d). This specific pattern is consistent with selection of an initial resistant subpopulation that spread upon antibiotic treatment because of its advantage over susceptible strains. The presence of both patterns may suggest that the two proposed processes acted in parallel in this patient.

A more precise reconstruction of the dynamics clearly warrants further research, using genome analysis of a more comprehensive sample per patient and day, and also assessment of phenotypic plastic responses (e.g. changes in gene expression), which we did not specifically address, although they could also contribute to resistance shifts.[37]

The possible importance of mutator strains during adaptation to antibiotics

Mutator strains show an increase in mutation rate (e.g. caused by mutations in DNA repair genes), which may accelerate adaptation.[38] *P. aeruginosa* mutator strains frequently occur in CF infections and are associated with antibiotic resistance.[39] An indication for mutators can be obtained from Etests through the presence of individual colonies with higher resistance within the zone of inhibition.[40] This phenotype was conspicuous for piperacillin/tazobactam in patient 11 (Figure 2), where we repeatedly observed colonies with stable higher resistance than the main population. Since the proportion of isolates with this behaviour increased over time, therapy may have selected for a mutator in this patient. Interestingly, we did not observe a general resistance increase in the main population (Figure 1b), in contrast to patients 2 and 5. Therefore, the putative mutator with high resistance was apparently not able to spread to fixation in patient 11, possibly because the increase in mutation rate comes at the cost of a higher abundance of deleterious mutations.[41]

Increased mutation rates may have contributed to the resistance dynamics in patients 2 and 5. Although the overall evidence is inconclusive, the possibly higher mutation frequency in the *P. aeruginosa* populations of these patients could have favoured the emergence of the selectively advantageous resistance mutations. The contribution of mutators to rapid adaptation of *P. aeruginosa* during therapy clearly deserves further analysis in the future.

The impact of antibiotic therapy on microbial populations is limited and patient specific

Treatment-induced reduction of bacterial load was overall limited across our cohort, consistent with previous studies.[42] Nevertheless, two patients turned *P. aeruginosa* negative. Treatment success may still not be permanent in these cases, as it has been repeatedly observed in CF patients that pathogen absence is transient and followed by later infection relapse.[43] In general, chronic *P. aeruginosa* bronchitis in CF appears to be difficult to eradicate completely.[43]

The airway microbiome proved to be similarly resilient to antibiotic therapy, in general agreement with previous work.[44] Patients differed substantially in microbiome composition, with some airways dominated by *Pseudomonas* and *Staphylococcus* spp. (airways of patients 2 and 8). These two patients suffered from advanced CF lung disease, but only patient 2 was severely ill upon hospitalization and showed reduced clinical status [low BMI and FEV_1 (forced expiratory repiration in 1 s) values for patient 2, but not patient 8]. The high abundance of *Pseudomonas* and *Staphylococcus* spp. could indicate that these patients were close to exacerbations, although an increase of *Pseudomonas* spp. does not necessarily precede an exacerbation.[45] While all patients had a history of *P. aeruginosa* infection, and although airway microbiome diversity is known to decrease with duration of *P. aeruginosa* presence,[46,47] we did not find a significant correlation between microbiome diversity and either patient age or any clinical parameter. Although metagenome analysis may be required for more detailed insight into these relationships,[48] our results may also indicate that microbiome characteristics alone do not determine disease outcome.

Implications for diagnostics and therapy

Our observation of rapid resistance shifts in two patients from a small cohort of eight individuals highlights the enormous adaptive potential of pathogens such as *P. aeruginosa*. It confirms our hypothesis that a single antibiotic treatment can favour resistance, but surprisingly this effect was both patient specific and very fast. Treatment could be optimized by improving diagnostics, for example by characterization of larger sample numbers than the commonly considered one or two isolates, in agreement with recommendations from several recent, related studies.[49,50] Mucoid subpopulations are sometimes tested separately due to their easy differentiation on agar, and our data indicate that this is useful, as the mucoid subpopulation did not follow the resistance shift in patient 2. A second resistance test after several days of therapy may be particularly valuable, because it can identify patients with rapid resistance shifts. For these patients, a drug switch is recommendable to counter novel antibiotic resistance. Moreover, it may generally be worth assessing treatment duration, because longer treatments usually help establishment of the resistant variants and cause more side effects, yet their effect on improving patient health is not always clear. Surprisingly, we also observed individual *P. aeruginosa* isolates with very high colistin resistance, a so-called last-resort drug, for which lower resistance rates are usually reported.[51] The fluctuation of colistin resistance in the first few days could indicate that a costly resistance mechanism can evolve,

but does not necessarily become fixed in the population. Nevertheless, the presence of such high resistance levels is worrisome and may suggest that colistin should be used with particular caution in CF patients.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 and Tables S1 to S9 are available as Supplementary data at *JAC* Online.

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