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Whole-Genome Sequencing Reveals Diversity of Carbapenem-Resistant *Pseudomonas aeruginosa* Collected through CDC's Emerging Infections Program, United States, 2016–2018

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ABSTRACT The CDC's Emerging Infections Program (EIP) conducted population- and laboratory-based surveillance of US carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) from 2016 through 2018. To characterize the pathotype, 1,019 isolates collected through this project underwent antimicrobial susceptibility testing and whole-genome sequencing. Sequenced genomes were classified using the seven-gene multilocus sequence typing (MLST) scheme and a core genome (cg) MLST scheme was used to determine phylogeny. Both chromosomal and horizontally transmitted mechanisms of carbapenem resistance were assessed. There were 336 sequence types (STs) among the 1,019 sequenced genomes, and the genomes varied by an average of 84.7% of the cgMLST alleles used. Mutations associated with dysfunction of the porin OprD were found in 888 (87.1%) of the genomes and were correlated with carbapenem resistance, and a machine learning model incorporating hundreds of genetic variations among the chromosomal mechanisms of resistance was able to classify resistant genomes. While only 7 (0.1%) isolates harbored carbapenemase genes, 66 (6.5%) had acquired non-carbapenemase β -lactamase genes, and these were more likely to have OprD dysfunction and be resistant to all carbapenems tested. The genetic diversity demonstrates that the pathotype includes a variety of strains, and clones previously identified as high-risk make up only a minority of CRPA strains in the United States. The increased carbapenem resistance in isolates with acquired non-carbapenemase β -lactamase genes suggests that horizontally transmitted mechanisms aside from carbapenemases themselves may be important drivers of the spread of carbapenem resistance in *P. aeruginosa*.

KEYWORDS *Pseudomonas aeruginosa*, antibiotic resistance, mechanisms of resistance, whole-genome sequencing

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The authors declare no conflict of interest.

Received 19 April 2022

Returned for modification 23 May 2022

Accepted 12 August 2022

Published 6 September 2022

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for more than 32,000 multidrug-resistant (MDR) infections per year in the United States, many of which are health care-associated infections (HAIs) (1). HAIs caused by *P. aeruginosa* have high levels of morbidity and mortality (2), especially among cystic fibrosis (CF) patients (3). Because *P. aeruginosa* is intrinsically resistant to many antibiotics (4), carbapenems are an important treatment option. However, nonsusceptibility to carbapenems has been observed in >20% of *P. aeruginosa* isolates from HAIs worldwide (5).

Outbreaks of carbapenem-resistant *P. aeruginosa* (CRPA) HAIs reported in the literature are often caused by bacteria carrying carbapenemase genes such as *bla*_{IMP} (6), *bla*_{KPC} (7), and *bla*_{VIM} (8, 9). However, a pilot surveillance study from 2015 suggested that carbapenemase-producing CRPA are relatively rare in the United States, occurring in only 2.3% of the isolates collected (10), a lower level than that reported in recent studies from other countries such as Germany (11), the Netherlands (12), and India (13). Carbapenem resistance in *P. aeruginosa* primarily arises from chromosomal mutations (14) driving three major mechanisms: a decrease in outer membrane permeability mediated by dysfunction of the porin OprD (15), overexpression (via derepression) of the MexAB-OprM efflux pump (16), and overexpression (via derepression) of the intrinsic β -lactamase AmpC (17).

To better understand the molecular epidemiology of CRPA and patterns of molecular mechanisms of resistance to carbapenems in the United States, we performed antimicrobial susceptibility testing (AST) and whole-genome sequencing (WGS) on 1,019 isolates collected as part of the CDC's Emerging Infections Program (EIP) surveillance of CRPA in eight sites from 1 August 2016 through 31 July 2018. This project was an expansion of a previous pilot study (10) and included a subset of isolates collected from CF patients.

RESULTS

Antimicrobial susceptibility testing. The 1,019 isolates collected through this study were tested against a panel of 12 antimicrobials and 3 combination agents from seven classes (File S2, Fig. S1) using reference broth microdilution. Applying definitions proposed by Magiorakos et al. (18), 709 (69.6%) isolates were determined to be MDR (non-susceptible to at least one agent from three or more classes of antimicrobials), and 400 (39.3%) were extensively drug-resistant (XDR, non-susceptible to at least one agent from all but one or two classes), as shown in Fig. S2b.

Three carbapenems (doripenem, imipenem, and meropenem) were included in the panel. All isolates were resistant to at least one carbapenem when tested at a local clinical laboratory, and 821 (80.6%) met that definition when tested at the CDC. Overall, 394 (38.7%) of the 1,019 EIP isolates were resistant to doripenem, 545 (53.5%) to meropenem, and 771 (75.7%) to imipenem (Fig. S2b).

Molecular epidemiology. The traditional, seven-gene multilocus sequence typing (MLST) scheme (19) and a core genome MLST (cgMLST) scheme (20) were applied to the WGS data to explore the phylogeny of all 1019 genomes. The set of genomes was diverse; it included 363 unique sequence types (STs), 147 (40.5%) of which were singletons (Fig. 1). The high-risk clone ST235 (21) was the most frequently occurring (94 isolates, 9.2% of the total) and was found at every EIP site along with two other STs previously identified as high-risk, ST155 (51, 5.0%) and ST298 (47, 4.6%), which are thus designated because they frequently cause MDR/XDR HAIs (22).

The diversity of the genomes was also reflected in the cgMLST (20) analysis. There was an average difference of 3,761 alleles between the genomes, representing 84.7% of the 4,440 genes included in the scheme (Fig. 1). The 1,019 EIP genomes were also compared to a set of 2,049 *P. aeruginosa* genomes from the NCBI RefSeq database (23). The genomes from this set differed from those from RefSeq by 78 (1.8% of cgMLST genes, $P < 0.05$, one-way analysis of variance [ANOVA]) more alleles, on average, than the number of alleles by which differed with each other (Table S1 and Fig. S3).

Mechanisms of carbapenem resistance. We analyzed the WGS data for markers of three chromosomal mechanisms known to contribute to carbapenem resistance (Table 1):

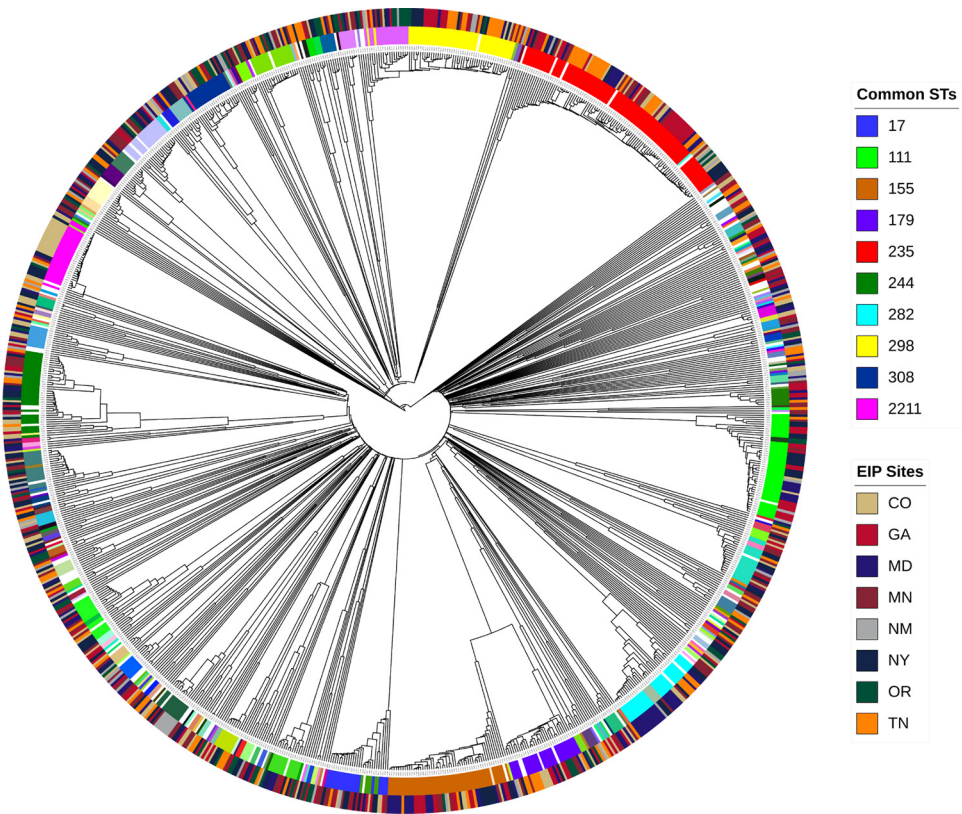


FIG 1 Dendrogram of the 1,019 Emerging Infections Program (EIP) *Pseudomonas aeruginosa* genomes from isolates made from a core genome multilocus sequence typing (cgMLST) scheme of 4,440 core genes. Inner circle indicates sequence type (ST); outer circle indicates EIP site from which the specimen yielding the isolate was collected. Tree branches are scaled based on allele differences between nodes.

downregulation or deleterious mutations of the porin OprD (usually insertions or deletions that result in truncations and changes the conformation of the protein, which prevents the channel from forming and functioning) (24), overexpression (via derepression) of the efflux pump MexAB-OprM (16), and overexpression (via derepression) of the β -lactamase AmpC (17). Mutations affecting OprD occurred the most frequently, found in 888 (87.1%) of the 1,019 genomes (Fig. S4a), and were largely driven by truncations of the *oprD* gene (Fig. S5a). Out of all the isolates, 964 (94.6%) had at least one chromosomal marker of resistance and 595 (58.4%) had more than one (Fig. S4b). Several hundred additional mutations were also identified in these genes (Table S2), but since their effects have not been experimentally validated, they were not counted as markers of carbapenem resistance.

TABLE 1 Chromosomal point mutations associated with carbapenem resistance

Mechanism	Gene	Mutations (reference)
OprD dysfunction	<i>oprD</i>	Truncation (49)
	<i>oprD</i> transcription promotion site	Point mutation (50, 51)
MexAB-OprM overexpression (derepression)	<i>mexS</i>	G78S (52), P254S (53)
	<i>mexR</i>	R21W (54), D56G (55), L57P/Q (56), G58E (56), R70Q/W (56), I72N/L (57), L75Q/R (56, 57), R83C/H/G (57), R85H (58), R91H (56, 57), L95F (56), G101R (59), A110T (56), R114C (60), T130P (56), V132A (59)
	<i>nalD</i>	T11N (61), D187H (62), F198L (63), G206S (64), T158P (65)
	<i>nalC</i>	E153Q (55)
ampC overexpression (derepression)	<i>ampR</i>	E88K (66), R244W (67)
	<i>ampD</i>	V10G (68), R11L (69), D28G (68), P41R/S (70), H77Y (70), G84D (70), A85G (71), F89S (70), H98Y (72), G121R (70), A134V (73), T139M/R (73), G156S (73), H157R (73, 74), S175L (74)
	<i>dacB</i>	E84K (17), A394P (69), G407S (59), T428P (75)

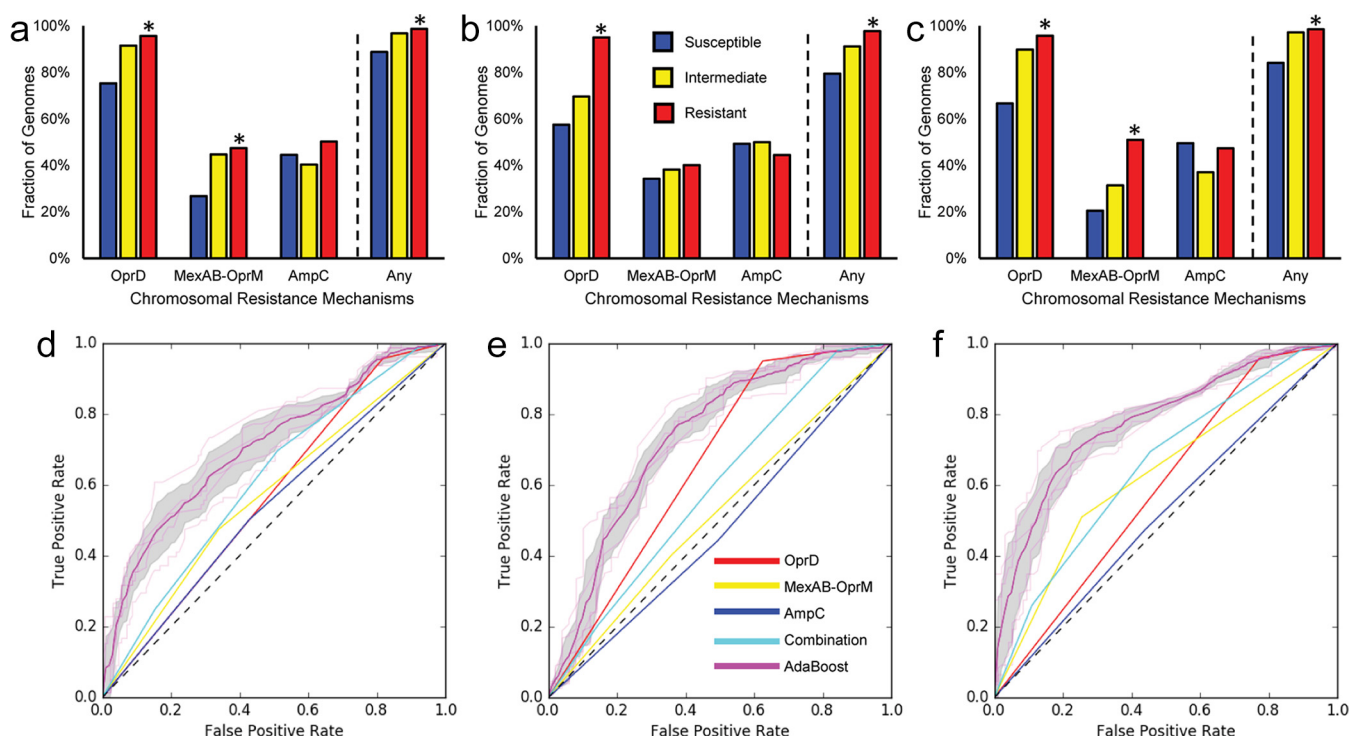


FIG 2 Frequency genetic markers of carbapenem resistance found in the EIP *P. aeruginosa* isolates categorized by their susceptibility to three carbapenems (a to c; statistically significant differences between the resistant and susceptible/intermediate isolates as determined by Fisher's exact test ($P < 0.05$) are shown with an asterisk [*]). Receiver operator characteristic (ROC) curves and area under the curve (AUC) values for the predictive power for carbapenem resistance of markers for the three chromosomal mechanisms of carbapenem resistance and combinations thereof, and a machine learning (ML) classifier made from the individual mutations on the genes associated with each mechanism (d to f). The ML line is the average of stratified, 5-fold cross-validations with shaded ± 1 standard deviation.

The resistant isolates were more likely to have any markers of chromosomal resistance mechanisms than susceptible or intermediate isolates for all three carbapenems (Fig. 2a to c, $P < 0.05$, Fisher's exact test). Markers for OprD overexpression were found more frequently in resistant isolates for all drugs, while MexAB-OprM overexpression markers were found more frequently in doripenem- and meropenem-resistant isolates. There was no difference in the occurrence of AmpC overexpression markers between the isolates which were resistant and non-resistant to each drug.

Despite the overall correlation between resistance markers and carbapenem resistance, none of the markers were strong predictors of resistance to individual carbapenems, as they all had receiver operating characteristic area under the curve (AUC) values of < 0.7 (Fig. 2d to f, Table 2). A machine learning classifier (25) incorporating all the genetic variations found on the genes of these three mechanisms was more successful at predicting resistance to each carbapenem. The most important feature for predicting imipenem resistance from this classifier was an OprD marker, and for meropenem resistance it was an MexAB-OprM marker (Table S3), consistent with previous work showing the specificity of these mechanisms to resistance to these individual carbapenems (26, 27). AmpC-related markers were also among the five most important features for the model to predict imipenem and meropenem resistance (Table S3).

TABLE 2 AUC ROC curve values for predicting resistance^a

Classifier	Doripenem	Imipenem	Meropenem
OprD dysfunction	0.570	0.663	0.593
MexAB-OprM overexpression	0.568	0.522	0.627
AmpC overexpression	0.537	0.474	0.517
Combination of mechanisms	0.612	0.592	0.652
AdaBoost (all unique markers)	0.704	0.736	0.781

^aAUC, area under the curve; ROC, receiver operating characteristic.

TABLE 3 Acquired *bla* genes detected by WGS^a

Allele	Family	Total isolates	STs (n)
CARB-2	<i>bla</i> _{CARB}	32	111 (29), 2875 (2), N/M
OXA-2	<i>bla</i> _{OXA}	19	179 (4), 308 (4), 282 (2), 298 (2), 309 (2), 463 (2), 235, 446
OXA-9	<i>bla</i> _{OXA}	13	111 (11), 235, N/M
OXA-141	<i>bla</i> _{OXA}	7	282 (7)
OXA-681	<i>bla</i> _{OXA}	2	282 (2)
VIM-4	<i>bla</i> _{VIM} ^b	2	357
KPC-3	<i>bla</i> _{KPC} ^b	2	235 (2)
GES-19	<i>bla</i> _{GES}	2	309
OXA-2 mutant	<i>bla</i> _{OXA}	1	845
OXA-10	<i>bla</i> _{OXA}	1	235
OXA-17	<i>bla</i> _{OXA}	1	27
OXA-415	<i>bla</i> _{OXA}	1	282
OXA-677	<i>bla</i> _{OXA}	1	357
CARB-3	<i>bla</i> _{CARB}	1	111
VIM-2	<i>bla</i> _{VIM} ^b	1	2555
KPC-2	<i>bla</i> _{KPC} ^b	1	235
GES-26	<i>bla</i> _{GES}	1	309
VEB-1	<i>bla</i> _{VEB}	1	357
VEB-5	<i>bla</i> _{VEB}	1	235
IMP-75	<i>bla</i> _{IMP} ^b	1	2,731
TEM-1A	<i>bla</i> _{TEM}	1	235
L2	<i>bla</i> _{L2}	1	3054

^aWGS, whole-genome sequencing.^bCarbapenemase.

Carbapenemase genes were found in seven (0.7%) isolates (Table 3) and included members from three gene families: IMP, KPC, and VIM. Two isolates had *bla*_{KPC-3} and two others had *bla*_{VIM-4}, but the overlapping alleles were found on otherwise unrelated contigs in the genome assembly, suggesting that they did not emerge from a common source such as a shared plasmid. The isolate with *bla*_{VIM-2} was not resistant to any carbapenems (it was intermediate to imipenem and susceptible to doripenem and meropenem) when tested by reference broth microdilution at the CDC.

β -Lactamase genes were detected in all of the sequenced isolates, and 985 (96.7%) had at least two, which was expected given that *P. aeruginosa* generally has two such genes on its chromosome (*bla*_{PDC} [28] and *bla*_{OXA-50} [29]). Sixty-six (6.5%) isolates had additional acquired, non-carbapenemase β -lactamase genes detected in their genomes (Table 3), including 19 with more than one. Isolates with these acquired *bla* genes were more likely to be resistant to all of the carbapenems than the isolates which lacked them (Fig. 3a, $P < 0.05$, Fisher's exact test). The isolates with acquired *bla* genes also had higher incidence of markers for OprD dysfunction, but decreased prevalence of markers associated with MexAB-OprM and AmpC overexpression (Fig. 3b). They were also more likely to be resistant to at least one carbapenem than acquired *bla*-negative isolates with OprD dysfunction (708 [86.6%] of 818, $P < 0.05$, Fisher's exact test). The most common acquired β -lactamase gene was *bla*_{CARB-2}, which was found in 32 (3.1% of the 1019 isolates) isolates, and 29 of these (90.6%) were ST111.

Enriched core gene alleles. We analyzed the results from the cgMLST scheme to determine whether specific alleles were enriched (i.e., found at a higher frequency, see Materials and Methods section for details) in this set of genomes versus those from publicly available RefSeq genomes (a diverse set that includes resistant and susceptible isolates from both human and non-human sources), which could indicate that any such alleles were involved in pathogenesis or drug resistance. Eighteen enriched gene alleles were identified (Table S4), including 11 genes with defined functions. These included an *xcpP* allele enriched at 73.0% which is part of the Type II secretion system involved in pathogenesis by *P. aeruginosa* (30), and an allele for a gene from the YggU family (enriched at 60.3%) which has been shown to be involved with biofilm formation (31) and infections caused

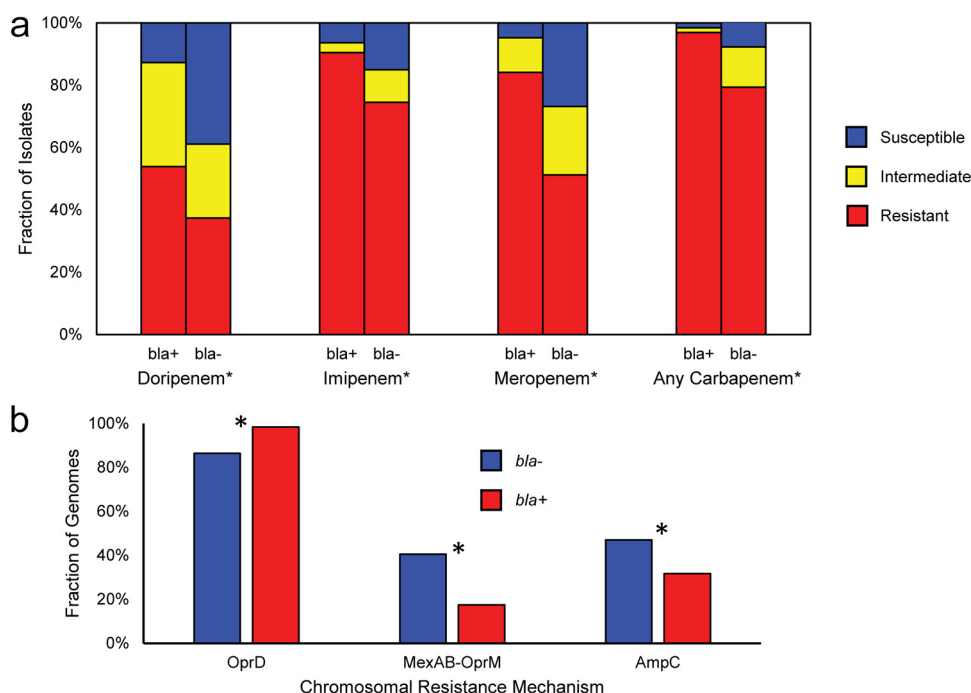


FIG 3 Comparison of carbapenem susceptibility between 65 isolates with acquired *bla* genes (not including carbapenemases) and those without. (a) The *bla*-harboring genomes (*bla*+, in red) had different prevalence of markers of chromosomal carbapenem-resistance mechanisms. (b) Statistically significant differences as determined by Fisher's exact test ($P < 0.05$) are shown with an asterisk (*).

by other bacterial species (32). These alleles were found at the same frequency in both the carbapenem-resistant and non-resistant genomes.

Isolates from cystic fibrosis patients. Fifty-two (5.1%) of the *P. aeruginosa* isolates were collected from CF patients, representing 44 different STs, 41 of which were found only in the CF isolates. Isolates from CF patients varied by 3,745 cgMLST alleles (84.3% of 4440 genes) among themselves and by an average of 3,799 (85.6%) alleles from the non-CF genomes (Table S5, Fig. S6 and 7). There were no cgMLST allele sequences enriched (using the definition given above) in the CF versus the non-CF isolates.

The CF isolates were more likely to be XDR (34, 65.4%) than the non-CF isolates (366, 37.8%; Fig. S8a, $P < 0.05$, Fisher's exact test). This was due to increased rates of resistance to drugs from four of the seven antimicrobial classes (cephems, β -lactam combination agents, quinolones, and aminoglycosides, Fig. S9). Forty-five (86.5%) of the CF isolates were resistant to at least one carbapenem when tested at the CDC, which was not a statistically significant difference from the non-CF isolates. These were more likely to be resistant to doripenem compared to the non-CF isolates (Fig. S8c). There was no significant difference in the prevalence of any markers of carbapenem-resistance mechanisms found in their genomes, and only one had an acquired β -lactamase (*bla*_{OXA-2}).

Hypermutator genotypes commonly associated with chronic lung infection (33) were identified in 19 (36.5%) CF isolates; this is consistent with previous studies of *P. aeruginosa* in CF patients, as the genotype can drive adaptation for long-term colonization in the face of competition and drug pressure (34, 35). The incidence of hypermutators was significantly higher than that observed in isolates from patients without CF (36.5% versus 8.1% [$P < 0.05$, Fisher's exact test], Fig. S8b).

DISCUSSION

The multiple pathways through which carbapenem resistance can arise in *P. aeruginosa* demonstrate the convergent evolution of this phenotype emerging from diverse

genotypes, reflected by the fact that no single ST constituted more than 10% of this set. While three high-risk STs were found in every participating EIP site, these together made up <20% of the isolates collected. The genomes varied from one another by an average difference of ~85% of the cgMLST alleles, less than they did compared to the *P. aeruginosa* genomes available from RefSeq, likely because these were all clinical isolates with similar resistance profiles, collected from a limited geographic range and time span.

Although all 1,019 of the *P. aeruginosa* isolates were resistant to a carbapenem when tested at local labs, 198 (19.4%) were not when tested at the CDC. This discrepancy could be due to the different antimicrobial susceptibility testing (AST) methods used at the EIP labs (36, 37), the degradation of resistance from multiple passages (38), and the variability in broth microdilution results for β -lactams (39) occurring around SIR threshold values, which could be the case for the isolate with carbapenemase (*bla*_{VIM-2}) which was not resistant to any carbapenems but was intermediate to one. The ambiguity in resistance could help explain why the enriched alleles identified in these more frequently than in the RefSeq genomes were found in both the resistant and non-resistant isolates, and why the majority (160, 80.8%) of the non-resistant isolates still had chromosomal markers of carbapenem resistance (though less frequently than those that were found to be resistant by testing at CDC).

The analysis of these chromosomal markers of resistance should include several additional caveats beyond the fact that all these isolates were resistant to at least one carbapenem at the time of initial collection. The nuances of the individual contributions of any specific mutations or their additive effects are lost when they are abstracted into three broad categories. The presence of specific markers is also related to the phenotypic profiles of the isolates, and this set included many more isolates which were resistant to imipenem than those which were resistant to the other carbapenems. This bias in resistance rates could help explain the high frequency of markers for OprD dysfunction, as this mechanism is specifically associated with imipenem resistance. Furthermore, the markers themselves were likely undercounted, as we only included previously identified genetic markers and did not perform any additional measures (such as gene expression studies) to validate the hundreds of novel mutations we found on genes associated with resistance.

Accordingly, a machine learning classifier that included all the genetic variations (including the uncharacterized mutations) found on the genes associated with these mechanisms had more predictive power for resistance to individual carbapenems than the broader mechanistic categories. This analysis also identified several AmpC-related features that enhanced the power of the model to predict resistance, even though as a class those mechanisms were not found at higher rates among the resistant isolates. Also, previously uncharacterized mutations could help explain the 17 isolates that were resistant to at least one carbapenem yet had no known resistance markers. Future work to better understand these markers could enhance genotype-phenotype predictions.

While very few known carbapenemase genes were found in this study, the presence of acquired, non-carbapenemase β -lactamase genes was correlated with an increased probability of resistance to all carbapenems tested. While some of these β -lactamases may hydrolyze carbapenems at low levels (40), the isolates with these genes were more likely to have mutations associated with OprD dysfunction and less likely to have any other markers of chromosomal resistance. But the increase in markers of OprD dysfunction alone does not fully explain the observed resistance, as isolates with the combination of OprD dysfunction markers and non-carbapenemase acquired β -lactamases were more likely to be carbapenem-resistant than those with OprD markers but no acquired β -lactamases, suggesting an additive effect. In addition, because these β -lactamase genes can be acquired horizontally, they can contribute to the non-clonal spread of carbapenem resistance in *P. aeruginosa*.

The genomes of the isolates collected from CF patients showed no difference in chromosomal markers of resistance mechanisms, nor did they have more acquired

β -lactamase genes than the non-CF genomes. They were more likely to have the hypermutator genotype, which could help explain their higher incidence of resistance to four of the seven antimicrobial classes tested.

The heterogeneity in the phylogeny and resistance mechanisms in this geographically diverse set of isolates underscores the complexity of the public health threat CRPA represents, making it difficult to diagnose with any single rapid molecular assay. Although most of the detected mechanisms of carbapenem resistance were chromosomal and therefore not readily spread through horizontal transfer (which could confer resistance even in the absence of antibiotic pressure), their variety suggests that such mechanisms emerge from convergent evolution under antibiotic pressure often found in the health care setting. The release of the full AST and WGS data from these 1,019 clinically relevant *P. aeruginosa* isolates (NCBI BioProject ID [PRJNA288601](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA288601)) can be used as a basis for future genotype-phenotype analyses beyond those presented here to better understand the mechanisms driving the evolution of resistance in CRPA.

MATERIALS AND METHODS

Isolate collection. A case was defined as the first isolate of *P. aeruginosa* resistant to imipenem, meropenem, or doripenem from the lower respiratory tract, urine, wound, or a normally sterile site identified from a resident of the EIP catchment area in eight states (Colorado, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee) over a 30-day period. The participating state labs conducted laboratory- and population-based surveillance for CRPA clinical isolates that met this definition from 1 August 2016 through 31 July 2018. The second year of surveillance also included CRPA isolates collected from throat swabs from CF patients. Each EIP site selected a systematic random sample of 5 to 8 isolates from patients without CF each month to submit to CDC for further characterization and submitted up to 10 CF patient isolates during the surveillance period. Confirmatory species identification by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF) was performed using the Biotyper 3.1 MALDI-TOF System (Bruker Daltonics, Billerica, MA).

Antimicrobial susceptibility testing. AST was performed by broth microdilution with in-house frozen reference panels with 12 antibiotics and 3 combination agents from seven classes (carbapenems [doripenem, imipenem, and meropenem], monobactams [aztreonam], cepheems [cefepime and ceftazidime], β -lactam combination agents [ceftazidime-avibactam, ceftolozane-tazobactam, and piperacillin-tazobactam], lipopeptides [colistin], quinolones [ciprofloxacin and levofloxacin], and aminoglycosides [gentamicin, amikacin, and tobramycin]) according to CLSI guidelines (41, 42). In-house reference panels were prepared using 96-well sterile microtiter plates (Capiugs, Rancho Dominguez, CA) with cation-adjusted Mueller-Hinton Broth (BD, Franklin Lakes, NJ) and stored at -70°C until testing. Each isolate was grown on Trypticase soy agar with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ) for 18 to 24 h at 37°C . Following CLSI guidelines (41), each isolate was suspended in 0.85% saline to match a 0.5 McFarland turbidity standard and further diluted 1:20 in sterile 0.85% saline. In-house panels were inoculated with $10\ \mu\text{L}$ of the 1:20 dilution using a sterile 95-pin inoculator (Capiugs, Rancho Dominguez, CA). The MIC plates were incubated at 35°C for 16 to 20 h. The following quality control isolates were tested along with the clinical isolates: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* strains BAA-2146, ATCC 700603, and BAA-2814.

Whole-genome sequencing. DNA was extracted using a Maxwell 16 MDx instrument (Promega; Madison, WI) with the Promega Maxwell 16 Cell Low Elution Volume (LEV) DNA purification kit. DNA was fragmented using a Covaris ME220 Focused-ultrasonicator (Covaris, Woburn, MA). Sample libraries were prepared with the NuGEN Ovation Ultralow System V2 assay kit (San Carlos, CA). Whole-genome sequencing was done on an Illumina MiSeq instrument (San Diego, CA) to generate 250-bp paired-end reads. The sequenced reads have been deposited in NCBI BioProject ID [PRJNA288601](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA288601).

Bioinformatic analysis. Initial analysis was performed using the PHoeNix pipeline (<https://github.com/CDCgov/phoenix>). The pipeline includes identification of MLST using PubMLST definitions (19) and antibiotic resistance (AR) genes via GAMMA (43), using a database constructed from the nonredundant entries in the ARG-ANNOT (44), NCBI AMRFinder (45), and ResFinder (46) AR gene databases.

Chromosomal markers of carbapenem resistance mechanisms from the three major categories were also assessed using GAMMA and are included in File S2, which includes a description of the mutations found when the genes were compared to those from the *P. aeruginosa* PAO1 strain (43, 47): a decrease in outer membrane permeability mediated by the porin OprD (15) considered truncations of the *oprD* gene itself and mutations of its transcription binding region and the *mexS* transcription factor; overexpression of the MexAB-OprM efflux pump via derepression (16) included an analysis of the *mexR*, *nalD*, and *nalC* repressor genes; and overexpression of the intrinsic β -lactamase AmpC by derepression (17) was determined by assessing mutations of the *ampR*, *ampD*, and *dacB* repressor genes. Only gene truncations of $>10\%$ or mutations previously associated with carbapenem resistance (Table 1) were counted as markers of resistance, although other mutations to these genes which have not been characterized in the literature were counted, added to Table S2, and included in the machine learning data set. Hypermutator genotypes were identified as frameshift mutations, deletions, or truncation of the DNA mismatch-repair genes *mutL*, *mutM*, *mutS*, or *uvrD* (34).

Genome phylogeny was assessed using *P. aeruginosa* MLST (19) and cgMLST (20) schemes. The

cgMLST scheme included 4,440 alleles, and considered any overlapping alleles to be different between two genomes if they differed by at least one base in their sequence. The dendrograms were made using the unweighted pair group method and annotated with iTOL (42). The scheme was also used to identify gene alleles enriched in the genomes from this set versus 2,049 *P. aeruginosa* genomes from RefSeq downloaded on 13 December 2018. Enriched alleles were defined as specific gene sequences found at 50% or higher frequency in the genomes collected in this study than in those from RefSeq.

Statistical analysis. The prevalence of categorical variables (i.e., presence/absence of gene markers, resistant/non-resistant, etc.) was evaluated using Fisher's exact test implemented through SciPy (<https://scipy.org/>), with a Bonferroni correction applied to a *P* value threshold of ≤ 0.05 to determine statistical significance. A one-way ANOVA technique was used to analyze allele differences determined from the *P. aeruginosa* cgMLST scheme. Supervised machine learning classification using an AdaBoost ensemble model was performed with all the individual mutational changes to the genes associated with the three chromosomal mechanisms of carbapenem resistance and assessed with a stratified, *k*-fold cross validation (*k* = 5) using scikit-learn (<https://scikit-learn.org/>) (48). Receiver operator characteristic (ROC) curves and AUC analyses were performed with scikit-learn to determine the predictive power of the resistance mechanisms and the AdaBoost model.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.9 MB.

ACKNOWLEDGMENTS

This work was supported by the Centers for Disease Control and Prevention's internal funding. The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

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