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The Resistome of *Pseudomonas aeruginosa* in Relationship to Phenotypic Susceptibility

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Many clinical isolates of *Pseudomonas aeruginosa* cause infections that are difficult to eradicate due to their resistance to a wide variety of antibiotics. Key genetic determinants of resistance were identified through genome sequences of 390 clinical isolates of *P. aeruginosa*, obtained from diverse geographic locations collected between 2003 and 2012 and were related to microbiological susceptibility data for meropenem, levofloxacin, and amikacin. β-Lactamases and integron cassette arrangements were enriched in the established multidrug-resistant lineages of sequence types ST111 (predominantly O12) and ST235 (O11). This study demonstrates the utility of next-generation sequencing (NGS) in defining relevant resistance elements and highlights the diversity of resistance determinants within *P. aeruginosa*. This information is valuable in furthering the design of diagnostics and therapeutics for the treatment of *P. aeruginosa* infections.

Pseudomonas aeruginosa is a Gram-negative bacterium associated with nosocomial infections (1, 2). It is the major cause of morbidity and mortality among individuals afflicted with cystic fibrosis (CF) (3) and is the third most common bacterium isolated from infections acquired in intensive care units (4). Due to its effective intrinsic and acquired resistance mechanisms to different classes of antibiotics (5), infections caused by this opportunistic pathogen are difficult to treat. As reported in many epidemiological studies, *P. aeruginosa* clinical isolates are resistant to an increasingly wide variety of antibiotics, including "fourth-generation" cephalosporins. High levels of β-lactam resistance have been reported in the United States (6), Europe (7), and South America (8, 9). In many cases, only colistin, and to some extent amikacin, still exhibits good activity against these multidrug-resistant *P. aeruginosa* infections (4).

Knowing the susceptibility profile of a *P. aeruginosa* isolate associated with an infection before beginning treatment allows the optimization of clinical outcomes and minimizes the unintended consequences of antimicrobial use, which include the emergence of resistance, toxicity, and further sequelae, such as *Clostridium difficile* colitis (10). With the ever increasing incidence of bacterial drug resistance, the need for rapid and reliable methods to predict antimicrobial susceptibility early in the course of treatment is ever more pressing (11). Advances in methodology and the decreasing cost of next-generation sequencing (NGS) have the potential to impact clinical microbiology ranging from species identification to antimicrobial susceptibility testing (12–14). Indeed, whole-genome sequencing studies of *Enterococcus* spp., *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Klebsiella pneumoniae* have demonstrated that reliable schemes can be generated for prediction of resistance phenotypes in these organisms (12, 15).

Accordingly, the study presented here used NGS to construct a comprehensive genomic analysis of 390 *P. aeruginosa* isolates. Whole-genome sequencing of these *P. aeruginosa* isolates was conducted to define the diversity and distribution of resistance mechanisms and to determine the extent to which NGS can reliably provide a genotype to support and better define a nonsusceptible phenotype.

MATERIALS AND METHODS

Isolates. A total of 390 *P. aeruginosa* isolates were obtained from the International Health Management Association (IHMA) and subjected to susceptibility testing (see Table S1 in the supplemental material). Two isolates repeatedly failed to grow in broth and were removed from the subsequent genotypic analysis ($n = 388$ isolates). Diversity was confirmed by the creation of a phylogenetic tree that was subsequently used to visualize the resistome-related data.

Whole-genome sequencing. Total DNA was extracted from several purified colonies of each isolate using the Maxwell 16 cell DNA purification kit (Promega) and prepared for multiplexing on either a HiSeq 2000 or MiSeq (Illumina). All genome assemblies were deposited at DDBJ/EMBL/GenBank and are associated with BioProject accession no. PRJNA264310.

Phylogenetic analysis. The complete genomes of 15 *P. aeruginosa* isolates used for reference purposes and orientation in the analysis were downloaded from NCBI (see Table S1 in the supplemental material). Shared open reading frames (ORFs) between all of the *P. aeruginosa* isolates were identified using the 5,570 ORFs of the reference genome PAO1 (AE004091) as a baseline comparator and extracted using tfastx36 (16, 17). ORFs found in all 390 isolates that share at least ≥90% coverage with the corresponding PAO1 genes (1,278) were used in phylogeny construction. Sequences were aligned using the MAFFT aligner (18), and the phylogenetic tree was generated using EXaML (19) with the parameters set to estimate a maximum likelihood. Branch support was estimated by 100 bootstrap replicates. Assemblies were completed in CLCGenomic Work-

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bench 6.5.1 (CLCBio). Integron analysis was conducted using Ray assemblies (20).

MLST analysis and *in silico* serotype. Multilocus sequence type (MLST) was determined using CLCBio Genomic Workbench 6.5.1 and the MLST schema for *P. aeruginosa* (www.pubmlst.org; downloaded April 2014) (21, 22). New sequence types were assigned a unique internal identifier. Serotyping was determined *in silico* (23, 24).

Identification of resistance genes. Resistance genes were identified using an in-house curated database followed by manual curation. This database consisted of 1,755 genes from various bacterial species associated with resistance to 14 different antibiotic classes as well as multidrug exporters. Accession numbers for the genomic elements associated with the nonsusceptible phenotypes identified among the 390 *P. aeruginosa* isolates are highlighted in Table S1 in the supplemental material. Quinolone resistance-determining regions (QRDRs) were assigned to amino acid positions 83 to 87 of the GyrA protein, positions 429 to 585 of the GyrB protein, positions 82 to 84 of the ParC protein, and positions 357 to 503 of the ParE protein (25). Stop codons that resulted in the production of a truncated protein and frameshifts in all chromosomally encoded resistome genes were considered to contribute to a susceptibility-related phenotype.

Susceptibility testing. MICs were determined using frozen Trek Sensititre custom plates (Thermo Scientific) following the Clinical and Laboratory Standards Institute (CLSI) guidelines (26, 27). Plates were custom ordered to contain an antibiotic panel consisting of amikacin, meropenem, and levofloxacin. These 3 antibiotics were chosen as genetic markers because their association with conferring nonsusceptibility has been clearly established. Susceptibility to colistin for a subset of the collection of isolates (362 of 390) was completed with the intention of potentially highlighting genetic polymorphisms that have been identified as contributing to nonsusceptibility observed against this last-line antibiotic. The quality control strain *P. aeruginosa* ATCC 27853 was routinely tested.

RESULTS

Diversity of *P. aeruginosa* population obtained through sampling. Phylogenetic analysis and assessment of genome size illustrated that a heterogeneous population of *P. aeruginosa* was obtained through sampling (Fig. 1A to C). Genomic size varied dramatically between isolates, ranging from ~6 to 7.4 Mbp (Fig. 1B), demonstrating the likely differences in genomic content between isolates, which were also independent of geographic or temporal distribution (data not shown). No phylogenetic correlation with anatomical site of infection could be demonstrated, with the exception of CF isolates (Fig. 1C), agreeing with previous observations (28). Additionally, the CF isolates typically exhibited reduced genome size as previously reported for the DK2 lineage (29) and other isolates collected in CF-related studies (30, 31).

The MLST alleles of 387 of the 390 isolates were sequenced with enough coverage to assign a sequence type (ST) and highlighted the large diversity in the *P. aeruginosa* clinical isolates, with 175 different assignable types (21, 22), 61 of which were not previously catalogued (see Table S1 and Fig. S1 in the supplemental material). MLST analysis suggested that specific clones were endemic to particular geographic locations, which may be reflective of environmental sources and transmission between patients (see Fig. S1). It was generally observed that any one center from which isolates were collected could contain a diverse set of isolates, although typical clonal associations were found, such as the ST277 SPM-1-carrying isolates being exclusively isolated in Brazil (32) and a majority of the ST175 clones coming from Spain (33) (see Fig. S1). Isolates belonging to the ST395 clade, which have previously been reported to be associated with water sources (34), were associated with several infections, suggesting that *P. aeruginosa* may readily be acquired from the environment. The globally dispersed clones (35, 36) belonging to either ST235 (37 isolates) or the ST111 (24 isolates) were well represented from the Americas, Europe, and Asia (see Table S1 and Fig. S1). These isolates also represented the dominant O serotypes, O11 and O12, respectively, with the O4 serotype also represented among the ST111 isolates (see Table S1).

Identification of integron arrangements associated with an array of β-lactamases. As horizontally acquired resistance genes are typically associated with integrons, an analysis aimed at identifying putative integron structures and in particular their associated β-lactamases was carried out (see Table S1 in the supplemental material). Class 1 integrons, which consist of a variable region bordered by conserved 5′ and 3′ ends, were identified in over 120 isolates. The majority were of the sul1 type (*qacEdelta1* and *sul1* in their 3′-CS); however, Tn*402*-type (*tniRQBA* in their 3′-CS) were also present. ST111 (O12) and ST235 (O11) isolates were found to harbor the majority of the integrons, although others were identified in multiple STs, representative mainly of the O6 and O4 serotypes. Some isolates contained two or three integrons (see Table S1). Many integrons were associated with genomic islands, but at least two were associated with plasmids that were assembled from the sequencing reads (AZPAE13872 and AZPAE14860). The contig associated with the plasmid of AZPAE13872, in addition to carrying the *sul1*-type integron associated with an IMP-15 metallo-β-lactamase (MBL), was identified on a contig that resembled a plasmid with the *oriT* from pSMC1 (AP013064.1) as well as genes conferring mercury resistance that have been identified in multiple *Pseudomonas* species, suggesting that it has moved across many species. Many of the integrons containing the MBL VIM and IMP genes and the serine β-lactamase GES gene also carried genes conferring amikacin resistance: e.g., *aacA7* and *aac(6′)-Ib*. Integron cassettes described over 20 years ago (OXA-2, OXA-10, *aadA*, and *cmlA*) (37, 38) were common, with OXA-2-associated integrons predominating in the ST235 isolates, suggesting that this configuration has been very successful in the face of selection by currently used antibiotic therapy. Genes for extended-spectrum β-lactamases that are not carbapenemases (e.g., OXA-17 and OXA-129) were also arranged in integrons and associated prominently with isolates from South America, potentially reflecting antibiotic treatment regimens. Three strains (AZPAE14700, -14703, and -14705) contained a novel group IIC-*attC* intron, a riboelement that may be involved in formation of integron cassettes (39). Many other resistance-related genes, in addition to those coding for the β-lactamases, were accumulated in these integron structures (see Table S1).

Horizontally acquired β-lactamases were detected in 92 isolates, of which 54 carried one β-lactamase gene (in addition to the chromosomal *ampC* and bla_{OXA-50}), 32 possessed two, and six harbored three β-lactamases (Fig. 2A and B) (see Table S1 in the supplemental material). The most prevalent acquired β-lactamases identified were VIM-2 (23 isolates), OXA-2 (22 isolates), and PSE-1 (13 isolates). The globally disseminated lineages of sequence types ST235 and ST111 carried a larger number of the horizontally acquired β-lactamases (Fig. 2B; see Table S1), potentially reflecting their success in hospital environments, where they are exposed to various drugs on a routine basis. Geographically, isolates from South America and India were more likely to carry multiple β-lactamases (see Table S1), potentially reflecting the relatively unregulated use of antibiotics in these regions.

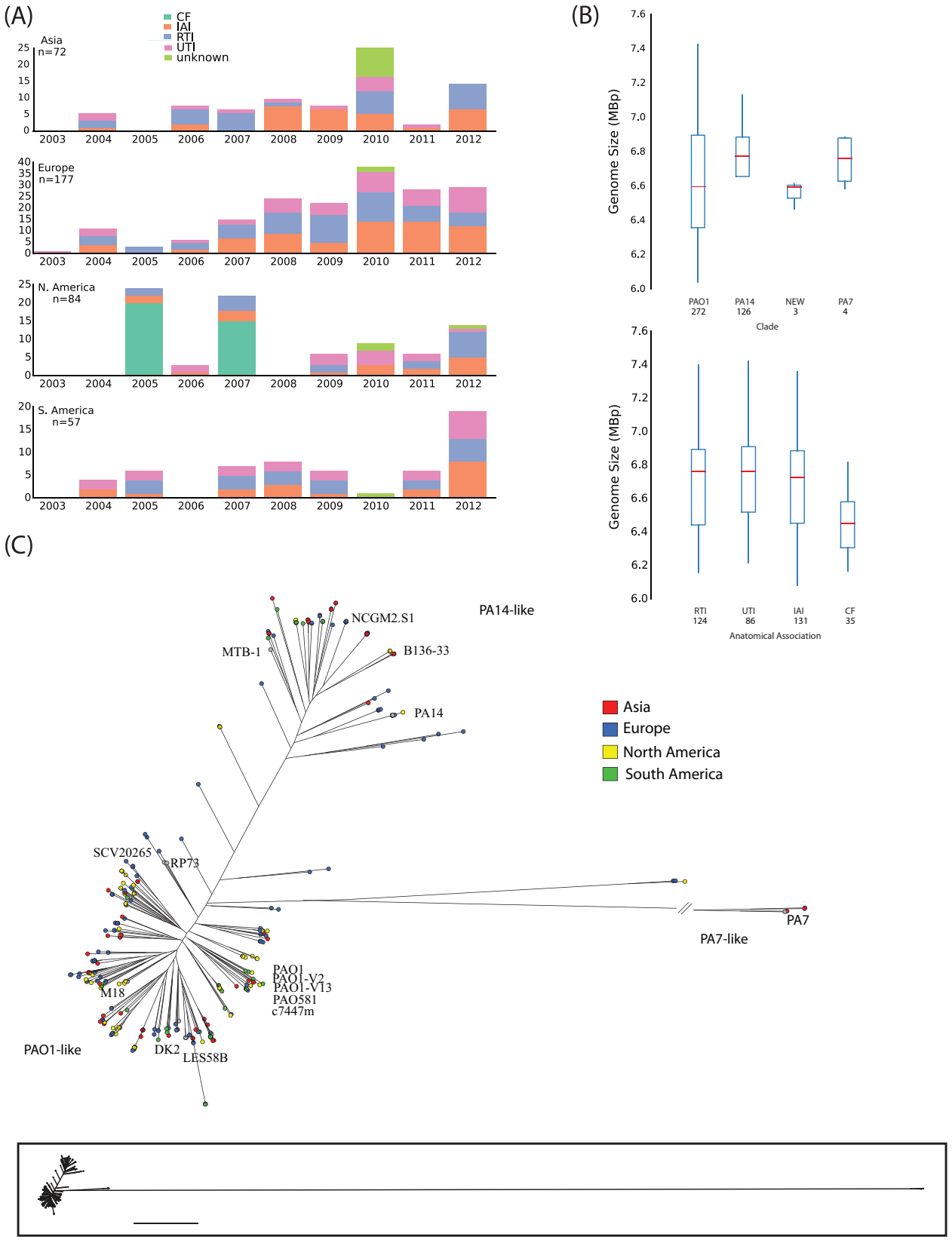


FIG 1 (A) Temporal, geographic and anatomical association of isolates used in this study. The majority of isolates came from sites in Germany, Spain, France, China, and the United States, while others were procured from hospitals in Argentina, Canada, Colombia, Croatia, Brazil, Greece, Italy, Israel, India, Portugal, Philippines, Romania, and Taiwan (see Table S1 in the supplemental material). (B) Box plot depicting size of draft genomes of *P. aeruginosa* associated with different clades and different anatomical association. CF isolates are distinguished as a separate group. Percentiles are marked. (C) Unrooted maximum-likelihood tree of 405 *P. aeruginosa* genomes based on single nucleotide polymorphisms within the core genome of 1,278 genes as defined by alignment to the PAO1 genome. Isolates are colored according to their continental origin. Representative genomes from NCBI are marked and colored gray. Isolates from Asia are colored red, isolates from Europe are blue, isolates from North America are yellow, and isolates from South America are green. The impression of the true distance of the phylogenetic tree is also provided.

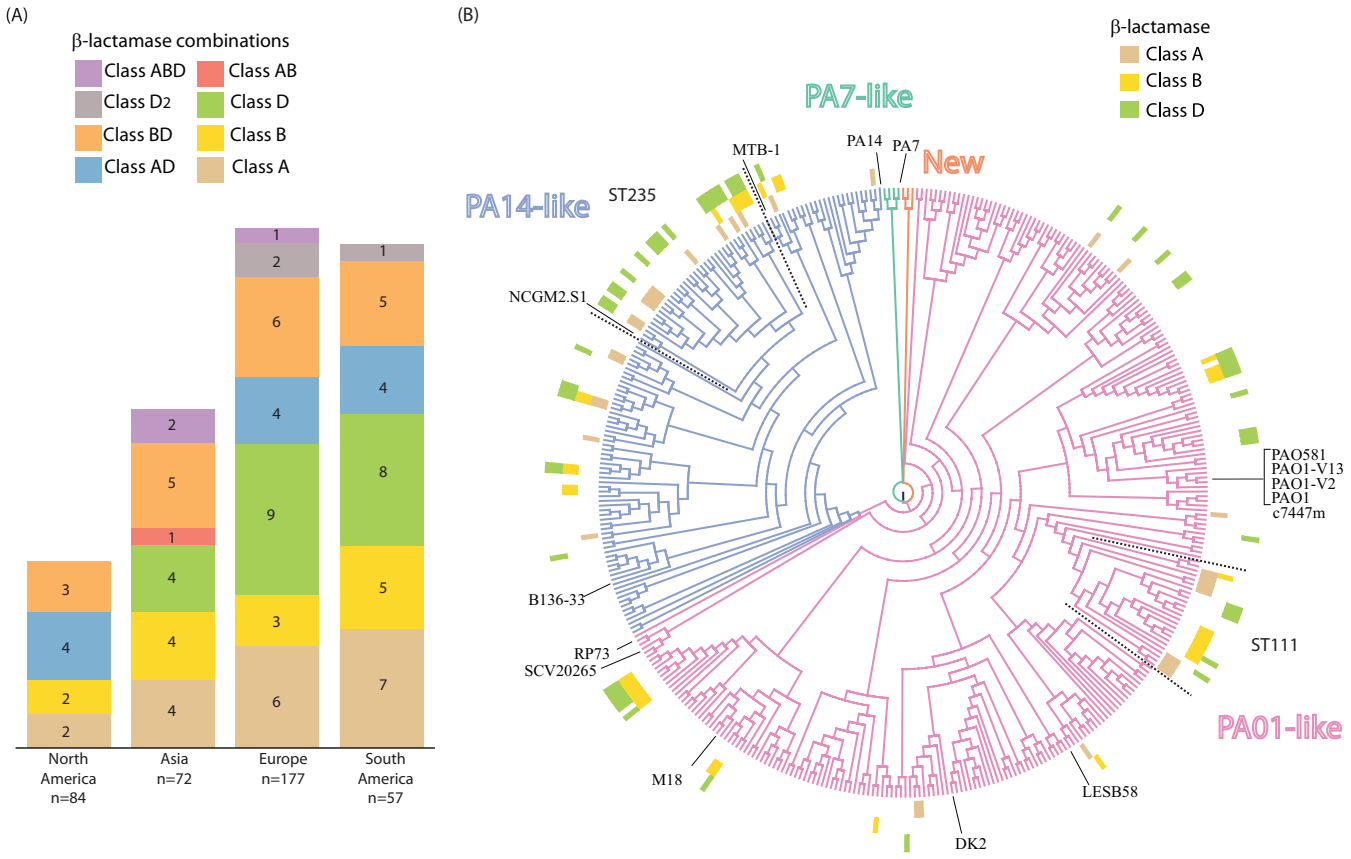


FIG 2 Summary of β-lactamase content. (A) Geographic association of β-lactamases among the collection of 390 *P. aeruginosa* isolates. Summary of class A (beige), B (yellow), and D (green) β-lactamase and cross-class combinations as marked: class D (2 types), gray; class AB, red; class AD, blue; class BD, orange; and class ABD, purple. Isolates that carried multiple β-lactamases from the same class are outlined in Table S1 in the supplemental material. Chromosomal *ampC* and bla_{OXA-50} genes were accounted for in the isolates except where noted (see Fig. S2A in the supplemental material). (B) Phylogenetic association of class A (beige), B (yellow), and D (green) β-lactamases. Clades are colored accordingly (PAO1-like, PA14-like, PA7-like, and new). Reference isolates are highlighted as are the isolates corresponding to ST235 and ST111, which were the largest groups enriched with β-lactamases.

A novel β-lactamase showing 81% identity to AER-1 was observed in two isolates, one from Spain (AZPAE14914) and the other from Brazil (AZPAE14926). Additionally, point mutations were identified in several of the β-lactamases that may alter their activity (see Table S1 in the supplemental material). Interestingly, no OXA-type carbapenemases or New Delhi metallo-β-lactamases were detected in any of the isolates.

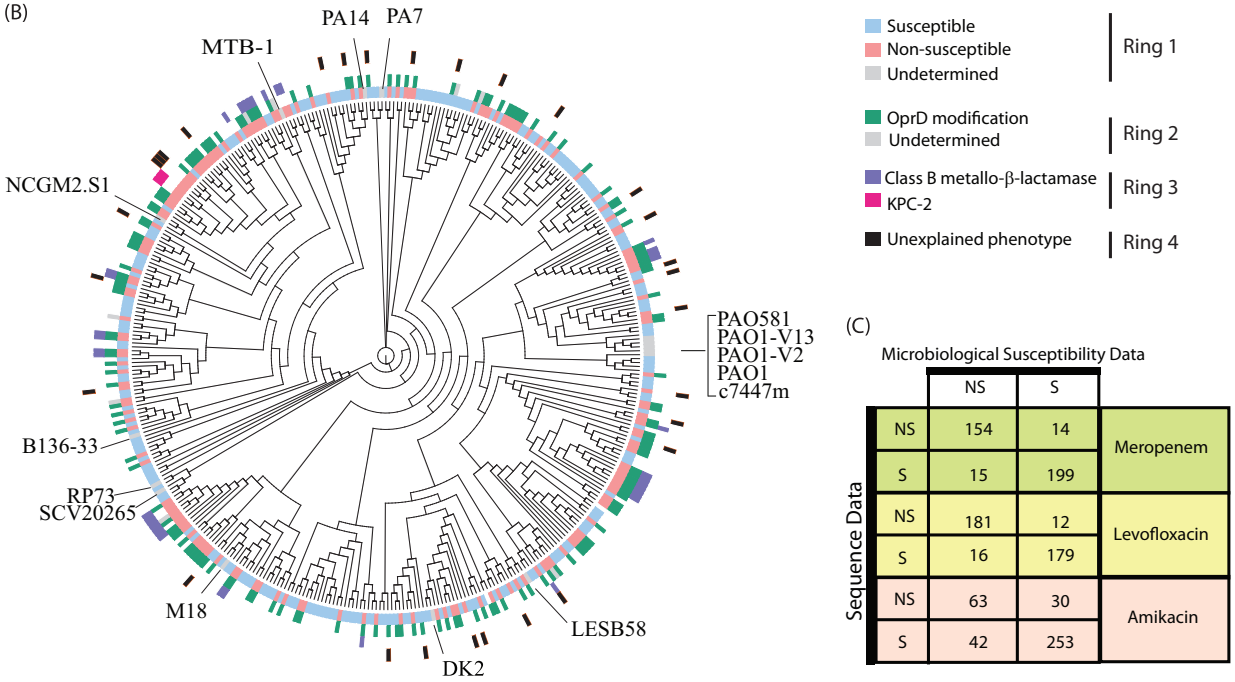
Genomic analysis of meropenem related resistance factors illustrates that mutations in OprD are the major driver of resistance. A total of 172 (44.3%) of the isolates were found to be nonsusceptible to meropenem (MIC, ≥4 μg/ml), and 216 (55.7%) of the isolates were susceptible (MIC, ≤2 μg/ml) (Fig. 3). OprD, in addition to its primary function in arginine import, is known to be the major entry point for meropenem (40). Mutations in *oprD* that result in the loss of porin function are known to reduce the uptake of the carbapenems and therefore confer resistance (41, 42). Of the ~100 porins identified in the PAO1 genome (43), OprD is the only porin that has a significant association with antibiotic susceptibility. We therefore started with a directed analysis of this candidate resistance gene, followed by identification of class B MBL and carbapenemases (Fig. 3A).

Analysis of OprD revealed that two distinct genotypes, differing mainly in loop 7 of the protein, exist in the population. These OprD types, by themselves, were not associated with a susceptibility phenotype and were classified either as type I, when showing similarity to the PAO1 parent sequence (146 isolates), or type II, when similar to the LESB58 genotype (241 isolates [3 not evaluable]) (see Table S1 in the supplemental material). Regardless of the type, classification by the presence of mutations of *oprD* appearing to affect OprD function could be used to assign an isolate as being nonsusceptible to meropenem. Such modifications, with the corresponding nonsusceptible phenotype, were seen in 143 isolates (28 of which additionally carried class B MBLs) (Fig. 3B) (see Table S1 and Fig. S2 in the supplemental material). In six isolates, MBL genes appeared to be the sole determinant of the genotypic definition mediating meropenem resistance. The class A carbapenemase, KPC-2, accounted for the resistance phenotype in another three isolates which were all from the same clinic in Colombia (Fig. 3B and C) (see Table S1). The OprD sequences of an additional 8 isolates were not evaluable, and these isolates were not assigned as susceptible or nonsusceptible—with the exception of 2 of these isolates that were found to carry the MBL VIM-2 and were therefore genotypically assigned as nonsusceptible. Genotypic assessment of full-length intact OprD and the absence of class B MBL or carbapenemases identified 201 isolates that agreed with the microbiologically susceptible phenotype. Twenty-nine

(A)

Meropenem Susceptibility	OprD mutations	OR	Class B β-lactamases (VIM, IMP, SPM)	OR	KPC-2, OXA-23, OXA-48, OXA-58		Efflux Mechanisms Regulatory Effects Unkown?
Levofloxacin Susceptibility	Mutations in QRDR *gyrAB* (Primary)	AND	*parCE* (Secondary)	OR	*aac('6)-Ib(cr)*		
Amikacin Susceptibility	ANY	*aph(3')-Vib* *aph(6')-Id*	*aph(3')-15* *aac(6')-Iai*	*aac(6')-31* *aac(6')-Ib*	*aacA29* *aacA7*	*ant(4')- IIb* *rmtD*	

(B)



(C)

Microbiological Susceptibility Data

Sequence Data	NS	S	
NS	154	14	Meropenem
S	15	199	
NS	181	12	Levofloxacin
S	16	179	
NS	63	30	Amikacin
S	42	253	

FIG 3 (A) Model identifying genetic determinants that were attributed to explaining the genetic susceptibility profile of the *P. aeruginosa* isolates. Meropenem susceptibility was genotypically assigned primarily based upon sequence modifications of OprD. Class B β-lactamases were also associated with a genotypic nonsusceptible phenotype when present in the absence of OprD modifications. Similarly, carbapenemases such as KPC-2 were considered to assign an isolate as nonsusceptible by genotype. For levofloxacin, combinations of mutations within the QRDR of *gyrA*/*B* and *parC*/*E* were primarily used to assign genotypic susceptibility. Amikacin genotypic susceptibility was assigned by identifying any of the contributing aminoglycoside resistance genes in any combination. Additional factors that may complicate the analysis and further contribute to resistance are also highlighted. (B) Comparison of the susceptibility profiles with the genetic determinants that classify an isolate as susceptible or nonsusceptible overlaid on the phylogenetic tree. The innermost ring (ring 1) illustrates the phenotypic susceptibility profile, where light red represents a nonsusceptible isolate and blue a susceptible isolate. Reference isolates are highlighted in gray as they serve for orientation and were not included in the analysis. Ring 2 highlights isolates that had changes in OprD (green) that would hinder the transport of meropenem through this porin. Isolates for which information could not be deduced for OprD due to incomplete sequence coverage are highlighted in gray. The third ring (ring 3) highlights the isolates that were found to carry class B MBL (purple) and those found to carry the carbapenemase KPC-2 (pink). The outermost ring (ring 4) highlights those isolates for which a discrepancy arose between the phenotypic susceptibility data and the genotypic analysis of the resistance-related components (black). (C) The 2-by-2 table summarizes the overlap in phenotypic (susceptibility) versus genotypic characterization for meropenem, levofloxacin, and amikacin.

isolates had susceptibilities to meropenem that could not be explained by OprD, MBLs, or KPC2 alone. Of the microbiologically susceptible meropenem population, 13 isolates were genotypically determined to have modifications in the *oprD* sequence. One isolate that was phenotypically susceptible to meropenem but would be classified as nonsusceptible by the chosen genotypic predictive criteria, carried an IMP-13 β-lactamase. The susceptible phenotype may be a result of this β-lactamase not being expressed, or it may have a reduced catalytic efficiency against meropenem (44). Finally, 15 isolates that lacked *oprD* modifications or class B MBL were microbiologically determined to be nonsusceptible, suggesting that additional factors, yet to be determined, contribute to their overall susceptibility profile.

Analysis of additional genomic determinants implicated in meropenem resistance. Many other β-lactamases, penicillin-binding proteins (PBPs), efflux pumps, and corresponding regulatory regions have been implicated in β-lactam resistance. We identified isolates containing AmpC variants (including Ala 105 variants) (45) and mutations leading to AmpC derepression (47), OXA-50 polymorphisms (48), PBP 5 polymorphisms (49), and multiple variants in efflux mechanisms (50, 51), such as MexAB-OprM, MexXY-OprM, and the regulator MexZ (see Fig. S2A in the supplemental material). While all may have contributory effects, no one particular variant was exclusive to the meropenem-nonsusceptible population of isolates whose phenotype was not explained by OprD and the class B MBL or KPC-2.

Genomic analysis of levofloxacin resistance mediated by type II topoisomerases. MIC determinations demonstrated that 198 (50.7%) isolates were nonsusceptible to levofloxacin (MIC, ≥4 μg/ml) and 190 (48.9%) could be categorized as susceptible (MIC, ≤2 μg/ml). We performed genomic analysis of the quinolone resistance-determining regions (QRDRs) (52, 53) of the 390 isolates. First-step mutations occur in the *gyrA* QRDR, while subsequent mutations are believed to further decrease susceptibility levels. Many secondary mutations alone are thought to be ineffective to render nonsusceptibility to levofloxacin, as the wild-type GyrA target would still be the most susceptible (25, 54, 55). Based on this premise (Fig. 3A), known *gyrA* mutations were used to classify an isolate as genotypically resistant to levofloxacin. Isolates with mutations in *gyrB* or *gyrB* plus *parE* have also been reported to be phenotypically nonsusceptible (25), and accordingly, isolates with these mutations were considered genotypically nonsusceptible in the analysis. Of the 198 phenotypically resistant isolates, *gyrA* mutations in the QRDR were identified in 163 isolates (see Table S1 in the supplemental material). Several combinations of mutations were identified within the QRDRs, with the corresponding amino acid changes in GyrA (T83I) and ParC (S87L) being the most prominent combination found in 82 of the nonsusceptible isolates (Table S1). Three isolates were identified with mutations in the QRDR of GyrB and ParE and also classified as nonsusceptible. Additional mutations in the nonsusceptible population that did not have QRDR *gyrA* mutations included 14 isolates with single mutations within the QRDR of *gyrB* (corresponding to 3 S466Y, 8 S466F, 1 S466F I487T, 1 Q467-, and 1 P749S). An additional 17 microbiologically nonsusceptible isolates did not harbor mutations in the QRDR of *gyrA*/*B*, but four harbored single mutations in *parC* or *parE* (2 ParC [S87L] and 2 ParE [A473V]).

Of the 190 phenotypically susceptible isolates, 174 isolates had no mutations within the QRDRs of any of the type II topoisomerases. One of these isolates had a G81D substitution in GyrA that fell just outside the QRDR, but this isolate was phenotypically susceptible, suggesting that the change did not confer nonsusceptibility. Five susceptible isolates did have GyrA mutations (3 T83I, 1 T83A, and 1 D87N) and one had a GyrA (T83I) plus ParE (D419N) substitution. An additional five microbiologically susceptible isolates harbored single mutations in the QRDR of *gyrB*, resulting in the following amino acid changes: 1 G712R, 3 I529V and S578A, and 1 S466F. One susceptible isolate had changes that resulted in GyrB (S466F) plus ParE (S87L). This illustrates the added value of sequencing information, in that these isolates that would be microbiologically susceptible may in fact be nonsusceptible given the right conditions.

Single changes were found in ParE (3 A473V and 2 A342V) of five phenotypically susceptible isolates. These single point mutations, typically considered second step mutations in mediating fluoroquinolone resistance, may have been acquired horizontally through recombination, as is commonly observed in the laboratory setting. Additional substitutions in the type II topoisomerase sequences were noted outside the QRDR; however, they were present in both the susceptible and nonsusceptible populations or were specific to a lineage (as determined by MLST), making it unlikely that they contribute to a susceptibility phenotype.

The *aac(6′)-Ib-cr* cassette that aids in mediating resistance to both aminoglycosides and fluoroquinolones (56) was identified in 10 isolates (see Table S1 in the supplemental material). Of these, 9 isolates had acquired QRDR mutations and were nonsusceptible. Screening for the point mutations in the QRDR was overall supportive of the phenotypic data for levofloxacin (Fig. 3C).

Genomic analysis of amikacin resistance in *P. aeruginosa* suggests genotypic analysis is further complicated by expression. A total of 105 (27%) of the isolates were found to be nonsusceptible to amikacin (MIC, ≥16 μg/ml), and 283 (73%) were susceptible to amikacin (MIC, ≤8 μg/ml). Aminoglycoside resistance in *P. aeruginosa* often arises via acquired aminoglycoside-modifying enzymes (AMEs), which are typically found in multiple combinations due to their substrate specificity, or 16S rRNA methyltransferases (RMTs) that provide high-level resistance to a broad range of aminoglycosides (57, 58). Efflux by the MexXY-OprM transporter is also a major contributor (59). Association of these genes and identification of mutations within the *mex*-related efflux components was used to genotypically assess the susceptibility of an isolate (Fig. 3A) (see Table S1 in the supplemental material).

Amikacin resistance-conferring AMEs encoded by *ant(4′)-IIb*, *aph15*, *aph(3′)-VIb*, *aph(6)-Id*, *aac(6′)-Ib*, *aac(6′)-Iai*, *aac(6′)-31*, *aacA29*, *aacA7*, and the RMT-encoding *rmtD* gene were all identified within select *P. aeruginosa* isolates (see Tables S1 and S2 in the supplemental material). Analysis aimed at associating the amikacin-related genes with the susceptibility data did not show a good correlation (Fig. 3C). Only 60% (63 of 105) of the isolates that were classified as nonsusceptible to amikacin contained a genomic element that would readily predict resistance (see Tables S1 and S2). The other 42 microbiologically nonsusceptible isolates did not contain any elements that would genotypically suggest amikacin resistance. Similarly, a majority of the susceptible isolates (89%) contained no genotypic markers associated with amikacin resistance. However, 30 microbiologically susceptible isolates did possess genes associated with conferring resistance, suggesting that expression data may also be required to properly interpret and predict susceptibility to this antibiotic.

Efflux mechanisms are another known major contributor to aminoglycoside resistance and were genetically recognizable in a number of these isolates (see Fig. S2B in the supplemental material). Upregulated expression of these efflux pumps could likely affect the susceptibility of an isolate without a clear genotypic explanation (59). Overall a number of nonsusceptible isolates had modifications that are predicted to alter the activity of the negative regulator MexZ of the MexXY-OprM efflux pump (see Fig. S2B). Patterns in the overall genotype of efflux components could be observed but appeared to be linked primarily with different MLST lineages. Interestingly, 24 of the isolates that were amikacin nonsusceptible were associated with CF infections, where MexXY hyperexpression has been shown to be a prevalent mechanism of resistance (60). The MexZ repressor was intact in these isolates; however, analysis of the two-component regulatory system ParRS, demonstrated to upregulate expression of *mexXY* (61), revealed that 6 (3 ParR and 3 ParS) of the CF isolates had premature stop codons (see Fig. S2B). The overrepresentation of aminoglycoside-nonsusceptible CF isolates without deleterious mutations in known aminoglycoside resistance-associated components likely reflects selection by the extended therapy required for these types of infections and potentially points to a novel mechanism of resistance.

Geographic associations could be made with several of the amikacin resistance cassettes. Approximately 50% of the *aph(6)-Id*

containing isolates were obtained from Argentina, the same geographic location where the PA7 strain, in which this element was first described, was isolated (62). A similar geographic association was identified for the *rmtD* gene, found in five isolates collected between 2004 and 2008 from a hospital in Brazil.

Colistin resistance identified in two CF isolates. Two isolates were identified as nonsusceptible to colistin among the 362 isolates tested (see Table S1 in the supplemental material). Both were from CF patients, consistent with the treatment regimens and reported resistance commonly among CF isolates. Studies initiated on defining the genetic basis of colistin resistance have implicated two-component regulatory systems that result in lipid A modifications as the principal resistance mechanism (63, 64)—the PmrAB and PhoQS systems (65). However, we identified multiple changes in these genes, previously associated with colistin resistance, in the susceptible population. Sequence analysis of the genes classically associated with colistin resistance did not identify any single mutation unique to the two resistant isolates.

DISCUSSION

With the diminishing supply of new drug candidates and the continuing rise in antimicrobial resistance, it is imperative that accurate susceptibility profiles are determined early and routinely in order to ensure that the correct treatment is administered. Current methods rely on susceptibility data generated by microbiological culture methods to select appropriate treatments, requiring a 24- to 48-h turnaround time and possibly missing any resistance potential carried by many strains as silent (or weakly expressed) genes. The application of genetics to defining the resistome of Gram-negative organisms has been focused until recently on PCR-based methods, but is insufficient given the growing number of novel resistance mechanisms now known to exist in different species. Expression methods based upon microarray technology have also been proposed, but the sensitivity and coverage of all the potential candidate genes are problematic (66). Recent advancements, improved turnaround time, and decreased cost of NGS technology have made genome sequencing a practical and promising method with potential as a diagnostic tool.

Few studies have tested NGS as a method for identification of resistance-related factors and correlation of this information with traditional susceptibility data (12, 15). Here we report the first steps toward this objective with the sequencing of 390 isolates of *P. aeruginosa* from diverse geographic locations and clinical infections. NGS was used to evaluate whether an isolate could be classified as susceptible or nonsusceptible based upon genome sequencing and to correlate resistance factors with traditional microbiological susceptibility data for three different antibiotics acting through different cellular processes.

Our analysis indicates that for meropenem and levofloxacin, the genome-based resistome and susceptibility data are in good agreement, suggesting that our understanding of the resistance mechanisms and their identification can readily be achieved by pure sequence analysis. Genome analysis showed that meropenem resistance was usually attributable to mutations in *oprD* or, to a lesser degree, the presence of MBLs. Used together these data provided a genetic test with 91% sensitivity and 94% specificity. Thus, in *P. aeruginosa*, genetic prediction of meropenem susceptibility is as effective as for fluoroquinolone susceptibility.

Efflux mechanisms and their relative expression level are known to be a major contributor to aminoglycoside resistance (67, 68), and genetic prediction of amikacin resistance proved less tractable. Intriguingly, isolates associated with CF represented a significant proportion of the amikacin-nonsusceptible strains that could not be correlated to a genotypic marker or markers, suggesting a unique expression-related mechanism induced by pressure from the selective niche environment in which these isolates dwell. Ongoing analysis is directed at using machine learning models to identify novel sequences that may confer amikacin resistance in these isolates, as well as alternative determinants of resistance in the entire population. Although our results suggest that NGS analysis is sufficient in identifying potential mechanisms associated with a nonsusceptible phenotype for *P. aeruginosa*, several isolates' phenotypes could not easily be explained by the presence or absence of functional genetic targets (Fig. 2) (see Table S1 and Fig. S2 in the supplemental material). This highlights one of the pitfalls of whole-genome sequencing in that it fails to capture the contribution of gene expression, an important contributor to resistance. Complementary analysis such as transcriptome sequencing (RNA-Seq) or microarray expression would be a practical "next step" in correlating the susceptibility phenotype of such isolates with genetic content (69). Nonetheless, NGS analysis permits not only the collection of information regarding the resistome but also the variability seen within the elements that may contribute, possibly in an additive manner, to produce higher MICs in the absence of known resistance determinants. Of the 390 isolates, 78 (20%) were determined to be nonsusceptible to all three antibiotics, highlighting the mounting concern of multidrug resistance.

P. aeruginosa, has a high genetic variability, and while numerous changes were observed in many genes associated with resistance (see Fig. S2 in the supplemental material), only those changes that would lead to a loss of function (i.e., a frameshift or premature stop) or genetically verified point mutations (based on literature review) were considered in this correlative analysis. Numerous other changes throughout the genome and the variability in genetic content due to acquisition of extrachromosomal elements, phages, and genomic islands, may also affect or contribute to the variable resistance levels observed with the test antibiotics. Some level of genomic commonality, such as that found in MLST defined clonal populations, is extremely helpful in dissecting phenotype-genotype correlations (70, 71), and undoubtedly we will uncover more resistance elements as more sequence data for *P. aeruginosa* further accrue. Such analyses will aid in highlighting elements of the accessory genome as well as lineage-specific genetic variants that may aid in adapting to the pressures of antibiotic exposure. Additionally, the contributions of restriction modification systems, clusters of randomly interspersed palindromic repeats (CRISPR) (72, 73), and phages (74–76) in mediating acquired resistance in *P. aeruginosa* are unknown, but it is likely that a combination of these restricts uptake and acquisition of exogenous elements. A search for known restriction modification and CRISPR-*cas* genes suggests that those isolates that are enriched in β-lactamases are devoid of such systems (data not shown). Similar to their Gram-positive counterparts, loss of these restrictive barriers may provide a niche advantage in that the isolates are better equipped to acquire not only mobile elements providing antibiotic resistance but also virulence-associated genes (77).

NGS has already been used in the clinic for real-time analysis of bacterial outbreaks, where it has aided in the identification of the index case and informed and guided infection control methods to prevent further dissemination (78–83). As NGS technology be-

comes integrated into the clinical setting, in conjunction with established susceptibility testing methods, antibiotic treatment will be provided in a more rigorous, rapid, and appropriate manner, thereby enhancing treatment. Such methods are established within the pharmaceutical industry, where NGS has greatly improved the turnaround time required in characterizing mutations associated with resistance-based screening assays and in identifying targets associated with the antimicrobial activity of novel compounds. Additionally, generation of large sets of genomic data provides relevant information pertaining to current and potential resistance to discovery compounds. The ability to determine the presence of an existing resistance mechanism in a population prior to drug exposure can greatly aid in modeling and predicting drug resistance rates. Knowledge of target diversity can also allow for better tailoring of drug design to overcome problems early in the discovery process. The potential of NGS to impact the multifaceted problems of antibiotic resistance is upon us. Our test case using NGS and *P. aeruginosa* illustrates the value in understanding the complexity of the resistome to better assess the susceptibility phenotype and will ultimately be reflected in more personalized treatment. The information gained from this diverse set of isolates will assist efforts in developing technologies and therapeutics for the diagnosis and treatment of infections.

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