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MECHANISMS OF RESISTANCE

Defects in Efflux (*oprM*), β-Lactamase (*ampC*), and Lipopolysaccharide Transport (*lptE*) Genes Mediate Antibiotic Hypersusceptibility of *Pseudomonas aeruginosa* Strain Z61

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ABSTRACT Antibiotic hypersensitive bacterial mutants (e.g., *Escherichia coli imp*) are used to investigate intrinsic resistance and are exploited in antibacterial discovery to track weak antibacterial activity of novel inhibitor compounds. *Pseudomonas aeruginosa* Z61 is one such drug-hypersusceptible strain generated by chemical mutagenesis, although the genetic basis for hypersusceptibility is not fully understood. Genome sequencing of Z61 revealed nonsynonymous single-nucleotide polymorphisms in 153 genes relative to its parent strain, and three candidate mutations (in *oprM*, *ampC*, and *lptE*) predicted to mediate hypersusceptibility were characterized. The contribution of these mutations was confirmed by genomic restoration of the wild-type sequences, individually or in combination, in the Z61 background. Introduction of the *lptE* mutation or genetic inactivation of *oprM* and *ampC* genes alone or together in the parent strain recapitulated drug sensitivities. This showed that disruption of *oprM* (which encodes a major outer membrane efflux pump channel) increased susceptibility to pump substrate antibiotics, that inactivation of the inducible β-lactamase gene *ampC* contributed to β-lactam susceptibility, and that mutation of the lipopolysaccharide transporter gene *lptE* strongly altered the outer membrane permeability barrier, causing susceptibility to large antibiotics such as rifampin and also to β-lactams.

KEYWORDS *Pseudomonas aeruginosa*, Z61, *ampC*, hypersusceptible, *lptE*, *oprM*, outer membrane

In addition to their cytoplasmic (inner) membrane, Gram-negative cells have an outer membrane (OM) that poses a significant permeability barrier. The OM barrier, in conjunction with active efflux, is effective at protecting these pathogens from many antibiotics, including members of our current antibacterial armamentarium (1). Importantly, this impermeability is recognized as perhaps the major impediment to the development of novel antibacterial drugs with sufficiently potent cellular activity (2), especially for pathogens such as *Pseudomonas aeruginosa*, which has a characteristically impermeable OM and a large complement of efflux pumps.

The Gram-negative outer membrane is a unique asymmetrical bilayer composed of an inner leaflet of phospholipid and an outer leaflet of lipopolysaccharide (LPS). LPS consists of a lipid anchor (lipid A) which forms the hydrophobic core of the outer leaflet, to which is attached core and O-antigen carbohydrate moieties that extend outward from the cell surface (3). Biosynthesis of the lipid A-core and O-antigen portions of LPS occur separately inside the cytoplasm, with lipid A-core assembly occurring at the inner leaflet of the inner membrane (3, 4). The lipid A-core is then flipped to the outer leaflet by the MsbA transporter, where it is linked to O-antigen in the periplasm and then transported into the OM by the Lpt transport system (5–7). Divalent cations cross-link

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phosphates on the sugar portion of lipid A (3) or the core regions (8–11) in the assembled OM, which packs the LPS layer, contributing to membrane integrity. The OM provides a significant permeability barrier because diffusion of hydrophobic molecules is hindered by the hydrophilic sugar regions while charged molecules are unable to efficiently diffuse through the hydrophobic bilayer segment of the OM. Certain polar molecules such as β-lactams can enter cells via water-filled porin channels, although porins also have various constraints limiting which compounds can pass through (12–15).

Working in conjunction with the limitations of compound influx afforded by the OM is active efflux of compounds from cells. There are several families of efflux pumps in bacteria that work together to protect cells from toxic insult (1). The resistance nodulation cell division (RND) family of efflux pumps is an important contributor to Gram-negative resistance because their tripartite architecture extends across the outer membrane, allowing extrusion of compounds across the OM and out of the cell (1, 16). RND family pumps are comprised of an inner membrane pump component and an outer membrane channel that are linked by a membrane fusion protein (8). The genome of *P. aeruginosa* encodes at least 12 RND family pumps (17), some of which have a broad and overlapping substrate recognition profile (1). Important among these for resistance to currently used antibiotics are the MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN pumps (18).

Gram-negative mutants compromised in either the membrane permeability barrier (e.g., *Escherichia coli imp* mutant [19], having a mutation in *lptD*, involved in LPS transport [20]) or efflux (e.g., *E. coli tolC* mutants) are often used in the study of intrinsic resistance but also to aid in identifying and characterizing weakly antibacterial molecules in drug discovery. Increased antibacterial activity of novel inhibitory compounds against these mutants can aid in their identification and determination of target identity and, compared to activity against unaltered parent strains, can provide insights into the impact of the membrane, efflux, or both on cellular antibacterial activity. An understanding of the factors mediating the level of susceptibility of these mutants (and whose inhibition could conceivably potentiate the activity of partner antibacterials) is of interest. A strain of *P. aeruginosa* (K799/61, commonly designated Z61) is a hypersusceptible mutant generated by chemical mutagenesis (18, 21) that has been extensively used in studies of antibiotic susceptibility and drug discovery and has been studied in some detail (22, 23), although the factors underlying drug hypersusceptibility are not yet fully understood.

P. aeruginosa Z61 is susceptible to a range of antibiotics with disparate physicochemical properties, suggesting that multiple factors mediating intrinsic resistance are compromised, consistent with its generation by chemical mutagenesis. Studies have implicated defects in active efflux, since the OprM component of the MexAB-OprM efflux pump is reduced or absent from the OM in this mutant (9). Defects in the outer membrane (LPS) leading to a reduced outer membrane permeability barrier and increased susceptibility to large or hydrophobic compounds and decreased inducibility of the chromosomally encoded AmpC β-lactamase have also been described, although the precise genetic explanation for these defects was not known (10, 11). Here, we used genome sequencing and isogenic mutant construction to identify the most important mutations causing susceptibility to a range of antibiotics in *P. aeruginosa* strain Z61.

RESULTS

Strain Z61 has a mutation encoding a premature stop in *oprM* that causes a defect in efflux. Whole-genome sequencing revealed single nucleotide polymorphisms (SNPs) for strain Z61 compared to its parent strain in 153 genes (see Table S1 in the supplemental material). This large number was expected given that strain Z61 was generated by multiple rounds of chemical mutagenesis (12). We prioritized mutations in genes related to efflux, OM permeability, or other drug resistance mechanisms, combined with possible relationships to phenotypes reported in previous studies. Drug hypersusceptibility in Z61 was originally attributed to a defect in OM permeability (10,

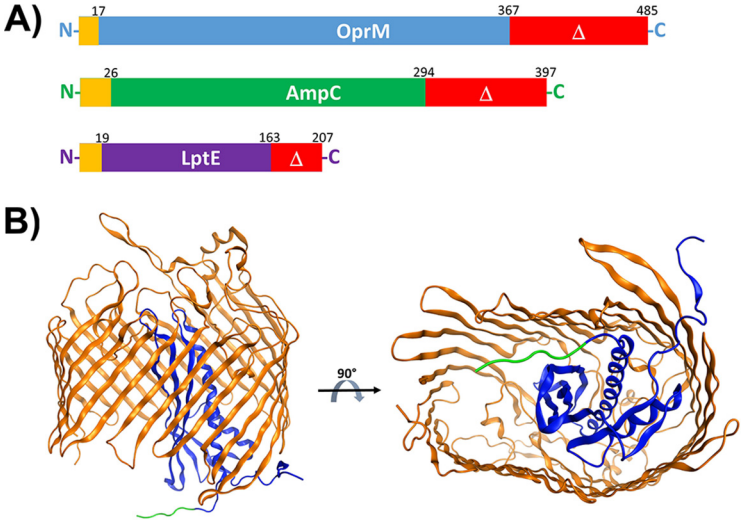

FIG 1 Impacts of Z61 mutations in *oprM* (PA0427, 485 amino acids), *ampC* (PA4110, 397 amino acids), and *lptE* (PA3988, 207 amino acids). (A) Schematic of the impact of the Z61 mutations on the protein sequences. OprM has a T123I amino acid substitution and premature stop at Q367. AmpC has a premature stop at Q294. LptE has a premature stop at Q163. The N-terminal signal sequences (orange boxes) of all three proteins are cleaved after secretion across the inner membrane. The truncated regions of all three proteins are shown in red boxes. (B) The X-ray crystal structure of *P. aeruginosa* LptDE is shown (PDB 5IVA). PaLptD is shown in orange, and PaLptE (residues 21 to 162) is shown in blue, and the Z61 C-terminal truncation (residues 163 to 168) is shown in green. Residues 169 to 207 were not in the structure because they were not resolved. The structure figures were generated with MOE 2018.0101.

11); however, it was later shown that Z61 also had defects in the efflux of radiolabeled tetracycline, which is a substrate of the MexAB-OprM and MexXY-OprM efflux pumps (13). Furthermore, it was demonstrated that mutant Z61, in contrast to the parent strain, did not express detectable amounts of the OprM protein (OM channel of MexAB-OprM and MexXY-OprM) in its OM, although the reason for this was not determined (9). Here we found that the *oprM* gene in Z61 harbored two SNPs (gene PA0427, Table S1), one encoding a T123I amino acid substitution and one encoding a premature stop at Q367 (Fig. 1). Previous work showed that truncation of the OprM C-terminal 23 amino acids (L463 to A485) or internal deletion of L463 to W467 abrogated OprM expression (14). The even larger OprM truncation in Z61 may explain the lack of detectable protein in the outer membranes of Z61 reported previously (9). It is noteworthy that previous studies mapped one of the mutations contributing to β-lactam hypersusceptibility in Z61 (dubbed *absB*) in proximity to *proC* (PA0393) on the *P. aeruginosa* genome (11). The *oprM* gene is located in proximity to *proC* (Fig. S1), which suggested the identity of *absB* is likely *oprM*.

Strain Z61 is hypersusceptible to a number of known MexAB-OprM substrate antibiotics such as tetracycline (TET), ampicillin (AMP), aztreonam (ATM), and carbenicillin (CAR) (Table 1). To confirm that the mutation identified in *oprM* played a role in Z61 drug hypersusceptibility, it was corrected on the genome of Z61 to that of the *P. aeruginosa* PAO1 wild-type sequence (15). A genetic deletion of *oprM* was also introduced into the parent NB52041 strain background to determine how the loss of OprM affected the parent strain.

AMP, ATM, and CAR are specific substrates of the constitutively expressed MexAB-OprM pump, although it was recently shown that a normally silent pump, MexMN-OprM, can also efflux β-lactams if MexMN is upregulated (16). Correction of the *oprM* defect in Z61 reduced susceptibility to AMP, ATM, and CAR by ca. 16-fold (strain Z61-CDY0025, Table 1). Rifampin (RIF) is a weak efflux pump substrate whose activity against *P. aeruginosa* as measured by standard MIC testing is not known to be substantially increased by genetic deletion of the major RND efflux pumps such as MexAB-OprM in *P. aeruginosa* (17, 18, 24). As expected, correction of *oprM* in Z61 or

TABLE 1 Antibiotic susceptibilities of *P. aeruginosa* Z61 and derivatives with mutational defects corrected on the genome

P. aeruginosa strain[a]	Genotype[b]	MIC (μg/ml)[c]						
		TET	RIF	CIP	GEN	AMP	ATM	CAR
NB52001	Control	32	32	0.25	1–2	4,096	8	64
NB52041	Parent of Z61	32	16	1	1	1,024	8	64
Z61		1	1	0.25	0.5	0.125	0.015	0.06
Z61-CDY0025	*oprM*	4	1	0.125	1	2	0.25	1
Z61-CDY0082	*ampC*	1	1	0.125	0.5	4–8	0.015	0.06
Z61-CDY0087	*ampC oprM*	4	1	0.125	1	16	0.125	1
Z61-CDY0083	*lptE*	4	8	1	0.5	2	0.25	0.5
Z61-CDY0085	*oprM lptE*	16–32	16–32	1	2	64	4	64
Z61-CDY0084	*ampC lptE*	4	8–16	0.5	0.5	128	0.5	0.5
Z61-CDY0086	*oprM lptE ampC*	32	16	0.5-1	2	128–256	4	32–64

[a]NB52001 is *P. aeruginosa* strain ATCC 12055.
[b]All of the genes listed here were restored to the wild-type PAO1 sequence.
[c]AMP, ampicillin; ATM, aztreonam; CAR, carbenicillin; CIP, ciprofloxacin; GEN, gentamicin; RIF, rifampin; TET, tetracycline.

deletion of *oprM* in NB52041 had no effect on susceptibility to RIF (strain Z61-CDY0025 [Table 1] and NB52041-CDY0175 [Table 2]). This supports a contribution of the *oprM* mutation to increased susceptibility to pump substrate antibiotics but indicates that there was an additional non-efflux-related defect mediating hypersusceptibility of Z61 to RIF. Indeed, correction of the *oprM* mutation alone in Z61 did not fully decrease susceptibility of Z61 to parental strain levels even for the MexAB-OprM-specific β-lactam pump substrates AMP, ATM, and CAR, also implying that an additional hypersusceptibility-related defect(s) was present on the genome of Z61. TET and CIP are structurally unrelated to β-lactams but are substrates of MexAB-OprM. They differ from β-lactams in that they are also substrates of several other efflux pumps including MexXY-OprM, MexCD-OprJ, and MexEF-OprN (1). Correction of the *oprM* defect decreased susceptibility of Z61 ~4-fold to TET but, as above, this was not enough to reach parental strain susceptibility levels (16-fold increase in susceptibility to TET upon deletion of *oprM* in NB52041 compared to a 4-fold shift seen for correcting *oprM* in Z61, Tables 1 and 2), again indicating that additional susceptibility defects were present in Z61. Interestingly, neither correction of *oprM* in Z61 or deletion of *oprM* in NB52041 significantly affected susceptibility to CIP. Deletion of both MexAB-OprM and MexXY-OprM (both OprM dependent) decreases the susceptibility of *P. aeruginosa* PAO1 ~8-fold to CIP (19). The lack of impact of *oprM* specifically on susceptibility to CIP in NB52041/Z61 seen here may be consistent with a previous report that Z61 overexpressed two other RND family pumps that can efflux CIP (MexCD-OprJ and MexEF-OprN), and this appeared to be a compensatory response to the loss of OprM expres-

TABLE 2 Antibiotic susceptibilities of *P. aeruginosa* NB52041 and mutants with specific Z61 genetic defects individually engineered into the genomes

P. aeruginosa strain[a]	Genotype[b]	MIC (μg/ml)[c]						
		TET	RIF	CIP	GEN	AMP	ATM	CAR
NB52001	Control	32	32	0.25	1–2	4,096	8	64
NB52041	Parent of Z61	32	16	1	1	1,024	8	64
NB52040	Z61	1	1	0.25	0.5	0.125	0.015	0.06
NB52041-CDY0175	NB52041 Δ*oprM*	2	16	1	0.5	512	0.5	2-8
NB52041-CDY0174	NB52041 Δ*ampC*	32	16	1	2	64	4	64
NB52041-CDY0176	NB52041 Δ*oprM* Δ*ampC*	2	16	1	0.5	2	0.25	0.5
NB52041-CDY0170	NB52041 *lptE*^{D}	4	1	0.125	1	32–64	0.125	1
NB52041-CDY0171	NB52041 *lptE*^{D} Δ*oprM*	0.5	1	0.06	0.25	8	0.015	0.06
NB52041-CDY0172	NB52041 *lptE*^{D} Δ*ampC*	2–4	1	0.125	0.5	8	0.125	1
NB52041-CDY0173	NB52041 *lptE*^{D} Δ*oprM* Δ*ampC*	0.25	1	0.03	0.5	0.06	0.0078	0.03

[a]NB52001 is *P. aeruginosa* strain ATCC 12055.
[b]A superscript "D" indicates a gene defect from strain Z61 encoding Q163* engineered into the genome of strain NB52041.
[c]AMP, ampicillin; ATM, aztreonam; CAR, carbenicillin; CIP, ciprofloxacin; GEN, gentamicin; RIF, rifampin; TET, tetracycline.

sion (9). Therefore, the impact of an OprM deficiency as regards susceptibility of Z61 to CIP may be difficult to clearly see in strain backgrounds capable of this compensatory response. This also suggests that a non-efflux defect plays a role in the 4-fold difference in CIP susceptibility between NB52041 and Z61.

Lastly, among the β-lactams tested here, AMP differed from ATM and CAR in that the fold change in susceptibility to AMP resulting from deletion of *oprM* in NB52041 was smaller (2-fold compared to ~16-fold) than was observed for correction of *oprM* in Z61 (16-fold for all). This suggested that an additional underlying non-efflux factor that was more specific to AMP was also defective in Z61. Overall, this clearly indicates that the defect in OprM is an important factor in Z61 hypersusceptibility to several pump substrate compounds in Z61 but implies that at least two additional defects are also in play: one that may be more specific to AMP and one that impacts a broader range of compounds, including RIF, TET, CAR, ATM, and possibly CIP. These factors are discussed below.

Strain Z61 has a mutation encoding a premature stop in *ampC*. *P. aeruginosa* harbors the inducible wide-spectrum class C β-lactamase, AmpC (20), also referred to as *Pseudomonas*-derived cephalosporinase-3 (PDC-3). This enzyme can decrease susceptibility to inducing and susceptible β-lactams, and mutants with constitutive upregulation of AmpC expression are emerging in hospitals (21). ATM is not reported to strongly induce expression of the chromosomally encoded AmpC β-lactamase (accounting in part for its relatively good activity against *P. aeruginosa*) (22). ATM is also not a good substrate of AmpC (although if AmpC expression is strongly upregulated due to regulatory mutations, it can bind ATM, affecting susceptibility via a titration effect), and CAR is also fairly stable to AmpC (22). In contrast, AMP can induce AmpC expression and is highly susceptible to degradation by this enzyme (22), and therefore AmpC is a much more important determinant of susceptibility to AMP in *P. aeruginosa* than to ATM or CAR. AMP, ATM, and CAR are all substrates of MexAB-OprM but, as mentioned above, correcting the *oprM* defect in Z61 shifted susceptibility to all three compounds by 16-fold but did not bring susceptibility back to near parental levels (Z61-CDY0025, Table 1), and deleting *oprM* in the parent NB52041 strain caused a 16-fold decrease in susceptibility to ATM and CAR but only a 2-fold shift for AMP (NB52041-CDY0175, Table 2). The impact of pump deletion alone on AMP activity in the absence of inhibition of AmpC was previously reported to be only ~2-fold in PAO1 (25), which is presumably related to the interplay of AmpC and MexAB-OprM when both factors effectively act on the β-lactam (23). This differential impact of the *oprM* defect on ATM and CAR compared to AMP therefore suggested that a specific defect in the chromosomally encoded AmpC β-lactamase existed in Z61.

Previous studies on strain Z61 found a defect in the inducibility of the chromosomally encoded β-lactamase (AmpC) in strain Z61 (10). The observed defect in AmpC inducibility was thought to be a possible artifact caused by enhanced periplasmic target exposure to inducing penicillins resulting from the defective outer membrane permeability barrier observed in Z61 (discussed in the next section). It was proposed that it would be difficult to establish a level of penicillin exposure that would not kill the cells prior to induction of AmpC (10). Here, we identified a mutation in the *ampC* structural gene (gene PA4110, Table S1) encoding a premature stop at Q294 (Fig. 1). This would be a significant truncation expected to substantially reduce or eliminate AmpC activity. Consistent with this, correction of the *ampC* mutation on the genome of Z61 (Z61-CDY0082, Table 1) decreased susceptibility to AMP ~32-fold (MIC shift from 0.125 μg/ml to 4 to 8 μg/ml) but had no effect on ATM or CAR, which are not as impacted by this β-lactamase (22). As expected, correction of the *ampC* defect had no effect on non-β-lactams such as TET and RIF. Deletion of *ampC* in the original parent strain NB52041 increased susceptibility to AMP ~16-fold (NB52041-CDY0174, Table 2) and did not appreciably alter susceptibility to ATM or CAR. Using a nitrocefin hydrolysis assay, we observed an ~32-fold-higher basal (uninduced) level of AmpC activity in the parent strain NB52041 than in Z61 (Table 3) and an ~>1,024-fold higher level of AmpC

TABLE 3 Comparison of basal and inducible AmpC activity in *P. aeruginosa* strains, as measured by hydrolysis of the chromogenic AmpC substrate nitrocefin

Strain comparison or strain[a]	AmpC activity ratio (nitrocefin hydrolysis), fold increase[b]		
	Induced/uninduced	Uninduced	Induced
Strain comparison			
NB52041/Z61		32	>1,024
Z61-CDY0082 (*ampC*)/Z61		1	1,024
Z61-CDY0086 (*ampC oprM lptE*)/Z61		16	>1,024
Strain			
NB52041	256		
Z61	2		
Z61-CDY0082 (*ampC*)	256		
Z61-CDY0086 (*ampC oprM lptE*)	256		

[a]All of the genes indicated in parentheses were restored to the wild-type sequence.
[b]"Induced" means induced by imipenem at 0.25× MIC for the corresponding strain.

(when induced with 0.25× MIC of the *ampC* inducing compound imipenem in each strain). Furthermore, we observed an ~256-fold induction of AmpC activity in NB52041 by imipenem (0.25× MIC) and only a 2-fold induction in Z61 (0.25× MIC) (Table 3). Correction of the *ampC* defect in Z61 brought the inducibility to 256-fold (Z61-CDY0082, Table 3), equal to the parent strain NB52041. Similarly, the level of basal (uninduced) *ampC* activity in Z61 restored for *ampC* was ~16-fold higher than for Z61 (Z61-CDY0082/Z61, Table 3), and the induced level of activity was 1,024-fold higher than for Z61, similar to the comparison between the parent strains NB52041 and Z61. This indicated that the structural mutation found in *ampC* profoundly reduced the level of AmpC activity in Z61.

Interestingly, restoration of both *ampC* and *oprM* to the wild type in Z61 only decreased susceptibility to AMP by ~2-fold compared to the impact of *ampC* restoration alone (Z61-CDY0087, Table 1). Restoration of *ampC* alone did not appreciably alter susceptibility to ATM or CAR, whereas restoration of *oprM* more substantially decreased susceptibility to ATM and CAR, similar to restoring *oprM* alone. This is in line with AmpC being relatively more important for resistance to AMP than ATM and CAR, allowing for the impact of efflux to be seen more clearly for ATM and CAR. Consistent with this, deleting *ampC* and *oprM* together in the original parent strain NB52041 (NB52041-CDY0176, Table 2) had a similar impact on susceptibility to ATM and CAR as the *oprM* defect alone, whereas both mutations together impacted AMP much more than either one alone. Importantly, the introduction of these defects into the parent NB52041 increased susceptibility to all three antibiotics more profoundly than the correction of these defects decreased susceptibility in Z61. This, along with the observation that correcting both *oprM* and *ampC* together had no effect on susceptibility to RIF, indicated that there were still other hypersusceptibility determinants in Z61 beyond the defects in *oprM* and *ampC*.

Strain Z61 has a mutation encoding a premature stop in *lptE* that mediates a membrane permeability defect. Strain Z61 is hypersusceptible to compounds such as RIF (Table 1) and argyrin B (26, 27), whose MICs are not appreciably affected by deletion of the major RND family efflux pumps or AmpC. This, along with previous observations of membrane permeability and/or LPS defects in strain Z61, suggested that a mutation associated with the outer membrane was likely involved in drug hypersusceptibility. We noted several mutations in genes involved in LPS biosynthesis and transport, including *lptE* (PA3988 encoding a component of the Lpt LPS transport machinery [28–31]), *waaL* (PA4999, encoding the ligase that attaches O-antigen to lipid A [32, 33]), and *wzz* (PA3160, encoding the determinant of O-antigen chain length [34]) (Table S1). Of these, the *lptE* gene was of primary interest for several reasons. First, disruptions of the more distal carbohydrate portions of LPS (O-antigen) have not generally been associated with the levels of drug hypersusceptibility seen in Z61 (25). As well, LptE appeared to

be the most profoundly affected, by being truncated at Q163 (45 of 207 amino acids) (Fig. 1). Lastly, a mutation (dubbed *absA*) affecting membrane permeability and susceptibility to hydrophobic antibiotics was previously mapped in proximity to *proA* (PA4007) on the *P. aeruginosa* genome (11). The *lptE* gene resides in this region (Fig. S1), and we therefore speculated that it might indeed be *absA*.

Restoration of *lptE* to the wild-type PAO1 sequence on the genome of Z61 decreased susceptibility to RIF almost to that of the parent strain of Z61, NB52041 (Z61-CDY0083, Table 1). RIF is a large hydrophobic antibiotic whose cellular activity in *P. aeruginosa* is not strongly impacted by efflux and that likely enter cells via diffusion across the membrane. As such, hypersusceptibility to RIF is often used as an indicator for defects in membrane permeability or OM disruption by compounds such as polymyxins (35, 36). Introduction of the *lptE* (Q163*) defect into NB52041 recapitulated the RIF susceptibility of Z61 (NB52041-CDY0170, Table 2). This confirmed a direct role for the *lptE* mutation in mediating the membrane permeability defect and hypersusceptibility to certain antibiotics of strain Z61 and indicates that it is likely the mutation previously referred to as *absA* (11). Intriguingly, correction of *lptE* in Z61 also reduced susceptibility to MexAB-OprM substrate antibiotics such as β-lactams and to the MexAB-OprM pump substrate antibiotic TET (Table 1), albeit not fully back to the parental (NB52041) levels. This reflects an interplay between membrane permeability and efflux for pump substrate antibiotics. Indeed, restoration of *oprM* (which would render both MexAB and MexXY functional) and *lptE* together (Z61-CDY0085, Table 1) shifted susceptibility to TET, ATM, and CAR back to, or near, parental strain levels, indicating that defects in efflux and membrane permeability are the main factors determining susceptibility to these antibiotics in Z61. Consistent with this interpretation, introduction of the *lptE* defect in NB52041 increased susceptibility to TET, ATM, and CAR, while introduction of the *lptE* mutation and deleting *oprM* increased susceptibility levels to that of Z61 (compare NB52041-CDY0171 and NB52041-CDY0170, Table 2). Susceptibility to AMP in the *lptE*-restored strain Z61-CDY0083 was also further decreased by correction of *oprM*, along with *lptE*, but not fully back to parental levels (Z61-CDY0085, Table 1). This suggested that the defect in AmpC was still a factor for AMP susceptibility. Consistent with this, restoration of *lptE* and *ampC* together (Z61-CDY0084, Table 1) also reduced susceptibility about the same as restoration of *lptE* and *oprM* together. Interestingly, restoring all three genes together only subtly reduced susceptibility to AMP (2-4-fold) over either pair, suggesting that there may be yet other defects affecting susceptibility to AMP in Z61. Introduction of the *lptE* defect into NB52041 increased AMP susceptibility (the MIC shifted from 1,024 μg/ml to 32 to 64 μg/ml, NB52041-CDY0170, Table 2), and the addition of either the *oprM* deletion (NB52041-CDY0171) or the *ampC* deletion (NB52041-CDY0172) further increased susceptibility (the MIC shifted from 32 to 64 μg/ml to 8 μg/ml). Introduction of all three defects (NB52041-CDY0173, Table 2) increased the susceptibility to AMP to levels similar to those of Z61. As discussed above, CIP is a substrate of MexAB-OprM, but the Z61 defect in *oprM* did not cause a measurable change in CIP susceptibility, possibly due to compensatory upregulation of additional efflux pumps. In addition to being a good pump substrate, CIP differs from compounds such as RIF by being smaller and more likely to enter cells via water-filled porins rather than diffusion across the membrane. Membrane permeabilization is thought to impact these molecules less than large and/or hydrophobic molecules (36). Nonetheless, the correction of the *lptE* defect in Z61 reduced susceptibility to CIP by 4-fold, while introduction of the *lptE* defect into NB52041 increased susceptibility by 8-fold. This suggests that the defect in *lptE* allows more rapid influx of CIP into cells or, alternatively, that it might destabilize to some extent the function of efflux pumps requiring an OM channel component. Lastly, it was previously reported that the lipid A core moiety (rough core) of LPS from strain Z61 exhibited higher mobility on SDS-PAGE gels than did that of the parent strain (11). Comparison of various mutants in that study indicated that the *absA* mutation, now identified here to be in *lptE*, was not responsible for this LPS defect. We also observed differential migration of Z61 LPS compared to its parent NB52041, and consistent with

this previous report, the LPS defect was not corrected upon restoration of *lptE* to wild type (data not shown). Therefore, additional mutations affecting LPS structure are also present in Z61.

DISCUSSION

Here we have characterized what appear to be the most important mutations mediating drug hypersusceptibility in the chemically mutagenized strain *P. aeruginosa* Z61, although some contribution to hypersusceptibility by other mutations in this highly mutagenized strain cannot be ruled out (Table S1). Most of the hypersusceptibility to a range of antibiotics with different properties, including β-lactams, TET, and RIF, can be largely explained by three genetic defects, located in *oprM* (affecting active efflux), *ampC* (eliminating the inducible β-lactamase AmpC), and *lptE* (affecting OM permeability). This provides the genetic explanation for previous observations that Z61 had defects in AmpC inducibility/activity (37, 38) and efflux of TET/chloramphenicol (9, 13), and had a defective OM permeability barrier (10, 11, 38). We created isogenic strains correcting these three defects alone and in combination in the Z61 strain background and introduced the *lptE* mutation with or without deletions of *oprM* and *ampC* into the parent strain to demonstrate the role of each of these functions in determining susceptibility levels to the compounds tested here. The impact of these mutations on susceptibility supported the notion of interplay among the OM and efflux in mediating intrinsic levels of susceptibility to many compounds and may suggest additional interrelationships between OM biogenesis and peptidoglycan biosynthesis that underlie the impact of *lptE* defects on β-lactam susceptibility shown here. The panel of defective strains made here may also be useful as tools for understanding the contribution of these factors in establishing the cellular activity/accumulation of other known or novel antibacterial compounds. Although the defect in *ampC* may be specific for β-lactams antibiotics, the defects in efflux and membrane permeability would be expected to impact a broad range of molecules.

Our findings regarding the interplay of efflux and membrane permeability mirror a previous report using engineered *P. aeruginosa* strains with defects in the LptD component of the Lpt LPS transport and assembly machinery (which interacts with LptE) and/or active efflux (39). In that study, the defects in LptD were based on similar defects described in *E. coli* LptD (40, 41), which were predicted to reduce the function of LptD and cause defects in the *P. aeruginosa* OM permeability barrier similar to what was observed in *E. coli*. Attempts to introduce another *E. coli*-based defect in LptD (42) were not successful (39), suggesting that it was lethal to *P. aeruginosa* and that there may therefore be differences in the LptE-LptD interaction/function between these bacteria. Alternatively, *P. aeruginosa* may simply not tolerate as much disruption of LPS biosynthesis or assembly as can *E. coli* and correspondingly cannot accommodate certain LptD alterations. Possibly supporting the latter, attempts to introduce an *lpxC* defect described in *E. coli* (43) were also not successful in *P. aeruginosa* (39). Intriguingly, this inability to generate LPS defects based on synonymous *E. coli* mutations also extended to LptE (28, 39). Here, we restored the wild-type *lptE* in *P. aeruginosa* Z61 and also introduced the Z61 LptE defect into its parent strain, creating an isogenic mutant with a permeability defect. However, multiple attempts to introduce this defect into a *P. aeruginosa* PAO1 (K767) strain (15) were unsuccessful, also suggesting that differential tolerance of cell envelope defects exists among different strains of *P. aeruginosa*.

The LptD protein component of the LptE-LptD translocon that mediates the final steps of LPS transport (7) is essential for growth in *P. aeruginosa*, as clearly demonstrated genetically and via specific chemical inhibition (44–46). It has recently been questioned whether LptE is also essential in *P. aeruginosa* (47) as it is in *E. coli* (48). Early studies using saturation transposon mutagenesis suggested an inability to inactivate *lptE* in *P. aeruginosa* PAO1 or PA14 (49, 50), whereas more recent experiments using transposon sequencing indicated the existence of insertions in *lptE* (51, 52). The *lptE* gene could not be deleted from the genome of PAO1 strain ATCC 15692 but a derivative of this strain with controlled expression of LptE was viable in the absence of

induction, prompting further speculation that LptE may indeed be dispensable for growth (47). However, it was not clear whether a small amount of uninduced expression of LptE may have been sufficient to support growth in that study. Downregulation of LptE did lead to a defect in membrane permeability, which is consistent with our observations here for the defect in Z61 LptE. The membrane defect resulting from LptE downregulation appeared to result at least in part from depletion of the LptD protein, which forms a complex with LptE, reflecting a compromised chaperone function of LptE in the formation of this active LptE-LptD complex rather than a direct role for LptE in LPS transport (47). This was proposed to lead to drug sensitivity based on the indirect effect of reduced LptD levels on LPS transport and cell division, as well as the possibility that nonstabilized LptD transporter barrel proteins lacking inserted LptE may circumvent the OM permeability barrier (47). Indeed, the dominant-negative *E. coli lptE14* allele was shown to be defective in plugging the lumen of the LptD β-barrel, resulting in a compromised permeability barrier to erythromycin and rifampin (53).

The crystal structure of *P. aeruginosa* LptD (5IVA, Fig. 1) indicates that *Pa*LptD has the largest luminal volume observed to date (54), so it is possible that the truncated Z61 LptE may open up a pore through LptD and the OM. Although the LptE amino acid sequences are generally conserved, the C-terminal ~40 amino acids are markedly less conserved (54). The crystal structure of *P. aeruginosa* LptDE (5IVA, Fig. 1) was obtained with the full-length LptE; however, the C-terminal ~40 amino acids (residues 169 to 207) were not well resolved and so were not included in the model (54). Considering that the Z61 LptE is truncated at Q163, the *Pa*LptDE structure may fairly well represent the Z61 LptE structure. The PaLptDE structure suggests that the C-terminal residues truncated in Z61 LptE are not located within the lumen (Fig. 1), and therefore their loss would not be expected to open up a pore through the LptD lumen. The large truncation of LptE (45 residues of 207) found here in Z61 (Fig. 1) and the significant phenotype of the single Z61 *lptE* mutation implied a correspondingly significant impact on LptE function, although we did not attempt a full deletion in strain NB52041 and therefore did not determine whether LptE is essential for growth or viability in this strain background. Further experiments will be needed to determine whether LptE is essential in NB52041 and whether the Z61 LptE defect causes membrane defects via reduced production of LptD or direct opening of the LptD pore.

MATERIALS AND METHODS

Whole-genome sequencing and bioinformatics analysis. *P. aeruginosa* strain NB52041 (ATCC 12055) and NB52040 (Z61, ATCC 35151) were obtained from the American Type Culture Collection. DNA isolation, fragment library preparation, and whole-genome sequencing of strains NB52041 (parent, ATCC 12055) and Z61 (hypersusceptible mutant, ATCC 35151) was conducted as previously described (55), except Illumina-compatible libraries were constructed using the Nextera DNA sample prep kit (Illumina, San Diego, CA). Velvet version 1.1.05 (56) was used to make an assembly from the reads of NB52041. Bwa version 0.5.9 (57, 58) was used to align the final sequence assemblies to the reference strain PAO1 (59). The gaps in the genome of NB52041 were completed with PAO1 sequence and this was then used as the reference sequence for variant calling. SAMtools version 0.1.17 (60) was used to convert the file from sequence alignment map (*.sam) into BAM (*.bam) format. SNPs were identified in Z61 using SAMtools mpileup. BLAST was used to conduct a comparative sequence analysis to identify homologous protein coding regions. Custom perl scripts were developed to annotate the identified SNPs using gene and protein annotations obtained from the NCBI gene database. A total of 387 SNPs were found in Z61 relative to the parent strain NB52041. Of these 387 regions, 321 contained SNPs that could be mapped to PAO1. Of these 321 regions, 168 harbored synonymous changes, while 153 harbored nonsynonymous changes. The total list of nonsynonymous changes identified in Z61 is provided in Table S1. The sequencing data were not examined for larger insertions or deletions.

Genetic manipulation of *P. aeruginosa* strains. *P. aeruginosa* strain NB52041 (ATCC 12055) and NB52040 (Z61, ATCC 35151) were obtained from the American Type Culture Collection. *Escherichia coli* strain S17-1 used for mobilizing gene replacement plasmids into *P. aeruginosa* was previously described (61). The mutations in *oprM*, *ampC*, and *lptE* in the hypersusceptible strain Z61 were corrected to the wild-type sequence as follows: the relevant regions were PCR amplified from the genome of strain PAO1 (15) using the primers listed in Table S2. The fragment encompassing *oprM* was inserted into the HindIII/PstI sites of the mobilizable gene replacement vector pEX18Tc-Gm (27). The *ampC* and *lptE* fragments were each inserted into the HindIII/KpnI sites of the gene replacement vector pEX18AP (62). Each vector was then transformed into the *E. coli* mobilizer strain S17-1 and mated into *P. aeruginosa* Z61 as previously described (55). Merodiploids were selected on *Pseudomonas* isolation agar (PIA) containing either 100 μg/ml gentamicin (pEX18Tc-Gm) or 100 μg/ml carbenicillin (pEX18AP). Resolvants that had

lost the plasmid backbone were selected on PIA containing 5% sucrose and gene replacements (correction of the sequence to wild type) were identified and confirmed by PCR amplification and sequencing. Combinations of gene corrections were done sequentially using the same protocol. A similar strategy was used to create defects in *oprM*, *ampC*, and *lptE* in strain NB52041, the parent strain of Z61. Gene replacement vectors were constructed as follows: a fragment harboring the specific defect in *lptE* (encoding Q163*) found in strain Z61 was PCR amplified from the genome of Z61 using the primers indicated in Table S2 and inserted into HindIII/PstI-digested pEX18AP using the Geneart Seamless cloning and assembly kit according to the provided instructions (Thermo-Fisher Scientific). Gene replacement vectors for generating unmarked deletions of *ampC* and *oprM* were created by PCR amplification of the upstream and downstream regions of each gene, using the primers listed in Table S2 and inserting them into HindIII/PstI-digested pEX18Tc-Gm using the Geneart reaction. These vectors were transformed into the mobilizer strain S17-1 and used to generate mutations in NB52041, alone and in combination, as described above.

Nitrocefin hydrolysis assays. *P. aeruginosa* cells were grown overnight in Mueller-Hinton broth (MHB) at 37°C. The overnight cultures were then diluted 1:50 in fresh MHB with or without 0.25× the MIC of imipenem (to induce AmpC expression), followed by incubation for 4 h at 37°C. Cells from 6 ml of each culture were then harvested by centrifugation and suspended in phosphate-buffered saline (PBS) to an optical density at 600 nm of approximately 8. The cell suspension was then lysed by sonication, and debris was cleared by centrifugation. Then, 200 μl aliquots of each lysate were transferred to column one of a 96-well microtiter plate. The remaining wells in the plate contained 100 μl of PBS with 50 μg/ml of nitrocefin (Calbiochem). Nitrocefin was then added to a final concentration of 50 μg/ml to the lysates in column one, which were then used for six serial 2-fold dilutions into the wells containing PBS-nitrocefin. The rates of nitrocefin hydrolysis were then monitored using a SpectraMax plate reader (Molecular Devices) at 490 nm at 5-min intervals for 2 h.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was conducted according to broth microdilution methodology described by the Clinical And Laboratory Standards Institute (63).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00784-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.03 MB.

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