

Mutant Alleles of *lptD* Increase the Permeability of *Pseudomonas aeruginosa* and Define Determinants of Intrinsic Resistance to Antibiotics

Carl J. Balibar,^a Marcin Grabowicz^b

Merck Research Laboratories, Kenilworth, New Jersey, USA^a; Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA^b

Gram-negative bacteria provide a particular challenge to antibacterial drug discovery due to their cell envelope structure. Compound entry is impeded by the lipopolysaccharide (LPS) of the outer membrane (OM), and those molecules that overcome this barrier are often expelled by multidrug efflux pumps. Understanding how efflux and permeability affect the ability of a compound to reach its target is paramount to translating *in vitro* biochemical potency to cellular bioactivity. Herein, a suite of *Pseudomonas aeruginosa* strains were constructed in either a wild-type or efflux-null background in which mutations were engineered in *LptD*, the final protein involved in LPS transport to the OM. These mutants were demonstrated to be defective in LPS transport, resulting in compromised barrier function. Using isogenic strain sets harboring these newly created alleles, we were able to define the contributions of permeability and efflux to the intrinsic resistance of *P. aeruginosa* to a variety of antibiotics. These strains will be useful in the design and optimization of future antibiotics against Gram-negative pathogens.

With emerging multidrug resistance and a limited number of treatment options, bacterial infections pose an ever growing threat to human health. Gram-negative bacteria are particularly recalcitrant to antimicrobial intervention due to their cell envelope structure. Possessing two membranes with different chemical properties (1) and containing an arsenal of efflux pumps (2), Gram-negative bacteria are capable of both excluding and expelling molecules, rendering them resistant to many drugs. Despite major Gram-negative pathogens being classified as urgent or serious threats by the CDC (3), no Gram-negative-active antibiotic with a new mechanism of action has been introduced in over 40 years. Of the 28 new antibiotics approved since 2000, only 18 are indicated for treatment of Gram-negative bacteria (4), and all of those are derived from the four legacy classes β -lactams, tetracyclines, macrolides, and fluoroquinolones, which were first introduced in 1942 (5), 1948 (6), 1952 (7), and 1967 (8), respectively. Novel derivatives of old antibiotics often have limited spectra of coverage and can only do so much to overcome existing mechanisms of resistance; therefore, introduction of novel classes of antibiotics is critical.

There is no shortage of potential targets in the antibacterial space given that the essential gene set for several Gram-negative pathogens has been defined (9–12). However, despite genetically demonstrating target essentiality, cognate inhibitors with exquisite *in vitro* activity often have no measurable cellular activity (13, 14). The reasons for this can be attributed to a lack of penetration of compounds into bacteria, efflux of molecules out of bacteria, insufficient inhibition of the target, metabolism within the cells, or a combination of the aforementioned factors. Understanding the reason for failure to translate enzymatic 50% inhibitory concentrations (IC_{50} s) into cellular MICs is paramount to an antibacterial research and development program and guides medicinal chemistry efforts. As a model organism for Gram-negative pathogens, many genetic tools have been developed for *Escherichia coli* in order to assess the contributions of efflux and permeability to intrinsic resistance. Strains exist with efflux pumps knocked out either individually or in combination, and the relative contribu-

tion of each system to the susceptibility to major classes of antimicrobials has been defined (15). For the most part, *tolC* is the major contributor to efflux in *E. coli*, and knockout of this gene is often used to assess whether novel compounds lack cellular activity due to efflux (16–24). Similarly, mutations in several genes—including *lpxC* (25–27), *lptD* (28, 29), and *lptE* (30, 31)—leading to increased permeability of *E. coli* have been described, and strains harboring such lesions are often used to assess the effect of increased cellular penetration on the bioactivity of molecules (32–37). These three genes in particular are often targeted because of their essential role in biosynthesis and biogenesis of lipopolysaccharide (LPS), which comprises the outer leaflet of the outer membrane (OM) in Gram-negative organisms and greatly contributes to reduced permeability. *LpxC* is a zinc-dependent deacetylase that forms UDP-3-O-(hydroxytetradecanoyl)glucosamine in the committed step of lipid A biosynthesis (38), and *LptD* and *LptE* constitute a β -barrel and plug, respectively, responsible for the final step of LPS transport to the OM (39–41).

Although *E. coli* serves as a powerful model for studying Gram-negative bacteria, findings are not always recapitulated in other more serious pathogens, in particular *Pseudomonas aeruginosa*. *P. aeruginosa* is a common nosocomial pathogen that often takes advantage of immunocompromised hosts to cause infections associated with high rates of mortality (42, 43). It is perhaps best

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Address correspondence to Carl J. Balibar, carl.balibar@merck.com.

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known as the major cause of declining lung function in cystic fibrosis (CF) patients (44). Among Gram-negative bacteria, *P. aeruginosa* is particularly resistant to antimicrobial intervention because it has a large and diverse set of multidrug efflux pumps (45, 46) and an especially impermeable OM (47, 48), and it can metabolize antimicrobial compounds (43). Although the permeability of the lipid bilayer domain of *P. aeruginosa*, as assessed by measuring the influx of hydrophobic steroid probes, is comparable to those of other Gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium and *E. coli* (48, 49), a limitation to the influx of molecules comes from OprF, the major porin of *P. aeruginosa*. Responsible for slow nonspecific diffusion of solutes into the bacterium, it has been shown that OprF folds into two conformations, with less than 5% of the porin present in the open-channel conformer, severely limiting penetration of molecules into the bacterium (50). It is for this multitude of reasons that often agents which are active on most other Gram-negative bacteria are less effective on *Pseudomonas aeruginosa* (13, 51–53), and finding compounds that inhibit this organism can be difficult. Again, it is imperative to understand the roles efflux and permeability play in the intrinsic resistance of this organism when developing the next generation of antibiotics. As with *E. coli*, collections of efflux pump-null strains of *P. aeruginosa* have been created which help to define the substrate specificities of the various pumps (43, 54, 55). However, *P. aeruginosa* efflux pumps are more numerous and redundant than those of *E. coli*, and often strains with multiple pump knockouts are necessary to assess the full effect of efflux on compound activity (13, 56–58). In contrast, techniques for assessing the effects of permeability in *P. aeruginosa* are less refined. Rather than single defined mutations, as utilized in *E. coli*, membrane permeabilization is often induced with chemical treatment using reagents such as polymyxin B nonapeptide (PMBN), compound 48/80, and EDTA (59–66). The latter two reagents can be harsh, inconsistent, and have incomplete effects, have intrinsic bioactivity, and can induce pleiotropic effects on the bacteria. PMBN at low concentrations is considered to be more specific, as it interacts with LPS to increase permeability, but the effects are dose dependent, and PMBN does have intrinsic growth-inhibitory activity at higher concentrations (67). A more elegant and defined system would be desired for assessment of the role of permeability in compound susceptibility in *P. aeruginosa*.

Herein we describe the creation of a suite of *Pseudomonas aeruginosa* mutants in which the final protein involved in transporting LPS for OM biogenesis was compromised in either a wild-type (WT) background or an efflux-deficient mutant. These strains were utilized to assess the activity of multiple classes of antibiotics and to determine the activity-limiting component in *P. aeruginosa*.

MATERIALS AND METHODS

General reagents. All DNA was purified using either the QIAprep spin miniprep kit, DNeasy blood and tissue kit, QIAquick gel extraction kit, or QIAquick PCR purification kit (Qiagen, Valencia, CA). Phusion high-fidelity polymerase and restriction enzymes PstI and BamHI were from New England BioLabs (Ipswich, MA). In-Fusion HD enzyme mix was from Clontech (Mountain View, CA). Electroporation was performed on a Gene Pulser II electroporator with Gene Pulser cuvettes (Bio-Rad, Hercules, CA). DNA sequencing was performed by Genewiz, Inc. (South Plainfield, NJ). LB, LB agar (LBA), super optimal broth (SOB) medium, cation-adjusted Mueller-Hinton broth (CAMHB), cation-adjusted Mueller-Hinton agar (CAMHA), tryptone, yeast extract, and the BBL Prompt

system were from Becton-Dickinson/Difco (Franklin Lakes, NJ). *E. coli* mating strain RHO3 (68), pEX18Ap plasmid (69), and associated protocols were provided by H. P. Schweizer (Colorado State University). All primers, diaminopimelate (DAP), sodium nitrate (NaNO₃), sucrose, EDTA, PMBN, ampicillin, clindamycin, linezolid, colistin, rifampin, tetracycline, ciprofloxacin, novobiocin, fusidic acid, and vancomycin were from Sigma-Aldrich (St. Louis, MO). Gentamicin was from Fisher Scientific (Waltham, MA). Carbenicillin was from Research Products International (Mount Prospect, IL). Azithromycin was from USP (Rockville, MD). Imipenem was from Merck (Kenilworth, NJ).

***Pseudomonas* strain construction.** Mutant alleles *lptE6* (30), *lpxC101* (25), *lptD4213* (28, 70), *lptD208* (28, 70), and *lptDΔ529–538* (71) were constructed in the suicide vector pEX18Ap (69). Mutations were incorporated into internal primers, which were used to PCR amplify the corresponding gene, in full, in two fragments composed of regions flanking the desired mutation. Upstream and downstream regions were amplified using primer pairs P1/P2 and P3/P4 for *lptE6*, P5/P6 and P7/P8 for *lpxC101*, P9/P10 and P11/P12 for *lptD4213*, P9/P13 and P14/P12 for *lptD208*, and P9/P15 and P16/P12 for *lptDΔ529–538* (Table 1). In a second-round PCR, splicing by overlap extension was used to combine up- and downstream products with external primers P1/P4, P5/P8, and P9/P12 for *lptE6*, *lpxC101*, and the *lptD* alleles, respectively. These complete alleles were then cloned into a BamHI/PstI-digested pEX18Ap vector using In-Fusion HD enzyme. After sequencing to confirm the correct alleles had been made, plasmids were mobilized from *E. coli* RHO3 (68) into a wild-type *P. aeruginosa* PAO1 strain termed MB5919 (56) and an efflux-deficient *P. aeruginosa* PAO1 $\Delta(mexAB-oprM)::FRT \Delta(mexCD-oprI)::FRT \Delta(mexXY)::FRT \Delta(mexJKL)::FRT \Delta(mexHI-opmD)::FRT \Delta(opmH)::FRT$ mutant termed MB5890 (57) (see Table S1 in the supplemental material). Briefly, overnight cultures of the RHO3 donor grown in SOB medium containing 400 μ g/ml DAP and 100 μ g/ml ampicillin were diluted 1/100 into 3 ml fresh culture medium and grown to mid-log phase. Overnight cultures of the *P. aeruginosa* recipient grown in SOB medium were diluted 1:1 in 500 μ l 20 mM NaNO₃ and incubated at 42°C for 3 h. Both cultures were subsequently washed with 1 ml SOB medium and resuspended in 200 μ l SOB medium. Ten microliters of recipient *P. aeruginosa* cells and 60 μ l donor RHO3 *E. coli* cells were mixed, plated as a dime-sized plane of growth on CAMHA plus 400 μ g/ml DAP, and incubated overnight at 37°C. The next day, a loop was dragged twice through the heaviest part of the plane of growth, resuspended in 200 μ l CAMHB, and diluted 1/100, and then 70 μ l was plated on CAMHA containing either 150 μ g/ml carbenicillin (for MB5919) or 40 μ g/ml carbenicillin (for MB5890). Exconjugates were confirmed for carbenicillin resistance and sucrose sensitivity. No exconjugates were ever obtained for the *lptE6* allele.

To resolve cointegrants to double-crossover mutants, single colonies were restreaked onto LB with no salt (LBNS: 10 g/liter tryptone, 5 g/liter yeast extract) with 10% (wt/vol) sucrose to select for loss of the *sacB*-containing vector backbone. The resulting colonies were patched onto LBNS, LBNS containing carbenicillin (40 μ g/ml for MB5890 and 150 μ g/ml for MB5919), and LBNS containing 6 μ g/ml rifampin in order to screen for growth, loss of the antibiotic resistance cassette-containing vector backbone, and increased permeability, respectively. Resolved mutants demonstrating the desired Carb^r Rif^r phenotype were obtained for the *lptD4213* and *lptD208* mutants, and sequencing across *lptD* confirmed mutant allele exchange. For the alleles *lpxC101* and *lptDΔ529–538*, hundreds of colonies were screened, and resolved mutants were found to be Carb^r but never Rif^r. Over 40 resolved clones for each of these constructs were sent for sequencing across *lpxC* or *lptD*, respectively, but only wild-type sequence was ever observed.

Unmarked *pagP* (PA1343) knockouts were constructed in MB5919 and MB5890 using the pEX18Ap suicide plasmid. Flanking regions encompassing 600 bp upstream (through the first 50 nucleotides [nt] of *pagP*) and 600 bp downstream (including the last 50 nt of *pagP*) were amplified using primer pairs P17/P18 and P19/P20, respectively, and then

TABLE 1 Primers used for strain construction

Gene and primer name	Sequence ^a
<i>lptE</i>	
P1	ccaagcttgcagcctgcagGCCAGCACCACAAGTTCAACAC
P2	TGCACCTGTACGCGCATCAGGACCAGGTCGTTGGC
P3	TGGTCCTGATGCGCGTACAGGTGCAGAAGGTCTAC
P4	cggtaccgggatccGGCGAATCCACTGCGGCAACTG
<i>lpxC</i>	
P5	ccaagcttgcagcctgcagCGCGGGTCCGGACCTGTCCCTG
P6	TTTTCCCCGAATACAGACCCGACGCCAGTAGCCCG
P7	GCGTCGGTCTGTATTGCGGGGAAAAGGTTTACCTG
P8	cggtaccgggatccAGGCATCGACCCAGTTGTCTGC
<i>lptD</i>	
P9	ccaagcttgcagcctgcagGCCGGTTCGAACAGCTCGTGC
P10	TTGGTTGACGTACGTACCTCGGCCAGCCAGCGCGA
P11	TGGCTGGCCGAGGTCACGTACGTCAACCAACGGGGC
P12	cggtaccgggatccTCTTGCCCATGTCCGAGGCAAG
P13	GCGATAGGTCAGAGTGATCCGCGTGTAGTCGACCTC
P14	GACTACACGCGGATCACTCTGACCTATCGCGGGGAC
P15	CTCGGCCACAGAGAGAAGACCGGCAGGCTGTCTTG
P16	AGCCTGCCGTTCTTCTCTGTGGCGGAGAACCGC
<i>pagP</i>	
P17	ccaagcttgcagcctgcagAAGGGAATCGAAGCGTCACGGTGAC
P18	CGAATTGCCGAGCAGCACCAGCCAGGAGGTCGCGGAGCAC
P19	GTGCTCGCCGACCTGCCTGGCTGGTGTCTCGGCAATTCG
P20	cggtaccgggatccGACACCGAGAGTTGGCGGTCTTGCTGAAG

^a Underlining denotes a restriction site. The transition from lowercase to capital letters denotes the boundary between the vector and gene sequence.

subsequently combined through splicing by overlap extension using the external primers P17/P20. The final PCR product was cloned into pEX18Ap, transformed into *E. coli* RHO3, mobilized into *P. aeruginosa*, and resolved to the double-crossover knockout as described above. MB5919 Δ *pagP* and MB5890 Δ *pagP* were confirmed by sequencing across the *pagP* gene.

MIC testing. MICs were determined by the broth microdilution method in CAMHB as recommended by the Clinical and Laboratory Standards Institute (72) in conjunction with the BBL Prompt system. The MIC was scored as the lowest concentration of compounds required to inhibit visible growth after 18 to 22 h of incubation while stationary at 37°C.

Lipid A palmitoylation assay. Overnight cultures were subcultured 1:100 into 5 ml of fresh CAMHB containing 5 μ Ci/ml ³²PO₄. Cultures were grown with aeration at 37°C until they reached an optical density at 600 nm (OD₆₀₀) of ~1; where indicated, cultures were then treated with 25 mM EDTA for 10 min. Lipid A was isolated as previously described (73). Briefly, cells were pelleted in a clinical centrifuge and washed with phosphate-buffered saline (PBS). Cells were resuspended in a single-phase Bligh-Dyer mixture of chloroform-methanol-water (1:2:0.8 [vol/vol]). Cells were lysed in the mixture for 10 min. The insoluble fraction was collected, resuspended in 12.5 mM sodium acetate (pH 4.5) and 1% SDS, and then incubated at 100°C for 30 min. Lipid A was then isolated from a two-phase Bligh-Dyer extraction and dried under a stream of nitrogen. Samples were resuspended in methanol-chloroform (1:4) mixture, and equivalent amounts were spotted onto a silica thin-layer chromatography (TLC) plate (Macherey-Nagel, Bethlehem, PA). TLC was developed using chloroform, pyridine, 88% formic acid, and water (50:50:16:5 [vol/vol]). Plates were dried and exposed to a phosphor storage screen overnight. Samples were visualized by imaging the screen using a Typhoon scanner (GE, Pittsburgh, PA).

RESULTS

Construction of *P. aeruginosa* mutants. In order to bridge the gap between *in vitro* potency and cellular efficacy in *P. aeruginosa*, matched isogenic mutants of *P. aeruginosa* were constructed containing mutations putatively leading to permeability defects in a wild-type and multiple-efflux-pump-knockout background. Previous studies have described mutations leading to hyperpermeable (antibiotic hypersusceptibility) phenotypes in *E. coli*, including mutations in *lptD*, *lptE*, and *lpxC*. Sequence alignment of these genes' proteins from *E. coli* and *P. aeruginosa* indicated a fair amount of homology, so five mutant alleles—*lptE6* (30), *lpxC101* (25), *lptD4213* (28, 70), *lptD208* (28, 70), and *lptD Δ 529–538* (71)—were engineered into *P. aeruginosa* in order to assess whether they would confer similar permeability defects (Fig. 1). Only two of these alleles, *lptD4213* and *lptD208*, were found to be viable. These results held true whether mutants were constructed in the wild-type MB5919 or efflux-deficient MB5890 strain background.

Assessing antibiotic susceptibility of *P. aeruginosa* *lptD* mutants. To assess the effect of the *lptD4213* and *lptD208* mutations on *P. aeruginosa*, strains were tested for susceptibility to a panel of antibiotics. A diverse array of scaffolds comprising a wide array of physicochemical properties were chosen, with compounds spanning a molecular size of 337 to 1,449 g/mol, a calculated value of overall lipophilicity (clogP) of -2.9 to 6.4 , a relative polar surface area of 19.5 to 48.3%, 3 to 36 free rotatable bonds, and 8 to 54 hydrogen bond donors and acceptors (74). Antibiotics included classes with activity on Gram-negative bacteria (β -lactam, polymyxin, tetracycline, fluoroquinolone, and aminoglycoside) and

lptE6 (PIS117-119R)

Ec LptE (82) IAKDTASVFRNGQTAEYQMIMTVNATVLIIP-GRDIYPISAKVFRSFFDNP
Pa LptE (78) NQQRIVSYTGSARGAEFELTNTINYEIVGANDLVLMNSQVQVKVYVHDE

lpxC101/envA (H19Y)

*

Ec LpxC (1) MIKQRTLKRIVQATGVGLHTGKKVTLTLRPAPANTGVIYR
Pa LpxC (1) MIKQRTLKNIIRATGVGLHSGEKVYLTLKPAPVDTGIVFC

imp4213 (Δ 330–352)*imp208* (Δ 335–359)

Ec LptD (321) MDQVWRFNVDYTKVSDPSYFNDFDNKYGSSTDGYATQKFSVGYAVQNFNA
Pa LptD (416) LDSRWLAEDYTRISDPYFQDLDTDLGVGSTTVNQRGTLTYRGDTFTG

lptD Δ 529–538

Ec LptD (506) QTLEPRAQYLYVPYRDQSDIYNYDSSLLQSDYSGLFRDRITYGGLDRIASA
Pa LptD (628) QTLEPRAMYLYVPYKQDQSLPVFDTSEPSFSYDSLWRENRFYTKDRIGDA

FIG 1 Alignment of LptE, LpxC, and LptD protein sequences from *E. coli* (*Ec*) and *P. aeruginosa* (*Pa*). Permeability-conferring alleles are listed with the resulting amino acid changes indicated in parentheses, and mutations are illustrated above the sequence with asterisks. Identical residues are highlighted in gray.

those which typically are inactive on Gram-negative bacteria (lincosamide, oxazolidinone, glycopeptide, rifamycin, aminocoumarin, fusidic acid, and macrolide). As can be seen in Table 2, both *lptD4213* and *lptD208* had a dramatic effect on the susceptibility to several antibiotics. MICs decreased by at least 32-fold for rifampin, ciprofloxacin, tetracycline, novobiocin, and azithromycin in the wild-type MB5919 background, and similar decreases could be observed for fusidic acid and carbenicillin when tested in the MB5890 efflux-null background. Although fold changes in MIC were difficult to assess for vancomycin because activity was not observed at the highest concentration tested (256 μ g/ml) in either MB5919 or MB5890, both *lptD4213* and *lptD208* led to

increased susceptibility of the strains and measurable MICs in the range tested. Notably, neither *lptD4213* nor *lptD208* had significant effects on the susceptibility of *P. aeruginosa* to gentamicin, clindamycin, or linezolid, with efflux contributing the majority of intrinsic resistance for the latter two. This might be expected given that these three antibiotics are rather small and polar and would not necessarily be subject to exclusion by the LPS barrier of the OM. Taken together, these results indicate that both *lptD4213* and *lptD208* successfully impart a permeability defect to *P. aeruginosa* and facilitate penetration of large hydrophobic antibiotics which normally are incapable of traversing the OM of this organism.

In *P. aeruginosa*, entry of imipenem is facilitated by the OM

TABLE 2 *In vitro* MICs of various antibiotics against wild-type and permeable *P. aeruginosa* strains

Antibiotic	MB5919				MB5890			
	MIC (μ g/ml)			Fold change	MIC (μ g/ml)			Fold change
	WT	Mutant			WT	Mutant		
		<i>lptD4213</i>	<i>lptD208</i>		<i>lptD4213</i>	<i>lptD208</i>		
Clindamycin	>256	>256	256	NC ^a	8	4	4	2
Linezolid	>256	>256	>256	NC	4	4	4	1
Imipenem	4	1	0.5	4–8	2	0.5	1	2–4
Vancomycin	>256	64	32	>4–8	>256	128	128	>2
Colistin	1	0.125	0.25	4–8	0.5	0.125	0.125	4
Rifampin	32	0.5	0.5	64	32	1	1	32
Tetracycline	16	2	0.5	8–32	0.25	0.125	0.125	2
Ciprofloxacin	2	0.0625	0.032	32–64	0.004	0.004	0.004	1
Novobiocin	>256	8	8	>32	64	1	2	32–64
Carbenicillin	16	2	4	4–8	4	0.125	0.125	32
Fusidic acid	>256	>256	128	>2	64	2	2	32
Azithromycin	256	2	2	128	8	0.125	0.125	64
Gentamicin	0.5	0.125	0.25	2–4	0.5	0.25	0.5	1–2

^a NC, not calculated due to lack of activity at the highest concentration tested.

TABLE 3 *In vitro* MICs of β -lactam antibiotics against *oprD* mutant *P. aeruginosa* strains with and without permeability-inducing alleles

Antibiotic	MB6477				MB6476			
	MIC ($\mu\text{g/ml}$)				MIC ($\mu\text{g/ml}$)			
	WT	Mutant		Fold change	WT	Mutant		Fold change
		<i>lptD4213</i>	<i>lptD208</i>			<i>lptD4213</i>	<i>lptD208</i>	
Imipenem	16	0.5	0.5	32	8	1	1	8
Carbenicillin	32	1	2	16–32	4	0.125	0.125	32

porin OprD. Altered expression levels (75), changes in amino acid sequence (76), and complete knockout (77) of this protein are frequently associated with carbapenem resistance. It was of interest to know whether the permeability defects imparted by the *lptD4213* and *lptD208* alleles could compensate for loss of OprD-mediated entry of imipenem. To this end, ΔoprD mutants of MB5890 and MB5919, termed MB6476 and MB6477, respectively, were constructed and subsequently engineered to introduce the two *lptD* mutations. When imipenem and a control β -lactam that is not specifically transported by OprD, the carbapenem carbenicillin, were tested for activity in these strains, OprD loss had the greatest effect on imipenem, resulting in a 4-fold increase in the MIC whether in an efflux-competent or -deficient background (Table 3). Introduction of either *lptD4213* or *lptD208* greatly increased susceptibility to imipenem, with resulting MICs of 0.5 to 1 $\mu\text{g/ml}$, irrespective of efflux capacity, which is equivalent to the MIC range for imipenem in the parental *oprD*⁺ MB5890 and MB5919 strains harboring the same permeability defects. Therefore, *lptD4213* and *lptD208* allow for greater penetration of imipenem than even that afforded by a known substrate-accepting porin.

Furthermore, we sought to compare the permeability defect caused by the *lptD4213* and *lptD208* alleles to the potentiation achieved with the chemical permeabilizer PMBN. As previously reported, PMBN had little intrinsic activity on the wild-type strain MB5919 (67) but had a MIC of 25.6 $\mu\text{g/ml}$ for the efflux-deficient MB5890 strain. Maximal antibiotic potentiation was essentially achieved with 4 $\mu\text{g/ml}$ PMBN, and this was sufficiently below the MIC for MB5890 to make comparisons. For the most part, MICs in the presence of PMBN (Tables 4 and 5) were within 2-fold of those achieved when strains harbored either the *lptD4213* or

lptD208 allele (Table 2). The most significant differences were that PMBN potentiated rifampin 16-fold more than either mutant *lptD* allele in both MB5919 and MB5890, fusidic acid 16-fold more in the MB5919 background, and ciprofloxacin up to 8-fold less in the MB5919 background. This may indicate that PMBN is somewhat better at potentiating certain hydrophobic compounds, but the mutant *lptD* alleles may be better at potentiating certain hydrophilic compounds.

Assessment of OM integrity in *P. aeruginosa* *lptD* mutants. We sought to determine if these *lptD* mutations caused LPS transport defects in the *P. aeruginosa* OM. In Gram-negative cells, the OM inner leaflet consists exclusively of phospholipids (PLs), while the outer leaflet is comprised primarily of LPS (78, 79). Mutations that cause defects in LPS transport allow PLs to mislocalize to the outer leaflet and accumulate (41, 80). These mislocalized PLs become available as the substrates of the OM enzyme PagP, which removes the PL *sn*-1 palmitoyl group and transfers it to the lipid A component of LPS (81, 82). The PagP palmitoylation reaction increases the acylation of LPS, and the presence of such LPS species is used diagnostically to identify compromised OM integrity (41, 80). For example, treatment of cells with EDTA causes loss of LPS from the cell surface, which allows PLs to mislocalize to the OM outer leaflet, triggering PagP palmitoylation of LPS (73).

P. aeruginosa has long been known to palmitoylate its LPS, but only recently has a *pagP* homolog (PA1343) been identified in PAO1 and shown to function as a palmitoyltransferase (83). We constructed a ΔpagP -null mutant and isolated the ³²P-radiolabeled lipid A component of LPS from this strain as well as from the *lptD4213* and *lptD208* mutants and the wild-type parent. Lipid A species were separated using thin-layer chromatography as described in Materials and Methods. Two major lipid A species were

TABLE 4 *In vitro* MICs of various antibiotics against wild-type *P. aeruginosa* strain MB5919 in the presence of various concentrations of PMBN

Antibiotic	MIC ($\mu\text{g/ml}$) at PMBN concn ($\mu\text{g/ml}$) shown							
	0	0.26	0.66	1.6	4.1	10.2	25.6	64.0
Clindamycin	>256	>256	>256	>256	256	128	128	64
Linezolid	>256	>256	>256	>256	>256	>256	>256	>256
Imipenem	4	4	4	1	0.5	0.5	0.25	0.063
Vancomycin	>256	>256	>256	64	32	32	16	16
Colistin	1	1	0.5	0.5	0.5	0.25	0.25	0.25
Rifampin	32	32	32	1	0.031	0.031	0.031	0.031
Tetracycline	16	16	16	2	1	1	1	1
Ciprofloxacin	2	2	2	0.5	0.25	0.25	0.25	0.125
Novobiocin	>256	>256	>256	256	4	4	4	1
Carbenicillin	16	16	16	2	2	2	2	1
Fusidic acid	>256	>256	>256	>256	8	8	4	1
Azithromycin	256	256	256	4	2	2	2	1
Gentamicin	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25

TABLE 5 *In vitro* MICs of various antibiotics against efflux-deficient *P. aeruginosa* strain MB5890 in the presence of various concentrations of PMBN

Antibiotic	MIC ($\mu\text{g/ml}$) at PMBN concn ($\mu\text{g/ml}$) shown							
	0	0.26	0.66	1.6	4.1	10.2	25.6	64
Clindamycin	8	8	8	2	2	2	NG ^a	NG
Linezolid	4	4	4	2	2	2	NG	NG
Imipenem	2	2	1	0.5	0.5	0.5	NG	NG
Vancomycin	>256	>256	>256	128	128	64	NG	NG
Colistin	0.5	0.5	0.25	0.25	0.25	0.125	NG	NG
Rifampin	32	32	16	0.031	0.031	0.016	NG	NG
Tetracycline	0.25	0.25	0.25	0.125	0.125	0.125	NG	NG
Ciprofloxacin	0.004	0.004	0.004	0.002	0.002	0.002	NG	NG
Novobiocin	64	64	64	16	1	1	NG	NG
Carbenicillin	4	4	4	0.5	0.25	0.25	NG	NG
Fusidic acid	64	64	64	8	8	1	NG	NG
Azithromycin	8	8	8	0.5	0.5	0.25	NG	NG
Gentamicin	0.5	0.5	0.5	0.25	0.25	0.25	NG	NG

^aNG, no growth.

produced by wild-type and ΔpagP strains (Fig. 2). We then isolated lipid A from these strains following treatment with 25 mM EDTA. Two novel lipid A species were produced by the wild type in response to EDTA treatment but were not produced by EDTA-treated ΔpagP cells. Clearly, the appearance of these novel lipid A species is PagP dependent, and these therefore represent palmitoylated lipid A species. The same PagP-modified lipid A species are highly abundant in *lptD4213*- and *lptD208*-harboring *P. aeruginosa* strains without the need for EDTA treatment (Fig. 2), confirming that the organization of the OM lipid bilayer is severely disrupted in these mutant strains.

DISCUSSION

Despite the need for novel Gram-negative antibiotics, new classes with alternative mechanisms of action have not been forthcoming.

Failure can often be attributed to an inability to achieve cellular activity with target-based *in vitro* inhibitors, perhaps caused by initial hits occupying the wrong physicochemical property space (13, 14, 74). When there is success in attaining cellular bioactivity, close attention must be paid to overcoming intrinsic resistance mechanisms such as efflux and permeability. Such efforts have shown promise in recent years with the development of hydroxamate LpxC inhibitors (84–86), novel bacterial type II topoisomerase inhibitors (NBTIs) (87, 88), tricyclic GyrB/ParE (TriBE) inhibitors (89), and pyrrolocytosine ribosome inhibitors (90). Although these classes of compounds have not yet reached clinical utility, they demonstrate progress and inspire confidence that novel Gram-negative antibiotics can be discovered.

One of the tools missing for assessment of determinants of compound activity in the major pathogen *P. aeruginosa* is a defined genetic means of measuring the contribution of permeability to intrinsic resistance. An antibiotic-hypersusceptible mutant, termed Z61, has been used to assess the effect of increased permeability on compound activity in *P. aeruginosa* (91–93); however this mutant was derived through multiple rounds of mutagenesis and contains hundreds of single nucleotide polymorphisms (SNPs) (94). Since numerous suppressors could be isolated with a spectrum of partial to full phenotypic reversion (91) and independent mutations could be identified leading to supersusceptibility to subsets of antimicrobial agents (95)—including *oprM*, *ampC*, *lptE*, and *amgRS* (94)—the Z61 strain is undoubtedly genetically complex, and hypersusceptibility cannot be solely attributed to defects in permeability. Additionally, the *E. coli* porin OmpF has been introduced into *P. aeruginosa*, resulting in strains significantly more susceptible to penem antibiotics (96). Given that porin expression was driven off a plasmid, results could vary due to expression level, and increased permeability is likely limited to smaller hydrophilic molecules.

Herein, we have created defined single mutations which lead to a general permeabilization of *P. aeruginosa*. This approach allows construction of isogenic strain sets that can separate the effects of compound influx from efflux and guide compound design to address the activity-limiting factor. The two mutations described herein, *lptD4213* and *lptD208*, are overlapping deletions in LptD, a β -barrel protein responsible for the final step of LPS transport and

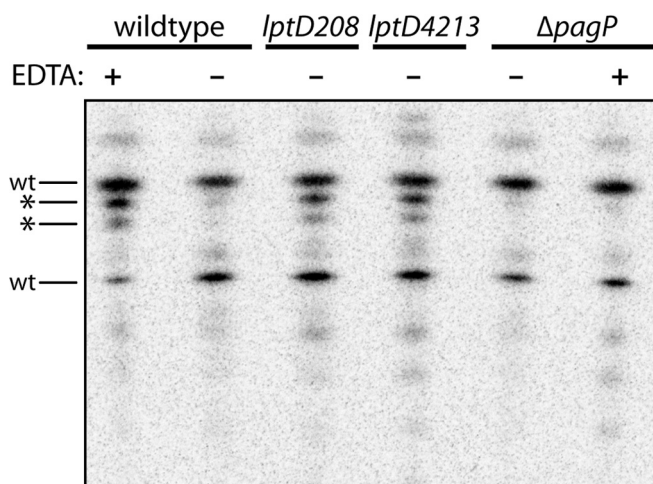


FIG 2 Mutations in *P. aeruginosa* *lptD* cause cells to produce LPS palmitoylated by PagP. LPS was extracted from stationary-phase cultures of MB5890 as well as ΔpagP , *lptD208*, or *lptD4213* derivative strains that were grown in CAMHB and radiolabeled with ^{32}P . Where indicated, cultures were treated with 25 mM EDTA for 15 min before sample collection. Samples were developed by TLC and visualized using a phosphor screen. The two major lipid A species produced in wild-type cells are denoted as “wt.” The two major PagP-dependent lipid A species are marked with asterisks.

assembly in the OM (40, 41, 70, 71). Based on recent crystal structures (97, 98), both mutations lead to the deletion of the alpha-helical loop L4 situated on the extracellular surface of the protein, as well as a portion of either $\beta 7$ or $\beta 8$, respectively. Deletion of this loop may impair LptD function by reducing a few hydrogen bond interactions with the LptE plug and increasing extracellular access to the lumen of the β -barrel. Interestingly, one of the attempted mutations that was found to be nonviable, *lptD* Δ 529–538, is also in LptD. However, this mutation leads to deletion of a large loop, L8, between $\beta 14$ and $\beta 15$, which extends much further into the lumen of the LptD β -barrel and makes extensive interactions with LptE. As was similarly suggested by the *lptE6* mutation, which was found to be dominant negative, the interactions between LptD and LptE may be particularly critical in *P. aeruginosa*, and significant disruption may be lethal.

In both the wild-type MB5919 and efflux-deficient MB5890 backgrounds, the *lptD4213* and *lptD208* alleles were demonstrated to impart significant permeability defects, as assessed by increased susceptibility to a range of antibiotics. The power of these defined genetic lesions and the use of isogenic strain sets are exemplified by the ability to define the activity-limiting component for the antibiotics tested. We determined that efflux was the major contributor to intrinsic resistance for clindamycin and linezolid, with the OM barrier providing minimal protection. Conversely, imipenem, vancomycin, colistin, and rifampin were found to be poor substrates for efflux, and their activity was mostly limited by poor entry into *P. aeruginosa*. For ciprofloxacin and tetracycline, efflux was the main factor limiting activity as *lptD4213* or *lptD208* mutations did not sensitize MB5890. However, in the wild-type MB5919 background, introduction of the *lptD4213* and *lptD208* alleles did reduce the MICs between 16- and 32-fold, indicating that increased penetration of ciprofloxacin and tetracycline into *P. aeruginosa* could overwhelm the efflux pumps and increase their bioactivity. For novobiocin, carbenicillin, fusidic acid, and azithromycin, both efflux and the OM barrier function contribute to intrinsic resistance, as mutations in either lead to additive improvements in potency. Finally, for gentamicin, neither efflux nor permeability appears to be a limiting factor for activity, as mutations in either did not significantly increase the susceptibility of *P. aeruginosa*.

LPS palmitoylation by PagP in strains harboring *lptD4213* and *lptD208* demonstrated that these alleles cause defects that disrupt the lipid asymmetry of the OM bilayer. Similar defects resulting in PagP-modified LPS can be observed upon depletion of the LptD and LptE proteins in *E. coli* (41, 80). The LPS perturbation observed in these mutants genetically phenocopies the results observed by chemical perturbation using reagents such as EDTA. As such, these strains will be extremely useful in antibacterial discovery to track activity of weakly bioactive compounds and to optimize hits from both phenotypic and biochemical screens.

In addition to being useful tools for delineating the factors affecting susceptibility to particular compounds, these permeability mutants will be useful in understanding basic biology related to outer membrane protein (OMP) assembly and LPS transport. Indeed, in *E. coli*, chemical conditionality was used in conjunction with the *lptD4213* allele to screen for suppressors of vancomycin (and related analogs) susceptibility, leading to identification of several components of the β -barrel assembly machinery, including *bamA* (99) and *bamB* (100, 101). Furthermore, it was the initial *E. coli lptD4213* strain that was used to determine that LptD

was involved in OM biogenesis (70) and ultimately responsible for LPS transport (39–41). To date, there have been limited studies exploring *P. aeruginosa* β -barrel assembly and the interplay with LptD function and LPS transport. As this study implies, the inability to engineer certain *lptE* and *lptD* alleles in *P. aeruginosa* that are viable in *E. coli* suggests that differences in the function of the LPS transport and β -barrel assembly machinery exist between organisms. This is further supported by the discovery of an extremely potent *P. aeruginosa*-specific LptD inhibitor that shows no activity on other Gram-negative organisms (102, 103). Clearly these systems are of importance and represent valuable targets for the development of novel antibiotics.

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