Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing

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An important question regarding the biologic implications of antibiotic-resistant microbes is how resistance impacts the organism’s overall fitness and virulence. Current evidence is generally thought that antibiotic resistance carries a fitness cost and reduces virulence. For the human pathogen Pseudomonas aeruginosa, treatment with carbapenem antibiotics is a mainstay of therapy that can lead to the emergence of resistance, often through the loss of the carbapenem entry channel OprD. Transposon insertion-site sequencing was used to analyze the fitness of 300,000 mutants of_P. aeruginosa_ strain PA14 in a mouse model for gut colonization and systemic dissemination after induction of neutropenia. Transposon insertions in the oprD gene led not only to carbapenem resistance but also to a dramatic increase in mucosal colonization and dissemination to the spleen. These findings were confirmed in vivo with different oprD mutants of PA14 as well as with related pairs of carbapenem-susceptible and -resistant clinical isolates. Compared with OprD+ strains, those lacking OprD were more resistant to killing by acidic pH or normal human serum and had increased cytotoxicity against murine macrophages. RNA-sequencing analysis revealed that an oprD mutant showed dramatic changes in the transcription of genes that may contribute to the various phenotypic changes observed. The association between carbapenem resistance and enhanced survival of _P. aeruginosa_ in infected murine hosts suggests that either drug resistance or host colonization can cause the emergence of more pathogenic, drug-resistant _P. aeruginosa_ clones in a single genetic event.

_Pseudomonas aeruginosa_ is a classical example of a bacterial pathogen that is often found associated with extremely difficult-to-treat infections that resist antibiotic therapies. This organism frequently emerges as a threat to neutropenic, immunosuppressed patients undergoing treatment for cancer wherein one usually observes the spread of antibiotic-resistant organisms from gastrointestinal (GI) sites into the blood stream. It has also been observed in the setting of cystic fibrosis (CF) that reversion or displacement of resident drug-resistant _P. aeruginosa_ strains does not occur even when antibiotic treatment is intermittent (7). This observation suggests that some mechanisms leading to antibiotic resistance could also enhance the fitness of _P. aeruginosa_ in vivo and thus contribute to persistent infections.

Transposon (Tn) insertion-site sequencing (Tn-seq) is a powerful analytical method that in various formats has been called “INSeq” (insertion-site sequencing) (8, 9), “Tn-seq” (10), or “high-throughput insertion tracking by deep sequencing” (HITS) (11). These methods allow one to measure the fitness of collections of insertion mutants under a given growth condition

**Significance**

> It is thought antibiotic resistance carries a fitness cost and reduces microbial virulence. Using high-throughput sequencing analysis of a transposon insertion bank in _Pseudomonas aeruginosa_, we found enhanced fitness for in vivo mucosal colonization and systemic spread of strains with transposon insertions in the oprD gene. This conferred resistance to carbapenem antibiotics as well as enhanced resistance to killing at acidic pH and by normal human serum along with increased cytotoxicity against murine macrophages. RNA-sequencing analysis revealed that oprD deficiency led to transcriptional changes in numerous genes that may contribute to the enhanced in vivo fitness observed. Thus, if carbapenem resistance develops during antibiotic therapy of _P. aeruginosa_ infections, it may lead to enhanced fitness and virulence in infected hosts.


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Results and Discussion

We have recently reported that antibiotic treated mice can be used in principle to analyze the GI fitness of P. aeruginosa Tn mutants (9). In brief, to establish P. aeruginosa GI colonization, mice received streptomycin and penicillin in sterile drinking water for 5 days to clear the indigenous commensal GI microbial flora, after which the PA14 TnSAMDGm insertion bank, grown overnight in lysogeny broth (LB, designated “input LB”) containing 15 mg gentamicin per L was added to sterile water containing penicillin and gentamicin. The drinking water containing bacteria was renewed after 72 h and administered to mice for a total of 6 days after which sterile drinking water was given for 24 h before mice were killed and their ceca removed. During this time, the strains with Tn insertions that could colonize and survive in the cecum of the GI tract were selected (designated “output cecum”). Following induction of neutropenia after 6 days of mucosal colonization, the strains with Tn insertions that were able to disseminate systemically over 24 h were recovered from the spleen (designated “output spleen”) (14). Splenic dissemination after induction of neutropenia was considered to indicate fitness for systemic dissemination.

Tn-seq analysis of the population of organisms derived from growing the Tn-insertion bank overnight in LB showed there were no Tn insertions mutants that yielded more than 8,000 sequencing reads out of 10⁸ normalized reads (0.8%) indicating that the Tn insertions were relatively evenly distributed in the genome and no Tn insertions were significantly overrepresented in the bank (Fig. 1C). However, strains with Tn insertions in 13 different genes were strongly overrepresented in the output cecum samples (Fig. 1B). Twelve of these were in genes resulting in the loss of type IVa pili (15) comprising 380,000 total reads (38%). Strikingly, strains bearing Tn insertions in the oprD gene, whose loss leads to acquisition of the resistance to the carbapenem antibiotics, represented 42% of the output cecum population. The number of sequencing reads of the various oprD mutants increased from 515 (0.05% of the total reads) in LB to 420,621 in the cecum (Fig. 1B). More dramatically, 94% of the strains that disseminated to the spleen of neutropenic mice (Fig. 1C) had Tn insertions in the oprD gene (947,397 sequencing reads in the spleen output group). Whereas a large number of Tn-insertion mutants showed a significant decrease in their recovery from each in vivo environment, mutants carrying Tn insertions in the oprD gene were dramatically increased in vivo fitness [Z = 972.29, P < 10⁻¹⁶, Kal’s statistical test (16)]. To avoid any bias due to an overrepresentation of the oprD mutants in water over time, we sequenced the bank of mutants after bacteria resided in water containing penicillin and gentamicin for 48 h at room temperature. Tn insertions in oprD represented less than 0.5% of the viable mutants present in such water samples. As the drinking water containing bacteria was changed after 72 h, the lack of overrepresentation of the oprD mutant strains at any time point in the water was confirmed by plating the bank of mutants.

![Fig. 1](https://www.pnas.org/ cgi/doi/10.1073/pnas.1221552110)

**Fig. 1.** Analysis of in vivo fitness of Tn insertions in the P. aeruginosa PA14 genome. (A) Circos plot of Tn insertions into strains grown overnight in LB with their ordered representation (inner blue circle). Gray circular lines represent 2,000, 4,000, 6,000, or 8,000 sequencing reads recovered from the input LB sample. Outermost circle represents the full PA14 genome. (B and C) Analysis of Tn insertions into genes within the PA14 chromosome revealed strains with increased in vivo fitness. (B) Ordered representation of the in vivo fitness for cecal colonization of all of the strains with Tn insertions able to grow overnight in LB. (C) Ordered representation of the in vivo fitness for splenic dissemination of all of the strains with Tn insertions able to colonize the output cecum. Total number of reads recovered from the input LB and output cecum and spleen samples were normalized to 1,000,000.
that survived for 72 h in water on plates with or without carbapenems (3 mg/L). We confirmed by PCR that only strains with a Tn inserted in the oprD gene were able to grow on the carbapenem-containing plates and they represented only 0.5% of the total strains. In parallel, we observed no difference in the growth of an oprD mutant strain in vitro (Fig. S1).

Increased fitness was not specific for Tn insertions into genes encoding for all outer membrane proteins (OMPs) although, as among the Tn insertions in the 262 genes predicted to encode for OMPs (17, 18), only 68 were able to colonize the cecum (Fig. 2A). Among them, only the Tn insertions in the oprD gene displayed an enhanced fitness for colonization (Fig. 2A).

To further analyze the comparative colonization and dissemination abilities of wild-type (WT) P. aeruginosa PA14 and strains with Tn insertions in the oprD gene, we characterized several oprD-deficient variants of strain PA14. Two of these strains, PA14_Tn-oprD:6-4 and PA14_Tn-oprD:8-1, were retrieved from an ordered Tn library (19) and had distinct Tn insertions in the oprD gene. An additional PA14 strain, PA14_Tn-oprD:Spleen, was recovered from a neutrophilic mouse spleen. All of these oprD Tn-insertion strains showed an enhanced resistance to the carbapenem antibiotic imipenem, with a minimum inhibitory concentration (MIC) of ≥6 mg/L compared with 1 mg/L for the WT PA14 strain.

To acquire correlative data from infected human patients, we obtained two additional strains of P. aeruginosa resistant to carbapenems from a collection of isolates. Pulsed-field gel electrophoresis (PFGE) (20) was used to identify related pairs of strains from two patients (Fig. S2A) in which earlier clinical isolates (strains 48-1 and 51-1) were carbapenem susceptible (imipenem MIC < 1 mg/L), and later isolates (48-2 and 51-2) were carbapenem resistant (imipenem MIC ≥ 32 mg/L). Sequencing of the oprD genes from these strains confirmed that the carbapenem-susceptible isolates had an intact oprD gene, whereas the carbapenem-resistant strain 48-2 had acquired the insertion sequence element ISPa132B at nucleotide 50 in the oprD gene and the carbapenem-resistant strain 51-2 had a 12-bp deletion (base pairs 579–590 included) and a single nucleotide change (G→A at position 1148 leading to a stop codon) in the oprD gene (Fig. S2B). We further constructed complemented strains for the PA14_Tn-oprD:Spleen strain and the 48-2 and 51-2 strains by conjugating into them a plasmid bearing a full-length oprD gene (PoprD). The complemented strains had MICs to imipenem of ≤1 mg/L. Finally, to further study the phenotypes associated with oprD deficiency, 22 additional clinical strains were selected (CS1–CS22): 10 strains (CS1–CS10) with a normal level of transcription of the oprD as determined by quantitative (q)RT-PCR, and 12 strains (CS11–CS22) with a reduced level of expression (Dataset S1). The three controls used for the qRT-PCRs were the PA14 WT strain, a strain with a clean deletion of the oprD gene, and a strain overexpressing oprD.

Analysis of OprD expression using SDS/PAGE showed that the 48.4 kDa OprD protein was readily seen in extracts from WT PA14, the clinical isolates 48-1 and 52-1 and the corresponding transcomplemented strains (Fig. 2B), whereas no evidence of an intact OprD protein was found in PA14 ΔoprD strain (used as a control), the Tn insertions designated 6-4, 8-1, Tn-oprD::Spleen, and the clinical strains 48-2 and 51-2 (Fig. 2B). This result was confirmed using a sensitive silver-staining reagent (Fig. S3) that also showed the lack of difference of expression in the other OMPs under these conditions, and suggested that OprD-truncated protein was not present in OprD mutant strains (Fig. S3).

As Tn insertions unable to produce type IVa pili were also positively selected for enhanced GI colonization, the swarming and the twitching motilities of P. aeruginosa PA14 Tn:oprD were assessed (15). No change in motility was associated with the loss of production of OprD (Fig. 2C and Figs. S4 and S5). Therefore,
the basis for enhanced fitness for GI colonization of oprD mutants in mice is not the result of a defect in the production of type IVa pili.

Attempts to mark WT *P. aeruginosa* PA14 with either streptomycin or tetracycline resistance for in vivo tracking resulted in a diminution in their ability to colonize the murine GI tract in comparison with unmarked WT *P. aeruginosa* PA14 (Fig. S6), emblematic of the fitness cost usually attributed to the acquisition of antibiotic resistance (6). Thus, to compare the oprD-deficient strains with WT PA14, the unmarked WT strain and the oprD-deficient strains (gentamicin resistant) or the WT and PoprD-complemented strains (also gentamicin resistant) were mixed together in drinking water at a ratio of ∼1:1 and the relative levels of WT and mutant strains in the output samples were determined by plating cultures on LB agar and LB agar with 15 mg gentamicin per L. We subtracted the colony forming units (cfu) determined from the latter plates from those on the non-selective plates to obtain the level of colonization with WT *P. aeruginosa* PA14. This ratio was further confirmed by sub-culturing 100 separate colonies that grew on the LB-agar plates with and without gentamicin. Analysis of the oprD gene by PCR for each gentamicin-resistant colony confirmed that they were oprD mutants and not spontaneous gentamicin-resistant strains. A similar approach was used to differentiate between oprD-deficient and PoprD-complemented strains, however, carbapenem plates were used instead of gentamicin plates as both strains were gentamicin resistant.

Comparing selective fitness during cecal colonization of the PA14_Tn-oprD::Spleen isolate with the WT PA14 strain and the complemented PA14_Tn-oprD::Spleen (PoprD) strains revealed that the PA14_Tn-oprD::Spleen strain out-competed both the WT and complemented strains, constituting >90% of the isolates recovered from the cecum (Fig. 3A). Comparing the WT PA14 strain and complemented PA14_Tn-oprD::Spleen (PoprD) strains in the cecal colonization setting showed that both colonized the mice at comparable levels (Fig. 3A), supporting the conclusion that the loss of expression of OprD conferred an in vivo GI colonization advantage to the PA14_Tn-oprD::Spleen strain over the WT PA14 (Fig. 3A). No effect of the empty vector used for complementation was found in the GI colonization model (Fig. S7).

Further confirmation that loss of OprD enhanced GI colonization was obtained by evaluating the competitive colonization efficacies of WT PA14 against the two carbapenem-resistant strains from the ordered Tn library, PA14_Tn-oprD6-4 and PA14_Tn-oprD8-1. The PA14_Tn-oprD6-4 and PA14_Tn-oprD8-1 strains were stronger cecal colonizers than the PA14 WT strain. Following induction of neutropenia, more than 90% of the isolates recovered from the spleen were oprD mutants (Fig. 3B and C). Comparative analysis of the competitive colonization capacities of the *P. aeruginosa* clinical isolates with an intact oprD gene, 48-1 and 51-1 with their corresponding related oprD mutants, 48-2 and 52-2, showed the oprD mutants constituted >95% of the recovered strains (Fig. 3B and C). Thus, in all circumstances, loss of OprD and the consequent acquisition of carbapenem resistance resulted in enhanced GI colonization and systemic dissemination during neutropenia. To ascertain whether the overrepresentation of the oprD mutants was not merely due to enhanced survival as opposed to enhanced fitness of these strains, we colonized antibiotic-treated mice with monocultures of either WT PA14 or streptomycin- or tetracycline-resistant variants, or with the PA14_Tn-oprD::Spleen strain, and found no difference in the levels of any of these variants in the ceca after 6 d of colonization (Fig. S8). Thus, in the absence of competition from the OprD-deficient strains, WT strains were able to achieve levels of GI colonization comparable to that of the oprD mutants.

We next evaluated several phenotypes that could be related to the oprD deficiency and account for the increased in vivo fitness of the oprD mutants by analyzing in vitro survival of WT PA14 or Tn-insertion mutants in the presence of major host innate immune factors in the serum (21), as well as survival in the acidic conditions found in the GI tract (pH 5 in mice) (22). After 180 min in 50% serum, all of the strains lacking OprD survived better than their isogenic or related parental or trans-complemented partners containing an intact oprD gene (Fig. 4A). Similarly, except for one strain (CS23 strain), all of the clinical strains with a low level of oprD transcription were significantly more resistant to serum-mediated killing compared with the clinical strains with a high level of oprD transcription (Fig. 4A). All of the oprD mutant strains tested were found to survive better at pH 5 than isogenic or related strains with an intact oprD gene (Fig. 4B). Furthermore, the PA14_Tn-oprD::Spleen strain displayed higher levels of cytotoxicity against murine macrophages than either this mutant complemented with PoprD or to the PA14 WT strain (Fig. 4C).

To define a mechanism that could explain all these phenotypes associated with oprD deficiency, we used RNA-seq (23) to determine the transcriptional profiles of WT PA14 and PA14_Tn-oprD::Spleen strains after in vitro growth. As shown Fig. 5, the PA14 in vitro transcriptome was quite uniform in that it showed little variability throughout the whole genome. In contrast, the PA14_Tn-oprD::Spleen transcriptome showed considerable variability with elevated and reduced transcriptional levels detected in many different chromosomal locations compared with WT PA14 (Fig. 5). In total, 97 genes that were clearly transcribed in WT PA14 were transcriptionally silent in PA14_Tn-oprD::Spleen when grown in vitro (Dataset S2). In contrast, 60 genes had transcription levels increased more than 10-fold in the PA14_Tn-oprD::Spleen strain compared with WT PA14 (Dataset S3).

Interestingly, increases in transcript levels were not found for the genes encoding most known virulence factors in the
Fig. 4. Phenotypes associated with increased in vivo fitness of oprD mutant strains. (A) Resistance to the antibacterial action of serum of P. aeruginosa strains with or without oprD deficiencies. OprD deficiency was confirmed by assessing the level of OprD expression (Fig. 2A) or the level of oprD mRNA transcription (strains CS1–S23; Dataset S1). The P values were determined by t test. (B) Survival for 60 min at pH 5 of either WT P. aeruginosa strains or those with mutations in the oprD gene. Bars indicate mean percent survival compared with isogenic or related strains with intact oprD. Error bars indicate a single SD of the data. *P value of less than 0.05 (t test) between the oprD intact and mutant strains; **P value of less than 0.05 between the oprD mutant and isogenic or related oprD complemented strain. (C) Role of the oprD gene in the cytotoxicity of PA14 against murine macrophages after 1 h of incubation. Two multiplicities of infection were tested: 20:1 (Upper) and 100:1 (Lower). The PA14 Δexou strain was used as a negative control. Error bars indicate the SD of the data.

P. aeruginosa genomes (www.mgc.ac.cn/VFs/main.htm). To validate RNA-seq results, we performed individual qRT-PCR determinations that confirmed the lack of significant increases in the transcription level between the WT PA14 and the PA14_Tn-oprD:Spleen strains for genes representative of the following virulence factors: type 1, 2, 3, and 6 secretion systems, type IVa oprD transcription level between the WT PA14 and the PA14_Tn-oprD mutants, carbapenem-resistant P. aeruginosa strains with MICs to imipenem or meropenem of 0.06–4 μg/mL, considered to be within the susceptible range (25), suggesting that increased fitness and full carbapenem resistance may be separable properties of the OprD protein. This might be explained by the findings of Eren et al. (29) who reported that multiple...
outer-membrane carboxylate channels (Occ) like OprD with different substrate specificities are found among various Gram-negative bacteria, raising the possibility that some strains of \textit{P. aeruginosa} might possess additional Occ channels involved in carbapenem uptake.

Although almost all prior reports indicated no obvious fitness cost for causing severe infections by OprD-deficient carbapenem-resistant \textit{P. aeruginosa}, our studies suggest there might be enhanced in vivo fitness of \textit{P. aeruginosa} as a result of acquisition of oprD-inactivating mutations. Mechanistically, this is likely due to the collective properties of OprD-deficient \textit{P. aeruginosa} including enhanced serum resistance, a better ability to survive in hostile environments such as the acidic pH of the stomach, increased cytotoxicity against phagocytes, and other yet-to-be-discovered properties contributing to enhanced fitness. Notably, OprD-deficiency does not appear to confer any enhanced fitness in noninfectious settings, as a previous study (50) on 328 unrelated \textit{P. aeruginosa} isolates from 69 localities in 30 countries on five continents collected from diverse clinical (human and animal) and environmental habitats over the last 125 y found no OprD-deficient strains among environmental isolates. However, the prevalence of OprD-deficient strains in CF and non-CF patients from the same study was 16% and 10%, respectively.

In a broader context, our findings imply that carbapenem treatment and the consequent selection of \textit{P. aeruginosa} oprD mutant strains can lead to enhanced in vivo fitness and potentially to an increase in virulence. If additional studies validate an increased fitness in noninfectious settings, such as in the murine GI tract colonization and systemic dissemination by \textit{P. aeruginosa} and the DNA preparation for illumina sequencing were as described (8, 14). The PFGE was interpreted according to previously used criteria (20). The bacterial OMPs were detected by SDS/PAGE. Twisting motility was assessed using 1.5% agar LB plates and swarming assays performed using supplemented fresh plates of M9 minimal medium. The serum killing experiments were performed with pooled human serum. The cytotoxicity experiments used RAW264.7 cells. The library for the RNA-seq was prepared using Encore Complete Prokaryotic RNA-Seq DR Multiplex Systems kit (NuGEN), and cDNA fragmentation was done using Qsonica Sonicator Q800R. A full description of methods is available in SI Materials and Methods.

Materials and Methods

The murine model of GI tract colonization and systemic dissemination by \textit{P. aeruginosa} and the DNA preparation for illumina sequencing were as described (8, 14). The PFGE was interpreted according to previously used criteria (20). The bacterial OMPs were detected by SDS/PAGE. Twisting motility was assessed using 1.5% agar LB plates and swarming assays performed using supplemented fresh plates of M9 minimal medium. The serum killing experiments were performed with pooled human serum. The cytotoxicity experiments used RAW264.7 cells. The library for the RNA-seq was prepared using Encore Complete Prokaryotic RNA-Seq DR Multiplex Systems kit (NuGEN), and cDNA fragmentation was done using Qsonica Sonicator Q800R. A full description of methods is available in SI Materials and Methods.

Supporting Information

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SI Materials and Methods

Murine Model of Gastrintestinal Tract Colonization and Systemic Dissemination by Pseudomonas aeruginosa. C57/HeN mice (female 6–8 wk old) were housed in groups of four in sterilized cages equipped with filter hoods. As described previously (1), mice (n = 8) received streptomycin (2 mg/mL) and penicillin (1,500 UI/mL) in sterile drinking water for 5 d to reduce the levels of the indigenous microbiota. The plasmid pSAMDGm (2), (3) derived from pSAMBt (4) was used to create a TnSAMDGm transposon (Tn) mutant library of 300,000 mutants in P. aeruginosa PA14. One aliquot of the of PA14 Tn insertions library was grown overnight in lysogeny broth (LB) (5) containing 15 mg gentamicin/L and then added to sterile water containing penicillin (1,500 UI/mL) and gentamicin (15 mg/L). The PA14 Tn-insertion population was administered to mice via drinking water for 6 d. To maintain the level of PA14 Tn insertions, the water was changed after 72 h. After 6 d, the drinking water was exchanged for sterile water containing penicillin (1,500 UI/mL) and gentamicin (15 mg/L) until the end of the experiment. Mice were colonized in two different groups: the gastrointestinal (GI) colonization group and the dissemination group. For ascertaining the TN insertions surviving the colonization period, mice (n = 4) were euthanized and ceca harvested 24 h after the exchange for sterile water. To induce systemic dissemination, mice (n = 4) were injected on the same day sterile water was provided with 250 µg of the neutrophil-depleting rat IgG2 monoclonal antibody RB6-8C5 that targets the Ly6G antigen. This dose of antibody renders mice severely neutropenic (polymorphonuclear leukocyte counts <100) for 5 d (1). When moribund (or 48 h after antibody injection), neutropenic mice were killed and spleens harvested, homogenized in LB containing 15 mg gentamicin/L and grown overnight at 37 °C. Genomic DNA was extracted from each sample of the initial and recovered bacterial populations using QIAamp DNA Mini Kit (Qiagen), quantified, and stored at −20 °C.

DNA Preparation for Illumina Sequencing. Illumina libraries were prepared as described (4) with the following exceptions: 20 µg of DNA from all samples were used at the start of library preparation; DNA was concentrated using a Speed-vac before gel loading and extraction; DNA from 1.2 to 1.5 kb was gel-extracted for further processing; and Kapa BioSystems High Fidelity Polymerase was used in the final PCR amplification step.

Determination of the Sequences at the Tn-Insertion Sites. All sequences retrieved from the Illumina sequencing reactions were trimmed to eliminate those reads with quality scores less than 0.05 and/or sequences with ambiguous nucleotides. All trimmed sequences from the LB and cecum and spleen output samples were mapped on the annotated genome of P. aeruginosa strain PA14, from -120 nucleotides (to include promoter regions) to the end of each ORF. Ambiguous reads were excluded, and no mismatches were allowed for the mapping.

Statistical Analysis. Data were analyzed using the RNA-sequence (RNA-seq) module of the Bioinformatics Workbench software package (CCL; www.clebio.com/index.php?id=1240). For each Tn insertion, the significance of the difference in proportion of reads from one environment to the other (LB to cecum and then cecum to spleen) was estimated using the statistical test defined by Kal et al. (6). The test is based on a statistical score Z that follows a normal distribution under the null hypothesis of no difference in proportions of read between the two environments that are compared. All P values were adjusted for multiple comparisons using Bonferroni correction.

Prevalence of the oprD Mutants in Water After 72 h. The bank of PA14 mutants was suspended in water, and after 72 h, an aliquot plated on LB with or without 5 µg imipenem/mL. The presence of a Tn inserted in the oprD gene in the mutants able to grow on imipenem-containing media was confirmed by PCR. The prevalence of the Tn-oprD mutants in the water after 72 h was determined by determining the ratio of the colony forming units (cfu) growing on LB to the cfu growing on LB with imipenem.

Competitive Assays. To assess the in vivo fitness of PA14 mutants, we obtained selected PA14 mutants from the PA14 Non-Redundant Transposon Insertion Mutant Set (7). The insertion sites for each Tn were confirmed by PCR. The GI tract decontamination described above using penicillin-streptomycin in the drinking water was used on four mice for each competition experiment. The drinking water was replaced with penicillin water containing a 1:1 ratio of WT PA14 and a single PA14 Tn-mutant (5 x 10^6 cfu/mL for each strain) for 6 d. Mice were then given sterile drinking water with penicillin alone, and 24 h later the mice were euthanized and the cecum harvested. Serial dilutions were plated on LB agar with or without antibiotics. To evaluate the final ratio of PA14 mutants (gentamicin-resistant) and WT PA14 (gentamicin susceptible), the samples were plated on LB agar and LB agar with 15 mg gentamicin/L. We subtracted the cfu determined from the latter plates from those on the nonselective plates to obtain the level of colonization with WT P. aeruginosa PA14. This ratio was further confirmed by subculturing 100 individual colonies that grew on the LB-agar plates on plates with and without gentamicin. Analysis of the oprD gene by PCR for each gentamicin-resistant colony confirmed that they were oprD mutants and not spontaneous gentamicin-resistant strains. LB agar with or without imipenem (3 µg/L) were used to distinguish between the mutated oprD isolates from the WT oprD isolates, when complemented strains (gentamicin resistant) were in competition with gentamicin-resistant mutants or when the two pairs of clinical P. aeruginosa isolates, confirmed to be related by pulse-field gel electrophoresis (PFGE) (see section PFGE), were in competition. The competitive index (CI) was defined as the ratio of cfu for the mutant compared with the cfu obtained for the WT strain in the output sample.

Selection of oprD Mutants from the PA14 Random Tn-Insertion Bank. Homogenized spleens from the neutropenic mice were streaked onto LB plates with gentamicin, and isolated colonies were screened for a Tn insertion into the oprD gene by PCR amplification of the entire oprD gene. Insertion of TnSAMDGm within the oprD gene would result in a 1.7-kb increase in the size of the 1.8-kb PCR product obtained from the WT oprD gene. This allowed us to identify Tn-oprD mutants in the spleen samples.

P. aeruginosa Clinical Isolates. Two pairs of clinical isolates, each containing an imipenem-susceptible variant and an imipenem-resistant variant, were used. These strains were isolated in 2011 at the University Hospital, Caen, France. One pair was isolated from the same stool specimen of a patient hospitalized in the Hematology Department, whereas the second pair was isolated from the same respiratory-tract specimen of a patient hospitalized in the medical intensive care unit. Sequencing of the oprD genes confirmed that the imipenem-susceptible strains had a WT oprD gene, whereas the imipenem-resistant isolates had mutated oprD sequences. A total of 22 other clinical strains were also
selected from the same Institute. The level of oprD transcription in these strains was determined by quantitative (q)RT-PCR. The study was approved by the Institutional Review Board of the Caen University Hospital, and informed consent was obtained in accordance with the Declaration of Helsinki.

qRT-PCR. From bacteria grown to late-exponential growth phase in LB, total RNA was extracted using the ZR Fungal/Bacterial RNA MiniPrep Kit (Zymo Research) as recommended by the manufacturer. Residual chromosomal DNA was removed by treating samples with the TURBO DNA-free kit (Life Technologies). DNase-treated RNA samples were quantified using a BioSpec-nano spectrophotometer (Shimadzu) and the integrity (RNA Integrity Number > 8) was assessed using the Agilent 2100 Bioanalyzer. qRT-PCR experiments were performed using the QuantCFast SYBR Green RT-PCR Kit (Qiagen) and the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories) according to manufacturers’ recommendations. Transcript levels in each sample were determined using gyrB and oprL as housekeeping reference genes. Each experiment was performed in triplicate. Primers used are presented in Dataset S5.

PFGE. PFGE analysis of genomic DNA fragments of the clinical isolates was carried out after digestion with the restriction endonuclease SpeI, the electrophoresis was performed with a CHEF-DRIII apparatus (Bio-Rad), and PFGE patterns were interpreted according to previously used criteria (8).

Outer-Membrane Protein Analysis. Bacterial outer-membrane proteins (OMPs) were detected by SDS/PAGE as previously described (9). Briefly, bacterial cells were broken by sonication, and membranes were collected by ultracentrifugation at 100,000 × g for 1 h. The inner membrane was solubilized with 1% sodium N-laurylsarcosinate. Proteins in the outer membrane were separated by SDS/PAGE and gels were stained with Comassie blue or Pierce Silver Stain Kit (Thermo) for visualization.

Imipenem Etest. Minimal inhibitory concentrations of imipenem were determined by using the Etest method (BioMérieux) according to the manufacturer’s recommendations.

Construction of the P. aeruginosa PA14 oprD Knock-Out Mutant. The oprD-deletion mutant (PA14 ΔoprD) was derived from WT PA14 using the replacement vector pEXG2 as described previously (10). Two fragments flanking the oprD gene were amplified by PCR with oprD_del primers (F1 and R1 for the upstream fragment and F2 and R2 for the downstream fragment; Tables S1 and S2) using chromosomal DNA of PA14 as template. Overlap-extension PCR was used to assemble these segments in a second reaction mixture by using upstream forward (oprD_del-F1–HindIII) and downstream reverse primers (oprD_del-R2–XbaI). The fusion product was cloned into pEXG2 as HindIII–XbaI fragment, resulting in pEXG2-oprDdel, and transformed in Escherichia coli Sm10pir with selection on LB agar containing gentamicin (15 μg/mL). For the isolation of a PA14 ΔoprD mutant, pEXG2-oprDdel was transferred from E. coli Sm10pir into PA14 by conjugation, followed by selection of gentamicin-resistant PA14 colonies on LB agar containing gentamicin (15 μg/mL) and irgasan (25 μg/mL). The merodiploid gentamicin-resistant PA14 strain was then streaked on LB agar containing 6% sucrose. Sucrose-resistant colonies were tested to confirm gentamicin susceptibility that confirmed excision from the genome of the pEXG2 backbone. The oprD deletion was confirmed by PCR (Tables S1 and S2), OMP analysis (Outer-Membrane Protein Analysis), and tested for imipenem-resistance by Etest (Imipenem Etest).

Single-Copy Complementation of oprD Mutants. Complementation was achieved by amplifying a 1.6-kb EcoRI–HindIII fragment containing the intact oprD gene from WT PA14 using the primers oprD_compF and oprD_compR. The oprD gene was cloned into mini-CTX1 (11) as an EcoRI–HindIII fragment, resulting in CTX1-oprD, and transformed into E. coli Sm10pir with selection on LB agar containing tetracycline (10 μg/mL). Following sequencing, CTX1-oprD was conjugated into the P. aeruginosa oprD mutant strains as described above. The oprD mutants complemented with CTX1-oprD were selected on LB agar containing irgasan (25 μg/mL) and tetracycline (75 μg/mL). Complementation was verified by PCR and OMP analysis and tested for imipenem susceptibility by Etest.

Overexpression of the OprD in P. aeruginosa. To construct the OprD overexpressing vector, the oprD gene from strain PA14 containing its own Shine–Dalgarno sequences was cloned into pMMB67EH using In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.), transformed into E. coli DH5α, and mobilized into P. aeruginosa PA14. OprD protein was overexpressed at late exponential growth phase by adding 2 mM (final) of isopropyl thigalactoside for 30 min at 37 °C.

Isolation of Tetracycline- and Streptomycin-Resistant Mutants. To create a tetracycline-resistant variant of strain PA14, the tetA gene was inserted between PA14-59170 and PA14-51960 on the Pseudomonas aeruginosa Pathogenicity Island (PAPI)-1 island. The PA14 streptomycin-resistant mutant was obtained by selection during passage on LB agar plates containing increasing concentrations of streptomycin.

Measurements of Growth Kinetic. P. aeruginosa strain PA14 WT, Tn insertions in the oprD gene, Tn_oprD-6–4 and Tn_oprD-8–1, the Tn_oprD::Spleen strain, and the Tn_oprD::Spleen strain complemented with an empty vector [Tn_oprD::Spleen(EV)] or with the cloned oprD gene [Tn_oprD::Spleen(oprD)] were initially grown on LB agar, then cells from these plates were used to inoculate LB medium that was placed at 37 °C with shaking at 250 rpm. The OD₅₆₀ nm was recorded every 30 min.

Twitching Motility and Swarming Assays. Twitching motility was assessed using 1.5% agar LB plates dried for 3 h at 37 °C. Strains were inoculated at the very bottom of the LB plates and incubated at 37 °C for 24 h and then left at room temperature. Swarming assays were performed using fresh plates of M9 minimal medium supplemented with amino acids (0.5%), dextrose (11 mM), CaCl₂ (1 mM), and MgSO₄ (1 mM) and solidified with agar (0.5%).

pH Sensitivity. Strains were grown overnight in LB medium at 37 °C for 20 h with shaking. Bacteria were diluted 1:200 to an OD₅₆₀ nm of 0.4 in HBSS+0.1% gelatin (~5 × 10⁵ cfu/mL), adjusted to pH 5 or pH 7 with HCl or sodium hydroxide, respectively, and incubated for 60 min at 37 °C with rotation. Serial dilutions of the samples were spread on LB plates and counted after overnight growth at 37 °C.

Serum Killing. Strains were grown in LB harvested during early logarithmic phase (OD₅₆₀ = 0.4) and adjusted in physiological saline to ~2 × 10⁸ bacteria per mL (dilution ~1:100). Each strain was incubated in pooled human serum (final concentration of the sera: 50%) for 180 min at 37 °C with rotation. Serial dilutions of the different samples were spread onto LB plates and cfu counted after overnight growth at 37 °C.

Cytotoxicity Experiments. RAW264.7 cells (ATCC TIB-71) were grown in DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS. Cells were maintained at 37 °C and 5% CO₂. Host cells were seeded in 24-well plates at a density of 2 × 10⁵ cells per well in

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drug-free media. The following day the cells were washed two times with PBS and infected with *P. aeruginosa* strains that were grown in LB until early logarithmic phase and washed with DPBS. Multiplicity of infection of 20 or 100 were used. Cytotoxicity was assessed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Uninfected cells, as well as cells infected with the PA14 ΔexoU strain, were used as negative controls. A positive control (i.e., 100% death) were noninfected macrophages lysed with Lysis Solution. Experiments were performed in quadruplicate and repeated twice. Representative experimental results are shown as means ± SD.

**RNA Extraction and RNA-Seq of *P. aeruginosa* PA14 WT and PA14_Tn oprD::Spleen.** RNA was extracted from three independent replicates of WT *P. aeruginosa* PA14, and the Tn-oprD::Spleen strain grown to early log phase in LB. The Ribopure-Bacterial Kit (Invitrogen) was used for RNA purification per manufacturer recommendations. DNaseI treatment was performed to remove DNA traces. The quality of RNA was analyzed using NanoDrop 1000 (Thermo Scientific). An RNA-seq library was prepared using Encore Complete Prokaryotic RNA-Seq DR Multiplex Systems kit (NuGEN) according to manufacturer instructions. cDNA fragmentation was done using Qsonica Sonicator Q800R. Sequencing was performed using Illumina HiSeq2000 platform in the Biopolymers core facility at Harvard Medical School. CLC Genomics Workbench software was used to map RNA reads to PA14 genomic DNA.

**Circos Plot Figures.** Circos figures were drawn following the instructions provided at www.circos.ca.

**Ethics Statement.** All animal studies conducted in this research were approved by the Harvard Medical Area Institutional Animal Care and Use Committee under protocol number 02791.

Fig. S2. (A) PFGE of clinical strains of *P. aeruginosa*. (B) *oprD* sequences from two pairs of isogenic clinical strains. The carbapenem-resistant strain 48-2 has acquired the insertion sequence element ISPa1328 (green highlighted portion) and the carbapenem-resistant strain 51-2 has a 12-bp deletion and a single nucleotide change (yellow highlighted portions) in the *oprD* gene. M, molecular mass standard.

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Fig. S3. Analysis of the OMP expression. OprD protein (arrow) is seen in the outer membrane extracted from WT *P. aeruginosa* PA14 in the clinical strains 48-1 and 51-1 and in the complemented (*PoprD*) strains. No difference in expression in the other OMPs from related strains was seen.
**Fig. S4.** Swarming of *P. aeruginosa* strain with intact oprD (strains PA14 WT and clinical strains 48-1 and 51-1), mutated oprD (strains PA14_Tn-oprD:6-4, 8-1, and clinical strains 48-2 and 51-2), or complemented in trans with the oprD gene (PorpD).

**Fig. S5.** Twitching motility of *P. aeruginosa* strains with intact oprD (strains PA14 WT and clinical strains 48-1 and 51-1), mutated oprD (strains PA14_Tn-oprD:6-4, 8-1, and clinical strains 48-2 and 51-2), or complemented in trans with the oprD gene (PorpD).
Fig. S6. Relative competitive fitness of *P. aeruginosa* PA14 WT strain versus antibiotic-marked PA14 strain for GI tract colonization. Error bars represent the SD. *strept R*, streptomycin resistance; *tet R*, tetracycline resistance.

Fig. S7. Lack of a role for the empty vector in the fitness for the colonization of the GI tract of the mice. Competition in cecal colonization between WT *P. aeruginosa* PA14, an oprD Tn-insert recovered from the spleen (PA14_Tn-oprD::Spleen), this oprD Tn-insert complemented by an empty vector [PA14_Tn-oprD::Spleen (EV)], and this oprD Tn insert complemented with oprD gene [PA14_Tn-oprD::Spleen (P oprD)].

Fig. S8. Level of *P. aeruginosa* present in the ceca of mice singly colonized for 5 d by different strains of *P. aeruginosa* PA14: PA14 WT, PA14 tet R, PA14 streptomycin resistance (strept R), and PA14_Tn-oprD::Spleen.
Table S1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli Sm10·pir</td>
<td>thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA integrated RP4-2 Tc::Mu Kmr λpir</td>
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<tr>
<td>PA14</td>
<td>Wild-type strain</td>
<td>Ref. 2</td>
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<tr>
<td>PA14 oprD:·Tn_6.4</td>
<td>Gent^{a}; oprD:·MrT7 at base 318</td>
<td>Ref. 3</td>
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<tr>
<td>PA14 oprD:·Tn_6.4 poprD</td>
<td>Tet^{b}; PA14 oprD:·Tn_6.4 attB::CTX1- oprD</td>
<td>This study</td>
</tr>
<tr>
<td>PA14 oprD:·Tn_8.1</td>
<td>Gent; oprD::MrT7 at base 1144</td>
<td>Ref. 3</td>
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<td>This study</td>
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<td>PA14 oprD:·Tn_spleen</td>
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<td>This study</td>
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<td>PA14 oprD:·Tn_spleen poprD</td>
<td>Tet^{b}; PA14 oprD:·Tn_spleen attB::CTX1- oprD</td>
<td>This study</td>
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<tr>
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<td>This study</td>
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<td>48.1 Imi^{b}; clinical isolate</td>
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<tr>
<td>48.2 Imi^{b}; mutant derived from the strain 48.1, with the ΔISPa1328 inserted at bp 51 relative to the start site of translation</td>
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<td>48.2 poprD</td>
<td>Tet^{b}; 48.2 attB::CTX1- oprD</td>
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<td>51.1 Imi^{b}; clinical isolate</td>
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<td>This study</td>
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<td>51.2 Imi^{b}-mutant derived from the strain 51.1, with a 12 bp deletion (bases 579–590) and a point mutation at bp 1017 relative to the start site of translation resulting in a stop codon</td>
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<td>51.2 poprD</td>
<td>Tet^{b}; 48.2 attB::CTX1- oprD</td>
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<td>PA14 derivative with an in-frame markerless deletion of oprD</td>
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<td>Gent; pilE::MrT7 at base 46</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pSAM_Bt</td>
<td>Amp^{a}; Ery^{a}; suicide delivery vector with mariner transposase himar1c9, Illumina bridge PCR priming sites (P7), bla, ermG, rp4 oriT/oriEsk</td>
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<td>pSAM_DGm</td>
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<tr>
<td>pEXG2-oprDdel</td>
<td>Gent^{a}; 1.05 kb fragment composed of sequences flanking 5′ and 3′ ends of oprD, cloned into pEXG2 as a HindIII–XbaI fragment</td>
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<td>CTX1-oprD</td>
<td>Tet^{b}; oprD from PA14 cloned into mini-CTX1 at EcoRI–HindIII sites</td>
<td>This study</td>
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</tbody>
</table>

Amp^{a}, ampicillin resistant; Ery^{a}, erythromycin resistant; Gent^{a}, gentamicin resistant; Imi^{a}, imipenem resistant; Imi^{b}, imipenem susceptible; Tet^{a}, tetracycline resistant.

3. Liberati NT, et al. (2006) An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proc Natl Acad Sci USA 103(8):2833–2838.
Table S2. Primers

<table>
<thead>
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<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Position*</th>
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<tr>
<td>oprD_F</td>
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<td>oprD_R</td>
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<td>oprD-int-F</td>
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<td>oprD-int-R</td>
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<td><strong>Deletion of oprD</strong></td>
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<tr>
<td>oprD_del-F1-HindIII</td>
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<td>oprD_del-R2-XbaI</td>
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<td><strong>Cis complementation of oprD</strong></td>
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</table>

Restriction sites are underlined. F, forward; R, reverse.

*Position according to the first nucleotide of the oprD coding sequence.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)
Dataset S5 (XLSX)