Involvement of two related porins, OprD and OpdP, in the uptake of arginine by *Pseudomonas aeruginosa*

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Abstract

The OprD family of specific porins in *Pseudomonas aeruginosa* comprises 19 members, some of which have been demonstrated to facilitate the uptake of specific compounds into the cell. The members of this family share considerable amino acid sequence similarity (46–57%), which is unusual among porin molecules. In this work, we sought to establish whether this sequence conservation was the basis for other shared aspects of this family. The transcriptional profiles of eight relatively well-characterized OprD homologs were assessed in cells grown on a variety of carbon compounds. The expression of these paralogous proteins correlated with their phylogenetic distribution into two subfamilies in that the three members of the OpdK subfamily were induced by their specific (organic acid) substrates while the five members of the amino-acid/peptide-specific OprD subfamily appeared to be constitutively expressed. Functional overlap with respect to arginine transport was observed between two members of the latter subfamily, the basic amino acid-specific porin, OprD, and the glycine-glutamate-specific porin, OpdP. The impact of this apparent functional redundancy on the genetic fitness of *P. aeruginosa* is discussed.

Introduction

Pseudomonas aeruginosa is a ubiquitous organism capable of living in a wide variety of terrestrial and aquatic environments. In addition, this organism is capable of infecting a range of animal and plant hosts, and in humans is most prevalently associated with lung infections in cystic fibrosis (CF) patients. This versatility is partially attributable to the large metabolic potential of *P. aeruginosa*. Roughly, 10% of the *P. aeruginosa* genome encodes transporter genes; another 8% is devoted to genes responsible for the metabolism of various carbon compounds, amino acids, fatty acids, phospholipids, and nucleotides (Stover *et al.*, 2000).

One of the more striking features of the *P. aeruginosa* genome is the presence of a large number of distinct gene families. Approximately 40% of the genes in this organism have at least one paralog; almost double the number that would be expected based on the size of the genome alone (Stover *et al.*, 2000). The large number of gene families in *P. aeruginosa*, is consistent with the likelihood that this organism preferentially used gene duplications to acquire novel cellular functions. Once duplicated, one gene copy would be proposed to retain the original function, whereas the other copy would, through a series of mutations, diverge with respect to function and/or regulation to create a paralog. Such divergence provides the cell with a new capability, allowing it to adapt and thrive in changing environments (Ohno, 1970). However, it is likely that the gene copies will continue to share some common features, enabling them to complement each other in the event of a gene loss or mutation, contributing to the overall fitness of the organism. This functional compensation has been studied most extensively in the yeast *Saccharomyces cerevisiae*, where it has been shown that the deletion of genes that have at least one paralog in the genome is only lethal in 12.9% of cases, whereas the proportion of lethal deletions for genes that do not have copies on the genome is 29% (Gu *et al.*, 2003).

In *P. aeruginosa*, the average gene family contains 2.7 members. Of particular note, then, are three large families of outer membrane proteins (Hancock & Brinkman, 2002). The TonB-dependent family of gated porins has 32 members, the OprM family of efflux channel-tunnels has 18 members, and the OprD family of specific porins consists of 19 members. The large number of paralogous proteins in the outer membrane underscores their important contribution to cellular physiology, particularly when considering the intrinsic impermeability of this structure.

The outer membrane of *P. aeruginosa* is a formidable barrier to antibiotics having a permeability coefficient of 1–8% of that of *Escherichia coli* (Hancock, 1997). The basis of this impermeability lies in the highly anionic nature of the lipopolysaccharide molecules comprising the outer surface of the outer membrane (Nikaido & Hancock, 1986) and the poor channel forming activity of the major porin OprF (Hancock, 1985). The majority of the small, hydrophilic molecules that enter the cell do so through specific porins. These channels are generally narrow but possess substrate-specific binding sites that selectively facilitate the uptake of structurally related classes of molecules (Hancock & Brinkman, 2002). However, the nonspecific diffusion of structurally diverse molecules may be permitted, provided that they are small enough to traverse the pore. In *Pseudomonas*, this general uptake activity has been observed with the basic amino acid-specific porin, OprD and with the glucose specific channel OprB, which respectively permit the passage of gluconate and a variety of carbohydrates (Huang & Hancock, 1993; Adewoye *et al.*, 1998).

The *P. aeruginosa* outer membrane is rich in specific porins including the 19 members of the OprD family. It has been proposed that the members of this family arose from an ancestral gene, through a series of duplication and divergence events, to permit facilitated uptake of a variety of unique substrates (Stover *et al.*, 2000). Indeed, this has been shown for eight of the 19 OprD homologs (Tamber *et al.*, 2006). However, in most cases single mutations in each of these proteins had only a modest influence on growth on the respective specific substrates. This indicates the potential for functional overlap whereby more than one OprD homolog has the ability to mediate permeation of a particular substrate across the outer membrane. This was investigated here with respect to the diffusion of arginine through the outer membrane.

Arginine is believed to traverse the outer membrane through OprD. This has been shown through the use of the liposome swelling assay, planar bilayer experiments, and competition experiments wherein basic amino acids compete with the structurally related carbapenems, imipenem and meropenem for the OprD-binding site thus raising the MIC of those antibiotics towards *P. aeruginosa* (Trias & Nikaido, 1990a, b; Huang & Hancock, 1993). In addition, OprD is positively regulated in response to arginine via the ArgR regulator (Ochs *et al.*, 1999a). However, despite these observations, mutants lacking OprD are not compromised whilst growing on arginine (Tamber *et al.*, 2006). It is demonstrated here, that the unhindered growth phenotype of the *oprD* mutants is due, in part, to the overlapping activity of the glycine-glutamate-specific porin, OpdP, implying that this porin may play a greater role in outer membrane permeability than previously thought.

Table 1. Bacterial strains and plasmids used in this study

	Description	References
Strain		
PAO1	Wild-type strain	Tamber *et al.* (2006)
PAK	Wild-type strain	Tamber *et al.* (2006)
opdP	PAK *opdP*::miniTn*5*-Tc^r	Tamber *et al.* (2006)
oprD	PAK *oprD*::*xylE*-Gm^r	This work
oprD/opdP	PAK *opdP*::miniTn*5*-Tc^r *oprD*::*xylE*-Gm^r	This work
Plasmid		
pC831	pEX100T/*oprD*::*xylE*-Gm^r	Chuanchuen *et al.* (2002)

Materials and methods

Bacterial strains and growth conditions

The *Pseudomonas aeruginosa* strains used in this study are listed in Table 1. Strains were maintained on Luria–Bertani (LB) agar plates. Antibiotics were provided at the following concentrations for plasmid selection and maintenance: tetracycline – 50 μg mL^{-1}, gentamicin – 50 μg mL^{-1}. Strains were grown on either Mueller–Hinton (MH) or BM2 liquid media [62 mM potassium phosphate buffer (pH 7), 0.5 mM $MgSO_4$, 20 μM $FeSO_4$] containing the specified carbon sources. The carbon source concentrations used were as follows: growth curves and quantitative PCR – 1 mM, all other manipulations – 10 mM. Growth curves were carried out as described by Tamber *et al.* (2006).

Primers and reagents

A list of primers used is available on request from the authors. All chemicals were obtained from either Sigma or Fisher with the exception of glycine-glutamate and ^{14}C-arginine, which were purchased from Bachem (Torrance, CA) and American Radiolabelled Chemicals Inc. (St Louis, MO), respectively. Imipenem was obtained from Merck-Sharp-Dohme (West Point, PA) and meropenem obtained from ICI Pharmaceuticals (MacClesfield, England).

Molecular biology reagents were all purchased from Invitrogen (Carlsbad, CA), with the exception of the SYBR-Green Master Mix which was purchased from Applied Biosystems (Foster City, CA).

Construction of mutants

The plasmid, pC831 (Ochs *et al.*, 1999b), was used to mobilize a copy of the *oprD*::*xylE*-Gm^r cassette, into *P. aeruginosa* parental strain PAK (to create an isogenic *oprD* mutant) or mutant strain PAK *opdP* (to create the double mutant). The cells were made competent through repeated washes with $MgCl_2$ and $CaCl_2$, and transformed by heat shock according to the protocol of Chuanchuen *et al.*

(2002). The resulting transformants were plated onto LB agar containing the appropriate antibiotics.

As pC831 harbors a copy of the counter-selectable gene *sacB*, replacement of the native *oprD* gene with the interrupted copy was selected for by plating single colonies from the transformation onto LB agar plates containing the appropriate antibiotics plus 5% sucrose. The presence of the interrupted copy of *oprD* was verified by adding a solution of 10 mM catechol to a portion of the plate and observing a yellow color, which is indicative of the 2-hydroxymuconic semi-aldehyde produced by the XylE enzyme catechol 2,3-dioxygenase. The insertions in *oprD* and *opdP* were further verified by the amplifying each gene by PCR and confirming that the resulting products were of the expected size by agarose gel electrophoresis.

Quantitative and semi-quantitative PCR

RNA extraction from mid-logarithmic phase cultures, reverse transcription into cDNA and semi-quantitative PCR were carried out as described previously (Tamber *et al.*, 2006). The amplification reactions were cycled for 25 rounds with the exception of the control *rpsL* gene, which was cycled 20 times and the *opdB* and *opdT* genes which were amplified for 30 cycles.

Quantitative real-time PCR was carried out in an ABI Prism 7000 sequence detection system (Applied Biosystems) in 1 × SYBR-Green Master Mix using 200 nM of each forward and reverse primer. All reactions were normalized to the *rpsL* gene which encodes the S12 protein of the 30S ribosome.

Radioactive arginine uptake assays

Exponentially growing cultures (with an absorbance at 600 nm of 0.4–0.6) of PAK, and its isogenic *opdP*, *oprD*, and *opdP*/*oprD* mutants grown in BM2 media containing 10 mM glucose were harvested and washed twice with fresh medium. Cells were resuspended in prewarmed fresh medium to a final absorbance of 1.0 and allowed to equilibrate for 20 min in a 37 °C water bath that was shaking at 250 r.p.m. After this incubation period 100 μM total arginine (1 : 100 ^{14}C-arginine, 300 mCi mmoL^{-1} : unlabelled arginine) was added to the cells. Aliquots of 50 μL were removed at the specified time points and placed in 1 mL ice cold 1 M LiCl. These aliquots were immediately filtered through Whatman GF-C filters and washed with 5 mL LiCl. The filters were then placed in scintillation vials containing 5 mL ReadySafe scintillation fluid (Beckman, Fullerton, CA) and counted using a Beckman LS 500TA liquid scintillation counter.

Before the addition of arginine, 1 mL of cells were removed, pelleted and stored at − 20 °C. The pellets were resuspended in 0.5 mL 1% sodium dodecyl sulfate followed by a 30 min incubation at 37 °C. The protein content of the cell lysates was then determined by a modified Lowry procedure (Sandermann & Strominger, 1972).

Results and discussion

Expression profiles of eight OprD homologs

Members of the OprD porin family facilitate the diffusion of specific classes of compounds into the cell. To date, substrates specific to eight of the 19 porins have been assigned and relative to their expression levels on glucose, were induced to a higher copy number upon exposure to their respective substrates (Hancock & Brinkman, 2002; Tamber *et al.*, 2006). Despite this divergence, the members of this family share a considerable amount of primary sequence similarity (46–57%), indicating that they may exhibit some functional redundancy by permitting low levels of nonspecific diffusion.

To determine whether any of the eight characterized members of the OprD family were available to take up molecules nonspecifically, their expression patterns on a variety of carbon sources were assessed by semi-quantitative PCR using primer pairs specific for the eight OprD homolog genes and cDNA generated from wild-type *P. aeruginosa* PAO grown on either glucose, *cis*-aconitate, arginine, glycine-glutamate, pyroglutamate, tyrosine, vanillate, proline or histidine (Fig. 1).

The *oprD*, *opdP*, *opdC*, *opdB*, and *opdT* porin genes were transcribed on all nine carbon sources tested. In contrast, the *opdK* and *opdO* genes were only transcribed on their respective substrates, vanillate, and pyroglutamate. OpdH was expressed on the presence of two compounds, *cis*-aconitate and vanillate, with its expression level being approximately threefold higher on *cis*-aconitate.

Although the expression profiles of the 11 remaining members of this family remain to be determined, the data presented here, when examined in the context of the OprD family phylogenetic tree (Fig. 1a), revealed an interesting pattern. Members of the OpdK subfamily that promote the utilization of organic acids had a narrow expression profile. The three porins tested were expressed most highly on their respective substrates. Based on this profile, it is unlikely that these channels can complement a defect in another related porin, with the possible exception of OpdH that was transcribed on vanillate.

In contrast, the OprD subfamily members, which assist in growth on various amino acids or peptides, were expressed on all of the carbon sources tested. The regulation of this group of porins appears to be complex. As described previously, these channels are induced by their respective substrates (Fig. 1, compare grey bars to lane G); however, some were also induced by a number of other structurally

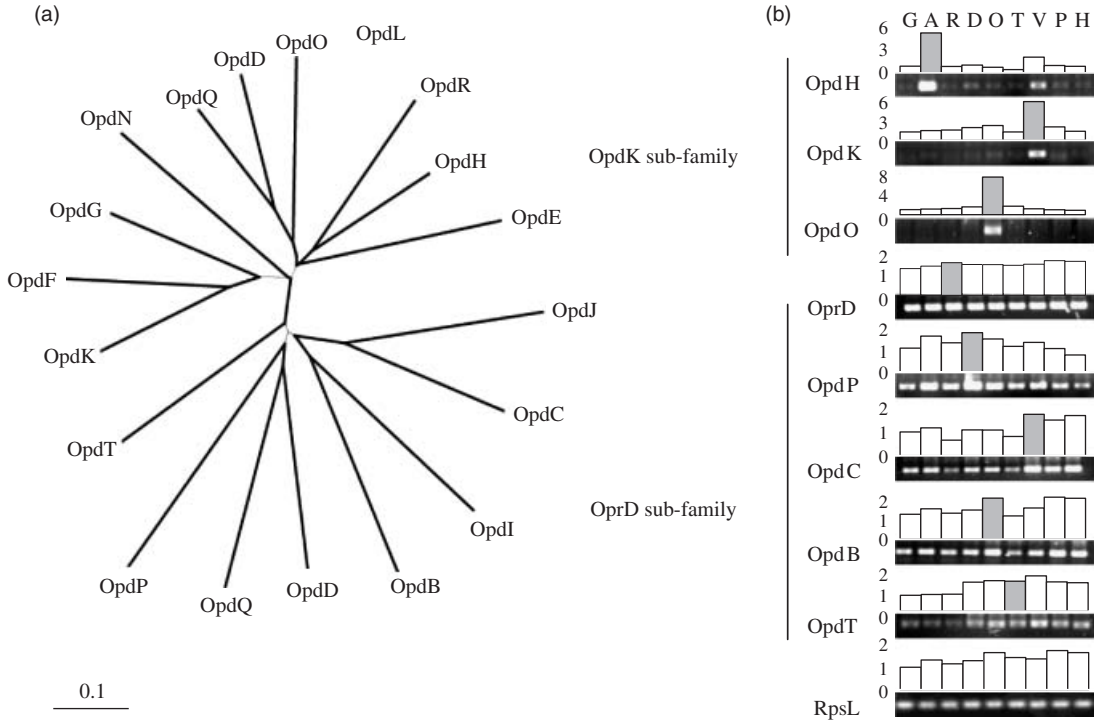

Fig. 1. Expression profile of eight OprD homologs. (a) Unrooted phylogenetic tree of the 19 members of the OprD family in *Pseudomonas aeruginosa*. The tree was constructed using the neighbour-joining distance matrix method in ClustalX (Thompson *et al*., 1997) as described in Tamber *et al* (2006). Bootstrap values were all over 800 (of 1000 trials) with the exception of the branches indicated by thinner lines. (b) Semi-quantitative PCR analysis of OprD homolog transcription on various carbon sources. mRNA was isolated from mid-logarithmic phase *P. aeruginosa* PAO1 cells grown on BM2+10 mM carbon source, reverse transcribed and used as the template for PCR. Lane designations are as follows: G, glucose; A, *cis*-aconitate; R, arginine; D, the dipeptide glycine-glutamate; O, pyroglutamate; T, tyrosine; V, vanillate; P, proline; H, histidine. The bar graphs above the gel images depict the gene expression values standardized to those of the *rpsL* control gene on each substrate. Grey bars correspond to the specific substrate of each porin. Data shown are representative of two separate experiments.

unrelated compounds (Tamber *et al*., 2006). This observation is consistent with a previous report demonstrating the nonspecific induction of OprD by a variety of amino acids (Ochs *et al*., 1999a). Based on the regulatory and sequence similarities shared among the OprD subfamily members, it seemed possible that they, like OprD, exhibit some general uptake capabilities (Huang & Hancock, 1993).

Arginine-mediated induction of OpdP

Of the four OprD homologs that were expressed on arginine, the level of *opdP* transcription was the highest (Fig. 1, lane R) and it shared the highest degree of sequence similarity with OprD (51%). Therefore, this porin was predicted to share some functional overlap with OprD and act as a conduit for the diffusion of arginine.

Transcriptional profiling of *opdP* indicated that compared with its levels on glucose, the porin was induced twofold in the wild-type strain when grown on arginine (Fig. 2). This pattern was conserved in an *oprD*-deficient mutant; however, the extent of *opdP* induction on arginine was doubled to levels that were at least fourfold higher than on glucose. Although the physiological significance of overexpressing a protein in response to the loss of a related one is apparent, the mechanism by which this could occur is not clear.

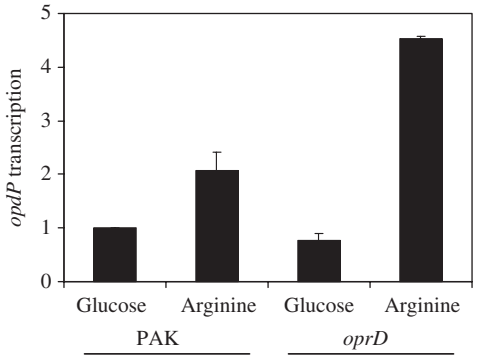

Fig. 2. Transcription of *opdP* in wild-type *Pseudomonas aeruginosa* and an *oprD*-deficient mutant. mRNA was isolated from mid-exponential phase cells grown on BM2+1 mM glucose or arginine, and reverse transcribed into cDNA, which was then used as the template for PCR. The levels of *opdP* transcription shown are relative to the value obtained from strain PAK grown in BM2+glucose. Data are from two separate experiments with at least four replicates.

However, this is not an unusual phenomenon as the compensatory expression of other outer membrane proteins has been reported in both *Escherichia coli* and *Pseudomonas aeruginosa* (Bavoil *et al.*, 1977; Li *et al.*, 2000; Rebiere-Huet *et al.*, 2002).

Arginine-mediated induction of the *oprD* gene is carried out via the ArgR regulator. To determine whether this protein might be directly involved in the regulation of *opdP*, the promoter region of the porin gene was examined for the ArgR consensus-binding site (Lu *et al.*, 2004). However, no significant similarities were found implying another mechanism may be responsible for the observed induction of OpdP.

Involvement of OpdP in the compensatory uptake of arginine

Various groups have shown that the OprD channel has a specific binding site for basic amino acids and thus facilitates their diffusion through the outer membrane (Trias & Nikaido, 1990a; Huang & Hancock, 1993). Despite these data, an OprD-deficient mutant grows as well as its isogenic wild-type strain on limiting arginine concentrations as low as 1 mM (Tamber *et al.*, 2006). Given the presence of OpdP in the outer membrane of the *oprD*-deficient strain, it seemed likely that this channel could participate in the uptake of arginine. To test this possibility, an *oprD*/*opdP* double mutant was constructed and its growth on BM2+1 mM arginine was compared with the wild-type PAK strain, the *oprD*$^{-}$ mutant and the *opdP*$^{-}$ mutant (Fig. 3a). On this medium, after an initial lag phase of approximately 3 h, the wild-type strain and the two single mutants grew at approximately the same rate. The *oprD*/*opdP* double mutant however exhibited a modest growth defect that was specific to arginine as all four strains grew equally well on BM2+glucose (data not shown).

To investigate the cause of the double mutant's growth defect, the rate of radioactive arginine uptake by the four strains was determined (Fig. 3b). Over a period of 5 min, the wild-type strain, the *opdP* mutant, and the *oprD* mutant transported arginine to similar extents and at similar rates (362, 439, and 319 nmol μg min^{-1}, respectively). In contrast, the double mutant had a lower uptake rate (201 nmol μg min^{-1}) implying that both channels are involved in arginine transport.

The observed redundancy of the two channels was limited to the uptake of arginine as the susceptibility of the *opdP* mutant to the basic amino acid analogs, imipenem and meropenem, was similar to that of the wild-type strain (0.5 and 0.125 μg mL^{-1}, respectively). In contrast, the *oprD*-deficient mutant as well as the *oprD*/*opdP* double mutant were eightfold more resistant to these antimicrobial agents. Given the size and charge of arginine (MW = 174) compared

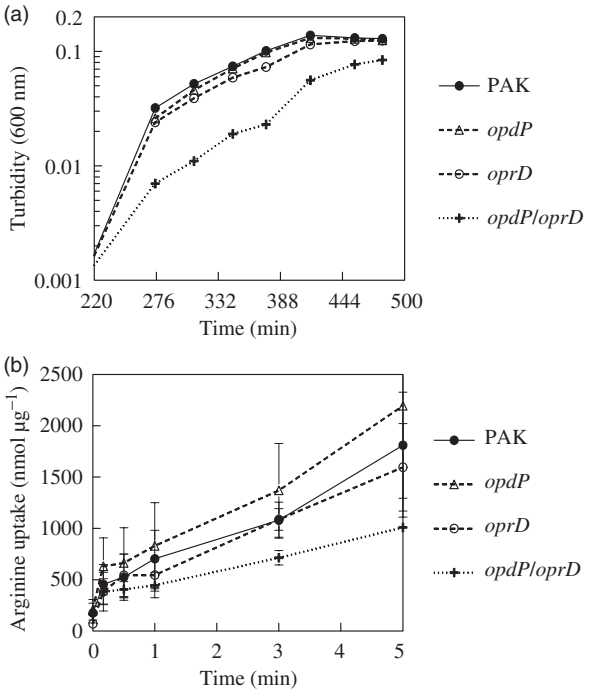

Fig. 3. Uptake of arginine by an *oprD*/*opdP*-deficient mutant. The wild-type *Pseudomonas aeruginosa*, an *oprD*-deficient mutant, an *opdP*-deficient mutant and an *oprD*/*opdP* double mutant were assessed for their ability to grow on BM2+1 mM arginine (a) and to take up radiolabelled arginine (b). Growth data shown are representative of three separate experiments, $n = 3$ for the transport assay.

with that of glycine-glutamate (MW = 204), it is likely that the passage of this amino acid through OpdP occurred by simple, nonspecific diffusion. Imipenem (MW = 317) and meropenem (MW = 438) are considerably larger than both arginine and glycine-glutamate and thus may not be able to traverse OpdP as it presumably lacks the specific-binding sites required to facilitate their uptake (Trias & Nikaido, 1990b).

The observation that the growth on or transport of arginine was not completely abrogated in the *oprD*/*opdP* double mutant indicates that other OprD homologs, particularly OprQ and the remaining five members of the OprD subfamily, may have been contributing to arginine uptake. Thus, with respect to this group of porins at least, transport through the outer membrane may be a collaborative effort with each protein contributing to uptake in small increments, providing that these porins exhibit overlapping functionality. This situation is analogous to the one observed with the efflux pumps of Gram-negative bacteria, wherein multiple homologous systems, which demonstrate a broad, often overlapping specificity for antimicrobial agents, organic solvents, and other noxious agents, are present. Mutations in multiple efflux systems are often required to completely inactivate the efflux of a specific

molecule (Rojas *et al.*, 2001; Poole, 2004). Indeed, this redundancy may be a common theme with *P. aeruginosa* outer membrane proteins as many examples of overlapping function have also been observed among the 32 members of its TonB-dependent gated porin family (Dean & Poole, 1993; Ochsner *et al.*, 2000; Ghysels *et al.*, 2004, 2005; Llamas *et al.*, 2006).

Given the selective pressures exerted on the outer membrane, it is not surprising that *Pseudomonas* sp. and other closely related bacteria have retained multiple gene copies with overlapping functions. Many outer membrane proteins can serve as phage receptors, are recognized by bacteriocins and/or the immune system, or can be used as portals for the transport of toxic agents. Thus, there are considerable advantages to altering a surface exposed protein or removing it completely. For *P. aeruginosa*, this strategy is particularly relevant in the CF lung where following prolonged treatment with carbapenem antibiotics, mutants in OprD are frequently isolated (Quinn *et al.*, 1986; Pirnay *et al.*, 2002). These strains are invariably resistant to imipenem and/or meropenem. However, the presence of 18 related proteins in the outer membrane ensures that the uptake of nutrients is not hindered, allowing *P. aeruginosa* to thrive despite the loss of an important transport protein.

The data presented here are also consistent with a model whereby most small substrates (including ones for which no specific channel is available) pass through the relatively weakly expressed channels of OprD family members rather than the highly expressed general (nonspecific) porins of *P. aeruginosa*, such as OprF. This strategy then permits *Pseudomonas* to take up a broad range of growth substrates while maintaining high intrinsic antibiotic resistance owing to low outer membrane permeability.

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