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# Role of the Novel OprD Family of Porins in Nutrient Uptake in *Pseudomonas aeruginosa*

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**To circumvent the permeability barrier of its outer membrane, *Pseudomonas aeruginosa* has evolved a series of specific porins. These channels have binding sites for related classes of molecules that facilitate uptake under nutrient-limited conditions. Here, we report on the identification of a 19-member family of porins similar to the basic-amino-acid-specific porin OprD. The members of this family fell into one of two phylogenetically distinct clusters, one bearing high similarity to OprD and the other bearing most similarity to the putative phenylacetic acid uptake porin PhaK of *Pseudomonas putida*. Analysis of the genome context, operon arrangement, and regulation of the PhaK-like porin OpdK indicated that it might be involved in vanillate uptake. This result was confirmed by demonstrating that an *opdK* mutant had a deficiency in the ability to grow on vanillate as a carbon source. To extrapolate these data to other paralogues within this family, the substrate specificities of 6 of the 17 remaining OprD homologues were inferred using an approach similar to that used with *opdK*. The specificities determined were as follows: OpdP, glycine-glutamate; OpdC, histidine; OpdB, proline; OpdT, tyrosine; OpdH, *cis*-aconitate; and OpdO, pyroglutamate. Thus, members of the OprD subfamily took up amino acids and related molecules, and those characterized members most similar to PhaK were responsible for the uptake of a diverse array of organic acids. These results imply that there is a functional basis for the phylogenetic clustering of these proteins and provide a framework for studying OprD homologues in other organisms.**

*Pseudomonas aeruginosa* is an extremely versatile organism that grows in many diverse terrestrial, marine, and freshwater habitats. It is also capable of forming intimate associations with plants and animals. Consistent with this ecological diversity, *P. aeruginosa* can utilize a wide variety of compounds, such as carboxylates, small aromatic compounds, amino acids, and amino acid derivatives, as carbon sources (31).

*P. aeruginosa* is noted for having 12- to 100-fold-lower outer membrane permeability to various compounds than *Escherichia coli*, a feature that is central to its high intrinsic resistance to antimicrobials (7). While this decreased permeability permits the organism to resist attack by antibiotics produced by competitors in the environment, it creates potential problems when substrate uptake at low concentrations is considered.

In general, hydrophilic molecules traverse the outer membrane barrier via water-filled channels called porins. The two major classes of porins, general and specific, differ primarily with respect to substrate selectivity. Many gram-negative species, with the exception of the pseudomonads, possess general porins in high copy numbers that provide an aqueous environment for small, structurally unrelated, hydrophilic molecules to diffuse through. The kinetics of diffusion through general porins is driven largely by the physicochemical properties (i.e., size, shape, charge, and polarity) and concentration gradient of the molecule in question.

Specific porins, in contrast, possess stereo-specific substrate binding sites that serve to facilitate the diffusion of structurally related classes of molecules by orienting them into energetically favorable positions as they cross the narrowest regions of the channel (29, 40). This facilitated uptake is necessary with nutrient-deficient conditions when uptake through the outer membrane becomes rate limiting for growth and simple diffusion through general porins becomes inefficient. The binding sites of specific porins do not preclude the entry of structurally unrelated compounds, and thus, these channels also display some characteristics common to general porins (11). Specific porins are often induced to higher copy numbers by their substrates.

Unlike other gram-negative organisms, such as *Escherichia coli*, which possesses a large number of general porins and a few specific ones, *Pseudomonas* sp. and other closely related soil organisms are unique in that they almost exclusively use specific porins for uptake through the outer membrane. The major general porin of *P. aeruginosa*, OprF, for example, forms an inefficient uptake pathway in that the majority of the OprF channels are too small to allow sufficient passage of molecules (7, 8). The specific porins of *P. aeruginosa* characterized to date include the glucose-specific porin OprB (*o*uter membrane *pro*tein *B*), the phosphate- and polyphosphate-specific porins OprP and OprO, respectively, and the basic-amino-acid-specific porin OprD, which also takes up the structurally related antibiotics imipenem and meropenem (7, 35, 36).

In addition, OprD is the prototype of a large paralogous family of porins (32). There are 18 OprD homologues in *P. aeruginosa* whose primary amino acid sequences are 46 to 57% similar to that of OprD. Members of this family are also found

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TABLE 1. OprD homologues in *P. aeruginosa* and other bacteria

| Organism | No. of OprD homologues | % Similarity to OprD[a] |
|---|---|---|
| *P. aeruginosa* PAO1 | 19 | 46–59 |
| *P. putida* KT2440 | 21 | 44–63 |
| *Pseudomonas fluorescens* PfO-1 | 14 | 47–74 |
| *Pseudomonas syringae* pv. tomato strain DC3000 | 10 | 45–74 |
| *Azotobacter vinelandii* | 29 | 43–54 |
| *Acinetobacter* strain ADP1 | 4 | 40–50 |
| *Burkholderia cepacia* | 3 | 46–48 |
| *Burkholderia pseudomallei* | 1 | 49 |
| *Xanthomonas campestris* | 1 | 46 |
| *Bradyrhizobium japonicum* USDA 110 | 1 | 33 |
| *Shigella flexneri* | 1 | 36 |
| *Yersinia* species | 3 | 34 |
| *Escherichia coli* | 1 | 37 |
| *Salmonella* species | 1 | 38 |

[a] Percent similarity at the level of the primary amino acid sequence.

in other *Pseudomonas* species and closely related soil bacteria, such as *Acinetobacter* strain ADP1, as well as more divergent bacterial species, such as *Escherichia coli* (4), *Shigella flexneri* (13), and *Yersinia pestis* (24) (Table 1).

A few OprD homologues have been implicated in nutrient uptake based on their placement in operons containing genes for the metabolism and transport of certain growth substrates. For example, the gene encoding the putative vanillate uptake porin VanP, in *Acinetobacter*, lies in the middle of the vanillate degradation operon (16). The *benF* and *phaK* putative porin genes of *Pseudomonas putida* (17) lie in the middle of operons responsible for the degradation of benzoate and phenylacetic acid, respectively. In addition, a number of OprD homologues have been observed during the investigation of global gene expression patterns of *Pseudomonas* grown under specific conditions (2, 5, 15, 37, 38). In this study, we hypothesize that in *P. aeruginosa*, the OprD family members are expressed under specific conditions and are responsible for the uptake of a variety of metabolites. The substrate selectivities of 7 of the 18 novel OprD homologues are described and discussed in the context of the phylogenetic framework of this family.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, primers, and media.** All strains and plasmids used in this study are listed in Table 2. The sequences of the DNA primers used in this study are available from the authors. Strains were maintained on Luria-Bertani agar. Antibiotics for either selection or maintenance were supplied at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 300 μg/ml for *P. aeruginosa*; gentamicin, 10 μg/ml for *P. aeruginosa*; and tetracycline, 100 μg/ml for *P. aeruginosa*. All other manipulations were done with strains grown in BM2 minimal medium (62 mM potassium phosphate buffer [pH 7], 0.5 mM $MgSO_4$, 10 μM $FeSO_4$) supplemented with specific carbon sources. All chemicals used were obtained from either Sigma or Fisher with the exception of glycine-glutamate, which was obtained from Bachem.

**Genetic manipulations.** Routine molecular biology techniques were carried out according to standard protocols. Enzymes and cloning kits were provided by Invitrogen. An *opdK* transcriptional fusion was constructed by amplifying the gene from *P. aeruginosa* strain H103 using *Taq* polymerase in 1× reaction buffer containing 2 mM $MgCl_2$, 5% dimethyl sulfoxide, 0.4 mM deoxynucleoside triphosphates [dNTPs], and 1 μM of each primer. After an initial denaturation step at 94°C for 2 min, the amplification mixture was incubated for 25 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min, followed by a final extension step at 72°C for 5 min. The resulting amplicon was then cloned into pCR2.1 using the TA Cloning kit and transformed into chemically competent *E. coli* DH5α.

TABLE 2. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description[a] | Reference or source |
|---|---|---|
| Strains | | |
| *P. aeruginosa* | | |
| H103 | Wild-type strain | |
| H859 | H103 *opdK*::*xylE*-Gm$^r$ | This study |
| PAO1 | Wild-type strain | Pathogenesis Corp. |
| PAK | Wild-type strain | Pathogenesis Corp. |
| *oprD* strain | PAO *oprD*::mini-Tn*5*-Tc$^r$ (624/1,439) | Pathogenesis Corp. |
| *oprE* strain | PAK *oprE*::mini-Tn*5*-Tc$^r$ (650/1,382) | Pathogenesis Corp. |
| *oprQ* strain | PAK *oprQ*::mini-Tn*5*-Tc$^r$ (264/1,277) | Pathogenesis Corp. |
| *opdB* strain | PAO *opdB*::mini-Tn*5*-Tc$^r$ (135/1,307) | Pathogenesis Corp. |
| *opdC* strain | PAK *opdC*::mini-Tn*5*-Tc$^r$ (1013/1,334) | Pathogenesis Corp. |
| *opdD* strain | PAK *opdD*::mini-Tn*5*-Tc$^r$ (394/1,250) | Pathogenesis Corp. |
| *opdF* strain | PAK *opdF*::mini-Tn*5*-Tc$^r$ (126/1,265) | Pathogenesis Corp. |
| *opdG* strain | PAK *opdG*::mini-Tn*5*-Tc$^r$ (977/1,250) | Pathogenesis Corp. |
| *opdH* strain | PAK *opdH*::mini-Tn*5*-Tc$^r$ (181/1,283) | Pathogenesis Corp. |
| *opdI* strain | PAK *opdI*::mini-Tn*5*-Tc$^r$ (417/1,358) | Pathogenesis Corp. |
| *opdJ* strain | PAK *opdJ*::mini-Tn*5*-Tc$^r$ (617/1,418) | Pathogenesis Corp. |
| *opdK* strain | PAK *opdK*::mini-Tn*5*-Tc$^r$ (966/1,253) | Pathogenesis Corp. |
| *opdL* strain | PAK *opdL*::mini-Tn*5*-Tc$^r$ (553/1,256) | Pathogenesis Corp. |
| *opdN* strain | PAK *opdN*::mini-Tn*5*-Tc$^r$ (560/1,295) | Pathogenesis Corp. |
| *opdO* strain | PAK *opdO*::mini-Tn*5*-Tc$^r$ (822/1,229) | Pathogenesis Corp. |
| *opdP* strain | PAK *opdP*::mini-Tn*5*-Tc$^r$ (773/1,454) | Pathogenesis Corp. |
| *opdQ* strain | PAK *opdQ*::mini-Tn*5*-Tc$^r$ (224/1,265) | Pathogenesis Corp. |
| *opdR* strain | PAK *opdR*::mini-Tn*5*-Tc$^r$ (907/1,250) | Pathogenesis Corp. |
| *opdT* strain | PAK *opdT*::mini-Tn*5*-Tc$^r$ (466/1,346) | Pathogenesis Corp. |
| *E. coli* | | |
| DH5α | General cloning strain | 6 |
| S17-1 | Mobilizing strain | 23 |
| Plasmids | | |
| pCR2.1 | TA Cloning vector, Ap$^r$ Km$^r$ | Invitrogen |
| pEX100T | Suicide vector containing *sacB* gene, Ap$^r$ | 30 |
| pX1918GT | Source of *xylE*-Gm$^r$ cassette, Ap$^r$ Gm$^r$ | 30 |

[a] Numbers in parentheses indicate ratios of the insertion point of the transposon to the total number of base pairs within the open reading frame.

The *opdK* gene was excised from pCR2.1 using EcoRI, and blunt ends were created by using the large fragment of Klenow DNA polymerase. This fragment was cloned into the SmaI site of pEX100T. A 400-base-pair fragment of *opdK* was then excised by digestion with PstI, and a *xylE*-Gm$^r$ cassette from pX1918GT was cloned into this site. The orientation of the *xylE*-Gm$^r$ cassette was confirmed by restriction analysis. This plasmid was transformed into electrocompetent *E. coli* S17-1 and then mobilized into *P. aeruginosa* H103 by biparental mating, followed by successive selection on gentamicin and 5% sucrose. The replacement of native *opdK* with *opdK*::*xylE*-Gm$^r$ in the resulting strain was confirmed by Southern blotting.

**Sequence analysis.** Sequences of the *P. aeruginosa* OprD homologues and neighboring genes were obtained from the most-recent versions of the *Pseudomonas* genome database at www.pseudomonas.com. The corresponding amino acid sequences were aligned with all of the microbial genomes in the nonredundant database using the Basic Local Alignment Sequence Tool (1) hosted by the NCBI. Genes were considered homologous if they were at least 30% similar over the length of the entire protein.

SignalP was used to predict the signal peptides of the OprD homologues (3). The protein sequences without the signal peptides were multiply aligned using CLUSTAL_X (34) and manually edited using GeneDoc (http://www.psc.edu/biomed/genedoc). Phylogenetic trees were made in CLUSTAL_X and viewed with TreeView (22).

**Reporter gene assays.** The catechol-2,3-dioxygenase (XylE) activity of the *opdK* transcriptional fusion was assayed as described in reference 30. Briefly, cells from overnight cultures were collected by centrifugation, resuspended in 50 mM potassium phosphate buffer (pH 7.5) plus 10% acetone, and disrupted by sonication. Unbroken cells and other debris were removed by centrifugation. Aliquots of the cell lysates were added to 50 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM catechol, and the increase in absorbance at 375 nm was monitored. The change in absorbance was then used to calculate the amount of 2-hydroxymuconic semialdehyde produced using a molar extinction coefficient of 44,000 $M^{-1}$ $cm^{-1}$. The protein content of the cell extracts was determined by a modified Lowry method (28).

**Growth assays.** The *Pseudomonas* strains were grown overnight in BM2 media supplemented with the specific carbon sources as indicated in the text. The overnight cultures were subcultured to a starting optical density at 600 nm of 0.01 into prewarmed fresh BM2 media containing the specific carbon sources. These cultures were incubated in a 37°C water bath with an orbital shaker shaking at 200 rpm. To ensure that the cultures received sufficient aeration, the 10 ml of growth cultures was grown in 125-ml flasks. Aliquots of the growth cultures were taken at specified time intervals, and the turbidity at 600 nm was determined using a spectrophotometer. The number of CFU was determined by serially diluting the aliquots to $10^{-4}$ to $10^{-6}$ in Luria-Bertani broth and then plating onto Luria-Bertani agar plates.

**Semiquantitative (SQ)-PCR.** mRNA was isolated from exponential-phase *P. aeruginosa* PAO1 cells grown in BM2 medium plus 10 mM glucose or the specified carbon source using an RNeasy mini RNA isolation kit (QIAGEN). Contaminating genomic DNA was removed using a DNA-free kit (Ambion). The RNA was quantified by determining its absorbance at 260 nm. RNA quality was assessed by comparing its absorbance at 260 nm with its absorbance at 280 nm and by examining its appearance on a 2% agarose-Tris-acetate-EDTA (TAE) gel. RNA aliquots were stored at −80°C.

Reverse transcription was carried out by combining 4 μg of total RNA with 750 ng of the random decamer $(NS)_5$ and incubating the mixture at 70°C for 10 min, followed by a10-minute incubation at 25°C. A mixture containing 1× reaction buffer, 10 μM dithiothreitol, 0.5 μM dNTPs, 500 units/ml of SUPERase-In (Ambion), and 10,000 units/ml of SuperScript II reverse transcriptase (Invitrogen) was then added to the RNA and incubated for an hour at 37°C and 2 hours at 42°C. The RNA was destroyed by the addition of 170 mM NaOH and incubation at 65°C for 15 min. The mixture was neutralized by the addition of 170 mM HCl.

One microliter of the resulting cDNA was used as the template for SQ-PCR using primers designed for the internal regions of the OprD-homologous genes, as well as the control gene, *rpsL*. The utility of all primer pairs was checked by using genomic DNA template controls. Amplification reactions were carried out using *Taq* polymerase (Invitrogen) in 1× reaction buffer containing 2 mM $MgCl_2$, 5% dimethyl sulfoxide, 0.4 mM dNTPs, and 40 nM of forward and reverse primers. The reaction mixtures were cycled for 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. All reaction mixtures were amplified for 25 rounds, with the exception of *oprD* and *rpsL*, which were amplified for 20 cycles. The reaction mixtures using the *opdB* primers were cycled 35 times, and the *opdT* reaction mixture was cycled 40 times. The amplicons (ranging from 85 to 100 base pairs in length) were resolved by electrophoresis on 2% agarose-TAE gels stained with 50 μg/ml ethidium bromide. The intensities of the resulting bands on the agarose gels were quantified using ImageJ (http://rsb.info.nih.gov/ij/). All reported values were normalized to the levels of *rpsL*.

## RESULTS

**A novel family of specific porins in *P. aeruginosa*.** The genome of *P. aeruginosa* encodes 18 proteins that share considerable amino acid similarity (ranging from 46 to 57%) with the basic-amino-acid-specific porin OprD. Included in this family are the previously identified porins OprE (39), OprE3 (which has been renamed to OprQ) (21), and OprD3 (now known as OpdT) (32). The 15 hitherto-unknown OprD homologues were named with the prefix Opd (for *o*uter membrane *p*rotein *D* family). Phylogenetic analysis of this family (Fig. 1) showed that it is comprised of two distinct groups. The smaller group, the OprD subfamily, consisted of eight members in *P. aeruginosa*, including OprD, OprQ, OpdT, and five additional OprD homologues. The other group, the OpdK subfamily, contained the anaerobically induced porin OprE plus 10 uncharacterized OprD homologues. Members of the OpdK subfamily of porins demonstrated a higher degree of amino acid similarity to the phenylacetate uptake porin PhaK of *P. putida* than they did to OprD (57 to 69% similar to PhaK versus 47 to 51% similar to OprD).

Eleven of the 19 channels in *P. aeruginosa* have clear orthologues in *P. putida* and are likely to share the same function in the two organisms. Six proteins in *P. aeruginosa* (OpdJ, OpdI, OpdR, OpdO, OpdD, and OpdQ) did not have any orthologues in *P. putida* and thus might have arisen from a postspeciation duplication event and would be proposed to exhibit functions unique to *P. aeruginosa*.

A protein sequence alignment of the OprD homologues (available online at http://cmdr.ubc.ca/bobh/omps/alignment.htm) indicated that this family of porins might share similar secondary structures. When overlaid with the OprD amino acid sequence, the alignment shows that the highest degree of conservation lies in the regions that correspond with the 16 putative transmembrane β-strands and periplasmic turns of OprD (12, 19). The lowest degree of sequence conservation lies in the blocks corresponding to the eight surface loops of OprD. Thus, it is postulated that as with OprD (12, 19), these putative loop regions are involved in determining which substrates pass through these novel porins.

**Involvement of OprD in arginine uptake.** The sequence similarity of the OprD homologues to that of OprD suggested that the members of this family were specific porins. Mini-Tn*5*-Tc$^r$ insertion mutants in each of the OprD-homologous genes were obtained from Pathogenesis Corporation (now Chiron Inc.). To determine whether any of the channels were involved in antibiotic uptake, the mutants were first screened for resistance to all of the major classes of antibiotics, including β-lactams, carbapenems, cephalosporins, quinolones, macrolides, aminoglycosides, chloramphenicol, trimethoprim, nitrofurantoin, and rifampin. With the exception of the OprD mutant, for which the MICs to imipenem and meropenem increased eightfold, none of the other mutants demonstrated increased resistance to any antibiotic tested (data not shown).

The work of Trias and Nikaido demonstrated that OprD possesses a binding site specific for the basic amino acids arginine and lysine, dipeptides containing these residues, and structural analogues, such as carbapenem antibiotics (35, 36). When present as a mixture, the amino acids and imipenem compete for the binding site within OprD and raise the MIC to imipenem 16-fold, emphasizing the role of this porin in amino acid uptake (11).

The lack of a resistance phenotype of the remaining OprD homologue mutants precluded the use of competition assays to aid in the determination of substrate specificity. Therefore, an *oprD*-deficient mutant was used as the prototype to develop a growth assay with which to test the remaining mutant strains.

Specific porins are required to facilitate diffusion in nutrient-limited environments (18). Otherwise, the rates of diffusion through the outer membrane would not be sufficient to saturate the high-affinity cytoplasmic membrane transporters, making outer membrane permeability rate limiting for growth (10). The contribution of an individual specific porin is not seen in nutrient-sufficient environments, as other porins are present in the outer membrane to serve as nonspecific diffusion channels. Thus, to find the optimal substrate concentration at which the loss of a specific porin would compromise growth, the OprD mutant was grown in minimal medium supplemented with arginine in concentrations ranging from 10 mM to 0.1 mM. At higher substrate concentrations, (i.e., 1 mM or greater) (Table 3), no growth differences between the mutant and wild-type strain were observed. Although the growth yield was limited at the lower carbon source concentrations, we were able to reliably follow growth as the optical density measurements correlated

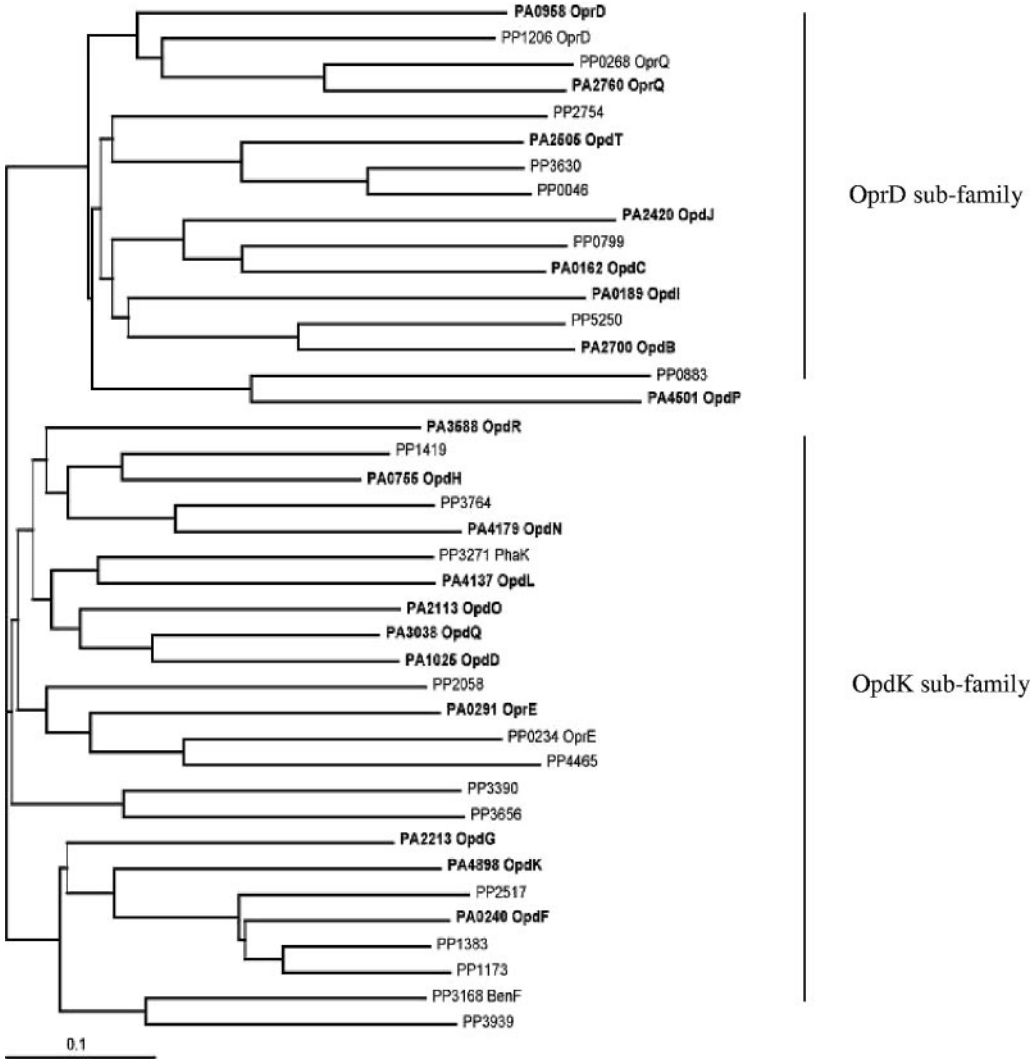



FIG. 1. Phylogenetic analysis of the OprD family in *P. aeruginosa* and *P. putida*. Phylogenetic trees were constructed using the neighbor-joining distance matrix method in CLUSTAL_X. All bootstrap values were over 700 (out of 1,000 trials), with the exception of the branches indicated by thinner lines. In *P. aeruginosa* (boldface type), OprD, OprE, OprQ, and OpdT (originally described as OprD3) (32) had previously been identified. The remaining homologues were given names beginning with Opd (for outer membrane protein, OprD-like). The *P. putida* OprD homologues were labeled according to their open reading frame (PP) numbers, including the previously named OprD, OprQ, OprE, BenF, and PhaK porins (indicated with lightface type).

with CFU/ml determinations ($R^2$ = 0.948; data not shown). Cultures grown in arginine concentrations below 1 mM quickly formed aggregates, obscuring quantitation by either spectroscopic or plate-counting methods. This aggregation was specific to arginine as it was not observed when glucose or numerous other carbon sources were used.

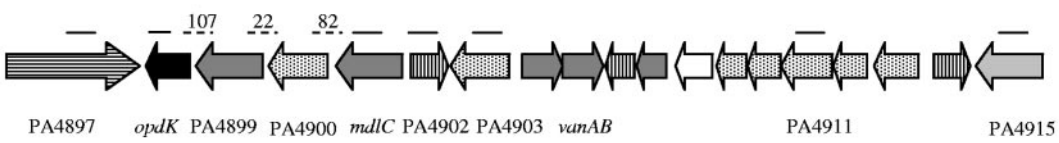
TABLE 3. Doubling times of *P. aeruginosa* strains (PAO, PAK, an *oprD* mutant, and an *opdK* mutant) grown in BM2 medium plus either arginine or vanillate[a]

| Carbon source concn (mM) | Doubling time in arginine (min) | | Doubling time in vanillate (min) | |
|---|---|---|---|---|
| | PAO | *oprD*::Tn*5* strain | PAK | *opdK*::Tn*5* strain |
| 0.5 | —[b] | — | 63 ± 1 | 122 ± 0.5 |
| 1 | 34 ± 5.0 | 40 ± 2.4 | 34 ± 1 | 49 ± 3 |
| 2 | 43 ± 0.6 | 41 ± 3.2 | 40 ± 1.5 | 36 ± 1.7 |

[a] Results shown are the means ± standard errors from two independent experiments.
[b] Cultures formed clumps and did not grow reproducibly at this concentration.

**Involvement of OpdK in vanillate uptake.** The phenotype of the *opdK*-deficient mutant was then investigated to determine whether there was a functional basis for the observed phylogenetic clustering of the OprD family. In bacteria, genes involved in the same metabolic pathway tend to be adjacent in the genome (14). Therefore, the substrate specificity of OpdK was investigated by first examining the functions of its neighboring genes. The *opdK* gene is located in the midst of genes putatively involved in the metabolism of vanillate and related compounds (Fig. 2A). The genes immediately downstream of *opdK* include one that is homologous to a *P. putida* aldehyde dehydrogenase gene, a putative major facilitator superfamily (MFS) transporter that is 57% similar to the BenK gene of *Acinetobacter* sp. strain ADP1, and a probable benzoyl formate decarboxylase gene (*mdlC*). Also found in this region are two

A




B

| Gene | Putative function |
|---|---|
| PA4897 | TonB-dependent family outer membrane protein |
| *opdK* | Porin |
| PA4899 | Aldehyde dehydrogenase |
| PA4900 | Major facilitator superfamily transporter |
| *mdlC* | Benzoylformate decarboxylase |
| PA4902 | Transcriptional regulator |
| PA4903 | Major facilitator superfamily transporter |
| *vanAB* | Vanillate O-demethylase oxidoreductase |
| PA4911 | Permease of ATP binding cassette transporter |
| PA4915 | Chemotaxis transducer |

C

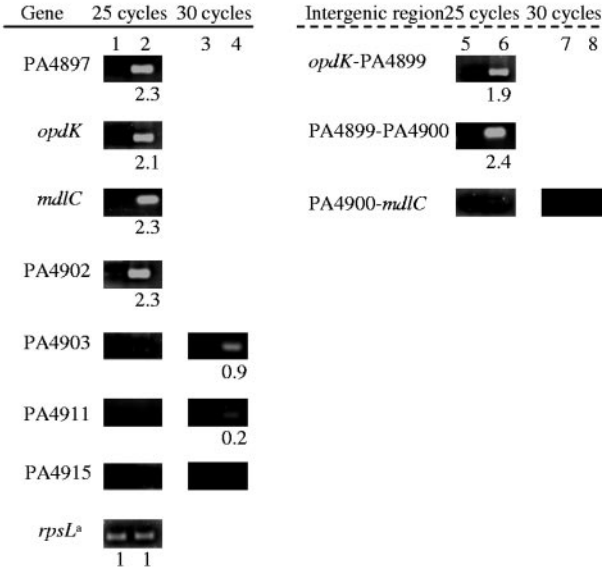



FIG. 2. (A) Genomic context of *opdK*. Lines above the genes indicate the locations of the intragenic (solid lines) and intergenic (dashed lines) regions that were amplified by SQ-PCR. Numbers above the dashed lines indicate the sizes, in base pairs, of the intergenic regions. Functional classes of the genes are indicated by the following colors or patterns: porin, black; TonB-dependent family member, horizontal stripes; cytoplasmic membrane transporters, stippled; transcriptional regulators, vertical stripes; enzymes involved in carbon catabolism, dark gray; chemotaxis transducer, light gray; and unknown function, white. (B) Probable gene functions of some of the open reading frames in the *opdK* gene cluster. (C) Transcription of regions within the *opdK* gene cluster in response to vanillate. mRNA was isolated from exponential-phase cells grown in BM2 medium plus 10 mM glucose (lanes 1, 3, 5, and 7) or BM2 medium plus 10 mM vanillate (lanes 2, 4, 6, and 8), reverse transcribed into cDNA, and used as the template for SQ-PCR. Numbers underneath the gels indicate levels of gene expression relative to *rpsL* (20 cycles); the absence of a number indicates that gene expression was negligible (i.e., <0.05).

genes highly similar to the *vanAB* genes, which encode the subunits of vanillate *o*-demethylase oxidoreductase from *Pseudomonas* species strain HR199 (25). Thus, the context of the *opdK* gene suggested that this porin may be involved in the uptake of small aromatic molecules.

As specific porins tend to be induced by their substrates, an *opdK* transcriptional fusion was grown in BM2 medium plus a 10 mM concentration of a carbon source (Table 4). Other than glucose and succinate, the carbon sources consisted of benzoate derivatives with one or two more polar substituents and for the most part are metabolized by *Pseudomonas* species through the β-ketoadipate pathway (9). The induction of *opdK* was quite specific and was expressed highly in both vanillate and its aldehyde derivative vanillin (Table 4). With the exception of *p*-hydroxybenzoate and anthranilate, the catechol-2,3-dioxygenase activity of the *opdK* transcriptional fusion grown in the remainder of the carbon sources was less than 10 pmol/mg/min protein and thus considered insignificant.

Primers flanking the intergenic regions of the putative four-gene *opdK* operon and primers to 100-base-pair regions within other adjacent genes were designed to permit the evaluation of mRNA levels in glucose and vanillate by SQ-PCR (Fig. 2C). In

TABLE 4. Catechol-2,3-dioxygenase activities of an *opdK*::*xylE*-Gm[r] transcriptional fusion grown in various aromatic compounds

| Carbon source | Mean catechol-2,3-dioxygenase activity (pmol/mg/min) ± SD[a] |
|---|---|
| Vanillate | 556 ± 37 |
| Vanillin | 387 ± 18 |
| *p*-Hydroxybenzoate | 20 ± 6 |
| Anthranilate | 15 ± 3 |
| Benzoate | 5.6 ± 2 |
| Glucose | 1.4 ± 0.6 |
| Succinate | 0.3 ± 0.1 |
| *p*-Hydroxybenzaldehyde | <0.01 |
| Phenylacetate | <0.01 |
| Hydroxyphenylacetate | <0.01 |
| Toluate (*o*- and *m*-) | <0.01 |
| Coumarate | <0.01 |
| Ferulate | <0.01 |
| 2,4-Dihydroxybenzoate | <0.01 |
| *p*-Aminobenzoate | <0.01 |
| Salicylate | <0.01 |
| Chlorobenzoate (*o*-, *m*-, and *p*-) | <0.01 |
| Tyrosine | <0.01 |
| Quinate | <0.01 |

[a] $n = 3$.

TABLE 5. Genomic contexts of *P. aeruginosa* OprD homologues

| Porin | Locus tag | Probable function(s) of adjacent genes/operon[a] | Predicted substrate(s) |
|---|---|---|---|
| OpdK subfamily | | | |
| OprE | PA0291 | Agmatine ureohydrolase | Arginine or proline |
| OpdD | PA1025 | Short-chain dehydrogenase and coenzyme A hydratases | Short-chain fatty acids or dicarboxylates |
| OpdF | PA0240 | Hexuronate transport | Hexuronates |
| OpdG | PA2213 | Phthalate transport | Phthalate |
| OpdH | PA0755 | Tricarboxylate transport | Tricarboxylates |
| OpdK | PA4898 | Vanillate metabolism and transport | Vanillate |
| OpdL | PA4137 | Unknown; PP3271, phenylacetate metabolism | Phenylacetate |
| OpdN | PA4179 | Unknown; PP3764, cobalamin synthesis | 5-Aminolevulinate or glutamate |
| OpdO | PA2113 | Lactam utilization | Lactam or other five-membered rings |
| OpdQ | PA3038 | Unknown; PP3656, nitrobenzoate reductase | Nitrobenzoate |
| OpdR | PA3588 | Phenylacetate metabolism | Phenylacetate |
| OprD subfamily | | | |
| OprD | PA0958 | Unknown | Basic amino acids |
| OprQ | PA2760 | Unknown | |
| OpdB | PA2700 | Diterpenoid transport | Diterpenoids |
| OpdC | PA0162 | Cation efflux | Basic compounds |
| OpdI | PA0189 | Unknown | |
| OpdJ | PA2420 | Isochorismatase | Aromatic amino acids |
| OpdP | PA4501 | Dipeptide transport | Dipeptides |
| OpdT | PA2505 | Catechol degradation | Aromatic compounds |

[a] Where applicable, the context of the orthologous protein in *P. putida* (indicated with a PP number) is listed.

vanillate, PCR products overlapping the intergenic regions between *opdK*, the putative aldehyde dehydrogenase (PA4899), and the probable MFS transporter (PA4900) were generated, implying that these three genes form an operon. The *mdlC* gene was apparently not cotranscribed with this operon, but the gene itself was strongly induced by vanillate. The putative regulator PA4902 was also highly expressed in vanillate. The probable cytoplasmic transporter 4903 (83% similar to the VanK MFS transporter of *P. putida*) was also induced by vanillate (amplified product detectable after 30 cycles) but to a much lesser extent than PA4900 (amplified product detected after 25 cycles). The expression of PA4911 (67% similar to BraE of *P. aeruginosa*, the permease component of an ATP binding cassette [ABC] transporter specific for a branched-chain amino acid) was not induced in vanillate. An unexpected result was the strong positive regulation of the gene for an outer membrane protein belonging to the large TonB-dependent family of porins (32), as these genes are often regulated by extracytoplasmic function sigma factors under limiting iron conditions.

In comparison to its isogenic wild-type strain, the *opdK* mutant was compromised in growth at low concentrations of vanillate (Table 3). At 0.5 mM vanillate, the mutant grew at approximately half the rate of the wild type. Similar to the results obtained with the *oprD* mutant, with a 2 mM carbon source, the growth rate of the *opdK* mutant was identical to that of the wild-type strain; at 1 mM vanillate, the mutant exhibited a very slight growth defect. The growth defect appeared to be specific to vanillate as no difference in growth between the two strains was observed when the other aromatic compounds listed in Table 4 were used as carbon sources (including the inducing molecule vanillin) (data not shown). Thus, over a restricted concentration range, OpdK afforded a growth advantage on vanillate.

**Growth phenotypes of the other OprD homologues.** To determine whether the apparent specificity of OpdK for an aromatic compound and OprD's specificity for basic amino acids could be generalized to other members of their respective subgroups, the substrate specificities of the 17 remaining OprD homologues were investigated. By an approach similar to that used for OpdK, the genomic contexts of the OprD-homologous genes were investigated to see if they were associated with any putative metabolic pathways (Table 5). The genes for 12 of the 17 novel OprD homologues were adjacent to genes involved in the transport or degradation of a variety of compounds, such as amino acids, carboxylic acids, and small aromatic molecules. Therefore, we were able to predict the putative substrates for these porins. The five remaining OprD homologues, like OprD itself, were neighbored by hypothetical proteins. However, substrates were ascribed to three of these remaining porins by examining the genomic contexts of their orthologous porins in *P. putida*. Of these three homologues, the orthologous genes for two homologues, *opdL* and *opdQ*, were adjacent to genes involved in the degradation of phenylacetic acid and nitrobenzoate, respectively. The orthologous gene of the third homologue, OpdN, lay directly downstream of a *cobF* homologue. In prokaryotes, *cobF* is involved in cobalamin biosynthesis (26). Therefore, it was postulated that OpdN might take up a vitamin $B_{12}$ precursor, such as glutamate or 5-aminolevulinic acid. Strains with mutations in the 17 remaining OprD homologues were then assessed for growth in BM2 minimal medium supplemented with a single carbon source as discussed above. The carbon sources used included those listed in Table 5, as well as their structural analogues and metabolic precursors. The genomic contexts of *oprQ* and *oprI* in both *P. aeruginosa* and *P. putida* did not suggest any potential substrates; however, since both porins belonged to the

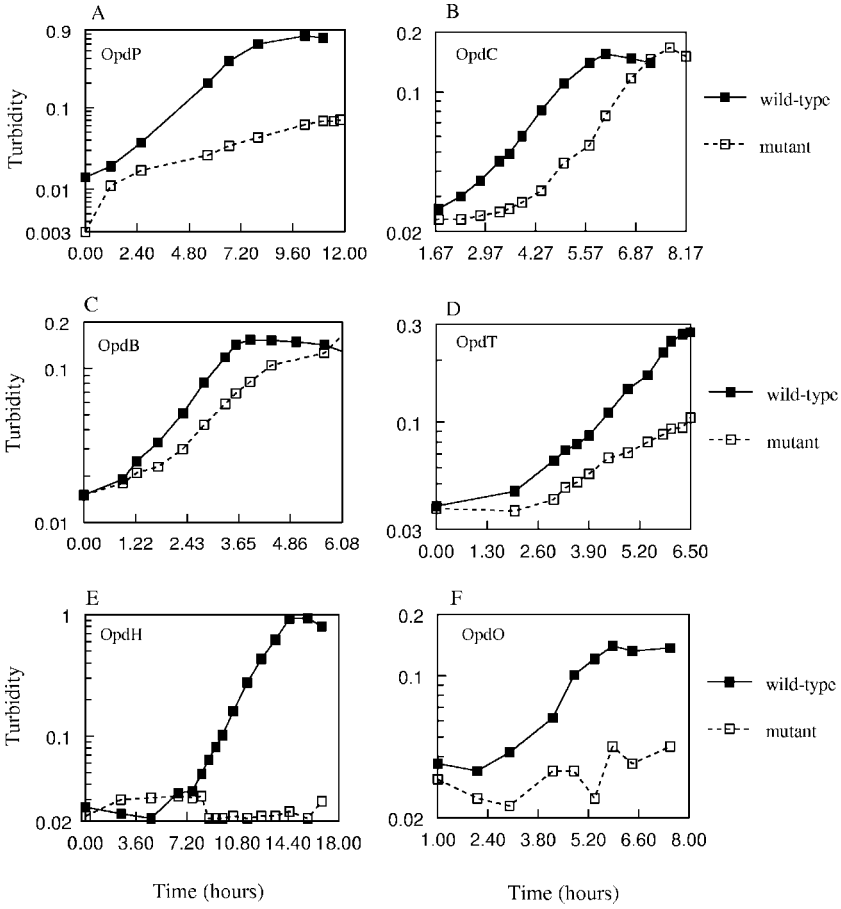



FIG. 3. Growth phenotypes of *P. aeruginosa* OprD homologue mutants. Eight OprD-homologous gene mini-Tn*5*-Tc$^r$ mutants as well as their isogenic wild-type PAO1 and PAK parent strains were grown in BM2 minimal medium supplemented with the porin's putative substrate as the carbon source. The mutants and carbon sources include *opdP*::Tn*5* in 5 mM glycine-glutamate (A), *opdC*::Tn*5* in 1 mM histidine (B), *opdB*::Tn*5* in 1 mM proline (C), *opdT*::Tn*5* in 1 mM tyrosine (D), *opdH*::Tn*5* in 10 mM *cis*-aconitate (E), and *opdO*::Tn*5* in 1 mM pyroglutamate (F). Data shown are representative of at least three separate experiments.

OprD subfamily, *oprQ* and *oprI* mutants were tested for growth in the 20 standard amino acids.

Growth in batch cultures demonstrated inherent variability from day to day, but the reported differences between mutants and wild-type strains were consistently observed. Thus, the growth curves representative of three separate experiments are shown in Fig. 3. In addition to the reported selectivity of OprD for arginine and the growth deficiency of the *opdK* mutant in vanillate, growth defects were observed for 6 of the 17 remaining OprD homologue mutants in the following substrates: *opdP*, glycine-glutamate; *opdC*, histidine; *opdB*, proline; *opdT*, tyrosine; *opdH*, *cis*-aconitate; and *opdO*, pyroglutamate. The extent of the growth defect varied among the six mutants compromised in growth, ranging from a very weak phenotype for the *opdC* and *opdB* mutants (Fig. 3B and C, respectively) to a very strong phenotype for the *opdP*, *opdH*, and *opdO* mutants (Fig. 3A, E, and F, respectively). In contrast, when 1 mM glucose or numerous other control substrates were used as carbon sources, the growth of these mutants was indistinguishable from that of the wild-type strains. When the other 12 *oprD* homologue mutants were grown in their predicted substrates as listed in Table 5, they did not consistently show any obvious growth defect compared to that of the wild-type strain (data not shown).

**Induction of OprD homologues by their substrates.** Since specific porins tend to be induced by their substrates (7), we sought to support the proposed substrate specificity of the eight OprD homologues by looking at their expression levels.

mRNA was isolated from wild-type *P. aeruginosa* grown in BM2 minimal medium using a 10 mM concentration of the putative substrate as the carbon source or 10 mM glucose as the control substrate. The mRNA was reverse transcribed into cDNA, which was then used as the template for PCR using specific primers to small internal fragments of the respective OprD homologues predicted to take up the compound in question. The resulting amplicons were resolved on 2% agarose-TAE gels. The data shown in Fig. 4 are representative of two separate experiments.

In glucose, the OprD homologues were either not expressed or expressed at low levels. OprD, OpdC, OpdB, and OpdT, all members of the OprD subfamily, showed higher levels of background expression than the members of the OpdK subfamily. In their respective substrates, the porins showed higher levels of induction. OpdH was more highly expressed in *cis*-aconitate,

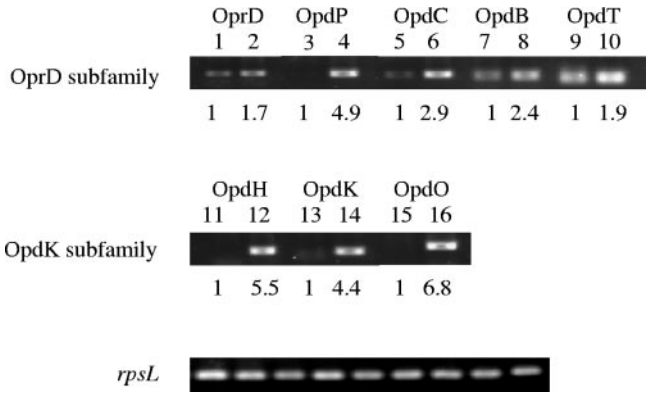



FIG. 4. Expression of OprD homologues in their putative substrates. mRNA was isolated from exponential-phase *P. aeruginosa* PAO1 grown in BM2 medium plus a 10 mM concentration of a carbon source, reverse transcribed into cDNA, and used as the template for SQ-PCR. The top panel shows the expression patterns of the OprD subfamily members: OprD (lane 1 [glucose] and lane 2 [arginine]), OpdP (lane 3 [glucose] and lane 4 [glycine-glutamate]), OpdC (lane 5 [glucose] and lane 6 [histidine]), OpdB (lane 7 [glucose] and lane 8 [proline]), and OpdT (lane 9 [glucose] and lane 10 [tyrosine]). The middle panel shows the expression patterns of the OpdK subfamily: OpdH (lane 11 [glucose] and lane 12 [*cis*-aconitate]), OpdK (lane 13 [glucose] and lane 14 [vanillate]), and OpdO, (lane 15 [glucose] and lane 16 [pyroglutamate]). The bottom panel shows the expression levels of the control gene *rpsL* in glucose, arginine, glycine-glutamate, histidine, proline, tyrosine, *cis*-aconitate, vanillate, and pyroglutamate. Numbers indicate the ratios of porin gene expression in glucose to that in the putative substrate. All values were normalized to the levels of *rpsL* produced in each substrate. Data shown are representative of two separate experiments.

OpdO in pyroglutamate, OpdK in vanillate, OprD in arginine (confirming previous results) (20), OpdP in glycine-glutamate, OpdC in histidine, OpdB in proline, and OpdT in tyrosine.

## DISCUSSION

*P. aeruginosa* is an extremely versatile organism able to metabolize over 100 different compounds (31). This versatility can be attributed partially to the large number of transporter genes encoded by this organism (32). Many of these genes, including 16 members of the OprD family of porins, have been identified based only on homology to known transporters. At the amino acid level, the OprD homologues are 46% to 57% similar to OprD. The similarities become more apparent when the protein sequences of this family are aligned (http://cmdr.ubc.ca/bobh/omps/alignment.htm), suggesting that like OprD, the homologues form channels in the outer membrane that are specific for the uptake of related classes of molecules. The OprD family was described first for *P. aeruginosa* (32), but since that time, it has become apparent that this family is found in many metabolically versatile soil bacteria and comprises over 100 members.

In *P. aeruginosa*, the 19 members of the OprD family cluster into two distinct subfamilies. This phylogenetic distribution is preserved when the OprD homologues of *P. putida* are included in the analysis. The majority of the homologues in the two organisms are orthologous (i.e., more similar in sequence to counterparts in the other species than to other porins within the same species), implying that the OprD gene duplication events took place prior to the speciation of these two organisms and may share the same function in the two species. Some of the channels, namely, OpdJ, OpdI, OpdR, OpdO, OpdD, and OpdQ in *P. aeruginosa*, as well as PP3390, PP3656, PP3168, and PP3939 in *P. putida*, lack a clear orthologue and may have arisen via postspeciation duplication events. Hence, these porins may have evolved to take up compounds found in niches unique to either *P. aeruginosa* or *P. putida*.

The specificity of the OprD homologues is implied by their genomic contexts. Sixteen of the 19 genes were proximal to genes implicated in the uptake or catabolism of unique compounds. Also, none of the mutants deficient in OprD homologues were resistant to multiple classes of antibiotics, indicating that the channels do not form general pores in the outer membrane.

The genomic context of the *opdK* gene indicated that it may be involved in the uptake of small, aromatic compounds. The list of potential substrates was shortened to vanillate and vanillin upon examination of the induction pattern of an *opdK*::*xylE*-Gm$^r$ transcriptional fusion grown in various aromatic compounds. The induction of *opdK* in vanillate was confirmed by SQ-PCR, and the coregulation of adjacent genes is consistent with the possibility that they may be involved in vanillate degradation. PA4902, a putative transcriptional regulator, was positively regulated by vanillate. In addition, vanillate induced the expression of two cytoplasmic membrane transporters, an MFS transporter, PA4900, which was highly expressed, and PA4903, which was expressed at a much lower level. The expression of the ABC transporter was negligible under the growth conditions tested. The TonB-dependent family member PA4897 was induced in vanillate. Members of this family of gated porins are responsible for the uptake of large compounds, such as iron-siderophore complexes, vitamin $B_{12}$, and sulfate esters (33). Therefore, PA4897 may be involved in the uptake of a large aromatic compound resembling vanillate, such as short lignin polymers.

Specific porins are required in nutrient-limited environments. In these dilute environments, outer membrane permeability becomes rate limiting for growth (18). We first tried to confirm this result for the well-characterized porin OprD. Despite the evidence suggesting a role for OprD in the uptake of basic amino acids (11, 20, 36), we were unable to detect a growth defect when the *oprD* mutant was grown in arginine. A growth defect was detected, however, for the *opdK* mutant grown in limiting concentrations of vanillate. The discrepancy between these results suggested that the growth assay was useful for detecting significant differences in growth but not sensitive enough to detect subtle growth changes between two strains and emphasizes the need for more-rigorous biochemical experiments to confirm the phenotypes discussed throughout this paper.

Combined with the substrate-specific SQ-PCR expression data, however, this approach led to the identification of six additional phenotypes. Including OprD, the probable specificities for the eight OprD family members are as follows: OprD, basic amino acids such as arginine; OpdP, the dipeptide glycine-glutamate; OpdC, histidine; OpdB, proline; OpdT, tyrosine; OpdH, *cis*-aconitate; OpdK, vanillate; and OpdO, pyroglutamate.

The 1 mM carbon source concentration used was in great

excess of the amounts of these compounds found in the natural environment and thus of the concentrations at which specific channels might be necessary. Therefore, it is possible that many compounds, in addition to binding to and being taken up by specific OprD homologues at low concentrations, may have been able to diffuse through other OprD homologues in a concentration gradient-dependent manner. Such compensatory uptake would obscure the contribution of a single channel and explain the relatively poor growth defects of some of the OprD homologue mutants and our inability to define phenotypes for 11 of the 19 mutants. Again, a more sensitive experimental system, perhaps involving radiolabeled substrates or model membrane systems, should be employed to fully understand the contribution of each of these porins to membrane permeability.

The members of the OprD subfamily that were expressed at background levels in glucose (Fig. 4), OprD, OpdC, OpdB, and OpdT, are likely candidates for permitting low levels of nonspecific uptake through the outer membrane. Indeed, it has already been shown that OprD can act as a nonspecific channel for the uptake of gluconate (11). Thus, in order to greatly decrease outer membrane permeability, mutants deficient in multiple OprD homologues would have to be generated. The Ttg efflux family of the solvent-tolerant bacterium *P. putida* DOT-T1E exhibits a similar type of compensatory activity. All three known efflux systems must be knocked out in order to completely abrogate toluene efflux (27). The redundancy of these homologous gene families confers a selective advantage to these *Pseudomonas* species, ensuring that in the event of a single-gene loss or mutation, the function of that gene is not completely abrogated.

The phenotypes that were found for OprD, OpdP, OpdC, OpdB, OpdT, OpdH, OpdK, and OpdO were consistent with the expression profiles found for these porins in their substrates by SQ-PCR. These data demonstrate that like the other specific porins of *P. aeruginosa*, these eight OprD homologues are positively regulated by their substrates (7). Thus, when a particular substrate is present in the environment, the expression levels and hence the uptake activity of the specific porin will be maximal. Limiting the numbers and types of channels in the outer membrane when they are not required, in turn, would prevent the entry of potentially toxic compounds into the cell.

Taken together, both the growth data and expression data indicate that the OprD family is comprised of semispecialized uptake channels. The substrate specificities of these porins may form the basis of the phylogenetic clustering of this family into two subfamilies. Members of the family in the OprD subgroup, OprD, OpdP, OpdC, OpdB, and OpdT, all take up amino acids and related molecules such as dipeptides. The members more similar in amino acid sequence to OpdK (OpdH, OpdK, and OpdO) take up a variety of structurally diverse carboxylic acids. The speculated substrate specificities for eight of the remaining OprD homologues are in agreement with the proposed basis of phylogenetic clustering. The exceptions to this proposal include OprE, which was predicted to take up either arginine or proline, and OprQ and OpdI, both of which are members of the OprD subfamily, for which no substrates were assigned.

OpdB and OpdC were originally predicted to take up a diterpenoid and cationic compound, respectively. However, these predictions were modified over the course of this investigation based on the sequence similarities of OpdB and OpdC to OprD. Thus, the *opdB* mutant was predicted to have a growth defect in a ringed amino acid (proline), and the *opdC* mutant was expected to grow more slowly in a positively charged amino acid (histidine). These modified predictions were verified, highlighting the power of combining the investigation of genomic contexts with phylogenetic analysis to predict gene function. Similar analyses can be applied to the OprD family members found in other soil organisms. We speculate that like *P. aeruginosa*, these organisms evolved a large repertoire of OprD homologues through a series of duplication events to specifically take up a vast array of metabolites while excluding other potentially harmful compounds. It would be interesting and most informative to determine whether the phylogenetic distributions of the OprD homologues of these organisms share the same functional basis as those of *P. aeruginosa*.

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