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Structure of a putative BenF-like porin from *Pseudomonas fluorescens* Pf-5 at 2.6 Å resolution

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Abstract

The X-ray structure of a putative BenF-like (gene name: PFL1329) protein from *Pseudomonas fluorescens* Pf-5 (PflBenF) has been determined at 2.6 Å resolution. X-ray crystallography revealed a canonical 18-stranded β-barrel fold that forms a central pore with a diameter of ~4.6 Å, which is consistent with the size and physicochemical properties of the presumed aromatic acid substrate, benzoate. Detailed comparisons with the previously-determined structure of *Pseudomonas aeruginosa* OpdK, a vanillate influx channel, revealed an arginine-rich aromatic acid selectivity filter of nearly identical structure composed of seven highly conserved residues Arg~Asp~Arg~Arg~Ser~Asp~Arg (R~D~R~R~S~D~R sequence motif, where ~ denotes intervening residues) that define the narrowest part of the pore.

Keywords

BenF-like; substrate specific porin; OprD superfamily; OprD subfamily; OpdK subfamily; benzoate; *Pseudomonas*; integral membrane protein

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Protein Data Bank Codes: Atomic coordinates and structure factors of PflBenF were deposited to the PDB (www.rcsb.org) on 14 September 2009 with PDB accession code 3JTY. The NYSGXRC target identifier for PflBenF in TargetDB (<http://targetdb.pdb.org>) is "NYSGXRC-10383p". Expression clone sequences and selected interim experimental results are available in PepcDB (<http://pepcdb.pdb.org/>).

Introduction

Gram-negative bacteria typically overcome poor permeability of outer membranes through general porins like OmpF and OmpC, which form water filled transmembrane pores permitting diffusion of hydrophilic molecules with no particular selectivity.¹ Many bacteria lacking such general porins utilize substrate-specific porins to overcome growth-limiting conditions and facilitate selective transport of metabolites. Exclusive reliance on substrate-specific porins yields lower membrane permeability to small molecules (<600 Da) *versus* that seen for *Escherichia coli*. In *Pseudomonads*, transit of most small molecules across the cell membrane is thought to be mediated by substrate-specific channels of the OprD superfamily.² This property explains, at least in part, the high incidence of *Pseudomonas aeruginosa* antibiotic resistance. High-throughput DNA sequencing of the *P. aeruginosa* chromosome revealed the presence of 19 genes encoding structurally related, substrate-specific porins (with 30-45% pairwise amino acid sequence identity) that mediate transmembrane passage of small, water-soluble compounds. The OprD superfamily encompasses the eponymous OprD subfamily, which includes 9 *P. aeruginosa* proteins that convey basic amino acids and carbapenem antibiotics,³ and the OpdK subfamily, which includes 11 *P. aeruginosa* proteins that convey aromatic acids and other small aromatic compounds.⁴ Genome sequencing of other Gram-negative bacteria has revealed additional members of the OprD and OpdK subfamilies in various organisms, including other pseudomonads. Among the many bacteria in which OprD superfamily members have been identified are *P. putida*, *P. fluorescens* Pf-5, *P. syringae*, and *Azotobacter vinelandii*, all of which share closely related genes that encode the so-called BenF-like porins. In *P. putida*, *benF* is part of an operon involved in benzoate catabolism regulated by *benR*.⁵ Within this operon *benK*, *benE*, and *benF* genes have been suggested to contribute towards either influx or efflux of benzoate.^{5,6} BLAST⁷ analysis of the amino acid sequence of *P. fluorescens* Pf-5 gene PFL1329 (Uniprot⁸ id: <http://www.uniprot.org/uniprot/Q4KH25>)⁹ against *P. putida* KT2440 strain¹⁰ identified twenty related porins. The top six hits include *P. putida* KT2440 genes PP1383 (annotated as BenF-like), PP2517 (annotated as BenF-like), and PP3168 (annotated as BenF), which share sequence identities of 76%, 66%, and 44% with PFL1329, respectively. The precise functions of these genes are not yet known. Therefore, we refer to the protein product of gene PFL1329 as PflBenF-like, which reflects its current annotation in the Uniprot database.

Crystal structures of OprD¹¹ and OpdK (vanillate specific porin)¹², both from *P. aeruginosa* (designated below as PaOprD and PaOpdK, respectively) have been determined. Herein, we report crystal structure of a putative BenF-like porin from *P. fluorescens* Pf-5 (PflBenF-like). For the sake of brevity, all subsequent references to the PflBenF-like will be made using PflBenF. X-ray crystallography revealed a canonical 18-stranded β -barrel fold that forms a central pore with a diameter of $\sim 4.6\text{\AA}$. We describe detailed comparisons of the PflBenF structure with those of PaOprD and PaOpdK.

Methods

Cloning and expression of PflBenF

The gene encoding putative *P. fluorescens* BenF-like porin was cloned from genomic DNA of the strain Pf-5 (American Type Culture Collection, USA). The desired truncation (encoding residues 30-420) was PCR amplified using GAGTCGGGCTTTCTCGAAGATGC and CCAGCAGGCTGAGGGGATAGC as forward and reverse primers, respectively. The purified PCR product was subsequently TOPO[®] (Invitrogen, USA) cloned into pSGX3, a derivative of pET26b(+), giving rise to a fusion protein with a non-cleavable C-terminal hexa-histidine tag. Plasmids were transfected into BL21(DE3)-Condon+RIL (Invitrogen, USA) cells for overexpression. Expression was

carried out at 22 °C in 4L of Terrific Broth¹³ supplemented with kanamycin (50 µg/ml) and chloramphenicol (35 µg/ml). Protein expression was induced by addition of 0.4 mM IPTG at an OD₆₀₀ of 1.0. Cells were harvested after 21 hours by centrifugation at 4 °C. Virtually all PflBenF protein was found in inclusion bodies.

Purification and refolding of PflBenF

The *E. coli* cell pellet was resuspended in 30mL of cold buffer (20 mM Tris HCl pH 8.0, 500 mM NaCl, 25 mM imidazole, and 0.1% Tween20) and cells were lysed *via* sonication. Inclusion bodies were pelleted by centrifugation at 4 °C and solubilized in 20 mM TrisHCl pH 8.0, 1 mM β-mercaptoethanol, and 6 M guanidinium chloride. Solubilized PflBenF was purified under denaturing conditions *via* immobilized metal ion affinity chromatography with NiNTA resin by washing the column with solubilization buffer plus 50 mM imidazole. Following elution with the solubilization buffer plus 400 mM imidazole, PflBenF was diluted to a concentration of ~1 mg/mL and refolded by drop wise dilution into a buffer containing 20 mM TrisHCl pH 8.0, 150 mM NaCl, 0.5 mM TCEP, and 0.5% (w/v) lauryldimethylamineoxide (LDAO) to a final protein concentration of ~250 µg/mL. The refolded protein was concentrated by immobilization on NiNTA resin followed by elution with 20 mM TrisHCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, 5 mM LDAO, and 400 mM imidazole. Refolded PflBenF was further purified by size exclusion chromatography using a 120 mL Superdex S200 column equilibrated with 20 mM TrisHCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, and 5 mM LDAO. SDS-PAGE analysis showed greater than 95% purity and protein fractions corresponding to the symmetric portion of the size exclusion chromatography profile were pooled and concentrated using spin filters. Size Exclusion Chromatography with Multi Angle Laser Light Scattering (SEC-MALLS) revealed PflBenF to be a monomer associated with one LDAO micelle (data not shown).

Crystallization, data collection, and structure determination

PflBenF (protein concentration ~9.0 mg/mL; 0.3 µL protein-containing solution + 0.3 µL reservoir solution) was subjected to crystallization screening with the Classics, Classics II, CompAS, and PEG kits (Qiagen, USA) using a Phoenix Liquid Handling System (Art Robbins Instruments, USA) *via* sitting drop vapor diffusion at 4 and 21 °C. Several conditions yielded thin needles and microcrystals. Subsequent optimization was performed with additive and detergent screening. A single crystal diffracting to ~2.6 Å resolution was obtained with 21% PEG 3350, 100 mM citrate, and 10% (w/v) ANAPOE-X-114 (from the detergent screen) at 21 °C. Diffraction data were recorded under standard cryogenic conditions using the LRL-CAT 31-ID Beamline at the Advanced Photon Source (APS) and processed with MOSFLM¹⁴ and SCALA (Collaborative Computing Project Number 4, 1994).¹⁵ A poly-alanine model of PaOpdK was used for molecular replacement with PHASER¹⁶ as implemented in CCP4. The atomic model of PflBenF was built manually by visual inspection with COOT¹⁷ and refined to convergence using REFMAC5¹⁸ [Table 1]. Structural analyses were carried out using COOT and CCP4 and illustrations were prepared using PyMol (<http://pymol.sourceforge.net>).

Results and Discussion

Overall structure of PflBenF-like porin

An N-terminal truncation of the putative BenF-like porin from *P. fluorescens* Pf-5 (PflBenF, residues 30-420) was over-expressed in *E. coli*, purified under denaturing conditions from inclusion bodies by immobilized metal ion affinity chromatography, refolded by dilution into a buffer containing LDAO, and further purified by size exclusion chromatography. PflBenF crystallized in the monoclinic space group *P*2₁ with four molecules in the

asymmetric unit [Table 1]. The PflBenF polypeptide chain could be continuously traced for one protomer (denoted chain A and used for all illustrations and analyses herein). In each of chains B, C, and D, residues 112-115 were disordered in the experimental electron density map and could not be modeled. Otherwise, structures of the four protomers are very similar [pairwise root mean square deviations (r.m.s.d.s) range from 0.31 to 0.39 Å for common α -carbon atomic pairs].

PflBenF adopts the canonical β -barrel porin fold consisting of 18 anti-parallel β -strands arranged in a cylinder [Figs. 1A and B]. Pairwise comparisons of the structure of PflBenF with those of PaOpdK and PaOprD using SSM19 revealed highly similar polypeptide chain folds (PflBenF *versus* PaOpdK: r.m.s.d. = 0.9 Å, sequence identity = 53% for 369 α -carbon pairs; PflBenF *versus* PaOprD: r.m.s.d. = 1.3 Å, sequence identity = 40% for 342 α -carbon pairs; PaOpdK *versus* PaOprD: r.m.s.d. = 1.3 Å, sequence identity = 39% for 382 α -carbon pairs). The β -strands of PflBenF are connected by shorter turns T1-T8 and longer loops L1-L7 on the periplasmic and extracellular faces of the barrel, respectively. Loops L3 and L7 fold into the interior of the β -barrel, where they help define the geometry of the pore [Fig. 1B]. As for PaOpdK and PaOprD, PflBenF also possesses short S5 and S6 β -strands, which are characteristic of porins that form trimers in the outer membrane.²⁰ Notwithstanding this similarity, PflBenF protomers do not make extensive intermolecular interactions within the crystal lattice. Recombinant forms of both PaOpdK and PaOprD also appear monomeric in the crystal lattice. However, recombinant PaOpdK inserted into artificial lipid bilayer oligomerizes and behaves as a trimeric ion-conductor.¹¹ Given the short lengths of the S5 and S6 β -strands in the PflBenF-like porin and the PaOpdK precedent, there is no compelling reason to believe that PflBenF functions as a monomer.

Structure of the PflBenF benzoate-specific channel

The structure of the PflBenF β -barrel revealed a quasi-circular pore [Fig. 1B] with a diameter of ~ 4.6 Å (as estimated with HOLE21). Sequence alignment of PflBenF, *P. putida* BenF, PaOpdK, PaOprD, and three other porins with various annotations (pairwise sequence identities with PflBenF ranging from 34 to 76%) revealed significant conservation for β -strand residues [Fig. 1C] and those within the loops L2, L3, and L7. The electrostatic properties of the periplasmic and extracellular faces of PflBenF are illustrated in Fig. 1D. On the periplasmic face, conserved basic and non-polar surface features are visible in the immediate vicinity of channel constriction. There is no corresponding concentration of polar surface features on the extracellular side of the channel immediately beyond the constriction. In fact, the extracellular face of the protein is largely devoid of polar features [Fig. 1D lower panel]. The entire periplasmic face of PflBenF is also considerably more polar than the extracellular face of the protein.

Evidence for an aromatic acid selectivity filter shared by PflBenF and *P. aeruginosa* OpdK

The pore constriction of PflBenF is lined by residues Asp151 and Arg154 from loop L3; Ser310, Asp317, Ser318, and Tyr319 from loop L7; Phe46, Phe48, and Arg50 from the S1 β -strand; Arg186 from loop L4; and Glu408 and Arg410 from the S18 β -strand [Fig. 2A]. These four Arg residues decorate the periplasmic lip of the pore giving rise to a discrete positively charged patch between 4 and 6 O'clock [Fig. 1D, upper panel]. Of these 12 pore forming residues, four arginines and an aspartate at position 317 are invariant among the six divergent sequences included in Fig. 1C. In addition, Asp151 and Ser310 are highly conserved.

Given that PflBenF and PaOpdK are thought to transport chemically similar substrates (putative benzoate selectivity *versus* experimentally confirmed vanillate uptake), it is not unexpected that their respective pore constrictions are essentially identical in size (diameters

of $\sim 4.6\text{\AA}$ and $\sim 4.4\text{\AA}$, respectively) and shape [Fig. 2B]. What is surprising, however, is the finding that the seven pore forming residues with sidechains that contribute directly to the constriction (or selectivity filter) are identical between PflBenF and PaOpdK [Figs. 1C and 2B]. Specifically, Arg50 (*Arg22*, italics denotes PaOpdK residues), Asp151 (*Asp123*), Arg154 (*Arg126*), Arg186 (*Arg158*), Ser310 (*Ser282*), Asp317 (*Asp289*), and Arg410 (*Arg381*) are invariant between these two porins [Figs. 1C and 2B]. The only notable difference is the sidechain conformation of Arg410, which brings its guanidinium group closer to the constriction in the case of PflBenF. In contrast, the PaOprD pore constriction is smaller than that of PflBenF and PaOpdK ($\sim 3.7\text{\AA}$ versus $\sim 4.5\text{\AA}$), differs in shape, and is defined by a different configuration of selectivity filter residues [Fig. 2C]. Thus, the selectivity determining residues of the vanillate and putative benzoate pores appear to be similar. Further characterization of the function of PflBenF and other porins sharing this sequence motif will be required to establish whether or not it is in fact a signature of aromatic acid channels.

One of the metrics guiding target selection in structural genomics is modeling leverage at the level of greater than 30% sequence identity (see Supplementary Methods).²² Searching of the UniProtKB database of all known protein sequences using the sequences of PflBenF, PaOpdK, and PaOprD identified 221 unique protein sequences with sequence identity greater than 30% to at least one of these three proteins. The inaugural structure of PaOprD, enabled modeling of 165 related proteins (Supplementary Figure S1). Subsequent determination of the structure of PaOpdK enabled homology modeling of an additional 3 protein sequences. In contrast, determination of the PflBenF structure enabled homology modeling of 53 additional protein sequences (Supplementary Figure S1). Thus, the structure of PflBenF, reported herein, expands significantly the number of homology models of OprD and OpdK family of substrate-specific porins. Experimental structures of additional OprD / OpdK subfamily members should provide useful guides for planning experiments aimed at defining the mechanisms governing pore selectivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure of PflBenF

A: Rainbow-colored ribbon drawing of PflBenF with a dot surface representation of the pore calculated using HOLE21 viewed parallel to the cell membrane.

B: Stereodrawing of PflBenF with the pore viewed from the periplasmic face of the protein (using the same color coding as in Fig. 1A).

C: Sequence alignment of PflBenF (gi|123657525; PDB Code 3JTY), *P. putida* BenF (gi|81441797), *P. aeruginosa* OpdK porin (gi|81539679; PDB Code 2QTK), *P. putida* outer membrane protein (gi|167033173), *Acinetobacter baumannii* VanP (gi|193076825), *A. sp. RUH2654* OprD family porin (gi|260549927), and *P. aeruginosa* D2 porin (gi|417518; PDB Code 2ODJ) generated using ClustalW23 and color coded with ESPRIPT24; numbers after “_” denote sequence identity with respect to the PflBenF and aromatic acid selectivity pore residues are marked with ▲.

D: Semitransparent representation of the solvent accessible periplasmic (upper panel) and extracellular (lower panel) surfaces of PflBenF, color coded for qualitative electrostatic potential (generated using PyMol) shown with polypeptide chain as ribbon, using the same orientation as in Fig. 1B.

Figure 2. Structure of a putative aromatic acid selectivity filter

A: Atomic stick figure representation of the PflBenF pore forming residues surrounding the dot surface of the pore21 viewed parallel to the cell membrane (carbon atoms color coded to correspond to the representation in Fig. 1A: blue denotes β 1, green denotes L3 and L4, light brown denotes L7, pink denotes β 10; nitrogen atoms-blue; oxygen atoms-red).

B: Stereoview overlay of selected pore forming residues of PflBenF with residues defining the aromatic acid selectivity pore (color coded as in Fig. 2A, labeled with PflBenF sequence numbering) and *P. aeruginosa* OpdK (carbon atoms color coded cyan).

C: Stereoview overlay of selected pore forming residues of PflBenF (color coded as in Fig. 2A) and *P. aeruginosa* OprD (carbon atoms color coded yellow).

Table 1

Crystallographic data collection and refinement statistics for PflBenF.

Data Collection	
PDB code	3JTY
Space group:	$P2_1$
Unit-cell dimensions ($\text{\AA}$, $^\circ$):	a=62.1, b=210.6, c=84.1, β =97.8
Resolution ($\text{\AA}$):	53.68-2.58 (2.72-2.58)*
Number of unique reflections:	67053 (9774)
Completeness (%):	100.0 (100.0)
R_{symm} (%):	14.0 (52.7)
Multiplicity:	3.9 (3.8)
$\langle I/\sigma(I) \rangle$:	6.1 (2.7)
Refinement	
Resolution ($\text{\AA}$):	35.11-2.58
R-factor (%):	22.0
R_{free} (%):	27.5
Number of non-hydrogen atoms	
Protein	11859
Ligands	32
Water molecules	25
Average B-factors ($\text{\AA}^2$)	
Protein	41.9
Ligands	75.4
Water molecules	35.2
RMS deviations from ideal values	
Bond length ($\text{\AA}$):	0.018
Bond angles ($^\circ$)	1.60
Ramachandran Plot ²⁵ :	
MolProbity ²⁶ Residues in	
Favored region (%):	95.6
Allowed region (%):	99.9

* Values in parenthesis correspond to highest-resolution shell