RESEARCH LETTER

The major outer membrane protein OprG of *Pseudomonas aeruginosa* contributes to cytotoxicity and forms an anaerobically regulated, cation-selective channel

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Abstract

OprG of *Pseudomonas aeruginosa* is a member of the very large and widely distributed but poorly characterized OmpW (PF0392) family of outer membrane proteins. It was established here that OprG was highly transcribed in anaerobic environments rich in iron via the ANR regulator. In the absence of OprG, *P. aeruginosa* was significantly less cytotoxic toward human bronchial epithelial cells. Planar bilayer studies indicated that purified OprG formed cationic-selective channels with a conductance of 500 pS in 1 M KCl; however, contrary to previous reports, OprG did not appear to be involved in either iron or antibiotic uptake.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium commonly found in soil and water. It is also found in a variety of man-made environments, and thrives especially in the hospital setting, where it is among the three leading causes of nosocomial infections (Govan *et al*., 2007). Two factors contributing to the ecological success of *P. aeruginosa* are its nutritional versatility supported by a vast array of transport proteins (Stover *et al*., 2000) and its inherent resistance to many antimicrobial agents, due in part to the low permeability of its outer membrane (Hancock & Brinkman, 2002).

Many molecules traverse the outer membrane through water-filled protein channels called porins. *Pseudomonas aeruginosa* encodes a large number of porins that are differentially expressed, depending on the culture conditions (Hancock & Brinkman, 2002). The major outer membrane proteins under common laboratory growth conditions include OprD, OprE, OprF, OprG, and OprH. Accordingly, these proteins have been extensively studied, with the exception of OprG (Gensberg *et al*., 1999), which is the focus of this communication.

OprG was first investigated by Yates *et al*. (1989) who showed that the protein was expressed under iron-rich conditions and proposed that OprG may function as a low-affinity iron transporter. Subsequently, various groups suggested a link between the presence of OprG and resistance of *P. aeruginosa* to antibiotics because increased resistance to norfloxacin, kanamycin, and/or tetracycline was associated with the disappearance or downregulation of OprG (Chamberland *et al*., 1989; Peng *et al*., 2005). Surveying microarray experiments from the literature, it is clear that the levels of OprG change substantially under a variety of different conditions.

OprG is a member of the OmpW family of eight-stranded β-barrel porins that is very widely distributed. For example, the KEGG database (http://www.genome.jp/kegg/) recognizes 461 homologs, while precomputed BLAST indicates 1429 hits in 468 species. There is minimal information

regarding the potential functions of this widely distributed protein family. *Escherichia coli* OmpW was originally identified as a receptor for colicin S4 (Pilsl *et al.*, 1999). The crystal structure of this protein was subsequently determined, and based on the hydrophobicity of the pore interior, it is proposed to be a channel for small hydrophobic compounds (Hong *et al.*, 2006). Consistent with this, the genes encoding homologs AlkL, NahQ, and DoxH lie in operons responsible for the respective metabolism of alkanes, naphthalene, and dibenzothiophene in various *Pseudomonas* sp. (van Beilen *et al.*, 1992; Denome *et al.*, 1993).

In this work, we sought to further define the contribution of OprG to the lifestyle of *P. aeruginosa*. In addition to environments rich in iron, we demonstrate that anaerobic conditions induce *oprG* transcription via the global regulator ANR. A purified preparation of OprG formed relatively large (500 pS), cation-selective channels. Moreover, *oprG* was found to contribute to cytotoxicity in a human bronchial epithelial cell line.

Materials and methods

Bacterial strains, growth conditions, and cell culture

The bacterial strains and plasmids used in this study are described in Table 1. Strains were routinely maintained on Luria–Bertani agar. A list of the oligonucleotide sequences used can be obtained from the authors. All chemicals used were obtained from either Sigma or Fisher. Antibiotics for selection and maintenance were used at the following concentrations: tetracycline, 100 μg mL^{-1} for *P. aeruginosa* and 12.5 μg mL^{-1} for *E. coli*; gentamicin, 50 μg mL^{-1} for *P. aeruginosa* and 15 μg mL^{-1} for *E. coli*; and carbenicillin, 500 μg mL^{-1} for *P. aeruginosa*; ampicillin, 100 μg mL^{-1} for *E. coli*. Experiments using defined media were carried out using either BM2 minimal medium (62 mM potassium phosphate buffer, pH 7.0, 0.5 mM $MgSO_4$, and 10 μM $FeSO_4$) or chemically defined media (CDM) (100 mM HEPES, pH 7.4, 10 mM potassium phosphate, 0.4 mM $MgSO_4$, 7 mM $(NH_4)_2SO_4$, and 0.1% v/v trace ion solution as described previously (Hancock & Carey, 1979), supplemented with specific carbon sources at the indicated concentrations. High-iron conditions were achieved by adding $FeCl_3$ to a final concentration of 100 μM to the medium. Low-iron conditions were created by culturing the bacteria in acid-washed glassware and adding dipyridyl to a final concentration of 0.3 mM. Anaerobic and microaerophilic conditions were created using either the BBL GasPak or BBL Campy-Pac systems, respectively (BD Canada, Oakville, ON). Anaerobic culture media was supplemented with 50 mM KNO_3.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source
P. aeruginosa		
H899	Wild-type, PAO1 laboratory strain	Stover *et al.* (2000)
H900	H899 *oprG*::*xylE*-Gm^r	This study
PAO6261	PAO1 *anr*	Ye *et al.* (1995)
H951	PA06261 *oprG*::*xylE*-Gm^r	This study
RM536	PAO1 *dnr*::Tc^r	Arai *et al.* (1995)
H952	RM536 *oprG*::*xylE*-Gm^r	This study
PA14	Clinical isolate, multihost pathogen	Liberati *et al.* (2006)
PA14 *oprG*	PA14 *oprG*:: *MAR2xT7*	Liberati *et al.* (2006)
E. coli		
DH5α	General cloning strain	Hanahan (1983)
S17-1	Mobilizing strain	Parke (1990)
Plasmids		
pTOPO	TA cloning vector; Ap^r Km^r	Invitrogen
pEX100T	Suicide vector containing *sacB* gene; Ap^r	Schweizer & Hoang (1995)
pX1918GT	Source of *xylE*-Gm^r cassette; Ap^r Gm^r	Schweizer & Hoang (1995)
pEX*oprG*::*xylE*-Gm^r	Suicide vector for *oprG*::*xylE* fusion	This study

The immortalized human bronchial epithelial cell line 16HBE4o- (HBE, a kind gift from D. Gruenert, USCF, CA) between passages 5 and 12 was cultured in minimal essential media with Earle's salts and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were routinely cultured to 85–90% confluency in 100% humidity and 5% CO_2 at 37 °C.

Genetic manipulations

Routine cloning procedures were carried out as described by Sambrook *et al.* (1989). Vent polymerase was purchased from New England Biolabs (Ipswich, MA). All other enzymes and cloning kits were purchased from Invitrogen. An *oprG* knockout and transcriptional fusion was constructed using the method of Schweizer & Hoang (1995), by amplifying the gene from *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000) with Vent polymerase. The resulting amplicon was subcloned into pKRX and sequenced before proceeding. A sequence-verified clone had the *oprG* gene removed with SphI and cloned into pEX100T, producing pEX100T:*oprG*. The *xylE*-Gm^r cassette from PstI-digested pX1918GT was then cloned into PstI-digested pEX100T:*oprG* to create pEX*oprG*::*xylE*. This plasmid was transformed into *E. coli* S17-1 by electroporation and then mobilized into *P. aeruginosa* H103 by biparental mating, followed by successive selection on gentamicin and 5% sucrose. The replacement of native *oprG* with *oprG*::*xylE*-Gm^r in the resulting strain was confirmed by PCR analysis. The *oprG anr* and *oprG dnr*

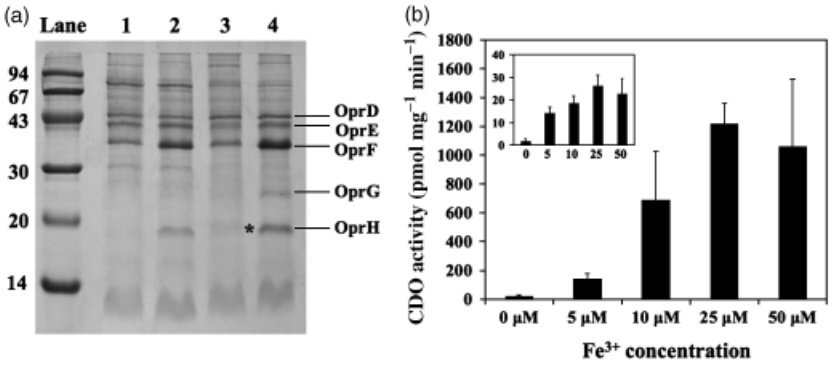

Fig. 1. Induction of OprG by growth in CDM supplemented with 100 μM $FeCl_3$. (a) Outer membrane protein profiles of *Pseudomonas aeruginosa* PAO1 grown in CDM with low or high iron. On the extreme left lane are shown molecular weight markers as defined on the left. Lanes 1 and 2, low iron; lanes 3 and 4, high iron. The proteins in lanes 1 and 3 were solubilized at 37 °C, whereas the proteins in lanes 2 and 4 were solubilized at 88 °C. The unheated form of OprG is visible as a diffuse band in lane 3 and indicated by an asterisk. Protein OprG was identified by its mobility when heated or not before electrophoresis and by its loss in the *oprG*-deficient mutant (data not shown). (b) *oprG*::*xylE* induction by Fe^{3+} under aerobic and anaerobic conditions. For aerobic conditions (inset), 10 mL of CDM containing the indicated concentration of $FeCl_3$ was shaken at 300 r.p.m. in 250-mL Erlenmeyer flasks to ensure good aeration before harvesting at the early stationary phase of growth. For anaerobic conditions, cultures were grown in CDM containing 50 mM KNO_3 for 48 h in GasPak jars before harvesting.

double mutants were constructed by introducing pH900 into PAO6261 (Ye *et al.*, 1995; a kind gift from Dr Dieter Haas) and RM536 (Arai *et al.*, 1995; kindly provided by Dr Hiroyuki Arai).

Assays

The catechol-2,3-dioxygenase activities of the *oprG*::*xylE*-Gm^r transcriptional fusion were determined as described previously (Schweizer & Hoang, 1995). Minimal inhibitory concentrations (MICs) were determined using standard broth microdilution procedures in BM2 minimal medium containing 20 mM glucose and 100 μM $FeSO_4$ with bacterial growth assessed following 24-h incubation at 37 °C.

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release from the immortalized human bronchial epithelial cell line 16HBE4o- (HBE) as described previously (Gooderham *et al.*, 2009).

Outer membrane preparations and electrophoresis

Outer membranes were isolated from stationary-phase cultures as described previously (Hancock & Carey, 1979). Proteins were resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Purification of OprG and channel studies

OprG was purified from stationary phase cultures of *P. aeruginosa* grown in BM2 medium with 0.4% (w/v) glucose and 50 mM KNO_3. Outer membranes were prepared as described above and the outer membrane proteins were crudely separated by successive solubilizations. The first solubilization step consisted of a 1-h incubation at room temperature in 10 mM Tris-HCl, pH 8.0, containing 3% *n*-octyl-polyoxyethylene (o-POE, Calbiochem, San Diego, CA), followed by centrifugation at 150 000 ***g*** for 1 h. The second step was carried out as above with the addition of 50 mM EDTA, pH 8.0, to the solubilization buffer. The supernatant was collected and mixed with an equal volume of 0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, and 20% (v/v) glycerol, and the proteins therein were separated on a 14% SDS-PAGE containing 70 mM NaCl (to assist outer membrane separation). The OprG band was excised from an unstained portion of the gel and eluted overnight at 4 °C into 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl. The presence and purity of OprG in the elution supernatant was confirmed by electrophoresis.

Planar bilayer experiments were performed as described previously (Benz *et al.*, 1985).

Results

Induction of OprG by iron and low oxygen tension

Previous work on OprG demonstrated that the porin was induced at high iron concentrations and upon entry into stationary phase (Yates *et al.*, 1989). The induction by iron was confirmed here using CDM in the presence of high iron concentrations (100 μM $FeCl_3$) (Fig. 1a). To permit more detailed assessment of the regulation of OprG in *P. aeruginosa*, an *oprG*::*xylE* transcriptional fusion/knockout strain H900 was constructed. Using this strain, the upregulation of

oprG in increasing iron concentrations (5–25 μM), by up to 26-fold (at 25 μM iron), was demonstrated to occur at the level of transcription (Fig. 1b, inset).

The induction of *oprG* upon entry into stationary phase, however, was not consistently observed (data not shown). We speculated that the reason for this inconsistency was that as batch cultures reach stationary phase, the metabolic activities of the highly dense population of cells would limit the available concentration of oxygen. When the concentration of oxygen was tightly controlled, by growing strain H900 under anaerobic conditions, the promoter activity of *oprG* increased by almost 50-fold from 25 U of XylE activity to 1200 U in 25 μM iron (Fig. 1b) suggesting that *oprG* was anaerobically induced and that this induction was facilitated by increasing concentrations of iron.

Comparison of the *oprG* promoter region with the promoter regions of the anaerobic regulators *anr*, *dnr*, and *azu* (a gene regulated by ANR) revealed some similarity (Fig. 2b). The ANR and DNR proteins are global transcriptional regulators that are involved in anaerobiosis and response to nitrogen oxides, respectively. Both regulators recognize the same binding site; however, because *P. aeruginosa* requires ANR to grow anaerobically (Ye *et al.*, 1995) and DNR (Arai *et al.*, 1995) to utilize the alternative electron acceptor, nitrate, we sought to determine whether *anr* or *dnr* were involved in the control of *oprG* transcription. Double mutants were constructed by introducing the *oprG*::*xylE* fusion construct into a *dnr*-deficient (Arai *et al.*, 1995) and an *anr*-deficient (Ye *et al.*, 1995) background. Analysis of these strains under microaerophilic conditions showed that the iron-regulated activation of *oprG* was dependent on ANR but not on DNR (Fig. 2a). Thus, it is likely that excess iron increases activation of ANR-regulated genes by increasing the efficiency of iron–sulphur cluster assembly in the ANR protein, a process that has been described previously (Blumer & Haas, 2000).

A decrease in OprG levels in the outer membrane has been correlated with an increase in fluoroquinolone resistance (Chamberland *et al.*, 1989); therefore, it was proposed that OprG may function as a low-affinity iron uptake system because quinolone antibiotics are known to be chelators of iron. However, the *oprG*-deficient strain exhibited similar growth kinetics as the parent when grown in CDM containing high ($T_d = 55$ min for both) or low ($T_d = 140$ for the parent and 145 min for *oprG*) concentrations of iron. Additionally, the susceptibility of the *oprG*-deficient strain to a panel of antibiotics was examined under both aerobic and anaerobic conditions in the presence or absence of iron. No significant differences compared with the parent strain were observed for any of the antibiotics examined under either condition with the MICs for both being ampicillin, $> 512\ \mu g\ mL^{-1}$; cefotaxime, 64; ceftazidime, 2; chloramphenicol, 64; ciprofloxacin, < 0.06; enoxacin, 2; kanamycin, > 32; methicillin, > 256; nalidixic acid, 256; norfloxacin, 0.5; polymyxin b, 1; streptomycin, 32; tetracycline, 32; and tobramycin, $0.5\ \mu g\ mL^{-1}$. These data were inconsistent with previous proposals hypothesizing a role for OprG in iron and/or antibiotic uptake (Chamberland *et al.*, 1989; Yates *et al.*, 1989).

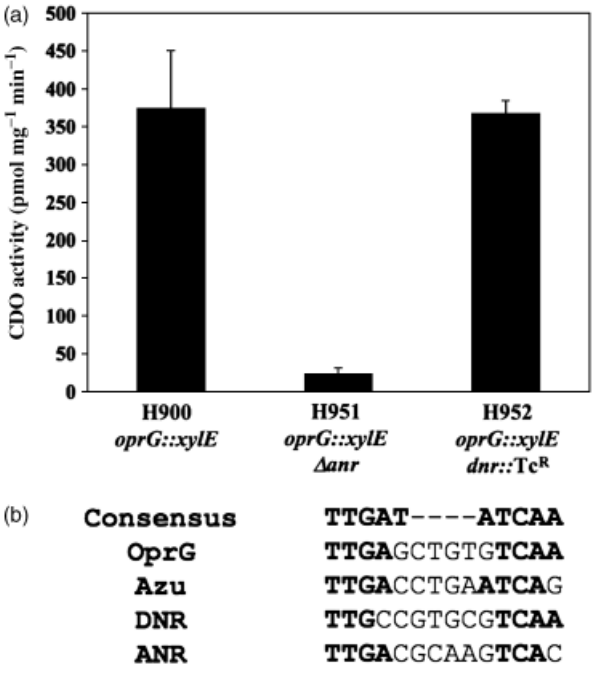

Fig. 2. Regulation of *oprG* by ANR. (a) Induction of *oprG*::*xylE* under high (50 μM) $FeCl_3$ in wild type (H900) or *anr* (H951) or *dnr* (H952) mutant backgrounds under microaerophilic conditions (Campy-Pak). (b) Alignment of the promoter regions of *oprG*, *azu* (which is regulated by *anr*), *anr*, and *dnr*. CDO, catechol-2,3-dioxygenase.

Role of OprG in cytotoxicity

Because of its location on the cell surface, the *oprG*-deficient mutant was also assayed for its ability to resist complement-mediated killing and to form biofilms. The behavior of the mutant was similar to the wild-type strain in these assays, suggesting that OprG does not appear to contribute to these roles (data not shown). To examine whether OprG might be involved in interactions with host cells, we examined the effect of its deletion on the cytotoxicity of *P. aeruginosa* toward the HBE cell line of human bronchial epithelial cells. The parent strain PA-14 (chosen because of its more robust toxicity) demonstrated substantial toxicity toward HBE cells, causing 42% lysis in 4 h and $> 80\%$ lysis after 7 h, as assessed by LDH release. In contrast, after incubation for 4, 7 and 9 h, an *oprG* mutant was found to be significantly ($P < 0.05$) less cytotoxic (> 3-fold after 4 h) to HBE cells than the parent strain (Fig. 3). This result indicated that OprG promotes host cell cytotoxicity during the early stages

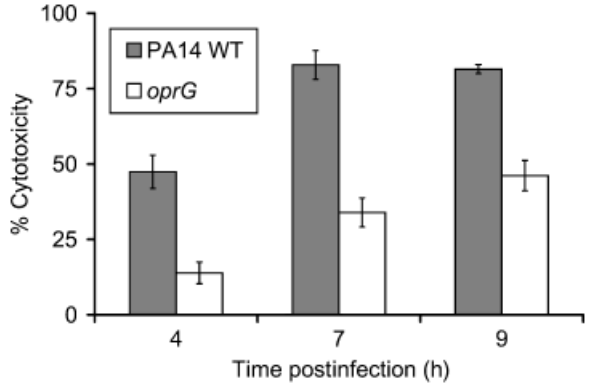

Fig. 3. Contribution of OprG to cytotoxicity. Monolayers of HBE cells were infected at an multiplicity of infection of 50 with either the wild-type strain (PA14; filled bars) or its *oprG* mutant (PA14 *oprG*::*MAR2xT7*; unfilled bars) (Liberati *et al.*, 2006). Supernatants were collected at 4, 7, and 9 h postinfection and cytotoxicity was assessed by measuring the release of cytosolic LDH. Results are expressed as the mean values of LDH released compared with the Triton X-100-treated control. $n = 4$. All results for the oprG mutant were statistically significantly different; $P < 0.05$.

of the host–pathogen interaction. A microarray experiment investigating the gene expression profile of *P. aeruginosa* adherent to HBE cells revealed that *oprG* was downregulated by 5.8-fold in adherent vs. nonadherent cells (data not shown). This result was confirmed by quantitative real-time PCR analysis which demonstrated that, after 4 h of interaction, *oprG* was 9.1-fold downregulated in adherent *Pseudomonas* bound to HBE cells as compared with its expression in nonadherent *Pseudomonas* from the same adherence experiment. This indicates that while in the parental strain OprG normally promotes host cell interactions leading to cytotoxicity, it is rapidly down regulated after initial interaction.

Channel properties of OprG

The structure of *E. coli* OmpW was recently solved (Hong *et al.*, 2006) and revealed that this protein forms an eight-stranded β-barrel with pore-forming properties conveyed by a long, narrow hydrophobic channel. Based on its similarity to OmpW (49% amino acid identity) and high proportion of hydrophobic residues (52%), it seemed probable that, like OmpW, OprG might be a porin responsible for the influx of small hydrophobic or amphipathic compounds. Therefore, the growth of the OprG-deficient mutant was assessed in BM2 minimal medium supplemented with ethanolamine, butanol, glycerol, and the detergent, sarcosine, as the sole sources of carbon. However, when compared with the wild-type strain, the growth of the mutant was not compromised with any of these compounds (data not shown). To gain further insights regarding the function of OprG, its channel-forming properties were studied.

OprG was purified by selective solubilization, followed by its excision from preparative nondenaturing SDS polyacrylamide gels. The protein was apparently in its native form (Hancock & Carey, 1979) because it remained heat-modifiable (Fig. 4a).

The addition of OprG to a planar lipid bilayer resulted in a series of step-wise increases in channel conductance (Fig. 4b), which represent individual channels entering the bilayer membrane. The median single-channel conductance of one hundred observed events was 500 pS in 1 M KCl, *c.* 30 times larger than reported for OmpW, but significantly less than OmpF (2 nS) and OmpC (1.5 nS), the major porins of *E. coli* (Benz *et al.*, 1985). The single-channel conductance of OprG was a linear function of the concentration of KCl, suggesting that the channel did not contain a binding site for either K^+ or Cl^- (Table 2).

Changing the anionic species in the bathing solution to acetate from chloride resulted in a minor change in the median single-channel conductance to 300 pS from 500 pS. Exchanging the cationic species potassium with hydrated lithium, however, dramatically decreased the observed conductance to 75 pS, suggesting that OprG was selective for cations. Indeed, the zero-current membrane potential

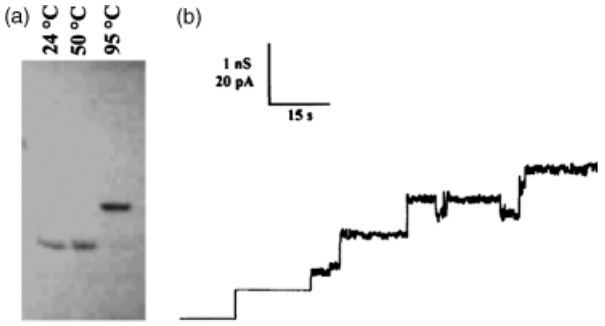

Fig. 4. Single-channel conductance of OprG in 1 M KCl. (a) Purified OprG separated by SDS-PAGE. The OprG band migrates at 20 kDa when unheated or heated to 50 °C, and 25 kDa (i.e. higher in the gel) when heated to 100 °C. (b) A chart recording demonstrating the step-wise increases in conductance formed upon the insertion of purified OprG into a bilayer composed of 1% diphytanoyl phosphatidylcholine.

Table 2. Single-channel conductance of purified OprG*

Salt solution	Conductance (pS)
0.1 M KCl	100
0.3 M KCl	200
1 M KCl	500
1 M KOAc	300
1 M LiCl	75

*Conductance values represent the median of 100 distinct single-channel events.

measurements revealed that the ratio of potassium ion to chloride ion permeability was 7.2 ± 0.7.

Discussion

This work describes the characterization of OprG, a major outer membrane protein of *P. aeruginosa* from the OmpW family, with an ill-defined role to date. Previous studies of OprG showed that its expression was regulated by growth in media containing high concentrations of iron and by growth into stationary phase (Chamberland *et al.*, 1989). Another study indicated that the presence of OprG correlated with susceptibility to fluoroquinolones (Yates *et al.*, 1989). It was therefore proposed that OprG may function as a low-affinity iron uptake system because quinolone antibiotics are known to be chelators of iron. However, the more detailed studies described here do not favor a role in either iron uptake or antibiotic susceptibility. Instead, we demonstrate that the apparent interaction with iron reflects a role for the anaerobic master regulator, ANR, the activity of which increases under iron-rich conditions as well as anaerobiosis (Blumer & Haas, 2000).

Comparison of the amino acid sequence of OprG with that of *E. coli* OmpW revealed a high degree of identity (49%). Much of the dissimilarity between the two porins was observed in the putative extracellular loops and in the periplasmic turns, which, in OmpW, are involved in substrate binding (Hong *et al.*, 2006). Therefore, it was not surprising that the growth of the *oprG* mutant was not compromised in small hydrophobic or amphipathic compounds. It should be noted, however, that we only tested a small subset of likely compounds. However, we were able to demonstrate here that OprG is a porin like OmpW. The differences in the single-channel conductances observed between OmpW (17 pS in 1 M KCl; Hong *et al.*, 2006) and OprG (500 pS in 1 M KCl) may be attributable to differences in their respective channel architectures. For example, the narrowest region of OmpW is defined by a hydrophobic gate comprised of Leu_{56} and Trp_{155} (Hong *et al.*, 2006). In OprG, the leucine is replaced by a valine (Fig. 4). Thus, it may be possible that the loss of a methylene group may make the constriction of the OprG pore larger than that of OmpW, as modest structural changes can result in large changes in conductivity (Brinkman *et al.*, 2000). Another major change might be in the disposition of the loop regions overlaying the channel because in OprD (Huang & Hancock, 1996), for example, it could be demonstrated that the loops can dramatically restrict channel conductance.

In summary, this manuscript further advances our understanding of the OprG porin of *P. aeruginosa*. Collectively, the studies described above indicate that this porin is tightly regulated by anaerobiosis and contributes to the cytotoxicity of *P. aeruginosa* during the early infection.

Acknowledgements

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