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## Highlights

- Vitronectin is a glycoprotein that is produced by the liver and respiratory epithelial cells.
- The production of vitronectin is upregulated in patients with cystic fibrosis.
- Airway isolates from patients with cystic fibrosis bind more vitronectin than isolates cultured from blood.
- Porin D is a novel and major vitronectin-binding protein on the surface of *P. aeruginosa*.

# **Identification of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical isolates of *Pseudomonas aeruginosa***

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**Running Foot:** *Pseudomonas aeruginosa* and vitronectin

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## Abstract

### Background

*Pseudomonas aeruginosa* is a pathogen that frequently colonizes patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). Several pathogens are known to bind vitronectin to increase their virulence. Vitronectin has been shown to enhance *P. aeruginosa* adhesion to host epithelial cells.

### Methods

We screened clinical isolates from the airways of CF patients and from the bloodstream of patients with bacteremia for binding of vitronectin. Two-dimensional SDS-PAGE and a proteomic approach was used to identify vitronectin-receptors in *P. aeruginosa*.

### Results

*P. aeruginosa* from the airways of CF patients ( $n=27$ ) bound more vitronectin than bacteremic isolates ( $n=15$ ,  $p=0.025$ ). Porin D (OprD) was identified as a vitronectin-binding protein. A *P. aeruginosa oprD* transposon insertion mutant had a decreased binding to soluble and immobilized vitronectin ( $p \leq 0.001$ ).

### Conclusions

*P. aeruginosa* isolates obtained from CF patients significantly bound vitronectin. Porin D was defined as a novel *P. aeruginosa* vitronectin-receptor, and we postulate that the Porin D-dependent interaction with vitronectin may be important for colonization.

**Keywords:** adhesion molecules; extracellular matrix; Porin D; *Pseudomonas aeruginosa*; vitronectin

## **Abbreviations**

aa=amino acid, BSA=bovine serum albumin, CF=cystic fibrosis, COPD=chronic obstructive pulmonary disease, CPM=counts per minute, DBA=direct binding assay, ECM=extracellular matrix, FCS=fetal calf serum, HBD=heparin binding domain, IPTG=isopropyl-1-thio- $\beta$ -D-galactoside, LB=lysogeny broth, mAb=monoclonal antibodies, NHS=normal human serum, OMP=outer membrane protein, pAb=polyclonal antibodies, PBS=phosphate buffered saline, PBST=PBS with Tween, SEM=standard error of the mean, VAP=ventilator associated pneumonia, WT=wild type bacterium

## 1.1 Introduction

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is responsible for a substantial burden of disease and mortality, particularly for patients with other underlying diseases. *P. aeruginosa* causes various infections in humans, notably pulmonary infections, keratitis, wound infections and septicemia [1]. It has been shown to be the main pathogen causing increased morbidity and mortality in patients with cystic fibrosis (CF) [2]. *P. aeruginosa* also associated with a high morbidity in patients with advanced stage of chronic obstructive pulmonary disease (COPD) [3], and is reported as the most common bacterial species isolated during exacerbations [4]. In parallel, *P. aeruginosa* is often found as an infectious agent in patients suffering from ventilator associated pneumonia (VAP) [5].

Though *P. aeruginosa* primarily exists in mucus plugs and sputum plaques during long term colonization of CF patients, it is known to adhere to epithelial cells, exposed basement membrane or other proteins of the extracellular matrix (ECM) [6]. For both *P. aeruginosa* and *Pseudomonas fluorescens* it has been suggested that the ECM protein vitronectin is important for bacterial adherence to respiratory epithelial [7,8].

Vitronectin is a plasma protein that exists naturally as a 75 kDa intact protein and a truncated 65 kDa form. It was first discovered for its ability to stick to glass surfaces where subsequently human primary cells could grow [9]. It has later been found to be associated with cell to cell adhesion, wound repair and regulation of the complement system. Furthermore, vitronectin promotes coating on polymer surfaces and it may aid to build up biofilms, as exemplified by coagulase-negative staphylococci on cerebrospinal shunts [10]. During wound healing, vitronectin facilitates mammalian cell adhesion and forms an ECM with other glycoproteins that is attached to mammalian cells through  $\alpha_v\beta_5$  integrins, which are upregulated during inflammation [11]. Although

vitronectin is mainly produced by hepatocytes, it is also released by respiratory epithelial cells and present in the healthy lung [12]. The vitronectin mRNA expression is upregulated in neutrophils derived from patients with CF and the concentration of vitronectin in the airway lumen is increased in patients suffering from sarcoidosis and interstitial lung disease, which may reflect the level of inflammation [13,14].

The goal of this study was to investigate whether *P. aeruginosa* has vitronectin-binding surface receptors, and to evaluate the ubiquity of this interaction by screening clinical isolates. Moreover, we also aimed to identify vitronectin-binding proteins at the surface of *P. aeruginosa*, and to characterize the nature of this interaction in detail. We identified Porin D as the main vitronectin-binding receptor, which is a previously unknown function attributed to this outer membrane protein (OMP).

## 1.2 Material and methods

### 1.2.1 Bacterial strains and culture conditions

Forty-two clinical *P. aeruginosa* isolates and the reference strain PA01 were supplied by the Clinical Microbiology laboratory (Malmö, Sweden) and the Department of Clinical Microbiology at Rigshospitalet (Copenhagen, Denmark) [15]. All isolates were verified as *P. aeruginosa* by using MALDI-TOF. A transposon insertion mutant was obtained from the *P. aeruginosa* two-allele library (Washington university, Seattle, WA): PW2742 oprD-E12::IS<sub>phoA7</sub>/hah [16]. *P. aeruginosa* was grown on blood agar plates or in liquid Lysogeny broth (LB). *E. coli* DH5 $\alpha$  and *E. coli* BL21 (DE3) were also cultured in LB medium supplemented with appropriate antibiotics (Table 1).

### 1.2.2 Vitronectin direct binding assay (DBA)

Vitronectin was labeled with [<sup>125</sup>I] using the Chloramine-T method [17]. One loop of *P. aeruginosa* was taken from a glycerol stock kept at -80°C and grown overnight on blood agar plates. Bacteria were scraped from the plates and resuspended to OD<sub>600</sub>=1.0 in phosphate buffered saline (PBS) containing 1 % bovine serum albumin (BSA). After washing, 15 ng of [<sup>125</sup>I]-vitronectin in 100 µl PBS was added to the bacterial suspension and incubated for 1 h at 37°C. The unbound [<sup>125</sup>I]-vitronectin was removed by 2 washes with PBS. Bacterial surface-bound radioactivity was measured in a Tri-Carb liquid scintillation counter (Perkin Elmer, Waltham, MA).

#### *1.2.3 Outer membrane protein identification with two-dimensional (2D) gel electrophoresis*

Bacterial OMP fractions were prepared from overnight cultures based upon the method of Alteri and Moble and analyzed by 2D-SDS-PAGE [18].

#### *1.2.4 Expression and purification of recombinant proteins*

The full-length gene encoding for Porin D (*oprD*) were amplified from genomic DNA of *P. aeruginosa* PAO1 using primers 5'-CTGAGGATCCGGACGCATTCGTCAGCGATCAGGCC-3' and 5'-CTGACAAGCTTCAGGATCGACAGCGGATAGTCGACGATCAG-3'. The amplified gene products were cloned into the expression vector pET26b (Novagen, Darmstadt, Germany), and used for protein expression and purification [19]. The *oprD* gene was also cloned into pET16b (Novagen) for expression of proteins at the surface of *E. coli* using primers 5'-TATACGCATATGAAAGTGATGAAGTGGAGCGCCATTGCA-3' and 5'-TCAATTGGATCCTTACAGGATCGACAGCGGATAGTCGA-3'. For expression and purification, *E. coli* DE3 (Novagen) with the appropriate vector was used. Vitronectin fragments were expressed in HEK 293T cells and purified by a Ni-NTA resin [18].



### 1.2.5 Antibody production

Two rabbits were immunized with 200 µg of recombinant protein emulsified in complete Freund's adjuvant (CFA; Difco and BD Biosciences, Franklin Lakes, NJ). Booster doses were injected on days 18 and 36 with the same dose of protein in incomplete FA. Blood was drawn three weeks later. Antibodies were purified by CN-bromide agarose conjugated with OprD [20].

### 1.2.6 Enzyme-linked immunosorbent assay (ELISA)

Initial protein-protein interactions were analyzed with ELISA. Proteins (50 nM) were coated in 96-well PolySorp® plates (Thermo Fisher Scientific, Waltham, MA) in coating buffer (100 nM Tris-HCl, pH 9.0) and stored overnight at 4°C. Thereafter a standard protocol was followed [18]. *Haemophilus influenzae* hypothetical protein UHP\_03526 (GI:144986114) was included as a negative control. It is an OMP derived from non-typable *H. influenzae* 3655 and does not bind Vn (unpublished data). UHP\_03526 was expressed and purified using the same method as described for Porin D and was used to exclude the possibility of unspecific binding derived from vector sequences including the Histidine-tag (6 His), or from trace amounts of co-purified *E. coli* contaminants.

### 1.2.7 Porin D-vitronectin affinity measurements

Kinetic analysis was performed by Biolayer interferometry using a forteBio OctetRed96 platform (Pall, Menlo Park, CA). Vitronectin aa 80-396 was immobilized on AR2G sensors (Pall) by amino coupling. The analyte (Porin D) was serially diluted in running buffer (PBS) ranging from 0.016 µM to 1 µM. The experiments were conducted at 30°C. Data analysis was performed using the Fortebio Data Analysis software 8.1 (Pall).

Curves were fitted with 1:1 binding kinetics and the  $K_{\text{ass}}$ ,  $K_{\text{diss}}$ , and affinity ( $K_D$ ) was calculated.

#### 1.2.8 Western blot

OMPs were prepared by resuspending bacteria in 50 nM Tris-phosphate buffer containing 3% Empigen (Calbiochem, Merck Millipore, Darmstadt, Germany) and a protease inhibitor (Complete; Roche, Basel, Switzerland). This suspension was incubated at 37°C with glass beads and end-to-end rotation. Proteins were separated on a NuPAGE 4-12% or 10% Bis-Tris gels (Life technologies, Carlsbad, CA) and blotted as described [18].

#### 1.2.9 Flow cytometry

*E. coli* BL21 (DE3) containing pET16b *oprD* or pET16 without insert (negative control vector) were induced overnight with isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG). Bacterial pellets were washed and resuspended to  $OD_{600}=1.0$  in PBS and incubated with recombinant vitronectin aa 80-396 in PBS-2% BSA followed by addition of an anti-vitronectin monoclonal antibody (mAb) VN58-1 (Abcam, Cambridge, UK). After washes, FITC-conjugated rabbit anti-mouse polyclonal Abs (Dako, Glostrup, Denmark) was added. Finally, samples were analyzed in a flow cytometer EPICS-XL (Beckman Coulter, Pasadena, CA). Gates were set to include 2 % of the background and any reading above this was considered positive. Controls were prepared in the presence of primary and secondary Abs, but in the absence of vitronectin.

#### 1.2.10 Binding of *P. aeruginosa* to immobilized vitronectin

Glass slides were coated with 0.5 µg human plasma vitronectin and air-dried at 37 °C. Bacteria were grown in LB medium, washed and resuspended in PBS (OD<sub>600</sub>=0.5). The slide was submerged in the bacterial suspension and incubated at 37°C for 1 h. After washing and drying, the sample was Gram-stained and the main investigator and one independent researcher counted adherent bacteria in six randomly selected fields.

#### *1.2.11 Statistical analysis*

Comparisons of means were evaluated with unpaired Student's *t*-test. *P*-values ≤ 0.05 and were considered as statistically significant. Statistical analyses were performed using Graph-Pad Prism® version 6.0 (GraphPad Software, La Jolla, CA).

#### *1.2.12 Ethics statement*

Permit (M193-11) was obtained from Malmö/Lund District Court (Djurförsöksetiska nämnden, Lund, Sweden) for immunization of rabbits.

### **1.3 Results**

#### *1.3.1 P. aeruginosa isolated from the respiratory tract of patients with cystic fibrosis have increased vitronectin-binding capacity*

To analyse the vitronectin-binding capacity of clinical *P. aeruginosa* isolates, we performed a DBA with [<sup>125</sup>I]-labeled vitronectin. Isolates from the airway of patients with CF (*n*=27) and blood isolates from bacteremic patients (*n*=15) were selected. Binding of vitronectin was normalized to the reference strain *P. aeruginosa* PAO1 that was set to 1.0. Intriguingly, we found that airway isolates from CF patients bound significantly (*p*=0.025) more vitronectin in comparison to blood isolates (Fig. 1).

### 1.3.2 Proteomics reveals Porin D as a vitronectin receptor of *P. aeruginosa*

To identify vitronectin-binding proteins in the outer membrane of *P. aeruginosa*, OMPs from PAO1 and four selected clinical strains were separated by 2D-SDS-PAGE. A typical gel with PAO1 is exemplified in Figure 2A. Vitronectin was used as a probe and the vitronectin-binding proteins were identified by a far Western blot immunoassay (Fig. 2B). We observed a unique spot corresponding to a putative vitronectin-binding protein with molecular mass of 50 kDa in all strains. This spot was excised from the 2D-gel, analyzed by MALDI-TOF and subsequently identified as Porin D (GI: 15596155, PA0958 according to the *Pseudomonas* Genome Project [21]). This indicated that vitronectin acquisition at the surface of *P. aeruginosa* involved Porin D. The protein was thereafter recombinantly expressed in *E. coli* (Fig. 2C), and used for immunization of rabbits. Resulting pAbs recognized the recombinant protein (Fig. 2D).

### 1.3.3 Recombinant Porin D from *P. aeruginosa* has a high affinity for vitronectin

To confirm that the vitronectin-binding property of the putative vitronectin receptor was not an artifact, protein-protein interactions between vitronectin and recombinant Porin D were analyzed by ELISA. Porin D was coated on microtiter plates and increasing concentrations of vitronectin were added. A dose-dependent binding of vitronectin to Porin D was observed, that is, significantly more vitronectin was bound by Porin D in comparison to the negative control. (Fig. 3A). To further evaluate the kinetics of this interaction, we performed a Biolayer interferometry assay with vitronectin immobilized to the sensors. The interaction was analyzed with Porin D in serial dilutions starting with 1  $\mu$ M. Under these experimental conditions and a vitronectin concentration of 125

nM, the dissociation constant ( $K_D$ ) was calculated to 3.6 nM ( $K_D$  error  $1.09 \times 10^{-10}$ ) (Fig. 3B).

Vitronectin contains three heparin-binding domains (HBD) (Fig. 3C). We have previously shown that *Haemophilus* surface fibrils (Hsf) from *H. influenzae* bind to the HBD3 of vitronectin (amino acids 352-374) [22]. In the present study, we used truncated vitronectin fragments to in detail test the interaction between Porin D and vitronectin (Fig. 3C). Our results implied that Porin D bound to vitronectin between amino acid (aa) sequence 352 to 374, which corresponds to HBD 3 (Fig. 3D). Moreover, we found that heparin completely blocked the binding of vitronectin to Porin D (Fig. 3E), which confirmed involvement of HBDs in the Porin D-dependent vitronectin binding.

#### 1.3.4 Porin D is functional and binds vitronectin at the surface of *E. coli*

The specific function of Porin D was further investigated using *E. coli* as a heterologous host. After transformation, porin D-expressing *E. coli* was analyzed by flow cytometry using anti-Porin D pAbs (Fig. 4A). Porin D was readily expressed at the surface of the *E. coli*-OprD transformant as opposed to the control with an empty expression vector. Vitronectin binding was confirmed by flow cytometry after incubation with increasing concentrations of vitronectin (0-128 nM). A significantly higher vitronectin-binding capacity was observed with *E. coli*-OprD as compared to the negative control (Fig. 4B). Taken together, these results confirmed that Porin D is a surface-exposed protein that significantly attracts vitronectin at the bacterial surface.

#### 1.3.5 Porin D is expressed in clinical *P. aeruginosa* isolates, and a Porin D-transposon insertion mutant has a decreased vitronectin binding capacity

To confirm expression of Porin D in 12 randomly selected clinical isolates, OMPs were isolated followed by Western blot using anti-Porin D pAbs. Four of those are presented in Fig. 5A. As seen in the Coomassie stained SDS-PAGE, approximately equal amounts of proteins were loaded in the gel (Fig. 5A, left panel). Porin D was expressed in the clinical isolates at similar levels as the reference strains tested (Fig. 5A, right panel), except in one strain in which no Porin D was detected (data n.s.). To further elucidate the role of Porin D as a vitronectin-binding protein, we used an *oprD* deficient transposon mutant. Porin D expression was completely abolished in the *P. aeruginosa* *oprD* mutant when compared to the wild type (WT) *P. aeruginosa* MPA01 in Western blot (Fig. 5A, right panel). Importantly, the growth rate of the *oprD* mutant was similar to that of the wild type *P. aeruginosa* MPA01 (Fig. 5B).

To further verify that Porin D in *P. aeruginosa* is important for vitronectin-binding, we analyzed bacteria in a DBA using [<sup>125</sup>I]-vitronectin. The *oprD* mutant bound significantly less [<sup>125</sup>I]-vitronectin than the Porin D-expressing wild type counterpart ( $p \leq 0.001$ ) (Fig. 5C). Moreover, the importance of Porin D-dependent vitronectin binding was further demonstrated when *P. aeruginosa* was added to vitronectin-coated glass slides. By counting the adherent bacteria after rinsing and Gram staining, we found that the *P. aeruginosa* *oprD* transposon insertion mutant adhered significantly less to the vitronectin-coated glass slides in comparison to the wild type strain ( $p \leq 0.001$ ) (Fig. 5D-F). Thus, Porin D mediates *P. aeruginosa* adhesion to soluble and to immobilized vitronectin.

## 1.4 Conclusions

The opportunist pathogen *P. aeruginosa* causes a great burden of disease, particularly to patients with CF. The host–pathogen interactions leading to the first stage of infection have not yet been fully elucidated. In the present study, we have shown that *P. aeruginosa* isolates bind the human ECM protein vitronectin and that isolates from the airway of CF patients have stronger vitronectin-binding phenotypes than isolates obtained from the blood stream. Moreover, we identified Porin D as the first known vitronectin-binding protein on the surface of *P. aeruginosa*.

Vitronectin is available in the lower respiratory tract as it is produced by respiratory epithelial cells. This production is upregulated in the airway of CF patients [13,23]. Despite adhesion may promote the host response and clearance, our observations suggest that vitronectin-dependent adhesion may be a virulence strategy promoting colonization of the airway of CF patients. Similar mechanisms are used by e.g. *Streptococcus pneumoniae* [24]. For *P. aeruginosa*, we speculate that adhesion is of importance at least during initial colonization and exacerbations.

To identify vitronectin-binding proteins in the outer membrane of *P. aeruginosa*, we used a proteomic approach with 2D-SDS PAGE using vitronectin as a probe. Following this, we confirmed the specific interaction between recombinant Porin D and vitronectin by ELISA and calculated the dissociation constant to 3.6 nM, which indicates a strong binding affinity. Surface expression of Porin D was confirmed in the outer membrane of several clinical *P. aeruginosa* isolates by Western blotting. Moreover, the vitronectin-binding function of Porin D was verified by heterologous expression on the surface of *E. coli*. Importantly, the vitronectin binding capacity was significantly

reduced in the Porin D deficient mutant, for which the interaction with vitronectin was decreased both with soluble vitronectin and immobilized vitronectin. Our current data thus clearly indicate that Porin D is a strong vitronectin-binding protein of *P. aeruginosa*. Vitronectin-binding is, however, likely to be multifactorial. We did not observe a complete reduction of the vitronectin binding capability in the transposon mutant and hence we would expect *P. aeruginosa* to possess other vitronectin-binding proteins. Consequently, it is likely that the clinical strains express multiple ECM binding proteins.

Though *P. aeruginosa* often exists in sputum plaques, adhesion to the epithelial surface is likely an important step for successful colonization of the airway. It has previously been shown that vitronectin bridges the *P. aeruginosa* surface and  $\alpha_v\beta_5$ -integrins of host lung epithelial cells, an interaction that ultimately contributes to the adherence and internalization of bacteria [7]. Supporting this observation, we were able to show that *P. aeruginosa* interacts with vitronectin at the C-terminal (HBD-3), leaving the RGD motif at the N-terminal free to bind integrins [25]. Recruitment of vitronectin by Porin D on the surface of *P. aeruginosa* may thus contribute to adherence to not only the ECM but also indirectly to the epithelial cell surface by using vitronectin as a bridging molecule.

Porin D is an OMP of approximately 50 kDa that is also known as OprD, PA0958, occD1 or Outer membrane protein D2. The crystal structure of Porin D has recently been solved [26]. Following the introduction of imipenem in clinical practice in the late 1980s, Porin D was identified as a channel for basic amino acids and imipenem [27]. Mutations in Porin D of *P. aeruginosa* have been shown to increase the bacterial resistance to imipenem, and worldwide it is one of the most commonly observed imipenem resistance mechanisms [28]. Porin D consists of 9 loops of which loops 2 and 3 have been most extensively studied. Deletions of loops cause conformational changes



that lower the sensitivity to imipenem. It has recently been reported that *oprD* deficient strains had an increase in *in vivo* fitness based on gastrointestinal tract colonization and systemic dissemination [29]. The mechanisms behind systemic dissemination from the gastrointestinal tract are likely to be different to the mechanisms promoting local colonization of the respiratory tract, which is why our results are not conflicting. We hypothesize that OprD binding to Vn is of biological importance for local colonization of the respiratory tract.

In conclusion, we found that clinical isolates from the airways of CF patients bind more vitronectin in comparison to isolates cultured from blood. Furthermore, we identified Porin D as the first known surface protein of *P. aeruginosa* that binds vitronectin. Our study sheds light upon the interaction between *P. aeruginosa* and the human ECM protein vitronectin. The findings also pave the way for further studies that aim to analyze the importance and function of this interaction in bacterial pathogenesis.

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## Legends

### FIGURE 1. *P. aeruginosa* binds vitronectin.

Clinical isolates **bind** vitronectin as determined by an [<sup>125</sup>I]-vitronectin DBA. *P. aeruginosa* from the lower airway of patients with cystic fibrosis ( $n=27$ ) and isolates cultured from blood from patients with bacteraemia ( $n=15$ ) were included. Binding (cpm) of replicate values were normalised to PAO1. **Mean value of airway isolates: 1.37  $\pm$ SEM 0.12, blood isolates: 0.98  $\pm$ SEM 0.12,  $p=0.025$ .** Error bars indicate the minimum and maximal values. Each experiment was repeated three times with triplicates.

### FIGURE 2. Porin D is identified as a vitronectin-binding surface protein of *P. aeruginosa*.

(A) The outer membrane proteome of PAO1 was separated by 2D-SDS-PAGE (pH 4-7) and stained with Coomassie-blue (left panel). (B) Another gel was in parallel blotted to a PVDF membrane and vitronectin binding was determined by Far-Western blotting using vitronectin as bait (right panel). The arrows point at a spot corresponding to a 50 kDa vitronectin-binding protein, which was subsequently identified by MALDI-TOF as Porin D. (C) Recombinant Porin D (5  $\mu$ g) was separated on a SDS-PAGE and stained with Coomassie-blue. (D) An identical gel as in (C) was blotted to a PVDF membrane that was incubated with anti-Porin D pAbs followed by incubation with HRP-conjugated secondary pAbs.

### FIGURE 3. Porin D interacts with vitronectin at the C-terminal HBD 3.

(A) Vitronectin bound to Porin D as shown by ELISA. *H. influenzae* UHP\_03526 was used as a negative control. Increasing concentrations of recombinant vitronectin aa 80-396 was added and bound vitronectin was detected by an anti-vitronectin mAb. (B) Kinetic

analysis was performed with Biolayer interferometry (Octet Red96). (C) Truncated vitronectin fragments were recombinantly expressed and purified by Ni-NTA affinity chromatography. The integrin binding domain (RGD) domain and heparin-binding domains (HBP) 1-3 are denoted in the figure. (D) ELISA showing binding of truncated vitronectin fragments (50 nM) to Porin D. (E) The interaction between Porin D and vitronectin was inhibited by heparin. Vitronectin at 50 nM was added together with increasing concentrations of heparin. In (A) and (D), mean values and SEM of three independent experiments are presented.

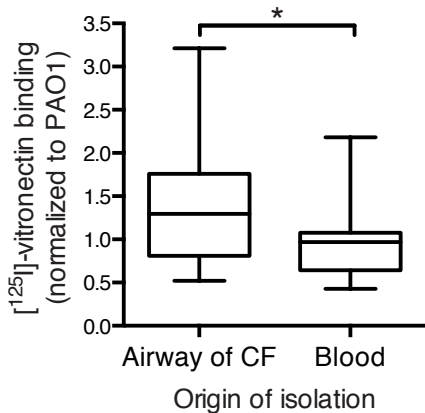
**FIGURE 4. Porin D expressed at the surface of *E. coli* binds vitronectin.**

(A) *E. coli* transformed with pET16-*oprD* expressed Porin D at the surface. *E. coli*-OprD was analyzed by flow cytometry after labeling with anti-Porin D pAbs. *E. coli* with an empty vector (pET16b) was used as a negative control. (B) Porin D was functionally active and bound more vitronectin at the bacterial surface than the control ( $p=0.037$  at vitronectin 64 nM). Mean values and SEM of three independent experiments are shown.

**FIGURE 5. Porin D is important for vitronectin binding in *P. aeruginosa*.**

(A) Analysis of Porin D expression in *P. aeruginosa* PAO1, four clinical *P. aeruginosa* isolates and confirmation of the *oprD* transposon insertion mutant. The *oprD* mutant is compared to the wild type (WT) counterpart *P. aeruginosa* MPAO1. OMP were loaded on NuPAGE 4-12% Bis-Tris gels that were Coomassie stained (*left*) and analyzed by Western blot (*right*) using anti-Porin D pAbs. The localization of Porin D is marked with an arrow. One representative experiment out of three performed is shown here. (B) Growth curve of *P. aeruginosa* WT (MPAO1) and the *oprD* insertion mutant cultured in LB medium. (C) The vitronectin-binding capacity of the *P. aeruginosa* *oprD* insertion

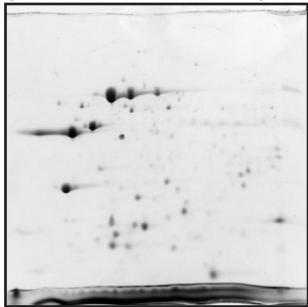
mutant and corresponding WT as determined by DBA using [<sup>125</sup>I]-vitronectin. CPM values were normalized to *P. aeruginosa* WT and presented as mean ± SEM of three separate experiments. **(D)** Adhesion of the *P. aeruginosa* WT and *oprD* mutant to immobilized vitronectin (0.5 µg) on glass slides. The bar diagram represents mean and the error bars SEM of three independent experiments. **(E-F)** Raw data for the results shown in panel D. Adherent bacteria were Gram stained and representative pictures were taken at 100x amplification.



**A**

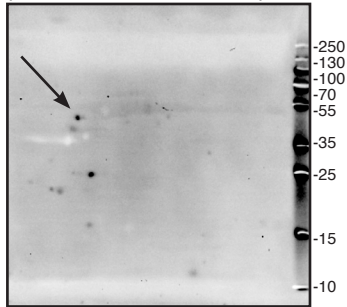
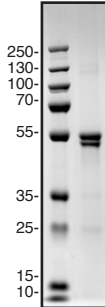
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pH 7

**B**

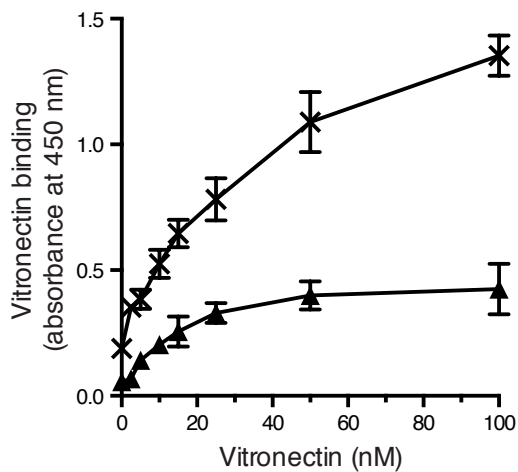
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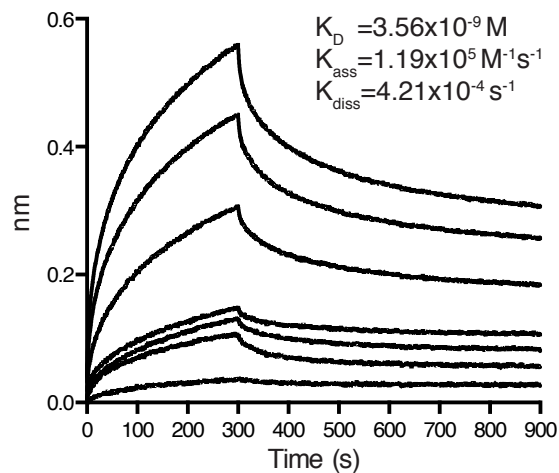
**C****D**



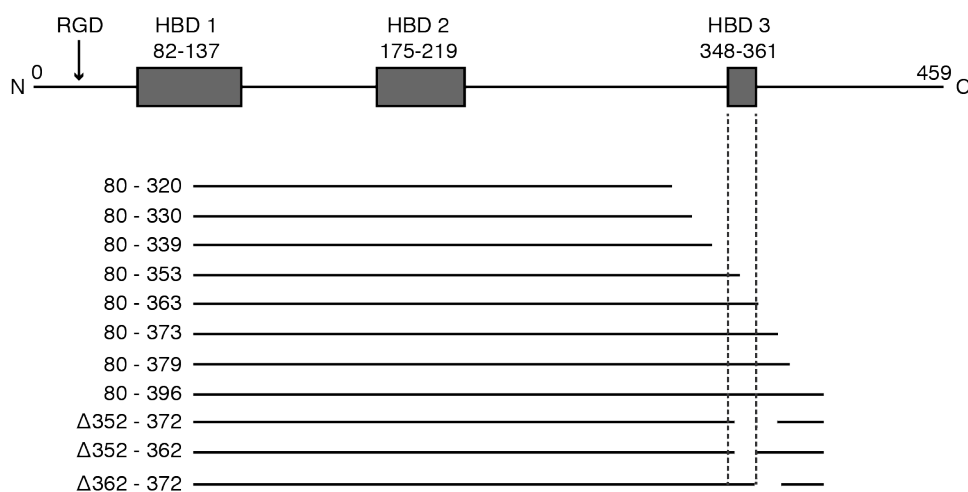
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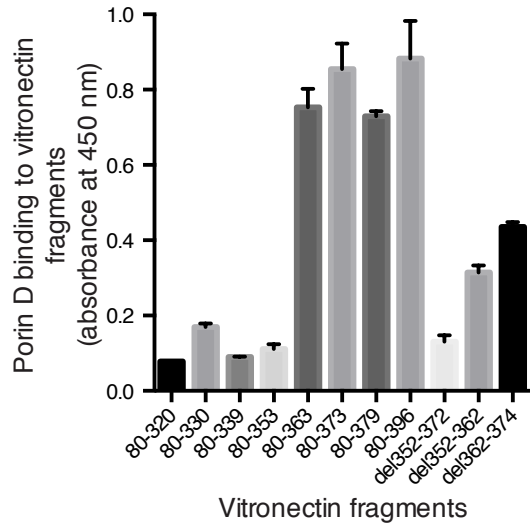
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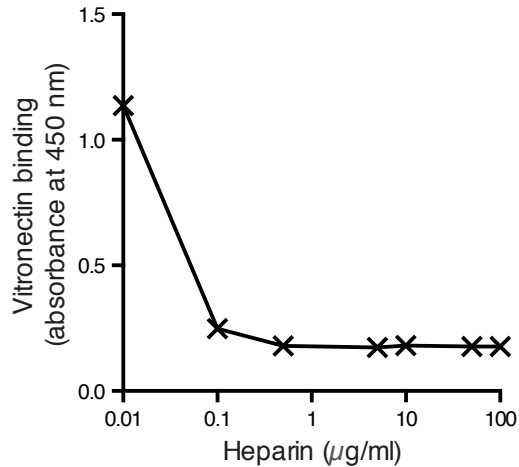
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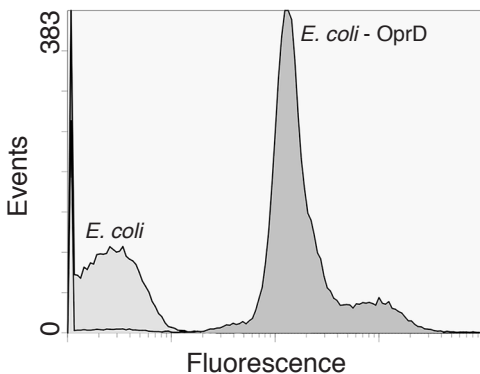
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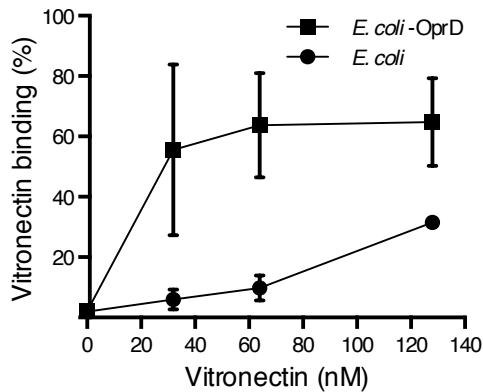
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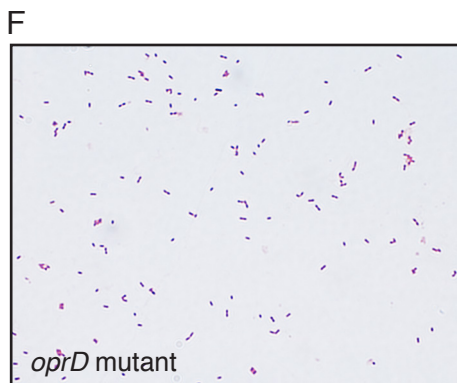
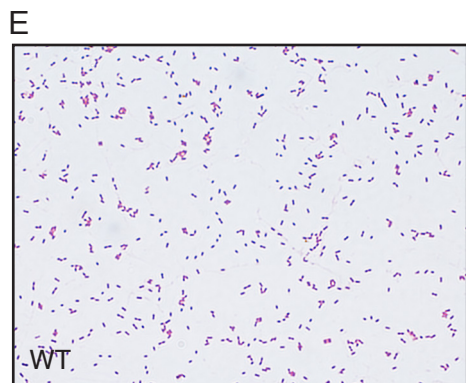
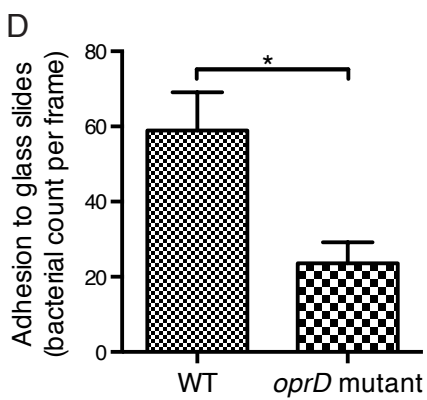
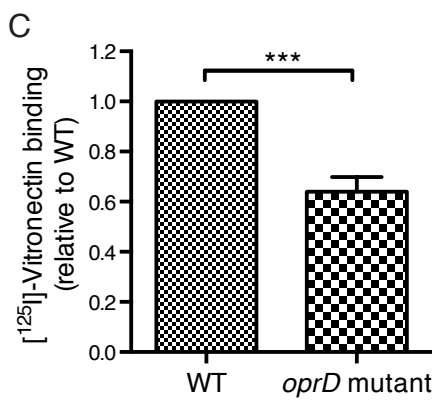
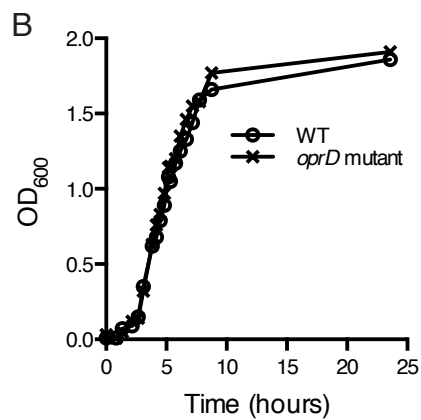
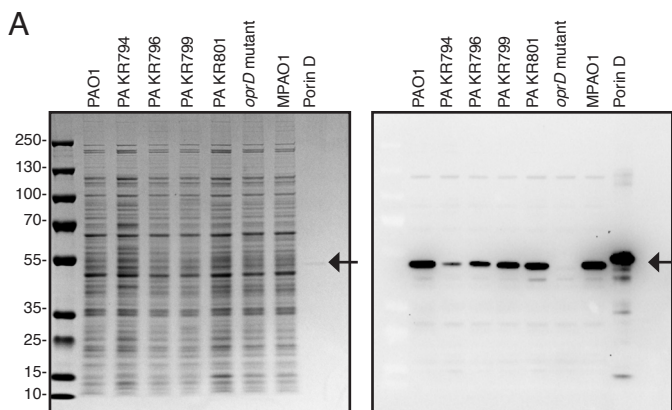


A



B





**TABLE 1.** Clinical *Pseudomonas aeruginosa* isolates and laboratory strains used in the present study.

<b><i>Name</i></b>	<b><i>Description/genotype</i></b>	<b><i>Reference</i></b>
<b>Clinical isolates</b>		
PA KR794	Urine isolate	This study
PA KR796	Airway isolate from patient with CF	This study
PA KR799	Blood isolate	This study
PA KR801	Airway isolate from patient with CF	This study
<b>Laboratory strains</b>		
PAO1	<i>P. aeruginosa</i> reference strain	[15]
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> laboratory strain	[19]
<i>E. coli</i> DH5 $\alpha$	<i>E. coli</i> laboratory strain	
<i>E. coli</i> - OprD	<i>E. coli</i> BL21(DE3) pET16b- <i>oprD</i>	This study
MPAO1	<i>P. aeruginosa</i> reference strain. Clone of PAO1	
	used in two allele transposon library.	[16]
PW2742 <i>oprD</i> -	MPAO1 mutant with transposon insert in <i>oprD</i>	
E12::IS <i>phoA7</i> /hah		[16]