

## Surfactant protein A recognizes outer membrane protein OprH on *Pseudomonas aeruginosa* chronic infection isolates.

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**ABSTRACT**

Surfactant protein A (SP-A) plays a critical role in the clearance of *Pseudomonas aeruginosa* from the lung. However, there is limited information about the interaction of this protein with *P. aeruginosa* cystic fibrosis (CF) isolates. We characterized the interplay between SP-A and a collection of isogenic sequential isolates from seven CF patients. We identified outer membrane protein OprH as a novel ligand for SP-A on *P. aeruginosa*. CF late isolates bound significantly less SP-A than their respective early isolates. This difference could be associated with a reduction in the expression of OprH. Binding of SP-A to OprH promoted phagocytic killing, thus late CF isolates were at least two-fold more resistant to SP-A mediated killing by human macrophages than their respective early isolates. We postulate that reduction of OprH expression is a previously unrecognized adaptation of *P. aeruginosa* to CF lung that facilitates the escape of the microorganism from SP-A-mediated phagocytic killing.

## INTRODUCTION

*Pseudomonas aeruginosa* is a major opportunistic pathogen and a leading cause of mortality and morbidity in patients with cystic fibrosis (CF). In these patients, after a period of intermittent colonization, the microorganism becomes permanently established and is rarely eliminated, despite an exuberant host inflammatory response [1]. One of the most striking features of the chronic lung infections by *P. aeruginosa* is that the establishment of the infection correlates with the display of a wide spectrum of colony variants. In particular, *P. aeruginosa* isolated from chronic lung infections includes otherwise isogenic variants that can be mucoid, dwarf, nonmotile, nonflagellated, lipopolysaccharide (LPS) deficient, auxotrophic, or resistant to antibiotics [2-4]. It is likely that this wide range of phenotypes is a result of the continuous adaptation of the microorganism to evade the detection and clearance by host defense mechanisms during the chronic phase of CF lung infection.

Surfactant protein A (SP-A), a large oligomeric protein member of the collectin subgroup of the mammalian C-type lectins, is a main component of the innate immunity in the lung. A number of studies have shown that SP-A contributes to killing of *P. aeruginosa* by several mechanisms. SP-A enhances pulmonary clearance of *P. aeruginosa* by stimulating phagocytosis by alveolar macrophages and by modulating the inflammatory response in the lungs [5, 6]. Accordingly, *P. aeruginosa* is cleared more readily from the lung of wild-type mice than from the lung of SP-A deficient mice [6]. In addition, SP-A directly inhibits the growth of the microorganism by permeabilizing the bacterial membrane [7].

Despite considerable evidences that SP-A has a role in host defense against *P. aeruginosa*, there is limited information about the ligand/s for this collectin on *P. aeruginosa* surface. It is generally accepted that, in Gram-negative bacteria, SP-A binds to the lipid A domain [8]. Hickling et al. demonstrated that binding of SP-A to *P. aeruginosa* purified LPS could be inhibited by 70% by competition with free lipid A [9]. Furthermore, SP-A binds preferentially to rough LPS strains containing truncated or absent O-antigen domains [8], a common feature among *P. aeruginosa* from CF patients. Other than these observations, there are few studies about the interaction of SP-A with *P. aeruginosa* chronic infection isolates. In the present study, we characterize the interaction between SP-A and a collection of chronic isogenic sequential isolates from CF patients in order to identify the SP-A-binding molecules present on the cell surface of *P. aeruginosa* and the mechanism adopted by these isolates to resist the SP-A mediated killing. As a result of this investigation we identify the outer membrane protein OprH as a new SP-A binding protein that promotes binding of SP-A and opsonophagocytosis of *P. aeruginosa* chronic infection isolates by human macrophages.

## MATERIALS AND METHODS

### Bacterial strains

The clinical isolates used in this study and their relevant features are listed in Table 1. They belong to a larger collection previously described [10]. The set of strains studied included the first available isolate and the last available isolate (when the project was initiated) from 7 CF patients who attended Hospital Son Espases, the reference hospital of the Balearic Islands, Spain. Each one of the isolates from the same patient included in the study were separated by at least a 3-year interval. All CF isolates were LPS O chain side deficient as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

*P. aeruginosa* reference strain PAO1 and its derived isogenic OprH deficient mutant PAO1 $\Delta$ OprH [11] were also used in this study. *Escherichia coli* strain S17- $\lambda$ pir was used in the cloning experiments.

Bacterial cells were grown in Luria Bertani (LB) (Scharlau) broth at 37°C with shaking or solidified with 1.5% agar.

### Purification and labeling of surfactant protein A

Native human SP-A was purified from bronchoalveolar lavage from patients with alveolar proteinosis following the method previously described [12]. Briefly, human bronchoalveolar lavage was made up to a final concentration of 20 mM Tris-HCl and 10 mM EDTA, pH 7.4 and centrifuged at 10,000 X *g* for 40 minutes at 4°C. Pellet was resuspended in 20 mM Tris HCl, 10 mM EDTA and 6 M Urea pH 7.4 and dialyzed against 20 mM Tris-HCl, 100 mM NaCl and 5 mM EDTA. The solution was then made up at 15 mM with respect to CaCl<sub>2</sub> and the content

of the sample separated by affinity chromatography. The SP-A eluted was then further purified via high resolution chromatography on a Superose-6 column (GE Healthcare). Purity of SP-A was confirmed by SDS-PAGE and Coomassie blue staining. Western blot analysis with antibodies specific for SP-A detected the same bands observed in the Coomassie blue stained gel. SP-A concentration was measured using the Coomassie-staining protein assay kit following the manufacturer's instructions (Biorad). We used the same stock of SP-A for all experiments.

SP-A was labeled with the Infrared Dye 800CW using the IRDye 800CW protein labeling kit (LI-COR) following the manufacturers' instructions.

### **SP-A binding assays**

Binding of SP-A to bacterial cells was determined using Infrared Dye 800CW conjugated SP-A. Briefly,  $2 \times 10^8$  bacteria were washed with phosphate-buffered saline (PBS) and incubated for 1 hour with agitation at 37°C with SP-A (250 ng/ml) in PBS containing 0.15 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{MgCl}_2$ . Cell-bound SP-A was determined, after exhaustive washing of the bacteria, using the Odyssey Infrared Imaging System.

To identify the SP-A binding proteins from *P. aeruginosa*, outer membrane proteins were resolved as describe below and transferred to INMOBILON-P membranes (Millipore). After transfer, membranes were blocked for 2 hours at room temperature with 1% bovine serum albumine (BSA) in PBS and incubated 1 hour with Infrared Dye 800CW conjugated SP-A (5  $\mu\text{g/ml}$ ) diluted in PBS-1% BSA. The membranes were subsequently washed and visualized with the Odyssey Infrared Imaging System.

### **Isolation, analysis, and identification of outer membrane proteins.**

Isolation of outer membrane proteins (OMP) were performed as previously described [13]. Cell envelopes were isolated from *P. aeruginosa* strains by centrifugation at 100,000 x *g* for 1 hour at 4°C after French press cell lysis. OMP were isolated as sodium lauryl sarcosinate-insoluble material, resuspended in Laemmli buffer, boiled for 5 minutes, resolved by SDS-PAGE and visualized by Coomassie blue staining. Gels were scanned with a Bio-Image densitometer (Millipore). Selected protein spots were excised from the gels, trypsin digested, and identified by tandem mass spectrometry, as described elsewhere [14]. The search for filtered peptides was performed using GPS Explorer v3.5 software with a licensed version 1.9 of MASCOT.

### **Phagocytosis assays**

Phagocytosis assays were performed using THP-1 cells (ATCC TIB- 202). THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% HEPES and 1% penicillin and streptomycin at 37°C and 5% CO<sub>2</sub>. Twenty four hours before use in experiments, cells were differentiated into adherent, well-spread macrophages by the addition of 1 nM phorbol myristate acetate (Sigma).

The phagocytosis rate of *P. aeruginosa* was determined as previously described [15]. Briefly, approximately  $5 \times 10^6$  bacteria cells were preincubated for 1 hour with agitation at 37°C with SP-A (5 µg/ml for CF isolates and 50 µg/ml for PAO1) in RPMI. The resulting mixture was then incubated with the differentiated THP-1 cells at a ratio of 10:1. After 1 hour at 37°C, the cells were exhaustively washed and finally lysed with 1% Triton X-100 solution. Bacteria

were quantified by plating appropriate dilutions on LB agar plates. The ratio of colony-forming units (CFU) between SP-A treated and untreated bacteria was computed for the fold of phagocytosis mediated by SP-A.

### DNA procedures

Sequencing of the *oprH-phoP-phoQ* operon from the clinical isolates FQSE12-1007 and FQSE12-1110 was done using Illumina technology. For the cloning of *phoP*, the gene was amplified by PCR from the genome of PAO1 using primers OPRHF1 (5'- CCTACGAGAACATCTCCGAC-3') and PHOQR1 (5'- CCTTGGCCTCCGGCAGGTTG-3') and the resulting PCR product was digested with PstI. A 0.9 Kb fragment containing *phoP* was cloned into the pBBR1MCS vector [16] to give plasmid pBBPhoP. Similarly, for the cloning of *oprH*, the gene was amplified as above using primers OPRHF2H (5'- GCAAACCTCGCCGAGCCGG-3') and OPRHR2B (5'- GGTGGTATTTCGCTGACCCGG-3') containing HindIII and BamHI sites, respectively. The resulting PCR product was digested with both enzymes and cloned into pBBR1MCS to give plasmid pBBOprH. Plasmids pBBPhoP and pBBOprH were transformed into *P. aeruginosa* or *E. coli* S17- $\lambda$ pir by electroporation. All molecular biology techniques were performed according to standard protocols as described previously [17].

## RESULTS

### **Binding of surfactant protein A to *P. aeruginosa* chronic infection isolates.**

To study the direct SP-A-binding capacity of a collection of chronic infection isolates sequentially isolated from different CF patients, we performed binding assays with Infrared Dye 800CW (IRD800CW) labeled SP-A. We observed a wide range of binding efficiencies among isolates, although the results obtained in different experiments were quite reproducible. We detected a significant reduction in the binding of SP-A in four of the late isolates compared to the early ones (Fig. 1). Thus, the late isolates of patients FQSE5, FQSE11, FQSE12 and FQSE15 bound 1.5 to 2.5 fold less SP-A than their respective early isolates. This phenomenon was predominant among the chronic isolates but not general, because we did not observe differences in the SP-A binding capacity between the early and late isolates of the rest of the patients.

### **Identification of the SP-A-binding protein of *P. aeruginosa*.**

To identify the SP-A binding protein of *P. aeruginosa*, outer membrane preparations were subjected to ligand blot analysis. OMPs from all clinical isolates were loaded on two parallel gels, one was stained with Coomassie blue (Fig. 2A) and the other one was transferred and incubated with IRD800CW labeled SP-A (Fig. 2B). A protein of approximately 21-kDa was recognized by SP-A in the OMPs of all strains. To identify the 21-kDa protein, the corresponding band was excised from the gel and the protein was subjected to mass spectrometry analysis. In all isolates, the band was found to correspond to the outer membrane protein OprH. To verify the identification of the 21-kDa SP-A binding protein, outer membrane preparations of PAO1 and its derived isogenic OprH-deficient mutant PAO1 $\Delta$ OprH were analyzed by ligand blotting

as described above. The 21-kDa protein from the outer membrane preparation of PAO1 reacted with SP-A, unlike purified OMPs from PAO1 $\Delta$ OprH, where no band was detected (Fig. 2B). Altogether these data indicate that outer membrane protein OprH binds SP-A.

### **OprH promotes binding of SP-A to *P. aeruginosa* chronic infection isolates.**

To investigate whether the differences in the binding of SP-A between the early and the late chronic infection isogenic isolates might be associated to changes in the expression of OprH, the amount of OprH present in the outer membranes of the chronic infection isolates were analyzed by SDS-PAGE (Fig. 2A) and densitometry (Fig. 3). The outer membranes of the early isolates from patients FQSE 11, 12 and 15 contained larger amounts of OprH than that of the late isolates. Densitometric analysis of the intensity of the band of OprH normalized for the band of OprF of four independent gels demonstrated that FQSE11-0603 and FQSE15-0803 produce  $1.8 \pm 0.02$  fold and  $2.1 \pm 0.09$ -fold more OprH than FQSE11-1010 and FQSE15-0110, respectively ( $P < 0.05$ , two-tailed  $t$  test). This difference was more dramatic between the isogenic isolates from patient FQSE12, where the expression of OprH in the late isolate FQSE12-1110 was completely abolished. There were no differences in the levels of OprH between the early and the late isolate from patient FQSE05 which exhibited a reduced binding of SP-A, suggesting that other bacterial components modulate this interaction in these isolates. In addition, we did not detect differences between the early and the late isolates from patients FQSE16, 21 and 24.

Sequence analysis of the *oprH-phoP-phoQ* operon from FQSE12-1110

detected two single nucleotide polymorphisms in the *phoP* gene that were not present in the early isolate FQSE12-1007 (Table S1), one of them leading to a missense mutation (N188S). Given that the expression of the response regulator PhoP is an absolute requirement for *oprH* transcription, FQSE12-1110 is unable to express OprH under either high- or low-Mg<sup>2+</sup> growth conditions (Fig. S1).

Interestingly, sequence analysis of the *oprH-phoP-phoQ* operon in the early isolated FQSE12-1007 identified an insertion of one nucleotide in *phoQ* which disrupted the translation of the sensor protein PhoQ leading to the overexpression of OprH in the presence of either high or low concentrations of Mg<sup>2+</sup> in the media (Table S1 and Fig. S1).

Sequence analysis of the *oprH-phoP-phoQ* operon from the early isolates FQSE11-0603 and FQSE15-0803 and their corresponding late isolates FQSE11-1010 and FQSE15-0110 which exhibited reduced binding of SP-A and decreased expression of OprH revealed no differences.

To confirm that OprH promoted the binding of SP-A to FQSE12-1007, we cloned the *phoP* gene from PAO1 in the OprH-deficient isolate FQSE12-1110. Cloning of *phoP* led to an overexpression of OprH (Fig. 4A) and increased the binding of SP-A to the same levels observed in the early isolate FQSE12-1007 (Fig. 4B). Given that PhoP may also affect the expression of other genes, we complemented the OprH-deficient isolate FQSE12-1110 with only the *oprH* gene from PAO1. Cloning of *oprH* restored the expression of OprH in FQSE12-1110 but not to the same levels seen in the early isolate FQSE12-1007 (Fig. 4A), perhaps because of the *phoP* mutation mentioned above. Accordingly, the binding of SP-A to FQSE12-1110 complemented with *oprH* did not increase

significantly compared to FQSE12-1110 (Fig. 4B).

To further confirm this result, we performed SP-A binding experiments with PAO1, its derived isogenic OprH deficient mutant PAO1 $\Delta$ OprH, and the PAO1 $\Delta$ OprH complemented with *oprH*. Since binding of SP-A to PAO1 was almost six-fold less efficient than to the chronic isolate FQSE12-1007 (data not shown), binding experiments were performed using a concentration of SP-A three times higher than that used for the CF isolates. Both PAO1 and the mutant complemented with *oprH* bound SP-A almost two-fold more efficiently than the OprH-deficient mutant (Fig. 4C).

Overall, these results indicate that OprH mediates the binding of SP-A to *P. aeruginosa*.

#### **Binding of SP-A to OprH promotes phagocytosis of *P. aeruginosa* chronic infection isolates.**

Since OprH on the *P. aeruginosa* surface binds SP-A, and SP-A serves as a potent opsonin, we determined whether OprH-SP-A interactions promote the phagocytosis of *P. aeruginosa* by human macrophages. Early isolates FQSE15-0803 and FQSE12-1007 and their corresponding isogenic late isolates FQSE15-1110 and FQSE12-1110 were opsonized with SP-A and incubated with human macrophages. Unattached bacteria were washed extensively and bacterial association and internalization assessed by plating on LB plates. Incubation with SP-A increased the phagocytosis of early isolates FQSE15-0803 and FQSE12-1007 but had reduce or no effect on the late isolates FQSE15-1110 and FQSE12-1110 (Fig. 5). Furthermore, phagocytosis of the FQSE12-1110 strain complemented with *phoP* was similar to that observed with the early isolate FQSE12-1007 and almost two fold more effective compared

with that of the late isolate FQSE12-1110. Similarly, SP-A increased the phagocytosis of PAO1 almost two fold but had a reduced effect on the OprH-deficient mutant. Thus, these results indicate that the interaction OprH-SP-A promotes the phagocytosis of *P. aeruginosa* CF isolates by human macrophages.

## DISCUSSION

SP-A plays a critical role in the clearance of *P. aeruginosa* from the lung. SP-A mediates the killing of this pathogen by increasing the permeability of the bacterial membrane and facilitating the recognition by the alveolar macrophages. SP-A binds preferentially to rough LPS strains containing truncated or absent O-antigen domains, a common feature among *P. aeruginosa* chronic infection isolates. Nonetheless, the interaction of SP-A with these isolates remains poorly investigated. In the present study, through a combination of approaches, including ligand blot, mass spectrometry and genetic loss of function/gain of function studies, we demonstrate that outer membrane protein OprH is a ligand for SP-A on the surface of *P. aeruginosa* from CF patients that mediates the binding of SP-A to intact bacterial cells and promotes the uptake of *P. aeruginosa* by human macrophages. Our results are consistent and extend those reported by Tan et al. which suggested that OprH might serve as SP-A ligand [18]. Another outer membrane protein, P2 of *Haemophilus influenzae*, have also been shown to bind SP-A [19]. Thus, it is likely that lipid A is not the only ligand for SP-A on the surface of the Gram negative organisms.

It would be interesting to know whether OprH also mediates the binding of SP-A to *P. aeruginosa* isolates from acute respiratory infections that usually display a complete LPS. However, our results using the reference strain PAO1 (serotype O5) suggest that OprH could be involved in this interaction, but only at high concentrations of SP-A, probably due to the barrier effect of the O side chain of the LPS.

OprH is repressed during growth in  $Mg^{2+}$  concentrations of approximately 2 mM or greater, a concentration that is similar to the physiologic  $Mg^{2+}$  levels in the lung [20]. Indeed, a global transcriptional analysis of differentially regulated genes in *P. aeruginosa* isolated from human sputum samples demonstrated that *oprH* was downregulated [21]. However, the low concentrations of  $Mg^{2+}$  at certain mucosal sites of the CF lung and in biofilms ensure that PhoQ will phosphorylate PhoP thereby permitting high-level expression of OprH. In fact, Mulcahy et al. demonstrated that expression of the *oprH-phoP-phoQ* operon is induced in the biofilm matrix, where the extracellular DNA chelates divalent cations such as  $Mg^{2+}$  [22]. Furthermore, Gellatly et al. provided evidence that *oprH* is rapidly transcribed after interaction of *P. aeruginosa* with human airway epithelial cells [23].

Altogether, these findings suggest that during the infection of the CF lung, sensing of  $Mg^{2+}$  by PhoQ may be an essential mechanism to reduce expression of OprH in planktonic cells to avoid the SP-A-OprH mediated killing. Once in the biofilm, although OprH may be overexpressed, *P. aeruginosa* remains protected against the humoral immune effectors such as SP-A.

Supporting this postulate we found that the mutation of the sensor PhoQ in the early isolate from patient FQSE12, which induces the constitutive expression of

PhoP and OprH and is involved in the resistance to colistin and polymixin [10, 24], forced the *in vivo* selection of a *phoP* mutant (late isolate) where the expression of OprH was completely abolished. Thus, it is likely that the accumulative mutations detected in *phoQ* and *phoP* in the Liverpool epidemic isogenic variant FQSE12-1110 resulted from both, the strong selective pressures exerted by the colistin treatment and the SP-A mediated effects. A similar feature was observed in three isolates from a Danish CF patient which exhibited mutations in both *phoQ* and *phoP* [24]. According to this idea and, given the crucial role of the SP-A in the host defense against *P. aeruginosa* infections, *phoQ* mutants will emerge rarely.

In conclusion, we have shown that OprH is a novel *P. aeruginosa* SP-A-acceptor molecule on CF isolates. We postulate that reduction of OprH expression is a previously unrecognized adaptation of *P. aeruginosa* to CF lung that facilitates the escape of the microorganism to the SP-A-mediated phagocytic killing.

**Potential conflicts of interest:** None to declare

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**Table 1.** *P. aeruginosa* sequential isolates from cystic fibrosis patients used in this study

Patient	Isolate	PFGE Clone (a)	MLST (b)	Isolation date	Relevant features
FQSE05	0403	E	1108	Apr 2003	New Sequence Type
	0111	E	1108	Jan 2011	
FQSE11	0603	K	701	Jun 2003	New Sequence Type
	1010	K	701	Oct 2010	
FQSE12	1007	B	146	Oct 2007	MDR Liverpool Epidemic Strain (LES-1)
	1110	B	146	Nov 2010	
FQSE15	0803	A	274	Aug 2003	Detected in CF patients in Australia, Austria and France
	0110	A	1089*	Jan 2010	
FQSE16	0803	M	1073	Aug 2003	New Sequence Type mutator
	0910	M	1073	Sep 2010	
FQSE21	1003	H	1088	Oct 2003	New Sequence Type
	0410	H	1088	Apr 2010	
FQSE24	0304	A	1089	Mar 2004	Detected in CF patients in Australia, Austria and France
	1010	A	1089	Oct 2010	

a) Clonal relatedness evaluated by Pulsed Field Gel Electrophoresis (PFGE)

b) Clonal relatedness evaluated by Multilocus Sequencing Typing (MLST)

\* ST1089 derives from ST274 [10].

**FIGURE LEGENDS**

**Figure 1.** Binding of surfactant protein A to chronic infection *P. aeruginosa* isolates.

Purified human surfactant protein A (SP-A) was labeled with IRDye800CW and incubated with  $2 \times 10^8$  bacterial cells of each isolate. Binding of fluorescent SP-A to each bacterial strain was measured after exhaustive washing of the cells. Data represents four experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were done with Student's unpaired two-tailed *t* test; \*  $P < 0.05$ .

**Figure 2.** Surfactant protein A binding analysis of *P. aeruginosa* chronic infection isolates outer membrane proteins.

Outer membrane proteins from each isolated, PAO1 and its derived OprH-deficient mutant PAO1 $\Delta$ OprH, were isolated, resolved, stained with Coomassie blue (A) or transferred to a membrane and incubated with IRD800CW labeled surfactant protein A (SP-A) (B). A band of approximately 21-kDa, identified by mass spectrometry analysis as OprH, reacted with the fluorescent SP-A.

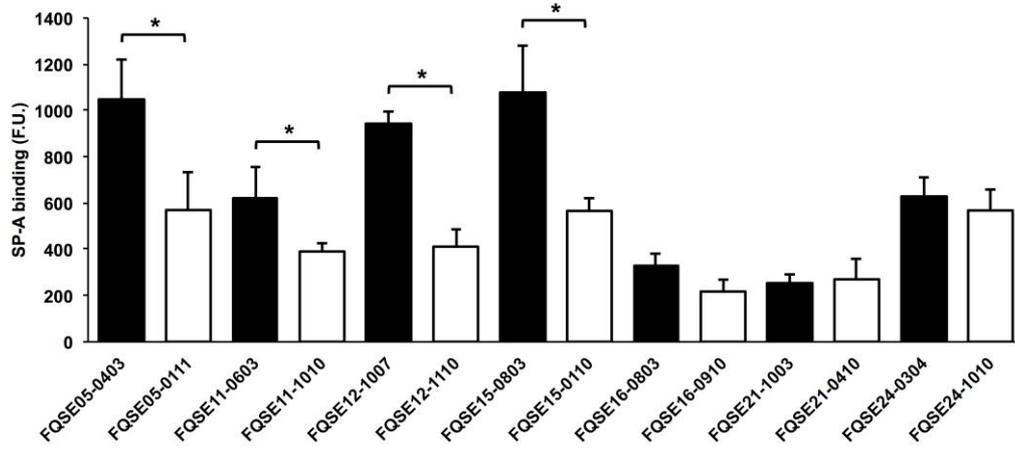
**Figure 3.** Densitometric analysis of the presence of OprH in *P. aeruginosa* chronic infection isolates. Outer membranes were isolated, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, stained with Coomassie blue and the intensity of the OprH band was analyzed by densitometry and normalized by the intensity of the OprF band. Results are of four independent experiments. Errors bars represent SEMs. Statistical analyses were done with Student's unpaired two-tailed *t* test; \*  $P < 0.05$ ; \*\* $P < 0.001$ .

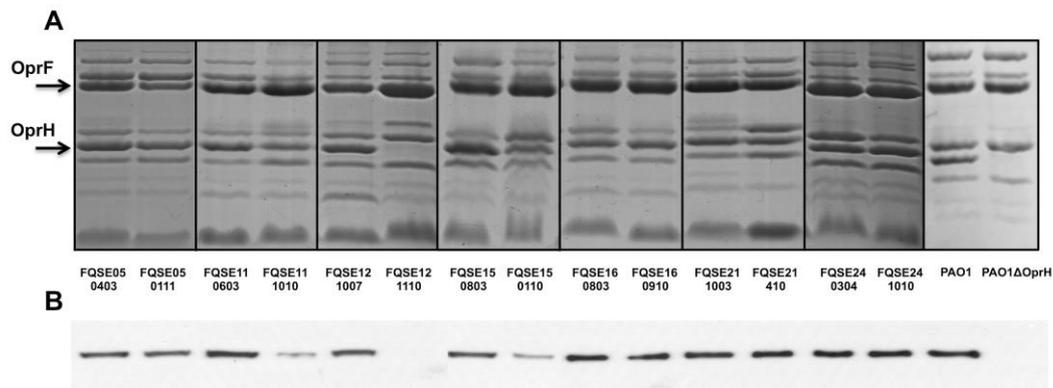
**Figure 4.** OprH promotes binding of surfactant protein A to *P. aeruginosa*.

Outer membrane proteins from different *P. aeruginosa* strains were isolated, resolved and stained with Coomassie blue (A). Fluorescent surfactant protein A (SP-A) (panel B; 250 ng/ml and panel C; 750 ng/ml) was incubated with  $2 \times 10^8$  bacterial cells of each strain. Binding of fluorescent SP-A to each bacterial strain was measured after exhaustive washing of the cells. Data represents three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were done with Student's unpaired two-tailed *t* test; \*  $P < 0.05$ .

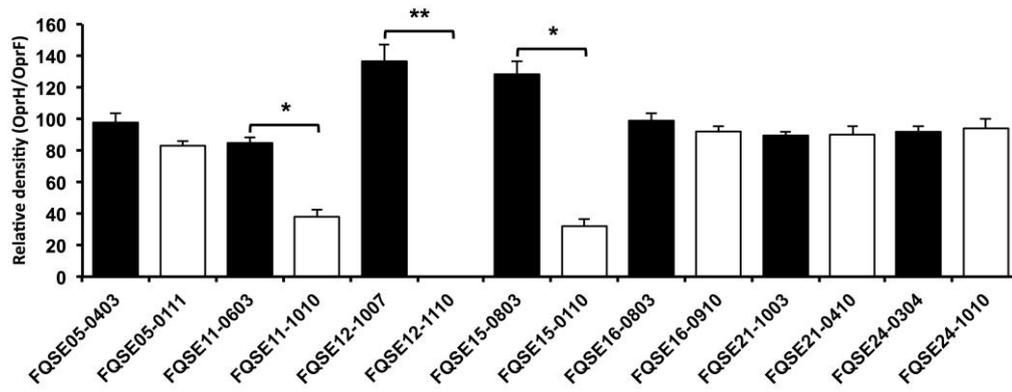
**Figure 5.** Binding of surfactant protein A to OprH mediates phagocytosis of *P. aeruginosa*.

*P. aeruginosa* strains were preopsonized with surfactant protein A (5  $\mu\text{g/ml}$  for the CF isolates and 50  $\mu\text{g/ml}$  for PAO1) or with bovine serum albumine (10  $\mu\text{g/ml}$ ) and subsequently incubated with human macrophages. Unattached bacteria were washed extensively and bacterial uptake was determined after lysis of the macrophages by plating on Luria Bertani plates. Data represents at least three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were done with Student's unpaired two-tailed *t* test; \*  $P < 0.05$ .

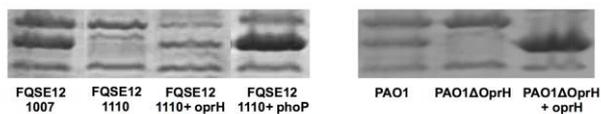
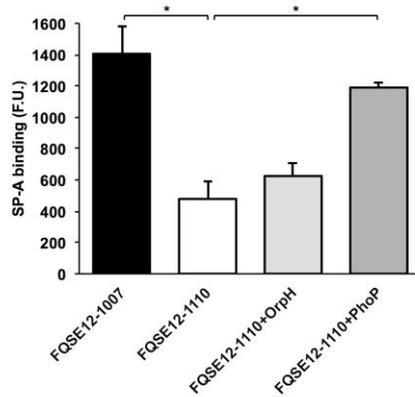
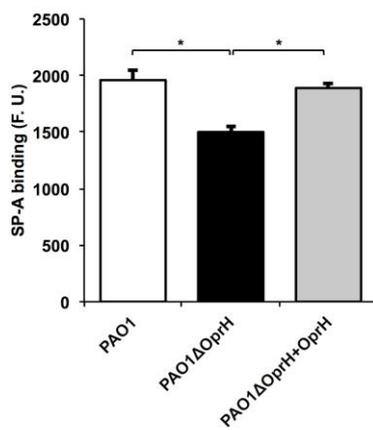




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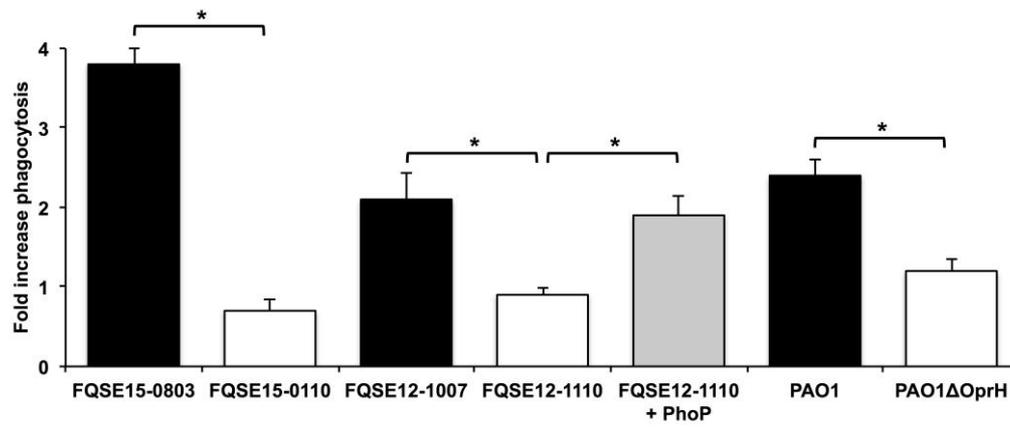


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