Universitat
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DOCTORAL THESIS

2017

IDENTIFICATION OF *P. aeruginosa* BINDING MOLECULES FOR COMPLEMENT COMPONENT C3 AND SURFACTANT PROTEIN A

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Doctor by the Universitat de les Illes Balears

Abbreviation	Full word
AP	Alternative pathway
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
Bp	Base pair
BSA	Bovine serum albumin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CFU	Colony forming unit
COPD	Chronic obstructive pulmonary disease
CP	Classical pathway
CRD	Carbohydrate recognition domain
DNA	Deoxyribonucleic acid
DPPC	Dipalmitoylphosphatidylcholine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Kbp	kilo base pairs
kDa	Kilodalton
LB	Luria-Bertani media
LP	lectin pathway
LPS	lipopolysaccharide
MAC	Membrane attack complex
MASP-1	mannose-associated serine protease 1
MASP-2	mannose-associated serine protease 2
MBL	Mannan-binding lectin
MBP	Mannose binding proteins
Mbp	Millions of base pairs
NHS	Normal human serum
ORFs	Open reading frames
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
pI	Isoelectric point
PMNs	Polymorphonuclear leukocytes
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNP	Single nucleotide polymorphims
SP-A	Surfactant protein A
SP-D	Surfactant protein D

List of publications

Surfactant protein A recognizes outer membrane protein OprH on *Pseudomonas aeruginosa* isolates from individuals with chronic infection.

Qadi M, Lopez-Causapé C, Izquierdo-Rabassa S, Mateu Borrás M, Goldberg JB, Oliver A, Albertí S.

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Sensing Mg^{2+} is essential for *Pseudomonas aeruginosa* to resist complement-mediated opsonophagocytosis.

Qadi M, Izquierdo-Rabassa S, Mateu Borrás M, Juan C, Goldberg JB, Hancock REW, Albertí S.

Submmited to Journal of Infectious Diseases.

Dedications

I dedicate my work to those dearest to me:

- My beloved country, PALESTINE.
- My dear mother, who faced a great deal of time and difficulties for the sake of my success and wellbeing.
- My kind father, whose advice has guided my path through difficult times.
- My late brother MAHMOUD, peace upon his soul, with whom I had the best companionship.
- My lovely sisters and their families, who supported my efforts.
- My supportive uncle ALI, who supported me in my duties.
- My pretty daughters ZAINHA and MINHA, who from their eyes I can see the sunshine of life.
- My great loving wife MARIAM, whose affection and efforts made this possible.

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1. SUMMARY

Introduction: *Pseudomonas aeruginosa* is a major opportunistic pathogen and a leading cause of severe nosocomial pneumonia and sepsis. Surfactant proteins and the complement system play a critical role in the host defense against *P. aeruginosa* infections. Nevertheless, the bacterial targets of the surfactant protein A (SP-A), the major surfactant protein in the lung, and the component C3, the central component of the complement system, remain poorly investigated. The objectives of this study were; i) to characterize the interaction between SP-A and a collection of chronic isogenic sequential isolates from cystic fibrosis (CF) patients to identify the SP-A-binding molecules of *P. aeruginosa* and the mechanism adopted by these isolates to resist the SP-A mediated clearance and ii) to identify novel C3 binding molecules of *P. aeruginosa* as well as to characterize new strategies that this pathogen uses to elude the complement system effects.

Research content: To achieve the first objective, we used a combination of in vitro approaches, including binding assays with human purified SP-A, mass spectrometry analysis, genetic loss of function/gain of function of selected bacterial genes and phagocytosis assays with human macrophages. 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*. Late isolates from CF patients 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 isolates from CF patients were at least two-fold more resistant to SP-A mediated killing by human macrophages than their respective early isolates. Our second objective, the identification of novel *P. aeruginosa* C3 binding molecules, arose from a study originally designed to investigate the role of PhoQ in *P. aeruginosa* bloodstream infections. We observed that a PhoQ-deficient mutant was avirulent in a murine model of systemic infection and did not cause bacteremia. In the presence of blood concentrations of Mg^{2+}, a PhoQ mutant bound more C3 and was more susceptible to complement-mediated opsonophagocytosis than the parent strain, suggesting a direct effect of the Mg^{2+} on the modulation of expression of a bacterial component by the PhoP/PhoQ system. Ligand blot analysis, C3 binding experiments and opsonophagocytosis assays identified this component as the outer membrane protein OprH.

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 from the SP-A-mediated phagocytic killing. Moreover, this study identifies PhoQ as an essential Mg^{2+} sensor for *P. aeruginosa* to avoid complement-mediated opsonophagocytosis due to the direct control that exerts on the expression of OprH, a previously unrecognized C3 binding molecule of *P. aeruginosa*.

Introducción: *Pseudomonas aeruginosa* es uno de los principales patógenos causantes de neumonía nosocomial grave y sepsis. Las proteínas surfactantes y el sistema del complemento juegan un papel crítico en la defensa del huésped frente a las infecciones por *P. aeruginosa*. Sin embargo, las dianas bacterianas de la proteína surfactante A (PS-A), la principal proteína surfactante del pulmón, y de C3, el componente central del sistema del complemento, no han sido investigadas adecuadamente. Los objetivos de este estudio fueron: i) caracterizar la interacción entre la PS-A y una colección de aislados crónicos isogénicos de pacientes con fibrosis quística (FQ) para identificar las moléculas de unión de la PS-A en *P. aeruginosa* y el mecanismo adoptado por estos aislados para resistir la muerte mediada por la PS-A e ii) identificar nuevas moléculas de *P. aeruginosa* que unen C3, así como también caracterizar las estrategias que este patógeno utiliza para evadir los efectos del sistema del complemento.

Contenido de la investigación: Para alcanzar el primer objetivo, se utilizó una combinación de distintas aproximaciones metodológicas in vitro incluyendo ensayos de unión con PS-A humana purificada, análisis de espectrometría de masas, pérdida genética de función / ganancia de función de genes bacterianos seleccionados y ensayos de fagocitosis con macrófagos humanos. Se caracterizó la interacción entre la PS-A y una colección de aislamientos secuenciales isogénicos de siete pacientes con FQ. Se identificó la proteína de membrana externa OprH como un nuevo ligando de PS-A en *P. aeruginosa*. Los aislados tardíos unieron significativamente menos PS-A que los aislamientos isogénicos tempranos, respectivos. Esta diferencia pudo asociarse a una reducción en la expresión de OprH. La unión de PS-A a OprH facilitó la eliminación de *P. aeruginosa* por fagocitosis. De esta forma, los aislados tardíos fueron al menos dos veces más resistentes a la eliminación por fagocitosis mediada por PS-A que sus aislados tempranos respectivos. La identificación de nuevas moléculas de *P. aeruginosa* que unen C3 surgió como resultado de un estudio diseñado para investigar el papel de PhoQ en las bacteremias causadas por *P. aeruginosa*. Se observó que un mutante deficiente en PhoQ era avirulento en un modelo murino de infección sistémica y no causaba bacteriemia. En presencia de concentraciones sanguíneas de Mg^{2+}, el mutante en PhoQ unió más C3 y fue más susceptible a la opsonofagocitosis mediada por el complemento que la cepa parental, lo que sugería un efecto directo del Mg^{2+} sobre la modulación de la expresión de un componente bacteriano por el sistema PhoP/PhoQ. Los experimentos de unión a C3 y los ensayos de opsonofagocitosis identificaron a este componente como la proteína de membrana externa OprH.

Conclusión: OprH es una nueva diana para la unión de PS-A en los aislados de *P. aeruginosa* de pacientes con FQ. La reducción de la expresión de OprH es una adaptación, previamente desconocida, que *P. aeruginosa* adopta en el pulmón con FQ para evadir la eliminación por la fagocitosis mediada por la PS-A. Hemos identificado a PhoQ como un sensor de Mg^{2+} esencial para que *P. aeruginosa* evite la opsonofagocitosis mediada por el complemento debido al control directo que ejerce sobre la expresión de OprH, una diana de C3, previamente desconocida, en *P. aeruginosa*.

Introducció: *Pseudomonas aeruginosa* és un dels principals patògens causants de pneumònia nosocomial greu i sèpsia. Les proteïnes surfactants i el sistema del complement juguen un paper crític en la defensa de l'hoste enfront de les infeccions per *P. aeruginosa*. Malgrat això, les dianes bacterianes de la proteïna surfactant A (PS-A), la principal proteïna surfactant del pulmó, i de C3, el component central del sistema del complement, no s'han investigat adequadament. Els objectius d'aquest estudi van ser: i) caracteritzar la interacció entre la PS-A i una col·lecció d'aïllats crònics isogènics de pacients amb fibrosis quística (FQ) per identificar les molècules d'unió de PS-A a *P. aeruginosa* i el mecanisme adoptat per aquests aïllats per resistir la mort mediada per la PS-A i ii) identificar noves molècules de *P. aeruginosa* que uneixen C3, així com caracteritzar les estratègies que aquest patogen utilitza per evadir els efectes del sistema del complement.

Contingut de la recerca: Per aconseguir el primer objectiu, es va utilitzar una combinació de diferents aproximacions metodològiques in vitro incloent assajos d'unió amb PS-A humana purificada, anàlisi d'espectrometria de masses, pèrdua genètica de funció / guany de funció de gens bacterians seleccionats i assajos de fagocitosis amb macròfags humans. Es va caracteritzar la interacció entre la PS-A i una col·lecció d'aïllaments seqüencials isogènics de set pacients amb FQ. Es va identificar la proteïna de membrana externa OprH com un nou lligant de la PS-A a *P. aeruginosa*. Els aïllats finals van unir significativament menys PS-A que els aïllaments isogènics inicials, respectius. Aquesta diferència va poder associar-se a una reducció en l'expressió de OprH. La unió de PS-A a OprH va facilitar l'eliminació per fagocitosi de *P. aeruginosa*. D'aquesta forma, els aïllats finals van ser almenys dues vegades més resistents a l'eliminació per fagocitosi mediada per la PS-A que els aïllats originals respectius. La identificació de noves molècules de *P. aeruginosa* que uneixen C3 va sorgir com a resultat d'un estudi originalment dissenyat per investigar el paper de PhoQ en les bacteremias causades per *P. aeruginosa*. Es va observar que un mutant deficient en PhoQ era avirulent en un model murí d'infecció sistémica i no causava bacteriemia. En presència de concentracions sanguínies de Mg^{2+}, el mutant de PhoQ va unir més C3 i va ser més susceptible a l'opsonofagocitosis mediada pel complement que la soca parental, la qual cosa suggeria un efecte directe del Mg^{2+} sobre la modulació de l'expressió d'un component bacterià pel sistema PhoP/PhoQ. Els experiments d'unió de C3 i els assajos d'opsonofagocitosis van identificar a aquest component com la proteïna de membrana externa OprH.

Conclusió: OprH és una nova diana per a la unió de PS-A en els aïllats de *P. aeruginosa* de pacients amb FQ. La reducció de l'expressió de OprH és una adaptació, prèviament desconeguda, que *P. aeruginosa* adopta en el pulmó amb FQ per evadir l'eliminació per la fagocitosi mediada per la PS-A. Hem identificat a PhoQ com un sensor de Mg^{2+} essencial per a que *P. aeruginosa* eviti l'opsonofagocitosis mediada pel complement a causa del control directe que exerceix sobre l'expressió de OprH, una diana de C3, prèviament desconeguda, en *P. aeruginosa.*

2. INTRODUCTION

2. INTRODUCTION

2.1. *Pseudomonas aeruginosa*: TAXONOMY AND CHARACTERISTICS

Pseudomonas aeruginosa is a Gram-negative bacillus that was first isolated from human infections in 1882 by Gessard, who called it *Bacillus pyocyaneus* (Bodey et al., 1983; Lyczak et al., 2000). This bacterium belongs to the phylum of proteobacteria, class of gamma proteobacteria, order of pseudomonadales, family of Pseudomonadaceae, and the genus of pseudomonas (Forbes et al., 2007; Garrity and Lilburn, 2005).

This bacterium is a straight rod, slightly curved, between 1.5 and 3 μm long and 0.5 to 0.8 μm wide. *P. aeruginosa* synthesizes many different pigments including pyocyanin (blue), pyoverdin (fluorescent green), pyorubin (dark red) and pyomelanin (dark-brown or black). The combination of these different pigments give to the bacterial colonies a particular color (Brooks et al., 2013; Forbes et al., 2007; Howarth and Dedman, 1964; Orlandi et al., 2015).

This bacterium is a common inhabitant of soil and water, and is able to colonize multiple environmental niches utilizing many compounds as energy sources. Its nutritional requirements are simple and it has a predilection for growth in moist environments. It is strictly aerobic but is able to use nitrate as electron receptor in anaerobic conditions. This microorganism produces oxidase and catalase, and grows on MacConkey medium as lactose non-fermenting colonies. *P. aeruginosa* grows at ambient temperature and at temperatures up to 42ºC, unlike most of the species of this genus. However, its optimal growth temperature is 37ºC (Brooks et al., 2013; Forbes et al., 2007).

The genome of the highly virulent reference strain, PA14 was sequenced almost 14 years ago (He et al., 2004). The genome of this microorganism is relatively large compared to the genome of other bacteria with around 6.35 Mbp and 5,873 predicted open reading frames (ORFs) on the chromosome which provides insights into the basis of the genetic complexity and ecological versatility of the bacterium (He et al., 2004; Stover et al., 2000). To date, the function of nearly 500 of the ORFs has already been identified. The genome of *P. aeruginosa* contains a high percentage of regulatory genes and genes encoding potential virulence factors, which, in contrast with other species, are distributed through the genome (Figure 1) (Skurnik et al., 2013).

2.2. *P. aeruginosa* INFECTIONS

P. aeruginosa is considered to be essentially an opportunistic pathogen that has emerged as one of the major nosocomial opportunistic human pathogens during the past decades, maybe as a result of its broad resistance to antimicrobial agents, which selects this bacterium against other environmental bacteria (Hoffken and Niederman, 2002; Meynard et al., 1999). *P. aeruginosa* causes infections in immunocompromised patients, such as burn patients, transplant recipients, neutropenic patients and patients with human immunodeficiency virus (Afessa and Green, 2000; Lyczak et al., 2000; Meynard et al., 1999). As a matter of fact, *P. aeruginosa* is responsible for 10% of the community-acquired infections caused by Gram-negative organisms, and for 10% of all nosocomial infections (Diekema et

al., 1999; Vincent, 2003). This pathogen causes a wide range of infections including dermatitis, keratitis, soft tissues infections, urinary tract infections, bacteremia, bone and joint infections as well as a wide variety of systemic infections (Diekema et al., 1999; Emori and Gaynes, 1993; Lyczak et al., 2000; Vincent, 2003).

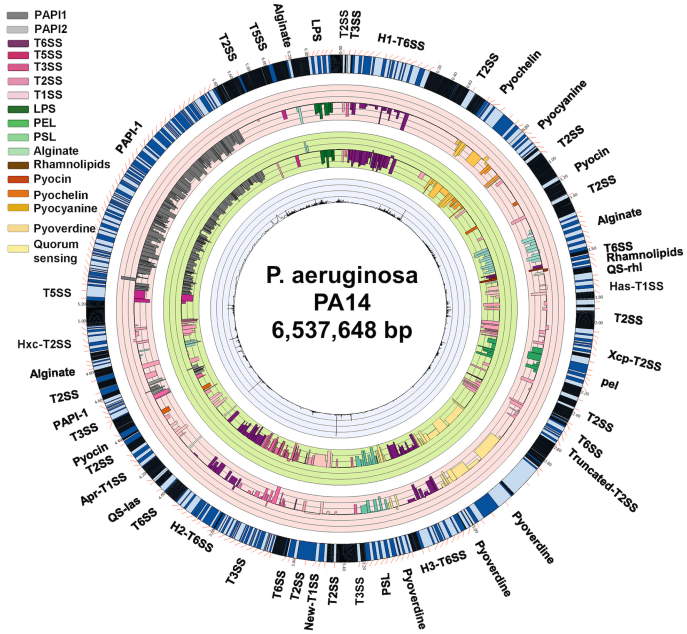

Figure 1. Representation of the *P. aeruginosa* PA14 genome and the position of genes encoding for identified virulence factors (adapted from Skurnik et al., 2013).

The virulence factors are ranked in 4 categories which are color-coded and depicted across a gradient of lighter to darker bars (upper left legend). Pathogenicity Islands PAPI-1 and -2 (grey bars); secretion systems (purple bars); exopolysaccharide/LPS (green bars), and others (brown bars) including pyoverdine, pyochelin, pyocyanine, pyocin, rhamnolipids, and quorum sensing (QS).

However, the predominant human diseases caused by *P. aeruginosa* are the respiratory infections in nosocomial settings and cystic fibrosis (CF) patients and the bacteremia in severe burn victims (Lyczak et al., 2000).

After *Staphylococcus aureus, P. aeruginosa* is the second cause of nosocomial pneumonia, and is isolated in 21% of the cases (Vincent, 2003). In addition, it is the most frequently isolated pathogen in patients under mechanical ventilation in the intensive care units where is associated with a high mortality rate (Rello et al., 2003).

P. aeruginosa also chronically infects patients with other pathologies such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) or bronchiectasis.

CF is a life-threatening disease caused by a genetic mutation that disrupts the cystic fibrosis transmembrane regulator (CFTR) protein. This mutation has an impact on the mucous secretions that become thicker and poorly hydrated, impeding the natural clearance of the colonizing microorganisms. Caucasians are the predominant population affected by mutations in CFTR and is one of the most common life-shortening genetic diseases in this race. CF lung is highly susceptible to *P. aeruginosa* infections and this microorganism plays a critical role in the development and progression of pulmonary disease in these patients (Lyczak et al., 2002). Chronic *P. aeruginosa* respiratory infections affect between 54% and 80% of adults with CF , and 97% of the children with CF are already colonized with *P. aeruginosa* by the age of 3 years (Lyczak et al., 2002). Chronic airway inflammation with recurrent *P. aeruginosa* infections is the major cause of morbidity and mortality of CF patients. In these patients, after a period of intermittent colonization, the microorganism becomes permanently established and is rarely eliminated, despite an exuberant host inflammatory response (Govan and Deretic, 1996). 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, or auxotrophic (Speert et al.,1994; Mahenthiralingam et al., 1994; Oliver et al., 2000). 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. In addition, these infections are highly difficult to eradicate, mostly due to the resistance of *P. aeruginosa* to antibiotics leading to pulmonary failure and death of the patient.

COPD is the 5th leading cause of death in the world. This disease is characterized by an abnormal inflammatory response of the lungs, predominantly induced by the tobacco smoke, resulting in a progressive limitation of the airflow. The evolution of the disease is marked by frequent acute exacerbations that cause significant worsening of symptoms. *P. aeruginosa* is frequently isolated in the sputum of COPD patients (7-12%) with advanced airflow obstruction, This frequency is even higher in COPD patients under mechanical ventilation (Lieberman and Lieberman, 2003).

In a longitudinal study, Martínez-Solano et al isolated genetically related *P. aeruginosa* strains from sequential exacerbation episodes of the disease in the same patient suggesting that this microorganism chronically infects patients with COPD. Moreover, during the chronic infection, each clone diversified, exhibiting an increased mutation rate, increased antibiotic resistance, and reduced production of proteases. As occurs in the *P. aeruginosa* isolates from CF patients, isolates from COPD patients showed lower cytotoxicity and higher biofilm production capacity (Martinez-Solano et al., 2008).

P. aeruginosa is also associated to bronchiectasis, an irreversible airway dilatation and inflammation, where it plays a critical role in the evolution of the disease, worsening the symptoms. 19% of the patients with bronchiectasis are infected/colonized by *P. aeruginosa* (Nicotra et al., 1995).

Finally, *P. aeruginosa* is the most common Gram-negative organism that infects burn wounds and one of the leading causes of mortality among severe burn patients with systemic infection (McManus et al., 1985). *P. aeruginosa* infection is a common complication of severe burns due to the extensive disruption of skin, which serves as the first defense line against infection, and due to a deficiency in the antibodies and complement components found locally at the burn site. In addition, this microorganism is so common in the environment that is extremely likely that an individual with severe burns will be challenged with this pathogen before the burns can heal. Exacerbating this situation, hospitals often harbor multidrug-resistant *P. aeruginosa* that can serve as the source of infection. In fact, *P. aeruginosa* has been found to contaminate the floors, bed rails, sinks of hospitals and the hands of the health care workers (Chitkara and Feierabend, 1981). Once the microorganism infects the burn wound, it proliferates rapidly, colonizing the devascularizated burnt tissue, which is rich in nutrients, and migrates to the hypodermis, invading the surrounding blood vessels. Finally, *P. aeruginosa* spreads systemically through the blood stream, causing bacteremia and eventually death.

2.3. TWO-COMPONENT REGULATORY SYSTEMS

As we mentioned above, *P. aeruginosa* is able to adapt and survive in a wide range of niches. It is found ubiquitously in the environment and can be isolated from water and soil but it is also a major opportunistic pathogen causing different types of infections. It is likely that this bacterium expresses a unique set of factors for each particular environment according to the specific stimulus that the microorganism senses through different systems. The two component regulatory systems serve as a basic stimulus-response coupling mechanism to allow *P. aeruginosa* to sense and respond to changes in many different environmental conditions. These systems are common in prokaryotes and are composed of a membrane-bound histidine kinase that senses a specific environmental signal and a corresponding response regulator located in the cytosol that mediates the cellular response through the activation or repression of target genes. Signal transduction is initiated by the transfer of phosphoryl group from ATP to a specific histidine residue in the histidine kinase. This is an autophosphorylation reaction. Phosphorylation causes the response regulator's conformation to change, usually activating an attached output domain, which then leads to the activation or repression of expression of target genes. Thus, the level of phosphorylation of the response regulator controls its activity. Histidine kinases and response regulators are encoded by genes usually located in the same operon. The two component systems are regulated and work cooperatively in a complex and integrative manner (Gooderham et al., 2009; Gooderham and Hancock, 2009).

At least 30 two-component regulatory systems contribute to the virulence and antimicrobial resistance of *P. aeruginosa*. The major two component regulatory systems of *P. aeruginosa* are listed in Table 1.

PA number	Gene product	Brief functional description
PA 0463/4	CreB–CreC	Catabolism. Swarming and swimming motility
PA 0929/30	PirR–PirS	Iron acquisition
PA 1099/8	FleR–FleS	Flagellar motility, adhesion to mucin. FleS likely cytoplasmic sensor
PA 1179/80	PhoP–PhoQ	Low Mg^{2+} signal. Polymyxin, antimicrobial peptide and aminoglycoside resistance. Virulence, swarming motility and biofilm formation
PA 2523/4	CzcR–CzcS	Metal and imipenem resistance
PA 2586	GacA	GacA–GacS system. Multihost virulence, quorum-sensing-dependent regulation of exoproducts and virulence factors, biofilm formation and antibiotic resistance, swarming motility, type III secretion
PA 2686/7	PfeR–PfeS	Iron acquisition
PA 2809/10	CopR–CopS	Metal and imipenem resistance
PA 3192/1	GltR	Glucose transport, type III secretion cytotoxicity
PA 3702/4	WspR–WspE	Wsp chemosensory system. Biofilm and cyclic-di-GMP level regulation, autoaggregation. WspR contains GGDEF output domain, WspE is CheA-type sensor
PA 3879/8	NarL–NarX	Nitrate sensing and respiration. Biofilm formation, swimming and swarming motility
PA 3947	RocR (SadR)	RocS1/R/A1 (SadA/R/S) system. Cytotoxicity, virulence, regulation of fimbriae adhesins, type III secretion and biofilm formation. RocA1 contains EAL output domain, RocR is RocA1 antagonist
PA 4296/3	PprB–PprA	Outer-membrane permeability and aminoglycoside resistance. PprA sensor likely cytoplasmic
PA 4547/6	PilR–PilS	Type IV fimbriae expression, twitching and swarming motility, biofilm formation
PA 4726/5	CbrB–CbrA	Carbon and nitrogen storage, cytotoxicity, swarming motility, nematode virulence
PA 4776/7	PmrA–PmrB	Induced by low Mg^{2+} and cationic antimicrobial peptides. Polymyxin B and antimicrobial peptide resistance
PA 5261/2	AlgR–FimS (AlgZ)	Virulence, alginate biosynthesis, twitching and swarming motility, biofilm formation, cyanide production, cytotoxicity and type III secretion system gene expression
PA 5360/1	PhoB–PhoR	Phosphate level regulation, low phosphate signal, quorum sensing

Table 1. Major *P. aeruginosa* virulence and antibiotic resistance-associated classical type response regulators and sensor histidine kinases (adapted from Gooderham and Hancock, 2009).

2.3.1. The PhoP-PhoQ regulatory system

The PhoP-PhoQ is, probably, the best well studied two-component regulatory system of *P. aeruginosa*. This system is composed of two proteins, the sensor histidine kinase PhoQ, facing outside and integrated in the inner membrane, and the response regulator PhoP, located in the cytoplasm of the cell (Macfarlane et al., 1999). Genetic studies performed by Macfarlane et al. in 1999 demonstrated that PhoQ and PhoP are encoded by two genes arranged in one operon that also includes the outer membrane protein gene *OprH* (Macfarlane et al., 1999) (Figure 2). In this operon, *PhoP* and *PhoQ*, which are overlapped 4 bp, are located downstream of the *OprH* gene. Both genes are transcribed as a single transcriptional unit. Primer extension analysis demonstrated that only one promoter, located upstream of OprH, drives the transcription of the entire operon. PhoP is composed of 225 amino acids residues and shows a 54% and 53% of similarity to PhoP of *Escherichia coli* and *Salmonella typhimurium*, respectively. PhoQ has an unusual start codon (GTG) and is composed of 448 amino acids residues. BLASTX analysis indicates that this histidine kinase is 33% and 34% similar to PhoQ of *E. coli* and *S. typhimurium*, respectively. It contains a histidine residue at position 271, which is conserved amongst sensor kinases and believed to be the site of autophosphorylation. In addition, there is an ATP-binding domain located at the C-terminal region of the protein (Macfarlane et al., 1999).

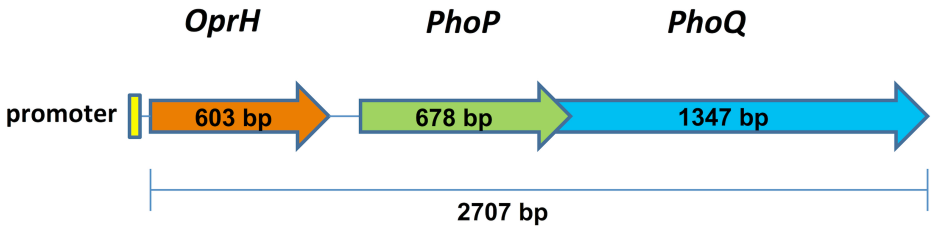

Figure 2. Schematic representation of the *oprH-phoP-phoQ* operon showing promoter location, structural arrangement and the size of each gene in the operon.

P. aeruginosa PhoQ responds to Mg^{2+} ion concentration and to acidic pH (Macfarlane et al., 1999; Wilton et al., 2015). Moreover, PhoQ is activated during adherence to epithelial cells (Gellatly et al., 2012). The concentration of the divalent cation Mg^{2+} regulates the expression of this operon. Thus, low concentrations of Mg^{2+} (< 1 mM) are sensed by PhoQ, which phosphorylates and activates PhoP which in turn transcriptionally activates or represses its target genes. As a result, PhoP upregulates the expression of the entire operon and OprH becomes the major protein in the outer membrane (Macfarlane et al., 1999). Macfarlane et al. used PhoP and PhoQ null mutants to investigate the role of each component in the *oprH-phoP-phoQ* activation. Their results shown that PhoP by itself is capable of activating a very high level of *oprH-phoP-phoQ* transcription under both high- and low- Mg^{2+}

conditions, whereas the presence of PhoQ restores regulation, and generally reduces this level of activation. Thus, in a PhoQ-deficient mutant, the PhoP modulated genes are dysregulated and expressed constitutively (Macfarlane et al., 1999). In view of the fact that the active form of response regulators is generally accepted to be the phospho-form, a second kinase for PhoP may exist in *P. aeruginosa* capable of phosphorylating this response regulator irrespective of Mg^{2+} levels in the growth medium. The main role of PhoQ would then be to act as a phosphatase that dephosphorylates, and therefore deactivates, PhoP selectively, in response to Mg^{2+} concentrations (Gooderham and Hancock, 2009; Macfarlane et al., 1999).

When *P. aeruginosa* grows under Mg^{2+} concentrations higher than 1 mM, like those found in the blood or in the human lung (2-4 mM), the expression of *oprH-phoP-phoQ* is downregulated. Accordingly, transcriptomic analysis of *P. aeruginosa* directly isolated from CF lung, human burned wound or murine burned wound revealed that the expression of both, *oprH* and *phoQ*, were downregulated (Bielecki et al., 2013; Turner et al., 2014). In contrast, in vitro studies performed by Mulcahy et al., demonstrated that the cation chelating property of the extracellular DNA present in the matrix of *P. aeruginosa* biofilms creates a cation-limited environment that results in induction of PhoP-PhoQ (Mulcahy et al., 2008). Furthermore, more recently Gellatly et al., found that PhoP-PhoQ was induced upon the interaction with the airway epithelial cells (Gellatly et al., 2012). Altogether these observations suggest that PhoP-PhoQ may be induced during the biofilm mode of growth and repressed in the planktonic cells.

Induction of PhoP-PhoQ modulates the expression of other genes besides *oprH-phop-phoQ* (Figure 3). Overall, the PhoPQ regulon affects the expression of 474 genes (Gooderham et al., 2009). One of the main targets of this two component regulatory system is the arnBCADTEF operon (Gooderham and Hancock, 2009). The activation of the PhoP-PhoQ and PmrA-PmrB systems leads to the activation of the arnBCADTEF operon that mediates the synthesis and transfer of 4-amino-L-arabinose to the lipid A of the lipopolysaccharide (Raetz et al., 2007). The addition of 4-amino-L-arabinose to lipid A confers resistance to the polycationic antibiotics gentamicin and polymyxin B and to polycationic peptides due to the modification of the charge of the lipid A (Gooderham and Hancock, 2009; Macfarlane et al., 2000). Inactivation of PhoQ leads to the activation of PhoP, which strongly upregulates arnBCADTEF conferring resistance to the polycationic antimicrobial agents (Macfarlane et al., 2000). In fact, PhoQ mutations are commonly found in *P. aeruginosa* clinical isolates resistant to polymixyn B (Barrow and Kwon, 2009; Miller et al., 2011).

Transcriptomic analysis of *P. aeruginosa* grown under Mg^{2+}-limited and Mg^{2+}-replete conditions determined that under Mg^{2+}-limited conditions, approximately 3% of the *P. aeruginosa* genes were differentially expressed compared to the expression in bacteria grown under Mg^{2+} replete conditions. However, only a modest subset of the Mg^{2+}-regulated genes were regulated through PhoP (Figure 3) (McPhee et al., 2006). To date, none of the genes regulated by PhoP, except PhoQ, have been involved in the virulence of *P. aeruginosa*. Moreover, to our knowledge, there is few data in the literature about the contribution of PhoP to *P. aeruginosa* pathogenesis.

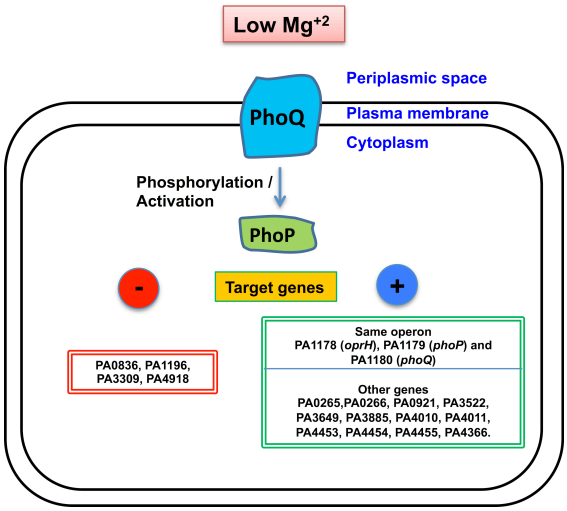

Figure 3. *P. aeruginosa* PhoP–PhoQ regulatory system.

P. aeruginosa PhoP–PhoQ regulatory system in Mg^{2+} limitation leads to the activation (phosphorylation) of the PhoP response regulator which positively autoregulate the transcription of its respective operon, as well as other genes outside the operon (adapted from Gooderham and Hancock, 2009). PA number: Annotated gene identification number (PA) and protein names according to the PAO1 genome at http://www.pseudomonas.com.

The expression profile of the genes controlled by PhoQ is consistent with the phenotype of the PhoQ null mutant. In this regard, a PhoQ deficient mutant derived from PAO1 exhibited a reduced virulence in a plant model of lettuce leaf and in rat model of chronic respiratory infection (Gooderham et al., 2009). It is likely that the attenuated phenotype of the mutant might have been associated with the down regulation of genes involved in the synthesis of important virulence factors (e.g; LPS, alginate, exopolysaccharide, type IV secretion system) (Gooderham et al., 2009), in the formation of the biofilm (Mulcahy and Lewenza, 2011), which facilitates the persistence of *P. aeruginosa* in the lung, or in the reduced ability to interact with epithelial cells (Gellatly et al., 2012). However, the bacterial components and the host mechanisms involved in the reduced virulence exhibited by the PhoQ mutant remain inadequately investigated.

2.3.2. The outer membrane protein OprH

OprH is a 21 kDa outer membrane basic protein (pI≈9) composed of 178 residues of amino acids (Rehm and Hancock, 1996). This protein stabilizes the outer membrane and is strongly bound to the LPS of *P. aeruginosa* (Bell et al., 1991; Edrington et al., 2011; Rehm and Hancock, 1996; Young et al., 1992). Membrane topology studies using PCR-mediated site-directed insertion and deletion

mutagenesis determined that OprH consists of eight transmembrane β-strands, four loops exposed to the cell surface, (L1-L4) and three periplasmic β-turns (T1-T3) (Figure 4) (Rehm and Hancock, 1996). OprH may form a β-barrel structure in the outer membrane, like the classical porins. But, in contrast to the porins, OprH showed no channel-forming activity in lipid bilayer experiments (Bell et al., 1991). It has been proposed that OprH forms a gated pore, which is normally closed unless activated by a certain molecule (Rehm and Hancock, 1996; Young and Hancock, 1992).

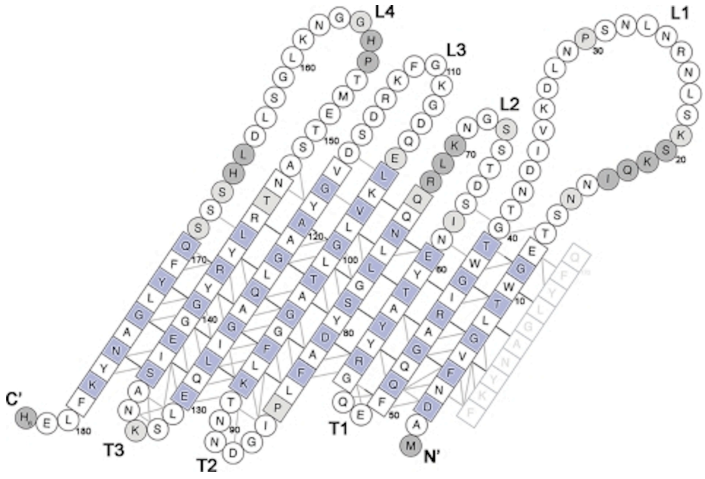

Figure 4. Schematic topology of OprH.

Residues that face the lumen of the barrel are colored light blue. β-Strand residues are denoted as squares. Loop and turn residues are denoted as circles (adapted from Edrington et al., 2011).

Initial evidence determined that OprH contributed to resistance to polycationic antibiotics, such as polymixyn and gentamicin as well as to the chelator agents like EDTA, by blocking the interaction of these compounds with the divalent cation binding sites of LPS. In fact, when grown under Mg^{2+} starvation conditions *P. aeruginosa* exhibits resistance to EDTA and the polycationic antibiotics (Brown and Melling, 1969; Nicas and Hancock, 1980). Moreover, an OprH deletion mutant was more susceptible to polymixyn B and gentamicin than the parent strain (Young et al., 1992). However, later experiments conducted by Macfarlane et al., using an OprH deletion mutant without polar effects on the PhoP-PhoQ system, clearly demonstrated that OprH is not directly involved in the resistance to the polycationic antibiotics (Macfarlane et al., 2000). Indeed, as we mentioned before, *P. aeruginosa* resistance to amynoglicosides and polymixyn B is mainly based on the activation of the arnBCADTEF operon through PhoP-PhoQ and PmrA-PmrB. OprH does not contribute to resistance to other commonly used antibiotics such as β-lactams antibiotics and tetracycline (Young and Hancock, 1992).

2.4. HUMORAL INNATE IMMUNITY IN *P. aeruginosa* INFECTIONS

The first challenge for microorganisms entering the respiratory tract or the bloodstream, such as *P. aeruginosa*, is to overcome the innate immune system of the host. In the lung, humoral mediators of the innate immunity consist of components present in the epithelial lining fluid that can selectively attack the bacterial cell walls and membranes, sequester microbial nutrients or act as decoys for microbial attachment. Soluble components of airway secretions that participate in innate immune response include antimicrobial factors, such as the lysozyme, cathelicidin, lactoferrin, defensins, secretory leukoprotease inhibitor, and surfactant proteins. In the blood, the major effector of the innate immune system is the complement system.

Innate immune system plays a critical role in the infections caused by *P. aeruginosa*. For this reason, this section will describe the current knowledge about the role of the main soluble components of the innate immune system, the surfactant proteins and the complement system, in the *P. aeruginosa* respiratory and bloodstream infections.

2.4.1. Pulmonary surfactant proteins

Pulmonary surfactant is produced mainly by the epithelial alveolar type II cells. Although Clara cells and submucosal cells also contribute to the production of surfactant. Pulmonary surfactant is secreted as a complex mixture of phospholipids (90%) and proteins (10%) packed together in a unique secretory organelle known as the lamellar-body (Olmeda et al., 2017). The main function of the surfactant is to reduce surface-tensions, and therefore keep alveoli from collapsing at the end of expiration. However, pulmonary surfactant also is involved in the defense of the respiratory tract against pathogens (Olmeda et al., 2017).

Around 80% of surfactant lipids are phospholipids and cholesterol comprises the largest amount of neutral lipids. Dipalmitoylphosphatidylcholine (DPPC), which has an essential function in reducing surface tension of the air-liquid interface, is the major phospholipid of surfactant. The second major phospholipid in surfactant is phosphatidylglycerol, and the other surfactant phospholipids include phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine (Batenburg and Haagsman, 1998). The protein content of surfactant is mainly composed of hydrophilic surfactant proteins A and D which play an important role in the innate immune response of the lung, and hydrophobic surfactant proteins B and C, which are crucial in surfactant homeostasis and in lowering surface tension at the air-liquid interface (Kuroki and Voelker, 1994).

Surfactant protein A (SP-A) is the most abundant protein in the pulmonary surfactant. In a bronchoalveolar lavage (BAL) from a healthy subject the concentration of this protein range from 1 to 12.35 µg/ml. On the other hand, surfactant protein D (SP-D) concentrations vary between 0.6 and 1.3 µg/ml (Honda et al., 1996; Postle et al., 1999; Shijubo et al., 1998). Surfactant composition in phospholipids and hydrophilic surfactant protein concentrations are significantly decreased in CF

patients (2.65 µg/ml SP-A and 0.1 ng/ml SP-D in BAL) compared to healthy patients (Postle et al., 1999).

SP-A and SP-D are large hydrophilic proteins with similar structural characteristics (Figure 5). Both proteins have an N terminal region containing a short inter-subunit disulfide bond formation, which is critical to stabilize the oligomeric structure, a collagen-like domain consisting of Gly-X-Y repeats (where X can be any amino acid while Y is often a hydroxyproline), a neck region and a carbohydrate recognition domain (CRD). The CRD regions of surfactant proteins are calcium-dependent and are mainly involved in pathogen recognition. This region recognizes with high affinity some oligosaccharides, such as N-acetylmannosamine L-fructose or inositol, but has a low affinity for single monosaccharides (Haagsman et al., 1987; Lim et al., 1994; Nayak et al., 2012; Persson et al., 1990). Indeed, it seems that this characteristic is important to distinguish between non-self from self, as most carbohydrates in animals are terminated by sugars, such as galactose or sialic acid, that are poorly recognized by SP-A and SP-D. On the other hand, the collagenous tail interacts with cell surface receptors such as the C1q receptor (Malhotra et al., 1992). Due to their structural similarity, SP-A, SP-D and mannose binding proteins (MBP) have been grouped into the collagen-lectin family or collectins.

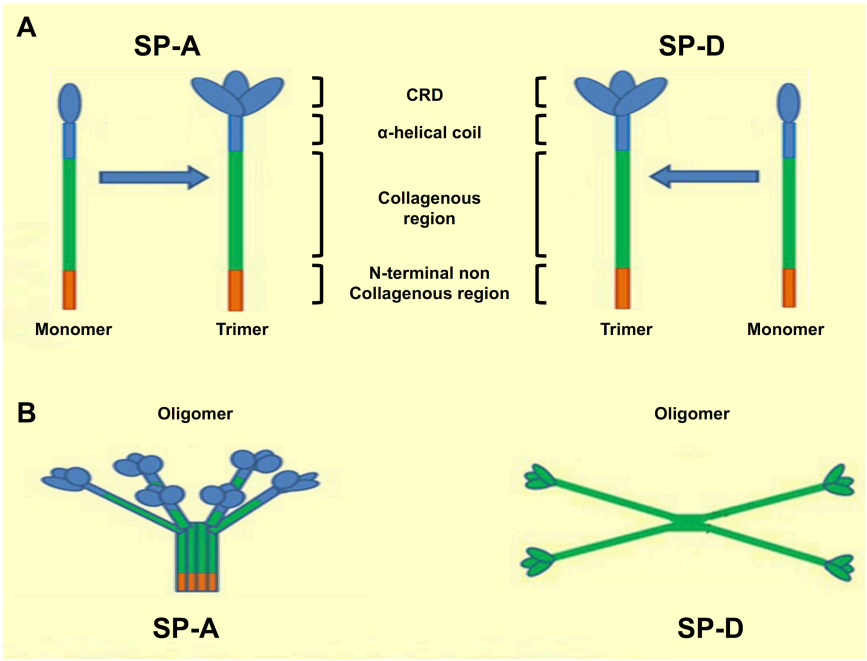

Figure 5. Structure of surfactant protein A and D (Adapted from Nayak et al., 2012).

SP-A and SP-D are synthesized as primary translation products of approximately 26-36 kDa and 45 kDa, respectively. Trimers are then formed by folding of the collagenous domains into triple helices and coiled-coil bundling of the α-helices in the neck (Figure 5A). The oligomeric structure of SP-A consists of an octadecamer formed by 6 trimeric subunits assembled in a bouquet-like manner (Figure 5B) (Bernhard, 2016). In contrast, SP-D forms a dodecamer in a cruciform manner composed of four trimeric subunits radiating from a disulfide-linked hub. Both proteins are synthesized in the endoplasmic reticulum and transported to lamellar body via the Golgi apparatus. Secretion occurs through exocytosis of lamellar body (Bernhard, 2016).

Both in vitro and in vivo studies show that SP-A and SP-D contribute to the elimination of particles and pathogens through at least three different mechanisms: by direct killing of the bacterium, opsonization or through modulation of the cellular immune system.

2.4.2. Surfactant proteins and *P. aeruginosa* infections

The role of the surfactant proteins in *P. aeruginosa* infections has been studied in detail using genetically engineered experimental animal models. SP-A (-/-) null mice have essentially normal lung function (Korfhagen et al., 1996), whereas SP-D (- /-) null mice present a progressive accumulation of lipid in the air-spaces after birth (Botas et al., 1998). Both, SP-A- and SP-D-deficient mice have an increased susceptibility to *P. aeruginosa* respiratory infections (Giannoni et al., 2006; LeVine et al., 1998). In addition, infected SP-A-deficient mice present higher levels of inflammatory mediators and nitric oxide metabolites in their lavage fluids with earlier neutrophil infiltration than infected wild-type mice (Giannoni et al., 2006). The results of these in vivo experiments support the critical role of surfactant proteins to innate pulmonary defense and orchestration of inflammatory response in the respiratory infections by *P. aeruginosa*.

Surfactant proteins contribute to the clearance of *P. aeruginosa* from the lung mainly through two different mechanisms. The first mechanism occurs through the direct binding of SP-A and SP-D to the microorganism resulting in the growth inhibition and the direct killing of the pathogen by permeabilization of the bacterial membrane (Wu et al., 2003). The second mechanism occurs through opsonization. Interaction of the surfactant proteins with various immune cells, mainly, neutrophils and alveolar macrophage results in enhanced phagocytosis of the collectin- associated pathogen (Hartshorn et al., 1998; Mariencheck et al., 1999). Alternatively, SP-A or SP-D aggregates the pathogen, which results in enhanced ingestion without the need of a direct interaction of the surfactant proteins with the phagocytic cell (LeVine et al., 1998).

The binding of SP-A and SP-D to *P. aeruginosa* occurs through the CRD region of surfactant proteins. Numerous studies have shown that SP-A and SP-D interact with bacterial LPS of different Gram-negative organisms in a calcium dependent manner. SP-A binds lipid A whereas SP-D binds the core oligosaccharide or the O-chain of bacterial LPS (García-Verdugo et al., 2005; Sano et al., 1999; Van Iwaarden et al., 1994). 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 (Hickling et al., 1998). Furthermore, Van Iwaarden shown that *P. aeruginosa* strains expressing rough LPS, a common feature of the strains isolated from CF patients, bound SP-A more efficiently than strains displaying a smooth LPS phenotype (Van Iwaarden et al., 1994). These findings suggest that the lipid A is the region of the LPS involved in the binding of SP-A to *P. aeruginosa*. In addition to lipid A, *P. aeruginosa* might bind SP-A through several outer membrane proteins. Ligand blot experiments using outer membrane proteins isolated from *P. aeruginosa* detected three proteins of 17, 22 and 25 kDa that reacted with SP-A (Mariencheck et al., 1999; Tan et al., 2014). However, these proteins remain unidentified and their role in SP-A binding to *P. aeruginosa* is unknown.

The major strategy used by *P. aeruginosa* to counteract the effects of the surfactant proteins is blocking the binding of these proteins to the microorganism. As we mentioned above, the O side chain of the LPS impedes the binding of SP-A (Van Iwaarden et al., 1994). Alginate polysaccharide also blocks the binding of SP-A to *P. aeruginosa* (Barbier et al., 2012). Accordingly, mucoid isolates, often isolated from CF patients with *P. aeruginosa* chronic infections, bind SP-A less efficiently than non-mucoid isolates.

Surfactant protein deficient mice has been used to investigate in detail novel microbial factors that contribute in vivo to the resistance of *P. aeruginosa* to SP-A mediated permeabilization and opsonization. Lau's group, using genetically-engineered SP-A mice and a library of signature-tagged *P. aeruginosa* mutants, demonstrated that the flagellum, type IV pilus, and genes such as *pch* (required for salicilate biosynthesis) and *ptsP* (phosphoenolpyruvate-protein phosphotransferase) are required to resist membrane permeabilization by SP-A (Tan et al., 2014; Tan et al., 2015; Zhang et al., 2005; Zhang et al., 2007). Disruptions of these genes impair the integrity of the bacterial membrane increasing the sensitivity of *P. aeruginosa* to SP-A permeabilization.

Other studies with mice have revealed that *P. aeruginosa* may influence the surfactant functions during the process of respiratory infections. Indeed, in humans, chronic *P. aeruginosa* infection is associated with decreased surfactant phospholipids levels and higher protein levels (Griese et al., 2004; Mander et al., 2002). Experiments conducted in vivo using mice demonstrated that *P. aeruginosa* is able to decrease surfactant levels in DPPC repressing the expression of a key enzyme in DPPC biosynthesis at the transcriptional level (Wu et al., 2007). Furthermore, different authors have shown that *P. aeruginosa* secretes various proteases involved in SP-A degradation, such as elastase and proteinase IV (Beatty et al., 2005; Kuang et al., 2011; Malloy et al., 2005; Mariencheck et al., 2003). Most of the *P. aeruginosa* strains isolated from the respiratory tract produce elastase that contributes to the low levels of surfactant proteins found in the infected patients and to the tissue damage (Haddad et al., 1994; Hamood et al., 1996). Furthermore, neutrophil recruitment into the airways by both host and bacterial chemotactic factors participates in decreasing surfactant concentration in lung through secretion of oxygen species and neutrophil elastase that degrades SP-A and damage epithelial cells, responsible for surfactant production (Haddad et al., 1994).

Finally, *P. aeruginosa* alginate polysaccharide reduces the levels of SP-A produced by the airway epithelial cells in vitro in a process mediated by the SP-A receptor CKAP4/P63 (Barbier et al., 2012).

2.4.3. The complement system

The complement system is an important effector of the innate immune system and it is responsible for the defense against invading microbes, inflammation and homeostasis of the host (Merle et al., 2015). During an infection, the complement system is essential to opsonize bacteria for phagocytosis, lyse them directly via pore formation and to recruit phagocytes to the site of the infection. The importance of this system is evident in the individuals with deficiencies in some or several of the components of the system that suffer severe and recurrent infectious diseases (Merle et al., 2015).

At least 20 distinct soluble plasma proteins and more than 10 receptors present in different cell populations have been identified as belonging to this system. Activation of this system occurs via three different pathways; the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). All activation pathways converge in a common central component, C3 (Figure 6) (Merle et al., 2015).

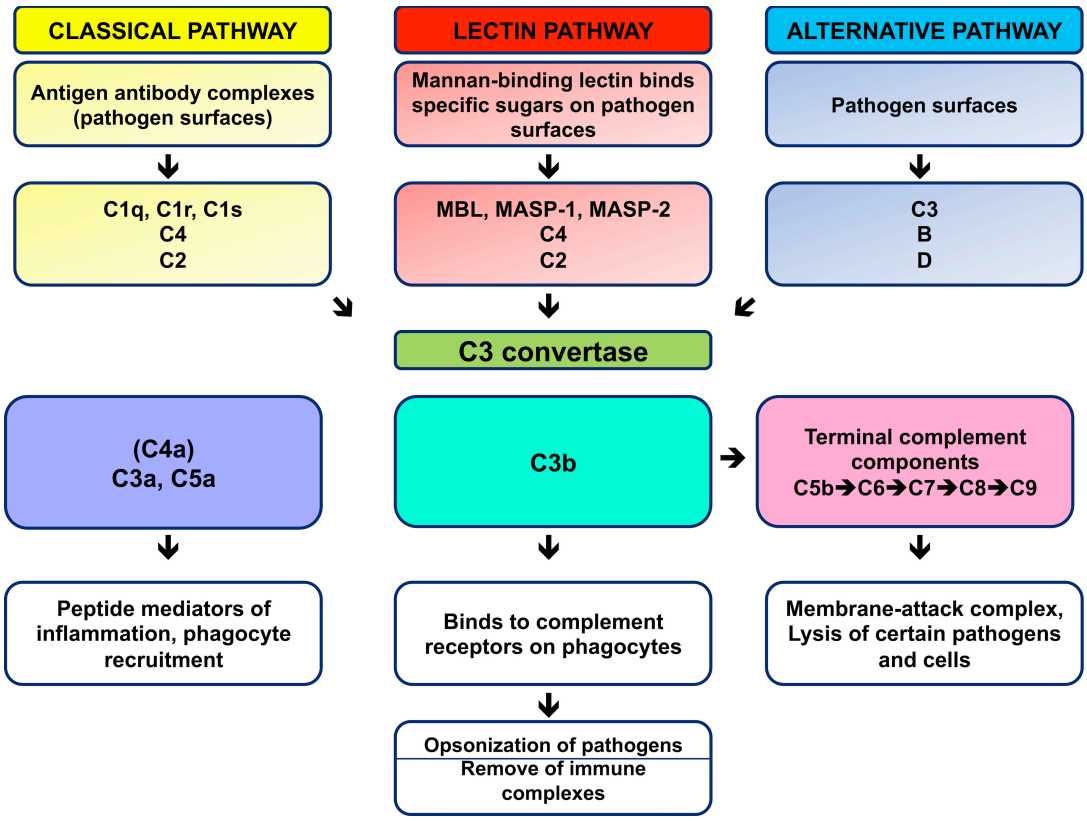

Figure 6. Overview of the complement system (Adapted from Janeway CA, 2001).

The CP is activated when IgG or IgM bound to the bacterial surface is recognized by C1q. The binding of this subcomponent of C1 to antibodies results in activation of the two C1 associated serine proteases C1r and C1s. Activated C1s cleaves C4 into C4a and C4b, of which the later will attach to the bacterial surface. Subsequently, C2 binds to C4b and is cleaved by C1s into C2a and C2b resulting in the formation of the CP C3 convertase C4b2a (Janeway CA, 2001).

The same C3 convertase results from the activation of the LP, however the initiation of this pathway differs from the CP. The LP is triggered when the first component of this pathway, the Mannan-binding lectin (MBL), recognizes specific carbohydrates on the bacterial surface. The binding of MBL to the bacteria results in the activation of the MBL- associated serine protease MASP-2, which is responsible for cleavage of C4 and C2, and MASP-1, which only cleaves C2 (Janeway CA, 2001).

Both, CP and LP C3 convertases, cleave C3 to yield the anaphylotoxin C3a and C3b, which is covalently attached on the bacterial envelope via a thioester domain (Janeway CA, 2001).

The AP is activated when C3b, which results from the spontaneous hydrolysis of C3, binds covalently to the microbial surface. Factor B stabilizes this binding and is cleaved by factor D into Bb and Ba, resulting in formation of the AP C3 convertase C3bBb. Thus, AP mainly functions as an amplification loop of the CP and the LP. The AP is the major responsible for the total complement activation (Janeway CA, 2001).

All complement activation pathways results in the formation of the C3 convertases, which cleave C3 into C3a and C3b. This last component is incorporated into the C3 convertases to give two different C5 convertases, the CP/LP C4b2aC3b and the AP C3bBbC3b. Both convertases cleaves C5 in to the major anaphylatoxin C5a and C5b. C5b forms a complex with C6, which in turn can bind C7 to form the membrane C5b67 complex. Subsequently, C8 binds to this complex and together with multiples copies of C9 forms a tubular pore, called the membrane attack complex (MAC) (Janeway CA, 2001).

The three major biological activities of the complement system are; i) recruitment and activation of phagocytes, including macrophages and neutrophils, ii) lysis of target cells, iii) and opsonization of microorganisms and immune complexes, so that they can be recognized by cells expressing complement receptors (Figure 6) (Janeway CA, 2001).

2.4.4. Complement and *P. aeruginosa* infections

The complement system plays a critical role to clear *P. aeruginosa* infections. It was first in 1978 when Gross et al. provided experimental evidences on the role of the complement system in a murine model of *P. aeruginosa* infection. These authors used cobra venom factor to deplete complement in mice that were infected by aerosolization with *P. aeruginosa*. The bacterial load of the lungs from complement-depleted animals was two-fold greater than those from saline-treated animals, indicating that hypocomplementemia predisposed to *P. aeruginosa* lung infection (Gross et al., 1978).

In 2003, Younger et al. used C5-deficient mice to investigate the role of complement in a murine model of lung infection. They demonstrated that the complement-deficient mice were more susceptible to *P. aeruginosa* infection than the normal mice. Furthermore, this difference did not seem to be a result of decreased inflammation because complement-deficient mice had normal neutrophil recruitment. These findings indicate that although *P. aeruginosa* is resistant to serum killing, C3 opsonization and C5 convertase assembly occur in its surface. This interaction in vivo plays a central role in host survival beyond just recruitment and activation of phagocytes and may serve to limit the inflammatory response to and tissue injury resulting from bacterial infection (Younger et al., 2003).

In 2004, Mueller-Ortiz et al. conducted a similar study to that reported by Younger one year before. However, these authors used C3-, C4- and Factor B-deficient mice instead of C5-deficient. All mice were challenged with *P. aeruginosa* via intranasal inoculation. After 7 days, C3-/- mice had a higher mortality rate than C3+/+ mice. Factor B-/- mice, but not C4-/- mice, infected with *P. aeruginosa* had a mortality rate similar to that of C3+/+ mice, indicating that in this model the AP of complement activation was required for the host defense against *Pseudomonas* infection. In vitro phagocytic assays demonstrated that the phagocytic cells from C3-/- or C3+/+ mice had a reduced capacity to ingest *P. aeruginosa* in the presence of C3-deficient serum compared to phagocytic cells in the presence of serum with C3. Overall these results indicated that the AP is essential for the survival of mice infected with *P. aeruginosa* and that the protection provided by the complement is due to C3-mediated opsonophagocytosis of *P. aeruginosa* (Mueller-Ortiz et al., 2004).

These studies demonstrate that complement system is critical to protect the host against *P. aeruginosa* infections, but also imply that this pathogen displays on its surface targets that are recognized by the activating components of the complement system. To date, two molecules have been identified on *P. aeruginosa* surface as binding molecules of C3, namely the lipopolysaccharide (Jensen et al., 1993) and the outer membrane protein OprF (Mishra et al., 2015). It is well established that several Gram-negative organisms, including *P. aeruginosa*, can activate complement through the lipid A of the LPS. Thus, polymyxin B inhibits complement activation by *P. aeruginosa,* suggesting that LPS is the major mediator of complement activation (Jensen et al., 1993). However, recently Mishra et al. identified OprF as a novel binding acceptor molecule of C3. C3 binding experiments and opsonophagocytosis assays demonstrated that an OprF-deficient mutant bound less C3 and was ingested by human neutrophils less efficiently than the wild-type strain. Interestingly, binding of C3 to OprF is mediated by the activation of the CP since there were no differences in the binding of C3 between the mutant and the parent strain when they were incubated in the presence of a C1q-deficient serum (Mishra et al., 2015).

P. aeruginosa have developed several mechanisms to interfere with the complement system in order to survive in the host. The first mechanism of complement evasion described here is blocking the binding of the complement activating proteins to the microorganism. The O side chain of the LPS is the best paradigm of this mechanism. Most *P. aeruginosa* isolated from clinical specimens express long O side chains (smooth LPS) that confer to the microorganism resistance to the bactericidal effect

of the human serum. By contrast *P. aeruginosa* isolated from chronic infections in CF patients are often serum sensitive and LPS rough (short or no O side chains) (Hancock et al., 1983). Another bacterial component that impedes the activation of the complement system is the PsI polysaccharide. Mishra et al. demonstrated that a PsI-deficient mutant bound more C3, C5 and C7 than the isogenic wild-type strains. This increased deposition of complement components led to a higher opsonophagocytosis rate of the mutant compared with the parent strain (Mishra et al., 2012). In addition, *P. aeruginosa* produces an alginate polysaccharide that forms a mechanical barrier that reduces the accessibility and the action of host complement proteins (Kharazmi, 1991).

A second mechanism that is used by *P. aeruginosa* to evade the complement system is the expression of proteases that can cleave complement components. *P. aeruginosa* secretes two proteases that cleave complement components namely, elastase and alkaline protease. Both purified proteins degrade C1q and C3 enhancing the virulence of this microorganism by aborting the complement-mediated killing (Hong and Ghebrehiwet, 1992). Furthermore, AprA degrades both C1s and C2 leading to the blocking of CP- and LP-mediated C3b deposition, preventing the opsonophagocytic killing of the pathogen (Laarman et al., 2012).

A third mechanism that is used by *P. aeruginosa* to avoid complement activation is the recruitment of complement regulatory proteins on the bacterial surface. *P. aeruginosa* binds Factor H, Factor H-like protein-1 and complement Factor H-related protein 1 through the dihydrolipoamide dehydrogenase (Hallstrom et al., 2012) and the elongation factor Tu (Kunert et al., 2007). These three proteins inhibit the activation of the AP by avoiding the formation of the C3 convertase. As a result, the deposition of C3b on the bacterial surface is impaired.

Vitronectin is another complement regulatory protein bound by *P. aeruginosa* through the dihydrolipoamide dehydrogenase (Hallström et al., 2015) and the porin D (Paulsson et al., 2015). This regulatory protein inhibits the membrane-damaging effect of the terminal cytolytic complement pathway. Thus, *P. aeruginosa* can evade the effects of the complement system by inhibiting its activation at different levels.

3. OBJECTIVES

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3.1. Identification of *P. aeruginosa* binding molecules for SP-A

Despite considerable evidence that SP-A has a role in host defense against *P. aeruginosa* infections, there is limited information about the ligand/s for this protein on *P. aeruginosa* surface. Furthermore, SP-A binds preferentially to rough LPS strains containing truncated or absent O-antigen domains, a common feature among *P. aeruginosa* from CF patients. However, there are few studies about the interaction of SP-A with *P. aeruginosa* chronic infection isolates.

The aim of this study was to 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.

3.2. Identification of *P. aeruginosa* binding molecules for complement component C3

Resistance of *P. aeruginosa* to complement-mediated effects is a major virulence trait that allows this pathogen to survive within the bloodstream and inflamed lung. To date, the actual mechanism for complement interaction with the *P. aeruginosa* surface remains inadequately investigated.

The objective of this project was to identify novel complement component C3 binding molecules on the outer surface of *P. aeruginosa* to understand the consequences of the interaction of this innate immune host component with this microorganism as well as to identify new strategies that *P. aeruginosa* uses to elude the complement system effects.

4. MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Bacterial strains

The clinical isolates from CF patients used in this study and their relevant features are listed in Table 2. They belong to a larger collection previously described (Lopez-Causape et al., 2013). 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 assesed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

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	

Table 2. *P. aeruginosa* sequential isolates from cystic fibrosis patients used in this study

a) Clonal relatedness evaluated by Pulsed Field Gel Electrophoresis (PFGE)
b) Clonal relatedness evaluated by Multilocus Sequencing Typing (MLST)
* ST1089 derives from ST274 (Lopez-Causape et al., 2013).

P. aeruginosa reference strain PAO1 and its derived isogenic PhoQ-deficient mutant H854 (Macfarlane et al., 1999), OprH deficient mutant PAO1ΔOprH (Edrington et al., 2011) were used in this study. The complemented OprH-deficient mutant was constructed for this study as we describe below. Four clinical isolates from different patients with bacteremia caused by *P. aeruginosa* were also included in the study. *E. coli* strain S17-λpir was used in the cloning experiments.

Bacterial cells were grown in Luria Bertani (LB) (Scharlau) broth at 37ºC with shaking or solidified with 1.5% agar. In some experiments LB was supplemented with Mg^{2+} (3 mM final concentration) by adding $MgSO_4$.

4.2. 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 (Strong et al., 1998). 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 4ºC. 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 $CaCl_2$ 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.

4.3. Human reagents

A pool of normal human sera (NHS) was obtained from blood of consenting healthy volunteers. Human C3-deficient serum and purified human complement component C3 were purchased from Sigma. C3 was labeled with the Infrared Dye 800CW using the IRDye 800CW protein labeling kit (LI-COR) following the manufacturers' instructions.

4.4. Isolation, analysis, and identification of outer membrane components

Isolation of outer membrane proteins were performed as previously described (Garcia-Sureda et al., 2011). 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. Outer membrane proteins were isolated as sodium lauryl sarcosinate-insoluble material, resuspended in Laemmli buffer, boiled for 5 min, resolved by SDS-

PAGE and visualized by Coomassie blue staining. Selected protein spots were excised from the gels, trypsin digested, and identified by tandem mass spectrometry, as described elsewhere (Barbier et al., 2013). The search for filtered peptides was performed using GPS Explorer v3.5 software with a licensed version 1.9 of MASCOT.

LPS from *P. aeruginosa* PAO1 was isolated by the phenol-water method of Westphal and Jann (Westphal and Jann, 1965).

4.5. 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 (Kovach et al., 1994) to give plasmid pBBPhoP. Similarly, for the cloning of *oprH*, the gene was amplified as above using primers OPRHF2H (5'-GCAAAACCTCGCCGAGCCGG-3') and OPRHR2B (5'-GGTGGTATTCGCTGACCCGG-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-λpir by electroporation. All molecular biology techniques were performed according to standard protocols as described previously (Sambrook et al., 1989).

4.6. SP-A binding assays

Binding of SP-A to bacterial cells was determined using Infrared Dye 800CW conjugated SP-A. Briefly, 2 x 10^8 CFU 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 $CaCl_2$ and 0.5 mM $MgCl_2$. 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 above and transferred to Immobilon-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 µg/ml) diluted in PBS-1% BSA. The membranes were subsequently washed and visualized with the Odyssey Infrared Imaging System.

4.7. C3 binding assays

Binding of C3 to bacterial cells was determined using an enzyme-linked immunosorbent assay (ELISA). Briefly, 1x10^9 CFU were washed with PBS and opsonized for 30 min at 37°C with NHS or

C3-deficient serum, as control, diluted in PBS (20% final concentration). After exhaustive washing of the bacteria, cells were incubated for 2 h at 37ºC in 50 mM carbonate-bicarbonate buffer (pH 9.0) containing 1 M NH_4OH to disrupt ester bonds between C3 fragments and the bacterial surface. Cell-bound C3 was quantified by ELISA. For this purpose, microtiter plate wells were coated overnight at 4ºC with serial dilutions of the C3 fragment suspensions. Wells were blocked with PBS-1% BSA, incubated sequentially with anti-human C3 (Sigma) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma), and developed with p-nitrophenyl phosphate (Sigma) in 50 mM carbonate-bicarbonate buffer (pH 9.6) plus 5 mM $MgCl_2$.

To identify the C3-binding proteins from *P. aeruginosa*, outer membrane proteins were separated as described above and transferred to Immobilon-P membranes (Millipore). After transfer, membranes were blocked for 2 hours at room temperature with PBS-1% BSA and incubated for 30 min with Infrared Dye 800CW conjugated C3 (2 µg/ml). The membranes were subsequently washed and visualized with the Odyssey Infrared Imaging System. Alternatively, membranes were incubated in NHS (0.2 %) diluted in PBS-1% BSA, washed and incubated sequentially with polyclonal anti-human C3 (Sigma), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma) and developed with BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium (Sigma).

To study the binding of C3 to purified LPS, microtiter plate wells were coated with serial dilutions of LPS by overnight incubation at 4°C in 50 mM bicarbonate (pH 9.6). After being washed, wells were blocked for 2 hours at room temperature with PBS-1% BSA and sequentially incubated with 5% NHS, polyclonal anti-human C3 (Sigma), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma) and developed with p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 25 mM bicarbonate buffer (pH9.6)-1 mM $MgCl_2$.

4.8. Serum resistance assays

Complement-mediated serum bactericidal activity was determined, as previously described (Alberti et al., 1993). Briefly, 2 x 10^8 CFU were suspended in 20% NHS-PBS or 20% heat-inactivated serum, as control, and incubated at 37°C for 1 hour. Viable counts counts were made by dilution and plating.

4.9. Phagocytosis assays with SP-A

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 37ºC and 5% CO2. 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 (Khubchandani et al., 2001). Briefly, approximately 5 x 10^6 CFU 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. (CFU:cell) After 1 hour at 37ºC, 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 CFU between SP-A treated and untreated bacteria was computed for the fold of phagocytosis mediated by SP-A.

4.10. Complement opsonophagocytic assays

Opsonophagocytic assays were performed using human polymorphonuclear leukocytes (PMNs) isolated from consenting healthy adult donors by dextran sedimentation and Ficoll-Histopaque density gradient centrifugation (Mosca and Forte, 2016). Briefly, $1x10^8$ CFU were pre-incubated with NHS (30% final concentration) or PBS for 30 min at 37ºC. Freshly isolated PMNs were added at a ratio of 100:1 (CFU:PMN) to the bacterial suspension and the mixture was incubated at 37°C for 30 min with shaking on an orbital shaker at 360 rpm. After incubation, the mixture was incubated for 60 min at 37ºC with gentamicin (100 µg/ml) or amikacin (400 µg/ml) to kill extracellular bacteria. Finally, PMNs were washed with PBS and phagocytosed bacteria were released by the addition of 0.5% Triton X-100 and quantified by plating appropriate dilutions on LB agar plates. The ratio of CFU between opsonized and non-opsonized bacteria was defined as the fold change of phagocytosis that was mediated by NHS.

4.11. Murine model of systemic infection

Mouse lethality studies were performed with male CD1 mice, each weighing 16 to 20 g (Harlan Ibérica, S.L.). Mice (n=8) were infected by intraperitoneal injection with approximately 5×10^6 CFU of *P. aeruginosa* from an early log-phase culture in LB. The animals were monitored daily during a period of 3 days and bacteremia was assessed every 12 h by culturing 10 to 30 µl of tail vein blood on LB agar plates. All animal experiments were performed according to institutional and national guidelines and were approved by the Animal Care and Use Committees of the institutions.

5. RESULTS

5. RESULTS

This section has been divided into two parts that correspond to each one of the objectives of this thesis.

5.1. Identification of *P. aeruginosa* binding molecules for SP-A

5.1.1. 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 (Figure 7). 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.

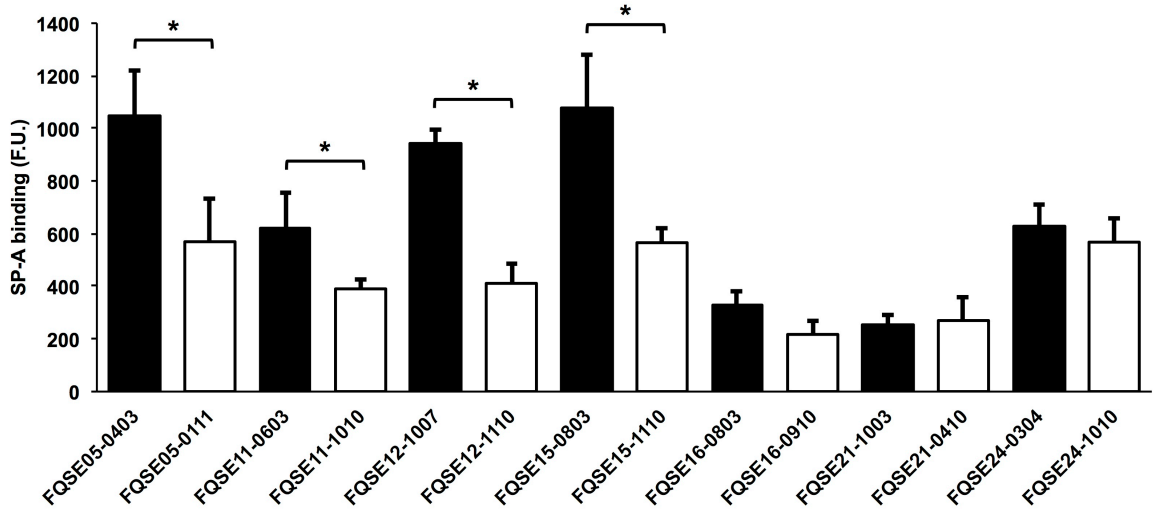

Figure 7. Binding of surfactant protein A to chronic infection *P. aeruginosa* isolates.

Purified human surfactant protein A (SP-A) was labeled with IRDye800CW and incubated with 2 x 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; * $P < 0.05$.

5.1.2. 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. Outer membrane proteins from all clinical isolates were loaded on two parallel gels, one was stained with Coomassie blue (Figure 8A) and the other one was transferred and incubated with IRD800CW labeled SP-A (Figure 8B). A protein of approximately 21-kDa was recognized by SP-A in the outer membrane proteins 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 PAO1ΔOprH 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 PAO1ΔOprH, where no band was detected (Figure 8B). Altogether these data indicate that outer membrane protein OprH binds SP-A.

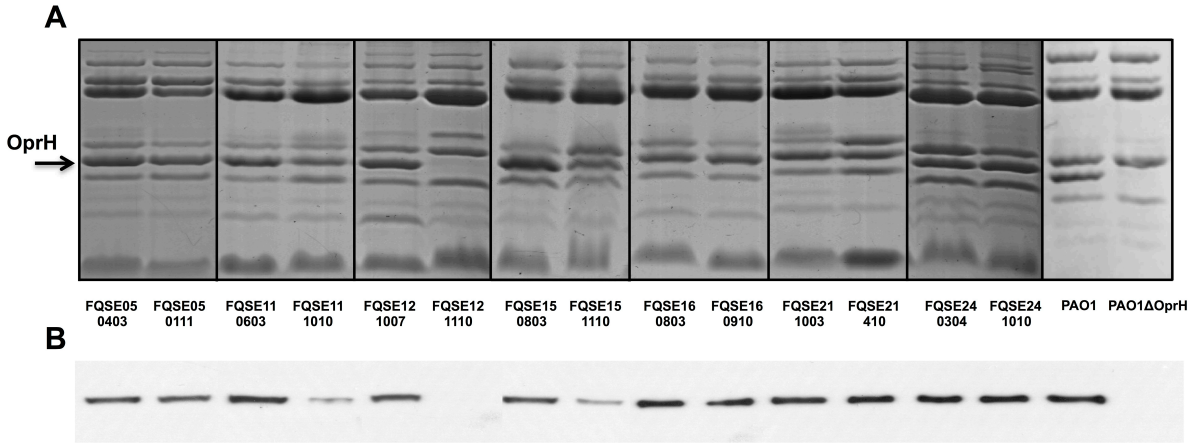

Figure 8. Surfactant protein A binding analysis of *P. aeruginosa* chronic infection isolates outer membrane proteins.

Outer membrane proteins from each isolate, PAO1 and its derived OprH-deficient mutant PAO1ΔOprH, 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.

5.1.3. 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

(Figure 8A) and densitometry (Figure 9). 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±0.02 fold and 2.1±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.

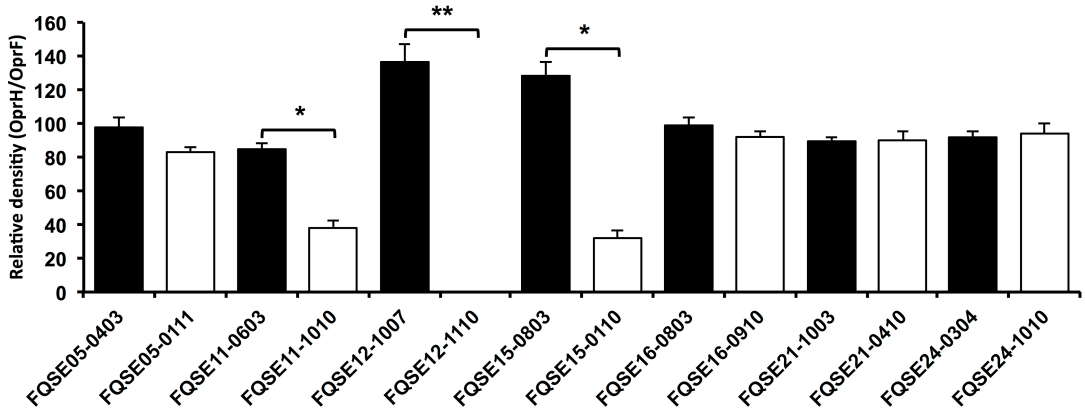

Figure 9. Densitometric analysis of the presence of OprH in *P. aeruginosa* chronic infection isolates.

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.

Sequence analysis of the *oprH-phoP-phoQ* operon from FQSE12-1110 detected two single nucleotide polymorphisms (SNPs) in the *phoP* gene that were not present in the early isolate FQSE12-1007 (Table 3), 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^{2+} growth conditions (Figure 10).

Gene	SNPs/Insertions encountered		Predicted effect	
	FQSE12-1007	**FQSE12-1110**	**FQSE12-1007**	**FQSE12-1110**
oprH	-	-	-	-
phoP	T537C	C138T T537C **A563G**	-	**N188S**
phoQ	T675G A702G T747C C1101T **nt1266$_{insG}$**	T675G A702G T747C C1101T **nt1266$_{insG}$**	**Different from I422 to V448**	**Different from I422 to V448**

Table 3. DNA sequence analysis of the *oprH-phoP-phoQ* operon.

Single nucleotide polymorphims (SNPs) or insertions detected in the *oprH-phoP-phoQ* operon from the early (FQSE12-1007) and late isolate (FQSE12-1110) and their predicted effect on the encoded protein. The numbering is based on *oprH-phoP-phoQ* operon sequence of PAO1.

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^{2+} in the media (Table 3 and Figure 10).

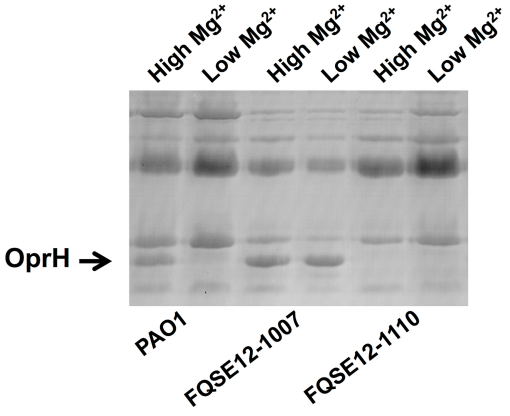

Figure 10. Effect of Mg^{2+} on the expression of outer membrane proteins of *P. aeruginosa*.

Representative sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the outer membrane proteins isolated from strains PAO1, FQSE12-1007 and FQSE12-1110 grown in LB with low Mg^{2+} (0.1 mM) or high Mg^{2+} (3 mM).

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 (Figure 11A) and increased the binding of SP-A to the same levels observed in the early isolate FQSE12-1007 (Figure 11B). 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 (Figure 11A), 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 (Figure 11B).

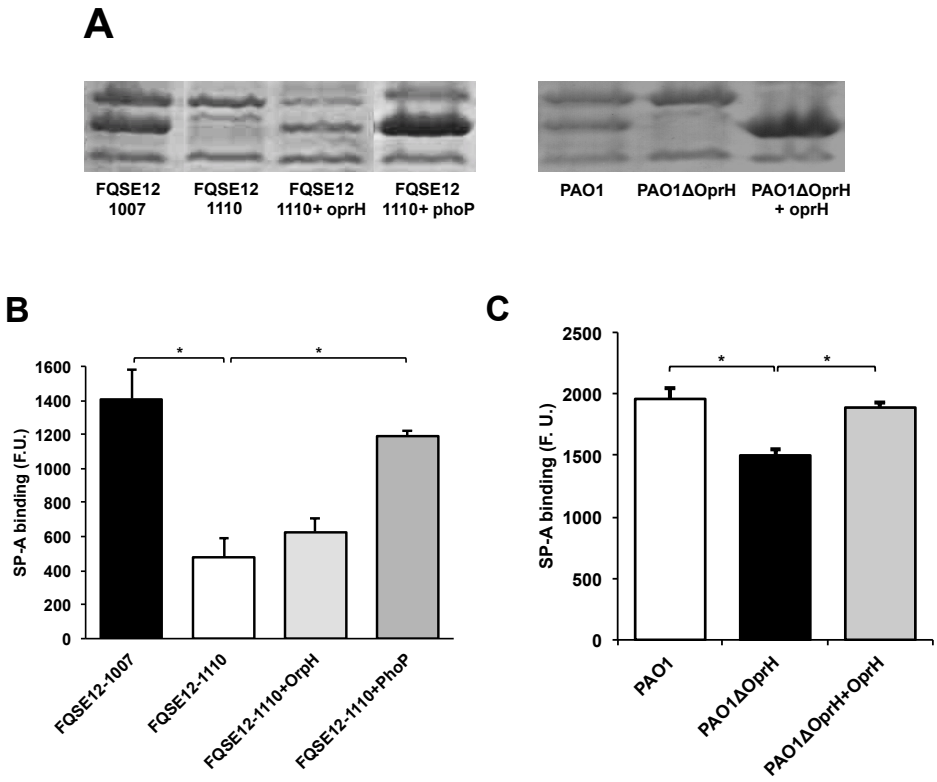

Figure 11. OprH promotes binding of surfactant protein A to *P. aeruginosa*.

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 x 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; * $P < 0.05$.

To further confirm this result, we performed SP-A binding experiments with PAO1, its derived isogenic OprH deficient mutant PAO1ΔOprH, and the PAO1ΔOprH 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 then the OprH-deficient mutant (Figure 11C). Overall, these results indicate that OprH mediates the binding of SP-A to *P. aeruginosa*.

5.1.4. 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 (Figure 12). 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.

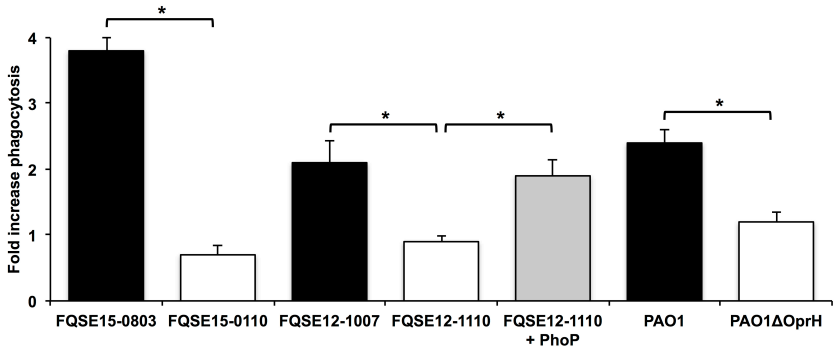

Figure 12. Binding of surfactant protein A to OprH mediates phagocytosis of *P. aeruginosa*.

P. aeruginosa strains were preopsonized with surfactant protein A (5 µg/ml for the CF isolates and 50 µg/ml for PAO1) or with bovine serum albumine (10 µg/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 LB 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; * $P < 0.05$.

5.2. Identification of *P. aeruginosa* binding molecules for complement component C3

In the course of a study to investigate the bacterial components and the host mechanisms involved in the reduced virulence exhibited by the PhoQ mutant, we identified a previously unrecognized C3 binding molecule of *P. aeruginosa*. In this section, we describe the experiments and results that led to the identification of this protein.

5.2.1. *P. aeruginosa* PhoQ mutant was avirulent in a murine model of systemic infection

To investigate the potential impact of the absence of PhoQ in the pathogenesis of *P. aeruginosa* sepsis, we tested the ability of the PhoQ-deficient mutant to cause bacteremia and fatal infection in a murine model of systemic infection. Mice were challenged intraperitoneally with strain PAO1 and the isogenic PhoQ-deficient mutant H854 and monitored for development of positive blood culture. All animals infected with the wild-type strain PAO1 developed bacteremia before 36 hours, while none of the mice infected with the mutant became bacteremic (Figure 13A). Analysis of survival indicated that bacteremia preceded fatal infection by 12–24 hours, and 100% of the animals infected with the wild-type strain died by day 3 with the majority of deaths occurring before 36 hours. By contrast, none of the mice infected with the PhoQ mutant died (Figure 13B).

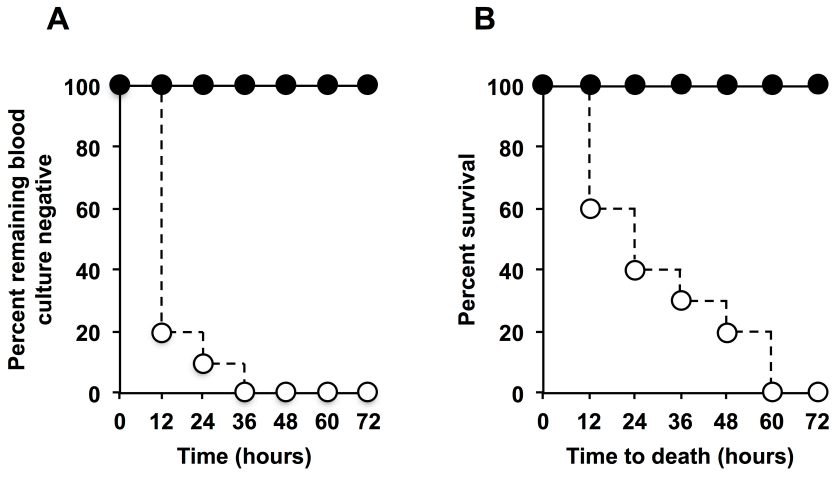

Figure 13. **Effect of PhoQ deficiency on *P. aeruginosa* systemic infection.**

Analysis of time to first positive blood culture (A) and survival curves over 3 days (B) of mice (n= 8) infected with ~ 5 x10^6 CFU of *P. aeruginosa* strain PAO1 (white circles) or its derived isogenic mutant PAO1ΔPhoQ (H854) (black circles). The time to first positive culture and the difference in survival between the two groups were significantly different by log rank test ($P < 0.0001$).

5.2.2. Role of complement in PhoQ-deficient mutant attenuated phenotype

Complement is the major soluble early host effector against blood infections and plays an important role in the clearance of *P. aeruginosa* by opsonizing this organism for phagcytosis (Mueller-Ortiz et al., 2004). To investigate the host defense mechanisms that led to clearance of the PhoQ-deficient mutant, we characterized the ability of the wild-type strain PAO1 and the isogenic PhoQ-deficient mutant to bind complement component C3. After 30 min of incubation in NHS the mutant grown under conditions of Mg^{2+} starvation (LB), bound similar amounts of C3 as did the wild-type strain (Figure 14A). However, in the presence of Mg^{2+} concentrations of 3 mM, a concentration that is similar to the physiological divalent cation levels in blood, the wild-type strain bound significantly less C3 than the mutant, which bound as much C3 as when it grew without Mg^{2+} (Figure 14A). These results suggest that divalent cations like Mg^{2+}, that are sensed by PhoQ, are a critical signal for suppressing the levels of C3 that can be deposited on the bacterial surface. Because the PhoQ sensor kinase is highly conserved amongst *P. aeruginosa* strains, we reasoned that Mg^{2+}-dependent binding of C3 should be demonstrable in other *P. aeruginosa* strains in addition to PAO1. To test this hypothesis, we determined the binding of C3 in a number of *P. aeruginosa* bloodstream isolates grown in LB supplemented or not with 3 mM Mg^{2+}.

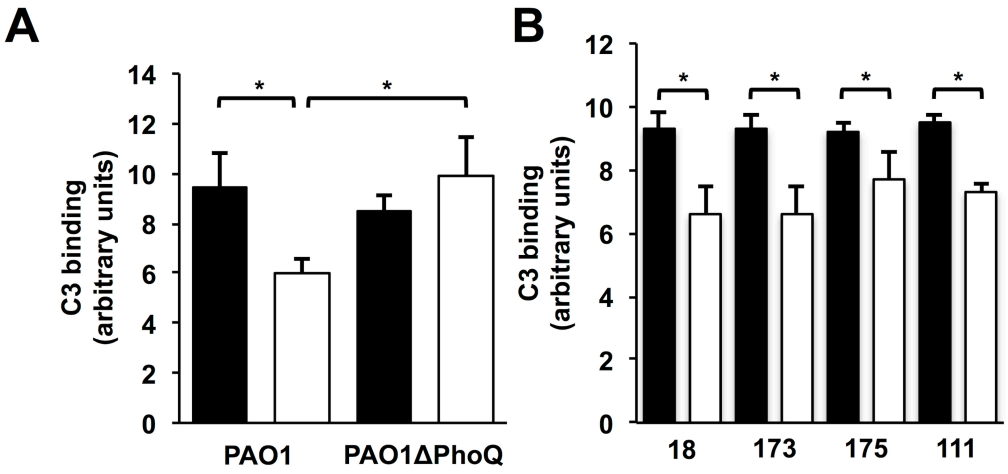

Figure 14. Effect of Mg^{2+} on the complement component C3 deposition on *P. aeruginosa*.

Cells of the wild-type strain PAO1 or the isogenic PhoQ-deficient mutant (panel A) or four different *P. aeruginosa* bloodstream isolates (panel B) grown in LB (black columns, low Mg^{2+}) or LB supplemented with 3 mM Mg^{2+} (white columns, high Mg^{2+}) were incubated in NHS or C3-deficient serum (as control). C3 deposited on the bacterial surface was determined by ELISA. Control values in C3-deficient serum were always <1 arbitrary unit and were substracted from the values obtained with NHS. Errors bars represent SEMs. Statistical analyses were performed using Student's unpaired two-tailed *t* test; * $P < 0.05$.

Similar to the results obtained for strain PAO1, C3 deposition in 4 additional *P. aeruginosa* strains decreased significantly during growth in LB broth supplemented with 3 mM Mg^{2+} (Figure 14B). Mg^{2+}-dependent reduction of C3 deposition did not confer increased resistance to the bactericidal effect of the complement. Both, PAO1 and the PhoQ-deficient mutant grown in high Mg^{2+} (3 mM) were as resistant to complement-killing as when they were grown in low Mg^{2+} (LB) (Figure 15A). However, it was crucial to reduce the recognition of the pathogen by human PMNs (Figure 15B). Phagocytosis of the wild-type strain grown in low Mg^{2+} (LB) and opsonized with NHS was almost three-fold more effective than was phagocytosis of cells grown in high Mg^{2+} (3 mM). By contrast, there was no difference between the opsonophagocytosis rate of the PhoQ-deficient mutant grown in either low or high Mg^{2+}. Furthermore, the mutant was phagocytosed by PMNs three-fold more efficiently than the wild-type strain when both were grown in high (3 mM) Mg^{2+} concentrations similar to those found in blood (high Mg^{2+}). Altogether, these results suggested that PhoQ is required for resistance to the early host defense mechanisms of blood, including complement and opsonized phagocytosis by PMNs.

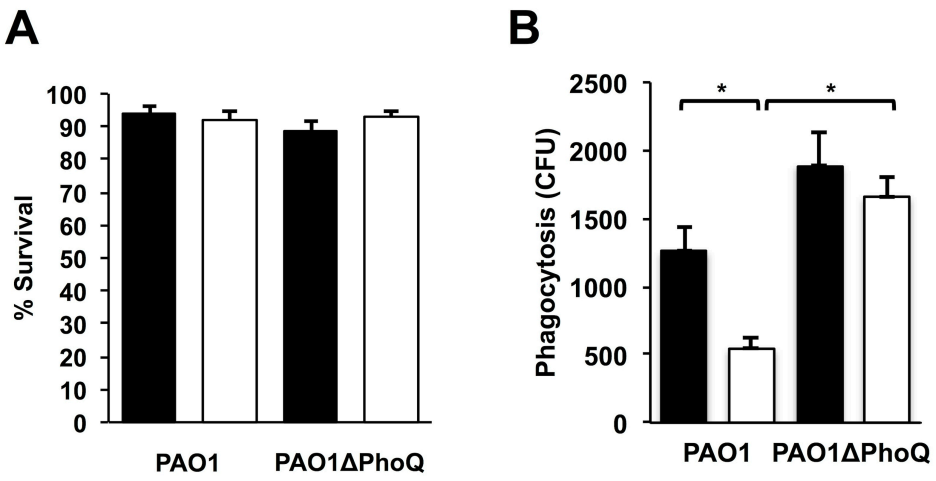

Figure 15. Effect of Mg^{2+} on the complement-mediated killing and complement-mediated opsonophagocytosis of *P. aeruginosa*.

Panel A: *P. aeruginosa* strains grown in LB (black columns, low Mg^{2+}) or LB supplemented with 3 mM Mg^{2+} (white columns, high Mg^{2+}) were incubated in 20% NHS or 20% heat-inactivated serum and after 1 hour the number of viable bacteria were determined by plating on LB plates. Percent survival was calculated as the number of viable bacteria in NHS relative to that in heat-inactivated serum. Panel B: *P. aeruginosa* strains, grown as described above, were preopsonized with NHS and subsequently incubated with freshly isolated human PMNs. Extracellular bacteria were killed with antibiotics and bacterial uptake was determined after lysis of the PMNs by plating on LB plates. Data represents three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were performed using Student's unpaired two-tailed *t* test; * $P < 0.05$.

5.2.3. Identification of a novel C3-binding protein of *P. aeruginosa*

To date, two C3 binding molecules have been identified on the *P. aeruginosa* surface, LPS (Jensen et al., 1993) and OprF (Mishra et al., 2015). To investigate whether the differences in the binding of C3 between PAO1 grown in low or high Mg^{2+} concentration were associated with changes in the LPS, the binding of C3 to LPS purified from PAO1 grown in LB supplemented or not with 3 mM Mg^{2+} was analyzed by ELISA. There were no differences in the binding of C3 to either LPS preparation (Figure 16). In addition, no difference was detected in the amount of OprF present in the outer membranes isolated from PAO1 grown in low or high Mg^{2+} concentration (Figure 17A).

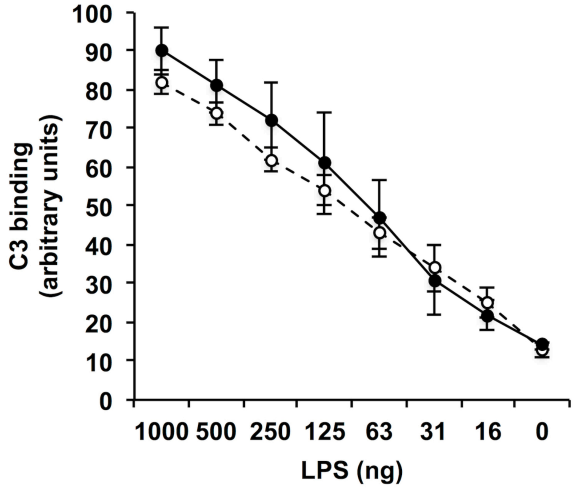

Figure 16. Effect of Mg^{2+} on the binding of C3 to *P. aeruginosa* LPS.

LPS from PAO1 grown in LB (low Mg^{2+}, black circles) or in LB supplemented with 3 mM Mg^{2+} (high Mg^{2+}, white circles) was purified and used to coat microtiter plate wells that were sequentially incubated with NHS (5% final concentration), rabbit anti-human C3, alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G and developed. Data represent three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were performed using Student's unpaired two-tailed *t* test.

These results suggested that other bacterial components modulated the Mg^{2+}-dependent deposition of C3 on *P. aeruginosa*. To identify this component, outer membrane preparations were subjected to ligand blot analysis. OMPs from PAO1 and the PhoQ-deficient mutant grown in LB or in LB supplemented with Mg^{2+} were loaded onto 3 separate gels, one of which was stained with Coomassie blue (Figure 17A), while the other two were electrophoretically transferred by Western blotting to Immobilon-P membranes and incubated with either IRD800CW labeled purified human C3 (2 µg/ml) (Figure 17B) or NHS (0.2% final concentration), rabbit anti-human C3 and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Figure 17C). In both blots, a protein of approximately 21-

kDa was recognized by C3 in the OMPs of both strains. To identify the 21-kDa protein, the corresponding band was excised from the gel and the protein was subjected to mass spectrometry analysis. The band was found to correspond to the outer membrane protein OprH, which is repressed during growth in Mg^{2+} concentrations of 2 mM or greater in PAO1, but not in the constitutive PhoQ-deficient mutant (Figure 17A).

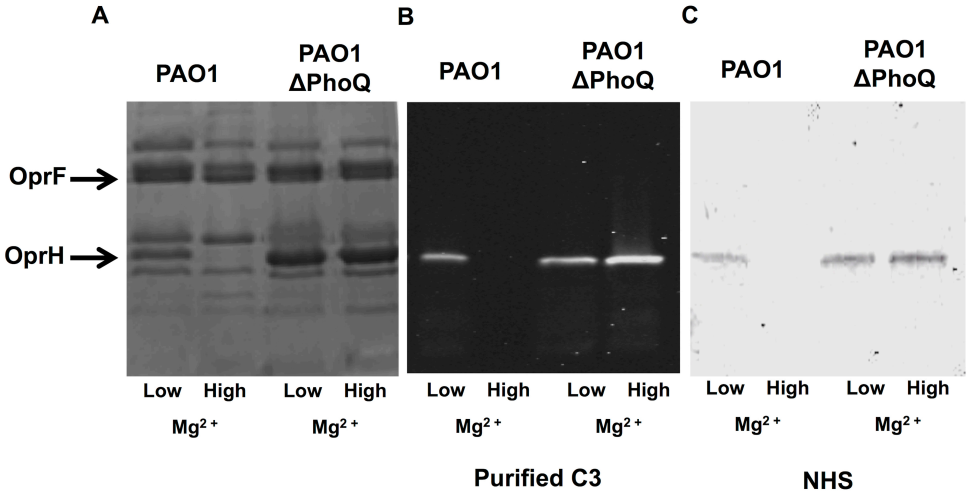

Figure 17. C3 binding analysis of *P. aeruginosa* outer membrane proteins.

Outer membrane proteins from PAO1 and its isogenic PhoQ-deficient mutant grown in LB (low Mg^{2+}) or in LB supplemented with 3 mM Mg^{2+} (high Mg^{2+}), were isolated, resolved, and either (A) stained with Coomassie blue: or transferred to an Immobilon-P membrane and (B) incubated with IRD800CW labeled C3 (2 µg/ml), or (C) NHS (0.2% final concentration), rabbit anti-human C3 and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (C). A band of approximately 21-kDa, identified by mass spectrometry analysis as OprH, reacted with C3.

5.2.4. OprH promotes binding of C3 and opsonophagocytosis of *P. aeruginosa*

Ligand blot results suggested that OprH mediated the Mg^{2+}-dependent C3 binding to *P. aeruginosa*. However, since PhoQ and Mg^{2+} modulate the expression of many other bacterial components (Gooderham et al., 2009), we investigated whether the Mg^{2+}-dependent C3 binding was exclusively due to the presence or absence of OprH in the outer membrane. For these experiments, we used the PAO1 derived isogenic OprH deficient mutant PAO1ΔOprH, and the PAO1ΔOprH complemented with *oprH*. Ligand blot analysis using purified fluorescent C3 shown that OprH, conditionally present in the outer membrane of PAO1 and constitutively present in the complemented mutant, but not in the *oprH* mutant, reacted with C3 (Figure 18A).

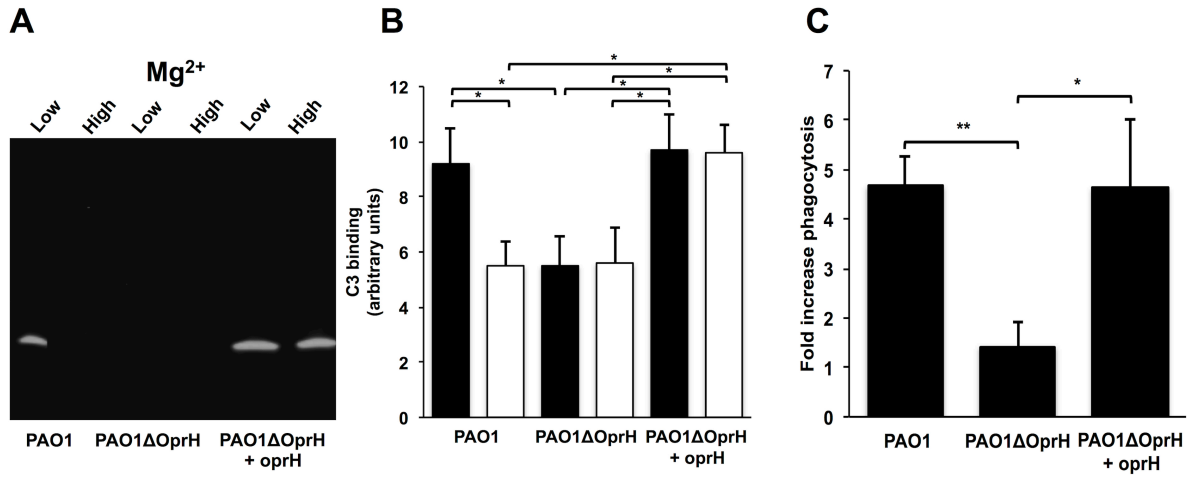

Figure 18. OprH promoted binding of C3 and opsonophagocytosis of *P. aeruginosa*.

Outer membrane proteins from the wild-type strain PAO1, the isogenic OprH deficient mutant OprH PAO1ΔOprH and the complemented mutant were isolated, resolved, transferred to an Immobilon-P membrane and incubated with IRD800CW labeled C3 (2 µg/ml) (panel A). Cells of the same strains grown in LB (black columns, low Mg^{2+}) or LB supplemented with 3 mM Mg^{2+} (white columns, high Mg^{2+}) were incubated in NHS. C3 deposited on the bacterial surface was determined by ELISA (panel B). *P. aeruginosa* strains were preopsonized with NHS or with PBS and subsequently incubated with freshly isolated human PMNs. Extracellular bacteria were killed with antibiotics and bacterial uptake was determined after lysis of the PMNs and plating on LB plates. The ratio of CFU between opsonized (NHS) and non-opsonized (PBS) bacteria was computed for the fold of phagocytosis-mediated by NHS (panel C). Data represent three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were performed using Student's unpaired two-tailed *t* test; * $P < 0.05$, ** $P < 0.01$.

C3 binding analysis to bacterial cells demonstrated that at low Mg^{2+} concentration, both PAO1 and the mutant complemented with *oprH* bound C3 almost two-fold more efficiently than the OprH-deficient mutant (Figure 18B). As expected, at the high Mg^{2+} concentration, the wild-type strain bound less C3 than at the low Mg^{2+} concentration. Conversely, the binding of C3 to the mutant was not affected by the Mg^{2+} concentration. The complemented mutant bound similar amounts of C3 at both Mg^{2+} concentrations due to the constitutive expression of *oprH*, that was driven by the *lac* promoter (Figure 18B). We next determined whether OprH-C3 interactions promoted the phagocytosis of *P. aeruginosa* by human PMNs. PAO1, the OprH-deficient mutant, and the complemented mutant were opsonized with NHS and incubated with human PMNs. Bacterial uptake was determined by plating on LB plates after killing extracellular bacteria with antibiotic. Incubation with C3 increased the phagocytosis of PAO1 and the complemented mutant by more than four-fold but had almost no effect on the OprH-deficient mutant (Figure 18C). Overall, these results indicate that OprH promoted the binding of C3 and serum-opsonized phagocytosis of *P. aeruginosa* by human PMNs.

6. DISCUSSION

6. 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 (Tan et al., 2014). Another outer membrane protein, P2 of *Haemophilus influenzae,* have also been shown to bind SP-A (McNeely and Coonrod, 1994). 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 (Reinhart, 1988). Indeed, a global transcriptional analysis of differentally regulated genes in *P. aeruginosa* isolated from human sputum samples demonstrated that *oprH* was downregulated (Bielecki et al., 2013). 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 permitttting 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+} (Mulcahy et al., 2008). Furthermore, Gellatly et al provided evidence that *oprH* is rapidly transcribed after interaction of *P. aeruginosa* with human airway epithelial cells (Gellatly et al., 2012).

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.

Suppporting 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 (Lopez-Causape et al., 2013; Miller et al., 2011), 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* (Miller et al., 2011). 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, in the first part of this study, 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 from the SP-A-mediated phagocytic killing.

The data presented in the second part of this study suggest that the suppression of sensing environmental magnesium by PhoQ, which is able to both phosphorylate and dephosphorylate PhoP (Macfarlane et al., 1999), is essential for *P. aeruginosa* to reduce the expression of a previously unrecognized complement target on the bacterial surface, OprH, and avoid opsonophagocytosis by human PMNs. The observation that high Mg^{2+} concentrations reduced the binding of C3 in the *P. aeruginosa* wild-type strain but not in an isogenic PhoQ mutant pointed to a direct effect of the divalent cation on the expression of a bacterial component, the expression of which is modulated by the PhoP/PhoQ system. Our experiments performed with a specific OprH-deficient mutant and the complemented mutant clearly demonstrated that the effect of Mg^{2+} on the binding of C3 relies on the expression of this outer membrane protein. Thus, it appears unlikely that bacterial component(s) modulated by PhoQ, other than OprH, are responsible for the effect of Mg^{2+} on the binding of C3. Indeed, although the levels of Mg^{2+} influence the lipid A structure (Gellatly et al., 2012), we did not detect differences in the binding of C3 to LPS from bacterial cells grown in low or high Mg^{2+} levels.

To our knowledge, OprH is the second *P. aeruginosa* outer membrane protein, together with OprF (Mishra et al., 2015), involved in the activation of the complement system. Conversely, the ligand blot experiments described here, failed to detect binding of C3 to OprF as described by Mishra et al. (Mishra et al., 2015). It is possible that the human serum used by these researchers contained specific antibodies against OprF. In fact, binding of C3 to OprF was markedly reduced when *P. aeruginosa* was incubated in a C1q-depleted serum, suggesting that binding of C3 to OprF was mediated by the activation of the classical complement pathway (Mishra et al., 2015). In contrast, in our experiments we used purified C3, and therefore excluded specific antibodies, suggesting that OprH mediated activation of the alternative complement pathway, which plays a role in resistance against *P. aeruginosa* infections (Mueller-Ortiz et al., 2004). Another explanation that may account for this discrepancy is based on the amount of complement used in the ligand blot experiments. Mishra et al. used 20% normal human serum (Mishra et al., 2015), while we used 2 µg/ml of C3 or 0.2% normal human serum. Indeed, in preliminary experiments using 20 µg/ml C3 or 20% normal human serum, we were able to detect OprF as a C3 binding molecule. Overall, this result suggests that both outer membrane proteins, OprF and OprH, bind C3 but with different affinity.

Here we have tested only the effect of Mg^{2+}, but *P. aeruginosa* PhoQ can sense other environmental signals in the body fluids to reduce OprH expression and evade opsonophagocytosis. These include other divalent cations such as Ca^{2+}, the concentration of which in the blood is in the range that reduces the expression of OprH, and polyamines (Kwon and Lu, 2006).

Although there are some parallels between the PhoP/PhoQ systems of *Salmonella* and *Pseudomonas*, there are many differences as well (Hancock and McPhee, 2005). For instance, in *P.* aeruginosa, a PhoP mutant is more virulent than the wild type-strain, while in *Salmonella* a PhoP mutant exhibits decreased virulence (Hancock and McPhee, 2005). This finding is consistent with our results since the expression of OprH, which confers increased susceptibility to opsonophagocytic killing, is abolished in the PhoP mutant. On the other hand, *P. aeruginosa* PhoP/PhoQ system is the uniquely part of a three-gene operon that includes the *oprH* gene encoding an outer membrane protein. Overall, it seems that the PhoPQ-dependent susceptibility to opsonophagocytic killing mediated by OprH may be exclusive to *P. aeruginosa*.

Although the experiments presented here were performed in vitro, Mg^{2+}-suppressed binding of C3 through OprH is expected to also operate in vivo during infection. In fact, expression of OprH is completely abolished when *P. aeruginosa* grows in human serum in vitro (Figure 19). In addition, previous studies demonstrated that expression of OprH is markedly reduced in a murine model of burn infection and in ex vivo samples from human burn infections compared to laboratory growth conditions (Bielecki et al., 2013; Turner et al., 2014).

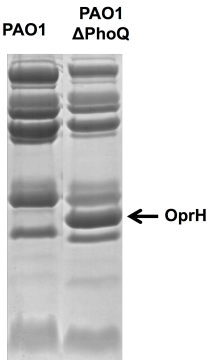

Figure 19. Representative sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the outer membrane proteins isolated from strains PAO1 and its isogenic PhoQ-deficient mutant grown in NHS.

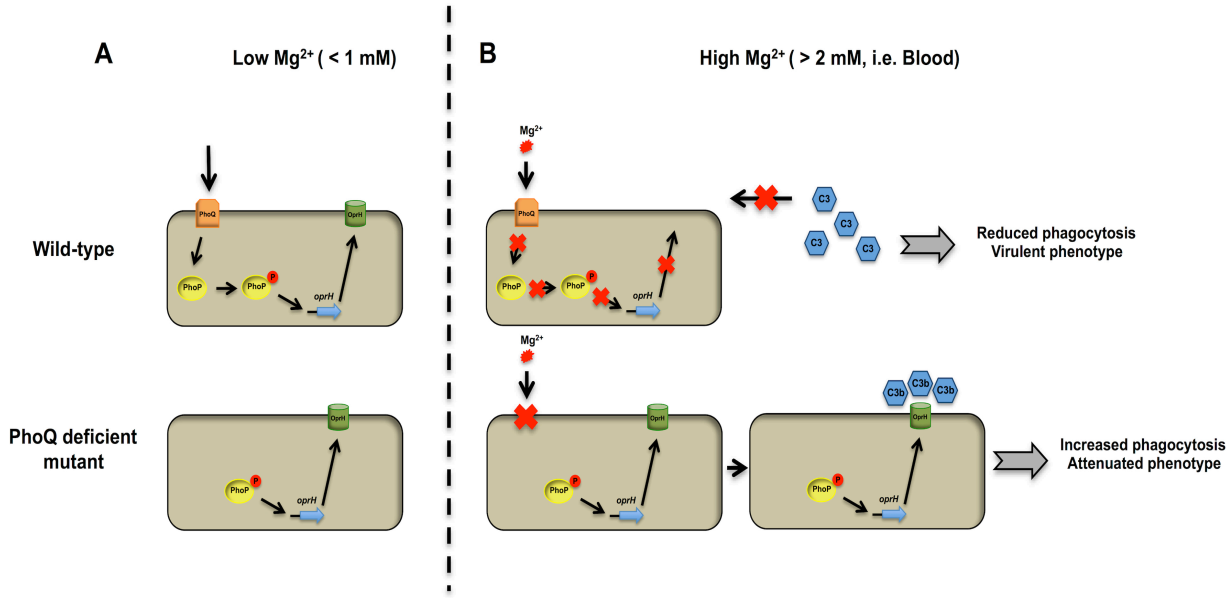

Figure 20. Sensing of Mg^{2+} by PhoQ and its effect on *P. aeruginosa* opsonophagocytic killing.

A) In the presence of low concentrations of Mg^{2+} (< 1mM), *P. aeruginosa* PhoQ phosphorylates PhoP, which in turns upregulates expression of OprH in the wild-type strain. In a PhoQ-deficient mutant, PhoP is phosphorylated, in consequence, OprH is constituvely expressed. B) In the presence of high concentrations of Mg^{2+} (> 2mM), similar to those found in blood during infection, in the wild-type strain, PhoQ senses Mg^{2+} and reduces PhoP phosphorylation, which results in downregulation of OprH expression. By contrast in a PhoQ-deficient mutant, expression of OprH, a novel C3 binding *P. aeruginosa* molecule, is constitutive. As a result, the PhoQ mutant binds C3 and is phagocytosed by PMNs more efficiently than the wild type strain.

In conclusion, this second part of the study identifies PhoQ as an essential sensor for *P. aeruginosa* to avoid complement-mediated opsonophagocytosis due to the direct control that exerts on the expression of OprH, a previously unrecognized C3 binding molecule of *P. aeruginosa* (Figure 20).

Interestingly, the results of our study demonstrate that the outer membrane protein OprH is the target for two of the most important soluble mediators of the innate immune system. We have not investigated the regions or domains of OprH involved in the interaction with SP-A and C3. Indeed, it might be possible that both host molecules share the same recognition domain in OprH.

It is well established that SP-A recognizes some specific sugars as well as some proteins on the bacterial surface. The interaction of the collectins, such as SP-A, through the C type lectin domain or the CRD with the corresponding target, is often of ionic nature (Casals, 2001). These interactions are sensitive to changes in ionic strengh and pH. According to the OprH model established by Edrington et al.(Edrington et al., 2011), the external loops of OprH contain a remarkable number of charged residues of amino acids (see introduction, Figure 4). This evidence suggests that the binding of SP-A to this outer membrane protein may be of ionic nature. It would be interesting to perform binding

experiments with purified SP-A and OprH in the presence of different concentracions of NaCl to confirm this hypothesis.

On the other hand, porins have been described as C3 binding molecules in *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Neisseria meningitidis* and *Legionella pneumophila* (Alberti et al., 1996; Bellinger-Kawahara and Horwitz, 1990; Ding et al., 2010; Lewis et al., 2013) However, there are no conserved domains within the structure of the external loops of those porins to establish a common region for the binding of C3. Moreover, other non-porin proteins have been identified as C3 binding molecules in other pathogens including a surface immunoglobulin binding protein and the extracellular fibrogen binding protein from *S. aureus* (Koch et al., 2012; Lee et al., 2004), the heparin binding agglutinin from *Mycobacterium tuberculosis* and *M. avium* (Mueller-Ortiz et al., 2001), and a phenolic glycolipid-1 from *M. leprae* (Schlesinger and Horwitz, 1991). Overall, all these studies showing C3 binding to diverse bacterial proteins support the notion that C3 has evolved to recognize different patterns present in the bacterial exposed proteins. Further studies with synthetic peptides covering residues of the external regions of OprH and site-directed mutagenesis of these regions are being conducted to identify the SP-A and the C3 binding domain.

To date, the function of OprH still remains poorly studied. In vivo and in vitro biochemical assays have demonstrated that OprH interacts directly with the LPS, suggesting that the function of OprH is to provide increased stability to the outer membrane of *P. aeruginosa* (Edrington et al., 2011). However, the absence of OprH does not impair the fitness of *P. aeruginosa*. Competition experiments performed with PAO1 and the isogenic OprH-deficient mutant, in the presence or absence of Mg^{2+}, did not reveal significant differences between both strains in any condition (data not shown). Nevertheless, since binding of SP-A and C3 to OprH facilitates recognition by macrophages and PMNs, the expression of this protein is tightly controlled by *P. aeruginosa* through the sensor kinase PhoQ. Thus, PhoQ may represent a promising target to develop new drugs against *P. aeruginosa* infections.

7. CONCLUSIONS

7. CONCLUSIONS

1. Outer membrane protein OprH is a target for the surfactant protein A on the surface of *P. aeruginosa* strains isolated from the lung of patients with cystic fibrosis.

2. The binding of SP-A to OprH enhances the opsonophagocytosis of *P. aeruginosa* isolates from cystic fibrosis patients by human macrophages.

3. Reduction of OprH expression is a previously unrecognized adaptation of *P. aeruginosa* to cystic fibrosis lung that facilitates the escape of the microorganism from the SP-A-mediated phagocytosis.

4. PhoQ expression is essential for *P. aeruginosa* virulence in the murine model of systemic infection.

5. Binding of the complement component C3 to *P. aeruginosa* is influenced by the concentration of Mg^{+2}. In the presence of physiological concentration of Mg^{+2}, *P. aeruginosa* binds C3 less efficiently than in the absence of this divalent cation.

6. Mg^{2+}-dependent binding of C3 to *P. aeruginosa* depends on the expression of PhoQ.

7. The outer membrane protein OprH mediates the Mg^{2+}-dependent binding of C3 to *P. aeruginosa* and promotes the opsonophagocytosis of this microorganism by human polymorphonuclear cells.

8. Overall, PhoQ is an essential sensor of Mg^{2+} for *P. aeruginosa* to avoid surfactant protein A- and C3-mediated opsonophagocytosis due to the direct control that exerts on the expression of OprH, a previously unrecognized surfactant protein A and C3 binding molecule of *P. aeruginosa*

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