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Sensing Mg^{2+} contributes to the resistance of *Pseudomonas aeruginosa* to complement-mediated opsonophagocytosis

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Summary

Pseudomonas aeruginosa **adaptation to survive in the host hinges on its ability to probe the environment and respond appropriately. Rapid adaptation is often mediated by two-component regulatory systems, such as the PhoP/PhoQ system that responds to Mg^{2+} ion concentration. However, there is limited information about the role of PhoQ in** ***P. aeruginosa*** **bloodstream infections. We used a murine model of systemic infection to test the virulence of a PhoQ-deficient mutant. Mutation of PhoQ impaired the virulence and the ability to cause bacteremia of** ***P. aeruginosa*****. 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 controlled by the PhoP/PhoQ system. Ligand blot analysis, C3 binding experiments and opsonophagocytosis assays identified this component as the outer membrane protein OprH, expression of which impaired the virulence of** ***P. aeruginosa*** **in a murine model of systemic infection. We demonstrate that expression of PhoQ is essential to detect Mg^{2+} and reduce the expression of OprH, a previously unrecognized C3 binding molecule that promotes the opsonophagocytosis of** ***P. aeruginosa*****.**

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Introduction

Pseudomonas aeruginosa is a ubiquitous microorganism widely distributed in many different ecological niches, and is also a major opportunistic pathogen. Indeed, *P. aeruginosa* is the most common Gram-negative organism causing nosocomial pneumonia, burn wounds infections and fatal bacteremia (Lyczak *et al.*, 2000). In addition, this pathogen chronically infects patients with significant underlying diseases such as cystic fibrosis, chronic obstructive pulmonary disease or bronchiectasis (Evans *et al.*, 1996; Hill *et al.*, 2000; Lyczak *et al.*, 2002).

The widespread occurrence of *P. aeruginosa* is due to several factors, including its ability to utilize many environmental compounds as energy sources and a large number of regulators enabling it to adapt rapidly by modulating the expression of gene products necessary for survival in specific environmental niches defined by the site of colonization. Therefore, adaptation and survival of *P. aeruginosa* hinge on its ability to probe the environment and respond appropriately.

Rapid adaptation to each environmental challenge is often mediated by two-component regulatory systems, such as the PhoP/PhoQ system. PhoQ is a membrane-associated sensor kinase, that in *P. aeruginosa*, 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). PhoQ phosphorylates its cognate response-regulator protein PhoP; this phosphoprotein in turn transcriptionally activates or represses its target genes by binding to specific upstream sequences. It has been proposed that PhoQ also acts as a phosphatase that dephosphorylates and therefore deactivates PhoP selectively. Thus, in a PhoQ-deficient mutant, the PhoP modulated genes are dysregulated and expressed constitutively (Macfarlane *et al*., 1999; Gooderham and Hancock, 2009).

The PhoPQ regulon comprises more than 474 genes that are dysregulated in a PhoQ-deficient mutant, including the outer membrane protein OprH gene, located in the same PhoP/PhoQ operon, and the *arnBCADTEF* operon that mediates the synthesis and transfer of 4-amino-L-arabinose to the lipid A of the lipopolysaccharide (LPS) (Gooderham *et al*., 2009). 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 lettuce leaf model of infection and in a mammalian 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 downregulation 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*, or in the reduced ability to interact with 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.

In this work, we used a murine model of systemic infection to test the virulence of a PhoQ-deficient mutant. Our findings demonstrated that PhoQ detected Mg^{2+} and was essential in vivo and reduced the expression of the outer membrane protein OprH, a previously unrecognized C3 binding molecule that promotes the opsonophagocytic killing of *P. aeruginosa*.

Results

Pseudomonas 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. Both strains showed similar in vitro growth rates; doubling times in mid-log phase in Luria Bertani (LB) were from 33 min to 36 min at 37°C (Fig. 1A). However, all animals infected with the wild-type strain PAO1 developed bacteremia before 36 h, while none of the mice infected with the mutant became bacteremic (Fig. 1B). Analysis of survival indicated that bacteremia preceded fatal infection by 12–24 h, and 100% of the animals infected with the wild-type strain died by day 3 with the majority of deaths occurring before 36 h. By contrast, none of the mice infected with the PhoQ mutant died (Fig. 1C).

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

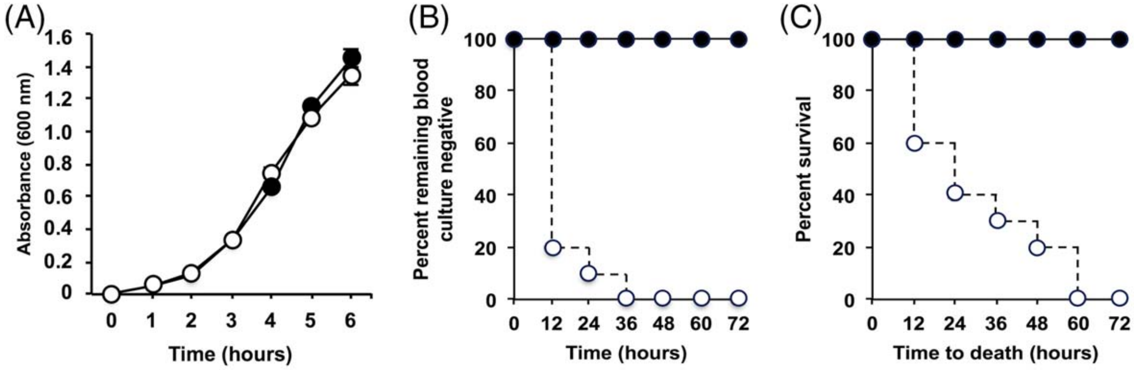

Fig. 1. Effect of PhoQ deficiency on *P. aeruginosa* systemic infection.
Growth curve at 37°C in LB of *P. aeruginosa* strain PAO1 (white circles) and its derived isogenic mutant PAO1ΔPhoQ (H854) (black circles) (A). Analysis of time to first positive blood culture (B) and survival curves over 3 days (C) of mice ($n = 8$) infected with $\sim 5 \times 10^6$ 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 ($P < 0.0001$).

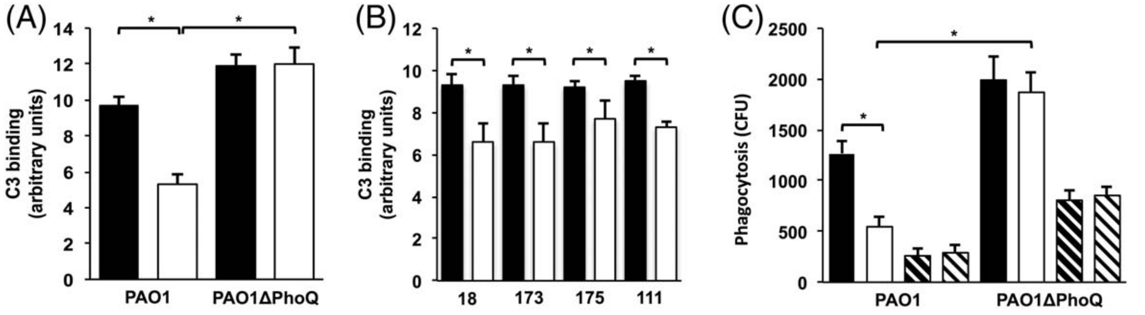

Fig. 2. Analysis of complement component C3 deposition and phagocytosis of *P. aeruginosa*.
Cells of the wild-type strain PAO1 or the isogenic PhoQ-deficient mutant (A) or four different *P. aeruginosa* bloodstream isolates (B) grown in LB (black columns, low Mg^{2+}) or LB supplemented with 3 mM Mg^{2+} (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 subtracted from the values obtained with NHS.
C. *Pseudomonas aeruginosa* strains grown in LB (black columns, low Mg^{2+}) or LB supplemented with 3 mM Mg^{2+} (white columns, high Mg^{2+}) were preopsonized with NHS (solid columns) or with PBS (striped columns) 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; $^*P < 0.05$.

clearance of *P. aeruginosa* by opsonizing this organism for phagocytosis (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 the complement component C3. After 30 min of incubation in normal serum sera (NHS) the mutant grown under conditions of Mg^{2+} starvation (LB), bound similar amounts of C3 as did the wild-type strain (Fig. 2A). 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+} (Fig. 2A). 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+}. 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+} (Fig. 2B). Mg^{2+}-dependent reduction of C3 deposition did not confer increased resistance to the bactericidal effect of the complement (data not shown). However, it was crucial to reduce the recognition of the pathogen by human polymorphonuclear leukocytes (PMNs) (Fig. 2C). Phagocytosis of the wild-type strain grown in low Mg^{2+} (LB) and opsonized with NHS was almost threefold more effective than was phagocytosis of cells grown in high Mg^{2+} (3 mM). By contrast, there was no difference between the phagocytosis rate of the wild-type strain grown in low or high Mg^{2+} when it was opsonized with PBS. However, there was no difference between the phagocytosis rate of the PhoQ-deficient mutant grown in either low or high Mg^{2+}, either preopsonized with NHS or PBS. Furthermore, the mutant was phagocytosed by PMNs threefold more efficiently than the wild-type strain when were both grown in high Mg^{2+} concentrations. Altogether, these results suggested that PhoQ contributes to resistance to the early host defense mechanisms of blood, including complement and opsonized phagocytosis by PMNs.

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 or LB supplemented with 3 mM Mg^{2+} was analysed by enzyme-linked immunosorbent assay (ELISA). There were no differences in the binding of C3 to either LPS preparation (Supporting Information Fig. S1). 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 (Fig. 3A). These results suggested that other bacterial components modulated the Mg^{2+}-dependent deposition of C3 on *P. aeruginosa*. To

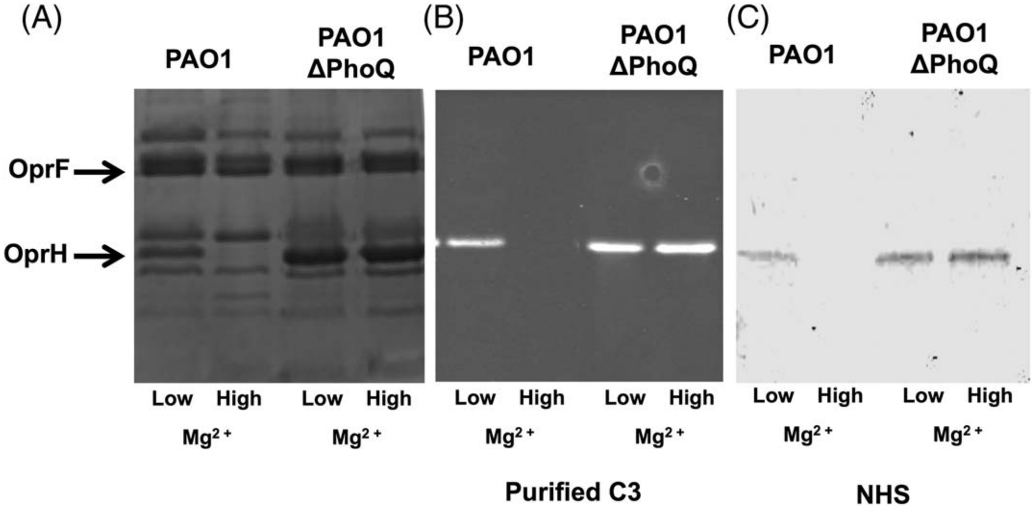

Fig. 3. C3 binding analysis of *P. aeruginosa* outer membrane proteins.
Outer membrane proteins from PAO1 and its isogenic PhoQ-deficient mutant grown in LB (low Mg^{2+}) or in LB supplemented with 3 mM Mg^{2+} (high Mg^{2+}), were isolated, resolved, and either (A) stained with Coomassie blue: or transferred 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.

identify this component, outer membrane preparations were subjected to ligand blot analysis. Outer membrane proteins from PAO1 and the PhoQ-deficient mutant grown in LB or in LB supplemented with Mg^{2+} were loaded onto three separate gels, one of which was stained with Coomassie blue (Fig. 3A), while the other two were electrophoretically transferred by Western blotting to Immobilon-P membranes and incubated with either IRD800CW labelled purified human C3 (2 μg/ml) (Fig. 3B) or NHS (0.2% final concentration), rabbit anti-human C3 and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Fig. 3C). In both blots, a protein of approximately 21-kDa was recognized by C3 in the outer membrane proteins 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 (Fig. 3A).

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 PAO1-OprH, and the PAO1ΔOprH complemented with *oprH*. Ligand blot analysis using purified fluorescent C3 showed reactivity with a band in the outer membrane preparations of PAO1 under low Mg^{2+}, but not high Mg^{2+} conditions, that was constitutively present in the complemented mutant, but was not present in the *oprH* mutant (Fig. 4A). Quantitative C3 binding analysis to bacterial cells demonstrated that at low Mg^{2+} concentration, both PAO1 and the mutant complemented with *oprH* bound C3 more efficiently than the OprH-deficient mutant (Fig. 4B). 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 (Fig. 4B).

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 or PBS 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 hardly any effect on the OprH-deficient mutant (Fig. 4C). Overall, these results indicate that OprH promoted the binding of C3 and serum-opsonized phagocytosis of *P. aeruginosa* by human PMNs.

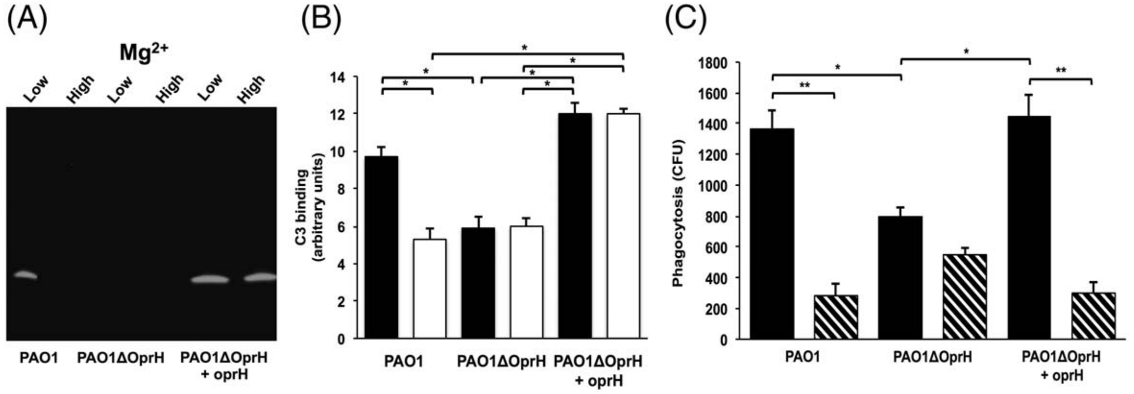

Fig. 4. OprH promoted binding of C3 and opsonophagocytosis of *P. aeruginosa*.
Outer membrane proteins from the wild-type strain PAO1, the isogenic OprH-deficient mutant OprH (PAO1ΔOprH), and the complemented mutant were isolated, resolved, transferred to an Immobilon-P membrane and incubated with IRD800CW labeled C3 (2 μg/ml) (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 (B). *P. aeruginosa* strains grown in LB were preopsonized with NHS (solid columns) or with PBS (striped columns) 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. Data represent three experiments done in duplicate. Errors bars represent SEMs. Statistical analyses were performed using Student's unpaired two-tailed *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$.

To establish the in vivo role of OprH in *P. aeruginosa* virulence, we tested the ability of the OprH-deficient mutant and the complemented mutant to cause fatal infection in a murine model of systemic infection. Both strains showed similar in vitro growth rates in LB with respect to the wild-type strain PAO1 (Fig. 5A). Analysis of survival indicated that constitutive expression of OprH impaired the virulence of *P. aeruginosa*. After 72 h, none of the animals infected with the OprH-deficient mutant complemented with *oprH* died. In contrast, 40% of the mice infected with the OprH-deficient mutant died by day 3 (Fig. 5B). Thus, in vivo expression of OprH impairs the ability of *P. aeruginosa* to cause systemic infection by promoting the binding of C3 and opsonophagocytosis.

Discussion

The data presented here suggests 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 to 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 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+}influences 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+}.

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. The ligand blot experiments described here failed to detect binding of C3 to OprF, as described 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

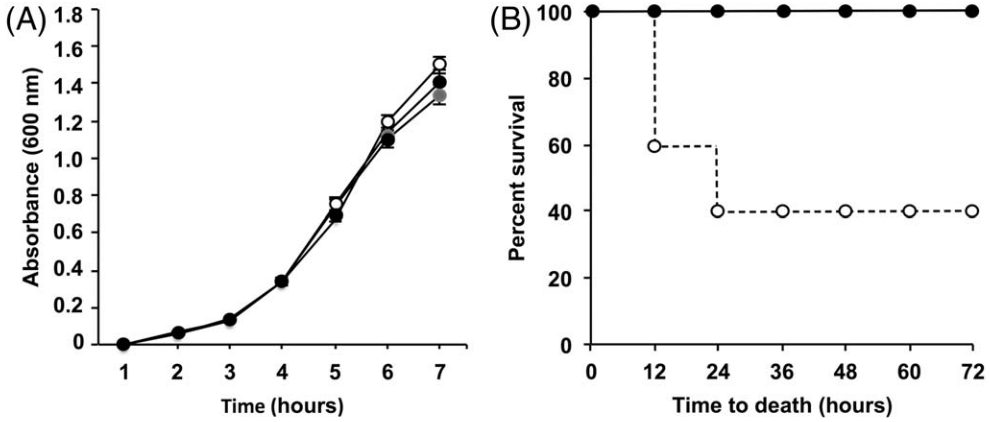

Fig. 5. Effect of in vivo expression of OprH on *P. aeruginosa* systemic infection.
A. Growth curve at 37°C in LB of *P. aeruginosa* strain PAO1 (grey circles), its derived isogenic mutant PAO1ΔOrpH (white circles) and the complemented mutant (black circles).
B. Analysis of survival over 3 days of mice ($n = 8$) infected with ~5×10^6 CFU of *P. aeruginosa* OprH-deficient mutant, PAO1ΔOrpH (white circles), or the complemented mutant (black circles). The difference in survival between the two groups were significantly different by log rank test ($P < 0.0001$).

different affinity. Thus, three different *P. aeruginosa* surface components bind C3, namely; LPS, OprF and OprH.

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).

However, *P. aeruginosa* has another two-component regulatory system that senses Mg^{2+}, PmrA-PmrB (McPhee *et al*., 2006). Both the PhoPQ and the PmrAB systems separately contribute to regulation of the *pmr*HFIJKLM-*ugd* operon in response to limiting concentrations of Mg^{2+}. However, the PmrAB system does not regulate OprH expression (McPhee *et al*., 2006), suggesting that this system probably is not involved in the Mg^{2+}-dependent deposition of C3 on *P. aeruginosa* we observed here.

Although there are some parallels between the PhoP/PhoQ systems of *Salmonella* and *Pseudomonas*, there are many differences as well (McPhee *et al*., 2003). For instance, *P. aeruginosa* PhoP/PhoQ system is the uniquely part of a three-gene operon that includes the *oprH* gene encoding an outer membrane protein. For this reason, it seems that the PhoPQ-dependent susceptibility to opsonophagocytic killing mediated by OprH may be exclusive to *P. aeruginosa*.

Our results obtained in vitro were supported by the mice infection study performed with the OprH-deficient mutant and the complemented mutant, suggesting that Mg^{2+}-suppressed expression of OprH in vivo is a key factor to avoid C3 mediated opsonophagocytosis of *P. aeruginosa*. Consistent with this finding, *P. aeruginosa* abolished or markedly reduced the expression of OprH when it grew in human serum (Supporting Information Fig. S2), which is in keeping with gene expression studies that showed decreased expression of *oprH* in a murine model of burn infection and in ex vivo samples from human burn infections compared with laboratory growth conditions (Bielecki *et al*., 2013; Turner *et al*., 2014).

In conclusion, this study identifies PhoQ as a critical 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* (Fig. 6). Thus, PhoQ may represent a promising target to develop new drugs against *P. aeruginosa* blood infections.

Experimental procedures

Bacteria strains

Pseudomonas aeruginosa reference strain PAO1 and its derived isogenic PhoQ-deficient mutant H854 (Macfarlane *et al*., 1999), OprH-deficient mutant PAO1ΔOprH (Edrington *et al*., 2011), and the complemented OprH-deficient mutant (Qadi *et al*., 2016), were used in this study. Four clinical isolates from different patients with bacteremia caused by *P. aeruginosa* were also included in the study.

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

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

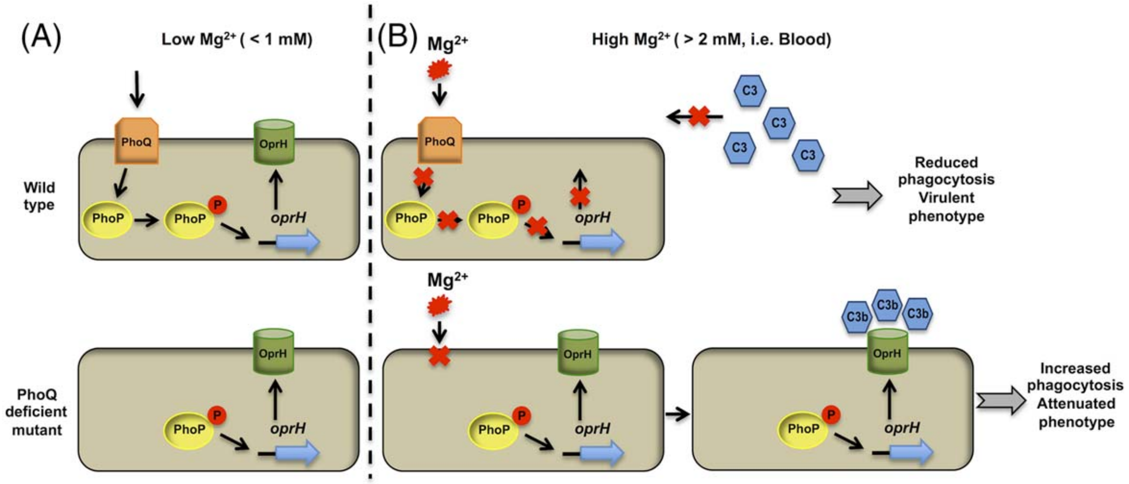

Fig. 6. Sensing of Mg^{2+} by PhoQ and its effect on *P. aeruginosa* opsonophagocytic killing.
A. In the presence of low concentrations of Mg^{2+} ($<$ 1 mM), *P. aeruginosa* PhoQ phosphorylates PhoP, which in turns upregulates expression of OprH in the wild-type strain. In a PhoQ-deficient mutant, PhoP is phosphorylated, therefore, OprH is constitutively expressed.
B. In the presence of high concentrations of Mg^{2+} ($>$ 2 mM), 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 resulting in attenuation.

were isolated from *P. aeruginosa* strains by centrifugation at 100 000 × *g* for 1 h 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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).

Murine model of systemic infection

Mouse lethality studies were performed with male CD1 mice, each weighing 16–20 g (Harlan Ibérica, S.L.). Mice ($n = 8$) were infected by intraperitoneal injection with approximately 5×10^6 colony forming units (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 10–30 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.

Human reagents

A pool of 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 labelled with the Infrared Dye 800CW using the IRDye 800CW protein labeling kit (LI-COR) following the manufacturers' instructions.

C3 binding assays

Binding of C3 to bacterial cells was determined using an ELISA. Briefly, 1×10^9 CFU were washed with phosphate-buffered saline (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 37°C 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 4°C with serial dilutions of the C3 fragment suspensions. Wells were blocked with 1% bovine serum albumin (BSA) in PBS, 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 h 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 h 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 $MgCl_2$.

Serum resistance assays

Complement-mediated serum bactericidal activity was determined, as previously described (Alberti *et al.*, 1993).

Opsonophagocytic assays

Opsonophagocytic assays were performed using human PMNs isolated from consenting healthy adult donors by dextran sedimentation and Ficoll-Histopaque density gradient centrifugation (Mosca and Forte, 2016). Briefly, 1×10^8 CFU were pre-incubated with NHS (30% final concentration) or PBS for 30 min at 37°C. Freshly isolated PMNs were added at a ratio of 1:100 to the bacterial suspension and the mixture was incubated at 37°C for 30 min with shaking on an orbital shaker at 360 r.p.m. After incubation, the mixture was incubated for 60 min at 37°C 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.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. 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.

Fig. S2. Representative SDS-PAGE analysis of the outer membrane proteins isolated from strains PAO1 and its isogenic PhoQ-deficient mutant grown in NHS and stained with Coomassie blue.