# iTRAQ-Based Quantitative Proteomic Analysis of *Acinetobacter baumannii* under Hypoxia and Normoxia Reveals the Role of OmpW as a Virulence Factor

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**ABSTRACT** *Acinetobacter baumannii* needs to adapt to hypoxia during infection. Understanding its proteome regulation during infection would allow us to determine new targets to develop novel treatments. iTRAQ proteomic analysis of A549 cell infection by the ATCC 17978 strain was performed. A total of 175 proteins were differentially expressed under hypoxia versus normoxia. We selected the hypoxia-downregulated protein OmpW to analyze its role as a virulence factor. The loss of OmpW decreased the adherence and invasion of *A. baumannii* in these host cells, without affecting its bacterial growth. Moreover, A549 cell viability with ΔOmpW infection was higher than that with the wild-type strain. ΔOmpW presented less biofilm formation. Finally, the minimum lethal dose required by the ΔOmpW mutant was higher than that of the wild-type strain in a murine peritoneal sepsis model, with lower bacterial loads in tissues and fluids. Therefore, OmpW seems to be a virulence factor necessary for *A. baumannii* pathogenesis.

**IMPORTANCE** *Acinetobacter baumannii* causes infections that are very difficult to treat due to the high rate of resistance to most and sometimes all of the antimicrobials used in the clinical setting. There is an important need to develop new strategies to combat *A. baumannii* infections. One alternative could be blocking specific bacterial virulence factors that this pathogen needs to infect cells. Pathogens modulate their protein expression as a function of the environment, and several studies have reported that hypoxia occurs in a wide range of infections. Therefore, it would be interesting to determine the proteome of *A. baumannii* under hypoxia in order to find new virulence factors, such as the outer membrane protein OmpW, as potential targets for the design of novel therapies.

**KEYWORDS** hypoxia, *Acinetobacter baumannii*, iTRAQ, virulence factors, OmpW

**A**cinetobacter baumannii is an aerobic Gram-negative bacillus that has become an increasingly important human pathogen responsible for nosocomial infections (1). *A. baumannii* can develop resistance to all classes of antimicrobial agents used in the clinical setting (2, 3). Thus, it is important to identify new virulence factors to characterize the pathogenesis and determine new therapeutic targets (4).

Several studies show that bacteria modulate their gene expression and protein levels, and thus the expression of virulence factors, in different environments (5–7). It is known that hypoxia occurs during infection, with oxygen levels in the infection focus being <1%; this is due to increased oxygen consumption and decreased perfusion (8, 9). Moreover, we previously determined that hypoxia decreases the adherence and

invasion of *A. baumannii* in human lung epithelial cells, without affecting bacterial growth (10). Thus, hypoxia might modify virulence factor expression during the course of infection.

Several genomic, transcriptomic, and proteomic analyses have identified different virulence factors that participate in the pathogenesis of *A. baumannii*, but we still know relatively few of them (1, 11–13). Techniques that use the relative quantification of the proteome, such as the isobaric tags for relative and absolute quantitation (iTRAQ) approach, have been used for characterization of bacterial proteomes, providing important information regarding virulence factors (7, 14–17). *A. baumannii* proteome analyses have also given new insights into the molecular mechanisms underlying its antibiotic resistance (18, 19), the growth-phase-dependent changes observed in its proteome (20), the molecular mechanisms involved in the response to desiccation and persistence (21), and proteins involved in biofilm formation (22). However, no study has investigated the influence of hypoxia on the *A. baumannii* proteome during infection.

The present study aimed to identify the *A. baumannii* proteins whose levels are regulated by hypoxia, to identify novel virulence factors. Thus, we compared protein levels in an infection model of human lung epithelial cells with *A. baumannii* ATCC 17978 under hypoxia (1% oxygen) and normoxia (21% oxygen) (10). We identified different upregulated and downregulated proteins under hypoxia. Further, we hypothesized that the outer membrane protein (OMP) most downregulated under hypoxia, OmpW (0.38-fold change), might be involved in the reduction in *A. baumannii* adherence to and invasion of host cells observed under hypoxia (10) and consequently in *A. baumannii* pathogenesis. We showed that an *ompW* deletion mutant presents less interaction with host cells *in vitro* and less virulence in an animal model, thus showing that OmpW is essential for *in vivo* infection by *A. baumannii*.

## RESULTS

***A. baumannii* protein expression profile.** To identify proteins associated with an inducible virulence response in *A. baumannii* during infection, we searched for proteins that were differentially expressed under hypoxia versus normoxia (10). A total of 175 proteins were differentially expressed under hypoxia (51 proteins downregulated [10] and 124 proteins upregulated) (see Table S1 in the supplemental material). This accounts for 4% of the ATCC 17978 proteome.

Most of the downregulated and upregulated proteins under hypoxia were localized in the cytoplasm (47.06 and 36.29%, respectively) and inner membrane (19.61 and 29.83%, respectively), although we also found proteins in the outer membrane, extracellular medium, and periplasm (Fig. 1). Downregulated proteins were mainly involved in metabolism and transport. Upregulated proteins were involved in metabolism, oxidative stress, DNA and RNA repair, transport, biogenesis of the fimbria, and transcriptional regulation. Furthermore, we observed that hypoxia modified the expression of OMPs. We found five downregulated OMPs, corresponding to OmpW (A1S_2325) and four ferric siderophores (A1S_3339, A1S_0474, A1S_0981, and A1S_1921) (10).

**Generation of OmpW mutants in *A. baumannii*.** The presence or absence of OmpW in the different strains was confirmed by PCR (see Fig. S1A) and quantitative reverse transcription-PCR (qRT-PCR) (see Fig. S1B). PCR showed that both wild-type strains (ATCC 17978 and ATCC 17978/p) and the complemented strain (ΔOmpW/pOmpW) yielded a band corresponding to the wild-type protein, while both mutants (ΔOmpW and ΔOmpW/p) yielded a band corresponding to *ompW* deletion (see Fig. S1A). We demonstrated by qRT-PCR that ATCC 17978 and ATCC 17978/p expressed similar levels of *ompW* RNA, while ΔOmpW/pOmpW showed overexpression of *ompW* due to the multicopy plasmid pUCp24 (see Fig. S1B). The ΔOmpW and ΔOmpW/p strains had no *ompW* expression.

**Effects of OmpW deletion on the growth of *A. baumannii* and on the bactericidal activity and bacterial cell adherence and invasion.** Growth curves under normoxia were analyzed to check ATCC 17978 and ΔOmpW growth rates. We showed that the
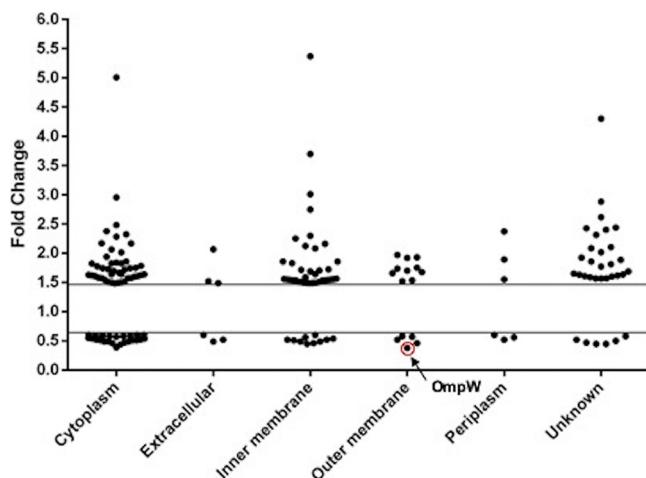
**FIG 1** *A. baumannii* protein expression profile. Classification of downregulated (<0.6-fold change) and upregulated (>1.49-fold change) proteins under hypoxia were compared to those under normoxia according to their subcellular localization. Fifty-one downregulated proteins were localized in the cytoplasm (47.06%), extracellular medium (5.88%), inner membrane (19.61%), outer membrane (9.80%), and periplasm (5.88%). A total of 124 upregulated proteins were localized in the cytoplasm (36.29%), extracellular medium (2.42%), inner membrane (29.84%), outer membrane (8.06%), and periplasm (2.42%).

growth of the two strains was indistinguishable. The complemented strain and strains harboring the empty pUCp24 plasmid had the same growth as the respective strains (Fig. 2A).

We wanted to determine whether the loss of OmpW affected the bactericidal activity of A549 cells against the mutant strain more than against the wild-type strain. Bacterial counts of ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains in the extracellular medium of A549 cells were the same at 2 h postinfection (Fig. 2B). There was a significant increase in bacterial counts of ΔOmpW/pOmpW strain in the extracellular medium at 24 h postinfection (Fig. 2B). These data confirmed that the loss of OmpW did not affect the bactericidal activity of A549 cells.

Next, we determined whether the loss of OmpW affected the adherence of *A. baumannii* to these host cells. The adherence of the ΔOmpW strain was significantly lower than that of the wild-type strain at 2 and 24 h postinfection (75.98% versus 100% [$P < 0.05$] and 79.29% versus 100% [$P < 0.05$], respectively) (Fig. 2C), and the same was true for ΔOmpW/p (70.06% versus 100% [$P < 0.05$] and 51.22% versus 100% [$P < 0.05$], respectively) (Fig. 2C). Complementation of ΔOmpW restored the wild-type strain adherence.

Moreover, we determined whether the loss of OmpW affected the invasion of *A. baumannii* in A549 cells. Bacterial counts of the mutant strain inside A549 cells showed a decrease, compared to the wild-type strain, at 2 and 24 h postinfection (43.96% versus 100% [$P < 0.05$] and 82% versus 100%, respectively) (Fig. 2D), and the same was true for ΔOmpW/p (52.90% versus 100% [$P < 0.05$] and 73.23% versus 100% [$P < 0.05$], respectively) (Fig. 2D). Complementation of ΔOmpW restored the wild-type strain invasion levels. These data indicated that the OmpW deletion diminished the adherence and invasion of *A. baumannii* in human lung epithelial cells.

**Role of OmpW in biofilm formation.** A biofilm assay was performed to determine whether the loss of OmpW affected the biofilm-forming potential of *A. baumannii*. The ATCC 17978 strain produced thick biofilm (Fig. 3A). However, the ΔOmpW strain demonstrated significantly less biofilm formation (6.89%), compared to the wild-type strain (100%), as did the ΔOmpW/p strain (31.64%) ($P < 0.05$). Complementation of the ΔOmpW strain restored the biofilm production to the levels of the ATCC 17978/p strain (97.30%).
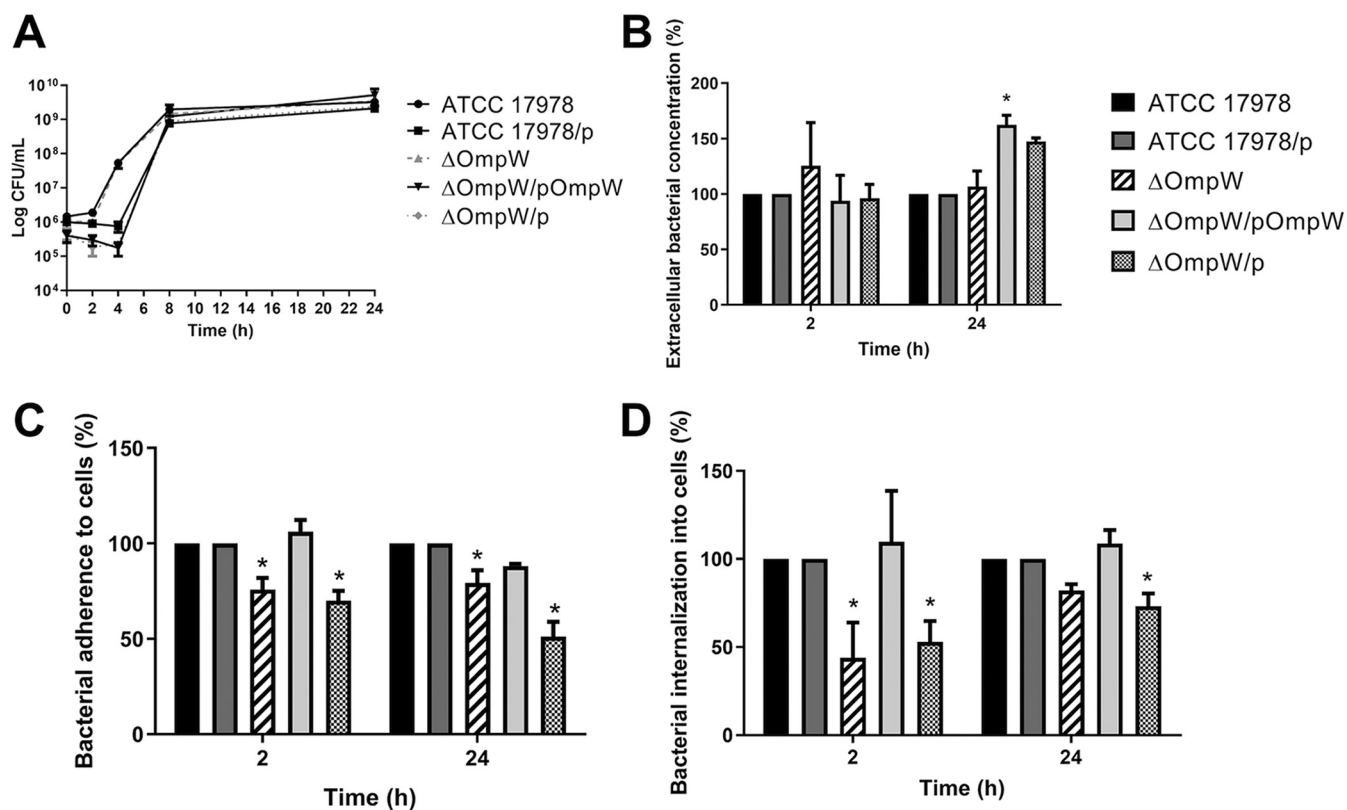
**FIG 2** Effect of OmpW deletion on the growth of *A. baumannii* and on the bactericidal activity and bacterial cell adherence and invasion. (A) Growth curves for *A. baumannii* ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains in MHB under normoxia. (B) Measurement of bacterial concentrations in the extracellular medium 2 and 24 h after A549 cell infection by *A. baumannii* ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains under normoxia. (C) Measurement of bacterial adherence 2 and 24 h after A549 cell infection by *A. baumannii* ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains under normoxia. (D) Measurement of bacterial invasion 2 and 24 h after A549 cell infection by *A. baumannii* ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains under normoxia. *, $P < 0.05$, versus the wild-type strain.

**Role of OmpW in cellular viability with *A. baumannii*.** We determined whether OmpW was involved in the cell death of A549 cells induced by *A. baumannii*. A cell survival assay showed that 24-h infection of A549 cells by the ATCC 17978 and ΔOmpW/pOmpW strains reduced cell viability to 74.41% and 76.94%, respectively. However, the ΔOmpW strain reduced the cell viability to 84.27% (Fig. 3B). These results showed that OmpW is involved in the cytotoxicity induced by *A. baumannii*.

**Effect of OmpW deletion on antibiotic susceptibility.** We wanted to determine how the deletion of OmpW in *A. baumannii* affected the susceptibility to some clinically relevant antibiotics. MIC values for the mutant strain were the same as those for the parental strain for all of the antibiotics tested (Table 1). These results indicated that deletion of OmpW did not affect the susceptibility of *A. baumannii* to these antibiotics.

**Role of OmpW in the virulence of *A. baumannii* in a murine peritoneal sepsis model.** To analyze the effect of OmpW on *A. baumannii* virulence, a murine model of peritoneal sepsis was used. The mortality rates were dependent on the concentration of bacteria in the inoculum for ATCC 17978, ΔOmpW, and ΔOmpW/pOmpW strains (Table 2). The 50% lethal dose ($LD_{50}$) and 100% minimum lethal dose ($MLD_{100}$) for the ΔOmpW strain were greater than those for the ATCC 17978 and ΔOmpW/pOmpW strains, i.e., 3.29 and 4.30 log CFU/mL versus 2.88 and 3.40 log CFU/mL, respectively, with ratios of 1.14 and 1.26, respectively. The 0% lethal dose ($LD_0$) values for the ΔOmpW, ATCC 17978, and ΔOmpW/pOmpW strains were the same, i.e., 2.30 log CFU/mL (Table 2). Kaplan-Meier analysis showed differences between animal groups receiving the same inoculum of ΔOmpW and ATCC 17978 strains of 3.2 log CFU/mL ($P < 0.01$). The group infected by the complemented strain had the same mortality rate as the wild-type strain (Fig. 4A and B).
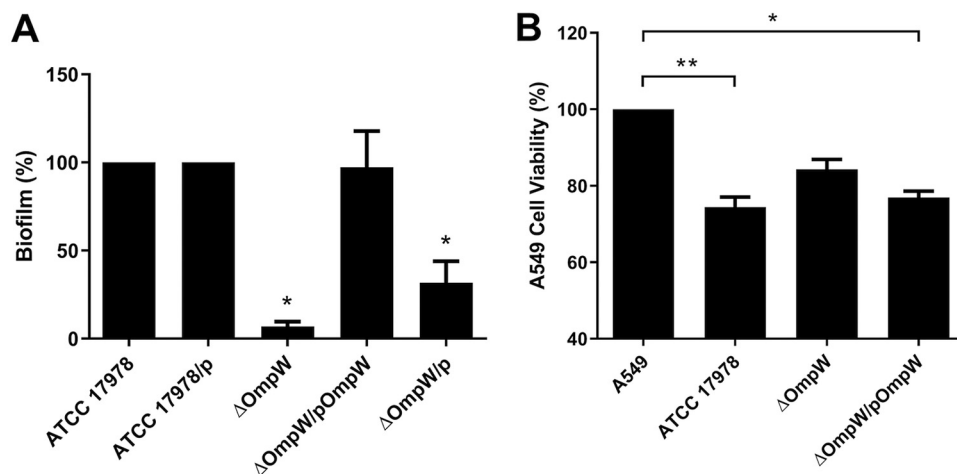
**FIG 3** Role of OmpW in biofilm formation and in cellular viability with *A. baumannii*. (A) Effect of the loss of OmpW on biofilm production. Biofilm formation was determined for *A. baumannii* ATCC 17978, ATCC 17978/p, ΔOmpW, ΔOmpW/pOmpW, and ΔOmpW/p strains. (B) Viability of A549 cells after 24 h of infection with *A. baumannii* ATCC 17978, ΔOmpW, and ΔOmpW/pOmpW strains. *, $P < 0.05$; **, $P < 0.01$, versus the wild-type strain.

Furthermore, we used the same murine model to compare the dissemination of the ATCC 17978, ΔOmpW, and ΔOmpW/pOmpW strains to different organs, inoculating mice intraperitoneally (i.p.) with each strain at 4.3 log CFU/mL. Collected tissues and fluids showed lower bacterial concentrations in mice infected by the ΔOmpW strain than in those infected by the ATCC 17978 strain (Fig. 4C). We found differences between the ΔOmpW and ATCC 17978 strains in liver (6.83 versus 8.33 log CFU/g [$P < 0.05$]), kidney (7.14 versus 8.66 log CFU/g [$P < 0.05$]), and peritoneal fluid (7.00 versus 9.19 log CFU/mL [$P < 0.05$]). In the case of the ΔOmpW/pOmpW strain, bacterial loads in tissues and fluids were similar to those with the wild-type strain except for the blood and lung samples. These data indicated that OmpW plays an important role in the *in vivo* infective capacity of and mortality rates with *A. baumannii*.

## DISCUSSION

In this study, we report for the first time the role played by OmpW in the interaction of *A. baumannii* with human host cells and in the infectivity in a murine peritoneal sepsis model. We demonstrate that OmpW is downregulated in *A. baumannii* during infection of hypoxic human lung epithelial cells. Pathogenic bacteria need to adapt to diverse host environments during infection, such as hypoxic environments. Environmental signals trigger adaptive responses and induce the expression of virulence proteins (23). For this reason, it is important to know the proteome of *A. baumannii* during infection to design new treatments.

**TABLE 1** MIC values for the OmpW deletion mutant and its wild-type and complemented strains

| Drug | MIC (mg/L) for strain: | | |
|---|---|---|---|
| | ATCC 17978 | ΔOmpW | ΔOmpW/pOmpW |
| Ceftazidime | 8 | 8 | 8 |
| Sulbactam | 2 | 2 | 2 |
| Imipenem | 0.125 | 0.125 | 0.125 |
| Amikacin | 2 | 1 | 1 |
| Azithromycin | 2 | 2 | 2 |
| Vancomycin | 64 | 64 | 64 |
| Rifampicin | 1 | 2 | 1 |
| Colistin | 0.125 | 0.125 | 0.125 |
| Ciprofloxacin | 0.5 | 0.5 | 0.5 |
| Tigecycline | 0.5 | 0.5 | 0.5 |

**TABLE 2** Seven-day mortality rates in the murine model of peritoneal sepsis with different inocula of *Acinetobacter baumannii* ATCC 17978 and its ΔOmpW mutant

| Strain | Mortality rate (%) with inoculum of: | | |
|---|---|---|---|
| | 4.3 log CFU/mL | 3.2 log CFU/mL | 2.3 log CFU/mL |
| Wild-type ATCC 17978 | 100 | 100 | 0 |
| ΔOmpW | 100 | 40 | 0 |
| ΔOmpW/pOmpW | 100 | 100 | 0 |

OmpW is an eight-stranded $\beta$-barrel porin that forms channels for the uptake of small hydrophobic molecules across the outer membrane (24). This protein is well conserved among *A. baumannii* laboratory and clinical strains (25) and in Gram-negative bacteria such *Escherichia coli*, *Pseudomonas aeruginosa* (OprG), *Vibrio cholerae* (26–28), *Klebsiella pneumoniae*, and *Shigella flexneri* (confirmed by BLASTp and ClustalW analyses) (see Fig. S2 in the supplemental material). It has been demonstrated to participate in bacterial adaptive responses to different environmental stresses (26, 28), although its function is not yet completely understood. OmpW participates in iron uptake in
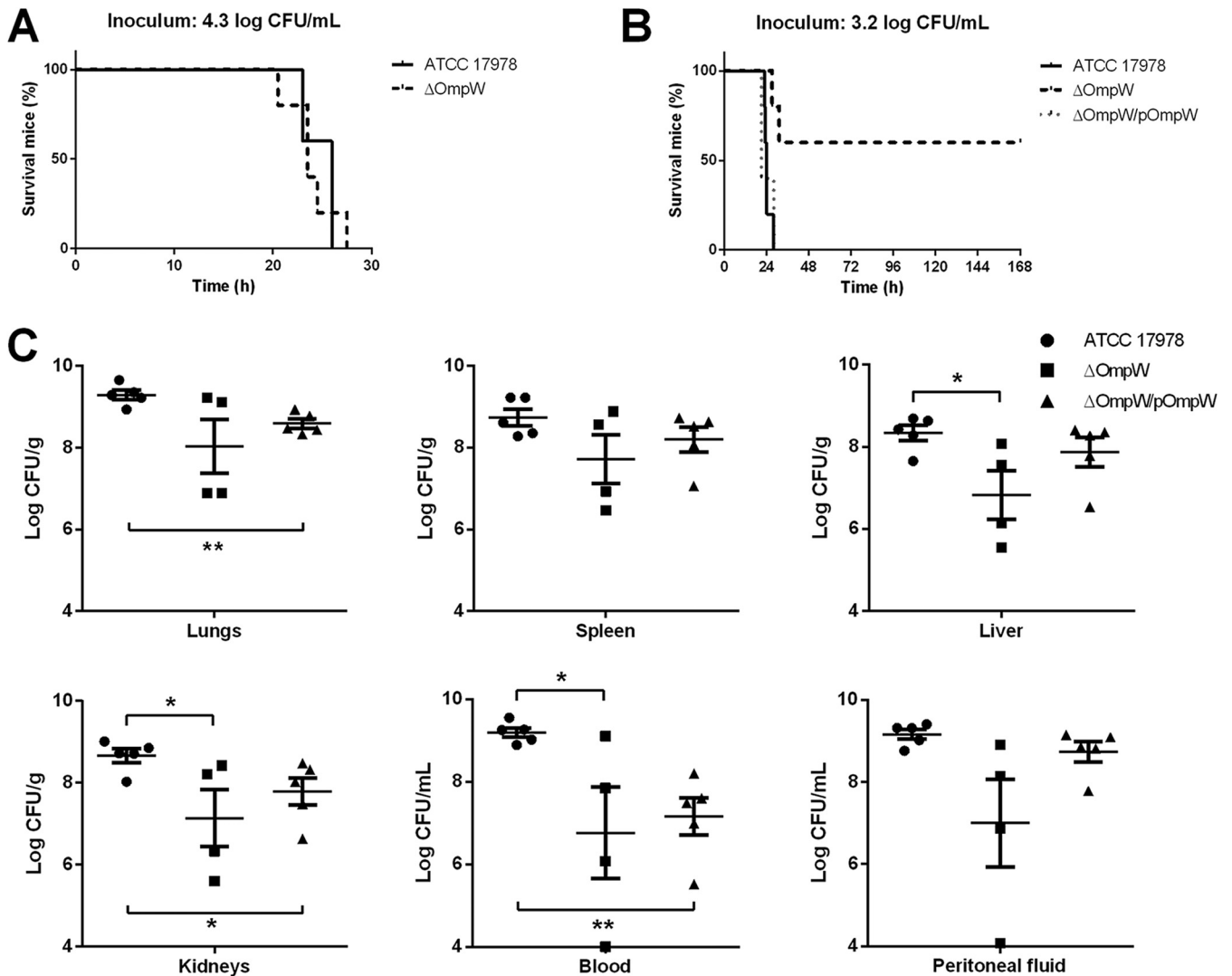


**FIG 4** Role of OmpW in the virulence of *A. baumannii* in the murine peritoneal sepsis model. (A and B) Mortality curves analyzed by Kaplan-Meier survival analysis. $P < 0.01$, versus the wild-type strain and for the mutant versus the complemented strain. (C) Bacterial loads in fluids and tissues in the murine peritoneal sepsis model with *A. baumannii* ATCC 17978, ΔOmpW, and ΔOmpW/pOmpW strains at 4.3 log CFU/mL. **, $P < 0.01$; *, $P < 0.05$, versus the wild-type strain.

*E. coli* and *A. baumannii* (26, 29), in the response to oxidative stress in *Salmonella enterica* serovar Typhimurium (30), and in *V. cholerae* growth under hypersaline conditions (28). OmpW is upregulated under oxygen-limiting conditions or anaerobiosis in *E. coli*, *Comamonas acidovorans*, and *P. aeruginosa* (27, 31, 32). However, we showed that OmpW is downregulated in *A. baumannii* under hypoxia, suggesting the existence of different regulation pathways in *E. coli* (a facultative anaerobic pathogen) and *A. baumannii* (a strictly aerobe pathogen). Moreover, OmpW is involved in iron uptake (26, 29), and iron levels are low during bacterial infection as a defense mechanism, which might also explain why OmpW is downregulated during hypoxic infection. Finally, a promoter analysis shows that the *ompW* promoter has a Lrp binding box (see Table S2). Lrp (A1S_3307) is a transcriptional regulator that either activates or represses transcription, and it is upregulated (1.94-fold change) during hypoxic infection (see Table S1). Therefore, Lrp might downregulate OmpW during hypoxic infection.

Previously, we demonstrated that hypoxia affected the adherence and invasion of *A. baumannii* in human lung epithelial cells and murine macrophages (10). We found that OmpW is the most downregulated protein under hypoxia. Therefore, our study suggests that OmpW might be involved in the interaction of *A. baumannii* with host cells. A previous study of proteins that are involved in the attachment of *Burkholderia cenocepacia* to human lung epithelial cells also showed that OmpW was involved in bacterial adherence (33), which supports our hypothesis. Bacterial adhesion to and invasion into host cells are important steps to cause *A. baumannii* cytotoxicity (34). We observed that deletion of *ompW* reduced *A. baumannii* adherence/invasion into host cells, as well as its cytotoxicity. Of note, the ATCC 17978/pUCp24, ΔOmpW/pUCp24, and ΔOmpW/pUCp24-ompW strains have similar growth curves and lag times. However, we observed a decrease in the adherence and invasion of the ΔOmpW/pUCp24 strain, compared to the ATCC 17978/pUCp24 and ΔOmpW/pUCp24-ompW strains, which confirms that the lag time does not have any impact on the rest of the assays. These results are consistent with previous observations that, in the absence of OprG, which is homologous to OmpW in *P. aeruginosa*, this pathogen was significantly less cytotoxic against human bronchial epithelial cells (27).

It is noteworthy that biofilm plays an important role in bacterial pathogenesis, making *A. baumannii* more resistant to environmental stresses (23). In this study, we demonstrated that the deletion of *ompW* affects the biofilm formation by *A. baumannii*. In the same way, Ritter et al. identified OmpW and its homologue as inducers of biofilm by *Pseudoalteromonas* species and *P. aeruginosa* (35).

Furthermore, we demonstrated that *ompW* deletion in *A. baumannii* did not affect the susceptibility to multiple antibiotics belonging to different families. Previous studies reported a downregulation of OmpW in carbapenem- and colistin-resistant *A. baumannii* (36, 37). In contrast, two other studies demonstrated that OmpW was upregulated in carbapenem-resistant *A. baumannii* and was not associated with colistin resistance (20, 25). However, the question of how OmpW is relevant for antibiotic susceptibilities of *A. baumannii* has not been resolved.

OmpW is essential for *A. baumannii* to disseminate between organs and to cause the death of mice, as observed for other pathogens such as *V. cholerae* (38). Motley et al. reported an increase in OmpW expression during *E. coli* infection in a murine granulomatous pouch model (39), and OmpW has been shown to protect *E. coli* against host responses, conferring resistance to complement-mediated killing and phagocytosis (40, 41). All of those previous studies indicated that OmpW could be a potential drug target in Gram-negative bacteria to develop new treatments. Moreover, mice immunized with OmpW were less infected by *B. cenocepacia*, *Burkholderia multivorans*, and *A. baumannii* in a sepsis model (25, 33).

To summarize, we have identified that OmpW is differentially regulated under hypoxia in *A. baumannii* infection. OmpW seems to be a promising virulence factor that is involved in *A. baumannii* interaction with host cells *in vitro* and in its pathogenicity *in vivo*, but it does not affect the antimicrobial resistance profile. Nevertheless, a better

understanding of the OmpW regulation is necessary to decipher its role in *A. baumannii* pathogenesis. Finally, inhibitors against this protein could be used as novel treatments in the future.

## MATERIALS AND METHODS

**Bacterial strain and growth curve assays.** The *A. baumannii* ATCC 17978 strain was used in the present study. Bacterial strains were grown overnight in Mueller-Hinton broth (MHB) (Sigma, Spain) under static conditions and then diluted 1:1,000 in a 40-mL culture in MHB. Bacterial strains were grown overnight in MHB at 160 rpm and 37°C for the eukaryotic cell culture experiments (human lung epithelial cell line A549), washed with phosphate-buffered saline (PBS) (Lonza, Spain), and suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Spain) before infection.

**A549 cell culture and bacterial infection.** A549 cells were grown in supplemented DMEM as described previously (10, 42). Briefly, in the case of hypoxia, cells were incubated in a hypoxic chamber (Coy Laboratories, USA) with 1% $O_2$ at 37°C for 6 h before bacterial infection. Then, cells were seeded for 24 h, washed three times with PBS, incubated in DMEM, and infected at a multiplicity of infection (MOI) of 500.

**iTRAQ assay.** We analyzed the differential protein expression profiles between hypoxia (1% $O_2$) and normoxia (21% $O_2$) in A549 cells infected with ATCC 17978 by iTRAQ as described previously (10). Briefly, cells were collected in a lysis buffer composed of 1 M triethylammonium bicarbonate buffer (Sigma), 1:100 protease inhibitor cocktail (complete mini EDTA-free; Roche, Spain), 1:100 phosphatase inhibitor cocktail (PhosSTOP EASYpack; Roche), 0.05% SDS, and 0.002% benzonase (Novagen, USA) 2 h after bacterial infection. The supernatant was removed, and the protein concentration of the pellet was quantified (Qubit Life Technologies, USA). An iTRAQ 4-plex (reporters at 114 to 117; AB Sciex, Spain) was used. Samples were analyzed by nano-liquid chromatography (Nano-LC 100; Thermo Fisher Scientific, USA) and tandem mass spectrometry (MS/MS) (Q Exactive Plus Orbitrap; Thermo Electron, USA). Protein identification was performed using Proteome Discoverer v1.4, and MS/MS fragmentation patterns were identified by mapping against the UniProt database. The quantifiable proteins were those that were identified through >2 peptides with a confidence level of ≥95%, a $P$ value of <0.05, and an error factor of <2 with every reference tag. Downregulated and upregulated proteins were considered when the fold change was <0.6 and >1.49, respectively. Subcellular localization was determined using the software PSORTb (http://www.psort.org/psortb).

**Mutant and complemented strain construction.** A stable, in-frame deletion mutant strain was constructed in ATCC 17978 by allelic exchange using the pMJG42 plasmid, which harbors the *sacB* gene for counterselection (43). For construction of the *ompW* deletion mutant (ΔOmpW), the 2,000 bp upstream and downstream of the open reading frame were amplified (see Table S3 in the supplemental material for primers). pMJG42 was digested with SpeI and NotI (New England Biolabs, USA). The Up insert was digested with SpeI and BamHI and the Down insert with BamHI and NotI. The plasmid and both inserts were ligated, and the construction was transformed into *Escherichia coli* DH5α λpir by electroporation before selection on LB agar plates with 5 mg/L tetracycline (Sigma). The construction was then transformed into *E. coli* MFD (44). The MFD donor strain harboring the respective pMJG42 gene (Up/Down) construct and the ATCC 17978 recipient strain were cultured overnight at 37°C. After 4 h of conjugation at 37°C, the cells were plated onto LB agar plates with 5 mg/L tetracycline. The colonies obtained were grown and plated on 10% sucrose plates. The OmpW deletion mutant was confirmed by PCR and sequencing using the primers OmpW-Out-F and OmpW-Out-R (see Table S3).

To complement the mutant, we followed the same protocol published by our research group (42). The open reading frame, 200 bp upstream, and 400 bp downstream were amplified with the primers NotI-OmpW-F and XbaI-OmpW-R. This DNA fragment was inserted into the pUCp24 plasmid and introduced by electroporation into the mutant strains before selection on LB agar plates with 10 mg/L gentamicin to obtain the complemented strain (ΔOmpW/pOmpW). Primers Seq-insert-pUCp24-F and Seq-insert-pUCp24-R were used to sequence the cloned gene. The wild-type strain and the deletion mutant strain were also transformed with the empty pUCp24 plasmid for use as a control (ATCC 17978/p and ΔOmpW/p, respectively).

**qRT-PCR assay.** Bacterial RNA was purified using the RNeasy minikit (Qiagen, Germany), and RT was carried out using the QuantiTect RT kit (Qiagen). We selected the primers 5′-AGCGGGTGGAGATATTC CTT-3′ (forward) and 5′-CACGCCAGCTCCGATATAAG-3′ (reverse) to amplify OmpW. We used the gene *rpoD* (A1S_2706) as a housekeeping gene (forward, 5′-CATGCGTGAAATGGGTACAG-3′; reverse, 5′-TTA CTGGCCAAATGCTGTTG-3′). The qRT-PCR was carried out with SYBR Premix *Ex Taq* (TaKaRa, Japan) using a MxPro 3005p system (Stratagene, USA). Three technical replicates for each sample were included. The amplification conditions were as follows: 95°C for 30 s, followed by 35 cycles of 95°C for 10 s, 56°C for 25 s, and 72°C for 25 s. Relative quantification of gene expression was performed with the comparative threshold cycle ($C_T$) method (Applied Biosystems guide).

**OmpW protein alignments and promoter analysis.** OmpW alignments between different bacterial pathogens were performed using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and represented with Clustal (https://www.ebi.ac.uk/Tools/msa/clustalo) and Jalview (https://www.jalview.org). The promoter of *ompW* was analyzed with BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) to find consensus boxes for transcription factors in its promoter region.

**Bactericidal activity and bacterial cell adherence and invasion.** After A549 cell infection with bacterial strains under normoxic conditions, extracellular medium was collected to determine bacterial concentrations at 2 and 24 h after bacterial infection (10).

Adherence and invasion assays were performed in triplicate as reported previously (10). Briefly, for adherence assays, cells were infected (MOI of 500), washed with PBS, and lysed with Triton X-100 (Sigma). The invasion assays were carried out in the same way as the adherence assay and included a treatment with 256 $\mu$g/mL tetracycline (Sigma) before lysis with Triton X-100.

**Cellular viability assay.** A549 cells were infected with an MOI of 500 for 24 h, as described previously (45). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) was performed in triplicate to determine the cell viability.

**Biofilm assay.** The biofilm assay was based on a previously described protocol (46). Briefly, strains were cultured overnight at 160 rpm and 37°C and diluted to $1 \times 10^5$ CFU/mL. Two hundred microliters of the suspension was added to a 96-well plate and grown overnight at 37°C. Every well was washed and filled with 0.4% crystal violet (Sigma) and then incubated for 10 min. Each well was then washed and filled with 96% ethanol. Biofilm formation was determined after 15 min by measuring the optical density at 580 nm (Asys UVM 340 microplate reader; Biochrom, USA). This assay was performed in triplicate.

**Antibiotic susceptibility testing.** MICs were determined by broth microdilution according to CLSI guidelines (47). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 strains were used as quality controls.

**Murine model of peritoneal sepsis. (i) Animals.** Immunocompetent C57BL/6 female mice (20 g) (Production and Experimentation Animal Center, University of Seville, Seville, Spain) were used. This study was performed following directive 2010/63/EU on the protection of animals used for scientific research. Experiments were approved by the Committee on the Ethics of Animal Experiments of the University Hospital of Virgen del Rocío (Seville, Spain) (approval number 20-05-14-84). All procedures were performed under sodium thiopental (B. Braun Medical S.A., Spain) anesthesia, and all efforts were made to minimize suffering.

**(ii) Experimental model.** A murine peritoneal sepsis model with *A. baumannii* was established by i.p. inoculation of bacteria (10). Animals were inoculated with 0.5 mL of the bacterial suspension, mixed 1:1 with a saline solution of porcine mucin (Sigma) at 10% (wt/vol). Groups of 5 mice for each strain were inoculated with different bacterial concentrations, and the survival of the mice was followed for 7 days. $LD_0$ and $MLD_{100}$ values were determined. $LD_{50}$ values were calculated using the probit method (48). To measure *in vivo* dissemination, groups of 5 mice were inoculated i.p. with 4.3 log CFU/mL. Mice were sacrificed by i.p. injection of 200 $\mu$L sodium thiopental after 12 h of infection. Spleens, lungs, kidneys, and livers were aseptically extracted and homogenized in 2 mL of saline solution using a Stomacher 80 homogenizer (Tekmar Co., USA). Bacterial loads in these tissues ($\log_{10}$ CFU per gram) and bacterial concentrations in blood and peritoneal fluid ($\log_{10}$ CFU per milliliter) were quantified.

**Statistical analysis.** Data are presented as means $\pm$ standard errors of the mean (SEMs). Significant differences were determined using the Kruskal-Wallis test with Dunn *post hoc* testing (IBM SPSS Statistics 22 software). For peritoneal sepsis survival, a Kaplan-Meier test was performed to determine mortality rate differences. Statistical significance was considered when *P* values were <0.05.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request. The proteomic data were deposited in the PeptideAtlas database with the identifier PASS01733.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
**SUPPLEMENTAL FILE 1**, PDF file, 0.8 MB.

## REFERENCES

1. Lee CR, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha CJ, Jeong BC, Lee SH. 2017. Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol 7:55. https://doi.org/10.3389/fcimb.2017.00055.

2. Nowak P, Paluchowska P. 2016. *Acinetobacter baumannii*: biology and drug resistance: role of carbapenemases. Folia Histochem Cytobiol 54: 61–74. https://doi.org/10.5603/FHC.a2016.0009.

3. European Centre for Disease Prevention and Control. 2015. Annual epidemiological report 2014: antimicrobial resistance and healthcare associated infections. European Centre for Disease Prevention and Control, Stockholm, Sweden. https://www.ecdc.europa.eu/sites/default/files/documents/antimicrobial-resistance-annual-epidemiological-report.pdf.

4. McConnell MJ, Actis L, Pachón J. 2013. *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. FEMS Microbiol Rev 37:130–155. https://doi.org/10.1111/j.1574-6976.2012.00344.x.

5. Cullen L, McClean S. 2015. Bacterial adaptation during chronic respiratory infections. Pathogens 4:66–89. https://doi.org/10.3390/pathogens4010066.

6. Méndez JA, Mateos J, Beceiro A, Lopez M, Tomás M, Poza M, Bou G. 2015. Quantitative proteomic analysis of host-pathogen interactions: a study of *Acinetobacter baumannii* responses to host airways. BMC Genomics 16: 422. https://doi.org/10.1186/s12864-015-1608-z.

7. Teng T, Xi B, Chen K, Pan L, Xie J, Xu P. 2018. Comparative transcriptomic and proteomic analyses reveal upregulated expression of virulence and iron transport factors of *Aeromonas hydrophila* under iron limitation. BMC Microbiol 18:52. https://doi.org/10.1186/s12866-018-1178-8.

8. Zinkernagel AS, Johnson RS, Nizet V. 2007. Hypoxia inducible factor (HIF) function in innate immunity and infection. J Mol Med (Berl) 85:1339–1346. https://doi.org/10.1007/s00109-007-0282-2.

9. Cummins EP, Keogh CE, Crean D, Taylor CT. 2016. The role of HIF in immunity and inflammation. Mol Aspects Med 47–48:24–34. https://doi.org/10.1016/j.mam.2015.12.004.

10. Gil-Marqués ML, Pachón-Ibáñez ME, Pachón J, Smani Y. 2018. Effect of hypoxia on the pathogenesis of *Acinetobacter baumannii* and *Pseudomonas aeruginosa in vitro* and in murine experimental models of infections. Infect Immun 86:e00543-18. https://doi.org/10.1128/IAI.00543-18.

11. Wong D, Nielsen TB, Bonomo RA, Pantapalangkoor P, Luna B, Spellberg B. 2017. Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. Clin Microbiol Rev 30:409–447. https://doi.org/10.1128/CMR.00058-16.

12. Leungtongkam U, Thummeepak R, Kitti T, Tasanapak K, Wongwigkarn J, Styles KM, Wellington EMH, Millard AD, Sagona AP, Sitthisak S. 2020. Genomic analysis reveals high virulence and antibiotic resistance amongst phage susceptible *Acinetobacter baumannii*. Sci Rep 10:16154. https://doi.org/10.1038/s41598-020-73123-y.

13. Le C, Pimentel C, Tuttobene MR, Subils T, Papp-Wallace KM, Bonomo RA, Actis LA, Tolmasky ME, Ramirez MS. 2021. Effect of serum albumin, a component of human pleural fluid, on transcriptional and phenotypic changes on *Acinetobacter baumannii* A118. Curr Microbiol 78:3829–3834. https://doi.org/10.1007/s00284-021-02649-9.

14. Xia K, Zang N, Zhang J, Zhang H, Li Y, Liu Y, Feng W, Liang X. 2016. New insights into the mechanisms of acetic acid resistance in *Acetobacter pasteurianus* using iTRAQ-dependent quantitative proteomic analysis. Int J Food Microbiol 238:241–251. https://doi.org/10.1016/j.ijfoodmicro.2016.09.016.

15. Sun L, Chen H, Lin W, Lin X. 2017. Quantitative proteomic analysis of *Edwardsiella tarda* in response to oxytetracycline stress in biofilm. J Proteomics 150:141–148. https://doi.org/10.1016/j.jprot.2016.09.006.

16. Kamath KS, Pascovici D, Penesyan A, Goel A, Venkatakrishnan V, Paulsen IT, Packer NH, Molloy MP. 2016. *Pseudomonas aeruginosa* cell membrane protein expression from phenotypically diverse cystic fibrosis isolates demonstrates host-specific adaptations. J Proteome Res 15:2152–2163. https://doi.org/10.1021/acs.jproteome.6b00058.

17. Wang Y, Li J, Zhang A, Zhu W, Zhang Q, Xu Z, Yan S, Sun X, Chen H, Jin M. 2017. iTRAQ-based quantitative proteomic analysis reveals potential virulence factors of *Erysipelothrix rhusiopathiae*. J Proteomics 160:28–37. https://doi.org/10.1016/j.jprot.2017.03.004.

18. Wang J, Zhang J, Fu Q, Guo S, Ta L, Sun P. 2016. Proteomic analyses uncover the mechanisms underlying antibiotic resistance differences among three *Acinetobacter baumannii* isolates. J Mol Microbiol Biotechnol 26:401–409. https://doi.org/10.1159/000447454.

19. Chopra S, Ramkissoon K, Anderson DC. 2013. A systematic quantitative proteomic examination of multidrug resistance in *Acinetobacter baumannii*. J Proteomics 84:17–39. https://doi.org/10.1016/j.jprot.2013.03.008.

20. Soares NC, Cabral MP, Gayoso C, Mallo S, Rodriguez-Velo P, Fernández-Moreira E, Bou G. 2010. Associating growth-phase-related changes in the proteome of *Acinetobacter baumannii* with increased resistance to oxidative stress. J Proteome Res 9:1951–1964. https://doi.org/10.1021/pr901116r.

21. Gayoso CM, Mateos J, Méndez JA, Fernández-Puente P, Rumbo C, Tomás M, Martínez de Ilarduya O, Bou G. 2014. Molecular mechanisms involved in the response to desiccation stress and persistence in *Acinetobacter baumannii*. J Proteome Res 13:460–476. https://doi.org/10.1021/pr400603f.

22. Cabral MP, Soares NC, Aranda J, Parreira JR, Rumbo C, Poza M, Valle J, Calamia V, Lasa I, Bou G. 2011. Proteomic and functional analyses reveal a unique lifestyle for *Acinetobacter baumannii* biofilms and a key role for histidine metabolism. J Proteome Res 10:3399–3417. https://doi.org/10.1021/pr101299j.

23. Fang FC, Frawley ER, Tapscott T, Vázquez-Torres A. 2016. Bacterial stress responses during host infection. Cell Host Microbe 20:133–143. https://doi.org/10.1016/j.chom.2016.07.009.

24. Catel-Ferreira M, Marti S, Guillon L, Jara L, Coadou G, Molle V, Bouffartigues E, Bou G, Shalk I, Jouenne T, Vila-Farrés X, Dé E. 2016. The outer membrane porin OmpW of *Acinetobacter baumannii* is involved in iron uptake and colistin binding. FEBS Lett 590:224–231. https://doi.org/10.1002/1873-3468.12050.

25. Huang W, Wang S, Yao Y, Xia Y, Yang X, Long Q, Sun W, Liu C, Li Y, Ma Y. 2015. OmpW is a potential target for eliciting protective immunity against *Acinetobacter baumannii* infections. Vaccine 33:4479–4485. https://doi.org/10.1016/j.vaccine.2015.07.031.

26. Brambilla L, Morán-Barrio J, Viale AM. 2014. Expression of the *Escherichia coli ompW* colicin S4 receptor gene is regulated by temperature and modulated by the H-NS and StpA nucleoid-associated proteins. FEMS Microbiol Lett 352:238–244. https://doi.org/10.1111/1574-6968.12385.

27. McPhee JB, Tamber S, Bains M, Maier E, Gellatly S, Lo A, Benz R, Hancock RE. 2009. The major outer membrane protein OprG of *Pseudomonas aeruginosa* contributes to cytotoxicity and forms an anaerobically regulated, cation-selective channel. FEMS Microbiol Lett 296:241–247. https://doi.org/10.1111/j.1574-6968.2009.01651.x.

28. Fu X, Zhang J, Li T, Zhang M, Li J, Kan B. 2017. The outer membrane protein OmpW enhanced *V. cholerae* growth in hypersaline conditions by transporting carnitine. Front Microbiol 8:2703. https://doi.org/10.3389/fmicb.2017.02703.

29. Lin XM, Wu LN, Li H, Wang SY, Peng XX. 2008. Downregulation of Tsx and OmpW and upregulation of OmpX are required for iron homeostasis in *Escherichia coli*. J Proteome Res 7:1235–1243. https://doi.org/10.1021/pr7005928.

30. Gil F, Hernández-Lucas I, Polanco R, Pacheco N, Collao B, Villarreal JM, Nardocci G, Calva E, Saavedra CP. 2009. SoxS regulates the expression of the *Salmonella enterica* serovar Typhimurium *ompW* gene. Microbiology (Reading) 155:2490–2497. https://doi.org/10.1099/mic.0.027433-0.

31. Xiao M, Lai Y, Sun J, Chen G, Yan A. 2016. Transcriptional regulation of the outer membrane porin gene *ompW* reveals its physiological role during the transition from the aerobic to the anaerobic lifestyle of *Escherichia coli*. Front Microbiol 7:799. https://doi.org/10.3389/fmicb.2016.00799.

32. Baldermann C, Lupas A, Lubieniecki J, Engelhardt H. 1998. The regulated outer membrane protein Omp21 from *Comamonas acidovorans* is identified as a member of a new family of eight-stranded $\beta$-sheet proteins by its sequence and properties. J Bacteriol 180:3741–3749. https://doi.org/10.1128/JB.180.15.3741-3749.1998.

33. McClean S, Healy ME, Collins C, Carberry S, O'Shaughnessy L, Dennehy R, Adams A, Kennelly H, Corbett JM, Carty F, Cahill LA, Callaghan M, English K, Mahon BP, Doyle S, Shinoy M. 2016. Linocin and OmpW are involved in attachment of the cystic fibrosis-associated pathogen *Burkholderia cepacia* complex to lung epithelial cells and protect mice against infection. Infect Immun 84:1424–1437. https://doi.org/10.1128/IAI.01248-15.

34. Smani Y, Docobo-Pérez F, López-Rojas R, Domínguez-Herrera J, Ibáñez-Martínez J, Pachón J. 2012. Platelet-activating factor receptor initiates contact of *Acinetobacter baumannii* expressing phosphorylcholine with host cells. J Biol Chem 287:26901–26910. https://doi.org/10.1074/jbc.M112.344556.

35. Ritter A, Com E, Bazire A, Goncalves MDS, Delage L, Le Pennec G, Pineau C, Dreanno C, Compère C, Dufour A. 2012. Proteomic studies highlight outer-membrane proteins related to biofilm development in the marine bacterium *Pseudoalteromonas* sp. D41. Proteomics 12:3180–3192. https://doi.org/10.1002/pmic.201100644.

36. Tiwari V, Vashistt J, Kapil A, Moganty RR. 2012. Comparative proteomics of inner membrane fraction from carbapenem-resistant *Acinetobacter baumannii* with a reference strain. PLoS One 7:e39451. https://doi.org/10.1371/journal.pone.0039451.

37. Vila J, Marti S, Sanchez-Cespedes J. 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. J Antimicrob Chemother 59:1210–1215. https://doi.org/10.1093/jac/dkl509.

38. Nandi B, Nandy RK, Sarkar A, Ghose AC. 2005. Structural features, properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*. Microbiology (Reading) 151:2975–2986. https://doi.org/10.1099/mic.0.27995-0.

39. Motley ST, Morrow BJ, Liu X, Dodge I, Vitiello A, Ward CK, Shaw KJ. 2004. Simultaneous analysis of host and pathogen interactions during an *in vivo* infection reveals local induction of host acute phase response proteins, a novel bacterial stress response, and evidence of a host-imposed metal ion limited environment. Cell Microbiol 6:849–865. https://doi.org/10.1111/j.1462-5822.2004.00407.x.

40. Li W, Wen L, Li C, Chen R, Ye Z, Zhao J, Pan J. 2016. Contribution of the outer membrane protein OmpW in *Escherichia coli* to complement resistance from binding to factor H. Microb Pathog 98:57–62. https://doi.org/10.1016/j.micpath.2016.06.024.

41. Wu XB, Tian LH, Zou HJ, Wang CY, Yu ZQ, Tang CH, Zhao FK, Pan JY. 2013. Outer membrane protein OmpW of *Escherichia coli* is required for resistance to phagocytosis. Res Microbiol 164:848–855. https://doi.org/10.1016/j.resmic.2013.06.008.

42. Gil-Marqués ML, Labrador-Herrera G, Miró-Canturri A, Pachón J, Smani Y, Pachón-Ibáñez ME. 2020. Role of PstS in the pathogenesis of *Acinetobacter baumannii* under microarobiosis and normoxia. J Infect Dis 222:1204–1212. https://doi.org/10.1093/infdis/jiaa201.

43. Jacobs AC, Thompson MG, Gebhardt M, Corey BW, Yildirim S, Shuman HA, Zurawski DV. 2014. Genetic manipulation of *Acinetobacter baumannii*. Curr Protoc Microbiol 35:6G.2.1–6G.2.11.

44. Ferrières L, Hémery G, Nham T, Guérout AM, Mazel D, Beloin C, Ghigo JM. 2010. Silent mischief: bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. J Bacteriol 192:6418–6427. https://doi.org/10.1128/JB.00621-10.

45. Smani Y, Domínguez-Herrera J, Pachón J. 2011. Rifampin protects human lung epithelial cells against cytotoxicity induced by clinical multi and pandrug-resistant *Acinetobacter baumannii*. J Infect Dis 203:1110–1119. https://doi.org/10.1093/infdis/jiq159.

46. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. 1999. Genetic approaches to study of biofilms. Methods Enzymol 310:91–109. https://doi.org/10.1016/S0076-6879(99)10008-9.

47. Clinical and Laboratory Standards Institute. 2017. Performance standards for antimicrobial susceptibility testing. Twenty-seventh informational supplement M100-S27. Clinical and Laboratory Standards Institute, Wayne, PA.

48. Tallarida R, Murray R. 1987. Manual of pharmacological calculation with computer programs, p 31–34. Springer-Verlag, New York, NY.