PhD Thesis

Integration of omics and bioinformatics to identify new therapeutic targets for *Acinetobacter baumannii*. Virulence role of CarO in *Acinetobacter baumannii* infections.

Gema Labrador Herrera

Doctoral Program of Molecular Biology, Biomedicine and Clinical Research
Department of Medicine
University of Seville

Seville 2020

DOCTORAL THESIS

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El Dr. Jerónimo Pachón Díaz, Profesor Emérito de Medicina de la Universidad de Sevilla e Investigador Responsable del Grupo de Enfermedades Infecciosas del Instituto de Biomedicina de Sevilla, como Director y Tutor de la Tesis Doctoral, la Dra. María Eugenia Pachón Ibáñez, Investigadora Posdoctoral perteneciente al programa "Nicolás Monardes" (C1-0038-2019), como Directora de la Tesis Doctoral, y el Dr. Alberto Acedo Bécares, Co-fundador y Director Ejecutivo de la empresa AC-Gen Reading Life, como Director de la Tesis Doctoral,

CERTIFICAN:

Que la Tesis para optar al grado de Doctor por la Universidad de Sevilla que lleva por título "Integration of omics and bioinformatics to identify new therapeutic targets for *Acinetobacter baumannii*. Virulence role of CarO in *Acinetobacter baumannii* infections." ha sido realizada por la Licenciada Doña Gema Labrador Herrera bajo nuestra supervisión, considerando que reúne los requisitos necesarios para su presentación.

Para que conste a los efectos oportunos, expiden la presente certificación en Sevilla, a 10 de abril de 2020.

Jerónimo Pachón Díaz	María Eugenia Pachón Ibáñez	Alberto Acedo Bécares
Director y Tutor	Directora	Director

DOCTORAL THESIS FUNDING

This work was supported by the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad (PI12/02553) and by Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015/0001; RD12/0015/0012; RD16/0016/0009) - cofinanced by European Development Regional Fund "A way to achieve Europe", Operative program Intelligent Growth 2014-2020.

G.L.H. is supported by the program i-PFIS (Doctorados IIS-empresa en Ciencias y Tecnologías de la Salud; IFI15/00128), Ministerio de Economía y Competitividad, Instituto de Salud Carlos III, Spain. M.E.P.I. is a postdoctoral researcher belonging to the program "Nicolás Monardes" (C1-0038-2019), Servicio Andaluz de Salud, Junta de Andalucía, Spain.

SCIENTIFIC PRODUCTION RESULTING FROM THE THESIS

Publications

1. **Labrador-Herrera G**, Álvarez-Marín R, López-Rojas R, Smani Y, Cebrero-Cangueiro T, Rueda A, Pérez-Florido J, Pachón J, Pachón-Ibáñez ME. Draft genome sequences of seven multidrug-resistant *Acinetobacter baumannii* strains, isolated from respiratory samples in Spain. Genome Announc. 2016; 4 (2). DOI: 10.1128/genomeA.00083-16.

2. **Labrador-Herrera G**, Pérez-Pulido AJ, Álvarez-Marín R, Casimiro-Soriguer CS, Cebrero-Cangueiro T, Morán-Barrio J, Pachón J, Viale AM, Pachón-Ibáñez ME. Virulence role of the outer membrane protein CarO in carbapenem-resistant *Acinetobacter baumannii*. Submitted to Journal of Infection.

Congress communications

1. (Oral presentation) **Labrador-Herrera G**, Álvarez-Marín R, López-Rojas R, Smani Y, Rueda A, P. Florido J, López FJ, Praena J, Pachón J, Pachón-Ibáñez ME, Pachón J. Estudio de los mecanismos de resistencia antimicrobiana mediante la secuenciación del genoma completo en cepas clínicas de *Acinetobacter baumannii*. XVI Congreso Sociedad Andaluza de Enfermedades Infecciosas. 11-13 December 2014; Almeria, Spain.

2. (Poster) **Labrador-Herrera G**; Álvarez-Marín R, López-Rojas R, Smani Y, Rueda A, Pérez-Florido J, López FJ, Praena, J, Pachón J, Pachón-Ibáñez ME. Study of the antimicrobial resistance mechanisms of *Acinetobacter baumannii* clinical isolates by whole-genome sequencing. 25th European Congress of Clinical Microbiology and Infectious Diseases. 25-28 April 2015; Copenhagen, Denmark.

3. (Oral presentation) **Labrador-Herrera G**, Pérez-Pulido A, Casimiro-Soriguer CS, Pulido-Fresneda M, Hermosín-Montes JM, Álvarez-Marín R, Smani Y, Pachón J, Pachón-Ibáñez ME. Integration of omics and bioinformatics tools to identify new

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4. (Oral presentation) **Labrador-Herrera G**, Pérez-Pulido AJ, Casimiro-Soriguer C, Álvarez-Marín R, Smani Y, Cebrero-Cangueiro T, Rodríguez-Villodres A, Pachón J, Pachón-Ibáñez ME. Identificación de proteína de la membrana externa asociada a mortalidad en pacientes con *Acinetobacter baumannii*. XIX Congreso Sociedad Andaluza de Enfermedades Infecciosas. 14-16 December 2017; Granada, Spain.

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6. (Oral presentation) **Labrador-Herrera G**, Cebrero-Cangueiro T, Smani Y, Pachón J, Viale AM, Morán-Barrio J, Pachón-Ibáñez ME. The *Acinetobacter baumannii* outer membrane protein CarO exhibits virulence factor roles in a murine infection model. 12th International Symposium on the Biology of *Acinetobacter*. 4-6 September 2019; Frankfurt, Germany.

OTHER SCIENTIFIC PRODUCTION ASSOCIATED WITH THE RESEARCH LINE

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3. (Poster) Cebrero-Cangueiro T, Iglesias-Guerra F, Sánchez-Céspedes J, Vega-Holm M, Smani Y, Candela-Lena JI, **Labrador-Herrera G**, Vega-Pérez JM, Pachón J, Pachón-Ibáñez ME. *In vitro* activity of a library of piperazine derivatives against clinical strains of panresistant *Acinetobacter baumannii*. 10th International Symposium on the Biology of *Acinetobacter*. 3-5 June 2015; Athens, Greece.

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9. (Poster) María Eugenia Pachón Ibáñez, **Gema Labrador Herrera**, Tania Cebrero Cangueiro, C. Díaz, Younes Smani, José Pérez del Palacio, Jesús Rodríguez Baño,

Álvaro Pascual Hernández, Jerónimo Pachón Díaz, M. Carmen Conejo. Efficacy of colistin, alone and in combination with rifampicin, in a murine sepsis model due to carbapenemase-producing *Klebsiella pneumoniae* clinical strains. 26th European Congress of Clinical Microbiology and Infectious Diseases. 9-12 April 2016; Amsterdam, Netherlands.

10. (Poster) Margarita Vega-Holm, Mª Eugenia Pachón-Ibáñez, José Manuel Vega-Pérez, Fernando Iglesias-Guerra, José Ignacio Candela-Lena, Tania Cebrero-Cangueiro, Sarah Mazzotta, Younes Smani, Francesca Aiello, **Gema Labrador-Herrera**, Inmaculada Fernández-Fernández, Jerónimo Pachón, Javier Sánchez-Céspedes. Synthesis of new piperazine derivatives and *in vitro* activity against clinical strains of panresistant *Acinetobacter baumannii*. 6th European Chemical Society Chemistry Congress. 11-15 September 2016; Seville, Spain.

11. (Poster) Cebrero-Cangueiro T, Mazzotta S, Carretero-Ledesma M, Iglesias-Guerra F, Jiménez-Baus A, Sánchez Céspedes J, Vega-Holm M, Smani Y, Candela-Lena J, **Labrador-Herrera G**, Vega-Pérez JM, Pachón J, Pachón-Ibáñez ME. *In vitro* activity of a library of piperazine derivatives against two clinical strain of colistin resistant *Acinetobacter baumannii*. 11th International Symposium on the Biology of *Acinetobacter*. 20-22 September 2017; Seville, Spain.

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13. (Oral presentation) R. Álvarez Marín, C. Ramírez Trujillo, T. Cebreiro Cangueiro, **G. Labrador Herrera**, Y. Smani, E. Cordero, M.E. Pachón Ibáñez. Actividad *in vitro* de pentamidina sola o combinada con antimicrobianos frente a cepas clínicas de enterobacterias productoras de carbapenemasas y/o resistentes a colistina. XXII Congreso Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. 24-26 May 2018; Bilbao, Spain.

14. (Poster) Tania Cebrero Cangueiro, **Gema Labrador Herrera**, Younes Smani, Jerónimo Pachón, María Eugenia Pachón Ibáñez. Efficacy of memory CD8 T cells in an experimental murine model of pneumonia caused by a clinical multidrug- resistant

Acinetobacter baumannii strain. 29th European Congress of Clinical Microbiology & Infectious Diseases. 13-16 April 2019; Amsterdam, Netherlands.

15. (Poster) Ildefonso Espigado, Nancy Rodríguez, **Gema Labrador**, Silvia Jiménez, Manuela Aguilar, Carlos Solano, José Falantes, Lourdes Vázquez, Cristina Fernández, Mi Kwon, Mª Carmen Limón, Francisco Márquez-Maraver, Olaya Fernández, Ariadna Pérez, Inmaculada García, Almudena Cabero, Nieves Dorado, Juan Cordero, Jose Molina, Mª Isabel Montero, José González, Clara Rosso, Jerónimo Pachón, José Antonio Pérez-Simón, Mª Eugenia Pachón Ibáñez. Reduced intensity conditioning and avoiding use of broad spectrum antibiotherapy active against gram-negative bacteria might have a cumulative impact in reducing intestinal microbiota misbalance. 24th European Hematology Association Congress. 13-16 June 2019; Amsterdam, Netherlands.

16. (Oral presentation) T. Cebrero-Cangueiro, **G. Labrador-Herrera**, M. Carretero-Ledesma, S. Herrera-Espejo, Y. Smani, J. Pachón, ME. Pachón-Ibáñez. Eficacia del tratamiento con células de memoria en modelos experimentales murino de neumonía por *Pseudomonas aeruginosa.* XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas. 21-23 November 2019; Seville, Spain.

17. (Oral presentation) T. Cebrero-Cangueiro, **G. Labrador-Herrera**, M. Carretero-Ledesma, S. Herrera-Espejo, Y. Smani, J. Pachón, ME. Pachón-Ibáñez. Eficacia del tratamiento con inmunoglobulinas enriquecidas en IgM en modelos experimentales murino de neumonía por *Pseudomonas aeruginosa.* XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas. 21-23 November 2019; Seville, Spain.

18. (Oral presentation) S. Herrera-Espejo, T. Cebrero-Cangueiro, **G. Labrador-Herrera**, M. Carretero-Ledesma, Y. Smani, J. Pachón, R. Álvarez-Marín, ME. Pachón-Ibáñez. Evaluación *in vitro* de la actividad de pentamidina asociada a otros antimicrobianos en el tratamiento de *Pseudomonas aeruginosa*. XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas. 21-23 November 2019; Seville, Spain.

RESEARCH STAYS PERFORMED DURING THE DOCTORAL THESIS

1. Group of Protein and Immunological Systems Biology (PISB) at the Department of System Biology of the Technical University of Denmark. 3 months (28 April–28 July 2015); Lyngby, Denmark.

2. Bioinformatics Department of the private company AC-Gen Reading Life. 1 year (10^{th} June 2019 – 10^{th} June 2020); Valladolid, Spain.

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AHL: acyl-homoserine lactone.

AME: aminoglycoside-modifying enzyme.

APACHE II: acute physiology and chronic health evaluation II.

ARDS: adult respiratory distress syndrome.

ATCC: American Type Culture Collection.

AUC: area under the curve.

bid: two times a day.

BLAST: Basic Local Alignment Search Tool.

BLASTN: Basic Local Alignment Search Tool for nucleotides.

BLASTP: Basic Local Alignment Search Tool for proteins.

bp: base pair.

BSA: bovine serum albumin.

BSI: bloodstream infections.

CAP: community-acquired pneumonia.

CC: clonal complex.

CFU: colony-forming units.

CGE: Center for Genomic Epidemiology.

CHDL: carbapenem-hydrolyzing class D β-lactamase.

CI (for competitive fitness studies): competition index.

CI: confidence interval.

CLSI: Clinical and Laboratory Standards Institute.

Cmax: maximum concentration.

CMS: colistimethate sodium.

CPIS: clinical pulmonary infection score.

CRAB: carbapenem-resistant *A. baumannii*.

DIP: 2,2'- dipyridyl.

DMEM: Dulbecco's modified Eagle's medium.

DMSO: dimethyl sulfoxide.

ECDC: European Centre for Disease Prevention and Control.

EDTA: ethylenediaminetetraacetic acid.

EUCAST: European Committee on Antimicrobial Susceptibility Testing.

FBS: fetal bovine serum.

FDA: Food and Drug Administration (United States of America).

FDR: false discovery rate.

GNB: Gram-negative bacilli.

HAI: healthcare-acquired infection.

HAP: hospital-acquired pneumonia.

IC: international clon.

ICU: intensive care unit.

IgG: immunoglobulin G.

IS: insertion sequence.

LB: Luria-Bertani.

LC-MSMS: liquid chromatography-tandem mass spectrometry.

LD: lethal dose.

LOS: lipooligosaccharide.

LPC: lysophosphatidylcholine.

LPS: lipopolysaccharide.

MATE: multidrug and toxic compound extrusion.

MBL: metallo-β-lactamase.

MDR: multidrug-resistant.

MHA: Mueller-Hinton agar.

MHB: Mueller-Hinton broth.

MHB II: cation-adjusted Mueller-Hinton broth.

MIC: minimum inhibitory concentration.

MIU: million international units.

MLD: minimal lethal dose.

MLST: multilocus sequence typing.

MW: molecular weight.

NCBI: National Center for Biotechnology Information.

OD: optical density.

OM: outer membrane.

OMP: outer membrane protein.

OMV: outer membrane vesicle.

OR: odds ratio.

PBP: penicillin-binding protein.

PBS: phosphate-buffered saline.

PCR: polymerase chain reaction.

PDB: protein data bank.

PDR: pandrug-resistant.

PFGE: pulsed-field gel electrophoresis.

PK/PD: pharmacokinetic/pharmacodynamic.

PNAG: polysaccharide poly-N-acetylglucosamine.

QC: quality control.

QQ: quorum quenching.

QRDR: quinolone resistance determining region.

qRT-PCR: quantitative reverse-transcription polymerase chain reaction.

QS: quorum sensing.

RND: resistance nodulation cell division.

SDS: sodium dodecyl sulfate.

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SEM: standard error of the mean.

SMR: small multidrug resistance.

ST: sequence type.

T2SS: type II secretion system.

T6SS: type VI secretion system.

TBA: tracheobronchial aspirate.

tid: three times a day.

TMT: tandem mass tag.

UK: United Kingdom.

U.S.: United States.

USA: United States.

VAP: ventilator-associated pneumonia.

VFDB: Virulence Factors of Pathogenic Bacteria database.

WGS: whole-genome sequencing.

WHO: World Health Organization.

XDR: extensively drug-resistant.

ABSTRACT

ABSTRACT

Acinetobacter baumannii remains a significant and difficult-to-treat pathogen that causes a range of interactions with the human host from asymptomatic colonization and carriage in the skin, intestinal tract, and respiratory tract to invasive infection, such as nosocomial pneumonia or bacteraemia. Especially, this pathogen affects critically-ill and immunocompromised patients admitted to Intensive Care Units (ICUs), causing them severe infections, which are associated with long hospital stay and high mortality rates. The success of this bacterium is due to a combination of several factors, highlighting its extraordinary ability to develop antimicrobial resistance that results in the rapid nosocomial spread of strains resistant to almost all known antimicrobials, including including the last reservoirs of our antimicrobial arsenal such as the carbapenems, worldwide. Thus, in the recent global priority list of antimicrobial-resistant bacteria of the World Health Organization, carbapenem-resistant *A. baumannii* (CRAB) is considered as a "critical priority" for the development of new antimicrobials, due to the lack of therapeutic options. This situation has promoted the search of new therapeutic strategies to deal with multidrug-resistant (MDR) *A. baumannii* strains displaying additional carbapenem-resistance, and non-antimicrobial approaches aimed at bacterial virulence factors may represent a promising alternative. Nevertheless, our knowledge on *A. baumannii* pathogenesis and virulence traits is still relatively scarce. In this Doctoral Thesis, and in order to stop the evolution of *A. baumannii* infections, we aimed to study more deeply the pathogenesis of CRAB infections using omics and bioinformatics to ultimately discover new therapeutic targets for anti-*A. baumannii* drugs.

Firstly, we studied MDR/CRAB isolates from tracheobronchial aspirate samples of ICU adult patients who suffered *A. baumannii* bacteraemic ventilator-associated pneumonia (VAP) or remained exclusively colonised by this pathogen. We analysed the *in vitro* and *in vivo* virulence of these isolates, in order to know if the invasive isolates exhibited higher virulence than the colonising ones, but no differences were found. Moreover, when the whole-genome sequencing (WGS) data of these isolates were analysed following different approaches, again we did not find any difference between both phenotypes regarding their clonal relationship, antimicrobial resistance mechanisms, or known virulence determinants. However, the invasive *A. baumannii* isolates exhibited higher levels of expression of the outer membrane protein (OMP) OmpA than the colonising ones, suggesting that those phenotypes depend on the regulation of already-known or still unknown virulence factors, instead of on the genomic content.

Secondly, six bacteraemic MDR/CRAB clinical isolates obtained from six clinically homogeneous ICU adult patients with bacteraemia secondary to VAP, who were subjected to optimal colistin treatment but with radically different clinical outcomes, were comparatively analyzed by WGS complemented with proteomic and immunoblot techniques. These analyses indicated that the *carO* gene, encoding for the second most abundant β-barrel protein of the *A. baumannii* outer membrane (OM), CarO, was interrupted by different disruptive events in the isolates from patients who recovered from infection, while it was intact in the isolates from patients who did not survive. When the virulence role of *A. baumannii* CarO was analyzed in model systems, an isogenic mutant lacking *carO* (ATCC 17978 Δ*carO*) showed lower ability *in vitro* to adhere and invade cultured human lung epithelial cells, and exhibited a higher minimum lethal dose and a lower dissemination potential into essential organs and fluids in a murine model of peritoneal sepsis. All of the above deficiencies were reverted in the ATCC 17978 Δ*carO* mutant transformed with a *carO* expression plasmid restoring OM CarO levels. Thus, the results presented here reveal a previously unnoticed virulence role for the *A. baumannii* OMP CarO, which may be responsible of the poor clinical outcome and therefore a potential target for the development of novel anti-*A. baumannii* drugs.

INTRODUCTION

1. Clinical relevance of *Acinetobacter baumannii*.

1.1. Epidemiological situation and most frequent clinical manifestations of *A. baumannii*.

1.1.1. Reservoirs, transmission mechanisms and epidemiological situation of *Acinetobacter* infections.

Acinetobacter spp. are ubiquitous in nature and can be found in different wet environments, such as moist soil/mud, wetlands, ponds, water treatment plants, fish farms, dump sites, and even seawater, but also on vegetables, animals, and humans (2-4). Nonetheless, despite non-*baumannii Acinetobacter* spp. have predominated in surveillance studies of skin colonisation, particularly among healthy individuals, more-pathogenic species, such as *A. baumannii*, has rarely been identified as a coloniser of skin among healthy patients (5, 6). *Acinetobacter* was initially considered a commensal opportunist, but the increasing utilization of complex intensive care since the 1960s (associated with an increasing ubiquity and intensity of mechanical ventilation, central venous and urinary catheterization, and antimicrobial therapy) has caused a surge in the frequency and severity of *Acinetobacter* infections (7, 8). Today, *Acinetobacter* infections have spread rapidly through hospitals across the globe, occurring the highest density of infections in intensive care units (ICUs). In the European Centre for Disease Prevention and Control (ECDC) point prevalence survey of healthcare-acquired infections (HAIs) and antimicrobial use in European acute care hospitals in the period 2011-2012, *Acinetobacter* spp. were the 11th most frequently isolated microorganisms (3.6%) in microbiologically documented HAIs, and more specifically, the 5th in pneumonia/lower respiratory tract infections, the 8th in bloodstream infections, the 9th in urinary tract infections, and the 11th in surgical site infections (9). These frequencies are similar in ICUs from United States (USA) (10), but in Asian and certain Latin American countries, *Acinetobacter* is one of the three most common causes of bacteraemia and nosocomial pneumonia (11-13). Thus, Spellberg *et al.* estimated that 1 million (range, 600,000 to 1,400,000) cases of *Acinetobacter* infections occur annually worldwide, according to published frequencies of HAIs (14).

Among *Acinetobacter* spp., *A. baumannii* is the most important pathogen associated with HAIs (15). In fact, most *A. baumannii* infections occur in critically ill patients and account

for up to 20% of infections in ICUs worldwide (16). *A. baumannii* is characterized by its great persistence in the environment, being able to survive for long periods on dry surfaces, its high tolerance to stressful or unfavourable situations, its relatively high virulence, and its extraordinary capability to develop resistance to antibiotics, as well as to disinfectants and biocides (7, 17, 18). These characteristics are closely related to its epidemiology and facilitate, within the nosocomial context, its localization in all types of medical and surgical material, furniture, surfaces and even in air near to carrier patients (19-22). The human being can also be a reservoir of *A. baumannii*. The oropharyngeal cavity and the respiratory tract of patients with mechanical ventilation are the locations where most frequently is isolated, followed by the gastrointestinal tract, which is considered a persistent reservoir of multidrug-resistant (MDR) strains (23-25). The main transmission mechanism of *A. baumannii* inside the hospital are health care personnel hands, which become contaminated after coming into contact with a carrier patient or with contaminated surfaces or fomites (26, 27). Outbreaks have also been reported from direct contact with medical procedures or instruments, such as unduly decontaminated respiratory support material (28, 29).

The occurrence and dissemination of *A. baumannii* poses major medical and organizational burdens to healthcare facilities since infections caused by *A. baumannii* are difficult to treat, and because eradication of this pathogen from the hospital environment and prevention of further spreading might require solid targeted measures, such as the isolation of patients and temporary closure of wards (30, 31). Moreover, the continuous appearance of nosocomial outbreaks worldwide stands out, as well as the presence of endemic patterns in some hospital centres, making control and eradication even more difficult and favouring the emergence of extensively drug-resistant (XDR) isolates or even pandrug-resistant (PDR) ones (26, 32-34). In fact, our hospital (University Hospital Virgen del Rocío, Seville, Spain), experienced a two decades-long hyperendemic situation with *A. baumannii*, with an outbreak of PDR *A. baumannii* in 2002 (35) and a second outbreak of XDR *A. baumannii* resistant to colistin in the adult ICU in 2012 (36). Despite several interventions along this long period, only a multicomponent intervention program, performed from January 2009 to August 2017 by a multidisciplinary team, were effective in controlling XDR *A. baumannii* outbreak and eradicating the endemic MDR *A. baumannii* in our hospital (36). This effective and necessary multifaceted program included the following measures: environmental

decontamination, hand hygiene instruction and surveillance, antimicrobial stewardship program, isolation and contact precautions, active surveillance for MDR *A. baumannii* colonisation, weekly report, and regular meetings with the staff of the affected areas.

Outbreaks characteristics vary depending on the conditions of the hospital (if there are one or multiple sources), and on the particularities of the strain, since some strains have greater tendency to spread in an endemic form than others (30). Moreover, very alike strains not epidemiologically related have been isolated from different places at different times. These clones are generally MDR, genetically stable and with high capacity to survive in hospital settings. The local, national, and international spread of these clones is facilitated by the increase of international travels, including the transfer of patients between hospitals and countries (37). Comparative typing of *A. baumannii* outbreak strains from geographically distributed European hospitals demonstrated the occurrence of three successful clones, originally named "European clones I–III" (38, 39). These clones have subsequently been identified worldwide and accordingly re-named as "international clones (IC) I–III" (40). In addition to these major clones, a wide geographic distribution of other clones has been reported (41, 42). The emergence of such highly successful clones seems to play a leading role in the increasing global occurrence of infections by this pathogen (40). Particularly, the international clone clonal complex (CC) 92 (Bartual/Oxford scheme (43)/CC2 (Pasteur scheme (40)) (44, 45) is by far the largest and most widely distributed one (Figure 1A), showing a broad international distribution among 34 countries (Italy, Spain, Germany, United Kingdom (UK), Greece, Netherlands, Denmark, Czech Republic, France, Poland, Turkey, Norway, Sweden, Portugal, Ireland, Belgium, Romania, Japan, China, Korea, Thailand, India, Malaysia, Philippines, Taiwan, Singapore, Vietnam, Lebanon, Brazil, South Africa, Reunion, Tahiti, New Caledonia, USA, and Australia) (Figure 1C) (46). CC92/CC2 extensively includes outbreak and MDR isolates (47). In these isolates, carbapenem resistance is mainly mediated by β-lactamases encoded by $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24/40-like}}$, and/or $bla_{\text{OXA-58-like}}$ genes (Figures 1D-E). Additionally, the clone included several isolates producing the 16S rRNA methylase ArmA (Figure 1F), as well as one isolate resistant to colistin (48). The intra-clonal diversity of phenotypic and genotypic resistance characteristics among isolates of CC92/CC2 could be due to the scattered occurrence of this clone worldwide, resulting in access to a wide range of varied pools of transmissible resistance elements (49). CC92/CC2 is closely linked with international clone II (Figure 1B) and the OXA-

66 cluster (the OXA-66, OXA-82, OXA-83, and OXA-109 variants) of the intrinsic *bla*$_{\text{OXA-51-like}}$ gene (50, 51). The apparent considerable capability of CC92/CC2 for epidemic clonal spread (47) could be explained by the biodiversity reduction and support for emergence of clonality produced by the two main stress conditions and crucial evolutionary bottlenecks for clinically relevant bacteria, which are human immune system and antibiotic treatment (52). Therefore, virulence and antibiotic resistance are vital adaptive mechanisms of selection used by particular bacterial strains and clones, such as the *A. baumannii* CC92/CC2 clone, to survive under stress conditions, thus avoiding competition with isolates from other strains and clones (52). As isolates from CC92/CC2 have repeatedly exhibited high resistance rates to all antimicrobial agent, it seems that the frequent acquisition of diverse antimicrobial resistance determinants is probably the main factor involved in its survival in the hospital setting (40, 53), providing them with more time for dissemination and further clonal expansion (48, 54). It is notable that the emergence of MDR isolates belonging to CC92/CC2 has most likely resulted from independent events involving acquisition of various resistance determinants associated with various mobile elements (55). Nevertheless, specific determinants for transmission, colonisation, and/or invasion, which may explain the epidemic behaviour of CC92/CC2, are still unknown (31, 54).

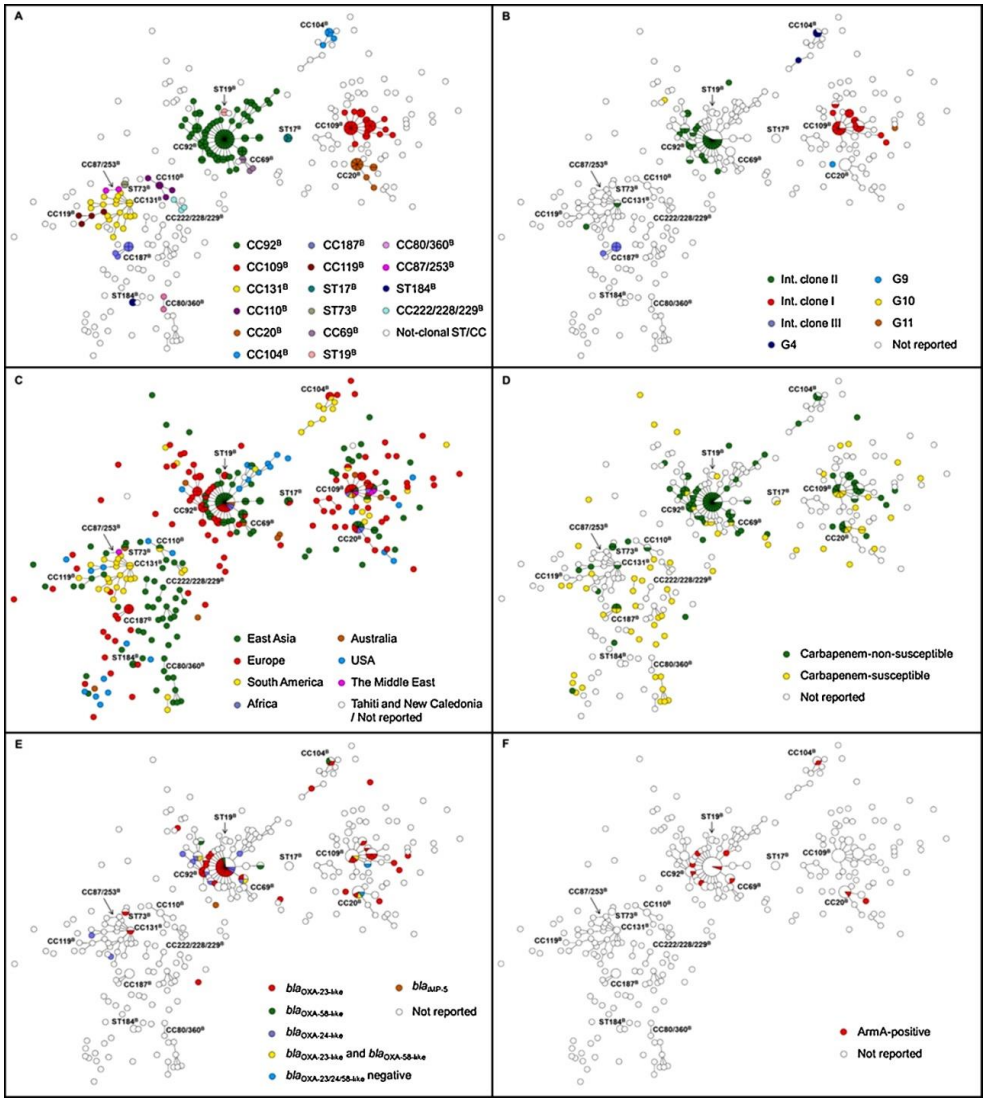

Figure 1. (A–F) Analysis of the current global epidemiology of *A. baumannii*, using a subset of 346 *A. baumannii* isolates, identified by previous studies and/or retrieved from the multilocus sequence typing (MLST) databases, and the Minimum Spanning Tree algorithm (47). Each circle corresponds to one allelic profile or sequence type (ST). Circle size increases logarithmically with the number of isolates of each ST. Each line indicates that the connected circles correspond to STs sharing 6/7 of the alleles. Each group of circles linked by at least one line corresponds to one clonal complex (CC). Distance between circles does not correspond to the number of allelic mismatches among the corresponding STs. (A) Circles highlighted in one colour correspond to one given clone. (B) Circles highlighted in one colour correspond to one given polymerase chain reaction (PCR)-based international

clone (Int. clone) or group (G). (C) Circles highlighted in one colour correspond to one given geographic area. (D) Circles highlighted in one colour correspond to one given carbapenem resistance status. (E) Circles highlighted in one colour correspond to one given mechanism of carbapenem resistance. (F) Circles highlighted in red correspond to ArmA-positive STs/CCs.

1.1.2. Most frequent clinical manifestations.

The ability of *Acinetobacter* to colonise any human epithelial surface explains why it can be isolated in a wide variety of biological samples, such as respiratory secretions, wound surface or urine. However, its isolation does not necessarily mean that there is an active infection (56). In fact, approximately half of the *A. baumannii* isolations from clinical samples are considered colonisations, as corroborated in a Spanish multicentre cohort with 221 clinical isolates of *A. baumannii*, where only a 52.9% of isolates were the cause of infection, while 47.1% were not (57). *A. baumannii* can cause infections in any system, apparatus or organ, especially when it is subjected to invasive manoeuvres that cause disruption of natural defense barriers. Although community infections by this microorganism have been described (58, 59), most are nosocomial, mainly in the ICUs. The most frequent are, in this order: respiratory tract infections, bacteraemia, urinary tract infection, surgical wound infection and postsurgical meningitis (60, 61). This study focuses on the most common infection by this pathogen, ventilator-associated pneumonia (VAP), as well as the bacteraemia associated with this respiratory infection.

1.1.2.1. Respiratory infections.

Respiratory tract infections, and in particular pneumonia and tracheobronchitis, are the nosocomial infections most frequently caused by *A. baumannii* (37, 60, 62). In countries were *A. baumannii* is spreading, it is the predominant pathogen isolated from patients with hospital-acquired pneumonia (HAP). Indeed, *A. baumannii* might be responsible of more than 36% of HAP cases in Asia (63). Nevertheless in some countries is responsible only of 1–2% of the nosocomial pneumonia episodes (64).

In nosocomial pneumonia by *A. baumannii*, the greatest risk factors are orotracheal intubation, tracheostomy, prior antibiotic therapy, a long period of ICU stay, recent

surgery, the presence of underlying chronic lung disease and propensity for aspiration of high secretions (27, 37, 65). Nosocomial pneumonia by *A. baumannii* is usually multilobar and may develop complications such as parapneumonic pleural effusion, cavitation and bronchopleural fistula. It is accompanied by bacteraemia in approximately 12% of the episodes, being pneumonia the most frequent clinical focus (66).

In the nosocomial environment, tracheobronchitis due to *A. baumannii* is also common. In recent years, more attention has been given to tracheobronchitis associated with mechanical ventilation as a clinical entity, although the diagnostic criteria are not completely agreed and its clinical impact is related, in many cases, to the further development of pneumonia (67).

1.1.2.2. Bacteraemia.

Bacteraemia is the second most frequent nosocomial infection caused by *A. baumannii* (27), being pneumonia often the clinical focus associated with it (66), followed by intravenous catheters (68, 69). Other origins, such as the urinary tract, skin and soft tissue infections, nosocomial meningitis or intra-abdominal infections are less frequent foci (68, 69). Moreover, in up to 44% of episodes, the bacteraemic origin is unknown (70). The frequency of bacteraemia by *A. baumannii* is a parameter that can serve as an expression of the increase in infections caused by this microorganism in recent years. Overall, *A. baumannii* is accountable for more than 12% of the cases of hospital-acquired bloodstream infections (BSI) in ICU, with wide geographic variations: it is frequent in Southern Europe, median Eastern countries, Asia, and South America, whereas it is rare in Northern European countries and Australia (71). In a study conducted in our centre in 1993, *A. baumannii* was the microorganism responsible for the 27% of bacteraemias by Gram-negative bacilli (GNB) (69).

Patients at high risk for bacteraemia by *A. baumannii* are those admitted to ICU, both adults and paediatric, with a Charlson comorbidity index > 3 points, previous hospitalizations, total parenteral nutrition, chemotherapy or have received antibiotic therapy during admission, especially β-lactams or carbapenems (69, 72). Among the risk factors for suffering bacteraemia by *A. baumannii*, the presence of invasive devices or procedures such as mechanical ventilation, intravascular catheter, bladder catheter or

nasogastric tube, are the most important, as reflected in multiple studies and in the systematic review performed by Zhou *et al.* (73).

1.1.3. Differentiation between *A. baumannii* colonisation and infection.

The respiratory tract, urinary tract, biological fluids, and surgical wounds, are the most likely locations for colonisation or infection by *A. baumannii*. As this pathogen is able to form biofilms on the surface of the endotracheal tube, high levels of colonisation in the lower part of the respiratory tract in intubated patients are frequent (74). In critically ill patients, up to half of the cases in which *A. baumannii* is isolated represents a mere colonisation (56, 57). Nonetheless, the behaviour of respiratory colonisation by *A. baumannii* is poorly described in the literature. Beyond its role as a previous step to respiratory infection, it is unknown if colonisation by *A. baumannii* has any clinical impact on its own. Moreover, owing to the characteristics of the patients from whom *A. baumannii* is isolated, it is often difficult to differentiate colonisation from infection (75). Thus, it is often challenging to differentiate colonisation from infection, especially in the critical care setting, and this situation needs to be considered to avoid diagnostic errors and unnecessary antibiotic therapy (75).

Several biomarkers, but especially procalcitonin levels, might have an important role in the process of discriminating the presence or absence of bacterial infections (76-79). However, the potential usefulness of procalcitonin, or other biomarker, as an instrument to distinguish colonisation from infection has not yet been shown (80). On the other hand, many studies have evaluated the value of bacteriological data in establishing the diagnosis of VAP compared to clinical criteria (81). Thus, a bacteriological threshold of ≥ 6 $\log_{10}$ colony-forming units (CFU)/mL in low respiratory samples, such as tracheobronchial aspirate (TBA), has been established to diagnose VAP (82). However, a study performed in our hospital showed that bacterial concentration in TBA samples was similar in patients with *A. baumannii* colonisation or VAP, being in both cases above the threshold of 6 $\log_{10}$ CFU/mL (83). Therefore, the bacterial concentration of *A. baumannii* in respiratory samples does not allow *per se* discriminating between *A. baumannii* colonisation and VAP. Thus, the exact mechanism by which the bacterium causes infection or remains as a mere coloniser is unknown. Although it is true that there may be factors associated with the host, it has been shown that different strains of *A. baumannii* have differences in

virulence (84). Multiple *in vitro* and *in vivo* studies have attempted to clarify the virulence mechanisms of this pathogen very recently, describing several elements associated with virulence factors, as will be detailed later. However, new studies are necessary to relate the expression of these virulence factors with the clinical behaviour of the strains.

1.2. Prognosis of infections caused by *A. baumannii*.

The crude mortality and morbidity associated with *A. baumannii* infections, although they vary depending on the time of infection and other factors, are high. In many cases, however, it is difficult to know the attributable mortality, since they occur mostly in patients with serious underlying diseases or critical situations.

In recent years, studies focused on prognostic factors of *A. baumannii* infections abound. These studies identified factors related to the host (severity of baseline disease, previous functional status, mechanical ventilation, malignancy, and immunosuppression), factors related to the infection (location, severity scores, septic shock, and respiratory distress), efficacy of the treatment used, and characteristics of the microorganism, such as infection by a specific clone or antimicrobial resistance (85-87).

Carbapenem resistance is one of the factors that has been most frequently associated with mortality in infections caused by *A. baumannii* (88, 89), quantified with a crude odds ratio (OR) of 2.22 (95% confidence interval (CI) = 1.66-2.98) (89). However, studies in this field are rather heterogeneous and it is still not clear the magnitude of the confusing factors, such as the proportion of inappropriate treatment.

1.2.1. Mortality and morbidity associated with pneumonia by *A. baumannii*. Prognostic factors.

Pneumonia caused by *A. baumannii* produce a high crude mortality, reaching up to 40-70% in VAP (57, 86, 90, 91). Nonetheless, if patients with *A. baumannii* VAP die as a result of or with the infection by this pathogen, is still a cause for controversy among specialists (57, 92, 93). Yet, several reviews described an attributable mortality of 8-23% for *A. baumannii* nosocomial pneumonia and 10-46% for *A. baumannii* VAP (94, 95).

The clinical relevance of colonisation, at least when it occurs in airways, is still unknown, but needs to be elucidated. In this context, it is worth noting a retrospective case-control study performed by Gkrania-Klotsas *et al.*, which compared all patients colonised or infected with MDR *A. baumannii* with all patients colonised or infected with MDR *Pseudomonas aeruginosa* in the same year (96). They observed a significantly higher mortality in the *A. baumannii* group, even after adjusting for stay in an ICU ($P = 0.0002$), concluding that colonisation or infection by MDR *A. baumannii* may be an independent risk factor for increased mortality.

Multiple risk factors for mortality of nosocomial pneumonia due to *A. baumannii* have been identified: underlying chronic diseases, clinical severity of the pneumonia or baseline situation measured with different scores, inappropriate empirical treatment or use of antimicrobials in the previous 28 days, are the most important (86, 97, 98). Carbapenem resistance has been linked to mortality in some studies, such as that of Zheng *et al.* (86), in which patients with nosocomial pneumonia caused by carbapenem-resistant *A. baumannii* (CRAB) had a higher mortality than those with pneumonia by carbapenem-susceptible *A. baumannii* (45.6% *vs.* 29.9%, $P = 0.02$). However, in this case, the resistance of the causal strain was not selected as a risk factor for mortality in the multivariate analysis, while the inappropriate treatment did; suggesting that the difficulty in establishing an adequate treatment is what increases the mortality of CRAB infections.

1.2.2. Mortality and morbidity associated with bacteraemia caused by *A. baumannii*. Prognostic factors.

Crude mortality rates in patients with *A. baumannii* bacteraemia are high, and varied between 30-76%, according to the case series and the distribution of risk factors (99, 100). Moreover, the prognosis of *A. baumannii* bacteraemia is worse compared to bacteraemia by other genospecies of the genus *Acinetobacter*. In this regard, Lee *et al.* detected a 30-day crude mortality of 29.7% for bacteraemia by *A. baumannii*, with an OR = 2.23 (95% CI = 1.053-4.821) with respect to the mortality of bacteraemia by other *Acinetobacter* species (101).

Nonetheless, it has been difficult to distinguish between attributable mortality and that attributed to the underlying illnesses and comorbid conditions of patients. On the one

hand, in a retrospective case-control study carried out by Blot *et al.*, no significant differences in mortality were found (102). Similarly, in a case control study, Jang *et al.* reported that the underlying illnesses seemed to play a more pivotal role than the infection itself as a cause of death (103). On the other hand, in another retrospective study comparing patients with *A. baumannii* bacteraemia with controls without bacteraemia, an attributable mortality of 36.5% was reported (104). Furthermore, in a recent study performed by Ballouz *et al.*, the crude mortality rate was 63.5% and a 70.3% of the deaths were attributed to the bacteraemia, showing the significant impact of the bacteraemia on patients' outcome (105). In addition, in a prospective cohort study performed by our group, patients with bacteraemic VAP had a tendency to higher mortality than VAP patients without bacteraemia (42.9% *vs.* 20.9%, $P = 0.1$) (106).

Multiple risk factors associated with mortality in patients with *A. baumannii* bacteraemia have been identified, including factors similar to those previously mentioned: serious underlying disease (Charlson index > 3, malignant neoplasm or other diseases such as chronic obstructive pulmonary disease or cerebrovascular disease) with a high degree of dependence, low levels of serum albumin, admission to ICU prior to bacteraemia, previous surgery or solid organ transplant at the same admission, mechanical ventilation, severe sepsis, elevated acute physiology and chronic health evaluation II (APACHE II), prior antibiotic exposure, inappropriate definitive treatment, and drug resistance, specially to carbapenems (101, 105, 107, 108). A study that specifically analysed patients with CRAB bacteraemia showed a 30-day mortality of 48%, being the severity of the baseline disease and the presence of immunosuppression the only predictors of mortality (109). In addition, Chopra *et al.* evaluated the sulbactam susceptibility (110) showing its importance, as the mortality for bacteraemia by carbapenem and sulbactam-resistant *A. baumannii* was 43%, while it was 20% for those by *A. baumannii* susceptible to any of these antibiotics.

1.3. Antimicrobial resistance: current epidemiological situation and resistance mechanisms.

A. baumannii exhibits a wide variety of resistance mechanisms to antimicrobial agents, with strains highly resistant to most clinically available antibiotics (15). Some European countries reported that more than half of *Acinetobacter* spp. isolates were resistant to all

antimicrobial categories under surveillance (carbapenems, fluoroquinolones and aminoglycosides) (9). This phenomenon has increasingly become a matter of great concern for stakeholders and the scientific community worldwide. Consequently, Tacconelli *et al*. in 2017 published a list of antibiotic-resistant "priority pathogens", which includes the 12 families of bacteria that pose the greatest threat to human health and for which development of new antibiotics is urgently needed. In this list, CRAB is considered as "priority 1" (critical) (111).

A. baumannii presents innate resistance mechanisms against multiple antimicrobials (including glycopeptides, lincosamides, macrolides, and streptogramins) on its core genome, but additionally, strains can easily accumulate new resistance mechanisms through chromosomal mutations and acquisition of resistance genes via horizontal transfer (112). Almost all antimicrobial resistance mechanisms so far described have been found in *A. baumannii*, such as drug-inactivating enzymes (β-lactamases, aminoglycoside-modifying enzymes (AMEs)…), modification of target sites (topoisomerase IV/DNA gyrase (*parC*/*gyrA*), ArmA, penicillin-binding proteins (PBPs), lipopolysaccharide (LPS)…), permeability defects (CarO, 33-36 kDa protein…), or active efflux pumps (AdeABC, TetB…) (Table 1) (15, 113, 114). Frequently, genes that confer resistance to antimicrobials are clustered together within an antibiotic-resistance island, which accumulates in specific genetic regions of the large accessory genome (115).

Table 1. Major resistance mechanisms found in *A. baumannii* (table modified from (1)).

Antimicrobial mechanism of action	Antimicrobial category/agent	Main resistance mechanisms	Examples
Inhibition of bacterial cell wall synthesis	**β-lactams**	Inactivating enzymes	β-lactamases: - Chromosomal cephalosporinase (AmpC) - Narrow spectrum: TEM, SHV - Extended spectrum: TEM, SHV, CTX-M, PER, VEB - Carbapenemases: * Class A: KPC * Class B: IMP, VIM, NDM * Class D: OXA-23-like, -24/40-like, -51-like, -58-like, -143-like, -235-like
		Target modification	Low affinity PBPs (mutations in PBP1, 2, or 3, or replacement)
		Decreased outer membrane permeability	Decreased expression of OMPs CarO, 33–36 kDa protein, OprD-like protein
		Overexpression of efflux pumps	AdeABC
β-lactamase inhibitor with additional antibacterial activity	**Sulbactam**	Inactivating enzymes	Multiple copies of $bla_{\text{TEM-1D}}$
			IS*Aba1* upstream *ampC*
		Target modification	Low affinity PBPs (mutations in PBP1 and PBP3)
		Overexpression of efflux pumps	AdeABC
Disruption of the outer membrane and alteration of the cytoplasmic membrane	**Polymyxins (colistin)**	Target modification	Plasmid-mediated *mcr* genes (LPS modification)
			Mutations in the PmrA/B two-component system (LPS modification)

			Mutations in LPS biosynthesis genes *lpxA, lpxC, lpxD* (LPS loss)
		Porin loss	OmpW
Inhibition of protein synthesis	**Aminoglycosides**	Inactivating enzymes	AMEs (AAC, ANT, APH)
		Target modification	Ribosomal methylation by ArmA
		Overexpression of efflux pumps	AdeABC, AdeM
	Tetracyclines	Inactivating enzyme	TetX
		Target protection	TetM
		Efflux pumps	Multidrug: AdeABC (overexpression); drug specific: TetA, TetB
	Glycylcyclines	Overexpression of efflux pumps	AdeABC
Alteration of nucleic acid structure or metabolism	**Quinolones**	Target modification	Mutations in quinolone resistance determining regions of *gyrA* and *parC*
		Target protection	Plasmid-mediated Qnr proteins (QnrA, QnrB, QnrS)
		Overexpression of efflux pumps	Class RND: AdeABC, AdeIJK and AdeFGH; class MATE: AbeM; class SMR: AbeS
	Rifampicin	Inactivating enzyme	Arr-2
		Target modification	Mutations in conserved regions of *rpoB*
		Modification of antibiotic permeability and overexpression of efflux pumps	Not defined
Blocking synthesis and utilization of metabolic factors	**Sulfonamides**	Target modification	Alternative drug-resistant variants of the target enzyme (Sul1, Sul2, Sul3)

PBP: penicillin-binding protein; OMP: outer membrane protein; LPS: lipopolysaccharide; AME: aminoglycoside-modifying enzyme; RND: resistance nodulation cell division; MATE: multidrug and toxic compound extrusion; SMR: small multidrug resistance.

1.3.1. Importance of carbapenem resistance in *A. baumannii*.

Carbapenems have been considered the treatment of choice for infections caused by MDR *A. baumannii*. However, an increasing number of *A. baumannii* isolates have developed carbapenem resistance in recent years, which has being strongly associated with prior use of carbapenems. A worldwide collection of 5127 *Acinetobacter* spp. collected between 2005 and 2009 from 140 hospitals in 32 countries in North America (17.1%), Europe (22.9%), Latin America (25.2%) and the Asia-Pacific region (34.8%), showed the overall nonsusceptibility rate to imipenem and meropenem to be 45.9 and 48.2%, respectively. However, the nonsusceptibility percentages had increased from 27.8 and 37.5% (for imipenem and meropenem, respectively) to 62.4 and 64.4%, when compared 2005 results with those from 2009 (116). Moreover, six countries (Belgium, Italy, Malta, Portugal, Slovakia and Spain) that collected during 2009 detailed resistance data from microorganisms associated with ICU-acquired infections reported CRAB isolates to be up to 80% (117). And, more recently, a study derived from the Magic Bullet clinical trial described that 97% of the *A. baumannii* isolates collected from patients from 15 hospitals in Greece, Italy and Spain during 2012-2015 were resistant to imipenem, and these were always associated with an acquired carbapenemase, OXA-23 (80%), OXA-24/40 (4.6%), OXA-58 (1.5%) or OXA-23/58 (1.5%) (118).

1.3.2. Main mechanisms of resistance to carbapenems in *A. baumannii*.

Carbapenem resistance mechanisms often work synergistically, including the production of antimicrobial-degrading enzymes, efflux pumps, and porin mutations (112). The most prevalent mechanism of carbapenem resistance in *A. baumannii* is enzymatic degradation by carbapenemases, mainly carbapenem-hydrolyzing class D β-lactamases (CHDLs), also named as OXA-type enzymes, and to a lesser content class B β-lactamases, also named as metallo-β-lactamases (MBLs) (119), and the class A β-lactamase KPC (120). More specifically, acquired OXA-type CHDLs of the OXA-23, OXA-24/40 and OXA-

58 subfamilies, and the intrinsic OXA-51-type are the most common among *A. baumannii* isolates (121). Furthermore, several insertion sequences (IS), mainly IS*Aba1* and IS*Aba 3*, have been described to play a major role in the overexpression of these carbapenemase-encoding genes.

Efflux pumps can extrude a variety of antimicrobial agents and compounds to the external environment, and therefore its overexpression can reduce the accumulation of antibiotics, being another efficient mechanism for carbapenem resistance (122). To date, the major efflux mechanism associated with carbapenem resistance in *A. baumannii* is the chromosomally encoded tripartite efflux pump, AdeABC (123-125). This pump is the first identified resistance-nodulation-cell division (RND)-type efflux pump and its overproduction confers resistance to aminoglycosides and decreased susceptibility to tetracycline, fluoroquinolones, chloramphenicol, erythromycin, trimethoprim and ethidium bromide, as well as to netilmicin and meropenem (126).

A. baumannii intrinsically have a smaller number and size of porins compared with other GNB, contributing to the intrinsic outer membrane (OM) impermeability (112). Porins are outer membrane proteins (OMPs) able to form channels allowing the transport of molecules, including antimicrobials, across lipid bilayer membranes. Moreover, variations in the structure or regulation of porin expression can provide another mechanism to become resistant to carbapenems (127). To date, three porins have been implicated in carbapenem resistance when their expression is reduced: CarO, Omp 33-36 and OprD (127), although a more recent work suggests the OprD may in fact not be involved (128). The carbapenem-associated OMP, also called CarO (a 29 kDa protein), is the most characterized porin in *A. baumannii* in this aspect. The analysis of strains with high minimum inhibitory concentration (MIC) to imipenem (up to 16 mg/L) showed disruptions in the *carO* gene by various insertion elements and thus loss of expression (129, 130). Alteration of the expression of CarO in the OM reduces the penetration of imipenem into the cell, therefore contributing to drug resistance (131). However, given the absence of a meropenem binding site on CarO, meropenem resistance may be mediated by another porin-mediated pathway (131), as is Omp33-36 (a 33–36 kDa protein) (132).

2. *A. baumannii* virulence factors.

Most infections and deaths associated with *A. baumannii* occur, as described before, in patients who are already seriously ill from another cause, have received antibiotic therapy and have acquired this bacterium in the hospital. These data contribute to the previous consideration of *A. baumannii* as a low-virulence pathogen in comparison with other GNB such as *P. aeruginosa* or *K. pneumoniae* (52, 94, 102). However, the number of studies reporting the importance of *A. baumannii* intrinsic virulence is increasing significantly (94, 133).

The pathogenic capacity of *A. baumannii* consists of multiple virulence factors, necessary for this bacterium to colonise and then infect the host (134). Nevertheless, only a relatively low number of virulence factors have been identified compared to other GNB (1). This trend is changing, thanks to the availability of genomic, transcriptomic and proteomic techniques that help to identify virulence factors that participate in *A. baumannii* pathogenesis (7, 113). Furthermore, the increasing antimicrobial resistance rates together with the lack of treatments options to combat infections, make necessary identify new virulence factors to characterize the pathogenesis and determine new therapeutic targets that allow the control and/or prevent these kinds of infections. The major virulence factors identified in *A. baumannii* are illustrated in Figure 2 and the functional aspects of these factors are described below.

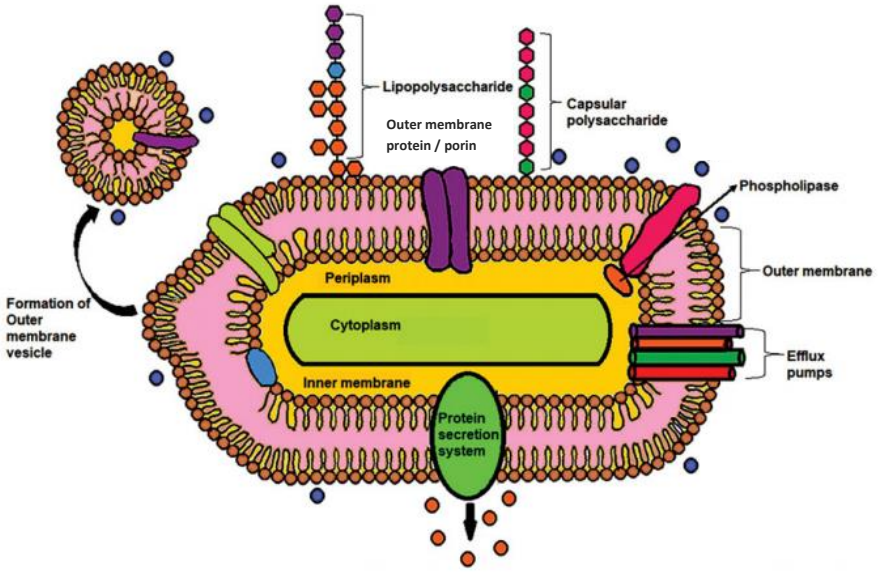

Figure 2. Main virulence factors involved in *A. baumannii* (135).

2.1. OMPs.

A. baumannii, like other GNB, contain a double membrane (OM and inner membrane) which serves as protection and provide nutrients for viability. The OM, besides being the first line of contact between the bacteria and its external environment, it acts as a protection mechanism against the entry of many toxic molecules into the cell (including many antibiotics) and allows the entry of nutrients required for cell survival (136). The OM has a unique composition and asymmetrical distribution of lipids, with the inner leaflet containing phospholipids, whereas the outer leaflet is composed of LPS (137). This membrane is also mainly composed of proteins, called OMPs, which have essential functions for the cell, including nutrient uptake, cell adhesion, cell signaling and waste export (138). The OMPs of Gram-negative bacteria are structured in the OM in a closed toroidal structure formed by antiparallel β-barrel sheets spanning the lipid bilayer in which the first strand is connected to the last by hydrogen bonding. The number of trans-membrane β-strands range from 8 to 24 depending on the OMP, and are connected by exposed loops of a length that also depends on the OMP and the species considered. In the case of pathogenic strains, many of these OMPs are involved in antimicrobial resistance as described before, but also serve as virulence factors for nutrient scavenging, induction of the expression of other virulence factors, cell adhesion and invasion, and evasion of host defense mechanisms by mutation, among others (137).

One of the main OMPs that has been characterized is OmpA, also called Omp38. OmpA is a 8-stranded β-barrel porin highly conserved among bacterial species and one of the most abundant porins in *A. baumannii* OM (139). It plays a considerable role in biofilm formation (140), adherence and invasion of epithelial cells (140-142), translocation into the epithelial cell nucleus (143), induction of epithelial cell apoptosis (144, 145), and binding to factor H, which may allow *A. baumannii* to develop serum resistance (146). Moreover, a recent study on patient cohorts suggested that *ompA* overexpression was an associated factor for pneumonia, bacteraemia, and death due to *A. baumannii* (147).

OmpW is another 8-stranded β-barrel OMP that is widespread in many GNB, such as *Vibrio cholerae*, *Escherichia coli*, and *A. baumannii* (148). OmpW protein is believed to be involved in the transport of hydrophobic or aromatic molecules across the OM (149-151), and it is implicated in iron homeostasis (152). Moreover, mutant strains of *V.*

cholerae, which expressed no OmpW, were shown to have reduced colonisation in the intestine of mice relative to those that expressed it (153).

Another important OMP is Omp33-36, which induces apoptosis and modulates autophagy in human cells (154). In addition, an isogenic knockout strain deficient in the *omp33* gene showed a reduction both in adherence and invasion of human lung epithelial cells, as in cytotoxicity of these cells and macrophages, and exhibited higher lethal doses (LD) LD_0, LD_{50}, and LD_{100}, and lesser dissemination in a murine peritoneal sepsis model (155).

Furthermore, it has been demonstrated that the lower expression of the porins CarO and OprD-like attenuates the virulence of a PDR *A. baumannii* strain (156). CarO is another 8-stranded β-barrel OMP and it is the most characterized porin in *A. baumannii*, together with OmpA, and it has been previously related to antimicrobial resistance, but its virulence role has not entirely being stated.

2.2. Capsular polysaccharides and LPS.

Capsular polysaccharides are involved in antimicrobial resistance and referred as target for defensive passive immunization in *A. baumannii*. It is known that mutants deficient in capsular polysaccharides have lower intrinsic resistance to peptide antibiotics, and that the presence of antibiotics induces hyperproduction of capsular polysaccharides (157). Moreover, it was observed that increased production of capsular polysaccharides causes an increase in virulence in a murine model (157). Hyperproduction of capsular polysaccharides depended on transcriptional increase in the *K*-locus gene where the expression of the gene is regulated mainly by the two-component system, such as BfmRS (158, 159). *bfmR* is important for persistence in the lung in a murine pneumonia model (160) and *bfmS* is involved in biofilm formation, adherence to eukaryotic cells, and resistance to human serum (158).

As previously mentioned, the major component of the outer leaflet of the OM in *A. baumannii* is LPS. Although historically the LPS was believed to be essential for OM stability and survival (161), *A. baumannii* strains lacking LPS have been isolated (162). Despite this, defects in the *lpxABCD* cluster (responsible for LPS biosynthesis) have been shown to clearly affect the ability of the organism to grow, thereby diminishing virulence

(7). Moreover, the LPS of *A. baumannii* has been shown to play crucial roles in cell motility (163), surface adhesion (164), stimulation of the proinflammatory immune response (162, 165), and resistance to opsonophagocytic killing (166) and to antimicrobial peptides in human serum (167), in addition to resistance to polymyxin antibiotics, as previously described. It should be noted that, although until now it has been considered that *A. baumannii* has LPS, recent studies have indicated that *A. baumannii* strains form only lipooligosaccharide (LOS), not LPS (168, 169). The difference between both OM components is that LPS is composed by three structural domains: a hydrophobic domain known as lipid A (or endotoxin), a core oligosaccharide and a highly variable O-antigen constituted of repeating oligosaccharide units (170), while LOS is composed only by the lipid A and a core oligosaccharide. Nonetheless, we will still mentioning it as LPS for the rest of the work.

2.3. Phospholipase.

Phospholipase is a lipolytic enzyme involved in the metabolism of phospholipid, and it acts as a virulence factor in *A. baumannii* (171). There are three classes of phospholipases: A, C, and D (171). The presence of phospholipase C has been related to an enhancement of adhesion and invasion ability (172) and cytolytic activity (173) of *A. baumannii*. Furthermore, a study revealed that disruption of *A. baumannii* phospholipase D caused a reduction in its ability to thrive in serum, a deficiency in epithelial cell invasion, and diminished pathogenesis in a murine pneumonia model (174).

2.4. Biofilm formation.

Biofilm refers to a complex group of microorganisms in which the cells are embedded in an extracellular polymeric substance, a self-produced matrix (175). The ability of *A. baumannii* to form biofilms allows it to grow persistently in unfavourable conditions and environments (176, 177), reduces antibiotic penetration (178), and plays an important role in immune evasion (179).

The biofilm cycle mechanism is composed of numerous steps (135, 175). The ability to adhere abiotic surfaces depends on the cell surface hydrophobicity followed by the

interference of the exopolysaccharide proteins and macromolecules in the intercellular aggregation. Further, quorum sensing (QS) occurs at a critical cell density, which acted as a communication pathway for cell coordination leading to the formation of biofilm. At later stage, the cells are detached and dispersed to colonise new regions due to the physical forces. Although there are several factors directly associated with the biofilm formation in *A. baumannii*, pilus formation and exopolysaccharide production are the main ones (177). The type I chaperone-usher pilus system (Csu pili), regulated by the BfmRS two-component system, are crucial for biofilm formation and maintenance on abiotic surfaces, constituting an *A. baumannii* virulence factor (180). Another virulence factor is the polysaccharide polymer poly-beta-1,6-N-acetylglucosamine, one of the most important components of exopolysaccharides constituting biofilm matrix that is crucial for maintaining the integrity of *A. baumannii* biofilm under environmental stresses (181) and is involved in the immune evasion and host-microbe interaction (135). In addition, biofilm-associated protein Bap functions in biofilm maturation and maintenance (175, 182).

2.5. QS.

QS is the cell to cell communication system used by the bacteria to perform and regulate various biological processes (183, 184), and plays an important role in regulating diverse virulence factors such as surface motility and biofilm formation (185). Moreover, a recent study (186) has suggested that QS of *A. baumannii* affects the development of secondary bacteraemia in pneumonia patients. In order to generate QS by the bacteria, small diffusible molecules called as “signals”, such as acyl-homoserine lactones (AHLs) are produced (187). When a threshold concentration is reached, the AHL molecules present inside the cell are transported to its AHL-receptor and transcriptional activator AbaR, which initiate molecular cascades that result in the synthesis of more AHL molecules by the AHL-synthase AbaI. These molecular cascades thereby cause the population to survive and proliferate (185).

2.6. Metal acquisition systems.

To overcome the iron limitation in the outside medium, most aerobic bacteria produce a high-affinity iron chelator known as a siderophore (188). One of the best-characterized *A. baumannii* siderophores is acinetobactin, a virulence factor that allows bacteria to persist within epithelial cells and cause cell damage and animal death (189). There are other metal acquisition systems such as the NfuA Fe-S scaffold protein (190), the metal-chelating protein calprotectin (191), and the zinc acquisition system ZnuABC (192), which are also described as virulence factors contributing to the pathogenesis of *A. baumannii*.

2.7. Outer membrane vesicles (OMVs).

OMVs are spherical nanovesicles secreted by the OM of various Gram-negative pathogenic bacteria (193). They are composed of LPS, OMPs, phospholipids, and DNA or RNA (194). These vesicles can transport virulence factors, such as OmpA, participate in biofilm formation, and may also be involved in QS and antimicrobial resistance gene transfer (195, 196).

2.8. Protein secretion systems.

There are several protein secretion systems identified in *A. baumannii* (197). The type II secretion system (T2SS), the type VI secretion system (T6SS) and the type V system autotransporter Ata, are some shown to mediate in the virulence of this pathogen (113). The T2SS is a multi-protein complex that translocates a wide range of proteins, including the lipases LipA and LipH and the protease CpaA, from the periplasmic space to the OM surface or to the extracellular medium (198). Moreover, a functional T2SS has been shown to be essential for a full virulence in *A. baumannii* using different experimental models (198). The T6SS is a multicomponent secretion machine used to inject effector proteins with the aim of killing competing bacteria (199) or enhancing colonisation during infection of eukaryotic hosts (200). *A. baumannii* uses its T6SS for bacterial competition (201), but in a strain-specific manner (202). Lastly, the type V system autotransporter Ata is a trimeric membrane protein involved in biofilm formation, adherence to extracellular matrix, and *in vivo* virulence (203).

3. Treatment of infections caused by *A. baumannii*.

Since its discovery in 1985, carbapenems have been the treatment of choice for infections caused by MDR *A. baumannii*, but the spread of their use has been rapidly followed by the emergence of strains resistant to these drugs worldwide (204). This worrying increase of antimicrobial resistance has seemingly outpaced the development of new antibiotics (205, 206), leading to the development and implementation of antimicrobial stewardship programmes to optimize the use of the existing antimicrobials (such as the PIRASOA programme, implemented in hospitals of the Public Health System of Andalusia (207)), the reintroduction of old antibiotics, alone or in combination, as well as the promotion of searching new therapeutic strategies, to deal with infections caused by MDR *A. baumannii* strains.

3.1. Current treatment and drug options.

3.1.1. Carbapenems.

Carbapenems (imipenem, meropenem and doripenem) are the drugs of choice for MDR *A. baumannii* isolates that retain susceptibility to carbapenems (208). The pharmacokinetic/pharmacodynamic (PK/PD) parameter that best relates to the efficacy of carbapenems is the time above the MIC (T > MIC) (209). For this reason, prolonged infusion has been studied as a strategy to improve this index (209). Imipenem is the one with the highest activity *in vitro* and it has shown activity in animal models against strains with intermediate susceptibility (MIC = 8 μg/mL) (210). Nevertheless, meropenem has a lower affinity for certain oxacillinase enzymes than imipenem (211). Moreover, its stability in extended infusion and a comparatively lower seizure threshold than imipenem makes meropenem a rational choice for *A. baumannii*. Unfortunately, rising MIC of meropenem substantially decrease the probability of reaching the optimal PK/PD index with the routine dosing regimens, making necessary its use in combination with other antimicrobials (80). Doripenem has shown greater *in vitro* activity than meropenem and imipenem against *A. baumannii* strains with a OXA-58 carbapenemase, remaining a therapeutic option for some strains resistant to imipenem (212). However, the clinical experience with this drug is still scarce. Therefore, carbapenems should not be used

empirically, at least in monotherapy, for severe infections in areas with a high rate of resistance to carbapenems.

3.1.2. Sulbactam.

Sulbactam, besides being a β-lactamase inhibitor, has an intrinsic inhibitory activity against *A. baumannii* and offers multiple advantages: good tissue penetration, its safety profile is excellent and is tolerated even in high doses, and the absence of activity in monotherapy against most others microorganisms cushion its impact on antibiotic pressure (213, 214). The efficacy of sulbactam against *A. baumannii* in clinical practice is sustained by several non-randomized studies that, in patients with severe infections (VAP, bacteraemia and meningitis, among others), have reported therapeutic success rates of 61-67.5% (215). A systematic review concluded that sulbactam treatments have the same efficacy as therapies based on other antimicrobials in *A. baumannii* infections (216). Furthermore, in a retrospective study that analysed infections caused by CRAB, polymyxin (colistin or polymyxin B) treatment was significantly associated with higher mortality than sulbactam (217). For all these reasons, it is considered the treatment of choice for infections caused by sulbactam-susceptible CRAB strains. Unfortunately, nowadays, the percentage of resistance to sulbactam has reached such a high level (218) that its empirical use against infections caused by *A. baumannii* is discouraged (219).

3.1.3. Polymyxins.

Only polymyxin B and polymyxin E (colistin) are used in clinical practice. Nonetheless, colistin is by far the most extensively used polymyxin. It is administered as colistimethate sodium (CMS), a prodrug that needs to be hydrolysed to its active form (colistin) (220). Although it was discovered in the late 1940s and used for several years in clinical practice, its popularity diminished because of reports of significant nephrotoxicity and neurotoxicity. Recently, colistin has resurfaced as a last-line treatment option for MDR Gram-negative pathogens (221).

Unfortunately, the emergence of strains resistant to colistin is worrisome and outbreaks caused by strains of PDR *A. baumannii* have been reported (35, 222), as well as the

appearance of isolates with colistin heteroresistance (221, 223). Its clinical relevance is not clear, but *in vitro* studies have shown that the heteroresistance development is related to subtherapeutic concentrations of colistin (224, 225). Until now, genetic alterations in the PmrAB two-component system and lipid A biosynthesis genes were the main mechanisms associated with colistin resistance in *A. baumannii* [205]. However, recent studies have reported the presence of plasmid-mediated *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4.3* genes in colistin-resistant *A. baumannii* isolates from different clinical and environmental samples in Pakistan, Baghdad-Iraq, and Brasil (226-228). Importantly, colistin resistance may be underestimated using automated testing methods (229, 230).

In the last years, our knowledge on the clinical pharmacokinetic of colistin has increased substantially but not enough. The lack of PK/PD studies and no universal harmonization of dose units have made it difficult to derive optimal dosing regimens and specific dosing guidelines for colistin (231). Nowadays, the ratio of the area under the curve (AUC) to the MIC (AUC/MIC ratio) is the best PK/PD index to describe its efficacy profile. Thus, a dosing regimen should allow for colistin plasma concentrations of about 2 μg/mL to assure the efficacy against colistin susceptible *A. baumannii* (80). With regard to colistin toxicity, most studies show that nephrotoxicity is reversible and less frequent than once thought, and neurotoxicity is rare (231). However, the traditional dosing regimen of colistin (6 million of international units [MIU] daily, three times a day [tid]) was considered insufficient to treat susceptible pathogens with a MIC in the upper limit of susceptibility, according to the results of multiple pharmacokinetics studies (232, 233). Consequently, higher dosages of colistin have been generally adopted (9 MIU daily, two times a day [bid]/tid), although a definitive consensus is lacking (234), and a loading dose is recommended to achieve early effective concentrations in critically ill patients (235). A meta-analysis of 32 studies evidenced, despite the low-quality of the data, a clinical benefit of high intravenous doses of colistin (236). In addition, the problem of heteroresistance to colistin can also be overcome with high doses of colistin or with the use of another active agent (237). Nonetheless, findings from the Magic Bullet study did not support the empirical use of colistin (4.5 MIU loading dose followed by a maintenance dose of 9 MIU daily) in the treatment of late VAP caused mostly by MDR-GNB, due to it demonstrated similar efficacy but greater nephrotoxicity than meropenem, when both were combined with levofloxacin (238). Moreover, our group demonstrated that colistin dosage (6 MIU of CMS daily, tid) without loading dose is efficacious when treating VAP

caused by CRAB strains with high susceptibility to colistin (106). Therefore, further investigations using higher CMS doses must be performed in critically ill patients to determine whether there is improved efficacy without increased toxicity.

3.1.4. Tetracyclines.

Minocycline and doxycycline exhibit *in vitro* and *in vivo* activity against *A. baumannii* (including CRAB, CRAB/sulbactam-resistant, and MDR strains), as well as synergistic effects when combined with different antimicrobials (239-241). Furthermore, the existing minocycline intravenous indication for treatment of *A. baumannii* infections has provided high rates of clinical success or improvement and, in addition, it was generally well tolerated by patients with MDR or CRAB infections (242, 243). Similarly, doxycycline has been successfully used to treat patients with VAP caused by MDR *A. baumannii* (244).

3.1.5. Tigecycline.

Tigecycline has activity against most of the XDR *A. baumannii* isolates, including those resistant to carbapenems. Based on clinical trials, its use is approved for skin and soft tissue infections, intra-abdominal infections and community-acquired pneumonia (245). However, when it has been used to treat other infections, therapeutic success has been inconstant (246, 247). Treatment of intravascular/bacteraemic infections by *A. baumannii* with tigecycline seems impossible with the approved regimen (100-mg loading dose followed by a 50-mg dose bid) because of the low maximum concentration (Cmax) achieved in serum (248). Similarly, tigecycline concentrations in pulmonary endothelial lining fluid with conventional dosing are insufficient to treat *A. baumannii* VAP (249). A matched cohort analysis concluded that the tigecycline-based therapy resulted in higher in-hospital mortality than the colistin-based therapy (61 *vs.* 44%, respectively) in critically ill patients with pneumonia caused by MDR *A. baumannii* (250). Two meta-analyses discourage the use of a tigecycline for the treatment of MDR-*A. baumannii* infections because, compared with other active antimicrobials, the use of tigecycline was associated with higher in-hospital mortality, lower microbial eradication rate and longer length of stay (251, 252). Nevertheless, a high-dose regimen (200 mg/day), usually in

combination with another antimicrobial, may be an effective and well-tolerated alternative for severe *A. baumannii* infections including HAP (253).

3.1.6. Combined therapy.

The use of combination therapy is an attractive approach justified by the high mortality rates associated to these infections, the lack of proven valid therapeutic options, and the rapid development of antimicrobial-resistance (254). Notwithstanding, most data regarding combination of antimicrobials rely on *in vitro* or animal studies, and its translation into the clinical practice is doubtful because even the same antimicrobial combinations have been reported to have different results (255, 256). Furthermore, most observational studies, randomized clinical trials, and meta-analyses coincide in the lack of clinical efficacy (cure rate or mortality) using combination therapy instead of monotherapy for *A. baumannii* severe infections (257, 258), although microbiological eradication rates are significantly higher with the use of two active antimicrobials in some cases (259-262). For example, a prospective observational study, conducted in 28 Spanish hospitals, that assessed the clinical efficacies of monotherapy (mainly colistin and carbapenems) and combination therapy (colistin/carbapenem plus tigecycline were the most used) in MDR *A. baumannii* sepsis concluded that there was no association of combination therapy with reduced mortality in this type of infection (257). A randomized open-label trial evaluated colistin monotherapy compared with the combination of colistin and fosfomycin for 7–14 days in patients infected with carbapenem-resistant *A. baumannii*. Microbiological response at the first 72 h and at the end of treatment was significantly higher in the combination group, but no differences in clinical cure rate or 28-day mortality were found (260). Similar results were obtained from another randomized open-label trial performed to compare colistin-rifampicin *vs.* colistin monotherapy groups in serious MDR-*A. baumannii* infections (262). Furthermore, a recent randomized controlled superiority trial conducted with 406 patients with severe infections caused by carbapenem-resistant Gram-negative bacteria (mostly *A. baumannii*) concluded that the treatment based on the combination of colistin plus meropenem did not result in better outcomes compared with colistin monotherapy (263). A systematic review and meta-analysis that included 12 controlled studies concluded that there was no strong evidence that combination regimen of polymyxins was superior to monotherapy

regimen (258). Therefore, most experts concluded that further studies were needed to better define the potential advantages and disadvantages of combination therapy for *A. baumannii* infections. Meanwhile, it seems reasonable to use monotherapy, at least in patients with less severe infections (257).

3.2. Novel and future treatment options.

3.2.1. New antibiotics.

Since 2017 eight new antibiotics have been approved (264). However, most of them are derivatives of known classes, such as the two tetracycline derivatives eravacycline and omadacycline, and furthermore, none of them is active against carbapenem-resistant *A. baumannii* (264). Alternatively, there are currently 32 antibacterial agents in clinical development Phases 1-3 targeting WHO priority pathogens (Table 2), being only four of these agents (cefiderocol, durlobactam [ETX-2514] + sulbactam, SPR-206, and TP-6076) active against MDR *A. baumannii (264).* Moreover, despite being promising options for the treatment of different infectious pathologies caused by MDR *A. baumannii*, these new antibiotics are also based on modifications of traditional antibiotic structures, leading to a possible cross-resistance to currently used antibiotics and therefore, challenging their longevity as therapeutic options (265). Thus, more investment is needed for the discovery, research and development of truly innovative and effective antibiotics.

Table 2. Antibiotics that are being developed against World Health Organization (WHO) priority pathogens (modified from (264).

Name (synonym)	Phase	Antibiotic class	Route of administration (developer)	Expected activity against priority pathogens			
				CRAB	CRPA	CRE	OPP
Lascufloxacin	NDA[1]	Fluoroquinolone	iv & oral (Kyorin)	❍	❍	❍	?
Cefiderocol[12]	NDA[2] MAA[2]	Siderophore cephalosporin	iv (Shionogi)	●	●	●	/
Sulopenem, Sulopenem etzadroxil/ probenecid	3	Penem	iv (Iterum) oral (Iterum)	❍	❍	❍[3]	/
Durlobactam (ETX-2514) + sulbactam	3	DBO-BLI/PBP2 binder + β-lactam-BLI/PBP1,3 binder	iv (Entasis)	●	❍	❍	/
Taniborbactam (VNRX-5133) + cefepime	3	Boronate-BLI + cephalosporin	iv (VenatoRx)	❍	?	●	/
Enmetazobactam (AAI-101) + cefepime	3	β-lactam BLI + cephalosporin	iv (Allecra)	❍	❍	❍[4]	/
Zoliflodacin	3	Topoisomerase inhibitor (spiropyrimidenetrione)	oral (Entasis/GARDP)	/	/	/	●
Gepotidacin	3	Topoisomerase inhibitor (triazaacenaphthylene)	iv & oral (GSK)	/	/	/	●
Levonadifloxacin Alalevonadifloxacin	3[5]	Fluoroquinolone	iv oral (Wockhardt)	❍	❍	❍	?
Cefilavancin (TD-1792)	3[6]	Glycopeptide-cephalosporin conjugate	iv (Theravance/R Pharm)	/	/	/	●
Solithromycin	3[7]	Macrolide	iv & oral (Melinta/Fujifilm Toyama Chemical)	/	/	/	●
Contezolid Contezolid acefosamil	2/3[8]	Oxazolidinone	oral (MicuRx) iv & oral (MicuRx)	/	/	/	●
Afabicin (Debio-1450)	2	FabI inhibitor	iv & oral (Debiopharm)	/	/	/	●
BOS-228 (LYS-228)	2	Monobactam	iv (Boston Pharmaceuticals)	❍	❍	●	/
Nafithromycin (WCK-4873)	2	Macrolide	oral (Wockhardt)	/	/	/	●
TNP-2092	2	Rifamycin-quinolizinone hybrid	iv & oral (TenNor)	/	/	/	?
Benapenem	2[9]	Carbapenem	iv (Sichuan Pharmaceutical)	❍	❍	❍	/
Zidebactam + cefepime	1	DBO-BLI/PBP2 binder + cephalosporin	iv (Wockhardt)	❍	?	●	/
Nacubactam + meropenem	1	DBO-BLI/PBP2 binder + meropenem	iv (NacuGen Therapeutics)	❍	❍	●[10]	/
ETX0282 + cefpodoxime	1	DBO-BLI/PBP2 binder + cephalosporin	oral (Entasis)	❍	❍	●[10]	/
VNRX-7145 + ceftibuten	1	Boronate-BLI + cephalosporin	oral (VenatoRx)	❍	❍	●	/
SPR-741 + β-lactam	1	Polymyxin (potentiator) + β-lactam	iv (Spero)	?	?	?	/
SPR-206	1	Polymyxin	iv (Spero)	●	●	●	/

Continuation of Table 2. Antibiotics that are being developed against World Health Organization (WHO) priority pathogens (modified from (264)).

Name (synonym)	Phase	Antibiotic class	Route of administration (developer)	Expected activity against priority pathogens			
				CRAB	CRPA	CRE	OPP
KBP-7072	1	Tetracycline	oral (KBP BioSciences)	○	○	○	●
TP-271	1	Tetracycline	iv & oral (Tetraphase)	?	○	○	●
TP-6076	1	Tetracycline	iv (Tetraphase)	●	○	?	/
EBL-10031 (apramycin)	1[10]	Aminoglycoside	iv (Juvabis)	?	-	?	/
AIC-499 + unknown BLI	1	β-lactam + BLI	iv (AiCuris)	?	?	?	/
TNP-2198	1	Rifamycin-nitroimidazole conjugate	oral (TenNor)	/	/	/	●
TXA-709	1	FtsZ inhibitor	oral & iv (Taxis)	○	○	○	●
BCM-0184	1	?	oral (Biocidium)	○	○	○	●
ARX-1796 (oral avibactam prodrug)	1	DBO-BLI + ¤-lactam	oral (Arixa Pharmaceuticals)	○	○	●[11]	/

CRAB: carbapenem-resistant *A. baumannii*; CRPA: carbapenem-resistant *P. aeruginosa*; CRE: carbapenem-resistant *Enterobacterales*; OPP: "high" and "medium" WHO priority pathogens.

Pathogen activity: ● active; ? possibly active; ○ not or insufficiently active; / activity not assessed, as the antibiotic is focused and developed for only either Gram-positive cocci or Gram-negative rods. The only agents assessed against OPPs were those that are not active against critical priority pathogens. OPP includes the high- and medium-priority pathogens.

[1] Clinical development only for Japan; registered on 20 September 2019 for CAP in Japan (oral).
[2] NDA submitted in December 2018 and MAA submitted in April 2019.
[3] Active against ESBL-producing cephalosporin-resistant but not carbapenem-resistant *Enterobacteriaceae*.
[4] Active against ESBL-producing cephalosporin-resistant and some KPC-producing CRE.
[5] Clinical development only for India.
[6] Clinical development only for Russia.
[7] Clinical development only for Japan.
[8] Contezolid acefosamil: Phase 2 in the United States. Contezolid: Phase 3 in China; NDA in China expected in 2020.
[9] Clinical development only for China.
[10] Previously used in animals.
[11] Active against KPC but not MBL-producing *Enterobacteriaceae*.
[12] FDA approval on 14 November 2019 for cUTI, which was after the cut-off date of this report.

3.2.2. Anti-virulence drugs.

As detailed above (section 2), recent whole-genome sequencing (WGS) studies involving *A. baumannii* have demonstrated not only a vast array of antibiotic drug resistance determinants but also many virulence factors (266). These studies demonstrate the contribution of a few factors in the pathogenesis of *A. baumannii*, suggesting that these mechanisms would be potential candidates for targets of novel antimicrobial agents. Thus, we could fight *A. baumannii* by blocking its virulence factors, relegating this bacterium to its prior role as opportunistic and rarely pathogenic bacteria. Moreover, as this strategy is not based on a bactericidal approach, the emergence of new resistant phenotypes may not be a limitation (267). In addition, this action would be improved by

combining this strategy with the classical antimicrobial agent approach, allowing us a potent defence–attack combination.

The experimental results demonstrating *in vitro* and *in vivo* efficacy of drugs able to block virulence factors (called "anti-virulence drugs") are hopeful, and the apparent advantages of the non-antimicrobial approaches described below are promising. However, caution is needed and further extensive studies have to be performed to elucidate the potential use of these new molecules as therapeutic alternatives (268).

3.2.2.1. Inhibition of OMPs.

Many Gram-negative pathogens, including *A. baumannii*, use the externally exposed loops of a number of OMPs to interact with their hosts to induce the expression of virulence factors, to invade tissues, and by mutation to escape the immune system (269-271). Thus, inhibitors of OMPs constitutes a recent and promising treatment alternative to fight against this pathogen. Until now, two OMPs have been chosen as drug targets for the development of such inhibitors, OmpA and OmpW.

A series of OmpA inhibitors were designed by virtual screening and tested *in vitro* and *in vivo* by Vila-Farrés X *et al.* (272). In this study, they reported that one of these inhibitors, the cyclic hexapeptide AOA-2, was able to inhibit the GNB adherence to host cells, reducing host cell death, and inhibit biofilm formation by these pathogens. Moreover, AOA-2 *in vivo* administration shows a large decrease in the bacterial concentration in spleen and lungs, together with a significant decrease in mouse mortality, especially in *A. baumannii* infections. Later, the synergistic activity of AOA-2 and colistin against colistin-resistant *A. baumannii* was described *in vitro* and *in vivo* (273).

Similarly, Soojhawon *et al.* used another *in silico* screening for the development of novel inhibitors for *A. baumannii* OmpW (148), reporting good results regarding antibacterial activity against a panel of clinical isolates of *A. baumannii* strains, for three of the tested compounds (D5, D12 and D6).

3.2.2.2. Inhibition of LPS synthesis.

The inhibition of the bacterial synthesis of LPS through the blocking of *lpx* genes could be another potential treatment approach (7). LpxC inhibition by LpxC-1 blocks LPS biosynthesis, blocking the ability of *A. baumannii* to activate the sepsis cascade and enhancing opsonophagocytic killing of the bacteria, resulting in the completely protection of mice from lethal infection (166). Moreover, no cross-resistance to the LpxC-1 was reported in this study. Advancement of novel classes of LpxC inhibitors has been performed, identifying a lead compound, LpxC-4, which is a potent broad-spectrum inhibitor against which spontaneous resistance arises fairly infrequently (274). In a similar strategy, the LpxA and LpxD structures have been recently crystallized and they will be tested as targets for new antibacterial agents (275, 276).

3.2.2.3. Inhibition of biofilm formation.

Several studies have focused on the inhibition of biofilm formation by different approaches, such as the use of garlic ointment (277, 278) or gaseous nitric oxide (278) to prevent biofilm formation in wounds or dressings. Another approach is DispersinB, an antibiofilm enzyme that alone or in a synergistic combination with the KSL-W antimicrobial peptide is able to disperse preformed biofilm and inhibit biofilm formation in chronic wound infection caused by *A. baumannii* without affecting bacterial growth (279). In addition, it has been developed a 2-aminoimidazole compound (2-AI) that inhibits and controls the biofilm development by targeting BfmR, a response regulator involved in biofilm development (280), or even a potent anti-biofilm peptide (1018) that inhibits biofilm formation and eradicates preformed biofilms formed by *A. baumannii* among others MDR Gram-negative pathogens by blocking (p)ppGpp, an important signal in biofilm development (281). Besides, four chimeric peptides have been reported to prevent biofilm formation by *A. baumannii* clinical isolates, in addition to its significant antibacterial effects, better than ampicillin, cefotaxime, ciprofloxacin, tobramycin, and erythromycin (282).

3.2.2.4. Inhibition of QS.

As many bacterial pathogens use QS to control virulence, the development of methods to intercept QS has attracted significant interest as a potential anti-infective therapy (187).

As previously described, *A. baumannii* uses AHLs as signal molecules and AbaR as the putative cognate receptor for QS, so that most novel anti-infective strategies are based on blocking this AbaR receptor with different compounds (268). Several non-native AHL were discovered as potent AbaR antagonists, which strongly inhibited *A. baumannii* motility and reduced biofilm formation by up to 40% (283). More recently, Nicol *et al.* demonstrated that unsaturated fatty acids were also able to decrease *A. baumannii* biofilm formation, presenting a biofilm dispersing effect and drastically reducing motility, by decreasing the expression of the regulator AbaR (284).

3.2.2.5. Iron chelation.

As iron is an essential cofactor for many bacterial processes, the use of iron chelators and iron competitors has been studied as an alternative to antibiotics or as a complement to antibiotic treatments. Some of the main iron chelators developed to fight against bacterial pathogens are deferoxamine, deferiprone, Apo6619, VK28 dihydrochloride, and 2,2'-dipyridyl (DIP). However, these iron chelators had only low or modest antibacterial activity *in vitro* against *A. baumannii* according, being DIP the one that showed the highest, and in addition, the antibacterial effect appeared to be dependent on the medium (285). Regarding iron competitors, gallium (Ga^{+3}) is the most studied for *A. baumannii*. It is a transition metal with a similar atomic radius and valence to iron (Fe^{+3}), allowing it to compete with Fe^{3+} for binding to iron-requiring enzymes, proteins, and microbial siderophores. Consequently, gallium could act as an inhibitor to these bacterial components upon binding because it cannot undergo oxidation–reduction cycles (286). Several *in vitro and in vivo* studies have reported promising results using gallium treatment alone or in combination with colistin against *A. baumannii* isolates (287, 288). Nevertheless, similar results to those observed with iron chelators were obtained, depending the effect of gallium nitrate on *A. baumannii* growth on the growth medium used (287). In summary, iron chelation and gallium-based therapies show good activity *in vitro,* but still additional work is needed to learn about their *in vivo* efficacy.

3.2.3. Repurposing drugs.

The repurposing of old U.S. Food and Drug Administration (FDA)-approved drugs as therapeutic alternatives for infections has recently gained interest. The advantages of this strategy are the availability of information about their pharmacological characteristics in preclinical and clinical trials, and consequently, the reduction of time, cost and risk required to evaluate these drugs for other therapeutic applications, such as the treatment of bacterial infections. A variety of drug families have been considered as potential candidates including: anthelmintics (salicylanilides and avermectins) (289, 290); antifungals (ciclopirox) (291), anticancer drugs (gallium nitrate and mitomycin C) (288, 292); anti-inflammatory/immunomodulatory drugs (celecoxib and glatiramer) (293, 294); and statins (simvastatin and atorvastatin) (295). Even though repurposed drugs showed promising preclinical data, its clinical use to combat infections caused by MDR *A. baumannii* could become a reality after performing further safety testing in a broader human population (296).

3.2.4. Antimicrobial peptides.

Antimicrobial peptides are key elements of the immune system of higher organisms, making them potential candidates for use as antibacterial agents (297). Most of these antimicrobial peptides share a cationic character and fold into amphipathic conformations that allow them to disrupt bacterial membranes, acting as direct microbicidal agents (298). Several antibacterial peptides have shown promising results *in vitro* and *in vivo* against *A. baumannii*, such as cecropin A–melittin hybrid peptides (299, 300), peptides derived from frog and toad skin (301, 302), and proline-rich antimicrobial peptides (303, 304). However, the failures of antimicrobial peptides in clinical development are countless, and experts have begun to doubt whether antimicrobial peptides will be viable therapeutic options for systemic infections (in contrast to topical applications (305)) without substantial further scientific advancements (306). Before peptide therapies can be reliably used systemically, they must overcome problematic pharmacology, toxicity, and inactivation in the context of biological matrices such as serum, surfactant, and other biological fluids and tissues (7). Studies aimed at developing peptide analogs through the introduction of structural changes that modify the stability and toxicity profiles of the peptide are addressing these limitations (7).

3.2.5. Phage therapy.

Bacteriophages, or phages, are viruses that infect, and in some cases lyse, bacterial cells. Recently, a substantial renewed interest in their potential has being witnessed (307, 308). Moreover, its use is supposed to be advantageous over antibiotics, due to phages are highly specific and have no effect against animal cells, and in addition, their isolation is a relatively rapid process (309). Numerous *in vitro* and *in vivo* studies have confirmed the lytic activity of phages against *A. baumannii* clinical isolates (310-316). While these studies illustrate that a potential limitation of phage therapy is the limited host range of *A. baumannii* phages, new studies overcome the limitations of each individual phage using a cocktail of multiple phages (317-320). Moreover, of particular relevance is the recent study of Schooley RT *et al.* (321) reporting the successful use of personalized bacteriophage-based therapeutic cocktails to treat a 68-year-old diabetic patient with necrotizing pancreatitis complicated by an MDR *A. baumannii* infection. Nevertheless, there are still some issues, such as the potential for secondary effects on human flora, appearance of resistant strains, and the inflammatory response in humans after administration of phage therapy, that need to be addressed (322).

3.2.6. Photodynamic therapy.

Phototherapy utilizes the combination of oxygen, infrared light, and a photosensitizer (a nontoxic, photoreactive dye) to generate reactive oxygen species that can damage DNA and disrupt cellular membranes. It was developed a long time ago to be used for other purposes such as cancer or ophthalmology, and for disinfection of clinical products (323). Nowadays, antimicrobial photodynamic therapy is starting to be considered as a promising alternative approach to resistant infections due to its advantages: it has been shown to destroy LPS (324), and it does not result in the selection of resistant strains after repeated cycles of treatment (325). However, potential limitations of photodynamic therapy are that it can only be used topically, and that the reactive oxygen species produced by this technique could potentially damage host as well as target cells; issues that must be taken into account when considering this treatment approach (322). Nonetheless, it appears to be suit for the treatment of superficial skin and soft tissue infections (326).

3.2.7. Immunotherapy.

The ability to modulate immune responses, by either suppression or enhancement depending on the need, has proved to be a useful therapeutic strategy in many contexts, including the prevention and treatment of infections (327). In addition, immunomodulation offers the advantage of avoiding selective pressure for the evolution of microbial resistance, by targeting the host rather than the pathogen (328). The novel immunomodulatory treatments that are under study as anti-infectives are largely designed as adjunctive therapies to support and extend the effectiveness of antibiotics and antivirals. For example, lysophosphatidylcholine (LPC), a major component of phospholipids in eukaryotic cells that is implicated in immune cell recruitment and modulation (329), has been demonstrated to be efficacious as a pre-emptive treatment alone and in combination with colistin, tigecycline, or imipenem treatment in experimental murine models of peritoneal sepsis and pneumonia caused by *A. baumannii* (330, 331). Vaccination can also be considered as a form of immunomodulatory therapy and is one of the most successful and cost-effective forms of medical intervention for the prevention of infectious diseases (332). Numerous studies have demonstrated the *in vivo* efficacy of different vaccines against *A. baumannii* infection and colonisation (333-340). Similar results were obtained in *in vivo* studies with passive immunization with antisera from immunized mice or monoclonal antibodies (341-344).

Therefore, immunotherapy represents an approach that could be highly effective for preventing and treating the most common and serious infections caused by *A. baumannii*. However, it presents some challenges, as targeting population selection, vaccine administration, antigen identification and control of the stimulation of protective immunity without an increase in systemic pro-inflammatory responses (345).

3. Omics techniques used to identify novel drug targets.

In the present post-genomics era, the possibilities of selecting targets using computational approaches with integrated omics data, such as genomics, transcriptomics, proteomics, and metabolomics (Figure 3) have been increasing continuously. As previously described, the discovery of new virulence factors of *A. baumannii* is making possible the

development of novel anti-virulence drugs or vaccines, which are promising alternatives to the classical antimicrobial approach.

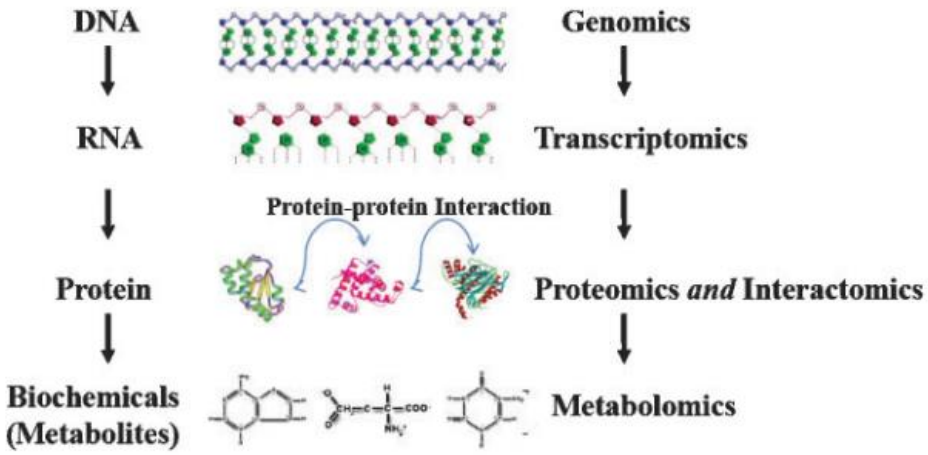

Figure 3. Schematic diagram of various omics technologies targeting different layers of cellular information (346).

In silico methods like comparative and subtractive genomics are being widely used for the prediction and identification of potential drug targets in numerous pathogenic bacteria (113, 347). Those methods relies on multiple approaches, being some of the main ones: a) genome comparison of strains with different virulence profile (54); b) comparisons between the genomic sequences of the pathogen with the host to include the protein-coding genes sequences that are absent in the host (non-homologous) and indispensable for pathogen survival (348-351); c) *in vivo* strain dominance using StrainSeq approach (352); d) search of orthologous proteins of a given virulence factor in other pathogenic bacteria (353); e) pangenome and coregenome analysis of all available genomes for detecting potential virulence factors (354).

Similarly, several transcriptomic studies have provided relevant information about *A. baumannii* virulence (17, 355). In fact, even *in vivo* studies have been performed using this type of techniques, describing the global gene expression profile of *A. baumannii* during infection and detecting potential therapeutic targets such as several iron uptake genes (356, 357).

Furthermore, proteomic techniques are being used following different approaches: a) proteome comparison of strains with different virulence potential (358, 359); b) comparison of the *ex vivo* proteomes of *A. baumannii* grown under conditions that simulate those found in the airways (360); c) proteome analysis under iron-limiting stage (361); d) subtractive proteomics to identify novel drug targets and reverse vaccinology (362).

Regarding the metabolomic techniques, a combination of a genome-scale metabolic network analysis and essentiality analyses was used for identifying drug targets candidates of the MDR *A. baumannii* AYE (363). Besides, Yeom *et al.* followed another metabolomic approach based on a comparison between planktonic and biofilm cells of *A. baumannii* 1656-2 (364).

Lastly, all these omics techniques are also being used in combination. For example, a combinatorial approach of pan-genomics, core genomics, proteomics and reverse vaccinology was used to find out potential vaccine candidates (365). Besides being used to discover new drug targets, omics analyses also provide information on the molecular mechanisms leading to antimicrobial resistance in *A. baumannii* (366-370).

Nevertheless, as these omics techniques are advancing and gaining accuracy, previous results are sometimes modified, which should be taken into account. For example, the complete genome sequences of *A. baumannii* strains AB307-0294 and ACICU, originally determined using 454 pyrosequencing technology, were re-determined by Hamidian *et al.* using Illumina MiSeq and MinION (Oxford Nanopore Technologies) technologies and a hybrid assembly generated using Unicycler (371). After comparing the resulting new high-quality genomes with the earlier 454-sequenced versions, a large number of nucleotide differences affecting protein coding sequence features were identified and corrected.

FUNDAMENTS

A. baumannii is a nosocomial pathogen that colonizes and infects, fundamentally, immunocompromised patients, with invasive devices and prior antibiotic therapy. Pneumonia especially that associated with mechanical ventilation, and bacteraemia especially that associated with respiratory focus, are the most frequent infections caused by microorganism.

In a significant number of clinical cases, colonisation occurs without developing infection, but the clinical relevance of respiratory colonisation by *A. baumannii* is still unknown. Moreover, the bacterial concentration of *A. baumannii* in respiratory samples, one of the parameters that is used to diagnose VAP, has recently reported to not allow *per se* discriminating between colonisation and VAP. For this reason, it is a challenge to try to differentiate colonisation from infection, especially in the critical care setting. Hence, it would be useful to study if there is any bacterial factor involved in it.

On the other hand, there are controversial results regarding the attributable mortality of VAP and bacteraemia caused by *A. baumannii*. Although the factors that have been most frequently associated with the development of clinical infection and mortality in *A. baumannii* infections are those related to the host (weakened baseline situation and clinical severity), recent studies suggest that differences in virulence factors among clinical isolates may explain the mixed results in terms of mortality of the different series. However, there are few data available regarding the relationship between virulence factors and clinical outcomes.

In addition, the increasing problem of multidrug resistance is not followed by the development of novel antimicrobials, so there is an important need to develop new strategies to fight against MDR and PDR *A. baumannii*. A new alternative to combat this kind of infections might be blocking specific bacterial virulence factors that bacteria need to infect. So the knowledge about virulence factors of MDR *A. baumannii* isolates that could explain the different clinical outcomes could also be used to design in the future novel inhibitors able to block them and fight against *A. baumannii* infections.

HYPOTHESES

The hypotheses of this Doctoral Thesis are:

Chapter I. Study of the mechanisms involved in the pathogenesis of colonising and bacteraemic VAP producing *A. baumannii* clinical isolates.

1. *A. baumannii* isolates causing bacteraemic VAP (invasive isolates) in ICU ventilated patients present higher *in vitro* and *in vivo* virulence than colonising *A. baumannii* isolates.

2. *A. baumannii* isolates that cause bacteraemic VAP in ICU ventilated patients have different virulence mechanisms than the isolates that only colonize the respiratory tract of those patients.

Chapter II. Identification of potential *A. baumannii* virulence factors of bacteraemic *A. baumannii* isolates recovered from clinically homogeneous adult patients with radically different clinical outcomes.

1. A clinically homogeneous group of patients with VAP and secondary bacteraemia caused by *A. baumannii* have different clinical outcome depending on the differential virulence factors of its bacteraemic isolates.

2. The presence of CarO increase the virulence of *A. baumannii*.

OBJECTIVES

The general aim of this Doctoral Thesis is to identify novel therapeutic targets in *A. baumannii* by finding new factors responsible for its virulence, through omics and bioinformatics tools.

The specific objectives of each chapter are the following:

Objectives of chapter I. Study of the mechanisms involved in the pathogenesis of colonising and bacteraemic VAP producing *A. baumannii* clinical isolates.

1. To evaluate the clonal relationship, *in vitro* and *in vivo* virulence, and antimicrobial susceptibility patterns of colonising and invasive isolates.
2. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the colonising and invasive isolates previously selected.
3. To find new mechanisms of infection by comparing colonising and invasive isolates.

Objectives of chapter II. Identification of potential *A. baumannii* virulence factors of bacteraemic *A. baumannii* isolates recovered from clinically homogeneous adult patients with radically different clinical outcomes.

1. To study the clonal relationship and antimicrobial susceptibility patterns in the selected bacteraemic *A. baumannii* isolates.
2. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the bacteraemic *A. baumannii* isolates.
3. To find new bacterial virulence mechanisms that may be influencing patient mortality: genomic, transcriptomic, and proteomic approaches.
4. To analyse the virulence role of CarO in *A. baumannii* using model systems.

MATERIALS AND METHODS

Chapter I. Study of the mechanisms involved in the pathogenesis of colonising and bacteraemic VAP producing *A. baumannii* clinical isolates.

Objective 1. To select colonising and invasive isolates according to specific criteria.

1.1. Patient cohort.

A previously described prospective, observational cohort of adult patients admitted to the ICUs of the University Hospital Virgen del Rocío (February 2010-June 2011) (106) was analysed in this study. This cohort consisted of one hundred patients on mechanical ventilation for more than 48 h and with at least one culture of TBA with *A. baumannii* isolation. TBA cultures were performed every three days while intubated until hospital discharge, death or 30 days, which ever occurred first. Each case was classified according to the clinical pulmonary infection score (CPIS) (372), as *A. baumannii* lower airway colonisation (CPIS < 6) or VAP (CPIS ≥ 6) and with an *A. baumannii* isolate recovered from TBA at 72 h before or after the diagnosis date (Table 3). Blood cultures were withdrawn when clinically indicated, to detect the presence of *A. baumannii* bacteraemia concomitant to the VAP diagnosis. Thus, 43 patients remained just colonised, 57 suffered VAP, and 15 VAP patients also developed bacteraemia. Length of the ICU stay, 30-days mortality, and clinical features including antibiotic therapy were recorded. ICU stay was measured from the first isolation of *A. baumannii*. Mortality was defined as death from any cause within 30 days after the onset of pneumonia.

Table 3. Clinical Pulmonary Infection Score (CPIS) used for the diagnosis of ventilator-associated pneumonia (372).

1. Temperature (°C)
≥ 36.5 and ≤ 38.4 = 0 point
≥ 38.5 and ≤ 38.9 = 1 point
≥ 39 or ≤ 36.0 = 2 point
2. Blood leukocytes (per mL)
≥ 4,000 and ≤ 11,000 = 0 point
< 4,000 or > 11,000 = 1 point
< 4,000 or > 11,000 + band forms ≥ 500 = 2 points
3. Tracheal secretions
Absence = 0 point
Non-purulent = 1 point
Purulent = 2 points
4. Oxygenation (Pa_{O2}/Fi_{O2}, mm Hg)
> 240 or ARDS = 0 point
≤ 240 and no evidence of ARDS = 2 points
5. Pulmonary radiography
No infiltrate = 0 point
Diffused (or patchy) infiltrate = 1 points
Localized infiltrate = 2 points
6. Culture of TBA
Pathogenic bacteria cultured ≤ 5 $\log_{10}$ CFU/mL = 0 point
Pathogenic bacteria cultured ≥ 6 $\log_{10}$ CFU/mL = 1 point
Pathogenic bacteria cultured ≥ 6 $\log_{10}$ CFU/mL and same bacteria on the Gram stain= 2 point

Total points = CPIS (varies from 0 to 12 points). ARDS: adult respiratory distress syndrome. TBA: tracheobronchial aspirate.

1.2. Selection criteria for colonising and invasive isolates.

The following criteria were used to select colonising and bacteraemic VAP-producing (since now named as invasive) *A. baumannii* isolates, obtained from TBA samples of patients from the previous described cohort. For colonising isolates: i) isolation from patients with no infection by *A. baumannii*, only colonisation; ii) pure colonising isolates (no isolation of another microorganism); and iii) inoculum > 6 $\log_{10}$ CFU/mL. In the case of invasive isolates: i) isolation from patients without previous colonisation by *A. baumannii*; ii) bacteraemia close to the pneumonia starting day; iii) pure invasive isolates; and iv) lowest inoculum possible but always ≥ 6 $\log_{10}$ CFU/mL. The threshold of 6 $\log_{10}$ CFU/mL is considered as the standard for the diagnosis of respiratory infection, considering infection an inoculum ≥ 6 $\log_{10}$ CFU/mL (82). However, in this cohort 43 patients remained exclusively colonised, despite having a TBA inoculum ≥ 6 $\log_{10}$ CFU/mL.

Moreover, the *A. baumannii* isolates recovered from blood samples of patients from which the selected invasive isolates belonged were also used for the pulsed-field gel electrophoresis (PFGE) studies to "confirmed" the secondary bacteraemia.

Objective 2. To evaluate the clonal relationship and *in vitro* virulence of colonising and invasive isolates. Selection of isolates for further studies.

2.1. PFGE typing.

The 16 colonising (C1-C16) and 7 invasive (IB1, IB2, IB3, IB4, IB6, IB7, and IB8) *A. baumannii* isolates from TBA samples, and the corresponding 7 *A. baumannii* isolates from blood cultures of the same patients as invasive isolates (HC1, HC2, HC3, HC4, HC6, HC7, and HC8) were typed by PFGE following the technique described by Seifert *et al.* with minor modifications (373). Briefly, each isolate was plated on sheep blood agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and incubated 18 h at 37 °C. The *A. baumannii* strain American Type Culture Collection (ATCC) 19606 was also plated and incubated, to allow later normalization of the electrophoretic patterns across the gel. Then, bacterial suspensions were performed using 2 mL of suspension buffer (100 mM Tris (Sigma-Aldrich, St. Louis, MO, USA); 100 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), pH 8.0), and then adjusted to

an optical density (OD) at 620 nm of 0.85-0.90 (9 $\log_{10}$ CFU/mL approximately). Afterwards, 0.5 mL of the adjusted bacterial suspension were incubated 10 min at 55 °C, and 25 µL of 20 mg/mL proteinase K (Roche, Madrid, Spain) were added. Plugs were formed by mixing the previous 0.5 mL of bacterial suspension with 0.5 mL of agarose solution (1% agarose (Pronadisa, Madrid, Spain) in TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0) with 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich)), and this mixture was then pipetted into plug molds (Bio-Rad Laboratories, Alcobendas, Spain). After the plugs solidified, each one was transferred to 5 mL of cell lysis buffer (50 mM Tris; 50 mM EDTA, pH 8; 1% sarcosine (Sigma-Aldrich); 25 µL of 20 mg/mL proteinase K) and were incubated at 55 °C in a water bath for 2 h with constant and vigorous agitation (150-200 rpm). After lysis, plugs were washed five times (15 min/wash) at 55 °C (twice with sterile ultrapure water and thrice with TE buffer; 10 mL for each washing step) in a shacking water bath. A slice from each plug (2/3 approximately) was cut and incubated at 25 °C for 15-20 min after adding 200 µL of the 1X restriction buffer provided with the enzyme (see below) and 100 µg/mL of bovine serum albumin (BSA) (New England Biolabs, Barcelona, Spain). Then, the restriction buffer was removed and replaced with 200 µl of restriction buffer with BSA containing 30 U of ApaI (New England Biolabs), and plugs slices were incubated at 25 °C for 4 h. Prior to casting of the gel, the restriction mixture was removed and replaced with 200 µl of 0.5X TBE (Roche). Plug slices were then loaded into the appropriate wells of a 1% agarose gel performed with Pulsed Field Certified Agarose (Bio-Rad Laboratories) and 0.5X TBE (Roche), and kept at 4 °C for 5 min. The wells were made by using a 15-slot comb, with each slot being 1.5 mm thick, and the *A. baumannii* strain ATCC 19606 was placed in the first and last wells of each gel. Electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF-DR II, Bio-Rad Laboratories) with the following conditions: running temperature of 14 °C, total run time of 19 h with switch times ranging from 5 to 20 s and linear ramping, and voltage for the run of 6 V/cm. The gels were stained for 30 min with 300 mL of ethidium bromide solution (1 µg/mL) (Promega, Alcobendas, Spain) and destained for 15 min in distilled water with gentle shaking. The gels were observed under UV illumination and photographed by the imaging system Gel Logic 200 (Kodak, Madrid, Spain). Digital images were stored electronically as TIFF files with an overall resolution of approximately 1,000 pixels per lane. PFGE-generated DNA profiles were entered into the Fingerprinting II v3.0 software (Bio-Rad Laboratories) using the band-based Dice coefficient and 0.5% of position tolerance. The PFGE profile of the *A. baumannii* strain

ATCC 19606 was used for normalization and a dendogram was created. Isolates that clustered together with a similarity > 85% were considered to belong to the same pulsotype (42, 374). Invasive isolates from TBA samples were also compared to bacteraemic isolates using a 100% PFGE cut-off level, in order to confirm that there was a high probability that bacteraemia was secondary to VAP in those patients, and therefore caused by the same *A. baumannii* strain.

2.2. *In vitro* virulence study.

2.2.1. Human lung epithelial cell cultures.

Human lung epithelial cells, line A549 (ATCC CCL-185), were grown in Dulbecco's modified Eagle's medium (DMEM, GE Healthcare Life Sciences, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Madrid, Spain), 1% HEPES (Sigma-Aldrich), vancomycin (50 µg/mL; Laboratorios Normon, Madrid, Spain), gentamicin (20 µg/mL; Laboratorios Normon) and amphotericin B (0.25 µg/mL; Gibco) in a humidified incubator at 37 ºC and 5% CO_2 (375). A549 cells were routinely passaged every 3-4 days, and for the cellular viability assays, they were seeded 24 h in 96-well plates (5×10^4 cells/well).

2.2.2. Cell viability assay.

The viability of A549 cells after infection with colonising or invasive isolates was studied using the MTT assay (Sigma-Aldrich). The MTT colorimetric assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilized and the resulting coloured solution is quantified using a scanning multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells, serving as an indirect measurement of cell growth/cell kill (376).

Following the MTT protocol, first, the 16 colonising (C1-C16) and 6 invasive (IB1, IB2, IB3, IB4, IB7, and IB8) isolates selected after PFGE analyses were grown overnight in 10 mL Mueller-Hinton broth (MHB, Sigma-Aldrich) at 37 ºC and 180 rpm. Then, cultured

bacterial cells were collected by centrifugation (15 min at 4,500 × g), rinsed in sterilized phosphate-buffered saline (PBS, Sigma-Aldrich), and resuspended in 10 mL DMEM before use in eukaryotic cell infection assays. A549 cells seeded in 96-well plates, as explained above, were rinsed twice with PBS immediately before infection, and infected with 8 $\log_{10}$ CFU/mL of the overnight culture resuspended in DMEM of each isolate (1 column of wells/isolate). A549 cells from a column of wells per each plate were not infected, serving as control for later analysis (healthy cells with 100% viability). Cell cultures were incubated for 24 h in a humidified incubator at 37 ºC and 5% CO_2. After the incubation period, the cells were washed 5 times with PBS, and 100 µl of a 1:10 dilution of MTT 5 mg/mL (final concentration 0.5 mg/mL) were added to each well. Multi-well plates were incubated for 3 h in a humidified incubator at 37 ºC and 5% CO_2. Then, the medium was removed from each well and replaced with 100 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich). Cell cultures were incubated 10 min in a humidified incubator at 37 ºC and 5% CO_2, and afterwards, the absorbance of each well was measured using a scanning multi-well spectrophotometer (Multiskan GO, Thermo Fisher Scientific) at 570 nm with a reference wavelength of 620 nm. An average of the absorbances was performed per each column. To calculate the percentage of A549 cell viability after incubation with the clinical isolates, the following formula was used:

$$A549\ cell\ viability\ (\%) = \frac{(OD570-OD620)A549\ cells\ infected\ with\ a\ specific\ isolate}{(OD570-OD620)healthy\ control\ A549\ cells}\ x\ 100$$

Assays were performed by triplicate in different days. The A549 cell viability (%) average of the 3 days for each isolate were represented on a graph, and also the average for colonising and invasive groups after the final selection (colonising isolates with the lowest *in vitro* virulence and invasive isolates with the highest).

Objective 3. To study the *in vivo* virulence and antimicrobial susceptibility patterns of colonising and invasive isolates.

3.1. *In vivo* virulence study.

3.1.1. Animals.

Immunocompetent adult C57BL/6J female mice (20 g, assessed for genetic authenticity and murine pathogen-free sanitary status) were obtained from the Production and Experimentation Animal Centre, University of Seville, Spain. Mice were housed in ventilated cages under specific pathogen-free conditions with water and food *ad libitum*. All procedures were performed following the recommendations from the Guide for the Care and Use of Laboratory Animals (377) in strict accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. Experiments were approved by the Committee on the Ethics of Animal Experiments of the University Hospital of Virgen del Rocío, Seville, Spain, and from the Ministry of *Agricultura, Pesca y Desarrollo Rural* (2012PI/246). Procedures were performed with all possible efforts made to minimize animal suffering.

3.1.2. Experimental murine model of peritoneal sepsis: determination of LD_0, LD_{50}, and LD_{100}.

The *in vivo* virulence of the selected *A. baumannii* clinical isolates, 5 colonising (C6, C7, C8, C11, and C12) and 6 invasive (IB1, IB2, IB3, IB4, IB7, and IB8), was assessed in a non-discriminative peritoneal sepsis murine model (330) measuring survival and calculating LD_0, LD_{50}, and LD_{100} (378). The LD_0 is the highest concentration of bacteria which allows 100% of the animals to survive. The LD_{50} is the dose of bacteria required to cause 50% mortality in the animals. The lowest concentration of bacteria at which 100% of inoculated animals die is the LD_{100} or minimal lethal dose (MLD). Briefly, for each isolate, bacterial cultures were serially diluted in MHB to obtain 10-fold decreases in the number of organisms. Groups of 6 animals were inoculated intraperitoneally (i.p.) with 0.5 mL of bacterial dilutions mixed 1:1 with a saline solution of porcine stomach mucin (type II, Sigma-Aldrich) at 10% (w/v), starting from an inoculum of 1.5 $\log_{10}$ CFU/mL approximately and ending with the first inoculum that cause 100% mice mortality (achievement of MLD). The animals were observed for 7 days, and the number of survivors at each dilution was noted.

3.2. Determination of the MIC of different antimicrobials.

The determination of the MIC of amikacin, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, rifampicin, sulbactam, tigecycline, and tobramycin was performed for the 5 colonising (C6, C7, C8, C11, and C12) and 6 invasive (IB1, IB2, IB3, IB4, IB7, and IB8) *A. baumannii* isolates by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (379). For this, stock solutions of antimicrobial agents were prepared using the standard laboratory powders (Table 4) and following the CLSI standards. Subsequently, 50 µl of MHB II (cation-adjusted, Sigma-Aldrich) were added to 96-well microtitre plates "U" form, and then, 50 µl of each antimicrobial solution were added to the first column of wells. Next, 1:2 serial dilutions were performed starting from the first column. Thus, 10 wells per antimicrobial, containing decreasing antimicrobial concentrations with different ranges of concentrations, were required (Table 4). Finally, 50 µl of each of the selected *A. baumannii* isolates, obtained from a MHB II culture in exponential growth phase equivalent to 0.5 McFarland standard and diluted 1:100, were added (final concentration of 5×10^5 CFU/mL). A growth control with bacterial inoculum but without antimicrobial agent, and a sterility control without bacterial inoculum or antimicrobial agent, were performed. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control (QC) strains, according to the CLSI guidelines. Microtitre plates were incubated at 37 ºC and aerobic conditions during 20-24 h. After incubation period, the CMI of each antimicrobial agent for each isolate were determined, defining CMI as the lowest concentration of an antimicrobial which prevents visible bacterial growth. MICs results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/) or CLSI (380) breakpoints when available. As tigecycline and rifampicin clinical breakpoints were not defined by EUCAST or CLSI, they were interpreted according to the FDA criteria (381) and PK/PD models with Monte Carlo simulation (382), respectively. Clinical isolates were defined as MDR when they had acquired non-susceptibility to at least one agent in three or more antimicrobial categories according to standard criteria (383). Experiments were performed in triplicate on different days.

Table 4. Antimicrobial brands and their concentration range used for the determination of their minimum inhibitory concentrations by broth microdilution method for colonising and invasive isolates.

	Antimicrobial brand	Antimicrobial concentration range tested (µg/mL)
Amikacin	Amikacin sulfate salt, Sigma-Aldrich	128-0.25
Ceftazidime	Ceftazidime hydrate, Sigma-Aldrich	256-0.5
Ciprofloxacin	Ciprofloxacin, Sigma-Aldrich	256-0.06 (2 consecutive microtitre plates)
Colistin	Colistin sulfate salt, Sigma-Aldrich	16-0.03
Imipenem	Imipenem monohydrate, Sigma-Aldrich	256-0.5
Meropenem	Meropenem trihydrate, Sigma-Aldrich	16-0.03
Rifampicin	Rifampicin, Sigma-Aldrich	256-0.5
Sulbactam	Sulbactam, Sigma-Aldrich	256-0.5
Tigecycline	Tigecycline, Sequoia Research (Pangbourne, UK)	8-0.016
Tobramycin	Tobramycin, Sigma-Aldrich	128-0.25

Objective 4. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the colonising and invasive isolates.

4.1. DNA extraction, WGS and data processing (read preprocessing, assembly, contig filtering, protein-coding gene prediction and annotation).

DNA of the 5 colonising (C6, C7, C8, C11, and C12) and 6 invasive (IB1, IB2, IB3, IB4, IB7, and IB8) isolates was extracted using QIAamp® DNA Mini Kit (QIAGEN, Madrid, Spain). Sequencing was performed using MiSeq platform (Illumina, Madrid, Spain), according to the standard protocol for WGS paired-end, producing 2 × 300 base pairs (bp) fragment reads. Regarding the bioinformatics analyses of the WGS data, first, reads were

preprocessed with Trimmomatic v0.32 (384) and *de novo* assembled with ABySS assembler v1.5.2 (385). Then, the assemblies were filtered for contigs larger than 500 bp. Gene prediction and annotation were performed utilizing GeneMark v2.5 (386) and RAST server v1.0 (387), respectively.

4.2. MLST typing.

MLST was performed by uploading the contig files obtained from the *de novo* assembly of the WGS data of each of the colonising and invasive isolates to the MLST web server MLST v2.0 from the Center for Genomic Epidemiology (CGE) (388). Both MLST schemes, Bartual/Oxford and Pasteur, were used.

4.3. Identification of antimicrobial resistance mechanisms.

The ResFinder v3.2 web server of the CGE (389) was used to identify antimicrobial resistance genes in the assembled WGS data from the colonising and invasive isolates, using a threshold of 100% identity for the genes encoding β-lactamases and 98% identity for all other genes. Mobile IS located upstream the β-lactamase-encoding genes were detected using the Basic Local Alignment Search Tool (BLAST) (390) for nucleotides (BLASTN) from the ISFinder database (https://isfinder.biotoul.fr/blast.php). Furthermore, point mutations in the quinolone resistance determining regions (QRDRs) of the genes associated with quinolone resistance (*gyrA* and *parC*) were identified in all the isolates comparing the predicted proteins encoded by *gyrA* and *parC* with the deduced amino acid sequences of *gyrA* (UniProt entry: D0CBH9) and *parC* (UniProt entry: D0CB90) from *A. baumannii* ATCC 19606 (ciprofloxacin-susceptible strain), and also with those from *E. coli* K12 (UniProt entries: P0AES4 and P0AFI2, for the deduced amino acid sequences of *gyrA* and *parC*, respectively) in order to define the position of the mutations according to the *E. coli* protein. The BLASTP (protein BLAST) tool from the National Center for Biotechnology Information (NCBI) was used for the performance of the comparisons. Similarly, point mutations in the conserved regions of *rpoB* gene, related with rifampicin resistance, was detected following the same procedure described for *gyrA* and *parC*, using the deduced amino acid sequences of *rpoB* (UniProt entry: A3M1G3) from *A. baumannii* ATCC 17978 (rifampicin-susceptible strain). Identification

of mutations in *pmrAB* and *lpx* genes was not performed due to all isolates were colistin-susceptible.

4.4. Identification of virulence factors.

Genes encoding known virulence factors were identified from the WGS data of the colonising and invasive isolates using two different strategies. For both strategies, virulence genes described in the MDR *A. baumannii* isolate AB0057 were used for the comparison, being the *A. baumannii* isolate with the higher number of virulence genes contained in the Virulence Factors of Pathogenic Bacteria database (VFDB) (391). The first strategy was based on the VFanalyzer option of the VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) (392). VFanalyzer is an automatic analysis pipeline for a systematic screen of known/potential virulence factors in given complete/draft bacterial genomes. So, draft genomes (contig files) were uploaded and Option 3 was selected ("Upload private data of raw or annotated genome in FASTA or GenBank format"). VFanalyzer performed an automatic prediction of protein-coding genes using GLIMMER3 and then ran the comparison analysis. For the second strategy, amino acid sequences of all the virulence factors of *A. baumannii* isolate AB0057 contained in VFDB were downloaded, and the inferred protein sequences from the six bacteraemic isolates were compared to them using the standalone version of BLASTP from the NCBI. A protein was related to a virulence factor present in the database when the BLAST hit indicated an identity ≥ 95% for a query coverage ≥ 95%. Thus, using both strategies, the ability to detect all virulence genes were higher than using one alone.

Objective 5. To find new mechanisms of infection by comparing colonising and invasive isolates.

5.1. Comparative genomic study.

Several bioinformatics approaches were used with WGS data to find any genomic difference between both groups of sequenced isolates (colonising and invasive).

In the first approach, performed with the nucleotide sequences (gene-level analysis), a multiple alignment with the 11 assembled genomes were performed using Mugsy v1.2.3 (393). Then, from this multiple alignment, genomic regions (contigs) that were presented in all colonising isolates but in none of the invasive isolates, and vice versa, were selected. Afterward, genes contained in those contigs were predicted and annotated with GeneMark v2.5 and RAST server v1.0, respectively. Moreover, the core- (common genes (≥ 90% identity) among all the isolates) and pan-genome (the entire gene set of all isolates: common + non-common genes) were identified from the multiple alignment of all isolates, and an additional analysis of point mutations in the core-genome was carried out comparing colonising *vs.* invasive isolates, and vice versa.

At the same time, a second approach was applied using the predicted amino acid sequences (protein-level analysis). All the predicted proteins, from all isolates, were compared using a global alignment based on the Needleman-Wunsch algorithm (https://www.ebi.ac.uk/Tools/psa/) in order to find the differential ones between both groups of isolates. Then, the predicted core-proteome (predicted proteins presented in all isolates with an identity ≥ 90%) was selected and predicted-protein variants between the colonising and the invasive groups were obtained.

5.2. Determination of *ompA* expression levels by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

The 5 colonising (C6, C7, C8, C11, and C12) and 6 invasive (IB1, IB2, IB3, IB4, IB7, and IB8) isolates were analysed for the expression of the ompA gene. Cultures were grown to an OD_{600} of 0.6 in Luria-Bertani (LB) broth (Sigma-Aldrich) and subsequently, the protocol of TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific) was followed to extract DNase-treated bacterial RNA from them. qRT-PCR was carried out with an MX3000P system (Stratagene, Barcelona, Spain). A total of 35 ng of RNA was used in the target gene studies. The primers and TaqMan probes (Thermo Fisher Scientific) used were: ompA forward: 5'-AGCTCTTGCTGGCTTAAACG-3'; ompA reverse: 5'-GAGCAACTGGAGTTGGTTCA-3'; ribosomal RNA forward: 5'-GTAGCGGTGAAATGCGTAGA-3'; ribosomal RNA reverse: 5'-CTTTCGTACCTCAGCGTCAG-3'; ompA probe: 5'-[DFAM]CAGCAGGCTTCAAGTGACCACCA[DTAM]-3'; and ribosomal RNA probe:

5'-[DFAM]CGAAGGCAGCCATCTGGCCT[DTAM]-3' (147). The experiment was performed in triplicate using independent RNA extractions in every assay, and the absence of contaminating DNA in the samples was confirmed by the use of controls without reverse-transcriptase. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. The 16S ribosomal RNA gene was used as housekeeping gene for normalization of gene expression (394) and *A. baumannii* ATCC 17978 was used as calibrator or reference sample (expression set to 1).

Statistical analyses of Chapter I

Differences in cell viability, LD, and *ompA* expression levels (Sections 2.2.2, 3.1.2, and 5.2 of Material and Methods from Chapter I, respectively) were determined using GraphPad Prism 6 (GraphPad Software Inc., California, USA). Specifically, Student's t-test and Mann-Whitney test were used when comparing two groups in which the dependent variable was normally and not normally distributed, respectively. A *P*-value of ≤ 0.05 was considered statistically significant. In addition, LD_{50} values for the isolates used were determined using the Probit method in IBM SPSS Statistics v17.0 (IBM, Madrid, Spain).

Chapter II. Identification of potential *A. baumannii* virulence factors of bacteraemic *A. baumannii* isolates recovered from clinically homogeneous adult patients with radically different clinical outcomes.

Objective 1. To select bacteraemic isolates according to specific criteria.

1.1. Selection criteria.

The following criteria were used to select bacteraemic *A. baumannii* isolates from patients from the previous described cohort: i) isolation from patients without comorbidities (Charlson index = 0), with APACHE II score ≥ 15 and similar CPIS in the moment of inclusion, similar ages, and no co-infections during their hospital stay, but with different clinical outcomes; ii) appropriate antimicrobial treatment of the VAP (106), being the isolates extremely susceptible to colistin (MIC range: 0.03-0.125 μg/mL) and lacking colistin-heteroresistance; and iii) same PFGE profile as their corresponding VAP isolates, confirming the source of bacteraemia.

Objective 2. To study the clonal relationship and antimicrobial susceptibility patterns in the bacteraemic *A. baumannii* isolates.

2.1. PFGE typing.

Molecular typing of the six bacteraemic isolates, using the PFGE method, was performed following the same protocol previously detailed in Section 2.1 of Materials and Methods (Chapter I).

2.2. Determination of the MIC of different antimicrobials.

Identical procedure as detailed before in Section 3.2 of Materials and Methods (Chapter I) was used to determine the MIC of amikacin, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, rifampicin, sulbactam, tigecycline, and tobramycin for the six bacteraemic isolates. Additionally, doripenem (doripenem hydrate, Sigma-Aldrich; range tested: 8-0.016 μg/mL) and doxycycline (doxycycline hyclate, Sigma-Aldrich; range tested: 128-0.25 μg/mL) susceptibilities were also determined.

Objective 3. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the bacteraemic *A. baumannii* isolates.

3.1. DNA extraction, WGS and data processing (read preprocessing, assembly, contig filtering, protein-coding gene prediction and annotation).

DNA of the six bacteraemic isolates was extracted using QIAamp® DNA Mini Kit (QIAGEN). Sequencing was performed using MiSeq platform (Illumina), according to the standard protocol for WGS paired-end, producing 2 × 300 bp fragment reads.

After comparing several bioinformatics tools, newer and more accurate bioinformatics analyses of the WGS data were performed in comparison with those performed with the clinical isolates from Chapter I. After carrying out a result comparison among different types of read trimming and *de novo* assemblers, such as ABySS, Velvet (395) and SPAdes (396) (results not shown), reads were directly *de novo* assembled into contigs using SPAdes v3.5, without preprocessing (raw reads), as it was the combination that resulted in the highest genome size assembled and the least number of contigs. In a similar way, different programs to predict genes (Prodigal (397) *vs.* GeneMark) and to annotate them (Sma3s (398) *vs.* RAST) were compared to choose the best in terms of highest number of protein-coding genes inferred and annotation accuracy (results not shown). As a result, protein-coding genes were finally predicted by Prodigal v2.6.3, an accurate and widely used bacterial gene finder, and the derived amino acid sequences were obtained. Moreover, the predicted proteins were functionally annotated by Sma3s v2 using the bacterial taxonomic division of UniProt database to improve the procedure specificity.

3.2. MLST typing.

MLST typing of the six bacteraemic clinical isolates from WGS data was performed following the same procedure as in Section 4.2 of Materials and Methods (Chapter I).

3.3. Identification of antimicrobial resistance mechanisms.

For the identification of the antimicrobial resistance genes contained in each of the six entirely-sequenced genomes, besides using ResFinder web server of the CGE and

BLASTP (as previously described in Section 4.3 of Materials and Methods, Chapter I), genes with functional annotations including either the term "resistance" or "antibiotic" were considered as resistance genes, and it included a manual review before its definitively confirmation.

3.4. Identification of virulence factors.

For the identification of the genes encoding known virulence factors from the WGS data of the six bacteraemic isolates, the same two strategies previously described in Section 4.4 of Materials and Methods (Chapter I) were used.

Objective 4. To find new bacterial virulence mechanisms that may be influencing patient mortality: genomic, transcriptomic, and proteomic approaches.

4.1. Comparative genomic study between isolates from non-surviving patients and isolates from surviving patients.

4.1.1. Differential protein-coding genes analyses.

Again, a newer and more accurate protocol was performed, in comparison with the one used with the clinical isolates from Chapter I. The amino acid sequences of all the predicted protein-coding genes were clustered using the CD-HIT tool (399) with an identity threshold and coverage for the longer sequence of 95%. It clusters the same protein from the different clinical isolates. So, if a specific cluster has no sequences coming from a specific isolate, it was considered that this isolate lacks the gene coding for that protein. And, if a cluster has a protein with a sequence identity lower than 100%, it will be considered that this isolate presents a variant for this protein-coding gene.

To refine the discovery of sequence variants between the different clinical isolates, a more specific protocol was designed using the standalone version of BLASTP from the NCBI with both identity threshold and query coverage of 100%. All the proteins from each isolate were independently compared to the proteins from the remaining isolates with the aim of discovering proteins with variants only found in one of the two groups of bacteria, that coming from the non-surviving patients or from the surviving patients. Each protein

found that presented a variant between both groups of isolates were subsequently further reviewed.

4.1.2. CarO sequence alignments and analysis.

The amino acid alignments of the inferred CarO proteins from the six bacteraemic isolates were performed using MAFFT v7.312 with default setting (400). When possible, the CarO protein sequence of a given isolate was assigned to a variant following the proposal of Mussi MA *et al*. (401). The secondary structure of CarO was obtained from the protein data bank (PDB) entry 4fuv.1.A, which presents an identical amino acid sequence. The multiple alignment with all data was depicted using Jalview v2.11.0 (402).

4.2. Comparative study of *ompA* expression levels between isolates from non-surviving patients and isolates from surviving patients by qRT-PCR.

The determination of the expression of the *ompA* gene in the six bacteraemic isolates was carried out following the protocol described in Section 5.2 of Materials and Methods (Chapter I).

4.3. Comparative proteomic studies between isolates from non-surviving patients and isolates from surviving patients.

4.3.1. OMP profiles and immunoblot analyses.

The OM fractions from the six bacteraemic isolates were isolated. Briefly, 50 mL of LB were inoculated with 500 µL of an overnight culture, and it was incubated during 4 h at 37 °C and 180 rpm. Bacterial cells were pelleted by centrifugation (4,600 × g at 4 °C, 15 min). Then, the pellet was resuspended in 25 mL of 10 mM sodium phosphate buffer (pH 7.2) to wash the cells. Bacterial cells were again pelleted by centrifugation and immediately resuspended in 5 mL of 10 mM sodium phosphate buffer (pH 7.2) and vortexed. Cells were lysed on ice by sonication for a total of 5-7 min in 30 s intervals. Unlysed cells were pelleted by centrifugation (4,600 × g at 4 °C, 5 min), and the supernatant was centrifuged at 20,000 × g for 45 min at 4 °C to pellet cell envelopes (inner

and outer membranes). Inner membranes from the resulting pellet were solubilized for 30 min at room temperature with 500 µL of 2% N-laurylsarcosinate in 10 mM phosphate buffer (pH 7.2). After solubilization, the insoluble outer membrane fraction was pelleted by centrifugation at 20,000 × g for 45 min at 4 °C. The outer membrane fraction was washed by resuspension in 1 mL 62.5 mM Tris-HCl (pH 6.8) and centrifuged at 20,000 × g for 45 min at 4 °C. The resulting pellet was resuspended in 60 µL of 62.5 mM Tris-HCl (pH 6.8) and stored at -80 °C until use. Immediately before use, proteins were quantified by the Bio-Rad Protein Assay, which is based on the method of Bradford and used the reagent Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories).

Then, the OMP profiles were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 4-15% SDS gels (Bio-Rad Laboratories) and 10 µg of OMPs, followed by SimplyBlue SafeStain staining (Thermo Fisher Scientific). The differential bands found between both groups of clinical isolates were analysed by liquid chromatography-tandem mass spectrometry (LC-MSMS) using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Proteins were identified using Proteome Discoverer 2.1 software (Thermo Fisher Scientific). This software used Sequest HT as its search engine and the database of *A. baumannii* in UniProt. Results were filtered by a false discovery rate (FDR) (also named *q-value*) of 1% (403).

Western blot analysis was conducted after by incubating the polyvinylidene difluoride membrane with polyclonal antibodies against *A. baumannii* CarO elicited in rabbits (129) and peroxidase-labelled anti-rabbit immunoglobulin G (IgG) antibody from donkeys (Sigma-Aldrich) (155).

4.3.2. Tandem mass tags-6 plex.

To quantify and compare the protein expression of both groups of isolates (those isolated from surviving patients *vs.* those isolated from non-surviving patients), a tandem mass tag (TMT)-6 plex was performed. Isolates were grown in MHB during 4 h at 37 °C and vigorous shaking (180 rpm). Then, cultures were lysed (8.4 M urea, iNtRON Biotechnology; 2.4 M thiourea, Sigma-Aldrich; 2% SDS, Sigma-Aldrich; 10 mM DTT, Sigma-Aldrich; and 5 µL of protease inhibitor cocktail, Sigma-Aldrich) and sonicated (40% ampl, 30 sec pulses for 10 min). For the quantitative studies, 40 µg of total proteins

from each isolate were digested with trypsine and peptides were marked with the TMT reagent for each sample. After 2 h of tagging, the peptides were pooled and cleaned with a SEP-PAK C18 cartridge and approximately 1 µg of peptide was analysed by LC coupled to Triple-qTOF MS. The data obtained by MS were analysed with 4 different search engines (MASCOT (Matrix Science, v.2.5), OMSSA (NCBI, v.2.1.9), X!Tandem2 (TheGPM, v.win-13-02-01-1), X!Tandem2 with k-score plugin (LabKey Software, v.2.3-7806), Myrimatch (Vanderbilt University, v.2.1), and MS-GF+ (CCMS-NIGMS, v.Beta v10072)) comparing to the database of *A. baumannii* in UniProt. Identifications and quantification data of the four engines were combined and FDRs were calculated and used for identification (FDR < 1% at the peptide level) and quantification (FDR ≤ 5%). The quantification analysis compared the proteins from the bacteraemic isolates of the surviving patients vs. the proteins from the bacteraemic isolates of the non-surviving patients, and it was expressed as $\log_2$ fold change for each protein identified (403). Proteins with a non-differential expression between both groups were those with a $\log_2$ fold change = 0, approximately. On the other hand, differentially expressed proteins were those with a $\log_2$ fold change > 0 (over-expressed proteins) or < 0 (down-expressed proteins).

Objective 5. To analyse the virulence role of CarO in *A. baumannii* using model systems.

5.1. Laboratory bacterial strains, growth conditions, and antibiotics.

After having found that CarO was the main differential protein between isolates from non-surviving patients and those from surviving patients, *A. baumannii* ATCC 17978 was selected as a model strain to analyse the possible role of this OMP as a virulence factor. The corresponding mutant strain lacking CarO (Δ*carO*) and the mutant strain complemented with plasmid pWH1266-*carO* (Δ*carO*/pWH1266-*carO*) (401) were generously ceded by Dr. Alejandro M. Viale (Institute of Molecular and Cellular Biology of Rosario (IBR), Argentina), under a collaborative work. The mutant strain lacking CarO (Δ*carO*) was constructed by replacing in the *A. baumannii* ATCC 17978 wild-type (wt) strain the *carO* gene by a *npt* (kanamycin resistance) cassette. The construction of plasmid pWH1266-*carO* expressing CarO (variant IV, from *A. baumannii* ATCC 17978) was done by cloning the corresponding gene and promoter region into the shuttle-vector

pWH1266. This plasmid was transformed into Δ*carO* cells (Δ*carO*/pWH1266-*carO*) to restore CarO production in these mutants. Wild-type and Δ*carO* strains were also transformed with the "empty" vector pWH1266 (wt/pWH1266 and Δ*carO*/pWH1266, respectively) to be used as controls. Strains were grown for 18-20 h in MHB at 37 ºC and 180 rpm. Kanamycin (20 µg/mL, Sigma-Aldrich) was added to the medium to select Δ*carO* strain, ticarcillin (80 µg/mL, Sigma-Aldrich) to select wt/pWH1266 strain, and both antibiotics to select Δ*carO*/pWH1266 and Δ*carO*/pWH1266-*carO* strains. Mueller-Hinton agar (MHA, Sigma-Aldrich) plus kanamycin and/or ticarcillin plates were used to select these strains, and sheep blood agar plates for wt strain.

5.2. OMPs profiles and immunoblot analyses.

Same protocols used for the bacteraemic strains (Section 4.3.1 of Materials and Methods from Chapter II) were used for the laboratory strains, but using 6 µg of OMPs.

5.3. Growth curves: separately and in competition.

The growth profiles of 17978 wt, Δ*carO*, Δ*carO*/pWH1266-*carO*, wt/pWH1266, and Δ*carO*/pWH1266 cells were compared. For individual growth curves, 5×10^5 CFU/mL were grown in 10 mL of MHB or in 10 mL of inactivated human serum (Sigma-Aldrich). At 2, 4, 8, 24, 48, and 72 h, the corresponding CFU/mL were determined by plating serial $\log_{10}$ dilutions on their corresponding plates.

Growth competition between wt and Δ*carO* cells was assessed in MHB or inactivated human serum by mixing 5×10^5 CFU/mL of each strain in the same culture. At the same time points as previously, serial $\log_{10}$ dilutions were plated on MHA plates and MHA plates containing 20 µg/mL of kanamycin to determine the CFU/mL of each strain. Both wt and Δ*carO* cells grew in MHA plates, while only Δ*carO* cells grow in MHA plates containing kanamycin. To determine the number of wt CFU, the calculated number of kanamycin-resistant Δ*carO* CFU was subtracted from the calculated number of total CFU estimated in MHA plates. Competition index (CI) was defined as the number of Δ*carO* CFU recovered/number of wt CFU recovered, divided by the number of Δ*carO* CFU

inoculated/number of wt CFU inoculated (222). Experiments were performed in triplicate in different days.

5.4. *In vitro* virulence study.

5.4.1. Human lung epithelial cells cultures and infection.

The same A549 cell line described previously in Section 1.4.1 of Materials and Methods from Chapter I was used for these experiments, and cells were seeded (10^5 cells/well) for 24 h in 24-well plates. Before infection, strains (17978 wt, Δ*carO*, Δ*carO*/pWH1266-*carO*, wt/pWH1266, and Δ*carO*/pWH1266) grown overnight were collected by centrifugation (15 min at 4,500 g), rinsed with sterilized PBS, and resuspended in DMEM, and A549 cells were rinsed twice with PBS. Then, A549 cells were incubated with a 1:1000 dilution of the overnight culture of each strain resuspended in DMEM (6.5 $\log_{10}$ CFU/mL, 3 wells/strain).

5.4.2. Bacterial adherence and invasion of cultured human lung epithelial cells.

Bacterial adherence and invasion assays were performed as previously described (155). To measure the number of adherent bacterial cells, A549 cells were incubated for 2 h with the different strains as described above, rinsed five times with PBS, and resuspended 10 min at 37 ºC in 200 µl of trypsin-EDTA (Gibco). Then, 200 µl of 0.5% Triton X-100 (Sigma-Aldrich) were added, and incubation continued for another 2 min. For bacterial invasion, A549 cells were incubated with the different bacterial strains as described above for 24 h, and 256 µg/mL gentamicin (Laboratorios Normon) was added during 30 min before the trypsin-EDTA addition. In all cases, serial dilutions of the Triton X-100 lysates were plated in their correspondent plates to determine the attached and internalized bacteria by A549 cells. Assays were performed by triplicate in different days.

5.5. *In vivo* virulence studies.

5.5.1. Animals and experimental murine model of peritoneal sepsis.

Same animals (immunocompetent adult C57BL/6J female mice, 20g), and non-discriminative murine model of peritoneal sepsis, detailed in Section 1.5 of Materials and Methods (Chapter I) were used.

5.5.2. Determination of MLD and survival analysis.

Same protocol as that explained in 1.5.2 of Materials and Methods (Chapter I) was used for the determination of the MLD of the strains 17978 wt and Δ*carO*. With the Δ*carO*/pWH1266-*carO* complemented strain, only the inoculum that would be used for the *in vivo* dissemination study (3.2 $\log_{10}$ CFU/mL) was tested, following the 3Rs principles (https://www.nc3rs.org.uk/the-3rs). Survival curves were represented graphically with mice infected with an inoculum of 3.2 $\log_{10}$ CFU/mL of 17978 wt, Δ*carO*, and Δ*carO*/pWH1266-*carO* strains.

5.5.3. *In vivo* dissemination.

Three groups of 14 mice each were inoculated i.p. with 0.5 mL of 3.2 $\log_{10}$ CFU/mL of 17978 wt, Δ*carO*, or Δ*carO*/pWH1266-*carO* strains. Eight and twenty-four hours post-inoculation, 7 animals of each group were randomly selected and sacrificed (200 µL sodium thiopental, i.p.). Then, bacterial loads were quantified in spleen, lungs, kidneys, liver, peritoneal fluid, and blood. For this purpose, immediately after the sacrifice the indicated organs were aseptically removed and homogenized (Stomacher 80; Tekmar Co., Newton Aycliffe, England) in 2 mL of sterile 0.9% NaCl solution. Quantitative and qualitative bacterial determinations of homogenized tissues ($\log_{10}$ CFU/g) and blood ($\log_{10}$ CFU/mL) were performed. To calculate the bacterial burden in peritoneal fluid ($\log_{10}$ CFU/mL), 2 mL of sterile 0.9% NaCl was injected i.p. in the sacrificed mice followed by 2-min abdominal massage. This fluid was then collected, serially diluted and plated.

Statistical analyses of Chapter II

Differences in *ompA* expression levels, bacterial adherence and invasion, mice survival, and bacterial load in mice tissues and fluids (Sections 3.1, 4.4.2, 4.5.2, and 4.5.3 of Material and Methods from Chapter II, respectively) were determined using GraphPad Prism 6 (GraphPad Software Inc., California, USA). Specifically, Student's t-test and Mann-Whitney test were used when comparing two groups in which the dependent variable was normally and not normally distributed, respectively. Survival curves and log-rank test were used to compare the survival distributions of mice. Statistical significance was established at a *P*-value of ≤ 0.05.

RESULTS

Chapter I. Study of the mechanisms involved in the pathogenesis of colonising and bacteraemic VAP producing *A. baumannii* clinical isolates.

Objective 1. To select colonising and invasive isolates according to specific criteria.

Sixteen colonising and seven invasive (bacteraemic VAP-producing) *A. baumannii* isolates, obtained from TBA samples of 23 different patients from the previous described cohort, were used (Table 5 and Table 6, respectively). These isolates were the only ones that fulfilled all the criteria set out in Section 1.2 of Materials and Methods from Chapter I. Also, the seven bacteraemic *A. baumannii* isolates recovered in the group of patients with bacteraemic VAP were used for the PFGE studies.

Table 5. Colonising clinical isolates selected.

Colonising isolates	
C1 (8.18 $\log_{10}$ CFU/mL)	C9 (7.48 $\log_{10}$ CFU/mL)
C2 (8.81 $\log_{10}$ CFU/mL)	C10 (8.45 $\log_{10}$ CFU/mL)
C3 (8.54 $\log_{10}$ CFU/mL)	C11 (8.56 $\log_{10}$ CFU/mL)
C4 (7.02 $\log_{10}$ CFU/mL)	C12 (6.62 $\log_{10}$ CFU/mL)
C5 (7.04 $\log_{10}$ CFU/mL)	C13 (9.13 $\log_{10}$ CFU/mL)
C6 (9.19 $\log_{10}$ CFU/mL)	C14 (6.66 $\log_{10}$ CFU/mL)
C7 (6.85 $\log_{10}$ CFU/mL)	C15 (7.48 $\log_{10}$ CFU/mL)
C8 (9.17 $\log_{10}$ CFU/mL)	C16 (7.95 $\log_{10}$ CFU/mL)

C: colonising isolate. In brackets: inoculum contained in the tracheobronchial aspirate sample from which each isolate was obtained.

Table 6. Invasive clinical isolates selected.

Invasive isolates
IB1 (8.62 $\log_{10}$ CFU/mL)
IB2 (6.32 $\log_{10}$ CFU/mL)
IB3 (8.75 $\log_{10}$ CFU/mL)
IB4 (9.22 $\log_{10}$ CFU/mL)
IB6 (8.32 $\log_{10}$ CFU/mL)
IB7 (8.59 $\log_{10}$ CFU/mL)
IB8 (8.75 $\log_{10}$ CFU/mL)

IB: invasive isolate. In brackets: inoculum contained in the tracheobronchial aspirate sample from which each isolate was obtained.

Objective 2. To evaluate the clonal relationship and *in vitro* virulence of colonising and invasive isolates. Selection of isolates for further studies.

2.1. Molecular typing of the isolates by PFGE. Selection of "true" bacteraemic VAP-producing isolates.

Applying the criterion of 85% similarity to the PFGE profiles of the first selected isolates obtained from TBA samples (16 colonising and 7 invasive isolates), we found that isolates from the colonising group belonged to 4 different pulsotypes (A-D), while invasive isolates belonged to two of these pulsotypes (B and C) (Figure 4 and Table 7).

Otherwise, when the criterion of 100% similarity was applied to the PFGE profiles of the 7 invasive isolates recovered from TBA samples and the 7 isolates obtained from blood cultures of the same bacteraemic VAP patients, we found that the invasive isolates IB1, IB2, IB3, IB4, IB7, and IB8 obtained from TBA had exactly the same PFGE profiles as

their corresponding *A. baumannii* isolates recovered from blood cultures (HC1, HC2, HC3, HC4, HC7, and HC8) (Figure 4). Therefore, once confirmed that these patients had a high probability of bacteraemia derived from VAP, we selected these 6 invasive isolates for the next experiments. However, the invasive isolate IB6 was discarded from the study for not having exactly the same PFGE profile as its corresponding bacteraemic isolate (Figure 4).

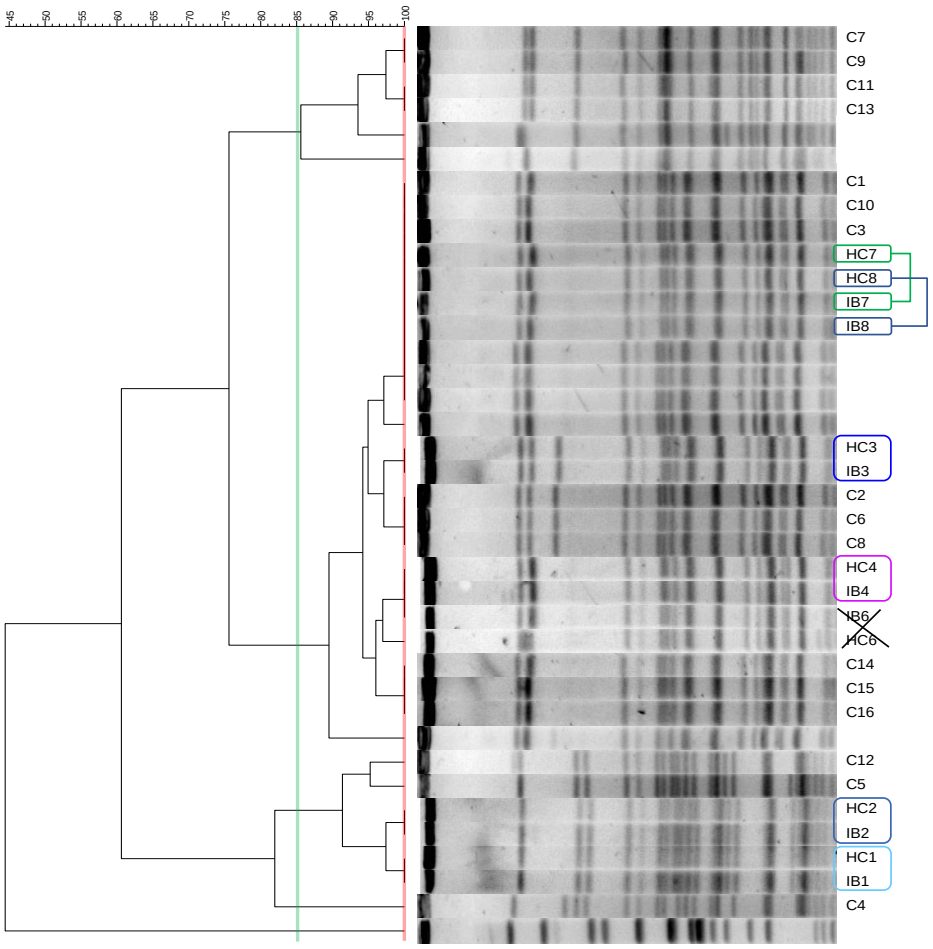

Figure 4. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of the first selected colonising (C-number) and invasive isolates (IB-number) obtained from tracheobronchial aspirate samples. PFGE patterns of the bacteraemic isolates (HC-number) from the same patients as invasive isolates were also presented. Green and red lines indicate 85% and 100% similarity among PFGE profiles, respectively. Pairs of invasive isolates from TBA and blood samples from the same patient are highlighted in coloured boxes when they presented exactly the same PFGE pattern, if not, these were marked with a cross.

Table 7. Distribution of the first selected colonising and invasive isolates according to pulsotypes.

Pulsotypes	Colonising isolates	Invasive isolates
A	C7, C9, C11, C13	-
B	C1, C2, C3, C6, C8, C10, C14, C15, C16	IB3, IB4, IB6, IB7, IB8
C	C5, C12	IB1, IB2
D	C4	-

2.2. *In vitro* virulence of the colonising and invasive isolates. Selection of the colonising and invasive isolates with the lowest and highest *in vitro* virulence, respectively.

At the beginning of the present work, it was proposed to assess the *in vitro* virulence of each isolate by the viability assay in A549 cell cultures, and based on the results, select the 5 colonising isolates with the lowest *in vitro* virulence and the 5 invasive isolates with the highest, in order to find more differences between them in further analyses. In the case of colonising isolates, 16 isolates m*et al*l the criteria specified previously (Section 1.2 of Materials and Methods, Chapter I). However, this number was lower than expected in the case of invasive isolates, 6 isolates only. Therefore, we decided to study the *in vitro* virulence of all isolates, 16 colonising and 6 invasive, but as a result select the 5 colonising isolates with the lowest *in vitro* virulence and the 6 invasive isolates directly for next experiments.

Results of the *in vitro* virulence of each isolate assessed are shown in Figure 5. Similar ranges of viability cell reduction were found when infected with colonising or invasive isolates: 59.82%-78.58% and 54.46%-73.82% of cell viability after infection with colonising and invasive isolates, respectively (Figure 5). Thus, when *in vitro* virulence was compared between groups of colonising and invasive isolates, no significant

differences were found (Figure 6A, averages of cell viability (mean ± SEM): 67.25% ± 0.83% for colonising and 65.41% ± 1.60% for invasive isolates, $P = 0.4104$). Even so, the 5 colonising isolates with the lowest *in vitro* virulence were selected for further experiments. These isolates were those that reduced cell viability to a percentage higher than 70% (Figure 5), namely isolates C6, C7, C8, C11, and C12. Nonetheless, a significant difference was found in *in vitro* virulence between both groups of isolates once only the colonising isolates with the lowest *in vitro* virulence were selected (Figure 6B, averages of cell viability (mean ± SEM): 72.22% ± 1.00% for colonising and 65.41% ± 1.60% for invasive isolates, $P = 0.0014$).

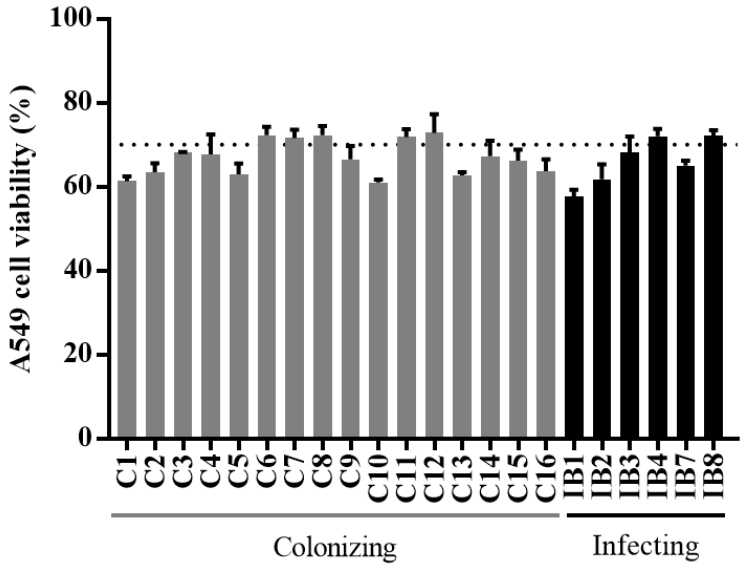

Figure 5. Cell viability assay results after A549 cell culture infection with colonising and invasive isolates. Data represented as mean ± standard error of the mean (SEM) (triplicate in 3 different days). Dashed line indicates the threshold of 70% A549 cell viability used for selecting the less *in vitro* virulent colonising isolates.

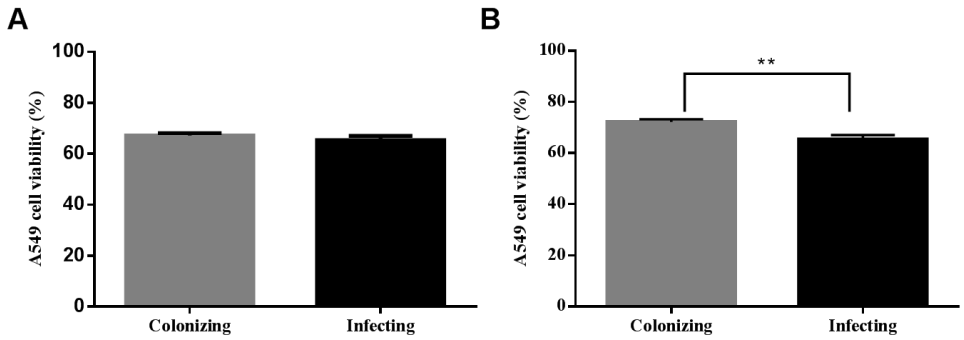

Figure 6 (A-B). Averages of cell viability after infection of A549 cell culture with colonising and invasive isolates. (A) Considering all colonising and invasive isolates (16 and 6 isolates, respectively) (*P* = 0.4104 for invasive *vs.* colonising isolates, Mann Whitney test); (B) considering only the colonising isolates with the lowest *in vitro* virulence and all the invasive ones (5 and 6 isolates, respectively) (*P* = 0.0014 for invasive *vs.* colonising isolates, Student's t-test). Data represented as mean ± SEM.**

Objective 3. To study the *in vivo* virulence and antimicrobial susceptibility patterns of colonising and invasive isolates.

3.1. *In vivo* virulence of the colonising and invasive isolates.

The *in vivo* virulence of the 5 colonising and 6 invasive isolates selected after the *in vitro* virulence studies was analysed in a non-discriminative peritoneal sepsis murine model. The LD_0, LD_{50}, and LD_{100} (or MLD) are shown in Table 8.

Table 8. Lethal doses (LD) of the colonising and invasive isolates in a non-discriminative peritoneal sepsis murine model.

Isolates		LD_0 (Log_{10} CFU/mL)	LD_{50} (Log_{10} CFU/mL)	LD_{100} (MLD) (Log_{10} CFU/mL)
Colonising	C6	2.20	3.77	5.43
	C7	3.30	4.65	5.42
	C8	2.23	3.32	4.38
	C11	1.40	3.32	5.30
	C12	2.27	3.42	4.28
Invasive	IB1	2.04	2.85	3.78
	IB2	2.11	3.81	5.26
	IB3	3.26	3.85	4.41
	IB4	3.70	4.43	5.66
	IB7	2.43	3.76	4.48
	IB8	2.50	4.06	5.36

There was no LD difference between colonising and invasive isolates, presenting a heterogeneous pattern independently of the studied group. Thus, no significant differences were found between both groups (Table 9).

Table 9. Averages lethal doses (LD) of the groups of colonising and invasive isolates in a non-discriminative peritoneal sepsis murine model.

Isolates	LD_0 (Log_{10} CFU/mL)	LD_{50} (Log_{10} CFU/mL)	LD_{100} (MLD) (Log_{10} CFU/mL)
Colonising	2.28 ± 0.30^a	3.70 ± 0.25^b	4.96 ± 0.26^c
Invasive	2.67 ± 0.27^a	3.79 ± 0.21^b	4.83 ± 0.29^c

Mann-Whitney test: $^aP = 0.5152$ for LD_0 invasive *vs.* colonising; $^bP = 0.5022$ for LD_{50} invasive *vs.* colonising; $^cP = 0.9004$ for LD_{100} invasive *vs.* colonising. Lethal doses expressed as mean ± SEM.

3.2. Antimicrobial susceptibility profiles of the colonising and invasive isolates.

All clinical isolates, colonising or invasive, were MDR, being resistant to carbapenems (CRAB), ceftazidime, and ciprofloxacin, but susceptible to tigecycline (MIC: 0.25 µg/mL) and colistin (0.06 µg/mL) (Table 10). The isolates exhibited variable MICs of sulbactam (4-32 µg/mL), amikacin (0.5-32 µg/mL), tobramycin (1- > 128 µg/mL), and rifampicin (2-128 µg/mL) (Table 10). No differential antimicrobial susceptibility pattern was observed between the colonising and invasive isolates.

Table 10. Minimum inhibitory concentrations (MICs) of different antimicrobials for the colonising and invasive isolates.

Isolates	Antimicrobial agent MICs (µg/mL) (SIR)[a]	Imipenem	Meropenem	Ceftazidime	Sulbactam	Amikacin	Tobramycin	Ciprofloxacin	Rifampicin	Tigecycline	Colistin
Colonising	C6	16 (R)	> 16 (R)	64 (R)	4 (S)	4 (S)	2 (S)	16 (R)	128 (R)	0.25 (S)	0.06 (S)
	C7	16 (R)	8 (R)	128 (R)	8 (I)	32 (R)	> 128 (R)	32 (R)	128 (R)	0.25 (S)	0.06 (S)
	C8	16 (R)	8 (R)	64 (R)	4 (S)	1 (S)	1 (S)	16 (R)	128 (R)	0.25 (S)	0.06 (S)
	C11	16 (R)	> 16 (R)	64 (R)	4 (S)	16 (R)	> 128 (R)	64 (R)	128 (R)	0.25 (S)	0.06 (S)
	C12	128 (R)	> 16 (R)	128 (R)	32 (R)	32 (R)	> 128 (R)	128 (R)	4 (S)	0.25 (S)	0.06 (S)
Invasive	IB1	128 (R)	> 16 (R)	64 (R)	32 (R)	32 (R)	> 128 (R)	128 (R)	2 (S)	0.25 (S)	0.06 (S)
	IB2	128 (R)	> 16 (R)	64 (R)	32 (R)	32 (R)	> 128 (R)	128 (R)	2 (S)	0.25 (S)	0.06 (S)
	IB3	16 (R)	8 (R)	32 (R)	4 (S)	2 (S)	2 (S)	16 (R)	16 (R)	0.25 (S)	0.06 (S)
	IB4	16 (R)	> 16 (R)	64 (R)	4 (S)	2 (S)	1 (S)	64 (R)	32 (R)	0.25 (S)	0.06 (S)
	IB7	64 (R)	> 16 (R)	32 (R)	4 (S)	0.5 (S)	1 (S)	16 (R)	16 (R)	0.25 (S)	0.06 (S)
	IB8	64 (R)	> 16 (R)	64 (R)	4 (S)	2 (S)	2 (S)	16 (R)	16 (R)	0.25 (S)	0.06 (S)

[a]S: susceptible; I: intermediate; R: resistant. Breakpoints, imipenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; meropenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; ceftazidime (CLSI): susceptible MIC ≤ 8 µg/mL, intermediate MIC = 16 µg/mL, and resistant MIC ≥ 32 µg/mL; sulbactam (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; amikacin (EUCAST): susceptible MIC ≤ 8 µg/mL and resistant MIC ≥ 16 µg/mL; tobramycin (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; ciprofloxacin (EUCAST): susceptible MIC ≤ 1 µg/mL and resistant MIC > 1 µg/mL; rifampicin (Rev Esp Quimioter 2012;25(2):134-138): susceptible MIC ≤ 4 µg/mL and resistant MIC > 4 µg/mL; tigecycline (FDA): susceptible MIC ≤ 2 µg/mL, intermediate MIC = 4 µg/mL, and resistant MIC ≥ 8 µg/mL; colistin (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 4 µg/mL.

Objective 4. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the colonising and invasive isolates.

4.1. WGS data processing.

Results obtained after WGS data processing, for the 5 colonising and the 6 invasive isolates are shown in Table 11. The mean coverage of the WGS was 23 ± 2-fold (mean ± standard error of the mean (SEM) of the 11 isolates). *De novo* assembly of the preprocessed reads resulted in 490 ± 51 contigs (mean ± SEM of the 11 isolates). Then, the assemblies were filtered for contigs larger than 500 bp, leaving 281 ± 32 contigs (mean ± SEM of the 11 isolates) with an N_{50} of 38,033 ± 4,458 bp (mean ± SEM of the 11 isolates). Despite the filtering, the number of contigs remained high (> 300 contigs) in the isolates IB2, IB4, IB7, and IB8. Notwithstanding, most of the genome was assembled in all cases, being the *A. baumannii* genome size ~ 4.0 Mb (266, 404) and the mean ± SEM of the contig total length of the 11 isolates 3,986,739 ± 37,072 bp. The G+C content was 39.18% ± 0.03% (mean ± SEM of the 11 isolates), a value corresponding to that reported for other *A. baumannii* strains or even other members of the *Acinetobacter* genus (404). Moreover, the number of protein-coding genes predicted and annotated was 3,819 ± 41 (mean ± SEM of the 11 isolates), similar to the 4,000 protein-coding genes usually reported for *A. baumannii* (405).

Table 11. Comparison of the general features of the 5 colonising and 6 invasive isolates after whole-genome sequencing data processing.

Isolates	Mean coverage (fold)	No. contigs[a]	Contig N_{50} (bp)[a]	No. contigs[b]	Contig N_{50} (bp)[b]	Contig total length (bp)[c]	G+C (%)	No. protein-coding genes
C6	21	343	55,866	175	55,866	3,892,724	39.0	3,728
C7	22	374	46,492	207	46,492	4,143,108	39.2	3,970
C8	27	317	47,748	180	47,748	3,949,375	39.1	3,788
C11	31	363	43,355	215	43,355	4,139,387	39.2	3,988
C12	18	443	48,899	223	48,899	4,128,796	39.2	3,996
IB1	32	368	58,150	196	58,150	4,122,522	39.2	3,972
IB2	11	809	18,541	463	18,541	3,974,939	39.3	3,765
IB3	25	459	30,711	276	30,711	3,876,316	39.1	3,692
IB4	22	741	18,979	456	18,979	3,820,049	39.3	3,631
IB7	21	603	23,292	358	23,292	3,886,324	39.2	3,715
IB8	22	572	26,333	347	26,333	3,920,594	39.2	3,761

C: colonising isolate; IB: invasive isolate. [a]Before filtering contigs > 500 bp. [b]After filtering contigs > 500 bp. [c]Total genome assembled with ABySS.

4.2. Molecular typing of the isolates by MLST.

Two different STs were found among colonising and invasive isolates according to the Bartual/Oxford MLST scheme: most isolates belong to ST208, except C12, IB1, and IB2 that belong to ST218 (Table 12). ST208 and ST218 share 6 out of 7 alleles with ST92 (CC92) differing both only at the *gpi* allele with ST92 (https://pubmlst.org/abaumannii/). Following the current definition of CC (47), all isolates were thus assigned to CC92 in the Bartual/Oxford scheme. According to the Pasteur MLST scheme, isolates C12, IB1, and IB2 belong to ST2 (CC2), isolates C7 and C11 belong to ST745, and the rest of the

isolates belong to ST187 (Table 12).. ST187 and ST745 share 6 out of 7 alleles with ST2 differing both only at the *rpoB* allele with ST2 (https://pubmlst.org/abaumannii/). All isolates were thus assigned to CC2 in the Pasteur scheme. It follows that all isolates belong to CC92 (Bartual/Oxford)/CC2 (Pasteur), the most widely disseminated *A. baumannii* clonal complex worldwide (47). Besides, when these results were compared with PFGE results, Pasteur MLST scheme was the one that exactly matched the PFGE results, having both in addition a greater discriminatory power than the Bartual/Oxford MLST scheme (Table 12). Thus, taking into account the MLST and PFGE results, there was no relationship between the ability to cause or not infection and the clone they belonged to.

Table 12. Summary of the multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) typing results for colonising and invasive isolates.

Isolates	MLST Bartual/Oxford scheme	MLST Pasteur scheme	PFGE
C6	ST208	ST187	Pulsotype B
C7	ST208	ST745	Pulsotype A
C8	ST208	ST187	Pulsotype B
C11	ST208	ST745	Pulsotype A
C12	ST218	ST2	Pulsotype C
IB1	ST218	ST2	Pulsotype C
IB2	ST218	ST2	Pulsotype C
IB3	ST208	ST187	Pulsotype B
IB4	ST208	ST187	Pulsotype B
IB7	ST208	ST187	Pulsotype B
IB8	ST208	ST187	Pulsotype B

C: colonising isolate; IB: invasive isolate.

4.3. Antimicrobial resistance mechanisms.

A wide variety of genes associated with resistance to *β*-lactams, aminoglycosides, sulfonamides, and tetracyclines, as well as point mutations in genes associated with quinolones and rifampicin resistance, were detected in most colonising and invasive isolates when WGS data was analysed with several bioinformatics approaches and tools (Table 13), explaining most of the antimicrobial susceptibility profiles. However, clear distinctions in the composition of antimicrobial resistance mechanisms among ST/PFGE types were observed, while no association with the colonising/invasive phenotypes was detected.

With respect to *β*-lactam resistance genes, both cephalosporinase- (class C *β*-lactamase) and OXA-type carbapenemase (class D *β*-lactamase or CHDLs)-encoding genes were identified in all isolates. Specifically, all isolates possessed the *bla*$_{\text{ADC-25}}$ gene, which is intrinsic to *Acinetobacter* spp. and encodes an AmpC-type cephalosporinase. This enzyme is constitutively expressed at low levels, but may be responsible for clinically significant resistance to third-generation cephalosporins, such as ceftazidime and cefotaxime, when expressed from the promotor provided by IS*Aba1* (406). Thus, IS*Aba1* was detected upstream of the *bla*$_{\text{ADC-25}}$ gene in all the isolates from ST745, 1 isolate from ST178 (C8), and 2 isolates from ST2 (C12 and IB1), mechanism that has been also associated with sulbactam resistance (218). Regarding the OXA-type carbapenemases, all isolates had a gene coding for a naturally occurring OXA-51-like *β*-lactamase: isolates from ST745 and ST187 possessed *bla*$_{\text{OXA-66}}$, while isolates belonging to ST2 had *bla*$_{\text{OXA-109}}$. Both OXA-51-like enzymes show weak carbapenemase activity, although the presence of IS*Aba1* upstream the *bla*$_{\text{OXA-51-like}}$ gene may provide a promoter that leads to overproduction, thus generating resistance to carbapenems (407, 408). Therefore, only in 2 of these isolates (C12 and IB1), the *bla*$_{\text{OXA-51-like}}$ gene was overexpressed due to the presence of an IS*Aba1* upstream, but the rest of the isolates had an additional acquired carbapenemase. Isolates from ST745 and ST187 carried *bla*$_{\text{OXA-58}}$ with IS*Aba3* located upstream and in the opposite orientation to this gene (associated with resistance to penicillins and carbapenems), and only the isolate IB2 from ST2 harboured *bla*$_{\text{OXA-24/40}}$ (associated with resistance to penicillins, cephalosporins, and carbapenems) (409). None of these isolates had any other β-lactamase-encoding genes registered at ResFinder database.

With respect to aminoglycoside resistance genes, all isolates possessed several AMEs. Isolates belonging to ST745 and ST2 had the acetyltransferase-encoding gene *aac(6')-Ip* (encodes resistance to amikacin and tobramycin, among others), and two phosphotransferase-encoding genes, *strA* and *strB* (both encodes resistance to streptomycin) (410). Moreover, isolates from ST2 has an extra acetyltransferase-encoding gene, *aac(3)-IIa*, which encodes resistance to gentamicin and tobramycin (410). On the other hand, isolates from ST187 harboured the acetyltransferase-encoding gene *aac(3)-Ia* (encodes resistance to gentamicin), the adenylyltransferase-encoding gene *aadA1* (encodes resistance to streptomycin and spectinomycin), *strA* and *strB* (410). None of the isolates had the 16S rRNA methylase-encoding gene *armA*.

Regarding the quinolone resistance mechanisms, all isolates had point mutations in the QRDRs of *gyrA* and *parC*, genes coding for the quinolone target enzymes DNA gyrase (GyrA subunit) and topoisomerase IV (ParC subunit), respectively. In particular, these identified mutations caused amino acid substitution in GyrA (Ser83Leu) and ParC (Ser80Leu). This combination of mutations has been previously associated with high level resistance to quinolones in *A baumannii* (411, 412). Moreover, none of the isolates possessed *qnr* genes.

Likewise, point mutations in the conserved regions of *rpoB* gene (encodes the *β*-subunit of the rifampicin target enzyme RNA polymerase) that resulted in the substitution of a single amino acid in the conserved domain II of the RNA polymerase *β*-subunit RpoB were found in isolates belonging to ST745 (mutation I581M) and ST187 (mutation S583L). In this case, only the mutation I581M has been previously described and associated with high resistance to rifampicin (413). No mutation was found in *rpoB* gene of the isolates from ST2. Moreover, none of the isolates had the *arr-2* gene.

Furthermore, the sulfonamide resistance genes *sul1* and *sul2* (encode forms of the target enzyme dihydropteroate synthase that are not inhibited by sulfonamides (414)) was found in isolates belonging to ST745 and ST187, while isolates from ST2 only harboured *sul1*.

Finally, all isolates possessed the *tet(B)* gene, which codes for an efflux protein that confers resistance to tetracycline, minocycline, and doxycycline, but not glycylciclines (tigecycline) (415, 416); and none of the isolates had *mcr* genes, associated with colistin resistance, as expected

Table 13. Antimicrobial resistance mechanisms found with ResFinder and BLASTP in the colonising and invasive isolates.

Isolates	*β*-lactams	Aminoglycosides	Quinolones	Rifampicin	Sulfonamides	Tetracyclines
ST745 (C7 and C11)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{OXA-58}}$	*aac(6')-Ip*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80L mutation in *parC*[b]	I581M mutation in *rpoB*	*sul1*, *sul2*	*tet(B)*
ST187 (C6, C8, IB3, IB4, IB7, and IB8)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{OXA-58}}$	*aac(3)-Ia*, *aadA1*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80L mutation in *parC*[b]	S583L mutation in *rpoB*	*sul1*, *sul2*	*tet(B)*
ST2 (C12, IB1, and IB2)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-109}}$ *bla*$_{\text{OXA-24/40}}$ (only in IB2)	*aac(6')-Ip*, *aac(3)-IIa*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80L mutation in *parC*[b]	-	*sul1*	*tet(B)*

C: colonising isolate; IB: invasive isolate. [a]Amino acid position equivalent to Ser-83 of *E. coli*. [b]Amino acid positions equivalent to Ser-80 and Ser-463 of *E. coli*.

4.4. Virulence factors.

Most of the virulence genes searched were found in all colonising and invasive isolates (64 out of the 82 genes contained in the reference *A. baumannii* isolate AB0057) (Table 14). It is worth noting that those genes found belonged to all virulence factor classes studied in this analysis: adherence, biofilm formation, phospholipase enzymes, immune evasion, iron uptake, regulation, and serum resistance. On the other hand, only two genes involved in heme utilization were identified in all isolates, in comparison with the ten genes found in AB0057. Moreover, the number of genes related to capsule formation were variable among isolates, but it was associated with the ST type, possessing isolates from ST2 the least number. Thus, the composition of known virulence genes had no association with the isolate phenotype (colonising/invasive).

Table 14. Virulence genes found in the colonising and invasive isolates.

Virulence factor class	Virulence factors[a]	Related genes[a]	Isolates presenting the corresponding gene										
Adherence	Outer membrane protein	*ompA*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		adeF	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		adeG	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AdeFGH efflux pump/transport autoinducer	*adeH*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	Biofilm associated protein	*bap*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		csuA/B	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		csuA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		csuB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		csuC	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		csuD	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	Csu pili	*csuE*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		pgaA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		pgaB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		pgaC	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
Biofilm formation	PNAG	*pgaD*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	Phospholipase C	*plC*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
Enzyme	Phospholipase D	*plD*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		lpsB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		lpxA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		lpxB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		lpxC	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
Immune evasion	LPS	*lpxD*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8

	lpxL	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	lpxM	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0091	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0092	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0093	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0094	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0095	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0096	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0097	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0098	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0099	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0100	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0101	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0102	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0103	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0104	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0105	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	AB57_0106	C6	C7	C8	C11	C12	IB1	-	IB3	IB4	IB7	IB8
	AB57_0107	C6	C7	C8	C11	C12	IB1	-	IB3	IB4	IB7	IB8
	AB57_0108	C6	C7	C8	C11	-	-	-	IB3	IB4	IB7	IB8
	AB57_0109	C6	C7	C8	C11	-	-	-	IB3	IB4	IB7	-
	AB57_0110	C6	C7	C8	C11	-	-	-	IB3	IB4	-	-
	AB57_0111	C6	-	C8	C11	-	-	-	-	-	-	-
	AB57_0112	-	-	-	-	-	-	-	-	-	-	-
	AB57_0113	-	-	-	-	-	-	-	-	-	-	-
Capsule	AB57_0114	-	-	-	-	-	-	-	-	-	-	-

		AB57_0115	-	-	-	-	-	-	-	-	-	-	-
		barA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		barB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basC	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basD	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basF	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basG	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basH	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basI	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		basJ	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauA	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauB	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauC	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauD	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauE	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bauF	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	Acinetobactin	*entE*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		hemO	-	-	-	-	-	-	-	-	-	-	-
		AB57_0984	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		AB57_0985	-	-	-	-	-	-	-	-	-	-	-
		AB57_0986	-	-	-	-	-	-	-	-	-	-	-
		AB57_0987	-	-	-	-	-	-	-	-	-	-	-
		AB57_0988	-	-	-	-	-	-	-	-	-	-	-
Iron uptake	Heme utilization	AB57_0989	-	-	-	-	-	-	-	-	-	-	-

		AB57_0990	-	-	-	-	-	-	-	-	-	-	-
		AB57_0992	-	-	-	-	-	-	-	-	-	-	-
		AB57_0993	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		abaI	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
	Quorum sensing	*abaR*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
		bfmR	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
Regulation	Two component system	*bfmS*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8
Serum resistance	Polysaccharide polymer	*pbpG*	C6	C7	C8	C11	C12	IB1	IB2	IB3	IB4	IB7	IB8

C: colonising isolate; IB: invasive isolate. [a]Virulence factors/genes described in the *A. baumannii* isolate AB0057. PNAG: polysaccharide poly-N-acetylglucosamine; LPS: lipopolysaccharide.

Objective 5. To find new mechanisms of infection by comparing colonising and invasive isolates.

5.1. Comparative genomic study between colonising and invasive isolates.

With the first bioinformatics approach, no genomic fragment (contig), and therefore no gene, was found exclusively in any of the studied groups of isolates (colonising or invasive). Then, core- and pan-genome were identified, consisting of 3,297 and 4,376 genes, respectively. However, there was no point mutation in the core-genome that was present in all colonising isolates but in none of the invasive isolates, or vice versa. Similarly, using the second bioinformatics approach, there was no predicted protein exclusive of the colonising or invasive isolates, and neither exclusive variants in the predicted core-proteome.

5.2. Comparative study of *ompA* expression levels between colonising and invasive isolates.

Having found no genomic difference between colonising and invasive isolates, a transcriptomic study based on the well-known *A. baumannii* virulence factor OmpA was performed. qRT-PCR analysis showed that *ompA* gene was transcribed at higher levels in all the invasive isolates in comparison with the colonising isolates (Table 15).

Table 15. *ompA* expression levels of the colonising and invasive isolates.

Isolates		*ompA* expression
Colonising	**C6**	0.42
	C7	0.35
	C8	0.37
	C11	0.09
	C12	0.22
Invasive	**IB1**	1.75
	IB2	1.04
	IB3	1.84
	IB4	2.94
	IB7	2.29
	IB8	3.02

In fact, when averages were performed, a significant *ompA* overexpression was confirmed in the invasive isolates group compared with the colonising isolates group, with a ratio of 2.15 *vs.* 0.29 (P = 0.0043) (Table 16).

Table 16. Average *ompA* expression of the groups of colonising and invasive isolates.

Isolates	*ompA* expression
Colonising	0.29 ± 0.06**
Invasive	2.15 ± 0.31**

***P* = 0.0043 for invasive *vs.* colonising isolates, Mann-Whitney test. Expression of *ompA* expressed as mean ± SEM.

Chapter II. Identification of potential *A. baumannii* virulence factors of bacteraemic *A. baumannii* isolates recovered from clinically homogeneous adult patients with radically different clinical outcomes.

Objective 1. To select bacteraemic isolates according to specific criteria.

Six *A. baumannii* isolates obtained from blood cultures of six patients with bacteraemia secondary to VAP from the cohort previously described (Chapter I) were selected according the pre-specified criteria. Clinical and demographic characteristics of these patients, as well as its *A. baumannii* bacteraemic isolate name, are detailed in Table 17. Despite their demographic and clinical homogeneity, including lack of comorbidities, and having reached the optimal therapeutic target for colistin, three patients died (patients 1-3, infected by B1, B4 and B7 *A. baumannii* isolates, respectively), and three survived (patients 4-6, infected by B8, B9 and B11 *A. baumannii* isolates, respectively).

Table 17. Patients' baseline characteristics, clinical features, antibiotic therapy received in the intensive care units (ICUs), and bacteraemic isolate name.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	34	48	41	35	48	26
Sex	Female	Male	Female	Male	Male	Male
Charlson index[a]	0	0	0	0	0	0
Diagnosis at ICU admission	CAP[b]	Sepsis	Stroke	Abdominal surgery	Fournier's gangrene	Cardiovascular surgery
APACHE II score[c]	23	20	20	15	17	23
CPIS score[d]	8	6	8	7	6	6
Pitt score[e]	6	12	6	7	6	3
Co-infection	No	No	No	No	No	No
Septic shock	Yes	Yes	Yes	Yes	No	Yes
Acute renal failure	Yes	No	Yes	Yes	No	Yes
Multiorganic failure	Yes	No	Yes	No	No	Yes
CMS[f] treatment (MIU[g]/24 h)	6	8	6	9	9	6
ICU length-of stay (days)	23	18	30	46	31	20
30-days mortality	Yes	Yes	Yes	No	No	No
Bacteraemic *A. baumannii* isolate (name)	B1	B4	B7	B8	B9	B11

[a]Charlson index: comorbidity index; [b]CAP: community-acquired pneumonia; [c]APACHE II score: acute physiology and chronic health evaluation II score; [d]CPIS score: clinical pulmonary infection score; [e]Pitt score: Pitt bacteraemia score; [f]CMS: colistimethate sodium; [g]MIU: million international units.

Objective 2. To study the clonal relationship and antimicrobial susceptibility patterns in the selected bacteraemic *A. baumannii* isolates.

2.1. Molecular typing of the isolates by PFGE.

Applying the criterion of 85% similarity to the PFGE profiles of the 6 bacteraemic clinical isolates, 3 different pulsotypes were found, being those named as A, B, and C in Chapter I. Pulsotype A comprised isolates B4 and B9, pulsotype B isolates B7, B8, and B11, and pulsotype C isolate B1only.

2.2. Antimicrobial susceptibility testing.

The six isolates were MDR, being all resistant to carbapenems (CRAB), ceftazidime, ciprofloxacin, and doxycycline; but susceptible to tigecycline and colistin (Table 18). Moreover, the isolates showed variable MICs for sulbactam, amikacin, tobramycin, and rifampicin (Table 18).

Table 18. Minimum inhibitory concentrations (MICs) of different antimicrobials for the six bacteremic isolates.

Isolates \ Antimicrobial agent MICs (µg/mL) (SIR)[a]	Imipenem	Doripenem	Meropenem	Ceftazidime	Sulbactam	Amikacin	Tobramycin	Ciprofloxacin	Rifampicin	Doxycycline	Tigecycline	Colistin
B1	64 (R)	> 8 (R)	> 16 (R)	64 (R)	32 (R)	128 (R)	> 128 (R)	256 (R)	4 (S)	16 (R)	0.5 (S)	0.06 (S)
B4	32 (R)	> 8 (R)	4 (I)	128 (R)	4 (S)	64 (R)	128 (R)	64 (R)	32 (R)	32 (R)	1 (S)	0.03 (S)
B7	8 (R)	4 (R)	4 (I)	128 (R)	4 (S)	2 (S)	128 (R)	32 (R)	16 (R)	32 (R)	0.5 (S)	0.06 (S)
B8	16 (R)	4 (R)	4 (I)	64 (R)	4 (S)	4 (S)	1 (S)	16 (R)	16 (R)	32 (R)	0.5 (S)	0.03 (S)
B9	8 (R)	4 (R)	4 (I)	128 (R)	8 (I)	64 (R)	128 (R)	64 (R)	32 (R)	32 (R)	1 (S)	0.12 (S)
B11	16 (R)	4 (R)	4 (I)	64 (R)	8 (I)	2 (S)	> 128 (R)	16 (R)	32 (R)	32 (R)	0.5 (S)	0.06 (S)

[a]S: susceptible; I: intermediate; R: resistant. Breakpoints, imipenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; doripenem (CLSI): susceptible MIC ≤ 2 µg/mL, intermediate MIC = 4 µg/mL, and resistant MIC ≥ 8 µg/mL; meropenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; ceftazidime (CLSI): susceptible MIC ≤ 8 µg/mL, intermediate MIC = 16 µg/mL, and resistant MIC ≥ 32 µg/mL; sulbactam (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; amikacin (EUCAST): susceptible MIC ≤ 8 µg/mL and resistant MIC ≥ 16 µg/mL; tobramycin (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; ciprofloxacin (EUCAST): susceptible MIC ≤ 1 µg/mL and resistant MIC > 1 µg/mL; rifampicin (Rev Esp Quimioter 2012;25(2):134-138): susceptible MIC ≤ 4 µg/mL and resistant MIC > 4 µg/mL; doxycycline (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; tigecycline (FDA): susceptible MIC ≤ 2 µg/mL, intermediate MIC = 4 µg/mL, and resistant MIC ≥ 8 µg/mL; colistin (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 4 µg/mL.

Objective 3. To perform WGS and data analysis (MLST, antimicrobial resistance and virulence determinants) of the bacteraemic *A. baumannii* isolates.

3.1. WGS data processing.

WGS data-processing results of the 6 bacteraemic isolates are shown in Table 19. The mean coverage of the WGS was 92 ± 6-fold (mean ± SEM of the 6 isolates). *De novo* assembly of the raw reads resulted in 173 ± 6 contigs (mean ± SEM of the 6 isolates), with an N_{50} of 101,802 ± 5,110 bp (mean ± SEM of the 6 isolates). The contig total length was 3,984,000 ± 29,848 bp (mean ± SEM of the 6 isolates), covering the total *A. baumannii* genome size (266, 404). The G+C content was 39.08% ± 0.02% (mean ± SEM of the 6 isolates), similar to the value usually reported for *A. baumannii* strains (376). Furthermore, the number of protein-coding genes predicted and annotated was 3,909 ± 35 (mean ± SEM of the 6 isolates), in range with the number of protein-coding genes usually reported for *A. baumannii* (405).

Table 19. Comparison of the general features of the bacteraemic isolates after whole-genome sequencing data processing.

Isolates	Mean coverage (X)	No. contigs	Contig N_{50} (bp)	Contig total length (bp)[a]	G+C (%)	No. predicted proteins
B1	109	187	121,094	4,079,150	39.13	4,038
B4	102	167	110,911	4,033,331	39.12	3,943
B7	88	197	91,138	3,938,157	39.03	3,890
B8	74	163	94,216	3,894,247	39.04	3,796
B9	80	168	103,992	4,029,100	39.12	3,940
B11	102	160	89,463	3,928,781	39.02	3,847

[a]Total genome assembled with SPAdes.

3.2. Molecular typing of the clinical isolates by MLST.

Bartual/Oxford MLST scheme assigned ST218 for B1 and ST208 for the other five isolates (Table 20). Therefore, all isolates were thus assigned to CC92 in the Bartual/Oxford scheme according to the current definition of CC, as explained in Section 2.2 of Results (Chapter I). Pasteur MLST scheme, assigned ST2 for B1, ST745 for B4 and B9, and ST187 for B7, B8, and B11 isolates. All isolates were thus assigned to CC2 in the Pasteur scheme (Section 2.2 of Results, Chapter I). So, these six isolates belong to CC92 (Bartual/Oxford)/CC2 (Pasteur), the largest and most widely distributed *A. baumannii* global clone (47), as isolates from Chapter I.

PFGE profiles were better accordance with the Pasteur MLST scheme, indicating also in this case a slightly better discriminatory power for these two methodologies as compared to the MLST Oxford scheme. Nevertheless, there was no association between the bacterial clonality and the clinical outcomes of the patients.

Table 20. Summary of the multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) typing results for the six bacteraemic isolates.

Isolates	MLST Bartual/Oxford scheme	MLST Pasteur scheme	PFGE
B1	ST218	ST2	Pulsotype C
B4	ST208	ST745	Pulsotype A
B7	ST208	ST187	Pulsotype B
B8	ST208	ST187	Pulsotype B
B9	ST208	ST745	Pulsotype A
B11	ST208	ST187	Pulsotype B

3.3. Antimicrobial resistance mechanisms.

Several genes related to resistance to *β*-lactams, aminoglycosides, sulfonamides, and tetracyclines, in addition to point mutations in target genes associated with resistance to quinolones and rifampicin, were detected in most isolates by WGS data analysis using ResFinder and BLASTP (Table 21), explaining most of the antimicrobial susceptibility profiles. In fact, these antimicrobial resistance mechanisms were exactly the same as those found in isolates from Chapter I, existing again an association between their composition of antimicrobial resistance mechanisms and the clone they belonged to, but no with the clinical outcomes of the CRAB bacteraemia.

Table 21. Antimicrobial resistance mechanisms found with ResFinder and BLASTP in the bacteraemic isolates.

Isolates	*β*-lactams	Aminoglycosides	Quinolones	Rifampicin	Sulfonamides	Tetracyclines
ST745 (B4 and B9)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$ *bla*$_{\text{OXA-58}}$	*aac(6')-Ip*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80L mutation in *parC*[b]	I581M mutation in *rpoB*	*sul1*, *sul2*	*tet(B)*
ST187 (B7, B8, and B11)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-66}}$ *bla*$_{\text{OXA-58}}$	*aac(3)-Ia*, *aadA1*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80Lmutation in *parC*[b]	S583L mutation in *rpoB*	*sul1*, *sul2*	*tet(B)*
ST2 (B1)	*bla*$_{\text{ADC-25}}$, *bla*$_{\text{OXA-109}}$ *bla*$_{\text{OXA-24/40}}$	*aac(6')-Ip*, *aac(3)-IIa*, *strA*, *strB*	S83L mutation in *gyrA*[a], S80L mutation in *parC*[b]	-	*sul1*	*tet(B)*

[a]Amino acid position equivalent to Ser-83 of *E. coli*. [b]Amino acid positions equivalent to Ser-80 and Ser-463 of *E. coli*.

On the other hand, with the additional bioinformatics analysis, in which genes with functional annotations including either the term "resistance" or "antibiotic" were considered as resistance genes, a more extensive list of resistance genes were obtained from the six bacteraemic isolates (Table 22), although there were still no differences between isolates from surviving and non-surviving patients. Most genes detected with ResFinder appeared in this list, although some genes did not. On the other hand, genes not considered by ResFinder, but identified within this analysis, were genes encoding multidrug efflux pumps, genes conferring resistance to antiseptics (acriflavine, for example) and antimicrobials not considered in this study (such as bicyclomycin or fosmidomycin), and heavy metal resistance genes. In addition, it is worth noting that isolate B1 contained a higher number of these resistance genes in comparison with the other bacteraemic isolates.

Table 22. Genes with "resistance" or "antibiotic" functional annotations found in the bacteraemic isolates.

Gene name	Description	Isolates presenting the corresponding gene					
mexB	Multidrug resistance protein MexB	B1	B4	B7	B8	B9	B11
nolG	Acriflavine resistance protein	B1	B4	B7	B8	B9	B11
yhcA	Putative multidrug resistance protein	B1	B4	B7	B8	B9	B11
emrY	Drug resistance transporter EmrB/QacA suBfamily	B1	B4	B7	B8	B9	B11
emrB	Multidrug resistance protein B	B1	B4	B7	B8	B9	B11
smvA	Major facilitator superfamily methyl viologen resistance protein	B1	B4	B7	B8	B9	B11
czcC	Cobalt-zinc-cadmium resistance protein CzcC	B1	B4	B7	B8	B9	B11
farB	MFS transporter, DHA2 family, multidrug resistance protein B	B1	B4	B7	B8	B9	B11
ypnP	Multidrug resistance protein, MATE family	B1	B4	B7	B8	B9	B11
norM	Multidrug resistance protein, MATE family	B1	B4	B7	B8	B9	B11
acrA	Acriflavine resistance protein A	B1	B4	B7	B8	B9	B11
fsr	Fosmidomycin resistance protein	B1	B4	B7	B8	B9	B11
mexB	Resistance-nodulation-cell division (RND) multidrug efflux transporter MexB	B1	B4	B7	B8	B9	B11
merA	Mercuric reductase	B1	-	-	-	-	-
tetB	Tetracycline resistance protein, class B	B1	B4	B7	B8	B9	B11
bcr	Bicyclomycin resistance protein	B1	B4	B7	B8	B9	B11
ABSDF2341	Putative multidrug resistance protein	B1	B4	B7	B8	B9	B11
ABAYE2235	Putative tetracycline resistance protein	B1	B4	B7	B8	B9	B11

emrA	Membrane fusion component of tripartite multidrug resistance system	B1	B4	B7	B8	B9	B11
ampC	Beta-lactamase	B1	B4	B7	B8	B9	B11
M3Q_pABCC8	Toxic anion resistance protein TelA	B1	-	B7	B8	-	B11
yBhR	Antibiotic transport system permease protein	B1	B4	B7	B8	B9	B11
emrA	Multidrug resistance protein A	B1	B4	B7	B8	B9	B11
pac	Penicillin amidase	B1	B4	B7	B8	B9	B11
emrA	Putative multidrug resistance efflux pump	B1	B4	B7	B8	B9	B11
arsB	Arsenical-resistance protein	B1	B4	-	-	B9	-
cacD	Cobalt-zinc-cadmium resistance protein CzcD	B1	B4	B7	B8	B9	B11
ABAYE0009	Putative RND type efflux pump involved in aminoglycoside resistance (AdeT)	B1	B4	B7	B8	B9	B11
ABAYE0008	Putative RND type efflux pump involved in aminoglycoside resistance (AdeT)	B1	B4	B7	B8	B9	B11
adeT	RND efflux pump involved in aminoglycoside resistance	B1	B4	B7	B8	B9	B11
ydhJ	Multidrug resistance protein A	B1	B4	B7	B8	B9	B11
adeT	RND type efflux pump involved in aminoglycoside resistance	B1	B4	B7	B8	B9	B11
folP	Dihydropteroate synthase	-	B4	B7	B8	B9	B11
aac(3)II	Aminoglycoside N(3)-acetyltransferase III	B1	-	-	-	-	-
mrcB	Penicillin-binding protein 1B	B1	B4	B7	B8	B9	B11
sul1	Dihydropteroate synthase	B1	B4	B7	B8	B9	B11
strB	Streptomycin resistance protein	B1	B4	B7	B8	B9	B11
cpo	Chloride peroxidase	B1	B4	B7	B8	B9	B11
uppP	Undecaprenyl-diphosphatase	B1	B4	B7	B8	B9	B11
blaoxa58	Beta-lactamase	-	B4	B7	B8	B9	B11
pcoB	Copper resistance protein B	B1	B4	B7	B8	B9	B11
gacA	Response regulator (Global antibiotic and cyanide control protein, LuxR/UhpA family)	B1	B4	B7	B8	B9	B11
A1S_0317	Putative fusaric acid resistance protein	B1	B4	B7	B8	B9	B11
yahN	Resistance factor to homoserine/threonine, RhtB family	B1	B4	B7	B8	B9	B11
yrhP	Resistance factor to homoserine/threonine, RhtB family	B1	B4	B7	B8	B9	B11
tetR	Tetracycline repressor protein	B1	B4	B7	B8	B9	B11
aac(6')I	Aminoglycoside acetyltransferase (6') type I	B1	B4	-	-	B9	-
ABAYE1699	Putative transcriptional repressor of multidrug resistance pump (MarR family)	B1	B4	B7	B8	B9	B11
tmrB	Tunicamycin resistance protein	B1	-	-	-	-	-
aac(3)I	AAC(3)-Ia aminoglycoside (3) acetyltransferase	-	-	B7	B8	-	B11
aadA1	Aminoglycoside-3"-adenylyltransferase ANT(3")-I	B1	B4	B7	B8	B9	B11
ABAYE1609	Putative transcriptional repressor of multidrug resistance pump (MarR family)	B1	B4	B7	B8	B9	B11
macB	Macrolide export ATP-binding/permease protein MacB3.6.3	B1	B4	B7	B8	B9	B11
merR	MerR regulatory protein	B1	B4	B7	B8	B9	B11

ohr	Organic hydroperoxide resistance protein	B1	B4	B7	B8	B9	B11
carO	Carbapenem-associated resistance protein	B1	B4	B7	-	-	-
merC	Mercuric resistance protein	B1	B4	B7	B8	B9	B11
sugE	Quaternary ammonium compound-resistance protein	B1	B4	B7	B8	B9	B11
merD	Mercuric resistance transcriptional repressor protein	-	B4	-	-	B9	-
AQ481_00120	Antibiotic biosynthesis monooxygenase	B1	B4	B7	B8	B9	B11
pcoB	Copper resistance protein CopB	B1	-	-	-	-	-
pcoA	Copper resistance protein A	B1	B4	B7	B8	B9	B11
eBr	Delta-quaternary ammonium resistance protein	B1	-	-	-	-	-
merA	Mercuric reductase	B1	B4	B7	B8	B9	B11
emrB	Drug resistance transporter EmrB/QacA suBfamily	B1	B4	B7	B8	B9	B11

3.4. Virulence factors.

Sixty-six out of the 82 known virulence genes contained in the reference *A. baumannii* isolate AB0057 were found in the six bacteraemic isolates (Table 23), belonging to all virulence factor classes analysed (adherence, biofilm formation, phospholipase enzymes, immune evasion, iron uptake, regulation, and serum resistance). Only two genes involved in heme utilization were detected. Moreover, the number of genes related to capsule formation were variable among isolates, but it was again associated with the ST type, possessing isolate B1, which belonged to ST2, the least number of these genes. Thus, the composition of known virulence genes had no association with the clinical outcomes of the CRAB bacteraemia.

Table 23. Virulence genes found in the bacteraemic isolates.

Virulence factor class	Virulence factors[a]	Related genes[a]	Isolates presenting the corresponding gene					
Adherence	Outer membrane protein	*ompA*	B1	B4	B7	B8	B9	B11
		adeF	B1	B4	B7	B8	B9	B11
		adeG	B1	B4	B7	B8	B9	B11
	AdeFGH efflux pump/transport autoinducer	*adeH*	B1	B4	B7	B8	B9	B11
	Biofilm associated protein	*bap*	B1	B4	B7	B8	B9	B11
		csuA/B	B1	B4	B7	B8	B9	B11
		csuA	B1	B4	B7	B8	B9	B11
		csuB	B1	B4	B7	B8	B9	B11
		csuC	B1	B4	B7	B8	B9	B11
		csuD	B1	B4	B7	B8	B9	B11
	Csu pili	*csuE*	B1	B4	B7	B8	B9	B11
		pgaA	B1	B4	B7	B8	B9	B11
		pgaB	B1	B4	B7	B8	B9	B11
		pgaC	B1	B4	B7	B8	B9	B11
Biofilm formation	PNAG	*pgaD*	B1	B4	B7	B8	B9	B11
	Phospholipase C	*plC*	B1	B4	B7	B8	B9	B11
Enzyme	Phospholipase D	*plD*	B1	B4	B7	B8	B9	B11
		lpsB	B1	B4	B7	B8	B9	B11
		lpxA	B1	B4	B7	B8	B9	B11
		lpxB	B1	B4	B7	B8	B9	B11
		lpxC	B1	B4	B7	B8	B9	B11
		lpxD	B1	B4	B7	B8	B9	B11
Immune evasion	LPS	*lpxL*	B1	B4	B7	B8	B9	B11

	lpxM	B1	B4	B7	B8	B9	B11
	AB57_0091	B1	B4	B7	B8	B9	B11
	AB57_0092	B1	B4	B7	B8	B9	B11
	AB57_0093	B1	B4	B7	B8	B9	B11
	AB57_0094	B1	B4	B7	B8	B9	B11
	AB57_0095	B1	B4	B7	B8	B9	B11
	AB57_0096	B1	B4	B7	B8	B9	B11
	AB57_0097	B1	B4	B7	B8	B9	B11
	AB57_0098	B1	B4	B7	B8	B9	B11
	AB57_0099	B1	B4	B7	B8	B9	B11
	AB57_0100	B1	B4	B7	B8	B9	B11
	AB57_0101	B1	B4	B7	B8	B9	B11
	AB57_0102	B1	B4	B7	B8	B9	B11
	AB57_0103	B1	B4	B7	B8	B9	B11
	AB57_0104	B1	B4	B7	B8	B9	B11
	AB57_0105	B1	B4	B7	B8	B9	B11
	AB57_0106	B1	B4	B7	B8	B9	B11
	AB57_0107	B1	B4	B7	B8	B9	B11
	AB57_0108	-	B4	B7	B8	B9	B11
	AB57_0109	-	B4	B7	B8	B9	B11
	AB57_0110	-	B4	B7	B8	B9	B11
	AB57_0111	-	B4	B7	B8	B9	B11
	AB57_0112	-	-	-	-	-	-
	AB57_0113	-	-	-	-	-	-
	AB57_0114	-	-	-	-	-	-
Capsule	AB57_0115	-	-	-	-	-	-

		barA	B1	B4	B7	B8	B9	B11
		barB	B1	B4	B7	B8	B9	B11
		basA	B1	B4	B7	B8	B9	B11
		basB	B1	B4	B7	B8	B9	B11
		basC	B1	B4	B7	B8	B9	B11
		basD	B1	B4	B7	B8	B9	B11
		basF	B1	B4	B7	B8	B9	B11
		basG	B1	B4	B7	B8	B9	B11
		basH	B1	B4	B7	B8	B9	B11
		basI	B1	B4	B7	B8	B9	B11
		basJ	B1	B4	B7	B8	B9	B11
		bauA	B1	B4	B7	B8	B9	B11
		bauB	B1	B4	B7	B8	B9	B11
		bauC	B1	B4	B7	B8	B9	B11
		bauD	B1	B4	B7	B8	B9	B11
		bauE	B1	B4	B7	B8	B9	B11
		bauF	B1	B4	B7	B8	B9	B11
	Acinetobactin	*entE*	B1	B4	B7	B8	B9	B11
		hemO	-	-	-	-	-	-
		AB57_0984	B1	B4	B7	B8	B9	B11
		AB57_0985	-	-	-	-	-	-
		AB57_0986	-	-	-	-	-	-
		AB57_0987	-	-	-	-	-	-
		AB57_0988	-	-	-	-	-	-
		AB57_0989	-	-	-	-	-	-
Iron uptake	Heme utilization	AB57_0990	-	-	-	-	-	-

		AB57_0992	-	-	-	-	-	-
		AB57_0993	B1	B4	B7	B8	B9	B11
		abaI	B1	B4	B7	B8	B9	B11
	Quorum sensing	*abaR*	B1	B4	B7	B8	B9	B11
		bfmR	B1	B4	B7	B8	B9	B11
Regulation	Two component system	*bfmS*	B1	B4	B7	B8	B9	B11
Serum resistance	Polysaccharide polymer	*pbpG*	B1	B4	B7	B8	B9	B11

[a]Virulence factors/genes described in the *A. baumannii* isolate AB0057. PNAG: polysaccharide poly-N-acetylglucosamine; LPS: lipopolysaccharide.

Objective 4. To find new bacterial virulence mechanisms that may be influencing patient mortality: genomic, transcriptomic, and proteomic approaches.

4.1. Comparative genomic study between isolates from non-surviving patients and isolates from surviving patients.

4.1.1. Differential presence of genes between *A. baumannii* isolates obtained from non-surviving and surviving patients.

In order to find any genomic difference between isolates from non-surviving and surviving patients, WGS data was analysed following several bioinformatics protocols.

First, the amino acid sequences of the predicted and annotated protein-coding genes (step 1 of Figure 7) of all the isolates were clustered by CD-HIT, obtaining 3444 clusters that had predicted protein-coding genes shared by all isolates (core-genome) (step 2 of Figure 7). Then, predicted proteins of each isolate were compared with predicted proteins from the core-genome, and differential proteins between both groups of isolates, were searched. However, no protein-coding gene presented in all isolates from non-surviving patients but absent in all isolates from surviving patients, or vice versa, was found (step 2 of Figure 7). Some couples of isolates from non-surviving patients had common genes not found in isolates from surviving patients, and vice versa, mainly annotated as uncharacterized, except for a gene encoding a ParB-like nuclease common between B1 and B7 isolates but not related to pathogenicity (step 2 of Figure 7).

Notwithstanding, variants of some proteins were identified as differential between both groups of isolates with this first protocol based on CD-HIT protein clustering. So, a more specific protocol was designed using BLASTP to study these variants with higher accuracy (step 3 of Figure 7). Thus, the most notable difference between both groups of clinical isolates was noted at the *carO* locus encoding the OMP CarO. While the *carO* genes present in the isolates B1, B4 and B7 from the non-surviving patients were intact, those of the B8, B9, and B11 isolates from the surviving patients were found to be prematurely interrupted by different mutational events (see below). Other differences between isolates involved loci corresponding to bacteriophage genes, and implied small changes in length either at the N-terminus or C-terminus of the encoded proteins, or disruptions in the case of the B1 isolate. In addition, differences in length were also observed in the gene encoding the giant biofilm-associated protein Bap. However, the

multiple arrays featuring immunoglobulin-like motifs common to this protein (417) made difficult the accurate assembly of the sequence data obtained for the different isolates, and whether significant differences exist at these loci between both groups of isolates is uncertain. Similar situation occurred for the locus encoding the pilus assembly protein FilE, which was found in both groups of isolates encoding a variable number of repeats of the tripeptide TAP in the different isolates (data not shown).

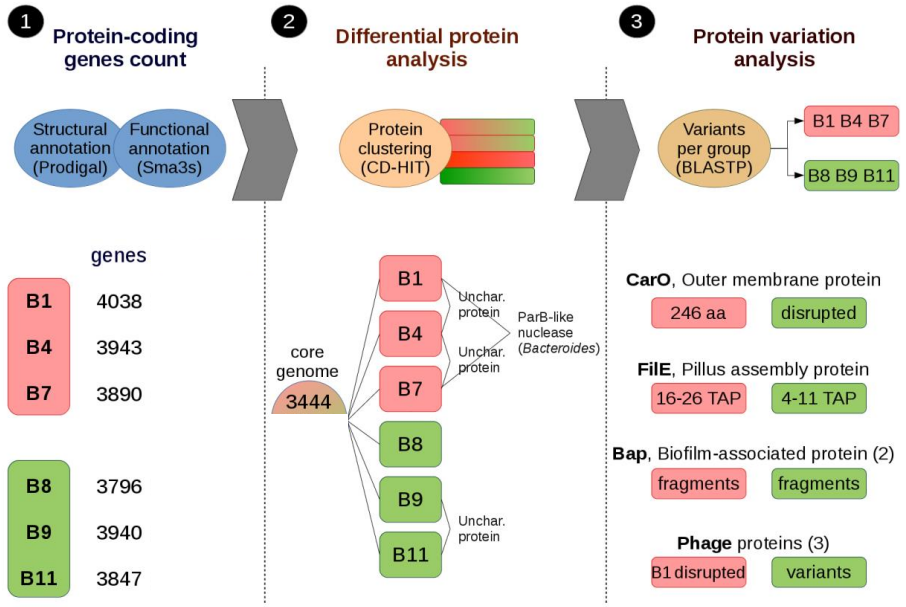

Figure 7 (steps 1-3). Designed protocol to discover differences between isolates from non-surviving and surviving patients. (1) All genomic assemblies were structural (by Prodigal) and functional (by Sma3s) annotated and the number of protein-coding genes for each isolate was counted. Isolates from non-surviving patients are highlighted in red colour, and isolates from surviving patients are highlighted in green colour. (2) Then, the proteins for all the isolates were clustered (by CD-HIT) to discover common proteins among the different isolates. 3444 clusters had proteins shared by all isolates (core genome). Additionally, some proteins are specific for several (not all) isolates from non-surviving patients or isolates from surviving strains. (3) Finally, variants between isolates from non-surviving patients and isolates from surviving patients were discovered (by BLASTP), and 7 proteins were found showing different versions between each isolate group: CarO, FilE, 2 Bap sequences, and 3 phage proteins.

Moreover, WGS proves that all isolates were different, presenting exclusive protein-coding genes that were not in other isolates (Figure 8). In fact, this reinforce our results, due to despite the high similarity between isolates B4 and B9, patient infected with B4 (which had a functional CarO) died, while patient infected with B9 (which had a truncated CarO) did not, and the same occurs with B7 and B8, or B7 and B11.

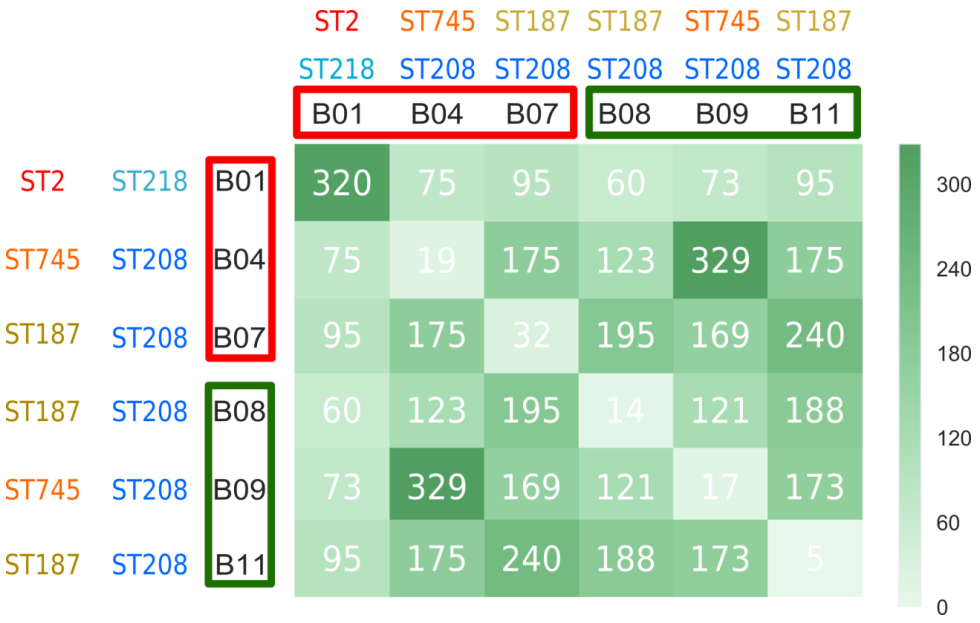

Figure 8. Number of protein-coding genes exclusively shared between couples of isolates, which were not in the other isolates. The higher is the number of shared protein-coding genes the greenest is the cell of the matrix. Isolates from non-surviving patients are highlighted in red colour, and isolates from surviving patients in green colour. Together the isolate names, both the Pasteur and Oxford MLST are shown.

4.1.2. CarO sequence characterization.

CarO protein sequences (complete and defective) inferred from the WGS data of the six isolates and their corresponding alignments are shown in Figure 9. As noted above, complete *carO* genes each encoding the same protein of 246 amino acids were found in the isolates from the non-surviving patients (B1, B4, and B7). In addition, the comparison of the amino acid sequences of these CarO proteins with the four allelic variants present

in the *A. baumannii* population (401) indicated that these isolates carried the same variant, designated CarOIII (401). In contrast, the *carO* genes in isolates B8, B9, and B11 from the surviving patients were all prematurely interrupted by separate mutational events. Isolate B8 showed a 2-bp insertion in the *carO* gene introducing a frame-shift resulting in a premature stop codon and a putative truncated protein of only 118 amino acids long, isolate B9 a 1-bp insertion resulting in a putative truncated protein of only 125 amino acids long, and in isolate B11 an IS*Aba1* insertion was detected inside *carO* resulting in a putative truncated protein of only 104 amino acids long. Therefore, from the eight antiparallel β-strands that conform the CarO protein, as judged by crystallographic analysis (418), isolates from non-surviving patients would generate, at best, only truncated CarO proteins ending shortly after the β-strand 4 (Figure 9).

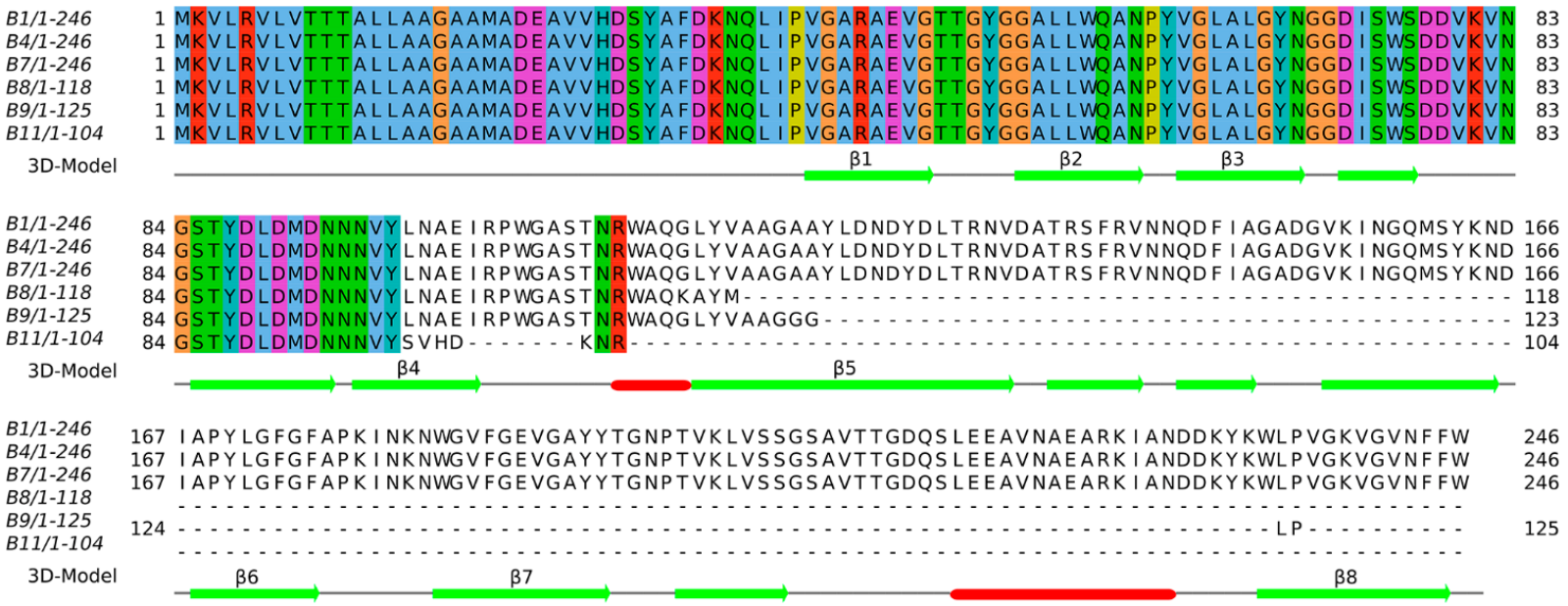

Figure 9. Alignment of CarO amino acids sequences from the six *A. baumannii* clinical isolates. The sequences have been aligned with MAFFT v7.312 using default setting. The numbers represent the positions of amino acids. CarO protein was intact in the isolates from non-surviving patients (B1, B4, and B7), while it was truncated in those from surviving patients (B8, B9, and B11). The secondary structure obtained from the PDB structure 4fuv.1.A is sh6own in the 3D-Model track: α-helices in red colour, and β-strands in green colour. The eight strands from the CarO β-barrel have been tagged (β1-β8).

4.2. Comparative study of *ompA* expression levels between isolates from non-surviving patients and isolates from surviving patients.

As the main difference found between colonising and invasive isolates from Chapter I was the level of expression of *ompA* gene, it was also determined in the bacteraemic isolates. In this case, the six bacteraemic isolates exhibited a higher *ompA* expression according to the qRT-PCR analysis, in comparison with the expression levels found in the isolates from Chapter I (Table 24), being even higher than in the invasive isolates. However, no significant differences were found between bacteraemic isolates from non-surviving and surviving patients (Table 24).

Table 24. *ompA* expression levels of the bacteraemic clinical isolates.

Isolates		*ompA* expression	*ompA* expression Mean ± SEM
From non-surviving patients	B1	4.07	5.38 ± 1.05[a]
	B4	4.63	
	B7	7.45	
From surviving patients	B8	2.12	4.82 ± 1.89[a]
	B9	8.47	
	B11	3.86	

[a]$P = 0.7000$ for isolates from non-surviving patients *vs.* isolates from surviving patients, Mann-Whitney test.

4.3. Comparative proteomic studies between isolates from non-surviving patients and isolates from surviving patients.

4.3.1. OMP profiles and detection of CarO by immunoblot and LC-MSMS analyses.

A comparative study of the OMP profiles were performed with the OM fractions of the six bacteraemic isolates with the aim of detecting any OMP difference between both

groups of isolates. Thus, in agreement with the WGS analysis of the isolates, a ~29 kDa protein band corresponding to the expected migration of CarO (129, 401) was observed only in the isolates from the non-surviving patients by SDS-PAGE/Western blot analyses (Figure 10A-B). LC-MSMS analysis confirmed the identity of this band as CarO (data not shown). Similar analysis failed to indicate the presence of CarO in isolates from the surviving patients (Figure 10A-B).

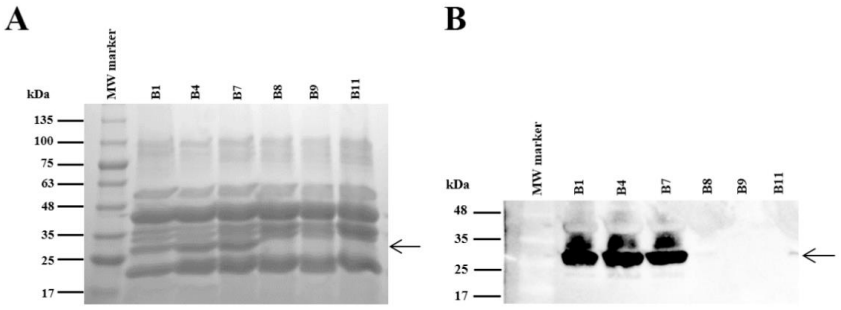

Figure 10 (A-B). Profiles of outer membrane proteins and immunodetection of CarO. Outer membrane fractions were extracted from the bacteraemic *A. baumannii* clinical isolates from the non-surviving patients (B1, B4, and B7) and from surviving patients (B8, B9, and B11), and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Simply Blue SafeStain (A) or immunoblotting with polyclonal rabbit antibodies toward *A. baumannii* CarO (B). Molecular mass standards (kDa) are shown on the left. Black arrows on the right indicate the final positions of CarO. Abbreviation: MW, molecular weight.

4.3.2. Quantitative proteomic study.

In addition to analysing genome- and OMP-level differences between both groups of isolates, a total protein-level comparison was also performed. A total of 1,988 proteins were identified by TMT-6 plex from the six isolates (data not shown). In the quantitative analysis, 57 proteins were over- or down-expressed in the isolates from surviving patients compared with the isolates from non-surviving patients with a *P-value* < 0.05 (data not shown). However, only one protein, CarO, was quantitatively different with a *P-value* < 0.05 and a *q-value* ≤ 0.05 (FDR ≤ 5%). CarO was "down-expressed" in the isolates from surviving patients with a $\log_2$ fold change of -2.003, *P-value* = 2E-05 and *q-value* = 0.039.

This result confirmed again the genomic results, although to be more exact, CarO was not "down-expressed", it was directly "non-expressed" by isolates from surviving patients. Nonetheless, besides CarO, there was no other protein with a differential expression between both groups of isolates.

Objective 5. To analyse the virulence role of CarO in *A. baumannii* using model systems.

5.1. OMP profiles and detection of CarO by immunoblot analysis.

CarO has been previously associated to the selective permeation of basic amino acids and structurally-related compounds across the *Acinetobacter* OM, such as the antibiotic imipenem (129-131, 401, 418, 419). Since the above results suggest that CarO could additionally play virulence functions in *A. baumannii*, we decided to analyse it further using model systems. We selected for this purpose *A. baumannii* ATCC 17978 (wt) (155, 353, 401, 420, 421), its isogenic Δ*carO* mutant strain (Δ*carO*), the complemented strain (Δ*carO*/pWH1266-*carO*), and two control strains (wt/pWH1266 and Δ*carO*/pWH1266). As expected, the presence of CarO was detected by SDS-PAGE/Western blot analyses in all the studied strains except in the Δ*carO* mutant lacking or containing the plasmid vector pWH1266 (Figures 11A-B).

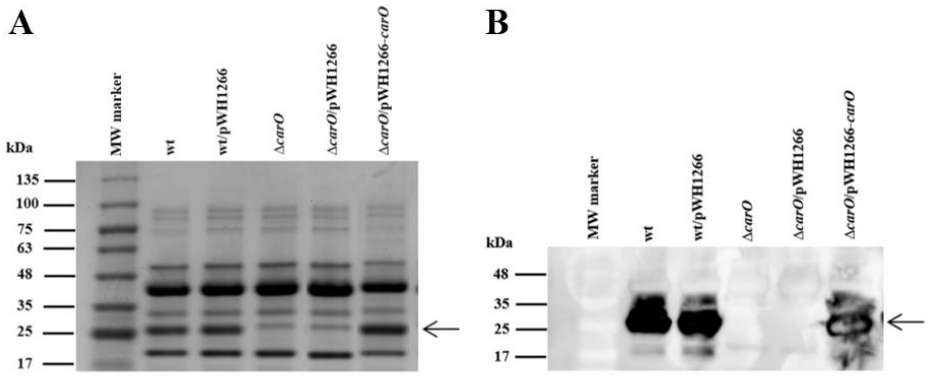

Figure 11 (A-B). Profiles of outer membrane proteins and immunodetection of CarO. Outer membrane fractions were extracted from the laboratory strains and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with

Simply Blue SafeStain (A) or immunoblotting with polyclonal rabbit antibodies toward *A. baumannii* CarO (B). Strains: *A. baumannii* ATCC 17978 wild-type (wt); wt with empty plasmid pWH1266 as control (wt/pWH1266); its isogenic *carO* deletion mutant (Δ*carO*); Δ*carO* mutant with empty plasmid as control (Δ*carO*/pWH1266); and, Δ*carO* mutant with plasmid pWH1266-*carO* expressing CarO (Δ*carO*/pWH1266-*carO*). Molecular mass standards (kDa) are shown on the left. Black arrows on the right indicate the final positions of CarO. Abbreviation: MW, molecular weight.

5.2. Effect of CarO on *in vitro* growth and bacterial fitness.

The effect of the absence of CarO on the growth and fitness of *A. baumannii* ATCC 17978 in rich media and in human serum was assessed as control for the rest of experiments (Figure 12). When wt, mutant, complemented and control strains were cultured independently on MHB, all showed similar growth profiles. Similar results were found when they were independently grown on inactivated human serum, although an extended lag period was observed compared to MHB growth. When wt and Δ*carO* strains were grown together on MHB, the Δ*carO* cells showed a modest loss of fitness compared to the wt cells (CI: 0.09, 0.63, 0.65, 0.69 at 2, 24, 48, and 72 h, respectively). On human serum, similar effect was detected at 2 and 8 h (CI: 0.21 and 0.55, respectively), while the Δ*carO* mutant showed a slightly higher fitness at the other time points. These results suggest that the loss of CarO does not significantly affect *A. baumannii* growth/fitness in both media.

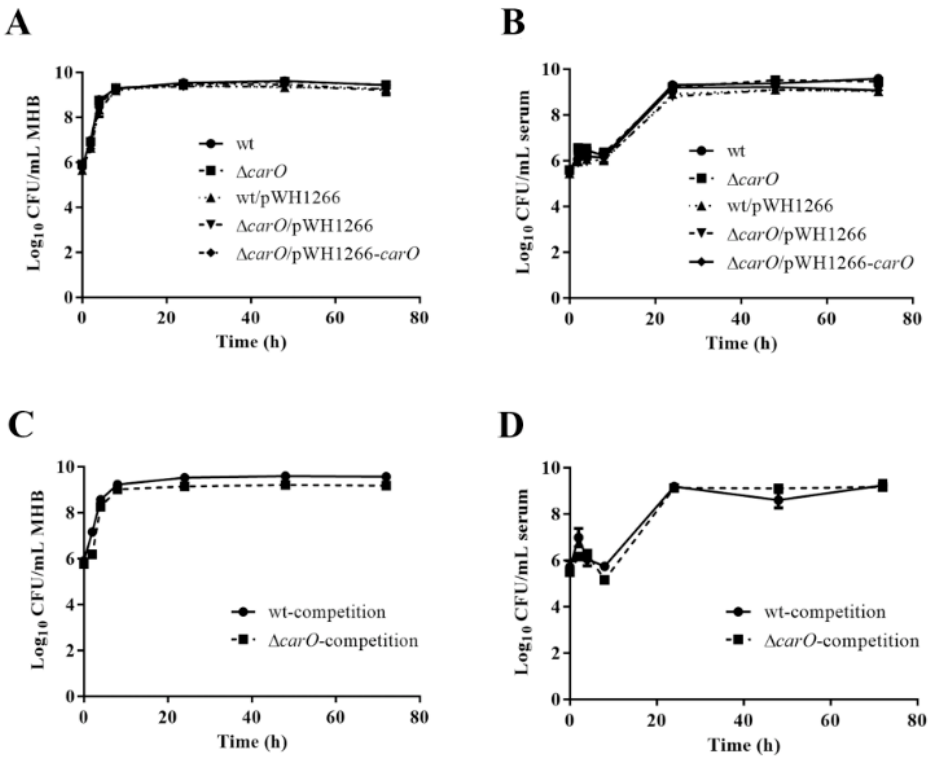

Figure 12 (A-D). *In vitro* bacterial growth and competition studies in MHB and serum. All laboratory bacterial strains: *A. baumannii* ATCC 17978 wild-type (wt); its isogenic *carO* deletion mutant (Δ*carO*); wt with empty plasmid pWH1266 as control (wt/pWH1266); Δ*carO* mutant with empty plasmid as control (Δ*carO*/pWH1266); and, Δ*carO* mutant with plasmid pWH1266-*carO* expressing CarO (Δ*carO*/pWH1266-*carO*) were grown in MHB (A) and inactivated human serum (B) for 72 h. In addition, wt and Δ*carO* mutant strains were grown together in competition also in MHB (C) and serum (D) during 72 h. Data are represented as mean ± SEM (n=3 replicates in different days). Abbreviations: MHB, Mueller-Hinton broth.

5.3. Effect of CarO loss on *A. baumannii* ATCC 17978 adherence and invasion of cultured human lung epithelial cells.

The loss of CarO reduced the adherence of 17978 wt cells to A549 human lung epithelial cells (31.56% ± 3.47% for Δ*carO vs.* 100% ± 5.45% for wt, $P < 0.0001$, Figure 13A). Moreover, adherence was recovered when CarO expression in the Δ*carO* mutant was

restored (Figure 13A). Similarly, the loss of *carO* reduced invasion of A549 cells by 17978 wt cells (32.85% ± 6.20% for Δ*carO vs.* 100% ± 2.46% for wt, $P < 0.0001$, Figure 13B). Again, complementation of Δ*carO* mutant restored invasion to wt levels (Figure 13B). These results suggest that CarO is involved in *A. baumannii* adhesion and invasion of human lung epithelial cells. On the other hand, the most simple explanation for the 30% residual binding of 17978 Δ*carO* mutants to cultured epithelial cells is that CarO is not the only factor involved in adherence and invasion, and that it plays a role in this process in conjunction with other proteins such as OmpA and Omp33 (144, 155). A similar explanation could apply for residual bacterial burden into mice tissues and fluids.

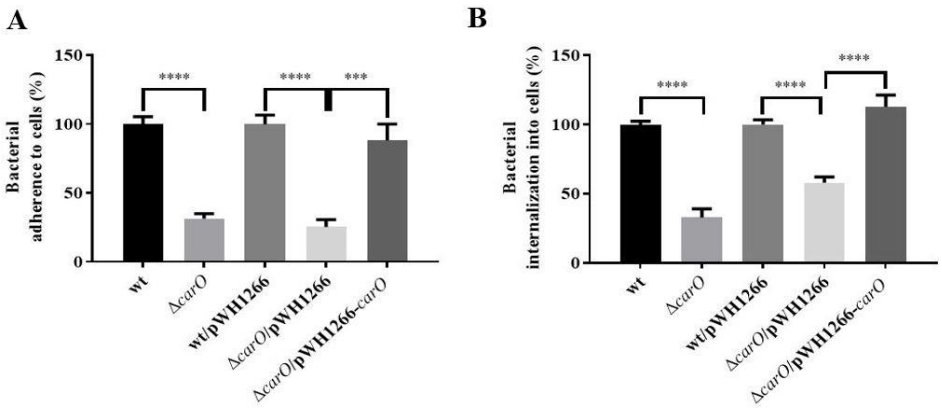

Figure 13 (A-B). *A. baumannii* adherence and invasion into A549 cells. A549 cell cultures were incubated with the different *A. baumannii* laboratory strains (ATCC 17978 wild-type (wt); its isogenic *carO* deletion mutant (Δ*carO*); wt with empty plasmid pWH1266 as control (wt/pWH1266); Δ*carO* mutant with empty plasmid as control (Δ*carO*/pWH1266); and Δ*carO* mutant with plasmid pWH1266-*carO* expressing CarO (Δ*carO*/pWH1266-*carO*)). The percentages of bacterial adherence (A) and invasion (B) were subsequently measured. Data are represented as mean ± SEM (n=3 replicates in different days). Asterisks indicate significant differences (** $P \leq 0.0001$; *** $P \leq 0.001$, Student's t-test and Mann-Whitney test) between means.**

5.4. Virulence roles of *A. baumannii* CarO evaluated by a non-discriminative peritoneal sepsis murine model.

5.4.1. Effect of CarO on the mortality rate induced in mice by *A. baumannii* 17978 cells.

We found differences in MLD between 17978 wt and Δ*carO* mutant strains (3.20 $\log_{10}$ CFU/mL *vs.* 4.30 $\log_{10}$ CFU/mL, respectively). Survival analyses (Figure 14) additionally revealed differences between mice groups inoculated with 3.2 $\log_{10}$ CFU/mL (MLD for the wt strain). All mice infected with the wt strain died within 24 h post-infection, while 67% of the animals infected with the Δ*carO* mutant died within 48 h, surviving the rest at least 7 days (P = 0.0289, wt *vs.* Δ*carO*). All mice infected with the complemented strain (Δ*carO*/pWH1266-*carO*) died within 24-48 h, similarly to the wt strain. These results indicated that CarO plays an important role in the infective capacity and mortality caused by *A. baumannii in vivo*.

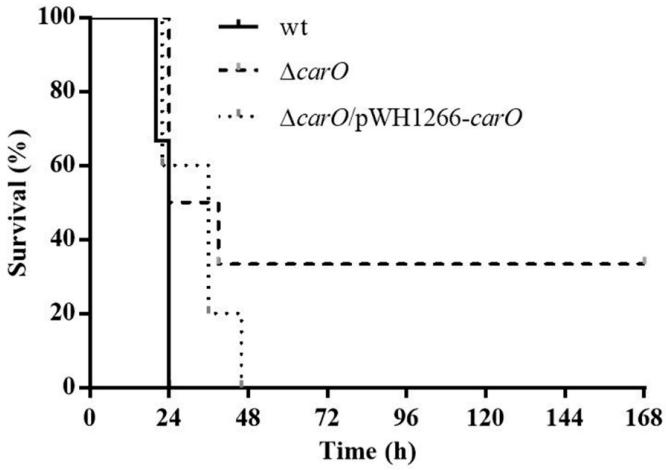

Figure 14. Mice survival. Analysis of survival time in the peritoneal sepsis model by 3.2 $\log_{10}$ CFU/mL of *A. baumannii* ATCC 17978 wild-type (wt), its isogenic *carO* deletion mutant (Δ*carO*), and its complemented strain (Δ*carO*/pWH1266-*carO*). Survival curves showed the percentages of mice survival during 7 days (n= 6 mice/strain). *P* = 0.0289 for wt *vs.* Δ*carO*, long-rank test.

5.4.2. Effect of CarO on the *in vivo* dissemination of *A. baumannii* 17978 cells into mice tissues and fluids.

We next compared the dissemination of the wt, mutant and complemented strains into different organs (spleen, lungs, liver and kidneys) and fluids (peritoneal fluid and blood) of mice inoculated i.p. with 3.2 $\log_{10}$ CFU/mL of each bacterial strain (Figure 15). Mice infected with the Δ*carO* strain showed at 8 h significant lower bacterial burden in all organs and fluids studied when compared with animals infected with the wt strain. Significant differences were found (Δ*carO vs.* wt) in spleen (5.78 ± 0.58 $\log_{10}$ CFU/g *vs.* 7.17 ± 0.34 $\log_{10}$ CFU/g; $P = 0.0175$, Figure 15A), lungs (5.28 ± 0.51 $\log_{10}$ CFU/g *vs.* 6.77 ± 0.40 $\log_{10}$ CFU/g; $P = 0.0175$, Figure 15B), liver (5.52 ± 0.50 $\log_{10}$ CFU/g *vs.* 6.72 ± 0.34 $\log_{10}$ CFU/g; $P = 0.0192$, Figure 15C), kidneys (5.25 ± 0.60 $\log_{10}$ CFU/g *vs.* 6.59 ± 0.49 $\log_{10}$ CFU/g; $P = 0.0175$, Figure 15D), and peritoneal fluid (5.68 ± 0.80 $\log_{10}$ CFU/g *vs.* 7.00 ± 0.48 $\log_{10}$ CFU/g; $P = 0.0175$, Figure 15E). These differences were greater after 24 h infection (Figure 15), finding significant differences (Δ*carO vs.* wt) in spleen (5.25 ± 0.99 $\log_{10}$ CFU/g *vs.* 9.90 ± 0.18 $\log_{10}$ CFU/g; $P < 0.001$, Figure 15A), lungs (5.38 ± 1.01 $\log_{10}$ CFU/g *vs.* 10.06 ± 0.17 $\log_{10}$ CFU/g; $P < 0.001$, Figure 15B), liver (5.09 ± 0.95 $\log_{10}$ CFU/g *vs.* 9.54 ± 0.10 $\log_{10}$ CFU/g; $P = 0.0006$, Figure 15C), kidneys (5.08 ± 0.89 $\log_{10}$ CFU/g *vs.* 9.56 ± 0.13 $\log_{10}$ CFU/g; $P = 0.0006$, Figure 15D), peritoneal fluid (4.24 ± 1.09 $\log_{10}$ CFU/g *vs.* 9.57 ± 0.09 $\log_{10}$ CFU/g; $P = 0.0004$, Figure 15E) and blood (3.32 ± 1.10 $\log_{10}$ CFU/g *vs.* 9.224 ± 0.23 $\log_{10}$ CFU/g; $P = 0.0012$, Figure 15F). Complementation of Δ*carO* mutant restored almost the wt count levels at 8 and 24 h. These results indicate that CarO affects the dissemination of *A. baumannii* in mice.

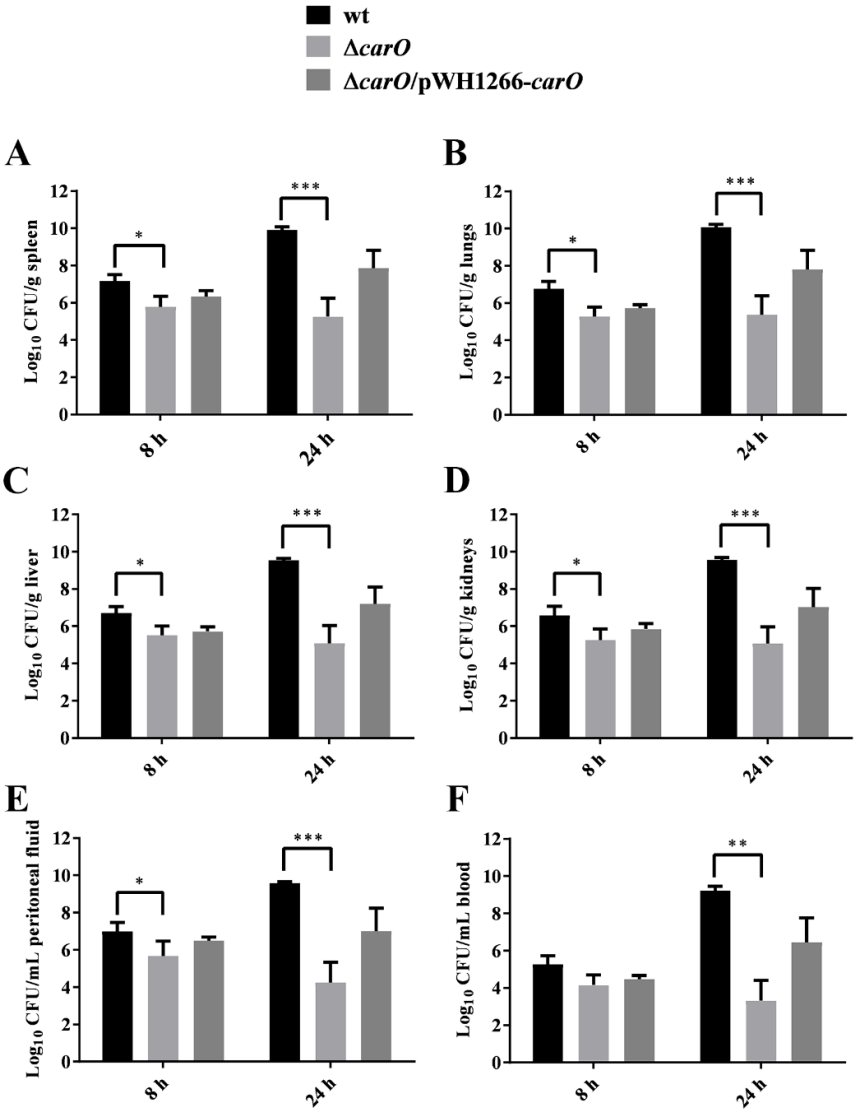

Figure 15 (A-F). Bacterial burdens in infected mice. Bacterial loads in spleen (A), lungs (B), liver (C), kidneys (D), peritoneal fluid (E), and blood (F), were determined at 8 h and 24 h after intraperitoneal infection with 3.2 $\log_{10}$ CFU/mL of *A. baumannii* ATCC 17978 wild-type (wt), its isogenic *carO* deletion mutant (Δ*carO*), and the Δ*carO* complemented strain (Δ*carO*/pWH1266-*carO*). Data are represented as mean ± SEM (n=7 mice/strain for each time point). Asterisks indicate significant differences (* $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$, Student's t-test and Mann-Whitney test) between means.**

DISCUSSION

Acinetobacter baumannii is undoubtedly one of the most successful pathogens responsible for hospital-acquired nosocomial infections in the modern healthcare system (30). This pathogen mainly affects critically-ill and immunocompromised patients admitted to ICU, causing infections such as pneumonia or, to a lesser extent, serious bloodstream infections, both associated with relatively long hospital stay and high mortality rates (422). The success of this bacterium as a nosocomial pathogen has been attributed to a combination of several factors: ability to tolerate and persist under unfavorable situations, relatively high virulence, and great ability to develop resistance to disinfectants, biocides and almost all available antimicrobial agents, including broad-spectrum antibiotics such as carbapenems (37, 113). Thus, the recent emergence of colistin resistance among CRAB strains has triggered a worldwide alert, considering the very few (or, in the worst case, lack of) therapeutic options left in these cases (11, 118, 423-425). For all these reasons, novel approaches are needed, such as the development of inhibitors blocking virulence factors, to treat *A. baumannii* infections (268, 322). However, the number of virulence factors described for *A. baumannii* is scarce, and moreover, they have not been usually studied in relation to the development of infection and mortality in patients (322). Therefore, the detection of novel microbiological factors involved in the pathogenesis of *A. baumannii* infections might help select new targets for the development of such therapeutic strategies (322), being this the main objective of the present Doctoral Thesis.

Chapter I. Study of the mechanisms involved in the pathogenesis of colonising and bacteraemic VAP producing *A. baumannii* clinical isolates.

A first step to achieve the main goal of this Doctoral Thesis is to have a better understanding of *A. baumannii* pathogenesis. In this context, due to the characteristics of the patients from whom this pathogen is isolated, it is often difficult to differentiate colonisation from infection (426), a situation that leads to diagnostic errors and unnecessary antibiotic therapy (75). In fact, many studies do not make the distinction between MDR *A. baumannii* colonisation and infection, and associate both status with higher mortality rates, compared to patients without evidence of *A. baumannii* (95, 427), or even to patients colonised or infected with MDR *P. aeruginosa* (96). Despite one study showed an increased mortality in ventilated patients with high endotracheal *P. aeruginosa*

colonisation burdens without clinical signs of infection (428), the majority of studies conclude that *A. baumannii* colonisation does not increase the mortality directly, as it does *A. baumannii* VAP and bacteraemia (66, 93, 429, 430). Nevertheless, *A. baumannii* colonisation prolongs mechanical ventilation and complicates its manipulation, and therefore extends ICU stays, thus weakening the patient even more, and this could ultimately be associated with a higher probability of death, but not the colonisation *per se*. Thus, in two of the few studies that distinguish *A. baumannii* colonisation from infection, performed by Álvarez-Marín *et al.* (106) and Martín-Aspas *et al.* (75), crude mortality rates of 27.9% and 22% in colonised patients was observed, respectively. Nonetheless, patients from these studies exhibited high Charlson index and APACHE II score, meaning that those mortalities could not be due to the *A. baumannii* colonisation, but because of the severity of their acute illnesses increased by their comorbidities. In addition, in the study of Álvarez-Marín *et al.*, patients with polymicrobial colonisation and infection were not excluded, as in most studies, whereby some patients with *A. baumannii* colonisation could have died because of other pathogen rather *A. baumannii*. The patient cohort described in this previous mentioned study carried out by Álvarez-Marín *et al.* is the one used in the present Doctoral Thesis. In both colonised and infected patients from this cohort, high *A. baumannii* burdens were presented in their lower respiratory tracts (≥ 6 $\log_{10}$ CFU/mL), and surprisingly, colonised patients with these high inoculums did not developed bacteraemic or non-bacteraemic VAP. Therefore, there are at least two possible explanations for this fact: first, *A. baumannii* colonizes or infects patients depending on the common and severe co-comorbidities they present, which weaken their immune systems and allow this pathogen to infect; and second, there are unique genetic features in *A. baumannii* that could be attributed to a specific pathogenic phenotype, colonising or invasive. Based on the second hypothesis, the exact mechanism by which *A. baumannii* causes infection or remains as a mere coloniser is unknown, although some virulence factors have been described, through *in vitro* and *in vivo* studies, to be involved in invasion of epithelial cells, such as OmpA (140) and Omp33 (155), among others (160).

Thus, in this Doctoral Thesis we selected colonising and invasive CRAB isolates from TBA samples of ICU adult patients with colonisation or bacteraemic VAP caused by this pathogen, respectively, and we studied *in vitro* and *in vivo* their virulence and searched for possible factors that could help to differ colonising from invasive isolates, that is,

those related to the development of the infection. It is worth mentioning that the *A. baumannii* clinical isolates selected were obtained from the same hospital setting in a limited timeframe that assure that same clinics with same criteria diagnose colonisation or infection. Moreover, strict criteria were used to select these isolates, assuring that only colonising isolates that exclusively acted as colonising in those patients, not presenting those patients VAP (CPIS ≥ 6) or bacteraemia during ICU stay, and invasive isolates that only acted as invasive, not presenting these patients previous positive surveillance cultures indicating *A. baumannii* colonisation, were selected. In addition, patients with polymicrobial colonisation or infection, situation that is frequent in UCI patients (431), were excluded, so patients' clinical outcomes could not be influenced by other pathogens different to *A. baumannii*. Additionally, selecting exclusively inoculums ≥ 6 $\log_{10}$ CFU/mL, we discarded possible invasive isolates that did not cause infection due to their low concentration in the lower respiratory tract, which could be mistakenly considered as colonising. Thus, colonising isolates that did not cause infection despite having an inoculum ≥ 6 $\log_{10}$ might be because they lack some important factors necessary to behave as an invasive isolate. To our knowledge, any previous study has taken into account these kinds of criteria for isolate selection. For example, in the study performed by Martín-Aspas *et al*., in patients from whom *A. baumannii* was recovered in several evolutionary moments, the possibility of infection or colonisation was considered in each of these moments, possibly including isolates that were invasive as colonising, and vice versa (75). In other study carried out by Sahl *et al.* (54), which is the only work that had a similar goal as ours, they compared colonising and invasive isolates but coming from diverse human sites. They considered as colonising those isolates coming from perinea or wounds, and as invasive those coming from blood, urine and cerebrospinal fluid. However, these isolates were obtained from different patient cohorts, and therefore in different hospital setting and time periods. As a result, they did not find any genomic region within the colonisation isolates that were absent in the invasion isolates, or vice versa.

However, when we analysed the *in vitro* virulence of all the initially selected colonising and invasive isolates, we did not found any significant difference between colonising and invasive isolates. This fact was due to the wide variability found within each group, seeming to be isolate-dependent instead of phenotype (colonising/invasive)-dependent, the *in vitro* virulence observed. Nonetheless, we selected for further experiments only the

colonising isolates with the lowest *in vitro* virulence, so a significant lower ability to induce cell death was then observed in these isolates when compared to invasive isolates. With this approach, a greater number of differences between both groups of isolates in the following experiments could be expected to be found. Nevertheless, similar results were obtained when we used a non-discriminative model of peritoneal sepsis to determine the virulence of the isolates. We failed to observe any significant difference *in vivo* according to the isolate phenotype (colonising/invasive). Again, a great variability in the LD results within both groups of isolates was observed, finding even colonising isolates with lower LD_0, LD_{50}, and MLD, and therefore higher *in vivo* virulence, than some invasive isolates. Recently, Shirazi *et al.* used a murine model of peritoneal sepsis to compare the virulence among MDR *A. baumannii* isolates from different sources, obtaining also results with high variability, not finding any association between bacterial concentrations in spleen and blood recovered from infected mice and the isolation source of the isolates, and therefore their phenotypes (432). Similarly, Antunes *et al.* described that clinical strains showed comparable virulence in the *Galleria mellonella* model of infection, irrespective of their status as outbreak or sporadic strains and their virulence potential (134).

On the other hand, we applied WGS and several bioinformatics tools to study if despite not having found differences in *in vitro* and *in vivo* virulence between both *A. baumannii* phenotypes, there would be any difference regarding their clonality, antimicrobial resistance mechanisms or virulence determinants. Thus, our study shows that all isolates belonged to the most widely disseminated *A. baumannii* clonal complex worldwide, CC92/CC2, which is highly linked with IC II and extensively includes outbreak and MDR isolates (47). However, two or three different STs, according to Bartual/Oxford or Pasteur MLST schemes, respectively, were found among our isolates, not finding any association with the colonising or invasive phenotypes. Is it noting that, in our case, PFGE and the Pasteur MLST scheme had higher discriminatory power than the Bartual/Oxford MLST scheme, unlike in other study (433). Our results were in agreement with those obtained in numerous previous studies, which demonstrated that clinical isolates, even those very successful at causing nosocomial outbreaks, have varying virulence phenotypes, thus finding isolates belonging to the same ST with different virulence profiles or even causing different clinical outcomes (54, 434-437). ST2 has been the most prevalent clone worldwide, including most Mediterranean countries, since 1999 until now (34, 426, 438,

439). ST2 has been found to be more frequently associated with OXA-23 (ST2/OXA-23), being considered as a high-risk clone due to its great ability to accumulate mechanisms of antimicrobial resistance and spread rapidly in a global or local way (440). However, our isolates belonged to the clone ST2/OXA-24/40, which was first identified in Andalusia in 2015 by the Andalusian Reference Laboratory PIRASOA (2015 PIRASOA Reference Laboratory Scientific Report: http://pirasoa.iavante.es/course/view.php?id=3&section=2), having caused two different outbreaks in two hospitals in Seville (2015 PIRASOA Reference Laboratory Scientific Report: http://pirasoa.iavante.es/course/view.php?id=3&section=2). The Andalusian Reference Laboratory PIRASOA is the reference laboratory for the molecular typing of nosocomial pathogens and the detection of antimicrobial resistance mechanisms of sanitary interest in Andalusia, which receives and analyses CRAB isolated in Andalusian hospitals, among other nosocomial MDR pathogens, and it is located in the Microbiology Service of the University Hospital Virgen Macarena. On the other hand, of the two other STs found among our isolates, only ST745/OXA-58, have been detected by the Andalusian Reference Laboratory, and it was in 2016, through isolates from four different Andalusian hospitals (2016 PIRASOA Reference Laboratory Scientific Report: http://pirasoa.iavante.es/course/view.php?id=3&section=2). The clone ST187/OXA-58 was similar to ST2/OXA-24/40 and ST745/OXA-58 (433), so probably it is also spreading among Andalusian hospitals along with the other clones, although nobody has yet sent it to the Andalusian Reference Laboratory to analyse. Recent data of the Andalusian Reference Laboratory (2018 PIRASOA Reference Laboratory Scientific Report: http://pirasoa.iavante.es/course/view.php?id=3&section=2), showed that clone ST745/OXA-58 was still found in several Andalusian hospitals, but no ST2/OXA-24/40. Nonetheless, in Andalusia the more predominant ST, as worldwide, was ST2/OXA-23, but surprisingly it was not found among the studied isolates in this project (2014, 2015, 2016, 2017, and 2018 PIRASOA Reference Laboratory Scientific Reports: http://pirasoa.iavante.es/course/view.php?id=3&section=2).

Similarly, the composition of antimicrobial resistance determinants did not show any association with the isolate phenotype (colonising/invasive). However, a clear distinction in antimicrobial susceptibilities and antimicrobial resistance mechanisms among STs was observed, as previously described (435). All isolates were CRAB and MDR, as expected when belonging to *A. baumannii* clonal complex CC92/CC2 (47), sharing also resistance

to ceftazidime and ciprofloxacin, as well as susceptibility to tigecycline and colistin, revealing the great similarity between the three STs, although the resistance mechanisms were not always the same. However, differences were found regarding other antimicrobial susceptibilities, such as aminoglycosides or rifampicin, that were ST-dependent. Thus, all isolates possessed the same resistance mechanisms for quinolones and tetracyclines, but differed in the others. Isolates from ST745 and ST187 shared the same β-lactamases, including the carbapenemases OXA-66 and OXA-58, and the same sulfonamide-resistance genes, but differed in AMEs composition and rifampicin resistance mechanism, while isolates from ST2 had different β-lactamases, lacked the sulfonamide-resistance gene *sul1*, and not possessed mutations conferring rifampicin-resistance, in comparison with isolates from the other two STs, but possessed similar AMEs to isolates from ST745. Furthermore, isolates belonging to the same ST could display different mechanisms, such as the presence of IS*Aba1* or OXA-24/40, demonstrating the plasticity of this microorganism (441) and its ability to adapt differently in different environments.

Despite sporadic studies suggested a correlation between the number and composition of already-known virulence genes with organism's phenotypic differences (160), we did not find it with our colonising and invasive clinical isolates. All isolates possessed most of the virulence genes examined, although a slight decrease in the number of virulence genes related to capsule formation were observed in isolates colonising and invasive isolates from ST2, but the exact function and relevance of these genes are still unknown, and besides that, these genes were not differentially presented in colonising or invasive isolates. In this context, although several studies demonstrated that some antibiotic resistance mechanisms caused a metabolic cost to the bacterium and hence the "anti-virulence" factor (7, 222), others reported that MDR and even PDR *A. baumannii* isolates could be hyper-virulent (442), containing a very high proportion of virulence genes (443). Our isolates might be not considered hyper-virulent, as the majority of the *A. baumannii* hypervirulent strains present a robust heme uptake system that included a putative heme oxygenase (*hemO*) (444), and it was absent in our isolates, but they displayed a great number of virulence genes, in addition to a high number of antimicrobial resistance genes. Kim *et al.* found similar results to ours, studying bacteraemic CRAB isolates that caused different clinical outcomes, finding no association between the number or composition of known virulence genes and the clinical outcomes (435). They suggested that the

mismatch between phenotypic and genotypic results could be due to individual virulence factors may not be important for *A. baumannii* virulence in human hosts, and that the same virulence factor may play different roles in different habitats. Further evaluation is therefore required regarding the authentic effects of these putative virulence genes, although all these data suggested that the key might be in virulence factors still unknown and/or virulence gene regulation (134).

Consequently, we performed a comparative genomic analysis to find possible genetic differences between colonising and invasive isolates that had not been described so far. But again, we fail to observe any genetic feature that could be correlated to each phenotype, colonising or invasive, not finding therefore any new virulence factor related to infection. Same results were found by Sahl *et al.*, as previously described (54).

On the other hand, as OmpA plays multiples roles in bacterial pathogenesis and it is one of the most studied virulence factor of *A. baumannii*, we measured its expression in our colonising and invasive isolates. To our surprise, invasive isolates exhibited a significantly higher *ompA* expression than the colonising ones, not being in addition clonal-dependent. Later, Smani *et al.* corroborated this phenomenon but using a higher number of TBA *A. baumannii* isolates from different clones, which belonged to two different clinical cohorts, one unicentre (the one used in this Doctoral Thesis) and one multicentre. They reported that isolates from non-bacteraemic or bacteraemic patients with VAP overexpressed more *ompA* than those from colonised patients from the both cohorts, and that isolates from bacteraemic VAP patients overexpressed non-significantly more *ompA* than those from non-bacteraemic VAP patients in both cohorts, suggesting that *ompA* overexpression is an associated factor for pneumonia, and even for bacteraemia and death due to *A. baumannii* (147). Recently, María Tomas *et al.* performed a similar study (186), using a smaller group of isolates from the just above mentioned OmpA study (from colonised, non-bacteraemic VAP, and bacteraemic VAP patients), and analyzed the expression of Quorum network (QS/QQ) genes of *A. baumannii*, *abaR* and *abaI* (QS system) and *aidA* (QQ mechanism), in relation to clinical features of pneumonia and bacteraemia. As a result, they observed that there was no difference in the expression of Quorum network genes between isolates from colonised and VAP patients, as previously described (445), but between isolates from non-bacteraemic VAP and bacteraemic VAP patients, exhibiting significant higher expression isolates from bacteraemic VAP patients. So, they suggested that the QS (*abaR* and *abaI* genes)/QQ (*aidA* gene) network affects

the development of secondary bacteraemia in VAP patients and also the virulence of *A. baumannii*.

Therefore, all these data suggested that expression of virulence-associated genes, already-known or still unknown, could be under different regulation in *A. baumannii* depending on its phenotype. Recently, some studies have focused on transcriptome and expression of virulence apparatus to clarify different virulence capability among clinical isolates (357, 446). However, drawing a complete picture of the host–pathogen interaction associated with *A. baumannii* remains to be explored and elucidated, because maybe host conditions also regulate bacterial gene expression in some way, thus determining its phenotype.

Nevertheless, newer and more accurate WGS technologies as well as bioinformatics tools should be used with a higher number of colonising and invasive *A. baumannii* clinical isolates in order to discard the hypothesis that there could be novel bacterial virulence factors still to be discovered, involved in the infection process. In addition, demographic and clinical characteristic of the patients from whom isolates were obtained should be taken into account for further studies, selecting isolates from clinically homogeneous patients, in order to avoid isolate selection errors due to the influence of patients' comorbidities and severity of their acute illnesses on the possible bacterial phenotype. Finally, despite being the non-discriminative murine model of peritoneal sepsis the most commonly used murine model to test virulence differences between bacterial strains or isolates (155, 222, 432), the pneumonia model could be more appropriate to compare colonising and invasive isolates, mimicking better what really happens in patients with lower airways colonisation or infection (84, 160).

Chapter II. Identification of potential *A. baumannii* virulence factors of bacteraemic *A. baumannii* isolates recovered from clinically homogeneous adult patients with radically different clinical outcomes.

Another issue that is still little studied is the possible involvement of *A. baumannii* molecular features in patient outcome. However, there are very few studies that have addressed this issue. As mentioned in the previous Chapter I, Kim *et al.* performed a comparative genomic study of bacteraemic CRAB isolates that caused different clinical

outcomes with the aim of finding any *A. baumannii* molecular feature that could have clinical implications (435). However, they failed to observe any significant differences in patient outcomes according to STs, and therefore known antimicrobial resistance and virulence genes. One of the main limitations of this study is that they only studied association between clinical outcomes and STs, not carrying out a robust analysis based on comparative genomics but considering all possible genomic features. In addition, they analysed bacteraemic *A. baumannii* isolates but from patients admitted to different hospital settings during a long period time, who additionally had a wide variety of co-morbidities and bacteraemia origin, as well as different antimicrobial treatments, so it might be possible that co-morbidities and other clinical aspects could be influencing mortality in those cases, more than the bacteria *per se*. Nevertheless, these limitations are regular when studying ICU patients. A second study that partially addressed this issue is the OmpA study performed by Smani *et al.* (147), in which they described the association of *ompA* overexpression in lower respiratory tract *A. baumannii* isolates with the development of pneumonia and bacteraemia, and even with death. Despite *ompA* overexpression is undoubtedly demonstrated to be associated with pneumonia and bacteraemia development, there were some limitations when related to death. The multivariate analysis of risk factors associated with death in both cohorts grouped together (colonised and VAP patients) showed that *ompA* expression was one of the factors independently associated with death (OR, 1.62 [95% CI, .98–2.69; P = .049]). However, when bacteraemia was included in the analysis, the *ompA* expression showed a trend association, although non-significant, with death (OR, 1.59 [95% CI, .94–2.69; P = .09]). In addition, there was no confirmation if the bacteraemia was secondary to VAP in the patients who suffered bacteraemic VAP, and neither were analysed the bacteraemic isolates.

Taking into account all the above, in this Doctoral Thesis, we first investigated the virulence factors of bacteraemic CRAB isolates from a group of clinically homogeneous ICU adult patients with bacteraemia secondary to *A. baumannii* VAP. Despite their demographic and clinical homogeneity, and having reached the optimal therapeutic target for colistin, three patients survived the infection while the others did not. Using a combination of experimental approaches including measurement of *ompA* expression levels, comparative WGS and proteomic analyses, and immunoblot analysis of OM fractions, we tried to find those molecular features of these bacteraemic *A. baumannii* that

could be associated with patient mortality. First, we fail to observe any significant difference in patient outcomes according to STs, antimicrobial susceptibilities, antimicrobial resistance mechanisms or known virulence genes. In fact, antimicrobial susceptibilities, antimicrobial resistance mechanisms or known virulence genes were clearly associated with ST types, as described in Chapter I and other studies (435). It is worth noting that these bacteraemic isolates were practically identical to the TBA isolates studied in the previous chapter, according these characteristics. Moreover, when *ompA* expression levels were measured in all isolates, no significant differences were found in relation with patient outcomes. In this case, all isolates exhibited high levels of expression of *ompA*, in comparison with those exhibited by the isolates from Chapter I and from the study of Smani *et al.* (147). As *ompA* overexpression was suggested to be associated with nosocomial bacteraemia development among other clinical feature (147), it could explained the high level of expression observed in our bacteraemic isolates, as in the previous studies only isolates from TBA samples were analysed. However, it could not explain the differential clinical outcome observed in our bacteraemic VAP patients, despite having also been described as a risk factor for mortality rate increase (147). On the other hand, performing WGS comparisons, proteomic, and immunoblot analysis of OM fractions, we detected that the OMP CarO was present in the CRAB isolates from the group of patients with the worst outcome, but missing in the isolates from the group of surviving patients. Moreover, we found that the absence of CarO in the OM of the latter isolates was due to the selection of different mutations affecting the *carO* gene, including point mutations introducing premature stop codons and an IS*Aba1*-mediated insertional inactivation of the gene.

In a second step, additional genetic evidence indicating a virulence role in *A. baumannii* for CarO was obtained by using the model type strain ATCC 17978 and its isogenic Δ*carO* mutant, as well as the Δ*carO* mutant complemented with a plasmid reinstating CarO expression. Significant reductions were observed both in adherence and invasion of cultured human lung epithelial cells in ATCC 17978 cells lacking CarO, which also exhibited higher MLD and lower dissemination into essential organs and fluids in a murine model of peritoneal sepsis.

CarO, together with OmpA, are among the most abundant and characterized *A. baumannii* OMPs (129-131, 140-145, 147, 401, 418, 419, 447). OmpA has been shown to be involved in adherence to epithelial cells (140-142), biofilm formation (140), translocation

into the epithelial cell nucleus (143), induction of epithelial cell death, and mice mortality (144, 145, 147). In the case of CarO, the loss or mutation of this protein among clonally-related MDR *A. baumannii* clinical strains was first associated with the acquisition of carbapenem resistance (129, 447). Later, the same authors reported that CarO participates in the selective permeation of imipenem and structurally-related basic amino acids across the *Acinetobacter* OM, with a notable preference for ornithine and arginine (129, 130, 401, 419). A number of *in vitro* studies made by different authors using purified recombinant CarO proteins reconstituted in artificial membranes supported in fact the notion that CarO may participate in the selective uptake of imipenem (131) and ornithine (418). However, further studies (401, 419) indicated the presence of imipenem channels other than CarO in the *Acinetobacter* OM and that, while *carO* loss may contribute to imipenem resistance, other mechanisms such as the presence of carbapenemases are probably more relevant to the carbapenem-resistance phenotype. The observation made in this work that the CRAB isolates with interrupted *carO* genes did not show higher MICs to imipenem than the isolates with intact *carO* genes, and the WGS analysis indicating the presence of acquired carbapenemases in all of them, agree with this interpretation and strongly suggest that these enzymes represent the main cause of carbapenem resistance among them.

As noted above, CarO is the second most abundant protein after OmpA in the *A. baumannii* OM (401) and, similarly to OmpA (140-145, 147), several evidences suggest that it could also play role(s) in pathogenesis. First, it has been noted that *A. baumannii* can catabolize both arginine and ornithine (130), two basic amino acids present in the blood of mammals in which the arginine-to-ornithine conversion is increased after severe skin injury. This, added to the observation that CarO levels in the *A. baumannii* OM are optimum at 37 ºC and its role as a basic amino acid OM channel, led to suggest that CarO may form part of a fine-tuned mechanism of response to specific signals when confronting a compromised host (130). Second, four well-defined allelic variants of CarO co-exist in the *A. baumannii* clinical population, and the cognate genes are frequently exchanged between different *A. baumannii* lineages by horizontal gene transfer and assortative recombination (401). This CarO exchanges could facilitate, among others, *A. baumannii* persistence by evading the host immune response (401). Third, increased expression of CarO has been described in *A. baumannii* cells during iron-limiting conditions (448) and biofilm formation (449, 450), also suggesting roles for CarO in pathogenesis. Fourth, we

reported previously that reduced expression of CarO and OprD in a clinical PDR *A. baumannii* isolate was concomitant with lower virulence, thus suggesting also roles for these OMPs in this process (156). Fifth, it has recently been shown using the *A. baumannii* type strain ATCC 19606 that CarO promotes bacterial adhesion and nasal colonisation in mice mainly through inhibiting host cell inflammatory immunity responses, again indicating roles for this OMP in pathogenesis (451). Thus, different lines of evidence and the results of this Doctoral Thesis point to CarO as an important factor involved in *A. baumannii* recognition and attachment to epithelial cells, in reaching the bloodstream with the potentiality of causing bacteraemia, and also in the invasion of different organs in mammals. Further work is certainly needed to clarify the exact role(s) of this small β-barrel OMP in *A. baumannii* pathogenesis.

OMPs functioning at the interface with the environment constitute prime candidates for the design of inhibitors aimed to disturb recognition of target cells by the pathogen (268, 272), especially in cases of infections caused by pathogens highly resistant to antimicrobial drugs as is the case of *A. baumannii*. Most commonly prescribed antibiotics are either bactericidal or bacteriostatic, and the majority work against a broad spectrum of bacteria. There are key approaches that could help to alleviate the problem of antibiotic resistance: first, the development of therapies focused on the specific treatment of infections caused by a single pathogen highly resistant to antimicrobial agents (14); and second, targeting bacterial virulence factors without inhibiting bacterial growth, which can slow the development of drug resistance by reducing the selective pressure on the bacteria (268). CarO presence is restricted to the OM of *Moraxellaceae* family members, to which the genus *Acinetobacter* belongs (129). Therefore, an inhibitor targeted towards CarO might provide us with a dedicated therapeutic agent, affecting not only *A. baumannii* but also other pathogenic species of the *Acinetobacter* genus and even the *Moraxellaceae* family. Until now, OmpA has been the *A. baumannii* OMP most studied as potential therapeutic target. Besides our group has shown that *ompA* overexpression is an associated factor for pneumonia, bacteraemia, and death due to *A. baumannii* (147), a great amount of challenging results have been achieved through different therapeutic strategies based on this OMP (452). On the one hand, active and passive immunization strategies using OmpA have postulated this protein as promising candidate antigen after demonstrating *in vivo* that it prevents *A. baumannii* infection (341, 453). On the other hand, our group together with Giralt E. *et al.*, developed a series of OmpA inhibitors, and

specifically, the cyclic hexapeptide (AOA-2) exhibited good *in vitro* and *in vivo* results against *A. baumannii*, as it reduced host cell death, inhibited biofilm formation, decreased bacterial loading in both spleen and lungs of infected mice, and reduced mouse mortality (272). Moreover, this OmpA inhibitor was effective in combination with colistin in an experimental model of severe infection with colistin-resistant *A. baumannii* strains (273).

Therefore, this drug discovery program is considered as an initial stage of a novel class of antimicrobial agents, such as anti-virulence drugs, and in this context CarO emerges as an attractive target for vaccine and inhibitor design.

Finally, highlight the importance of omics techniques applied to medical microbiology nowadays, especially WGS. WGS is increasingly being demonstrated to be a powerful tool in clinical microbiology (454), and it is being used in numerous reference laboratories, mainly applied to molecular epidemiology for the purposes of surveillance and outbreak investigation, as it possessed the highest possible discriminatory power (455-458). Apart from this, WGS of bacterial genomes is used to reveal the presence of resistance determinants or virulence attributes, as well as to discover new genetic mechanisms of resistance or virulence, as described in this Doctoral Thesis and other studies (459). However, whether or not it can entirely replace routine microbiology is a matter of debate.

On the one hand, the correlation between genotype and phenotype is still surrounded by controversy. Although preliminary studies have reported promising results inferring the MIC of bacteria from WGS data (460-465), in a recent review from EUCAST, Ellington *et al.* conclude that for most bacterial species there is currently insufficient evidence to support the use of antibiotic susceptibility testing inferred from WGS to guide clinical decision making (466). In our case, we found no discrepancies between phenotypic and genotypic results in terms of antimicrobial resistance, with the exception of sulbactam, and tobramycin for some bacteraemic *A. baumannii* isolates. As the presence or absence of antimicrobial resistance genes does not always guarantee a respective phenotypic resistance or susceptibility to a specific antimicrobial drug antimicrobial resistance, depending also on their transcriptional regulation, as well as the regulation of the expression efflux pumps and porins, traditional antimicrobial susceptibility testing should continue to be done, at least until more accurate omics techniques were developed. Nonetheless, WGS as a sole diagnostic method to detect resistance could be viable where

complete or near-complete congruence exists between phenotype and genotype, and where phenotypic testing is extremely slow, as the case of slow-growing bacteria, such as those belonging to the *Mycobacterium tuberculosis* complex (467). A similar situation occurs with the virulence factors, not always being correlated the genotypical results with the phenotypical characteristics, as shown on Chapter I. However, it could be practical for the detection of virulence genes with an undoubtedly role in pathogenesis, as toxin genes in *Clostridioides difficile* or *S. aureus* isolates, among others (468, 469).

On the other hand, the time from sample to result should be drastically reduced to obtain the result within a clinically relevant timeframe, as well as the costs, which should be reduced to be cost-effective in comparison with the techniques used in current laboratory practice. To achieve all these goals, further development is required to improve the workflow for WGS, especially on technology and bioinformatics. Thus, new WGS platforms, such as MinION, have overcome such limitations, as it generates long reads (which facilitate the downstream analysis of genomes) in relatively short runtimes of just a few hours, compared with platforms such as Illumina sequencers (470); and even other sequencers with the advantages of MinION but with higher throughput may be entering the market in the near future. In addition, bioinformatics scientists have also risen to the challenge and numerous commercial or user-friendly bioinformatics tools and databases have been produced and these continue to evolve with this rapidly advancing field. Nonetheless, more automated pipelines for data analyses and easy-to-use software for analysis must be developed. During the development of this Doctoral Thesis, new bioinformatics pipelines have been created to compare *A. baumannii* genomes following different approaches. These software tools were so useful, as it can be used in other similar studies, even for bacterial pathogens different to *A. baumannii*. In fact, some of these tools have been used in a recently published work in which our group have collaborated (354).

In conclusion, ensuring the quality of WGS may be a bottleneck for the transition from research use of WGS (with occasional *ad hoc* use in urgent situations like outbreak investigations) to routine implementation, but when this technique reaches maturity, routine patient management and infection control management will rely on WGS, enabling the transformation of clinical microbiology into a genome-based and personalized diagnostic field (470).

CONCLUSIONS

1. No differences were found *in vitro* or *in vivo* between colonising and invasive *A. baumannii* isolates obtained from respiratory samples regarding their virulence.

2. Colonising or invasive phenotypes do not depend on the clone they belong to, and neither on the number and composition of known antimicrobial resistance mechanisms and virulence determinants.

3. There is no genetic feature differential between colonising and invasive *A. baumannii* isolates according to whole-genome sequencing data, suggesting that those phenotypes depend on the regulation of already-known or still unknown virulence factors, instead of on the genomic content.

4. The outer membrane protein CarO is found only expressed in the bacteraemic *A. baumannii* isolates from the non-surviving patients with *A. baumannii* bacteraemia secondary to VAP, suggesting that it could be responsible of the poor clinical outcome.

5. The deletion of the *carO* gene decreases adherence and translocation *in vitro*, and reduces the virulence *in vivo*, supporting its role as *A. baumannii* virulence factor.

6. The results of this study suggest that CarO may be a target for the development of new inhibitors, which could be used as adjuvants for the treatment of *A. baumannii* infections.

7. Omics techniques are powerful tools to reveal the presence of resistance determinants or virulence attributes, as well as to discover new genetic mechanisms of resistance or virulence in *A. baumannii*.

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ANNEXES

Annex 1: Article 1 (Chapter I). Draft genome sequences of seven multidrug-resistant *Acinetobacter baumannii* strains, isolated from respiratory samples in Spain.

Most of the results described in the previous Sections 2.1, 3.2, 4.1, 4.2, and 4.3 of Results from Chapter I (PFGE typing, antimicrobial susceptibility, WGS data processing, MLST typing, and antimicrobial resistance mechanisms, respectively) were published in Genome Announcements journal (DOI: 10.1128/genomeA.00083-16). Nevertheless, as for this journal it was mandatory that isolates had ≤ 300 contigs once removed those with a length < 500 bp, only C6, C7, C8, C11, C12, IB1, and IB3 isolates were described in the article (named as MDR-GLH1, MDR-GLH2, MDR-GLH3, MDR-GLH4, MDR-GLH5, MDR-GLH6, and MDR-GLH8, respectively).

These WGS projects were deposited at DDBJ/EMBL/GenBank under the accession no. LIWE00000000 (AbMDR-GLH1), LIZZ00000000 (AbMDR-GLH2), LJAA00000000 (AbMDR-GLH3), LJAB00000000 (AbMDR-GLH4), LJAC00000000 (AbMDR-GLH5), LJAD00000000 (AbMDR-GLH6), and LJAF00000000 (AbMDR-GLH8).

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements

Draft Genome Sequences of Seven Multidrug-Resistant *Acinetobacter baumannii* Strains, Isolated from Respiratory Samples in Spain

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The draft genome sequences of seven multidrug-resistant *Acinetobacter baumannii* clinical strains belonging to sequence types ST-208 and ST-218 are reported in this study. They were isolated from tracheobronchial aspirate of mechanically ventilated adult patients admitted to the intensive care unit of a Spanish tertiary hospital during 2010 to 2011.

Received 21 January 2016 **Accepted** 12 February 2016 **Published** 31 March 2016

Citation Labrador-Herrera G, Álvarez R, López-Rojas R, Smani Y, Cebrero-Cangueiro T, Rueda A, Pérez Florido J, Pachón J, Pachón-Ibáñez ME. 2016. Draft genome sequences of seven multidrug-resistant *Acinetobacter baumannii* strains, isolated from respiratory samples in Spain. Genome Announc 4(2):e00083-16. doi:10.1128/genomeA.00083-16.

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Acinetobacter baumannii is among the leading etiologies of hospital-acquired infections, particularly in critically ill patients (1). The increasing prevalence of multidrug-resistance reduces available treatments and threatens public health (2). During 2010 to 2011, 207 multidrug-resistant (MDR) *A. baumannii* strains were isolated from 100 adult patients in the intensive care unit of a tertiary hospital in Seville, Spain.

In this study, we present the draft genome sequences of 7 of these MDR *A. baumannii* clinical strains (including resistance to gentamicin, imipenem, meropenem, ceftazidime, cefepime, ticarcillin, trimethoprim-sulfamethoxazole, tetracycline, levofloxacin, and ciprofloxacin; and susceptibility to colistin) isolated from tracheobronchial aspirates. Whole-genome sequencing was performed using the Illumina MiSeq platform with 300 bp paired-end reads, resulting in mean genome coverage of 25.2 ± 5.5-fold (mean ± SD of the 7 strains). Reads were preprocessed with Trimmomatic v0.32 (http://www.usadellab.org/cms/?page=trimmomatic) (3) and *de novo* assembled with ABySS assembler v1.5.2 (http://www.bcgsc.ca/platform/bioinfo/software/abyss) (4), resulting in 381 ± 52 contigs. The assemblies were filtered for contigs larger than 500 bp, leaving 210 ± 34 contigs with a total length of 4,036,456 ± 123,611 bp and an N_{50} of 47,317 ± 8,993 bp. The G+C content was 39.2 ± 0.1%. Gene prediction and annotation were performed utilizing GeneMark v2.5 (http://exon.gatech.edu/GeneMark/) and RAST server v1.0 (http://rast.nmpdr.org) (5), respectively, yielding a total of 3,876 ± 134 coding sequences and 70 ± 15 RNAs. Two different sequence types (ST) were found with MLST v1.8 using the Oxford database (https://cge.cbs.dtu.dk/services/MLST/) (6, 7). Most of the strains belong to ST-208, except AbMDR-GLH5 and AbMDR-GLH6 that belong to ST-218. With pulsed-field gel electrophoresis, 3 pulsotypes were found: pulsotype 1 (AbMDR-GLH2 and AbMDR-GLH4), pulsotype 2 (AbMDR-GLH1, AbMDR-GLH3, and AbMDR-GLH8), and pulsotype 3 (AbMDR-GLH5 and AbMDR-GLH6). To identify genes conferring antimicrobial resistance, the bioinformatic tool ResFinder v2.1 was used (https://cge.cbs.dtu.dk/services/ResFinder/) (8). The aminoglycoside resistance genes *aac(6′)-II*, *strB*, and *strA*, were detected in pulsotype 1 strains, while pulsotype 2 strains carry *aac(3)-Ia*, *aadA1*, *strB*, and *strA* genes, and pulsotype 3 strains carry *aac(6′)-II*, *aac(3)-IIa*, *strB*, and *strA* genes. Resistance genes *blaOXA-58*, *blaOXA-66*, and *blaADC-25*, which confer β-lactam resistance, were found in strains from pulsotypes 1 and 2; nevertheless, pulsotype 3 strains carry *blaOXA-109* and *blaADC-25* genes. Referring to sulfonamide resistance, all the strains have *sul1* gene, and strains from pulsotypes 1 and 2 also possess *sul2* gene. The gene *tet(B)*, related with tetracycline resistance, was found in all the strains. Furthermore, point mutations in genes associated with quinolone resistance were found in all the strains using BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (9) comparing with genes from susceptible strains.

Nucleotide sequence accession numbers. These whole-genome shotgun (WGS) projects have been deposited at DDBJ/EMBL/GenBank under the accession numbers LIWE00000000 (AbMDR-GLH1), LIZZ00000000 (AbMDR-GLH2), LJAA00000000 (AbMDR-GLH3), LJAB00000000 (AbMDR-GLH4), LJAC00000000 (AbMDR-GLH5), LJAD00000000 (AbMDR-GLH6), and LJAF00000000 (AbMDR-GLH8). The versions described in this report are the first versions, LIWE01000000, LIZZ01000000, LJAA01000000, LJAB01000000, LJAC01000000, LJAD01000000, and LJAF01000000.

ACKNOWLEDGMENT

This study was supported by the Instituto de Salud Carlos III, Proyectos de Investigación en Salud (grant PI12/02553).

FUNDING INFORMATION

This work, including the efforts of Jerónimo Pachón, was funded by MINECO | Instituto de Salud Carlos III (ISCIII) (PI12/02553).

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Annex 2: Article 2 (Chapter II). Virulence role of the outer membrane protein CarO in carbapenem-resistant *Acinetobacter baumannii.*

This manuscript has been submitted to Journal of Infection, and included all data from Chapter II.

The sequences reported in this paper have been deposited in the National Center for Biotechnology BioProject database (accession number PRJNA417465): PKON00000000 (B1), PKOO00000000 (B4), PKOP00000000 (B7), PKOQ00000000 (B8), PKOS00000000 (B9), and PKOR00000000 (B11).

Original article.

Title: Virulence role of the outer membrane protein CarO in carbapenem-resistant *Acinetobacter baumannii*.

Running title: *A. baumannii* CarO virulence role.

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Abstract

Objectives: To analyse the virulence role of CarO outer membrane protein in carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical isolates and in the strains ATCC 17978 and ATCC 19606.

Methods: Six CRAB clinical isolates causing bloodstream infections secondary to ventilator-associated pneumonia from demographics and clinically homogeneous adult patients, who received an optimal treatment but with different clinical outcomes, were comparatively analysed by whole-genome sequencing complemented with proteomic/immunoblot techniques. The virulence role of the putative virulence factor CarO was studied *in vitro* and *in vivo* using model systems.

Results: Bioinformatics analyses indicated that the *carO* gene was interrupted by different disruptive events in CRAB isolates from patients who survived, while it was intact in isolates from patients who died, and proteomic/immunoblot techniques corroborated it. When the virulence role of *A. baumannii* CarO was analysed in model systems, isogenic mutants lacking *carO* and a selected CRAB clinical isolate with truncated CarO, showed lower ability *in vitro* to adhere and invade human lung epithelial cells and lower *in vivo* virulence.

Conclusions: These results expose a previously unnoticed virulence role for the *A. baumannii* outer membrane protein CarO, which could be a potential target for the development of new therapies for carbapenem-resistant *A. baumannii* infections.

Keywords: *Acinetobacter baumannii*, bloodstream infections, ventilator-associated pneumonia, carbapenem-resistance, genomics, outer membrane protein, virulence factor, CarO.

Introduction

Acinetobacter baumannii is one of the most successful opportunistic pathogen responsible for healthcare-acquired infections worldwide (1). Of particular importance is its ability to cause bloodstream infections (BSI) in critically ill patients, mainly due to ventilated associated pneumonia (VAP), associated with septic shock (2). Crude mortality rates in these patients have been reported between 30-76% (3). This pathogen is endowed with an extraordinary capability to develop resistance to antibiotics, including carbapenems (1-3). This situation has prompted the search of new therapeutic strategies to deal with carbapenem-resistant *A. baumannii* (CRAB) infections, and non-antimicrobial approaches targeting bacterial virulence factors might represent promising alternatives (4). Nevertheless, the knowledge on *A. baumannii* virulence traits is relatively scarce (5). Whole-genome sequencing (WGS) comparisons between different strains of a given pathogen causing infections are employed for the identification of potential virulence factors (6). However, the origin of the strains and the patients' clinical characteristics should be carefully considered when drawing conclusions on possible virulence factors, or these might not be definite (7-9).

This study aimed to gain insight, using WGS complemented with proteomic and immunoblot analyses, and *in vitro* and *in vivo* model systems, into putative *A. baumannii* virulence factors associated with the different outcomes of clinically homogeneous patients with CRAB BSI secondary to VAP.

Materials and methods

Clinical isolates and clinical and demographic characteristics of the patients

Six CRAB clinical isolates (Supplementary material, Table S1) from blood cultures of six patients with BSI secondary to VAP were used. These patients were selected from a

cohort of adult patients with VAP admitted to the intensive care unit (ICU) of a tertiary hospital, fifteen of them with secondary BSI (10). Patients' clinical and demographic characteristics and isolates codes are detailed in Table 1. The criteria to select them were: i) isolation from young adult patients without underlying chronic comorbidities (Charlson index=0), acute physiology and chronic health evaluation (APACHE II) ≥15, similar clinical pulmonary infection score (CPIS) at the inclusion, and no co-infections during hospital stay; ii) appropriate antimicrobial treatment of the VAP (10), being all the isolates colistin-susceptible (MIC range: 0.03-0.125 µg/mL) and without colistin-heteroresistance; and iii) same pulsed-field gel electrophoresis pattern as their corresponding VAP CRAB isolates, confirming the source of BSI. Three patients died (patients 1-3, infected by B1, B4, and B7 *A. baumannii* isolates, respectively) and three survived (patients 4-6, infected by B8, B9, and B11 *A. baumannii* isolates, respectively). From now on we will refer to them as "both groups of clinical isolates".

DNA extraction, WGS and protein-coding gene annotation

DNA of the six CRAB clinical isolates was extracted using QIAamp® DNA Mini Kit. Sequencing was performed using MiSeq platform (Illumina), according to the standard protocol for WGS paired-end, producing 2×300 bp fragment reads. SPAdes v3.5 was chosen for *de novo* assembly of the reads into contigs (11). Then, protein-coding genes were predicted by Prodigal v2.6.3, and the derived amino acid sequences were obtained (12). The predicted proteins were functionally annotated by Sma3s v2 using the bacterial taxonomic division of UniProt database (13).

Multilocus sequence typing (MLST) and identification of known resistance and virulence mechanisms

MLST (Pasteur and Oxford MLST schemes) was performed by uploading the contigs files obtained from the *de novo* assembly of the WGS data of each of the six CRAB clinical isolates to the MLST web server v2.0 from the Center for Genomic Epidemiology (CGE) (14). Then, several strategies were used to identify resistance mechanisms using the assembled WGS data from the six CRAB clinical isolates. Thus, ResFinder web server v3.2 from CGE (15) was used for acquired antimicrobial resistance genes, functional annotations including either the term "resistance" or "antibiotic" for other resistance genes, and BLASTP (16) for point mutations in genes associated with quinolone and rifampicin resistance (Supplementary material, Text S1). Secondly, two different approaches were used for the identification of known virulence genes, both based on the Virulence Factors of Pathogenic Bacteria database (VFDB) (17) (Supplementary material, Text S1).

Differential protein-coding gene analyses

The amino acid sequences of all the predicted protein-coding genes of the six CRAB clinical isolates were clustered using the CD-HIT tool (18) with an identity threshold and coverage for the longer sequence of 95%. It brings together the same protein from the different isolates in the same cluster. So, if a specific cluster has no sequences coming from a specific isolate, it will be considered that this isolate lacks the gene coding for that protein. To determine protein variants between the isolates, a more specific protocol was designed using the standalone version of BLASTP (16) with identity threshold and query coverage of 100%. So, proteins from each isolate were independently compared to the proteins of the rest of isolates to discover variants. When a sequence showed or identity or query coverage between isolates different to 100% it was considered a differential protein-coding gene.

CarO sequence alignments and analysis

The amino acid alignments of the inferred CarO proteins from the six CRAB clinical isolates were performed using MAFFT v7.312 with default setting (19). When possible, the CarO protein sequence of a given isolate was assigned to a variant following the proposal of Mussi *et al*. (20). The secondary structure of CarO was obtained from the Protein Data Bank (PDB) entry 4fuv.1.A, which presents an identical amino acid sequence. The multiple alignment with all data was depicted using Jalview v2.11.0 (21).

Outer membrane protein (OMP) profiles and immunoblotting

The outer membrane (OM) fractions from the six CRAB clinical isolates were isolated as described previously (22). The OMP profiles were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15% SDS gels and the equivalent to 10 µg of total OM protein per sample, followed by SimplyBlue SafeStain staining. The differential bands found between both groups of clinical isolates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MSMS). Proteins were identified using Proteome Discoverer v2.1 software, and results were filtered by a false discovery rate of 1% (23). Western blot analysis was conducted after by incubating the polyvinylidene difluoride membrane with polyclonal antibodies against *A. baumannii* CarO elicited in rabbits (24), and peroxidase-labelled anti-rabbit immunoglobulin G (IgG) antibody from donkeys (25).

Strains and isolates selected as model systems

The type strains ATCC 17978 and ATCC 19606 were selected to analyse the role of CarO as a virulence factor in *A. baumannii*, as well as their corresponding mutants lacking CarO (Δ*carO*) and complemented strains with CarO expression restored (Δ*carO*/pWH1266-

carO, with variants CarOIV and CarOI, respectively) (20). Both wild-type (wt) strains and their Δ*carO* mutants were also transformed with the "empty" vector pWH1266 (wt/pWH1266 and Δ*carO*/pWH1266, respectively) and used as controls. Additionally, the two clinical isolates with the highest number of common genes (Fig. 1), but with different CarO expression and isolated from patients with different outcome (B4 and B9), were used. Isolates/strains were grown for 18-20 h in Mueller-Hinton broth (MHB) at 37 ºC and 180 rpm. Kanamycin (20 µg/mL) and ticarcillin (80 µg/mL) were used to select Δ*carO* strains and strains harbouring pWH1266 or pWH1266-*carO*, respectively. The cultured isolates/strains were collected by centrifugation (15 min at 4,500 g), rinsed with sterilized phosphate-buffered saline (PBS), and resuspended in Dulbecco's modified Eagle's medium (DMEM) before use in epithelial cell infection assays. All strains/isolates exhibited similar growth rates in Mueller-Hinton broth (MHB) (Supplementary material, Text S1 and Fig. S1).

A. baumannii adherence and invasion of cultured human lung epithelial cells

Human lung epithelial cells, line A549 (ATCC® CCL-185™), were seeded (10^5 cells/well) for 24 h in 24-well plates. Before infection, cells were rinsed twice with PBS and then incubated with a 1:1000 dilution of an overnight culture of all the strains and isolates described above. Bacterial adherence and invasion assays were performed as previously described (25). Assays were performed thrice in different days.

Peritoneal sepsis murine model

Experiments were approved by the Committee on the Ethics of Animal Experiments of the University Hospital of Virgen del Rocío, Seville, Spain, and from the Ministry of

Agricultura, Pesca y Desarrollo Rural (2012PI/246). A peritoneal sepsis murine model using immunocompetent adult C57BL/6J female mice (26) was used.

Determination of minimum lethal doses (MLD)

Briefly, mice were inoculated intraperitoneally (i.p.) with 0.5 mL of bacterial dilutions (of 17978 wt, 17978 Δ*carO*, B4, and B9) mixed 1:1 with a saline solution of porcine stomach mucin (type II) at 10% (w/v), starting from an inoculum of 1.0 $\log_{10}$ CFU/mL approximately and ending with the first inoculum that cause 100% mice mortality (achievement of MLD). Groups of 6 mice per inoculum was used for 17978 cells, and groups of 4 mice for B4 and B9 clinical isolates, and survival rates were monitored during 7 days. With the 17978 Δ*carO*/pWH1266-*carO* complemented strain, only the inoculum used for the *in vivo* dissemination study (3.2 $\log_{10}$ CFU/mL) was tested, following the 3Rs principles (https://www.nc3rs.org.uk/the-3rs).

In vivo dissemination

Three groups of 14 mice each were inoculated i.p. with 0.5 mL of 3.2 $\log_{10}$ CFU/mL of 17978 wt, 17978 Δ*carO*, or 17978 Δ*carO*/pWH1266-*carO* strains. Eight- and twenty-four-hours post-inoculation, 7 animals of each group were randomly selected and sacrificed (200 µL sodium thiopental, i.p.). Then, bacterial loads were quantified in tissues ($\log_{10}$ CFU/g) and fluids ($\log_{10}$ CFU/mL).

Statistical analysis

GraphPad Prism 6 (GraphPad-Software) was used. Student's t-test and Mann-Whitney test were used to compare variables normally and not normally distributed, respectively. Survival curves and log-rank test were used to compare the survival distributions of mice.

Significance was established at a *P*-value < 0.05. Error bars on graphs represent the standard error of the mean (SEM).

Results

MLST characterisation of the CRAB clinical isolates

Oxford MLST scheme assigned ST218 for B1 and ST208 for the other isolates. Following the current definition of clonal complex (27), all six isolates were thus assigned to CC92 in the Oxford scheme. Pasteur MLST scheme, assigned ST2 for B1, ST745 for B4 and B9, and ST187 for B7, B8, and B11 isolates. Therefore, all six CRAB isolates were thus assigned to CC2 in the Pasteur scheme. It follows that all six isolates belong to CC92 (Oxford)/CC2 (Pasteur), the largest and most widely distributed *A. baumannii* global clone (27).

Identification of known resistance and virulence mechanisms

All resistance and virulence mechanisms found in the six CRAB clinical isolates are described in the Data sets S1 and S2, respectively (Supplementary material). No differences were found between both groups of clinical isolates regarding these mechanisms. Carbapenem resistance could be attributed to the presence of different carbapenem-hydrolysing class D β-lactamases such as OXA-40/24 and OXA-109 in B1, and OXA-58 and OXA-66 in the other five clinical isolates.

Differential presence of genes between CRAB clinical isolates from non-surviving and surviving patients

WGS comparisons indicated that all CRAB clinical isolates were different (Fig. 1). When comparisons were performed between both groups of clinical isolates, some differences

in a few number of genomic loci were found (Supplementary material, Text S2). The most notable difference between both groups of clinical isolates was noted at the *carO* locus encoding the OMP CarO. While the *carO* genes present in the isolates B1, B4 and B7 from the non-surviving patients were intact, those of the B8, B9, and B11 isolates from the surviving patients were found to be prematurely interrupted by different mutational events (see below).

CarO sequence characterisation

CarO protein sequences (complete and defective) inferred from the WGS data of the six CRAB clinical isolates and their corresponding alignments are shown in Fig. 2. As noted above, complete *carO* genes each encoding the same protein of 246 amino acids were found in the isolates from the non-surviving patients (B1, B4, and B7). In addition, the comparison of the amino acid sequences of these CarO proteins with the four allelic variants present in the *A. baumannii* population (20) indicated that these isolates carried the same variant, designated CarOIII (20). In contrast, the *carO* genes in isolates B8, B9, and B11 from the surviving patients were all prematurely interrupted by separate mutational events. Isolate B8 showed a 2-bp insertion in the *carO* gene introducing a frame-shift resulting in a premature stop codon and a putative truncated protein of only 118 amino acids long, isolate B9 a 1-bp insertion resulting in a putative truncated protein of only 125 amino acids long, and in isolate B11 an IS*Aba1* insertion was detected inside *carO* resulting in a putative truncated protein of only 104 amino acids long. Therefore, from the eight antiparallel β-strands that conform the β-barrel protein CarO, as judged by crystallographic analysis (28), isolates from non-surviving patients would generate, at best, only truncated CarO proteins ending shortly after the β-strand 4 (Fig. 2).

OMPs profiles of CRAB clinical isolates and detection of CarO by immunoblot and LC-MSMS analyses

In agreement with the WGS analysis of the CRAB clinical isolates, a ~29 kDa protein band corresponding to the expected migration of CarO (24) was observed only in the isolates from the non-surviving patients by SDS-PAGE, and LC-MSMS analysis confirmed the identity of this band as CarO (data not shown). Western blot analysis confirmed these results and failed to indicate the presence of CarO in isolates from the surviving patients (Fig. 3).

Effect of CarO loss on *A. baumannii* adherence and invasion of human lung epithelial cells

The loss of CarO reduced the adherence of 17978 wt and 19606 wt cells to A549 human lung epithelial cells (Figs. 4A and 4C, respectively). Moreover, adherence was recovered when CarO expression in the Δ*carO* mutants was restored (Figs. 4A and 4C, respectively). Similarly, the loss of *carO* reduced invasion of A549 cells by 17978 wt (Fig. 4B) and 19606 wt cells (Fig. 4D). Again, complementation of Δ*carO* mutants restored invasion to the levels of their parental wt strains (Figs. 4B and 4D, respectively). In addition, the clinical isolate with a truncated CarO protein (B9) exhibited lower adherence to A549 cells than the one with a functional CarO (B4) (Fig. 4E), as well as lower ability to invade them (Fig. 4F). These results suggest that CarO is involved in *A. baumannii* adhesion and invasion of human lung epithelial cells.

Virulence roles of *A. baumannii* CarO evaluated by a peritoneal sepsis murine model

Effect of CarO in the mortality rate induced in mice by *A. baumannii* cells

Differences in MLD were found between the 17978 wt strain and its isogenic mutant 17978 Δ*carO* (3.20 $\log_{10}$ CFU/mL *vs.* 4.30 $\log_{10}$ CFU/mL, respectively), and also between the CRAB clinical isolate with a functional CarO protein, B4, *vs.* the one with a truncated CarO, B9 (4.00 $\log_{10}$ CFU/mL *vs*. 5.18 $\log_{10}$ CFU/mL, respectively). Moreover, survival analyses revealed differences between animals inoculated with the different strains/isolates using the MLD of those with complete *carO* gene (Fig. 5). These results indicated that CarO plays an important role in the infective capacity and mortality caused by *A. baumannii in vivo*.

Effect of CarO in *in vivo* dissemination of *A. baumannii* 17978 cells into mice tissues and fluids

We next compared the dissemination of the 17978 wt, mutant, and complemented strains into different organs and fluids in mice inoculated i.p. with 3.2 $\log_{10}$ CFU/mL of each bacterial strain (Fig. 6). Mice infected with the 17978 Δ*carO* strain showed at 8 h significant lower bacterial burden in all organs and fluids studied when compared with animals infected with the 17978 wt strain. These differences were even greater 24 h post-infection. Complementation of the Δ*carO* mutant restored almost the wt count levels at 8 and 24 h. These results indicate that CarO affects the dissemination of *A. baumannii* in mice.

Discussion

In this study, we first investigated the virulence factors of CRAB clinical isolates from six adult patients with BSI secondary to *A. baumannii* VAP. Despite their demographics and clinical characteristics homogeneity, as well as receiving optimal therapy with colistin, three patients survived while the others three did not. Using a combination of

experimental approaches, we detected that the OMP CarO was present in the isolates from non-surviving patients, while the isolates from the group of surviving patients lacked it due to the selection of different mutations affecting the *carO* gene. Then, additional genetic evidence indicating a virulence role in *A. baumannii* for CarO was obtained by using the model type strains ATCC 17978 and ATCC 19606, their isogenic Δ*carO* mutants, and the Δ*carO* mutants complemented with a plasmid reinstating CarO expression, as well as two of the CRAB clinical isolates. Significant reductions were observed in adherence and invasion of cultured human lung epithelial cells, as well as higher MLD and lower dissemination into essential organs and fluids in the peritoneal sepsis murine model, in strains/isolate lacking CarO.

CarO is the second most abundant protein after OmpA in the *A. baumannii* OM (20) and it has been previously associated to the acquisition of carbapenem resistance (when it is lost or mutated) due to its participation in the selective permeation of imipenem across the *Acinetobacter* OM (20, 24, 28, 29). Nevertheless, similarly to OmpA (30), several evidences exist suggesting that this protein could also play role(s) in pathogenesis. First, it has been noted that *A. baumannii* can catabolise both arginine and ornithine (29), two basic amino acids present in the blood of mammals in which the arginine-to-ornithine conversion is increased after severe skin injury. This, added to the observation that CarO levels in the *A. baumannii* OM are optimum at 37 ºC and its role as a basic amino acid OM channel, led to suggest that CarO may form part of a fine-tuned mechanism of response to specific signals when confronting a compromised host (29). Second, four well-defined allelic variants of CarO co-exist in the *A. baumannii* clinical population, and the cognate genes are frequently exchanged between different *A. baumannii* lineages by horizontal gene transfer and assortative recombination; an exchange that could facilitate,

among others, *A. baumannii* persistence by evading the host immune response (20). Third, increased expression of CarO has been described in *A. baumannii* cells during iron-limiting conditions (31) and biofilm formation (32, 33), also suggesting roles for CarO in pathogenesis. Fourth, we reported previously that reduced expression of CarO and OprD in a clinical PDR *A. baumannii* isolate was concomitant with lower virulence, thus suggesting also roles for these OMPs in this process (34). Fifth, it has recently been shown using the *A. baumannii* type strain ATCC 19606 that CarO promotes bacterial adhesion and nasal colonisation in mice mainly through inhibiting host cell inflammatory immunity responses, again indicating roles for this OMP in pathogenesis (35). Thus, different lines of evidence and the results of this work point to CarO as an important factor involved in *A. baumannii* recognition and attachment to epithelial cells, in reaching the bloodstream with the potentiality of causing BSI, and also in the invasion of different organs in mammals. Further work is certainly needed to clarify the exact role(s) of this small β-barrel OMP in *A. baumannii* pathogenesis.

OMPs functioning at the interface with the environment constitute prime candidates for the design of inhibitors aimed to disturb recognition of target cells by the pathogen (36, 37). Most commonly prescribed antibiotics are either bactericidal or bacteriostatic, and the majority work against a broad spectrum of bacteria. There are key approaches that could help to alleviate the problem of antibiotic resistance: first, the development of therapies focused on the specific treatment of infections caused by a single pathogen highly resistant to antimicrobial agents (38); and second, targeting bacterial virulence factors without inhibiting bacterial growth, which can slow the development of drug resistance by reducing the selective pressure on the bacteria (36, 39). CarO presence is restricted to the OM of *Moraxellaceae* family members, to which the genus *Acinetobacter*

belongs (24). Therefore, an inhibitor targeted towards CarO might provide us with a dedicated therapeutic agent, affecting not only *A. baumannii* but also other pathogenic species of the *Acinetobacter* genus and even the *Moraxellaceae* family. Recently, our group together with Giralt *et al.*, developed a series of OmpA inhibitors and tested their effectiveness *in vitro* and *in vivo* in preventing infection by the most prevalent gram-negative bacilli (GNB) in clinical settings including *A. baumannii* (37). One of these peptides, a cyclic hexapeptide (AOA-2) lacking bactericidal or cytotoxic activities, was able to inhibit GNB adherence to host cells and biofilm formation, thereby preventing the development of infection *in vitro* and in a murine model of peritoneal sepsis (37). A further study indicated that this OmpA inhibitor was effective in combination with colistin in an experimental model of severe infection with colistin-resistant *A. baumannii* strains (40). This drug discovery program is considered as an initial stage of the development of a novel class of antimicrobial agents, and in this context CarO emerges as an attractive target for drug design.

In summary, the results of the present study, both from the WGS complemented with proteomic and immunoblot analyses of carbapenem-resistant *A. baumannii* clinical isolates causing bloodstream infections and from *in vitro* and *in vivo* model systems, are consistent with the role of the protein CarO in the virulence of *A. baumannii*. Thus, CarO may be suggested as a new target for the development of inhibitors to deal with infections by carbapenem-resistant *A. baumannii*.

Data availability

The sequences reported in this paper have been deposited in the National Center for Biotechnology BioProject database (accession number **PRJNA417465**):

PKON00000000 (B1), **PKOO00000000** (B4), **PKOP00000000** (B7), **PKOQ00000000** (B8), **PKOS00000000** (B9), and **PKOR00000000** (B11).

Transparency declaration

All authors have no conflicts of interest to declare.

Funding

This study was supported by the *Instituto de Salud Carlos III, Proyectos de Investigación en Salud* (PI12/02553); by *Plan Nacional de I+D+i* 2013-2016 and *Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades* (grant BFU2016-77297-P); by *Plan Nacional de I+D+i* 2013-2016 and *Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades*, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0009) - co- financed by European Development Regional Fund "A way to achieve Europe", Operative program Intelligent Growth 2014-2020; and by PICT-2015-1072 to Alejandro M. Viale, PICT-2017-3536 to Jorgelina Morán-Barrio (*Agencia Nacional de Promoción Científica y Tecnológica, ANPCyT, Argentina*). G.L.H. is supported by the program i-PFIS (*Doctorados IIS-Empresa en Ciencias y Tecnologías de la Salud), Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades* (IFI15/00128). T.C.C. is supported by the *V Plan Propio* of the University of Seville with a postdoctoral contract. M.E.P.I. is a postdoctoral researcher belonging to the program "Nicolás Monardes" (C1-0038-2019), *Servicio Andaluz de Salud, Junta de Andalucía*, Spain.

Acknowledgments

We would like to thank Dr. Henrik Hasman (Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark) for the WGS, and C3UPO (Pablo de Olavide University, Seville, Spain) for the high-performance computing (HPC) support.

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Figures

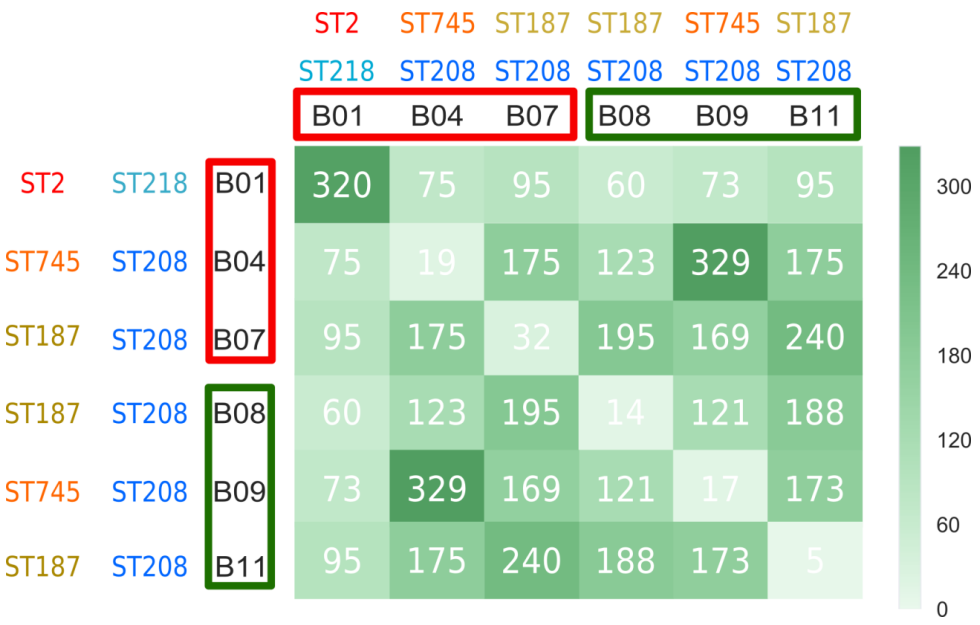

Fig. 1. Number of protein-coding genes exclusively shared between couples of CRAB clinical isolates, which were not in the other CRAB clinical isolates. The higher is the number of shared protein-coding genes the greenest is the cell of the matrix. Isolates from non-surviving patients and surviving patients are highlighted in red and green colours, respectively. Besides the isolate codes, both the Pasteur and Oxford STs are shown.

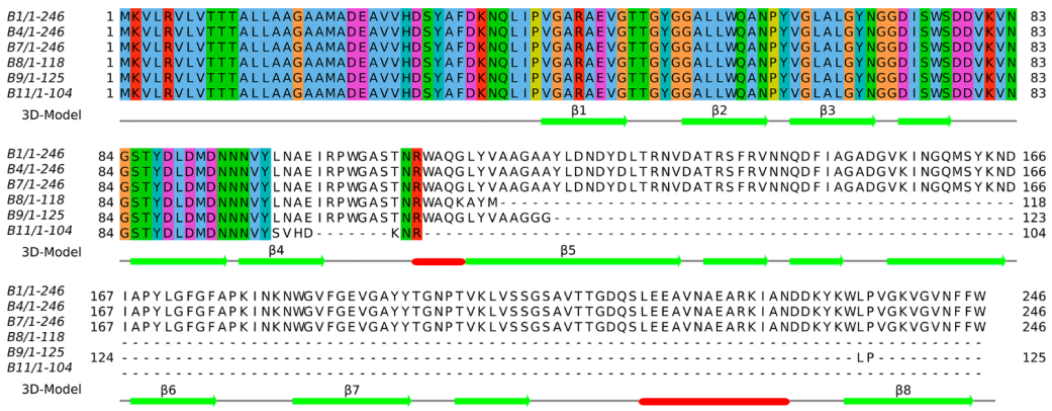

Fig. 2. Alignment of CarO amino acids sequences from the six CRAB clinical isolates. The numbers represent the positions of amino acids. CarO protein was intact in the isolates from non-surviving patients (B1, B4, and B7), while it was truncated in those from surviving patients (B8, B9, and B11). The secondary structure obtained from the PDB structure 4fuv.1.A is shown in the 3D-Model track: α-helices in red colour, and β-strands in green colour. The eight strands from the CarO β-barrel have been tagged (β1-β8).

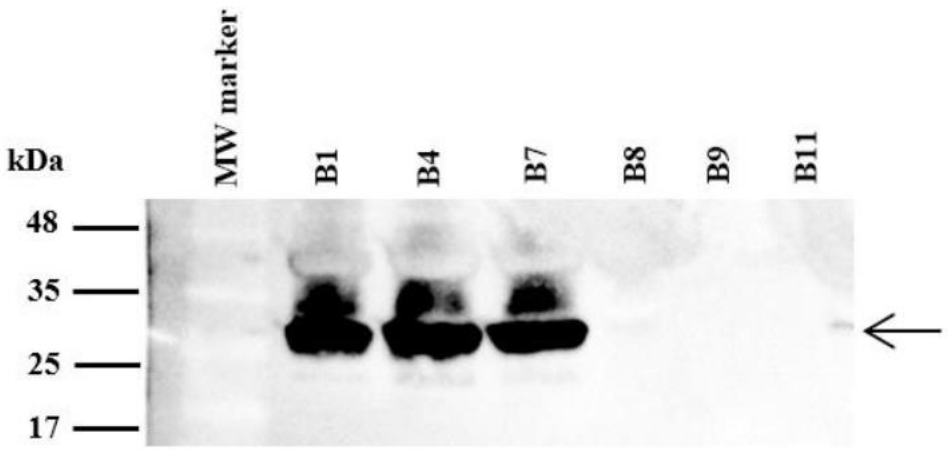

Fig. 3. Immunodetection of CarO in outer membrane protein profiles. Outer membrane fractions were extracted from the bacteraemic CRAB clinical isolates from non-surviving patients (B1, B4, and B7) and from surviving patients (B8, B9, and B11), and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with polyclonal rabbit antibodies toward *A. baumannii* CarO. Molecular weight standards (kDa) are shown on the left. Black arrow on the right indicate the final position of CarO. MW, molecular weight.

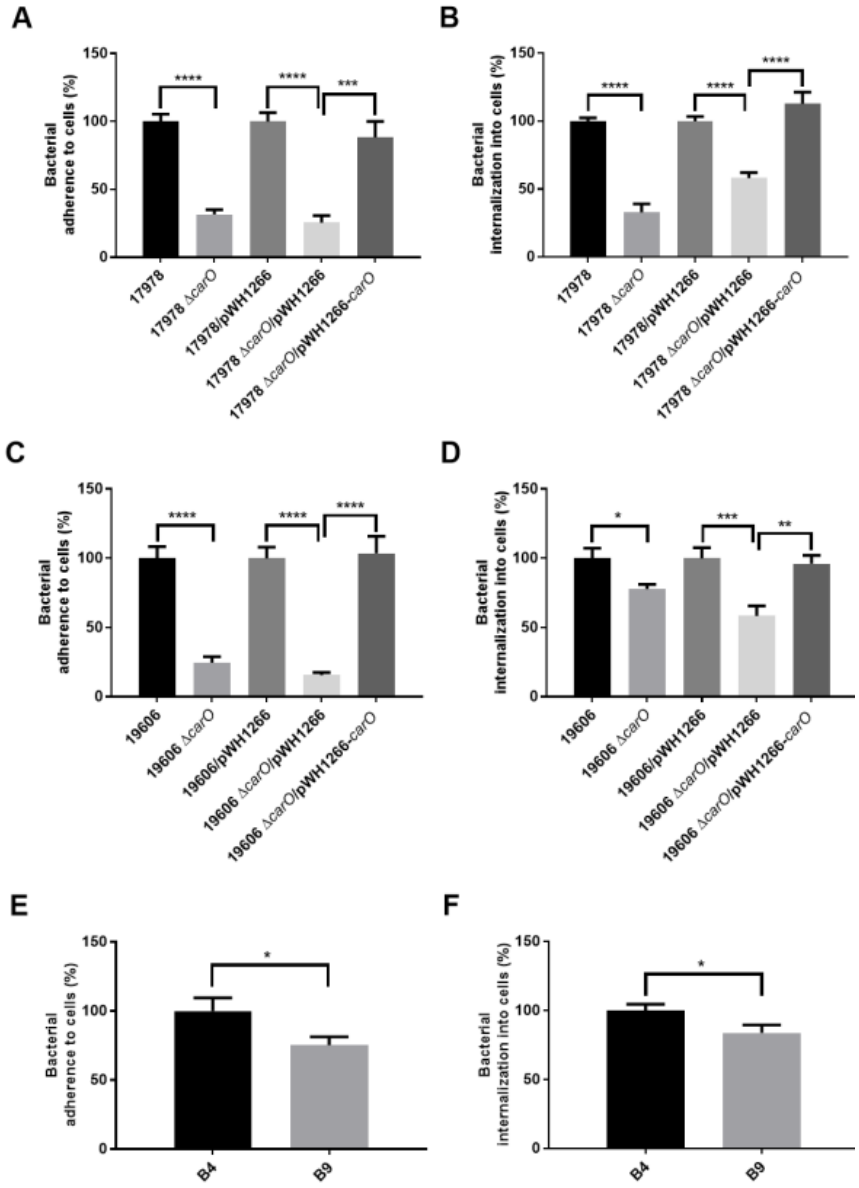

Fig. 4. *A. baumannii* adherence and invasion into human lung A549 cells. A549 cell cultures were incubated with two reference *A. baumannii* strains: ATCC 17978 and ATCC 19606 wild-type (17978 and 19606); their isogenic *carO* deletion mutants (17978 Δ*carO* and 19606 Δ*carO*); both 17978 wt and 19606 wt with empty plasmid pWH1266 as controls (17978/pWH1266 and 19606/pWH1266); both 17978 Δ*carO* and 19606

Δ*carO* mutants with empty plasmid as controls (17978 Δ*carO*/pWH1266 and 19606 Δ*carO*/pWH1266); 17978 Δ*carO* and 19606 Δ*carO* mutants with plasmid pWH1266-*carO* expressing CarO (17978 Δ*carO*/pWH1266-*carO* and 19606 Δ*carO*/pWH1266-*carO*) (A-D); and with the two CRAB clinical isolates B4 and B9 (E-F). The percentages of bacterial adherence (A, C, E) and invasion (B, D, F) were subsequently measured. Data are represented as mean ± SEM (n=3 replicates in different days). **P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001, Student's t-test and Mann-Whitney test.

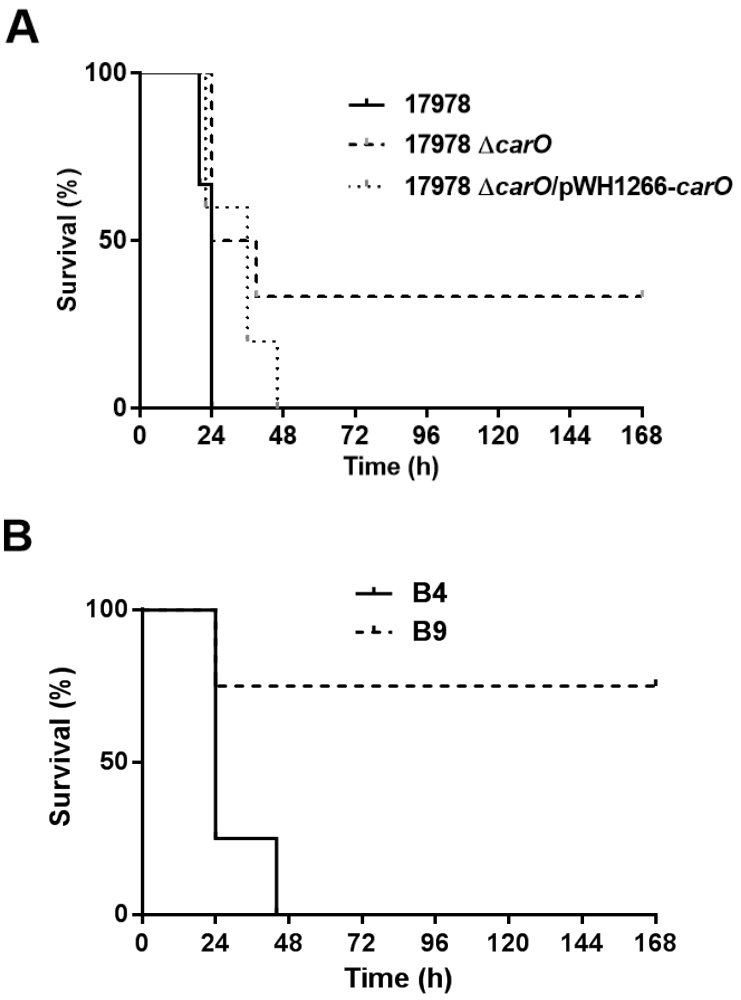

Fig. 5. Analysis of mice survival time in the peritoneal sepsis model by (A) 3.20 $\log_{10}$ CFU/mL of *A. baumannii* ATCC 17978 wt (17978), its isogenic Δ*carO* mutant (17978 Δ*carO*), and the complemented strain (17978 Δ*carO*/pWH1266-*carO*) (n = 6 mice/strain); and by (B) 4.00 $\log_{10}$ CFU/mL of the *A. baumannii* clinical isolates B4 and B9 (n = 4 mice/isolate). Survival curves showed the percentages of mice survival during 7 days. *P* = 0.03 for 17978 *vs.* 17978 Δ*carO*, and *P* = 0.04 for B4 *vs.* B9, log-rank test.

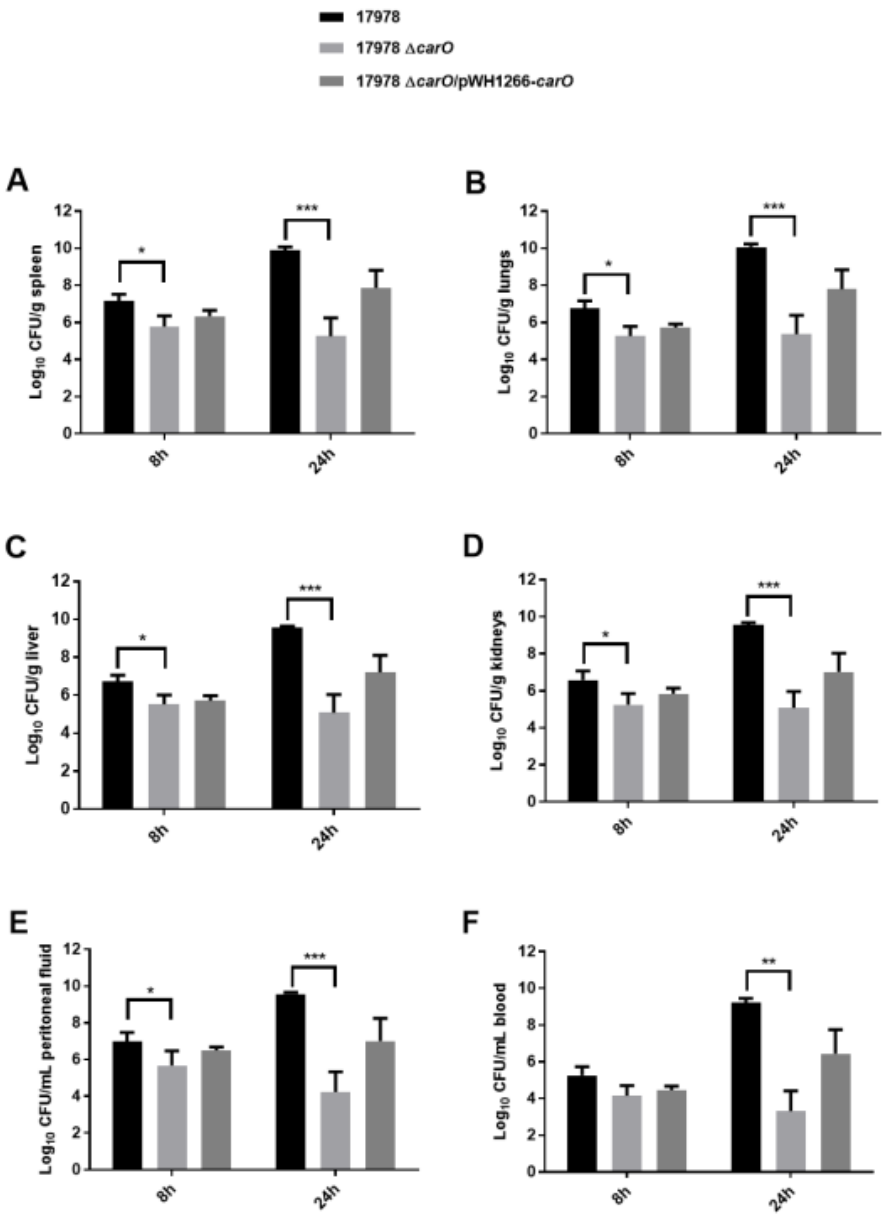

Fig. 6. Bacterial burdens in peritoneal sepsis murine model. Bacterial loads in spleen (A), lungs (B), liver (C), kidneys (D), peritoneal fluid (E), and blood (F), were determined at 8 h and 24 h after intraperitoneal infection with 3.2 $\log_{10}$ CFU/mL of *A. baumannii* ATCC 17978 wild-type (17978), its isogenic *carO* deletion mutant (17978 Δ*carO*), and the Δ*carO* complemented strain (17978 Δ*carO*/pWH1266-*carO*). Data are represented as

mean ± SEM (n=7 mice/strain for each time point). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t-test and Mann-Whitney test.

Table 1. Patients' demographics, clinical features and antibiotic therapy.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
BSI CRAB isolates (codes)	B1	B4	B7	B8	B9	B11
Age (years)	34	48	41	35	48	26
Sex	Female	Male	Female	Male	Male	Male
Charlson index[a]	0	0	0	0	0	0
Diagnosis at ICU admission	CAP[b]	Sepsis	Stroke	Abdominal surgery	Fournier's gangrene	Cardiovascular surgery
APACHE II score[c]	23	20	20	15	17	23
CPIS score[d]	8	6	8	7	6	6
Pitt score[e]	6	12	6	7	6	3
Co-infection	No	No	No	No	No	No
Septic shock	Yes	Yes	Yes	Yes	No	Yes
Acute renal failure	Yes	No	Yes	Yes	No	Yes
Multiorgan failure	Yes	No	Yes	No	No	Yes
CMS[f] treatment (MIU[g]/24 h)	6	8	6	9	9	6
ICU length-of stay (days)	23	18	30	46	31	20
30-days mortality	Yes	Yes	Yes	No	No	No

[a]Charlson: comorbidity index; [b]CAP: community-acquired pneumonia; [c]APACHE II: acute physiology and chronic health evaluation II score; [d]CPIS: clinical pulmonary infection score; [e]Pitt: severity bacteraemia score; [f]CMS: colistimethate sodium; [g]MIU: million international units.

Supplementary material

Text S1: Materials and methods

Identification of known resistance and virulence mechanisms

The ResFinder v3.2 web server (https://cge.cbs.dtu.dk/services/ResFinder/) (1) of the CGE was used to identify acquired antimicrobial resistance genes in the assembled WGS data from the six CRAB clinical isolates producing bloodstream infections, using a threshold of 100% identity for the genes encoding β-lactamases and 98% identity for all other genes. Moreover, genes with functional annotations including either the term "resistance" or "antibiotic" were considered as resistance genes. Furthermore, point mutations in the quinolone resistance determining regions (QRDRs) of the genes associated with quinolone resistance (*gyrA* and *parC*) were identified in all the isolates comparing the predicted proteins encoded by *gyrA* and *parC* with the deduced amino acid sequences of *gyrA* (UniProt entry: D0CBH9) and *parC* (UniProt entry: D0CB90) from *A. baumannii* ATCC 19606 (ciprofloxacin-susceptible strain), and also with those from *Escherichia coli* K12 (UniProt entries: P0AES4 and P0AFI2, for the deduced amino acid sequences of *gyrA* and *parC*, respectively) in order to define the position of the mutations according to the *E. coli* protein. The BLASTP (protein BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (2) tool from the National Center for Biotechnology Information (NCBI) was used for the performance of the comparisons. Similarly, point mutations in the conserved regions of *rpoB* gene, related with rifampicin resistance, was detected following the same procedure described for *gyrA* and *parC*, using the deduced amino acid sequences of *rpoB* (UniProt entry: A3M1G3) from *A. baumannii* ATCC 17978 (rifampicin-susceptible strain). Identification of

mutations in *pmrAB* and *lpx* genes was not performed due to all isolates were colistin-susceptible.

Genes encoding known virulence factors were identified from the WGS data of the clinical isolates using two different strategies. For both strategies, virulence genes described in the MDR *A. baumannii* isolate AB0057 were used for the comparison, being the *A. baumannii* isolate with the higher number of virulence genes contained in the Virulence Factors of Pathogenic Bacteria database (VFDB) (http://www.mgc.ac.cn/VFs/) (3). The first strategy was based on the VFanalyzer option of the VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) (4). VFanalyzer is an automatic analysis pipeline for a systematic screen of known/potential virulence factors in given complete/draft bacterial genomes. So, draft genomes (contig files) were uploaded and Option 3 was selected ("Upload private data of raw or annotated genome in FASTA or GenBank format"). VFanalyzer performed an automatic prediction of protein-coding genes using GLIMMER3 and then ran the comparison analysis. For the second strategy, amino acid sequences of all the virulence factors of *A. baumannii* isolate AB0057 contained in VFDB were downloaded, and the inferred protein sequences from the six CRAB clinical isolates were compared to them using the standalone version of BLASTP from the NCBI. A protein was related to a virulence factor present in the database when the BLAST hit indicated an identity ≥ 95% for a query coverage ≥ 95%. Thus, using both strategies, the ability to detect all virulence genes were higher than using one alone.

Growth curves

The growth profiles of all strains and isolates used for *in vitro* and *in vivo* studies were compared. Thus, 5×10^5 CFU/mL were grown in 10 mL of MHB (with antibiotic when

necessary). At 2, 4, 8, and 24 h, the corresponding CFU/mL were determined by plating serial log_{10} dilutions on the corresponding MH agar plates without or with antibiotic, depending on the strain, and on blood agar plates the clinical isolates.

Text S2: Results

Other genomic differences between CRAB clinical isolates from non-surviving and surviving patients

Despite CarO was the most notable difference between both groups of clinical isolates, other differences between isolates involving loci corresponding to bacteriophage genes, and implying small changes in length either at the N-terminus or C-terminus of the encoded proteins, or disruptions in the case of the B1 isolate, were found. In addition, differences in length were also observed in the gene encoding the giant biofilm-associated protein Bap. However, the multiple arrays featuring immunoglobulin-like motifs common to this protein (5) made difficult the accurate assembly of the sequence data obtained for the different isolates, and whether significant differences exist at these loci between both groups of isolates is uncertain. A similar situation occurred for the locus encoding the pilus assembly protein FilE, which was found in both groups of isolates to encode for a variable number of repeats of the tripeptide TAP in the different isolates (data not shown).

References in Texts S1 and S2

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Table S1. Minimum inhibitory concentrations (MICs) of different antimicrobials for the six CRAB clinical isolates.

Antimicrobial agent MIC (µg/mL) (SIR)[a] / Clinical isolates	Imipenem	Doripenem	Meropenem	Ceftazidime	Sulbactam	Amikacin	Tobramycin	Ciprofloxacin	Rifampicin	Doxycycline	Tigecycline	Colistin
B1	64 (R)	> 8 (R)	> 16 (R)	64 (R)	32 (R)	128 (R)	> 128 (R)	256 (R)	4 (S)	16 (R)	0.5 (S)	0.06 (S)
B4	32 (R)	> 8 (R)	4 (I)	128 (R)	4 (S)	64 (R)	128 (R)	64 (R)	32 (R)	32 (R)	1 (S)	0.03 (S)
B7	8 (R)	4 (R)	4 (I)	128 (R)	4 (S)	2 (S)	128 (R)	32 (R)	16 (R)	32 (R)	0.5 (S)	0.06 (S)
B8	16 (R)	4 (R)	4 (I)	64 (R)	4 (S)	4 (S)	1 (S)	16 (R)	16 (R)	32 (R)	0.5 (S)	0.03 (S)
B9	8 (R)	4 (R)	4 (I)	128 (R)	8 (I)	64 (R)	128 (R)	64 (R)	32 (R)	32 (R)	1 (S)	0.12 (S)
B11	16 (R)	4 (R)	4 (I)	64 (R)	8 (I)	2 (S)	> 128 (R)	16 (R)	32 (R)	32 (R)	0.5 (S)	0.06 (S)

[a]S: susceptible; I: intermediate; R: resistant. Breakpoints, imipenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; doripenem (CLSI): susceptible MIC ≤ 2 µg/mL, intermediate MIC = 4 µg/mL, and resistant MIC ≥ 8 µg/mL; meropenem (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 8 µg/mL; ceftazidime (CLSI): susceptible MIC ≤ 8 µg/mL, intermediate MIC = 16 µg/mL, and resistant MIC ≥ 32 µg/mL; sulbactam (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; amikacin (EUCAST): susceptible MIC ≤ 8 µg/mL and resistant MIC ≥ 16 µg/mL; tobramycin (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; ciprofloxacin (EUCAST): susceptible MIC ≤ 1 µg/mL and resistant MIC > 1 µg/mL; rifampicin (Rev Esp Quimioter 2012;25(2):134-138): susceptible MIC ≤ 4 µg/mL and resistant MIC > 4 µg/mL; doxycycline (CLSI): susceptible MIC ≤ 4 µg/mL, intermediate MIC = 8 µg/mL, and resistant MIC ≥ 16 µg/mL; tigecycline (FDA): susceptible MIC ≤ 2 µg/mL, intermediate MIC = 4 µg/mL, and resistant MIC ≥ 8 µg/mL; colistin (EUCAST): susceptible MIC ≤ 2 µg/mL and resistant MIC ≥ 4 µg/mL.

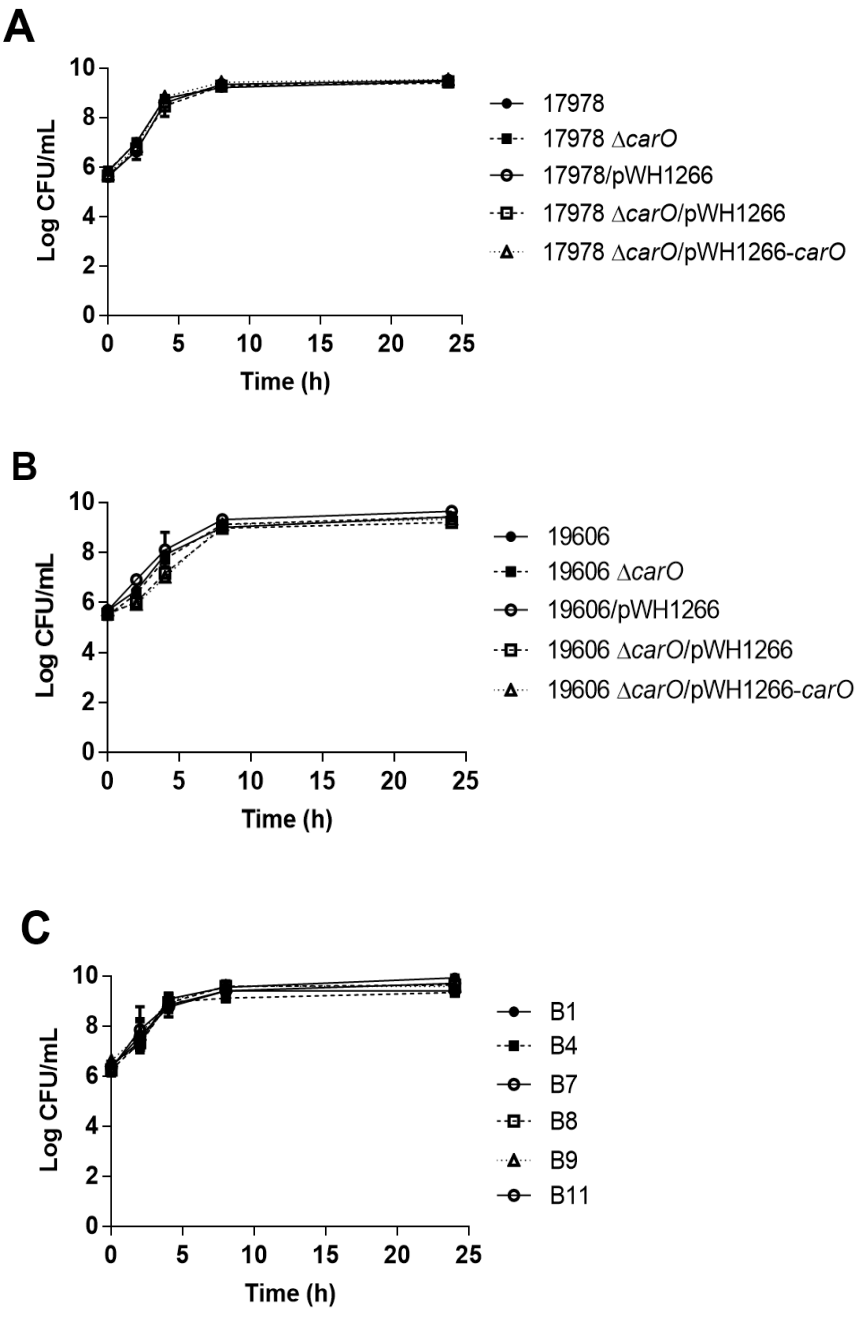

Fig. S1. *In vitro* bacterial growth in MHB. All laboratory bacterial strains: *A. baumannii* ATCC 17978 wild-type (17978), 17978 wt with empty plasmid pWH1266 as control (17978/pWH1266), its isogenic *carO* deletion mutant (17978 Δ*carO*), Δ*carO* mutant

with empty plasmid as control (17978 Δ*carO*/pWH1266), and Δ*carO* mutant with plasmid pWH1266-*carO* expressing CarO (17978 Δ*carO*/pWH1266-*carO*) (A); *A. baumannii* ATCC 19606 wild-type (19606), 19606 wt with empty plasmid pWH1266 as control (19606/pWH1266), its isogenic *carO* deletion mutant (19606 Δ*carO*), Δ*carO* mutant with empty plasmid as control (19606 Δ*carO*/pWH1266), and Δ*carO* mutant with plasmid pWH1266-*carO* expressing CarO (19606 Δ*carO*/pWH1266-*carO*) (B); and the six carbapenem-resistant *A. baumannii* clinical isolates from the present study (C), were grown in MHB for 24 h. Data are represented as mean ± SEM (n=3 replicates in different days).

Data set S1. Antimicrobial resistance mechanisms identified by ResFinder (A) and BLAST (B), or genes with functional annotations including either the term "resistance" or "antibiotic" (C), from whole-genome sequencing data of the 6 bacteraemic carbapenem-resistant A. baumannii clinical isolates.

	Isolates presenting the corresponding gene or mechanism						Gene names	Description	
A	B1	B4	B7	B8	B9	B11	*bla* $_{ADC-25}$	Beta-lactam resistance gene, AmpC	
	B1	-	-	-	-	-	*bla* $_{OXA-109}$	Beta-lactam resistance gene, carbapenemase	
	-	B4	B7	B8	B9	B11	*bla* $_{OXA-66}$	Beta-lactam resistance gene, carbapenemase	
	B1	-	-	-	-	-	*bla* $_{OXA-24/40}$	Beta-lactam resistance gene, carbapenemase	
	-	B4	B7	B8	B9	B11	*bla* $_{OXA-58}$	Beta-lactam resistance gene, carbapenemase	
	B1	B4	-	-	B9	-	*aac(6')-Ip*	Aminoglycoside resistance gene	
	-	-	B7	B8	-	B11	*aac(3)-Ia*	Aminoglycoside resistance gene	
	B1	-	-	-	-	-	*aac(3)-IIa*	Aminoglycoside resistance gene	
	-	-	B7	B8	-	B11	*aadA1*	Aminoglycoside resistance gene	
	B1	B4	B7	B8	B9	B11	*strA*	Aminoglycoside resistance gene	
	B1	B4	B7	B8	B9	B11	*strB*	Aminoglycoside resistance gene	
	B1	B4	B7	B8	B9	B11	*sul1*	Sulphonamide resistance gene	
	-	B4	B7	B8	B9	B11	*sul2*	Sulphonamide resistance gene	
	B1	B4	B7	B8	B9	B11	*tet(B)*	Tetracycline resistance gene	
B	B1	B4	B7	B8	B9	B11	S83L mutation in *gyrA*[a]	Quinolone resistance mechanism	[a] Amino acid position equivalent to Ser-83 of *E. coli*
	B1	B4	B7	B8	B9	B11	S80L mutation in *parC*[b]	Quinolone resistance mechanism	[b] Amino acid positions equivalent to Ser-80 and Ser-463 of *E. coli*
	-	B4	-	-	B9	-	I581M mutation in *rpoB*	Rifampicin resistance mechanism	
	-	-	B7	B8	-	B11	S583L mutation in *rpoB*	Rifampicin resistance mechanism	
	B1	B4	B7	B8	B9	B11	*mexB*	Multidrug resistance protein MexB	
	B1	B4	B7	B8	B9	B11	*nolG*	Acriflavine resistance protein	
	B1	B4	B7	B8	B9	B11	*yhcA*	Putative multidrug resistance protein	
	B1	B4	B7	B8	B9	B11	*emrY*	Drug resistance transporter EmrB/QacA suBfamily	
	B1	B4	B7	B8	B9	B11	*emrB*	Multidrug resistance protein B	
	B1	B4	B7	B8	B9	B11	*smvA*	Major facilitator superfamily methyl viologen resistance protein	
	B1	B4	B7	B8	B9	B11	*czcC*	Cobalt-zinc-cadmium resistance protein CzcC	
	B1	B4	B7	B8	B9	B11	*farB*	MFS transporter, DHA2 family, multidrug resistance protein B	
	B1	B4	B7	B8	B9	B11	*ypnP*	Multidrug resistance protein, MATE family	
	B1	B4	B7	B8	B9	B11	*norM*	Multidrug resistance protein, MATE family	
	B1	B4	B7	B8	B9	B11	*acrA*	Acriflavine resistance protein A	
	B1	B4	B7	B8	B9	B11	*fsr*	Fosmidomycin resistance protein	
	B1	B4	B7	B8	B9	B11	*mexB*	Resistance-nodulation-cell division (RND) multidrug efflux transporter MexB	
	B1	-	-	-	-	-	*merA*	Mercuric reductase	
	B1	B4	B7	B8	B9	B11	*tetB*	Tetracycline resistance protein, class B	
	B1	B4	B7	B8	B9	B11	*bcr*	Bicyclomycin resistance protein	
	B1	B4	B7	B8	B9	B11	*ABSDF2341*	Putative multidrug resistance protein	
	B1	B4	B7	B8	B9	B11	*ABAYE2235*	Putative tetracycline resistance protein	
	B1	B4	B7	B8	B9	B11	*emrA*	Membrane fusion component of tripartite multidrug resistance system	
	B1	B4	B7	B8	B9	B11	*ampC*	Beta-lactamase	
	B1	-	B7	B8	-	B11	*MSQ_pABCC8*	Toxic anion resistance protein TelA	

	B1	B4	B7	B8	B9	B11	*yBhR*	Antibiotic transport system permease protein
	B1	B4	B7	B8	B9	B11	*emrA*	Multidrug resistance protein A
	B1	B4	B7	B8	B9	B11	*pac*	Penicillin amidase
	B1	B4	B7	B8	B9	B11	*emrA*	Putative multidrug resistance efflux pump
	B1	B4	-	-	B9	-	*arsB*	Arsenical-resistance protein
	B1	B4	B7	B8	B9	B11	*cacD*	Cobalt-zinc-cadmium resistance protein CzcD
	B1	B4	B7	B8	B9	B11	*ABAYE0009*	Putative RND type efflux pump involved in aminoglycoside resistance (AdeT)
	B1	B4	B7	B8	B9	B11	*ABAYE0008*	Putative RND type efflux pump involved in aminoglycoside resistance (AdeT)
	B1	B4	B7	B8	B9	B11	*adeT*	RND efflux pump involved in aminoglycoside resistance
	B1	B4	B7	B8	B9	B11	*ydhJ*	Multidrug resistance protein A
	B1	B4	B7	B8	B9	B11	*adeT*	RND type efflux pump involved in aminoglycoside resistance
C	-	B4	B7	B8	B9	B11	*folP*	Dihydropteroate synthase
	B1	-	-	-	-	-	*aac(3)II*	Aminoglycoside N(3)-acetyltransferase III
	B1	B4	B7	B8	B9	B11	*mrcB*	Penicillin-binding protein 1B
	B1	B4	B7	B8	B9	B11	*sul1*	Dihydropteroate synthase
	B1	B4	B7	B8	B9	B11	*strB*	Streptomycin resistance protein
	B1	B4	B7	B8	B9	B11	*cpo*	Chloride peroxidase
	B1	B4	B7	B8	B9	B11	*uppP*	Undecaprenyl-diphosphatase
	-	B4	B7	B8	B9	B11	*blaoxa58*	Beta-lactamase
	B1	B4	B7	B8	B9	B11	*pcoB*	Copper resistance protein B
	B1	B4	B7	B8	B9	B11	*gacA*	Response regulator (Global antibiotic and cyanide control protein, LuxR/UhpA family)
	B1	B4	B7	B8	B9	B11	*A1S_0317*	Putative fusaric acid resistance protein
	B1	B4	B7	B8	B9	B11	*yahN*	Resistance factor to homoserine/threonine, RhtB family
	B1	B4	B7	B8	B9	B11	*yrhP*	Resistance factor to homoserine/threonine, RhtB family
	B1	B4	B7	B8	B9	B11	*tetR*	Tetracycline repressor protein
	B1	B4	-	-	B9	-	*aac(6')I*	Aminoglycoside acetyltransferase (6') type I
	B1	B4	B7	B8	B9	B11	*ABAYE1699*	Putative transcriptional repressor of multidrug resistance pump (MarR family)
	B1	-	-	-	-	-	*tmrB*	Tunicamycin resistance protein
	-	-	B7	B8	-	B11	*aac(3)I*	AAC(3)-Ia aminoglycoside (3) acetyltransferase
	B1	B4	B7	B8	B9	B11	*aadA1*	Aminoglycoside-3"-adenylyltransferase ANT(3")-I
	B1	B4	B7	B8	B9	B11	*ABAYE1609*	Putative transcriptional repressor of multidrug resistance pump (MarR family)
	B1	B4	B7	B8	B9	B11	*macB*	Macrolide export ATP-binding/permease protein MacB3.6.3
	B1	B4	B7	B8	B9	B11	*merR*	MerR regulatory protein
	B1	B4	B7	B8	B9	B11	*ohr*	Organic hydroperoxide resistance protein
	B1	B4	B7	-	-	-	*carO*	Carbapenem-associated resistance protein
	B1	B4	B7	B8	B9	B11	*merC*	Mercuric resistance protein
	B1	B4	B7	B8	B9	B11	*sugE*	Quaternary ammonium compound-resistance protein
	-	B4	-	-	B9	-	*merD*	Mercuric resistance transcriptional repressor protein
	B1	B4	B7	B8	B9	B11	*AQ481_00120*	Antibiotic biosynthesis monooxygenase
	B1	-	-	-	-	-	*pcoB*	Copper resistance protein CopB
	B1	B4	B7	B8	B9	B11	*pcoA*	Copper resistance protein A
	B1	-	-	-	-	-	*eBr*	Delta-quaternary ammonium resistance protein
	B1	B4	B7	B8	B9	B11	*merA*	Mercuric reductase
	B1	B4	B7	B8	B9	B11	*emrB*	Drug resistance transporter EmrB/QacA suBfamily

Data set S2. Virulence genes identified in the 6 bacteraemic carbapenem-resistant *A. baumannii* clinical isolates when compared their whole-genome sequencing data with the Virulence Factors of Pathogenic Bacteria database (VFDB).

Virulence factor class	Virulence factors[a]	Related genes[a]	Isolates presenting the corresponding gene					
Adherence	Outer membrane protein	*ompA*	B1	B4	B7	B8	B9	B11
Biofilm formation	AdeFGH efflux pump/transport autoinducer	*adeF*	B1	B4	B7	B8	B9	B11
		adeG	B1	B4	B7	B8	B9	B11
		adeH	B1	B4	B7	B8	B9	B11
	Biofilm associated protein	*bap*	B1	B4	B7	B8	B9	B11
	Csu pili	*csuA/B*	B1	B4	B7	B8	B9	B11
		csuA	B1	B4	B7	B8	B9	B11
		csuB	B1	B4	B7	B8	B9	B11
		csuC	B1	B4	B7	B8	B9	B11
		csuD	B1	B4	B7	B8	B9	B11
		csuE	B1	B4	B7	B8	B9	B11
	PNAG	*pgaA*	B1	B4	B7	B8	B9	B11
		pgaB	B1	B4	B7	B8	B9	B11
		pgaC	B1	B4	B7	B8	B9	B11
		pgaD	B1	B4	B7	B8	B9	B11
Enzyme	Phospholipase C	*plC*	B1	B4	B7	B8	B9	B11
	Phospholipase D	*plD*	B1	B4	B7	B8	B9	B11
Immune evasion	LPS	*lpsB*	B1	B4	B7	B8	B9	B11
		lpxA	B1	B4	B7	B8	B9	B11
		lpxB	B1	B4	B7	B8	B9	B11
		lpxC	B1	B4	B7	B8	B9	B11
		lpxD	B1	B4	B7	B8	B9	B11
		lpxL	B1	B4	B7	B8	B9	B11
		lpxM	B1	B4	B7	B8	B9	B11
	Capsule	AB57_0091	B1	B4	B7	B8	B9	B11
		AB57_0092	B1	B4	B7	B8	B9	B11
		AB57_0093	B1	B4	B7	B8	B9	B11
		AB57_0094	B1	B4	B7	B8	B9	B11
		AB57_0095	B1	B4	B7	B8	B9	B11
		AB57_0096	B1	B4	B7	B8	B9	B11
		AB57_0097	B1	B4	B7	B8	B9	B11
		AB57_0098	B1	B4	B7	B8	B9	B11
		AB57_0099	B1	B4	B7	B8	B9	B11
		AB57_0100	B1	B4	B7	B8	B9	B11
		AB57_0101	B1	B4	B7	B8	B9	B11
		AB57_0102	B1	B4	B7	B8	B9	B11
		AB57_0103	B1	B4	B7	B8	B9	B11
		AB57_0104	B1	B4	B7	B8	B9	B11
		AB57_0105	B1	B4	B7	B8	B9	B11

		AB57_0106	B1	B4	B7	B8	B9	B11
		AB57_0107	B1	B4	B7	B8	B9	B11
		AB57_0108	-	B4	B7	B8	B9	B11
		AB57_0109	-	B4	B7	B8	B9	B11
		AB57_0110	-	B4	B7	B8	B9	B11
		AB57_0111	-	B4	B7	B8	B9	B11
		AB57_0112	-	-	-	-	-	-
		AB57_0113	-	-	-	-	-	-
		AB57_0114	-	-	-	-	-	-
		AB57_0115	-	-	-	-	-	-
Iron uptake	Acinetobactin	*barA*	B1	B4	B7	B8	B9	B11
		barB	B1	B4	B7	B8	B9	B11
		basA	B1	B4	B7	B8	B9	B11
		basB	B1	B4	B7	B8	B9	B11
		basC	B1	B4	B7	B8	B9	B11
		basD	B1	B4	B7	B8	B9	B11
		basF	B1	B4	B7	B8	B9	B11
		basG	B1	B4	B7	B8	B9	B11
		basH	B1	B4	B7	B8	B9	B11
		basI	B1	B4	B7	B8	B9	B11
		basJ	B1	B4	B7	B8	B9	B11
		banA	B1	B4	B7	B8	B9	B11
		banB	B1	B4	B7	B8	B9	B11
		banC	B1	B4	B7	B8	B9	B11
		banD	B1	B4	B7	B8	B9	B11
		banE	B1	B4	B7	B8	B9	B11
		banF	B1	B4	B7	B8	B9	B11
		entE	B1	B4	B7	B8	B9	B11
	Heme utilization	*hemO*	-	-	-	-	-	-
		AB57_0984	B1	B4	B7	B8	B9	B11
		AB57_0985	-	-	-	-	-	-
		AB57_0986	-	-	-	-	-	-
		AB57_0987	-	-	-	-	-	-
		AB57_0988	-	-	-	-	-	-
		AB57_0989	-	-	-	-	-	-
		AB57_0990	-	-	-	-	-	-
		AB57_0992	-	-	-	-	-	-
		AB57_0993	B1	B4	B7	B8	B9	B11
Regulation	Quorum sensing	*abaI*	B1	B4	B7	B8	B9	B11
		abaR	B1	B4	B7	B8	B9	B11
	Two component system	*bfmR*	B1	B4	B7	B8	B9	B11
		bfmS	B1	B4	B7	B8	B9	B11
Serum resistance	Polysaccharide polymer	*pbpG*	B1	B4	B7	B8	B9	B11

[a]Virulence factors/genes described in the *A. baumannii* isolate AB0057. PNAG: polysaccharide poly-N-acetylglucosamine; LPS: lipopolysaccharide.