Not the usual suspects: membrane translocation, pathogenic potential and bacterial species of the *Acinetobacter baumannii* group

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Barcelona, enero de 2019

El Dr. **JORDI VILA ESTAPÉ**, Catedrático del Departamento de Fundamentos Clínicos de la Facultad de Medicina de la Universitad de Barcelona, Jefe del Servicio de Microbiología del Hospital Clínic de Barcelona y Research Professor y Director de la Iniciativa de Resistencia a antimicrobianos del Instituto de Salud Global de Barcelona (ISGlobal) y el Dr. **IGNASI ROCA SUBIRÀ** Assistant Research Professor, ISGlobal,

CERTIFICAN:

Que el trabajo de investigación titulado "**Not the usual suspects: membrane translocation, pathogenic potential and bacterial species of the *Acinetobacter baumannii* group"**, presentado por **CLARA COSGAYA CASTRO**, ha sido realizado en el Laboratorio de Microbiología de ISGlobal, dentro de las dependencias del Hospital Clínic de Barcelona, bajo nuestra dirección y cumple con todos los requisitos necesarios para su tramitación y posterior defensa ante el Tribunal correspondiente.

Barcelona, enero 2019,

Dr. Jordi Vila Estapé
Director de la tesis doctoral

Dr. Ignasi Roca Subirà
Director de la tesis doctoral

A mi abuelo Pepe

Solstici

Reconduïm-la a poc a poc, la vida,
a poc a poc i amb molta confiança,
no pas pels vells topants ni per dreceres
grandiloqüents, sinó pel discretíssim
camí del fer i desfer de cada dia.

Reconduïm-la amb dubtes i projectes,
i amb turpituds, anhels i defallences,
humanament, entre brogit i angoixes,
pel gorg dels anys que ens correspon de viure.

En solitud, però no solitaris,
reconduïm la vida amb la certesa
que cap esforç no cau en terra eixorca.
Dia vindrà que algú beurà a mans plenes
l'aigua de llum que brolli de les pedres
d'aquest temps nou que ara esculpim nosaltres.

Miquel Martí i Pol

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Abbreviations and Acronyms

%G+C content	Ratio of guanine and cytosine nucleosides in the genomic DNA
AAC	Aminoglycoside Acetyltransferases
AAD	Aminoglycoside Adenylyltransferase
Ab group	*Acinetobacter baumannii* group
ABC family	ATP-Binding Cassette family
ACB complex	*Acinetobacter calcoaceticus-Acinetobacter baumannii* complex
ADC	*Acinetobacter*-Derived Cephalosporinase
AFLP	Amplified Fragment Length Polymorphism
AI-2	Autoinducer-2
AI-2E superfamily	Autoinducer-2 exporter
AME	Aminoglycoside-Modifying Enzyme
AMK	Amikacin
AMP	Antimicrobial Peptide
ANI	Average Nucleotide Identity
ANIb	ANI based on BLAST algorithm
ANT	Aminoglycoside Nucleotidyltransferase
APACHE II score	Acute Physiology and Chronic Health Evaluation II score
APH	Aminoglycoside Phosphotranferases
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ARI	*Acinetobacter* Resistance Imipenemase
Ata	*Acinetobacter* Trimeric Transporter
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BAP	Biofilm Associated Protein
BJ	Bouvet & Jeanjean
bla	β-latamase
BLAST	Basic Local Alignment Search Tool
BlsA	Blue Light Sensing A
BLUF	Blue Light Using Flavin
CAZ	Ceftazidime
CC	Clonal Complex
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CFU	Colony Forming Units
CHDL	Carbapenem-Hydrolyzing class D β-lactamase
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute

CO_2	Carbon dioxide
COL	Colistin
DDH	DNA-DNA Hybridization
DNA	Desoxyribonucleic Acid
DNase	Desoxyribonuclease
EDTA	Ethylenediamine Tetraacetic Acid
EPI	Efflux Pump Inhibitor
EPS	Extracellular Polymeric Substance
ESBL	Extended Spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Cefepime
FIG	Figure
FMT	Fecal Microbiota Transplantation
GEN	Gentamicin
gen. sp.	Genomic species
GES	Guiana Extended-Spectrum
GIM	German Imipenemase
HGT	Horizontal Gene Transfer
H-NS	Histone-like Nucleoid Structuring
ICU	Intensive Care Unit
IMI	Imipenem
IMI	Imipenemase
Inf.	Infection
IS	Insertion Sequence
ITS	Internal Transcribed Spacer
KAN	Kanamycin
KPC	*Klebsiella pneumoniae* Carbapenemase
LB	Luria-Bertani, Lysogeny Broth
LPS	Lipopolysaccharide
LT50	Lethal time 50%
LVX	Levofloxacin
MAFFT	Multiple Alignment using Fast Fourier Transform
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight
MAPK	Mitogen Acitvated Protein Kinase
MATE family	Multidrug And Toxic compound Extrusion family
MBL	Metallo-β-lactamase
MDR	Multidrug Resistant
MEM	Meropenem
MFP	Membrane Fusion Protein
MFS	Major Facilitator Superfamily
MH	Mueller Hinton

MIC	Minimum Inhibitory Concentration
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
MOX	Moxifloxacin
MS	Mass Spectrometry
MUSCLE	Multiple Sequence Comparison by Log- Expectation
MVA	Multivariate Analysis
NCBI	National Center for Biotechnology Information
ND	Not Determined
NDM	New Delhi Metallo-β-lactamase
NF-κB	Nuclear Factor κB
NGM	Nematode Growth Medium
NLS	Nucleas Localisation Signal
NMC	Non-Metallocarbapenemase
NMP	1-(1-naphthylmethyl)-piperazine
NOD1/2	Nucleotide-binding Oligomerisation Domain containing protein 1/2
O_2	Oxygen
OD	Optical Density
OMC	Outer Membrane Complex
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicle
ON	Overnight
OPZ	Omeprazole
OXA	Oxacillinase
PAβN	Phenylalanine-β-naphthylamide
PACE family	Proteobacterial Antimicrobial Compound Efflux family
PAMP	Pathogen-Associated Molecular Patterns
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDR	Pandrug Resistant
PFGE	Pulsed Field Gel Electrophoresis
PRR	Pattern Recognition Receptor
QS	Quorum Sensing
RAPD	Random Amplification of Polymorphic DNA
REP-PCR	Repetitive Extragenic Palindromic PCR
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RND superfamily	Resistance-Nodulation-cell Division superfamily
rRNA	Ribosomal RNA
SD	Standard Deviation

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIM	Seoul Imipenemase
SME	*Serratia marcescens* Enzyme
SMR family	Small Multidrug Resistance family
SPM	Sao Paulo Metallo-β-lactamase
SS	*Salmonella-Shigella*
ST	Sequence Type
T2SS	Type II Secretion System
T5SS	Type V Secretion System
T6SS	Type VI Secretion System
TCS	Two Component System
TGC	Tigecycline
TLR	Toll-like Receptor
Tm	Melting/Annealing Temperature
TOB	Tobramycin
tRNA	Transfer RNA
TU	Tjenberg & Ursing
UPGMA	Unwighted Pair Group Method using Arithmetic Averages
VIM	Verona Imipenemase
WHO	World Health Organisation
WT	Wild Type
XDR	Extensively Drug Resistant
XLD	Xylose Lysine Deoxycholate

I. INTRODUCTION

1. Bacterial taxonomy

The biological diversity surrounding us is overwhelming, especially when we consider the microbial world, where tiny organisms challenge our knowledge beyond eyesight with their microscopic existence. Since ancient Aristotle's times, classification of all forms of living beings has helped us to understand biodiversity, and after the publication of *On the Origin of species* by Charles Darwin in 1859 [1], the sorting of organisms into different groups has also been tightly linked to our conception of evolution itself. Taxonomy stands, thus, as an essential field for our comprehension of the world.

Taxonomy is not just about naming organisms, it involves doing so in accordance with their most defining properties, at least up to the gender level. This discipline can be considered the study of biodiversity which also attempts to organise it, and it should be performed following a classification system that reflects to its best the natural order of the organisms [2]. For this purpose, the accurate description of the common properties of any group of organisms under study, thus is characterisation, becomes fundamental to properly classify such group within a determined taxon. If enough evidence of its uniqueness is found, the group can be named following the Linnaean nomenclature system. Therefore, characterisation, classification and nomenclature constitute interrelated and dependent fields that altogether shape taxonomy. Unfortunately, and although unarguably useful, this whole process is a mere artificial construct, especially for prokaryotes, where the lack of a consensus definition for the *species* concept [3] and the absence of an official system for bacterial characterisation and classification [4,5] hinder the establishment of the boundaries between taxa.

The *species* concept proposed by Ernst Mayr [6] for eukaryotes, defined as "reproductively isolated units of organisms" cannot be applied to bacteria, since even when "sexual" exchange of genetic material happens, i. e. horizontal gene transfer (HGT), it can occur beyond the species boundary and even beyond the *Bacteria* and *Archaea* domains [7]. The *species* concept in bacterial taxonomy is a controversial topic, where many definitions have been proposed, but no clear consensus has been achieved. Some authors have even claimed that the species unit cannot be defined for *Bacteria* and *Archaea* [8]. Ford Doolittle considers that the inability to find a biological process that clearly explains the speciation

process of bacteria "highlight[s] the ontological vacuity of that vexed word [species]" and thus that *species* do not exist [9]. However, Rosselló-Móra states that, indeed, it is a semantic problem [2], and while *species* are regarded by most taxonomists as the basic unit of biological diversity, the dispute lies within the different interpretation of *species* by taxonomists, ecologists and evolutionary biologists and how to describe them (**FIG 1**) [3].

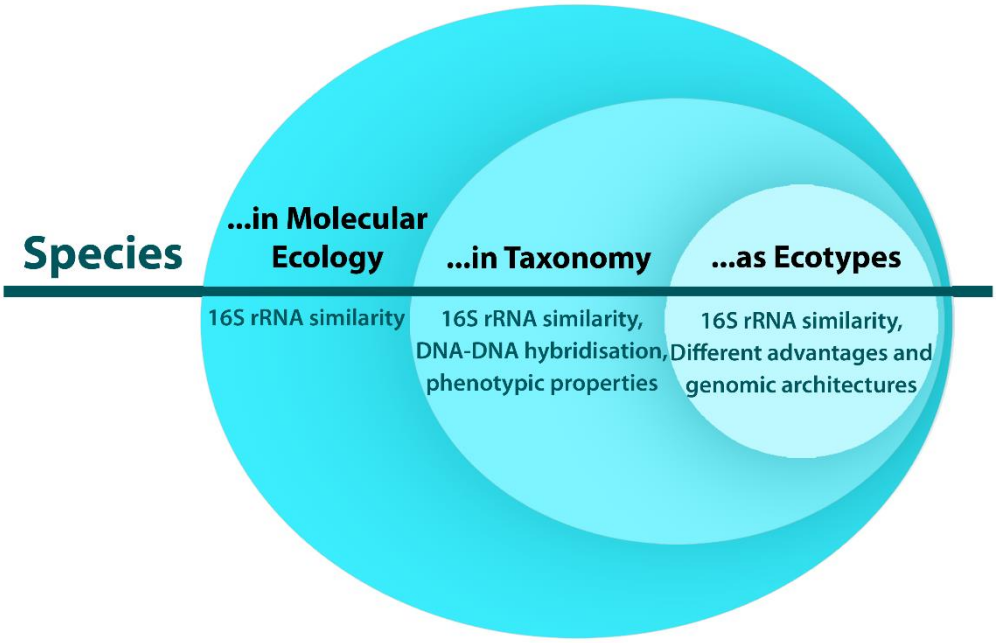


FIG1. Different interpretations of the *species* concept in microbiology according to different study fields. The narrowest concept, in which more recurrent patterns are considered, is the one used in genetic evolution, where species are seen as ecotypes; ecotypes consist in populations that are genetically cohesive but ecologically distinct due to a small number of extra genetic elements or mutations. Taxonomists have a less strict view, in which a compromise between finding accurate boundaries between species populations and coherence among genealogic, genomic and phenotypic traits is pursued. Finally, the most permissive interpretation is employed by molecular ecologists, who define new bacterial species based just on 16S rRNA gene sequence analyses. (Adapted from [2])

Rosselló-Móra and his colleagues have, over the years, proposed several *species* definitions, aiming to expose the concept in its most universal version [3,10–12]. Their last definition [3] stated the following:

> "[A species is] a category that circumscribes monophyletic and genomically and phenotypically coherent populations of individuals that can be clearly discriminated from other entities by means of standardized parameters"

According to the authors [3], monophyly is as an absolute premise for a group of organisms to be delineated as the same species. Moreover, for the characterisation of a new bacterial species the "standardized parameters" suggested have to include both genetic and phenotypic data. This characterisation system is known as *polyphasic* taxonomy [13,14], a multidimensional approach that arose after the popularisation of the molecular methods, and which is widely used nowadays by bacterial taxonomists to confront the fact that an officially recognised system does not exist.

The bacterial taxonomy field evolves along with the availability of new technological developments that allow the best resolution methods for the delineation of species (**TABLE 1**). However, as there have never been formal guidelines to characterise and classify new bacterial species, the techniques used depend greatly on the state-of-the-art methods and the type of organism; hence, contemporary publications can considerably differ in the methodologies employed. Between 1880 and 1900 the taxonomists' interests were focused on the description of pathogenic bacteria, which they performed mainly by comparing the morphology and growth requirements of a certain bacterial population. In the first half of the 20th century, the knowledge on the biochemical properties and physiology of bacteria increased and these data were also used to characterise bacteria. The introduction of molecular methods in the late 20th century allowed genetic characterisations of bacteria and represented an inflection point on the discovery rate of novel bacterial species, which rapidly increased (**FIG 2**). Furthermore, thanks to the introduction of genotypic analyses, the inference of the phylogenic relatedness between bacteria, and thus a proper classification, was finally possible, as the lack of bacterial fossil records and the unfeasibility to use comparative anatomy studies were restraining the establishment of evolutionary relations between microorganisms.

TABLE 1. History of the classification of *Bacteria* and *Archaea*. (Adapted from [15])

Period	Classification mainly based on
Late 19th century	Morphology, Growth requirements, Pathogenic potential
1900 – 1960	Morphology, Physiology, Biochemistry
1960 – 1980	Chemotaxonomy, Numerical taxonomy, DNA-DNA hybridization
1980 – Today	Genotypic analyses, Multilocus sequence analyses, Average nucleotide identity, Whole genome analysis

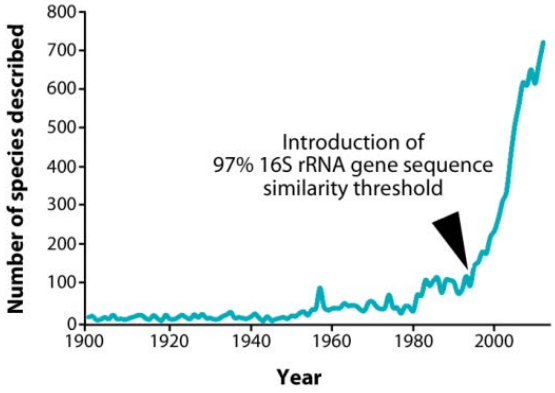


FIG 2. Number of prokaryotic species described per year since 1900 (Adapted from [16])

In the following sections, a detailed description of the characterisation and classifications methods that have been used in taxonomy will be provided, as well as an insight into the nomenclature system of bacteria and the current status of the taxonomy field beyond the polyphasic approach.

1.1. Characterisation

In order to properly characterise a novel species, one of the main objectives of the taxonomists should be to provide an accurate description of the distinguishing traits of a group of organisms to support its correct classification and indisputable identification. As already mentioned, taxonomy has varied over the decades as more discriminatory methods appeared (**TABLE 1**; **FIG 3**). Initially, the techniques employed were based on phenotypic characteristics, but later on, genetic analyses proved to be powerful methods for the characterisation of bacteria, and were integrated in taxonomy in the polyphasic approach. This term was introduced in 1970 [13] and the concept was further developed and adapted to newer techniques in the following years [14]. Since the recommendation of the inclusion of phylogenetic data [14,17], the polyphasic approach has been widely used in bacterial taxonomy, although the lack of official guidelines entails the problem of which techniques should be employed [4].

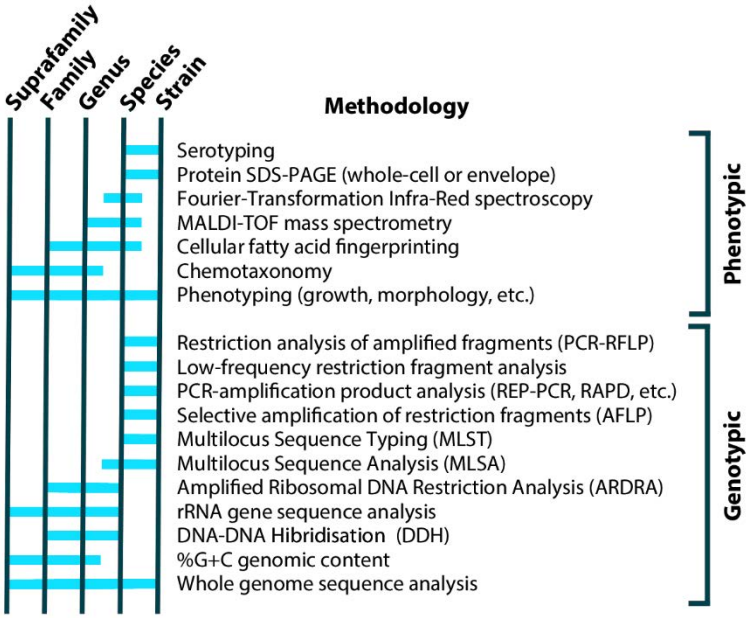


FIG 3. Different resolution levels of the different methods employed for new species characterisation. (Adapted from [18])

1.1.1. Phenotypic traits

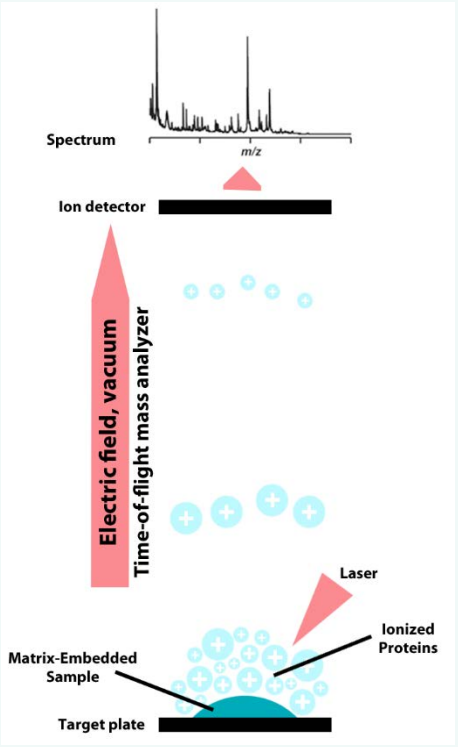
The phenotypic characterisation is considered as an observational approach and the starting point for the description of novel species. The phenotypic traits are the consequence of the expression of the genes of an organism, they include several features, from the growth conditions (O_2 and CO_2 requirements, temperature, components of the media…) to cell and colony characteristics (shape, size, colour, texture, opacity, odor…) [4,15,17]. These relevant features can suffice for a trained microbiologist to identify microorganisms according to the origin of the sample, especially in the clinical settings. Therefore, it is not surprising that this information has to be included in the protologue (the original description of a species, or taxon, which provides a detailed explanation of its defining characteristics in the publication where the species name is proposed) of the taxon being described. In addition, and in order to assess the variability of the phenotypic characteristics, it is recommended to use more than one representative strain of the new taxon and strains from the most closely related taxa for comparison purposes [4]. Other characteristics such as physiology (use of different substrates and metabolic activities) and chemotaxonomy,

which comprises the chemical composition of cell constituents (DNA composition, lipids, isoprenoids quinones, cytochromes, cell wall composition, peptidoglycan…) are also considered phenotypic traits [4,15]. The relevance of phenotypic data became evident after the rise of numerical taxonomy in the 1950s. After gathering the phenotypic information of several strains, matrices were built in order to detect pairwise similarities and this data was used to perform cluster analysis and to generate dendrograms that helped to determine the phenotypic consistency of the group under study [17].

Nevertheless, traditional phenotyping is nowadays regarded as a constraint to faster species descriptions because of its cost in time and resources, but also due to debatable reproducibility and minimized relevance after the introduction of molecular methods. Alternative and complementary methods do exist [2,17] as does the will of taxonomists to replace classical phenotypic characterisation [3,19]. Perhaps the most recognised alternative to biochemical methods is mass spectrometry (MS), particularly matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF). In the taxonomic field, MALDI-TOF MS is applied as a whole cell profiling methodology that detects macromolecular components (**BOX 1**). When whole-cell bacterial extracts are analysed, ribosomal and other structural proteins are detected as peaks in a spectral pattern for a given strain. Then, the spectra produced for every strain can be compared and used to infer similarities among them. Detection of species-specific peaks, or the absence of them, when analysing the spectral pattern of closely-related strains can help to elucidate if they belong to the same species. Interestingly, as the main fraction of proteins detected are ribosomal, the spectra generated for a given species show great stability and congruency with phylogenetic analyses (see below, **Section 1.2**). Altogether, the use of MALDI-TOF MS in taxonomy is not limited to the determination of phenotypic characteristics, but it also has an outstanding applicability as a reliable tool for routine bacterial species identification (as discussed in **Section 2.4.1**). Moreover, the generation of spectral patterns that can be uploaded to databases is valuable not only for identification, but also for the discovery of new bacterial species. Despite promising, MALDI-TOF MS has still to overcome some drawbacks that impair its extensive use such as the generation of non-comparable spectra from different commercial instruments and the lack of public databases.

BOX 1. Principles of MALDI-TOF MS in bacterial characterisation and identification.

Bacterial extracts from pure cultures, or direct colonies from agar plates, are deposited sterilely on a target plate and overlaid with a matrix solution composed of an organic acid, which co-crystalizes with the sample and lyses the organisms. Once the matrix is dry, the plate can be placed into the instrument, where the matrix-embedded samples are ionised by short laser pulses, and subsequently accelerated through an electric field in a vacuum tube. The differently charged particles will then move across the vacuum according to their mass and charge. The amount of time that each particle takes to reach the detector is called the "time-of-flight" (TOF), and can be used to infer the molecular mass of each particle. After all the abundant particles are detected, a spectral pattern is generated which is unique to the sample.



Mainly, the proteins detected are ribosomal but there are also highly abundant cytosolic proteins. When compared to other spectra, the differential peaks (proteins) in the spectral pattern can be used to determine phenotypic differences. For identification purposes, the software matches the spectral pattern produced against the reference database, allowing identification of the unknown organism.

1.1.2. Genotypic traits

In addition to the phenotypic characterisation, the genotypic traits, thus is, the features within the genetic material, can be employed to determine if two groups of organisms belong to the same or different species. Initially, the DNA base composition, particularly the ratio of guanine and cytosine nucleosides in the genomic DNA (%G+C content) was widely used despite its limited taxonomic resolution at the species level (**FIG 3**). Differences of more than 10% in the %G+C content indicate that the strains being compared belong to different genera, whereas within a well-defined species the %G+C content value should vary less than 3% [20]. Although, a similar %G+C content does not necessarily entail that the strains compared belong to the same species or are closely related, this characteristic, which nowadays is easily obtained after genome sequencing, is still provided as a defining characteristic in the protologue of the new species which is being described [11].

Until recently, the reference standard method for species genotypic characterisation was DNA-DNA hybridization (DDH). This technique, introduced in the late 1960s [21,22], consists in the fragmentation of the whole genome of two strains and subsequent pairwise hybridization. The outcome of DDH provides an indirect parameter of the sequence similarity between the two genomes that are being compared. In 1987, it was suggested that 70% or greater DDH relatedness between two strains showed that they belong to the same species [14]. This recommendation established a clear boundary for species delineation, which meant that using DDH for defining a new species became less subjective than the use of other characterising approaches without such a clear species threshold. Consequently, DDH rapidly became the method of choice to circumscribe new species, and it has been widely used over a period of 50 years, although, in fact, it was never meant to be a gold standard due to its weaknesses (**TABLE 2**) [15]. One of the main problems of the DDH technique lies behind its complexity and inter-laboratories irreproducibility [3]. For this reason, once genome sequencing became accessible to most of the laboratories, average nucleotide identity (ANI), which is obtained after the pairwise comparison of genome sequences, replaced DDH, now considered an outdated method. Actually, ANI is a great *in silico* alternative to DDH [23], since it has been experimentally demonstrated that ANI and DDH values correlate well, and that the cut-off value of 70% DDH for species demarcation corresponds to ANI values of ≈94% [23,24]. In 2009, Richter *et al.* developed the JSpecies software, this tool uses different genomic-based approaches, among which there is the calculation of the ANI value using different algorithms [25]. The ANI based on BLAST algorithm (ANIb) is the most used by the taxonomist community [16], although other genome-based approaches exist [25,26]. Of note, ANIb species boundary is set at ≈95-96%, and the algorithm allows successful analyses of genomes randomly sequenced to 20% coverage and can be used for uncultured microorganisms [25].

In addition to the genotypic methods mentioned above, there are other DNA typing methods whose discriminatory power goes beyond the species level and that have been widely used both for identification purposes and to divide species into different subtypes. These approaches, despite their high resolution (**FIG 3**), are less prevalent in taxonomy studies due to the lack of clear recommendations, and their use mostly depends on the time frame of the study, author preferences and/or available resources. Initially, some of the first genotypic procedures used, until PCR and DNA sequencing methods became routine techniques in the microbiology laboratories, were based on whole-genome restriction analysis (such as the restriction fragment length polymorphism (RFLP) and the low-frequency restriction fragment analyses coupled to pulsed field gel electrophoresis,

or PFGE) were used in order to obtain DNA band patterns that could be used to subtype species [4,17]. Once PCR was well established in the laboratories, PCR-based typing methods became the methods of choice. PCR-based approaches are sometimes combined with restriction enzyme methodologies like the amplified fragment length polymorphism (AFLP) technique or the amplified ribosomal DNA restriction analysis (ARDRA). These methodologies became popular because of their quickness and applicability to all sorts of bacteria, but nowadays their use in taxonomy is decreasing. Similar techniques, such as the restriction of genomic DNA by low frequency enzymes followed by PFGE, are still widely used by clinical microbiologist in order to determine the clonal relatedness of putative outbreak-causing isolates in the hospitals due to its high resolution power [17].

Of special relevance in the taxonomy field, and essential for classification purposes, was the combination of PCR and sequencing techniques. The concept that macromolecules such as proteins and DNA harbour in their sequence the record of the evolutionary process [27], together with the improvement of molecular techniques meant a revolutionary shift in taxonomy which allowed not only a thorough characterisation of new species, but also a classification system based on the genetic relationships among the taxa.

1.2. Classification

Classification is the process by which organisms are grouped into taxa according to their similarities and relationships in a hierarchical based scheme. Any classification system should provide scientifically based information, be stable and reproducible, and should try to mirror to its best the natural order of *Bacteria*. The phenotypic traits shared by a group of organisms provide useful information for characterisation and identification purposes, and could be used for classification, as in bacterial taxonomy; on the other hand, they do not contain phylogenetic information, that is, information about the ancestry of the organisms over time. The bacterial genotype, however, can be used as evidence of the rates of genetic change over time [27]. Therefore, after an appropriate analysis (**BOX 2**), bacterial taxa can be grouped according to their genetic similarities, which ultimately will lead to a less artificial classification in which the premise of monophyly, that is, having a common evolutionary history, is included.

TABLE 2. Advantages and disadvantages of the main methodologies employed for species delineation and phylogenetic studies. (Adapted from [15])

16S rRNA gene sequence analysis

Advantages	Disadvantages
• Fulfills properties of molecular markers (ubiquity, functionally constant, conserved: stable, poorly subjected to HGT) • Strains with sequence identity ≤98.7% can be generally considered as members of different species • Phylogenetically congruent with other methods and in good agreement with genome-based studies • Applicable for uncultivated bacteria • Data can be uploaded to databases	• Too conserved, usually insufficient resolution at the species level • Presence of multiple 16S rRNA genes, generally diverging 1-2% or higher • If sequence identity >97%, performing DDH is recommended • Often difficult to organise relative branching orders at the phylum level • Based on a single gene, does not necessarily reflect general phylogenetic relationships

DNA-DNA hybridization (DDH) studies

Advantages	Disadvantages
• Widely used reference standard for species delineation • Strains with DDH values >70% belong to the same species • Strains with DDH similarity values <70% correlate well with 16S rRNA	• Rough measurement of average genetic relationship • Only closely related species or subspecies can be distinguished (>90% genome similarity) • Tedious and time-consuming method with debatable inter-laboratory reproducibility • Databases cannot be developed • Not applicable for uncultivated bacteria

Multilocus sequence analysis

Advantages	Disadvantages
• Based on multiple genes, more phylogenetically informative and less subject to recombination effects • House-keeping genes are essential and evolve at slow rates, but faster than rRNA • Resolves at lower taxonomic levels than rRNA • Correlates well with classical species definitions • Amenable to automation and large curated databases exists or can be developed	• Different set of genes are required for different taxa, one set cannot fit all. Selection of proper genes is not clear • Difficulties in designing primers that match all strains • Only corresponds to a minor fraction of the genome • Depth of clustering that defines a taxon is not clear • Clustering may not occur or may include different groups of strains

Back in 1987, it was proposed that one of the most suitable molecular markers for bacterial phylogenetic analyses were rRNA, particularly 16S rRNA, because of their conserved functions and ubiquity [28]. The 16S rRNA gene is approximately 1500 bp, can be found in multiple copies in the bacterial genomes, and it is poorly affected by HGT. Initially, it was proposed that two strains with less than 97% 16S rRNA gene similarity should be considered as different species [29], later on, this threshold was increased to 98.7% [30]. In spite of the good correlation with the DDH similarity cut-off of 70%, DDH was still considered a superior method than 16S rRNA sequence analysis for species delineation [29], due to the different shortcomings of this method (**TABLE 2**), but mainly because of

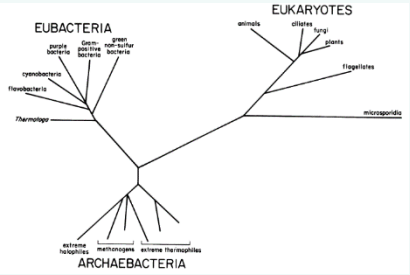
its limited resolution at the species level. In fact, 16S rRNA gene similarity values above the cut-off do not guarantee that the strains being compared belong to the same species [31] and, for this reason, Tindall *et al.* recommended the use of DDH when 16S rRNA similarity values were above 95% in order to better define the species status of the strains studied [4].

Nevertheless, the phylogenetic analysis of the 16S rRNA gene revolutionised bacterial taxonomy and has been the gold standard for deducing phylogenetic relationships during decades. Providing 16S rRNA sequence in new taxa descriptions has been compulsory for many years [11]. Nonetheless, single gene alternatives to the 16S rRNA gene do exist, such as those encoding other ribosomal proteins, RNA polymerases subunits, ATPase subunits, DNA gyrases and heat shock proteins, such as *rpoB*, *dnaJ*, *recA*, *gyrB*, *hsp60*, and *cpn60* among others. Their use on phylogenetic analyses depends on the taxa that are being studied, as some genes might be more suitable for one taxon than for other. Anyway, selection of target genes should always be made following the prerequisites of a good phylogenetic marker, i. e., ubiquity, functional constancy, and proper length and structure variation [2,32].

In order to overcome some of the weaknesses of DDH and 16S rRNA analyses (**TABLE 2**), the multilocus sequence analysis (MLSA) arose as an alternative method [11,33]. MLSA constitutes a compromise between whole genome and single gene analyses since it consists in the comparison of the partial sequences of several housekeeping genes, which are concatenated and utilised to construct phylogenetic trees (**BOX 2**). At least 5 genes sequences should be analysed in order to provide more powerful phylogenetic information than single gene studies [11], albeit a general consensus about the number of genes that should be utilised has never been achieved [2]. MLSA was seen as a good substitute with enough resolution at the species level while it was able to produce phylogenetic trees where species grouped into well-defined clusters. However, the lack of cut-off values for species demarcation and the unfeasibility of selecting universal genes, among other problems (**TABLE 2**), relegated this technique to an optional choice in the presence of more promising methods such as ANI. In contrast, this approach has a valuable use for determining intra-species genetic variability, in fact, it was one of the primary purposes of the method from which it is based on, multilocus sequence typing (MLST), which is used for assessing the clonality of pathogenic bacteria in epidemiological studies (**FIG 3**) [34].

BOX 2. Construction of phylogenetic trees.

The first comprehensive 16S rRNA phylogenetic tree was presented in 1987; in this tree, living organisms were divided into the three primary domains: the *Eukarya*, the *Bacteria* and the *Archaea* [28]. Trees are one of the best ways to represent possible events in the evolutionary story of the organisms by illustrating the clusters and relationships derived from sequence analyses.



However, since tree construction is a reductive process, we must assume that some information might be lost. Depending on which gene sequences are used, the model of evolution that it is used and how events are inferred, many different phylogenetic trees can be obtained. Firstly, nucleotide sequences must be aligned in order to emphasize their overall similarity, i. e. homology. This can be done using multiple alignment algorithms, such as ClustalW, MUSCLE or MAFFT, recommended for small, medium-large (up to thousand sequences) and large (up to 30000 sequences) alignments, respectively [650-652]. Then, the analysis of the multiple sequence alignments can be performed either by algorithmic methods that estimate the evolutionary distance between the sequences, such as the Unweighted Pair Group Method using Arithmetic averages (UPGMA) and the Neighbour Joining, or by other methods that apply a certain model of evolution to tree estimation, like the Maximum Parsimony and the Maximum Likelihood methods [653]. Whichever method is used to represent the phylogenetic tree, its reliability depends on the number of bootstrap repetitions [654-655], which usually range from 100 to 1000. Bootstraping consists in the resampling of data and repetition of the analysis, so a different tree might be generated each time. The number of times that a particular clade appeared in the bootstrap replicates is represented at the nodes of the best-fitted tree, providing a measure of confidence.

1.3. Nomenclature

Once there is enough evidence that a group of organisms is monophyletic and has distinctive and unique characteristics to be considered as a putative novel species, a species name can be proposed. The name of a species consists in a binary combination of the generic name followed by the specific epithet, in Latin, Greek or latinised words (**BOX 3**), that should follow the rules of the International Code of Nomenclature of Prokaryotes (latest version [35]). This code aims not only to regulate nomenclature but also to ensure stability in names, since nomenclature and the associated classifications are key elements for preserving the identity of the organisms.

In order to validly name a species, the International Code of Nomenclature of Prokaryotes only recognises a taxon name if it is *validly published*, which means that it has been effectively published, made available to the scientific community, and if it is accompanied by a description of the taxon or a reference to a previous description and other requirements that are gathered in the International Code of Nomenclature of Prokaryotes [35]. Novel species and nomenclature modifications are validly published in the International Journal of Systematic and Evolutionary Microbiology, and the official bacterial taxonomy is overviewed in the *Bergey's Manual of Systematic Bacteriology*. The updated list of validly published prokaryotic names with standing in nomenclature is available on the internet (http://www.bacterio.net/). This webpage also compiles the names of not effectively published or effectively but not validly published species names, as well as the list of *Candidatus* species for well characterised but yet uncultured organisms, whose nomenclature is not ruled by the International Code of Nomenclature of Prokaryotes.

BOX 3. The naming of taxa: α + κινητο + βακτηρ(ια).

An interesting fact in the context of this PhD dissertation is that according to the Chapter 3, Section 3, rule 6, recommendation 6(7) of the International Code of Nomenclature of Prokaryotes [35], *Acinetobacter*, which derives from the Greek [α + κινητο + βακτηρ(ια)] and means "non-motile bacteria", should be written as *Akinetobacter*, since it is advised that the greek K should be maintained in order to avoid confusion. However, this recommendation is not followed by the taxonomists nor the rest of the microbiologists and the search of "Akinetobacter" in the Pubmed database retrieved no results (last checked October 2018).

For the valid publication of a species name the International Code of Nomenclature of Prokaryotes requires that a type strain is made available by depositing it in at least two publicly accessible culture collections in different countries. The type strain should be the descendant from the strain designated as the nomenclature type, and should be maintained in pure culture. Moreover, it is recommended to base the description on as many strains as possible, and the minimum number of strains necessary for describing a new species should range between 5 and 10 [18,36]. However, single strains species descriptions are allowed [18], even when most of the taxonomic community do not recommend them because these type of descriptions are incomplete and neglect the diversity within a species.

Overall, nomenclature is essential for biological studies since it assigns the name by which a new taxon or species will be known by the scientific community. Thus, changes in nomenclature should not be taken lightly, since the attachment of a name to a bacterial strain has further effects than naming *per se*. Nomenclature places bacteria within a group in the hierarchical classification system, and consequently, certain characteristics are assumed, such as, for instance, pathogenicity. For this reason, the availability of accurate and feasible bacterial species identification tools become fundamental in order to link taxonomy with the rest of microbiological studies.

1.4. The current status of bacterial taxonomy

Similar to the revolution caused by the phylogenetic analysis of 16S rRNA gene sequences in the bacterial taxonomic field, nowadays the increasing numbers of genome sequences available in the public databases (**FIG 4**) have transformed microbiology. Since the sequencing of the first bacterial genome in 1995 [37], the introduction of next generation sequencing techniques and their recent affordability have made genome sequencing available to many laboratories. Therefore, unavoidably, genomics has also reached bacterial taxonomy, and its impact has been so deep that some authors claim that the polyphasic approach can no longer provide additional taxonomic information than genomics [38]. Interestingly, in 1987, Wayne *et al.* [14] already foresaw the importance that genome sequences will exert in the phylogeny and taxonomy of *Bacteria*:

> "the complete DNA sequence would be the reference standard to determine [bacterial] phylogeny and that phylogeny should determine taxonomy"

Many taxonomists are looking forward to the modernisation of the standards used for the description of species [39], since they consider that some of the methods currently used lack intra- and inter-laboratory reproducibility besides being time-consuming and expensive, and therefore, are not accessible to all researchers [40]. The use of the polyphasic approach is progressively being seen as obsolete due to the fact that phenotypic information might be extrapolated from genomic sequences using a genotype-to-phenotype strategy [41]. These authors propose that it is time to integrate genomics as a standard

into bacterial taxonomy, and that the incorporation of genome sequences data into the public repositories might not be sufficient [41]. In fact, Whitman recommended that genomic sequences should be allowed to serve as the type material instead of alive type strains [38,42]. However, there are still many drawbacks: from the lack of quality in the genomic sequences to the fact that the biological diversity encoded in the genome does not always translates into a given phenotype in a given environment [2,19].

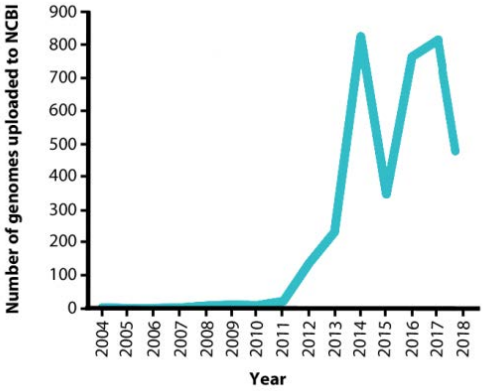


FIG 4. Number of *Acinetobacter* genomes uploaded to the NCBI genome database per year. From the first ever sequenced *Acinetobacter* spp. genome, uploaded in 2004 and corresponding to *Acinetobacter baylyi* ADP1, until the 30th June 2018, when the total number of *Acinetobacter* genomes was 3654. (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/Acinetobacter, 10/07/2018)

Despite these allegations, editors and reviewers of taxonomic journals still request a polyphasic approach for the description of novel species [19]. As said before, bacterial taxonomists are demanding a modernisation of their field and, among other requirements, they ask for the generation of public taxonomy databases, the replacement of chemotaxonomic techniques by newer methods, such as MALDI-TOF, and new publication formats [2,5,19,38]. Moreover, since there are no published guidelines for the description of a novel bacterial species, assessing how much characterisation is needed will remain a hot issue until a general agreement is achieved by the leading scientists on the field. Nevertheless, the implementation of some of the abovementioned requests and other changes, such as the digital protologue [43], are meant to renovate bacterial taxonomy in the future albeit they will still require some time to be fully adopted.

2. The *Acinetobacter* genus

Until the late 1980s, the organisms that we nowadays consider as members of the *Acinetobacter* genus have been taxonomic wanderers. Over the years, they have been described under at least 15 different genera and species names, although, until recently, most of the times they remained unclassified and were usually referred to as "unknown" organisms in the clinical setting. These taxonomic uncertainties were mainly due to the existing limitations in the characterisation and identification techniques available at that time, which rendered these bacteria as a heterogeneous group of non-motile, Gram-negative and strictly aerobic bacilli which, despite the divergences, could not be reliably classified.

Currently, the *Acinetobacter* genus comprises Gram-negative (although sometimes difficult to destain) strictly aerobic, non-fermenting, non-motile, catalase-positive and oxidase-negative coccobacilli with a DNA %G+C content ranging from 39 to 47% [44,45]. Members of the genus grow well between 20ºC and 37ºC, being 33 - 35ºC the optimal temperatures for most of the strains. The majority of *Acinetobacter* strains rarely reduce nitrates, but they can grow in simple mineral media supplemented with ammonium or nitrate salts and a single carbon and energy source [44]. Colonies are tipically smooth, but sometimes can be mucoid, and usually, when Gram-stained from agar plates, they display diplococcobacilli arrangements, which explains why they were once considered as *Neisseria* [46] or even designated as *Diploccoccus* [47]. Nowadays, *Acinetobacter* spp. are classified in the family of the *Moraxellaceae*, within the *Pseudomonadales* order and the *Gammaproteobacteria* class, together with genera such as *Moraxella* and *Psychrobacter* [48].

Despite their erratic taxonomy, since their final full acceptance in the *Bergey's Manual of systematic Bacteriology* in 1984 [49], the members of the *Acinetobacter* genus have gone from anonymity (and being unnamed) to being first in the list of antibiotic-resistant "priority pathogens" published by the World Health Organisation (WHO) in the beginning of 2017 [50]. Such success in attracting our attention is due to the fact that after the development of reliable identification methods for *Acinetobacter*, it was seen that these species were involved in severe infections and had increasing antibiotic resistance, especially in the hospital settings, where one species, *Acinetobacter baumannii,* stands out over the others.

In the following sections an overview of the history of the *Acinetobacter* genus and its most common habitats will be provided, followed by an outline of the species identification methodologies that have been employed over the years.

2.1. Taxonomic history

The first description of a member of the *Acinetobacter* genus was under the name of *Micrococcus calcoaceticus* back into 1911 [51] and was performed by Martinus Willem Beijerinck (1851–1931), a Dutch microbiologist and botanist. This group of organisms was recovered from soil after enrichment in a calcium acetate minimal medium. The publication *per se,* however, was not considered as a valid species description at the time. Indeed, this publication went unnoticed and lead to several later characterisations of similar organisms under different names, which were assigned to diverse genera and species, such as *Diplococcus mucosus* [47], *Alcaligenes haemolysans* [52], *Mima polymorpha* [46], *Moraxella lwoffii* [53], *Herellea vaginicola* [54], *Bacterium anitratum* [55], *Neisseria winogradskyi* [56], *Achromobacter anitratus* [57] and *Achromobacter mucosus* [58].

After reexamination of the strains described by Beijerinck in 1911 and the finding that they had the same characteristics as other species described afterwards, such as *B. anitratum*, it was proposed to include all of them in the *Achromobacter* genus [57]. Later on, the genus *Acinetobacter* was created in order to include the non-motile species of *Acrhomobacter* [59], and *Acinetobacter anitratus* (*calcoaceticus*) was selected as the type species of the genus [60]. Several alternatives about how to subdivide the *Acinetobacter* genus into different species were considered at that time, such as dividing it by the ability to produce acid from sugars or by proteolytic and non-proteolytic varieties [61]. Nevertheless, it was finally agreed that the genus should comprise only one species due to the inability to phenotypically distinguish among its members [61,62]. Many authors started using the name *Acinetobacter calcoaceticus* instead of *A. anitratus*, and finally the former name prevailed over and was accepted by the scientific community. In 1974, the *Bergey's Manual of systematic Bacteriology* [63] included for the first time the genus *Acinetobacter*, with the description of only one species, *A. calcoaceticus*, as its type species. In contrast, two *Acinetobacter* species were included in the "Approved List of Bacterial Names", i. e. *A. calcoaceticu*s and *Acinetobacter lwoffii,* classified according to their ability or inability to acidify glucose, respectively [64].

Overall, these taxonomic ambiguities underscore the complicated history that the *Acinetobacter* genus has underwent over the years, especially because of the lack of phenotypical differences among its members. Therefore, it is not surprising that *Acinetobacter* taxonomy was given a big impulse after the introduction of genotypic methods, such as DDH, which became key to delineate *Acinetobacter* species. In fact, in 1986, the use of DDH helped to distinguish 12 different DDH groups, also called "genomospecies" or genomic species (gen. sp.), four of which were given valid species names, *A. baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, and *Acinetobacter junii*, and the descriptions of *A. calcoaceticus* and *A. lwoffii* were also corrected [65]. Subsequent DDH studies revealed the existence of further genomic species. In 1988, gen. sp. 12 was designated *Acinetobacter radioresistens* [66]. The next year, Tjernberg and Ursing reported three additional genomic species, gen. sp 13, 14 and 15 (*sensu* Tjernberg & Ursing or TU) [67]. Later, Bouvet and Jeanjean described five proteolytic *Acinetobacter* genomic species, which they numbered from 13 to 17 (*sensu* Bouvet & Jeanjean or BJ) [68]. The overlapping numeration can lead to confusion, for this reason when talking about *Acinetobacter* genomic species from 13 to 15, the numbers are followed by the author's initials, TU or BJ. Of note, 13BJ and 14TU correspond to the same genomic species.

Almost 30 years later, thanks to the use of molecular methods, next generation sequencing and mass spectrometry tools, the number of validly published *Acinetobacter* species has increased up to 60. In fact, in the last five years 30 new species have been validly published. The use of these techniques has helped to overcome the delineation problems caused by the absence of classical phenotypical differences. Moreover, in addition to the validly published species, there are still 15 tentative species awaiting to be validly named (https://apps.szu.cz/anemec/Classification.pdf, last accessed 11/12/2018) (**TABLE 3**). These putative species were not named at the time of being described because of the low number of strains per group and/or the lack of phenotypic differences. However, these shortcomings have not refrained other authors to describe new species based only on few isolates, against the prokaryote's taxonomists recommendations [14]. Novel species description based on few isolates (**TABLE 3**), aside from the introduction of new characterising tools, might explain why the number of *Acinetobacter* species has increased so greatly in the last decades. Furthermore, there are, as well, 10 effectively but not validly published species, all of them based on single strain descriptions (https://apps.szu.cz/anemec/Classification.pdf, last accessed 11/12/2018).

TABLE 3. Classification and nomenclature of species from the *Acinetobacter* genus (Adapted from https://apps.szu.cz/anemec/Classification.pdf, last accessed 11/12/2018).

Validly published species name (n=60)	Former designation	Source
A. albensis [69]	Taxon 31	Soil, water
A. apis † [70]		Honey bee intestine
A. baumannii [65]	Genomic species 2	Human, warm-blooded animals
A. baylyi [71]		Activated sludge, soil
A. beijerinckii [72]	Phenon 7	Human, animals, soil, water
A. bereziniae [65,73]	Genomic species 10	Human
A. bohemicus [74]	Taxon 26	Soil, water
A. boissieri [75]		Floral nectar
A. bouvetii † [71]		Activated sludge
A. brisouii † [76]		Peat
A. calcoaceticus [65]	Genomic species 1	Soil, water, human
A. celticus [77]	Taxon 33	Soil, water
A. colistiniresistens [68,78]	Genomic sp. 13BJ/14TU	Human
A. courvalinii [68,79]	Genomic species 14BJ	Human, animals
A. defluvii †[80]		Hospital sewage
A. dijkshoorniae (= *A. lactucae*) ‡ [81,82]	NB14	Human, water
A. dispersus [79]	Genomic species 17	Soil, water, human
A. equi [83]		Horse
A. gandensis [84]	Taxon 30	Horse, cattle, water
A. gerneri † [71]		Activated sludge
A. grimontii † (= *A. junii*) ‡ [71,85]		Activated sludge
A. guangdongensis † (= *A. indicus*) ‡ [86,87]		Lead-zinc ore
A. guillouiae [65,73]	Genomic species 11	Soil, water, human
A. gyllenbergii [72]	Phenon 3	Human
A. haemolyticus [65]	Genomic species 4	Human
A. halotolerans † [88]		Soil
A. harbinensis † [89]		River water
A. indicus † [90]		Soil
A. johnsonii [65]	Genomic species 7	Soil, water, human, animals
A. junii [65]	Genomic species 5	Human, animals, water, soil
A. kookii [91]		Soil, water
A. lactucae † [82]		Lettuce
A. larvae † [92]		Moth larval gut
A. lwoffii ‖ [65,67]	Genomic species 9	Human, animals, soil, water
A. modestus [79,93]	Taxon 18	Human, water
A. nectaris [75]		Floral nectar
A. nosocomialis [67,94]	Genomic species 13TU	Human
A. pakistanensis † (= *A. bohemicus*) ‡ [95,96]		Wastewater
A. parvus [97]	Phenon 4	Human, animals
A. piscicola † [98]		Fish
A. pittii [67,94]	Genomic species 3	Human, soil, water
A. populi [99]		Populus bark
A. pragensis [100]	Taxon 28	Soil, water
A. proteolyticus [79,93]	Taxon 19	Human
A. puyangensis [101]		Populus bark
A. qingfengensis [102]		Populus bark
A. radioresistens [65,66]	Genomic species 12	Human, soil, cotton
A. rudis [103]		Raw milk, wastewater
A. schindleri [104]	Phenon 2	Human, animals
A. seifertii [105,106]	'Close to 13TU'	Human
A. sichuanensis † [107]		Hospital sewage
A. soli † [108]		Human, soil
A. tandoii † [71]		Activated sludge, water, soil
A. tjernbergiae [71]		Activated sludge
A. towneri [71]		Activated sludge, water, soil
A. ursingii [104]	Phenon 1	Human
A. variabilis [109]	Genomic species 15TU	Human, animals, soil
A. venetianus [110,111]		Salt water
A. vivianii [79,93]	Taxon 20	Human, soil, water
A. wuhouensis [112]		Hospital sewage

Tentative species designation (n=15)	Other designation	Source
Genomic sp. 6 [65]		Human
Genomic sp. 15BJ [68]		Human
Genomic sp. 16 [68]		Human
Taxon 21 [93]		Human
Taxon 22 [93]		Human
Taxon 24, 25, 27, 32, 34-39 [Nemec & Radolfova-Krizova, unpublished]		Mostly environmental
Effectively but not validly published species name (n=10)	**Other designation**	**Source**
'*A. antiviralis*' † [113]		Tobacco plant roots
'*A. kyonggiensis*' † [114]		Sewage treatment plant
'*A. marinus*' † [115]		Sea water
'*A. oleivorans*' † [116]		Soil
'*A. oryzae*' † (= *A. johnsonii*) [117]		Rice
'*A. plantarum*' † (= *A. junii*) § [118]		Wheat
'A. pseudolwoffii' [65,93] Nemec *et al.* in press	Genomic sp. 8, Taxon 23	Human, animals, soil, water
'*A. refrigeratoris*' † (= *A. variabilis*) [119]		Refrigerator
'*A. seohaensis*' † (= *A. towneri*) [115]		Sea water
'*A. septicus*' † (= *A. ursingii*) ‡ [120,121]		Human

† Species description based on a single strain only.

‡ The first name is a later heterotypic synonym of that shown in parentheses.

2.2. Habitat and sources

One of the most known characteristics about *Acinetobacter* species is their ubiquity. Different *Acinetobacter* species have been isolated from a huge variety of sources and have been found in many ecological niches (**TABLE 3**). However, ubiquity should be regarded as a property of the genus, not of each species, since not all the species show broad niches, and some are more prone to be isolated from environmental samples, such as *A. calcoaceticus*, and others are mainly known for their human origin, such as *A. baumannii*, whose natural habitat and reservoirs are poorly known [45] restraining the development of control measures of their spread.

In 1968, a study showed that *Acinetobacter* could be recovered, after enrichment culture, from almost all soil and water samples collected [122]. However, at the time of the study *Acinetobacter* was considered a single species genus, and thus the species identity of the isolates remains uncertain. Several posterior studies confirmed that *Acinetobacter* species are commonly found in soil and water, and even seawater (reviewed in [123]). In fact, the first documented *Acinetobacter*, i. e. *A. calcoaceticus*, was recovered from soil [51] and nowadays it is considered as an environmental species.

Interestingly, *Acinetobacter* was found to be the main bacterial taxa in floral nectar, and two novel species isolated from nectar plants have been described, *Acinetobacter boissieri* and *Acinetobacter nectaris* [75,124]. *Acinetobacter* species have also been detected in vegetables for human consumption, both from market and garden origin. In a study aimed to determine the isolation rates of *Acinetobacter* spp. from vegetables, Berlau *et al.* found among the most common species *A. baumannii* and *Acinetobacter guillouiae*, followed in abundance by *Acinetobacter pittii* and *A. calcoaceticus*, while, in another similar report, Houang *et al.* detected *A. pittii* in 75% of the vegetables that carried *Acinetobacter* bacteria (51%) [125,126]. Recently, a study identified at least 18 different *Acinetobacter* species in 77.9% of the lettuce and fruit samples, the most frequent species were *A. calcoaceticus* and *A. johnsonii*, but other species such as *A. baumannii*, *A. pittii*, *Acinetobacter nosocomialis* and *Acinetobacter seifertii* accounted for 11% of the species isolated [127].

Nevertheless, vegetables are not the only food sources where *Acinetobacter* species have been isolated from. The isolation of *Acinetobacter* species from dairy food is not uncommon [103,128–132], neither it is to find *Acinetobacter* in meat [131,133,134], with rates as high as 25% of raw meat samples carrying *A. baumannii* in Switzerland [135]. Interestingly, in a study carried out in Lebanon, the authors could not recover *A. baumannii* from soil samples, but they found this species in water, meat, cheese and milk [131]. In addition, in the same report, *A. calcoaceticus* and *A. pittii* were both isolated from soil as well as from meat and lettuce samples. Nevertheless, finding *Acinetobacter* species in meat is not unexpected, since their presence in cattle has been widely reported and, in some studies, the isolates recovered were carbapenem resistant and carried carbapenemase genes [84,136–140]. Noteworthy, *Acinetobacter* species have also been recovered from pets [131,141,142] and birds. One example are white storks, in which *A. baumannii* was found with a prevalence of 25% [143], although the authors claim that diversity among the isolates was so high that results point out to transient colonisation of the birds, and suggest that the feeding material of the storks might be the real source of *A. baumannii*. Interestingly, *A. baumannii* and some related species can grow at 41 - 44ºC [94], likely suggesting and adaptation to the higher body temperatures of birds. Altogether, these findings support that animals, either wild, domestic or for human consumption, might be potential reservoirs of *Acinetobacter* species, including the human pathogen *A. baumannii*.

A part from the abovementioned sources, the presence of *Acinetobacter* species in human specimens is also well acknowledged, especially the presence of *A. baumannii* as an aetiogical agent of nosocomial infections. Actually, some *Acinetobacter* species are part of the human skin microbiota [45]. Species such as *A. johnsonii*, *A. lwoffii* and *A. radioresistens* have

been detected as human skin colonisers. Skin and faecal carriage of the major human pathogens *A. baumannii* and *A. nosocomialis* is seldom reported in healthy humans, although faecal carriage can be high in hospitalised patients [144,145]. In contrast, *A. pittii*, which is also associated with nosocomial infections, is commonly recovered from skin samples [144]. Interestingly, a surveillance study determined that *A. baumannii* and *A. nosocomialis* were only found in patient samples and hospital fomites specimens, while *A. pittii*, in addition to those sources, could be also detected in food, soil, community volunteers and hospital staff members [146]. Nevertheless, other species different to the main pathogens of the genus, thus is *A. baumannii*, *A. nosocomialis* and *A. pittii,* have also been associated occasionally with infections, such as *Acinetobacter ursingii* and *Acinetobacter schindleri* [104] (see **Section 5**).

2.3. The Ab group

In 1989, DDH experiments showed that the *Acinetobacter* species *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* and *A. pittii* (formerly known as gen. sp. 2, 1, 13TU and 3 (**TABLE 3**), respectively, had a close relationship. Their DDH values ranged between 65-75% [67], just slightly above and below of the proposed species delineation DDH value of 70% [14]. In fact, Bouvet and Grimont already found in 1986 that representatives of these species were more closely related than any of the other *Acinetobacter* gen. sp. defined by DDH delineation [65]. Of note, it should be mentioned that at that time *A. nosocomialis* and *A. pittii* were still considered genomic species, since it was not until 2011 that their names were validly published [94]. The lack of phenotypic differences among these genomic species raised some concerns about their delineation as different species, and it was proposed that *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* and *A. pittii* should be rejoined into only one species under the original name of *A. calcoaceticus*, which had taxonomical priority over *A. baumannii* [67,106] or otherwise grouped together under the expression "*A. calcoaceticus-A. baumannii* (ACB) complex" [147], since they could be considered genotypically distinct but phenotypically similar. Nevertheless, the first opinion did not hold in time, and the ACB complex designation is still currently being employed when referring to these phenotypically alike species. Additional studies based on the comparison of housekeeping genes sequences or on whole genome analysis also support the tight phylogenetic relationship of these species within the *Acinetobacter* genus [93,94,148,149].

Identification of the members of the ACB complex still remains challenging for many routine microbiology laboratories in spite of the development of new identification techniques. Indeed, in 1992 the development of accurate identification tools for the ACB complex species was considered fundamental, since the clinical relevance *A. baumannii*, *A. nosocomialis* and *A. pittii* had already been recognised [150]. Nowadays, it is acknowledged that the species of the ACB complex may display different clinical outcomes [151], thus their right identification is necessary in the clinical diagnostic laboratories. For many years, species-level identification of the ACB complex species was not possible, and *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* and *A. pittii* have sometimes been referred as ACB complex. On the other hand, given the importance of *A. baumannii* in the healthcare settings, most of the identification systems still erroneously report ACB complex species homogeneously as *A. baumannii* [152]. Thus, whenever in a publication the identification methods are not clear or are known to be unable to discriminate among the members of the ACB complex, it should be assumed that behind the name of *A. baumannii,* the other pathogenic species of ACB complex are comprised too. This particular fact hinders the interpretation of the literature, and highlights the need of accurate species level identification of the ACB species in order to unravel the differences in the biology of these different species.

Aside from the limitations imposed by the identification methods, the use of the expression "ACB complex" in diagnostic and clinical laboratories can be considered misleading too, since *A. calcoaceticus* is an environmental species that is mainly isolated from soil and water and is seldom implicated in clinical diseases, while *A. baumannii*, *A. nosocomialis* and *A. pittii* are the most clinically relevant species of the genus and show elevated rates of resistance to antibiotics, and thus represent a higher threat for our health system. Therefore, referring to the clinical species as "*A. baumannii* (Ab) group", instead of ACB complex, consequently excluding the mainly environmental *A. calcoaceticus* from the group, seems more appropriate and meaningful [45] (**FIG 5**). In this dissertation the expression "Ab group" will be preferably used instead of "ACB complex", since it is focused on the healthcare-associated species. Whenever possible the species of the Ab group will be considered as different entities, although it can be a challenging task due to the fact that most studies prefer to simply refer to these species as a *A. baumannii* or are *A. baumannii*-centred.

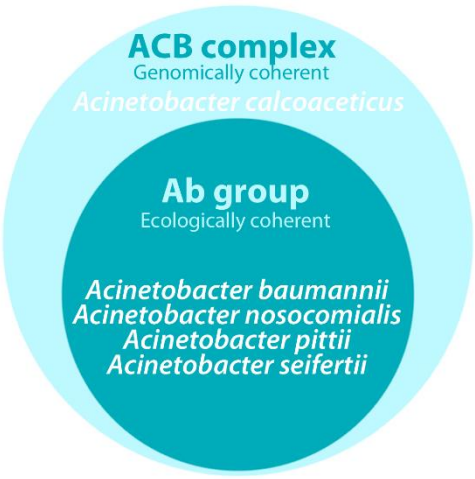


FIG 5. Distintion between the ACB complex and the Ab group. The genomically close related *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter nosocomialis*, *Acinetobacter pittii* and *Acinetobacter seifertii* are usually referred as *A. calcoaceticus-A. baumannii* (ACB) complex, however this designation is misleading since *A. calcoaceticus* is an environmental species. The use of the expression *A. baumannii* (Ab) group, comprising the clinical species only, seems more appropriate for the clinical settings.

Interestingly, in 1993 two new genomic species within the ACB complex were found in a clinical collection of 23 isolates from Denmark [106]. One of the groups has been designated gen. sp. "Between 1 & 3", because of its close relatedness to *A. calcoaceticus* and *A. pittii*, but so far its delineation as a novel species has not been possible [106]. On the contrary, the other group, which was provisionally designated gen. sp. "close to 13TU", was validly named as *A. seifertii* in 2015 [105]. This novel species has a close relationship to *A. nosocomialis* and is frequently isolated from human clinical samples, therefore it is considered as a novel Ab group species, which some authors picture as an emerging pathogen in Asia [153].

2.4. Species identification

For many years, routine identification of bacteria to the species level was usually performed by phenotypic methods. The phenotypic tests have proved useful to assign unknown organisms to previously described species in many genera, but this is not the case in the *Acinetobacter* genus. As mentioned before, *Acinetobacter* members are really difficult to distinguish by means of phenotypic methods. Indeed, even identification to the genus level by means of phenotypic tests is problematic, being one of the main reasons of the erratic taxonomy of the genus. The oxidase negative test helps to distinguish *Acinetobacter* spp. from other similar non-fermentative bacteria. Nonetheless, the transformation assay proposed by Elliot Juni in 1972 has been, for many years, the method of choice to reliably

identify strains to the genus level. This assay consists in the natural transformation of the mutant *Acinetobacter baylyi* strain BD413 *trpE27* by crude DNA, after which the tryptophan auxotrophy is only restored if the donor DNA comes from an *Acinetobacter* species strain [154]. However, it lacks applicability in the clinical microbiology laboratories.

In view of such drawbacks for unambiguous identification using phenotypic tests, the use of molecular techniques has become essential not only for new species delineation but also to develop accurate identification methods. However, many of these procedures are not suited for routine identification due to being laborious and time-consuming. On the other hand, spectrometric tools, such as MALDI-TOF MS, seem to overcome most of these problems and, at the same time, provide an accurate identification of *Acinetobacter* species.

Anyway, it should be taken into account that while taxonomy was slow in the past, nowadays has speeded up and is pushing forward the development of identification techniques. The description of a new species provides its discriminatory traits, however, characterisation techniques are not always suitable for routine identification purposes, and the optimization and update of the current identification methods, or even the development of new ones, becomes key in filling the gap between taxonomy and identification in the microbiology laboratories. Otherwise, accurate species designation might turn misleading due to obsolete methods and may lead to misrepresentation of new species. Thus, many of the identification methods that will be overviewed in the following sections are, or will soon be, unavoidably outdated as taxonomy advances. However, the different identification methodologies used over the years reflect how technology has evolved along with scientists' needs, and, albeit not being updated, many of them are still widely used for *Acinetobacter* species identification.

2.4.1. Phenotypic methods

Unfortunately for microbiologists working with *Acinetobacter*, there is no single metabolic test able to distinguish unambiguously the species belonging to the genus. In 1986, together with the first description of the 12 *Acinetobacter* genomic species based on DDH, the authors proposed a scheme of 28 phenotypic tests, able to distinguish between 11 of the 12 described genomic species [65]. One year later, this scheme was reduced to 16 tests,

which mainly consisted in carbon source utilisation and temperature of growth differentiation, besides production of acid from glucose and gelatin hydrolysis [155]. Validation studies [144,147] showed 78% and 95.6% correct identification, respectively, when compared to other identification reference standards, such as DDH. Interestingly, it should be noted that differentiation between *A. baumannii* and *A. nosocomialis* was not achieved in the first study [147], and the second one only included a few isolates of these species [144]. *A. calcoaceticus* and *A. pittii* were also problematic, since delineation between these species was only achieved by their ability to grow at different temperatures [147]. Although it cannot be considered as a gold standard for *Acinetobacter* species identification, this phenotypic test scheme used to be the standard phenotypic system for *Acinetobacter* spp. and was employed for the phenotypic characterisation of new species [85,104]. However, the scheme remains outdated for the identification of the most recently described species and, in addition, it is so laborious that cannot be applied in routine microbiology laboratories. Other carbon source assimilation tests exist, but they have been poorly used among microbiologists [71,156].

Manual and semi-automated commercial identification systems, such as API 20NE or Vitek 2, do not provide a reliable identification of *Acinetobacter* species, since their discriminatory power is insufficient [152,157]. In fact, many of these systems cannot distinguish among the most clinically relevant *Acinetobacter* species, i. e. the members of the Ab group, and even the current systems still identify these species as *A. baumannii* [152]. Such inaccurate identification systems misrepresent the prevalence and neglect the epidemiology of the non-*baumannii Acinetobacter* species in the clinical settings, where these systems are still widely used. Other systems, such as Biolog™, have been shown to accurately distinguish between certain *Acinetobacter* species [158], but they have never been widely used for routine identification.

As commented before, there are alternatives to classical phenotypic tests, which provide accurate species-level identification, such as the use of MALDI-TOF MS (**BOX 1**). In fact, nowadays MALDI-TOF MS has been shown to be highly reliable and to outperform conventional identification methods both in accuracy and rapidity, and many clinical microbiology laboratories have incorporated this identification method to their daily workflow. Nevertheless, its ability to correctly identify bacteria depends on the development of the reference databases against which the new spectra will be matched to and requires a high initial investment for the acquisition of the equipment. On the other hand, the associated long-term savings of using MALDI-TOF MS instead of conventional methods rapidly return the initial cost (**TABLE 4**).

Regarding identification of the *Acinetobacter* genus, in 2004 it was demonstrated that *Acinetobacter* species, together with *Escherichia coli* and *Salmonella*, could be identified after the use of the same standardized extraction protocol [159]. Later, the ability of MALDI-TOF MS to discriminate between different *Acinetobacter* species was confirmed [73], and *Acinetobacter* specialised protocols were suggested in order to increase the discriminatory power of MALDI-TOF MS [160]. Moreover, in 2011, the use of MALDI-TOF MS for identification of the members of the Ab group was validated and compared to other genotypic methods, demonstrating that MALDI-TOF MS was able to find enough differences in the spectral patterns of *A. baumannii*, *A. nosocomialis* and *A. pittii* species to allow correct identification of these species [161]. This study remarked that inclusion of *A. nosocomialis* reference spectra into the Bruker database was mandatory in order to correctly identify this species, which at that time was erroneously identified as *A. baumannii*, and also pointed out that due to score values below 2.30 when identifying *A. pittii* isolates (**BOX 4**), more diverse reference spectra of *A. pittii* should be included in the Bruker database [161]. After this study, some authors indicated that MALDI-TOF MS identification required the use of additional molecular techniques to properly differentiate among *Acinetobacter* species [162] and that the Bruker database should be updated [163,164]. However, further studies have supported the ability of MALDI-TOF MS to discriminate among *Acinetobacter* species [165,166]. On the other hand, it was noticed that misidentifications of *A. nosocomialis* and *A. pittii* still occurred after updating the Bruker database and some reports tried to improve MALDI-TOF MS identification by optimizing the extraction protocol [167] or using a combined MALDI-TOF MS and chemometric approach [168]. Interestingly, Sousa *et al.* [168] found that MALDI-TOF MS was unable to correctly identify *Acinetobacter* gen. sp. "Between 1 & 3" and *A. seifertii*, which were misidentified as either *A. pittii* or *A. calcoaceticus* and *A. baumannii*, respectively.

BOX 4. MALDI-TOF MS scores values and species identification.

According to the manufacturer, when identifying bacterial species different score values are given to provide a measure of the reliability of the identification. In the case of the MALDI Biotyper (from Bruker Daltonics), which is the equipment used at the Hospital Clínic of Barcelona, score values between the range of 2.300 – 3.000 point out to an excellent probability of identification to the species level; values between 2.000 - 2.299 show correct genus-level identification but probable species-level identification; values between 1.700 - 1.999 indicate probable genus-level identification; and values <1.700 mean non-reliable identification. Usually, low score values are obtained due to incorrect loading of the sample onto the target plate, but in other instances they indicate that the bacterial species is not present in the database, which can point to the need of improving the database or, less frequently but not rare, to a new bacterial species.

TABLE 4. Advantages and disadvantages of MALDI-TOF MS for bacterial identification

Advantages	Disadvantages
Speed, results obtained in minutes, high throughput	For some organisms, protein extraction is required before analysis
Low cost per sample	Expensive equipment, requires high initial investment
Reliability and reproducibility	Databases need updating and improvement
Easy to use, minimal isolate required (single colony), can be used directly from specimen	Requires pure cultures
Single platform for broad range of microorganisms identification	Difficulties to identify highly related organisms
Previous phenotypic tests unnecessary, but if performed errors do not impact the identification outcome	Cannot be used to quantitate bacterial load
Can be developed for organisms difficult to identify by conventional methods	Absence of validation and implementation guidelines
New organisms can be incorporated to the databases	

2.4.2. Molecular methods

Many different molecular methods have been proposed in order to identify *Acinetobacter* species (**TABLE 5**), and although some looked promising, they were never widely used and rapidly became outdated, thus, they will not be covered in this section. Moreover, it should be mentioned that many of the methodologies that have been employed have never been properly validated for species-level identification of *Acinetobacter*. Techniques based on DNA restriction such as AFLP analysis and ARDRA became reference validated methods for *Acinetobacter* species identification and were widely accepted by the scientist community, however, sequencing methods, which allowed uploading and sharing information in the databases, quickly replaced AFLP and ARDRA.

The AFLP methodology is a universal DNA fingerprinting method that consists in the digestion of total genomic DNA, ligation of adaptors to the cohesive ends, selective amplification of a subset of fragments with specific primers targeting the adaptors/restriction-site-sequences and electrophoretic separation of the PCR products, which will produce a distinctive band pattern for each species. This technique was shown to have enough discriminatory power to distinguish different *Acinetobacter* species, and also the

members of the ACB complex, that is *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* and *A. pittii* [169,170]. AFLP has proved to be excellent at species identification, although it requires technical expertise and it is arduous and time-consuming. Moreover, the equipment necessary to perform this technique is expensive, only allows the generation of local databases, and provides machine-dependent results. Therefore, the results obtained by different laboratories cannot be compared. Nowadays, this methodology is rarely used.

TABLE 5. List of the major molecular methods used for identification of *Acinetobacter* species. AFLP, Amplified Fragment Length Polymorphism; ARDRA, Amplified rRNA Restriction Analysis.

Restriction-based	Sequence-based
AFLP [169,170]	16S rRNA
ARDRA [171,172]	*recA* [173]
16S and 23S rRNA gene intergenic spacer [174,175]	16S and 23S rRNA gene intergenic spacer [176]
tRNA spacer fingerprinting [177]	*rpoB* [178,179]
	gyrB [180–183]

Conversely, ARDRA is considered a rapid and reliable method, and has the advantage that results can be compared between laboratories [171,172]. For *Acinetobacter* identification this technique is based on the sequence polymorphism of the 16S rRNA sequence. It consists in the PCR amplification of the 16S rRNA gene and subsequent digestion with different restriction enzymes, namely CfoI, AluI, MboI, RsaI and MspI. After gel electrophoresis, a number is assigned to the band pattern generated by each enzyme (**FIG 6**), and used to assign an ARDRA profile to the strain tested, which can be then compared to the library of ARDRA profiles of the different species (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html). Some species might require additional restriction by BfaI and BsmAI enzymes. Nevertheless, if the ARDRA profile cannot be allocated, it might indicate that the strain belongs to not-yet described species. Interestingly, ARDRA can distinguish between members of the ACB complex, including *A. seifertii*, whose ARDRA profiles were provided before its valid name publication, when it still was designated gen. sp. "close to 13TU". For many years, ARDRA has been the reference standard for reliable identification of *Acinetobacter* species, however, since 2009, the library of ARDRA profiles has not been updated.

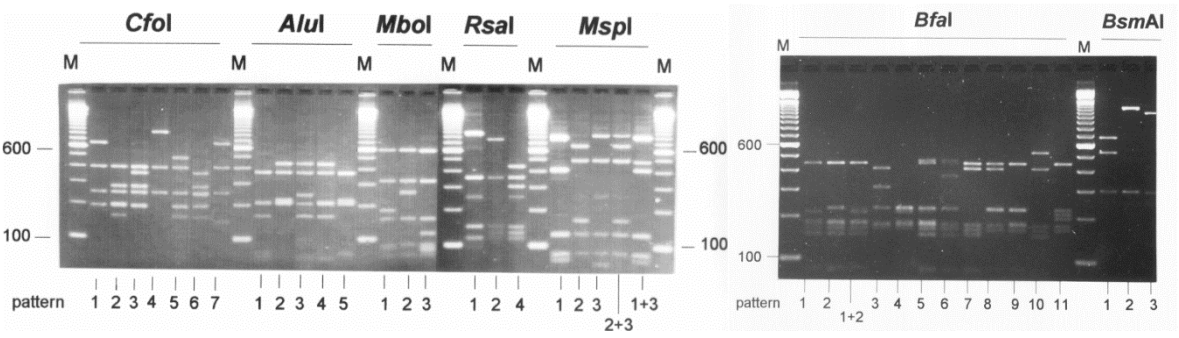


FIG 6. Overview of the ARDRA restriction patterns obtained after digestion with different restriction enzymes of the 16S rRNA of different *Acinetobacter* species. A) Standard panel enzymes: CfoI, AluI, MboI, RsaI and MspI, and the corresponding pattern number; B) Additional enzymes, BfaI and BsmAI, and the corresponding pattern number. (adapted from [172])

Regarding sequence-based methodologies, as seen before, 16S rRNA is one of the most widely used genes for classification of bacteria (See **Section 1.2**). but it is also used for bacterial identification to the species level. In the case of *Acinetobacter* species, however, this gene suffers from not having enough discriminatory power [85]. For instance, 16S rRNA similarity values between the species of the ACB complex are above the species threshold (97.8%), and the 16S rRNA gene can be found in several copies in their genomes, consequently the use of this gene is not appropriate for species identification in *Acinetobacter*. Other genes have been used for identification purposes, such as the ubiquitous and conserved *recA* [173], *rpoB* [178,179], and *gyrB* [180]. All these genes showed the close relationship between the ACB complex species in cluster analyses, but at the same time provide enough resolving power to help delineate these species. Of note, based on *gyrB* heterogeneity, specific *gyrB* primers yielding different size amplicons have been designed to easily differentiate *A. baumannii* from *A. nosocomialis* [181] and *A. pittii* from *A. calcoaceticus* [182]. In addition, a *gyrB* multiplex PCR has recently been developed in order to rapidly identify the species of the Ab group [183].

3. Antimicrobial resistance of the Ab group

Antimicrobial resistance is a common characteristic found among *Acinetobacter* species, but especially in the pathogen *A. baumannii*, and thus, it poses a serious challenge when managing infections caused by these bacteria. Resistance to antimicrobial agents is determined according to certain guidelines, such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI) [184,185], that establish the clinical breakpoint values that allow the classification of bacteria into susceptible or resistant to different antibiotics based on the likelihood of treatment success. Depending on the susceptibility testing method, the breakpoint values correspond to a concentration, which is usually referred as the minimum inhibitory concentration (MIC, which is the lowest concentration of an antibiotic that inhibits the visible growth of a microorganism after overnight incubation with media, mg/L or μg/mL) or the inhibition zone diameter (mm) [186]. In addition to the demarcation of bacteria into susceptible and resistant, bacteria can also be sorted according to their resistance to multiple antibiotic in: multidrug-resistant (MDR), if resistant to one or more agents in more than three antimicrobial categories; extensively drug resistant (XDR), if resistant to one or more agents in all but two or less categories; and pandrug-resistant (PDR), if resistant to all antimicrobial agents [187]. The antimicrobial categories and agents that should be considered vary from organism to organism, and for *Acinetobacter* species the appropriate antibiotics to look at are as follows: aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem, doripenem), fluoroquinolones (ciprofloxacin, levofloxacin), antipseudomonal penicillins plus inhibitors (piperacillin-tazobactam, ticarcillin-clavulanic acid), penicillins plus inhibitors (ampicilin-sulbactam) extended-spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime), folate pathway antagonists (trimethoprim-sulphamethoxazole), polymyxins (colistin, polymyxin B), and tetracyclines (tetracyclines, doxycycline, minocycline) [187] (**TABLE 6**). Nevertheless, it should be noted that the absence of breakpoints for *Acinetobacter* species in the EUCAST guidelines [184] when considering some antibiotics such as, for example, penicillins and tetracyclines, is due to these species usually displaying high intrinsic or unreliable MICs for these antimicrobial categories, and thus, they are inappropriate as therapeutic options for *Acinetobacter* (see **Section 5.2** below).

TABLE 6. Comparison of the different antimicrobial agents' breakpoints for *Acinetobacter* spp. in 2018 according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) guidelines [184,185].

	EUCAST breakpoints (mg/L)			CLSI breakpoints (μg/mL)		
Antimicrobial category and agents	**S**	**I**	**R**	**S**	**I**	**R**
Penicillins						
Piperacillin	ND	ND	ND	≤ 16	32-64	≥ 128
β-lactams + inhibitors						
Ampicillin-sulbactam*	ND	ND	ND	≤ 8/4	16/8	≥ 32/16
Piperacillin-tazobactam*	ND	ND	ND	≤ 16/4	32/4-64/4	≥ 128/4
Ticarcillin-clavulanate*	ND	ND	ND	≤ 16/2	32/2-64/2	≥ 128/2
Cephalosporins I, II, III and IV generation						
Ceftazidime*	ND	ND	ND	≤ 8	16	≥ 32
Cefepime*	ND	ND	ND	≤ 8	16	≥ 32
Cefotaxime*	ND	ND	ND	≤ 8	16-32	≥ 64
Ceftriaxone*	ND	ND	ND	≤ 8	16-32	≥ 64
Carbapenems						
Doripenem*	≤ 1	-	> 2	≤ 2	4	≥ 8
Imipenem*	≤ 2	-	> 8	≤ 2	4	≥ 8
Meropenem*	≤ 2	-	> 8	≤ 2	4	≥ 8
Polymyxins/Lipopeptides						
Colistin*	2	-	2	≤ 2[a]	-	≥ 4[a]
Polymyxin B*	ND	ND	ND	≤ 2	-	≥ 4
Aminoglycosides						
Gentamicin*	≤ 4	-	> 16	≤ 4	8	≥ 16
Tobramycin*	≤ 4	-	> 4	≤ 4	8	≥ 16
Amikacin*	≤ 8	-	> 4	≤ 16	32	≥ 64
Netilmicin*	≤ 4	-	> 4	≤ 8	16	≥ 32
Tetracyclines						
Doxycycline*	ND	ND	ND	≤ 4	8	≥ 16
Minocycline*	ND	ND	ND	≤ 4	8	≥ 16
Tetracycline*	ND	ND	ND	≤ 4	8	≥ 16
Fluoroquinolones						
Ciprofloxacin*	≤ 1	-	> 1	≤ 1	2	≥ 4
Levofloxacin*	≤ 0.5	-	> 1	≤ 2	4	≥ 8
Gatifloxacin	ND	ND	ND	≤ 2	4	≥ 8
Folate pathway antagonists						
Trimethoprim- sulfamethoxazole*	≤ 2[b]	-	> 4[b]	≤ 2/38	-	≥ 4/76
Miscellaneous						
Daptomycin	≤ 2	-	> 2	ND	ND	ND

*Antimicrobial agents to be considered in order to refer to isolates as multidrug, extensively drug or pandrug resistant according to Magiorakos *et al.* [187]; [a] Applies only to ACB complex species; [b] Refers to trimethoprim concentration;
S, susceptible; I, intermediate; R, resistant; ND, not determined

Acinetobacter species have been reported to be resistant to almost all antimicrobial agents. Of special relevance is the case of *A. baumannii*, which has an outstanding ability to survive in the nosocomial environment, where antibiotic pressure and circulation of other pathogens enhance the innate ability of this species to accumulate resistance mechanisms. *A. baumannii* isolates of clinical origin are frequently MDR and show higher resistance rates than the other species of the Ab group [151,188–195], however, acquisition of resistance mechanisms has been frequently described in the former members of the Ab

group, *A. nosocomialis* and *A. pittii*, and some reports describing acquired resistance in *A. seifertii* have arisen after its valid designation as a distinct species [196,197], while others already existed [198].

Antimicrobial resistance occurs whenever bacterial organisms harbour specific genetic determinants that allow the survival of bacteria in the presence of a given antimicrobial drug. Sometimes, the resistant phenotype is considered to be intrinsic to the species, in those cases the species harbours *per se* the mechanisms that confer resistance to a determined antibiotic, such as in the case of *Acinetobacter colistiniresistens* [78], whose name clearly refers to the inherent resistance to colistin of this species. In other occasions, resistance develops through the acquisition of mutations in pre-existing DNA content and/or the acquisition of novel genetic determinants that confer resistance to certain antibiotics. Acquired resistance usually arises after antibiotic exposure as a result of selective pressure. The coexistence of several mechanisms of resistance to the same antibiotic is also common, which ultimately leads to the development of increasing levels of resistance.

3.1. Intrinsic resistance

In *Acinetobacter* species, especially among those of the Ab group, the intrinsic mechanisms of resistance comprise the interplay between low membrane permeability and the presence of constitutively expressed active efflux systems that can extrude antimicrobial drugs outside the bacterial cell as well as the presence of chromosomally encoded enzymes with the ability to degrade β-lactams, such as AmpC-type *Acinetobacter*-Derived Cephalosporinases (ADCs), encoded by the *bla*$_{ADC}$ gene [199], and also the carriage of intrinsic oxacillinases or OXA-type enzymes, encoded by the *bla*$_{OXA}$ gene (**BOX 5**). These mechanisms can be considered intrinsic since they are already encoded in the bacterial genomes, with some exceptions such as the Tet efflux pumps (see below, **Section 3.1.2**), which are included in this section for convenience. Furthermore, in contrast to other acquired mechanisms, changes in their expression, rather than alteration of their activity or of the antibiotic target, are responsible for the development of the resistant phenotype. Altogether, the intrinsic resistance mechanisms of *Acinetobacter* species can confer resistance to antimicrobial agents such as, penicillins, cephalosporines, carbapenems, aminoglycosides, fluoroquinolones and tetracyclines.

3.1.1. Intrinsic β-lactamases

The intrinsic β-lactamases of *Acinetobacter* comprise the ADC enzymes, which belong to the Ambler class C, and the intrinsic OXA enzymes, which comprise class D oxacillinases (**BOX 5**). Both β-lactamases are commonly encoded in the chromosome and usually confer low β-lactam levels of resistance, however, the presence of upstream insertion sequence (IS) elements, such IS*Aba1*, IS*Aba125* or IS*Aba9*, can provide strong alternative promoters that increase their expression, resulting in decreased susceptibility to β-lactams, including carbapenems [200–204].

The ADCs, also known as AmpC chromosomal cephalosporinases, are frequent mechanisms of resistance to penicillins, cephalosporines and β-lactam-β-lactamase inhibitor combinations in *Acinetobacter* [199]. Interestingly, ADCs differ from other Gram-negative class C cephalosporinases in the fact that, instead of being inducible, they are constitutively expressed by its native promoter, and, in addition, they possess more different active site residues than other AmpC enzymes [205–207]. The presence of AmpC in *A. baumannii* was first described in 2000 [208]. The ADC designation was proposed due to its unique characteristics and, since its discovery, several allelic variants have been described [148,199,209]. Their presence in non-*baumannii* species has also been detected, including the remaining species of the ACB complex and the closely related genomic species "Between 1 & 3" and *A. pittii*-like and *A. calcoaceticus*-like strains, whose ADC sequences formed a tight cluster when analysed phylogenetically [148,210–213]. The role of the insertion of insertion sequences upstream the ADC gene, which may provide a strong promoter for the ADC gene and increase its expression, has been mainly studied in *A. baumannii* [200,201,214]. Nevertheless, enhanced expression of ADC thanks to upstream insertion of IS*Aba1* and consequent cephalosporine resistance has also been detected in *A. nosocomialis* [212].

BOX 5. β-lactamases: molecular classification of Ambler.

According to the molecular classification of Ambler, β-lactamases can be classified in four different classes based on their protein sequence: class A (penicillinases), class B (metalloenzymes), class C (cephalosporinases) and class D (oxacillinases). From a functional point of view, classes A, C, and D comprise enzymes that hydrolyze their substrates through the participation of a serine residue in the active-site, whereas class B metalloenzymes hydrolyze their substrates by using at least one zinc ion as a catalytic cofactor [656]. β-lactamases can hydrolyze β-lactam antibiotics such as penicillins, carbapenems, cephalosporines and monobactams, and can be located in the chromosome or in plasmids, sometimes embedded in integrons or as part of mobile elements such as transposons.

In 2004, the intrinsic OXA enzymes of *A. baumannii*, the OXA-51-like group, was identified in *A. baumannii* [215] as a chromosomally encoded class D enzyme. This oxacillinase group shows carbapenem hydrolyzing activity. In particular, OXA-51-like enzymes can hydrolyze imipenem but not meropenem, and they are active also against penicillins, oxacillin, cloxacillin, and a few cephalosporines, such as cephaloridine [215]. Certain allelic variants display specific substitutions that seem to enhance carbapenem activity [216]. Nevertheless, in the absence of ISs upstream from the OXA-51-like gene, the activity of these enzymes against β-lactams is very weak [203,217]. Interestingly, insertion of IS*Aba1* upstream the OXA-51-like gene can occur during imipenem treatment, leading to *in vivo* selection of clones with the IS integrated upstream the OXA-51-like gene, and thus, overexpressing this β-lactamase [218]. This group was initially thought to be *A. baumannii* species-specific [219], and, for this reason, the detection of the OXA-51-like gene by PCR has been widely used to identify *A. baumannii* isolates [220]. Nevertheless, the enzymes of the OXA-51-like group are not unique to *A. baumannii*. A couple of studies carried out with isolates from Taiwan, identified the OXA-51-like gene with upstream IS*Aba1* insertion in a plasmid in *A. nosocomialis* and *A. seifertii* and showed that the presence of the plasmid correlated with carbapenem resistance [221,222]. These reports were among the first to identify this kind of class D enzyme in non-*baumannii* species, and Lee *et al.* suggested that, likely, the OXA-51-like gene encoded in the plasmid came from *A. baumannii*, as they also found similar plasmids spread among *A. baumannii* isolates from Taiwan [223].

Posteriorly, it has been shown that almost each *Acinetobacter* species possesses its own intrinsic group of OXA enzymes. For example, the OXA-211-, OXA-213-, OXA-214-, OXA-229- and OXA-235-like groups are naturally occurring in *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus*, *Acinetobacter bereziniae*, and *A. schindleri*, respectively [224–226], and recently, a study found that OXA-665-, OXA-296-, OXA-664-, OXA-274- and OXA-286-like groups are intrinsic to *Acinetobacter rudis*, *Acinetobacter bohemicus*, *Acinetobacter tandoii*, *Acinetobacter guillouiae* and *Acinetobacter gyllenbergii*, respectively [227]. It should be noted, though, that the OXA-235-like group was initially described in *A. baumannii* [228], and thus, this underscores that cross transmission of the intrinsic OXAs between different *Acinetobacter* species can happen, like the plasmid-borne presence of OXA-51 from *A. baumannii* in *A. nosocomialis* [221,222]. In 2014, Périchon *et al.* described many of the OXA groups intrinsic to several *Acinetobacter* species, including *A. calcoaceticus*, *A. pittii*, the genomic species "Between 1 & 3", and the closely related *A. pittii*-like and *A. calcoaceticus* strains [148]. However, this study, which used whole genome sequences, could not detect any intrinsic OXA in the chromosomes of *A. nosocomialis* and *A. seifertii* [148], and to date none of the available genome sequences of these two species seem to carry an intrinsic OXA-like gene.

3.1.2. Efflux pumps and porins

The permeability of the bacterial envelope also plays a central role in conferring low intrinsic resistance to antibiotics, as it mediates the transport between the extracellular milieu and the bacterial cytoplasm. Many antibiotics have intracellular targets, and thus, need to be transported across the bacterial cell wall and membranes. The outer membrane proteins (OMPs), also called porins, are proteins that form channels through which molecules can passively diffuse across the outer membrane. Decreased permeability, caused by low number and small-sized channels of porins, can hinder the entrance of antibiotic molecules into bacteria and reduce the intracellular antibiotic concentration, leading to the emergence of the resistant phenotype in bacteria. However, hindering the influx of antibiotics is not the only non-enzymatic way that bacteria have to decrease antibiotic concentration. The active secretion of antibiotics from the inner cell to the environment by efflux pumps can also play a key role in resistance to certain antibiotics (**TABLE 7**).

Few studies have examined the permeability defects in *Acinetobacter* species other than *A. baumannii*. At the beginning of the 1990s it was suggested that *A. calcoaceticus* had lower permeability to antibiotics when compared to other Gram-negative bacteria due to small-sized channels and decreased porin production [229,230]. Posterior studies have revealed that downregulation and absence of some OMPs do, in fact, decrease antibiotics entry in *A. baumannii* (**TABLE 7**). In contrast to the other porins, OmpA seems to play an opposed role in antimicrobial resistance, because its disruption decreases aztreonam, chloramphenicol and nalidixic acid MICs, suggesting its involvement in the extrusion of antimicrobial agents in collaboration with active efflux systems [231].

Meanwhile, efflux pumps confer resistance to antibiotics if overexpressed, since they translocate certain antibiotics outside the bacterial cell. An increased production of efflux pumps translates in higher export rates from the inner side of the bacterial cell to the extracellular environment, thus reducing the intracellular concentration of antibiotics and allowing the emergence of resistance. Many efflux pumps can extrude a wide range of antibiotics, so their overexpression can result in clinically significant levels of multidrug resistance (**TABLE 7**). Efflux pumps are classified into 6 families (**FIG 7**) that can be further divided into two groups on the grounds of their energy source. The first group is composed by a single efflux family, the ATP-binding cassette (ABC) family, which obtains the energy from ATP hydrolysis. In Gram-negative bacteria, however, this family is seldom involved in antimicrobial resistance.

TABLE 7. Efflux pumps and porins involved in antimicrobial resistance in the Ab group and their corresponding antimicrobial agent substrates.

Protein	Substrates
Efflux pumps	
ATP-binding cassette (ABC) family	
ErmAB-like	Colistin [232]
Multidrug and toxic compound extrusion (MATE) family	
AbeM	Aminoglycosides, Fluoroquinolones, Chloramphenicol, Erythromycin and Trimethoprim [233,234]
Major facilitator Superfamily (MFS)	
AbaF	Fosfomycin [235]
AmvA	Erythromycin [234,236]
CmlA	Chloramphenicol [237]
CraA	Chloramphenicol [238]
TetA	Tetracycline [239]
TetB	Tetracycline, minocycline [239]
Tet39	Tetracycline [240]
Proteobacterial antimicrobial compound efflux (PACE) family	
AceI	Chlorhexidine [241]
Resistance-nodulation-cell division (RND) superfamily	
AdeABC	Aminoglycosides, Cefotaxime, Tetracyclines, Erythromycin, Chloramphenicol, Rifampicin, Trimethoprim, Tigecycline, Fluoroquinolones and some β-lactams, including meropenem [182,242–246]
AdeDE	Amikacin, Ceftazidime, Chloramphenicol, Ciprofloxacin, Erythromycin, Meropenem, Rifampin and Tetracycline [247]
AdeFGH	Chloramphenicol, Trimethoprim, Ciprofloxacin, Clindamycin, Tetracyclines, Tigecycline and Sulfamethoxazole [234,248]
AdeIJK	β-lactams, Chloramphenicol, Tetracycline, Erythromycin, Lincosamides, Fluoroquinolones, Fusidic Acid, Novobiocion, Rifampin and Trimethoprim, monobactam, tigecycline [246,249]
ArpAB	Amikacin and Tobramycin [250]
Small multidrug resistance (SMR) family	
AbeS	Chloramphenicol, Fluoroquinolones, Erythromycin and Novobiocin [234,251]
Porins	
AbuO	Amikacin, Carbecillin, Ceftriaxone, Meropenem, Streptomycin and Tigecycline [252]
CarO (Omp29)	Imipenem [253–255]
OmpA [a]	Aztreonam, Chloramphenicol, Nalidixic Acid [231]
Omp22	Imipenem, Meropenem [256,257]
Omp33-36	Imipenem [256–258]
Omp37, -44, -47	Carbapenems [259]
OprD-like	Imipenem [260,261] [b]
VacJ	Colistin [262]

[a] In contrast to the rest of porins, its absence reduces the MICs of the antimicrobial agents
[b] Both in *A. baumannii* and *A. nosocomialis*

The remaining families are ion-coupled transporters, which usually employ Na^+, H^+ or other metabolites to facilitate the transport and include the resistance-nodulation-cell division (RND) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the proteobacterial antimicrobial compound efflux (PACE) family.

The most extensively studied efflux pump family in *Acinetobacter* has been the RND family, whose involvement in antimicrobial resistance has been mainly investigated in *A. baumannii*, even though RND pumps are widely distributed in *Acinetobacter* [263]. To date five different RND efflux systems have been described in *Acinetobacter* species, the AdeABC, the AdeDE, the AdeFGH, the AdeIJK and the AdeXYZ, although sequence similarity analyses indicate that the latter two are likely to be the same proteins [264]. The RND efflux pumps are tripartite systems composed of an OMP, a transporter protein located in the inner membrane, and a membrane fusion protein (MFP) that connects the two other components [242]. All three proteins are usually encoded as an operon, although the porin might be missing sometimes [247,265] and its role can be exerted by other unspecific porins.

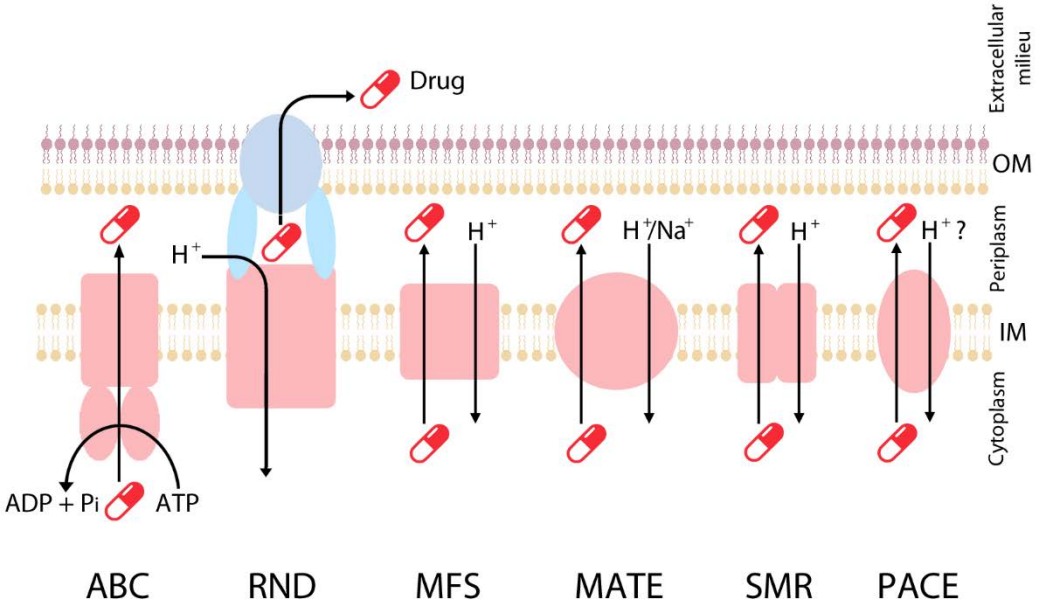


FIG 7. Schematic representation of the different efflux pumps families. ABC, ATP-binding cassette family; RND, resistance-nodulation-cell division superfamily; MFS, major facilitator superfamily; MATE, multidrug and toxic compound extrusion family; SMR, small multidrug resistance family; PACE, proteobacterial antimicrobial compound efflux family; OM, outer membrane; IM, inner membrane.

The first RND efflux system described in *A. baumannii* was AdeABC [242], which was shown to be involved in the extrusion of aminoglycosides, cefotaxime, tetracyclines, erythromycin, chloramphenicol, trimethoprim and fluoroquinolones [242], although usually the levels of resistance conferred by the AdeABC system do not reach clinical resistance breakpoints [265]. The contribution of this system to last resort antibiotics such as meropenem (but not imipenem) and tigecycline has also been observed [243,244]. Interestingly, hydrophilic antibiotics seem to be less efficiently transported by AdeABC, since hydrophilic aminoglycosides and fluoroquinolones are worst substrates for this efflux pump than their less hydrophilic counterparts [242,265]. This efflux system is regulated by the two component system (TCS) AdeRS, which is encoded usptream of the *adeABC* operon and divergently transcribed [266], and mutations or insertions in *adeRS* can lead to AdeABC overexpression [267,268]. Several studies demonstrated that under *in vitro* and *in vivo* antibiotic pressure, *A. baumannii* can become resistant to certain antibiotics, such as tigecycline, by overexpressing this efflux pump system [182,245]. AdeABC has been thought to be *A. baumannii* species-specific for a long period of time [269,270], and although not all *A. baumannii* isolates carry this efflux pump, it is highly widespread in this species [264]. Nevertheless, in 2011 its presence was empirically detected in *A. nosocomialis* [271] and *in silico* analyses revealed that it might be not exclusive to *A. baumannii* and *A. nosocomialis*, but also present in other *Acinetobacter* species [264]. Recently, AdeABC overexpression was found to be the underlying mechanism behind resistance to tigecycline in *A. nosocomialis* [272]. Interestingly, there are no studies reporting the presence of the *adeABC* genes in *A. pittii* [264,269].

The AdeDE system was first identified in *A. pittii* in 2004 [247]. This system, which does not encode for its own porin, is involved in the extrusion of amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampin and tetracycline [247]. It is highly prevalent in *A. pittii* species but it has also been detected *A. nosocomialis* [247,264,269,271,273] and only one study found *adeDE* in *A. baumannii* [273], although *in silico* analyses differ and were not able to identify *adeDE* in the available genomes of *A. baumannii* [264], thus pointing to incorrect species identification in the original study, since the isolates were identified using phenotypic methods only [273].

In 2008, the AdeIJK efflux system of *A. baumannii* was described for the first time [249], two years later than the description of AdeXYZ in *A. pittii* [269]. Although they are quite likely the same protein but different species variants (with 93% identity at the nucleotide level and 99% similarity at the protein level) [264], the involvement in antibiotic resistance of AdeXYZ could not be determined due to the impossibility to construct an *adeY* mutant

[269] and might be the reason why its finding went overlooked. AdeIJK was found in all the *A. baumannii* isolates tested, and consequently it was suggested that it might be intrinsic and specific to this species [243], although this initial conclusion has been refuted since the *adeIJK* operon has been found empirically in all the ACB complex species as well as other *Acinetobacter* species [264,271,274]. The AdeIJK system is regulated by AdeN, a TetR-like regulator which is not directly adjacent to the *adeIJK* operon [243], instead, it is located upstream from the *argF* gene in all the *Acinetobacter* species tested [274]. The AdeIJK basal expression is involved in low level resistance to β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocion, rifampin and trimethoprim [249], while aminoglycosides are not substrates of this efflux pump [275].

The AdeFGH efflux system has only been reported in *A. baumannii* so far, and it is also thought to be intrinsic of this species [234,248,276]. Its expression is modulated by the Lys-R type transcriptional regulator AdeL, which is located upstream the *adeFGH* operon and divergently transcribed [276]. Overexpression of this pump, usually by mutations in *adeL* [276], confers resistance to chloramphenicol, trimethoprim, ciprofloxacin, clindamycin, tetracyclines, tigecycline and sulfamethoxazole [234,248].

The AdeABC and AdeFGH systems are thought to be the main efflux pumps contributing to multidrug resistance in *A. baumannii* [234], and their contribution is regarded mainly as adaptative, while AdeIJK is seen as responsible for low intrinsic levels of resistance in *A. baumannii*. Although tightly regulated [277], insertion of IS elements and point mutations in their respective regulators (*adeRS*, *adeN* and *adeL*) can lead to increased antimicrobial resistance of certain antibiotics, such as tigecycline [268].

Recently, a novel RND efflux pump system has been described in the *A. baumannii* AB5075 strain while trying to determine the mechanisms controlling phase variation, thus is the interconversion between opaque and translucent colonies, a phenomenon occurring in many *A. baumannii* strains but greatly observed in the AB5075 strain (**BOX 6**). They found that mutants for the genes encoding the novel efflux pump ArpAB, which stands for aminoglycoside resistance pump, showed decreased switch from opaque to translucent colonies, and, in addition, that the mutants had increased motility, decreased virulence in the *Galleria mellonella* infection model, and increased susceptibility to the aminoglycosides amikacin and tobramycin [250].

In addition to RND efflux pumps, several pumps of the remaining families have been described, although their involvement in antimicrobial resistance has been studied to a lesser extent and reports are mainly focused on *A. baumannii*. Within the MFS family, the tetracycline efflux pumps TetA confers resistance to tetracycline and the TetB to tetracycline and minocycline. Both pumps have been located in transposon and plasmids, indicating that they might have been acquired from other species [239]. The Tet39 pump, conferring resistance to tetracycline but not to minocycline, has also been found in transferable plasmids [240], and *tetG* and *tetA* were detected as part of a resistance island in the *A. baumannii* strain AYE [237]. An additional MFS pump, CmlA, was also found to be encoded in the same resistance island and confers resistance to chloramphenicol [237]. The CraA efflux pump, a homolog of *E. coli* MdfA belonging to the MFS family, also contributes to chloramphenicol resistance in *A. baumannii* [238]. AmvA is also a member of the MFS family, it is highly prevalent, if not intrinsic, in *A. baumannii*, and its overexpression correlates to erythromycin resistance, which is the only antibiotic which was affected when the *amvA* gene was disrupted [234,236]. In addition, AbaF, another MFS efflux pump, has been suggested to be involved in fosfomycin resistance [235]. From the MATE family, only AbeM has been associated with antimicrobial resistance. This efflux pump confers low level of resistance to aminoglycosides, fluoroquinolones, chloramphenicol, erythromycin and trimethoprim [233,234]. There are several efflux pumps from the SMR family in *A. baumannii*, all chromosomally encoded and mostly involved in the removal of ammonium quaternary compounds, except for AbeS, a homologue to EmrE from *E. coli*, which in *A. baumannii* participates in the extrusion of chloramphenicol, fluoroquinolones, erythromycin and novobiocin, as well as to dyes and detergents [234,251]. Lastly, the AceI transporter confers tolerance to chlorhexidine, in contrast to other non-*Acinetobacter* species [241]; this pump belongs to the PACE family, which has been recently described.

Despite all the efflux pumps functionally characterised in *A. baumannii*, the pool of putative efflux systems encoded in its genome [237,278] and in the genomes of the different *Acinetobacter* species is still much larger and obscures our comprehension of the biology of this pathogen. Indeed, further efforts should be focused on assessing the role of the different pumps in *Acinetobacter* species because, in addition to antimicrobial agents, efflux pumps can extrude a wide range of substrates and are known to participate in the pathogenesis of other bacterial species [279].

BOX 6. The *A. baumannii* AB5075 strain and the phase variation.

In 2014, the *A. baumannii* AB5075 strain was postulated as an excellent model strain because of its recent isolation in 2008 from a tibia osteomyelitis, its MDR phenotype and its high virulence in both invertebrate and mammal infection models (*Galleria mellonella* and mice, respectively) [626]. Furthermore, the AB5075 strain can be genetically manipulated with relatively easiness, and a transposon mutant library has been created which is available to the scientific community [625].

Phase	Opaque 	Translucent 
Cell morphology	Cocci	Rods
Biofilm	↓	↑
Motility	↑	↓
Virulence (*G. mellonella*)	↑	↓
Capsule	↑	↓
Resistance to		
Aminoglycosides	↑	↓
Innate immune antimicrobials	↑	↓
Hospital disinfectants	↑	↓
Desiccation	↑	↓
Survival/Replication during infection (mice)	↑	↓
Isolation from clinical samples	↑	↓

However, the AB5075 strain displays a phenomenon called phase variation, which consists in the interconversion between opaque and translucent colony variants in a cell-density dependent and unstable manner, which seems to be related to the accumulation of a yet undetermined extracellular factor [657]. This phenomenon is common in *A. baumannii* strains, but not universal, and it provokes changes in cell morphology, biofilm formation, antibiotic resistance and virulence in the *G. mellonella* infection model [657].

In 2016, the ArpAB RND efflux system and the OmpR/EnvZ transcription regulator were found to participate in the unstable switch between opaque and translucent forms [250,658]. Recently, a further study found that opaque colonies showed a thicker capsule and were more resistant to diverse innate immune antimicrobials, hospital disinfectants and desiccation than the translucent ones [659]. Moreover, Chin *et al.* only detected opaque colonies from clinical samples, and showed that in a murine model of infection, opaque cells outcompeted the translucent ones, leading to the hypothesis that the opaque variant is the one causing diseases, since translucent cells cannot replicate/survive during infection and seem to be better adapted for natural environments outside the host [659]. Besides, they identified a key TetR-type transcriptional regulator able to lock *A. baumannii* cells in the avirulent translucent phase when expressed, which they suggest that can be potentially used to develop a live-attenuated vaccine for *A. baumannii* [659].

3.2. Acquired resistance

As seen in the previous section, both the accumulation of mutations in transcriptional regulators and the insertion of ISs, can lead to resistance by overexpressing some of the intrinsic mechanisms, whose activity, otherwise, would not be enough to reach clinical significant resistance levels. Thus, the MDR phenotype in *Acinetobacter* is usually caused by the synergistic action of intrinsic and acquired genetic determinants.

3.2.1. Target alteration

Error prone polymerases abound in *Acinetobacter* genomes [93]. This kind of polymerases might be responsible for the diversification of the genetic pool of the bacteria under antibiotic pressure, allowing the development of resistance not only by affecting gene expression by mutation of transcriptional regulators, but also by altering the structure of some antibiotics targets.

Fluoroquinolone resistance. In addition to being conferred by diverse efflux pumps, fluoroquinolone resistance can also be caused by mutations within the genes encoding the DNA gyrase and the topoisomerase IV, which are the targets for these antibiotics. Particularly, it has been shown in *Acinetobacter* that amino acid substitutions in *gyrA* (Ser83Leu, Gly81Val/Lys, Ala84Pro and Asp87Tyr) and *parC* (Ser80Leu/Trp/Tyr, Gly87Cys and Glu/Ser84Lys) can lead to fluoroquinolone resistance [280–286]. Some of these substitutions have also been found in quinolone resistant *A. nosocomialis* and *A. pittii* isolates [283,287].

Polymyxin resistance. Similar to fluoroquinolone resistance, resistance to polymyxin B and colistin (polymyxin E) in *Acinetobacter* can appear as a result of the antibiotic pressure during *in vivo* treatment with polymyxins by alteration or loss of the lipopolysaccharide (LPS) (**TABLE 8**). In *A. baumannii,* resistance to polymyxins is mainly due to mutations in the *pmrAB* TCS (**BOX 7**) and nucleotide substitutions or insertion of IS elements that inactivate the *lpxACD* operon, which synthetizes the lipid A [288–296]. Some of the most frequent substitutions found in *pmrB* in colistin resistant *A. baumannii* isolates are Pro233Ser, Pro360Gln and Leu208Phe [297]. In contrast, little is known about the colistin

resistance mechanisms of the non-*baumannii* species of the Ab group, despite the fact that *A. nosocomialis* and *A. seifertii* polymyxins resistance rates can be high [283,298–300] and that some authors even claim that *A. seifertii* might be intrinsically resistance to colistin [196,197]. Only few reports have studied the mechanisms behind colistin resistance in the species of the Ab group. Kim *et al.* focused on the interspecies sequence divergence of the *pmrCAB* operon, but did not look for amino acid substitutions related to colistin resistance in the non-*baumannii* species [301]. Vila-Farrés *et al.* found that *in vitro*-developed colistin resistance in one *A. nosocomialis* strain was due to LPS loss caused by mutations in the *lpxACD* operon [296].

TABLE 8. Main polymyxin resistance mechanisms described in *Acinetobacter* spp. (adapted from [299]).

Gene(s)/ Reference(s)	Alteration	Function	Phenotype	Clinical prevalence
pmrCAB [262,293–295]	Mutation, overexpression	PmrAB, two component system; PmrC, lipid A phosphoethanolamine transferase	Reduced negative charge of LPS	Yes, most frequent
lpxACD [288,289,296,302]	Mutation, truncation	Biosynthesis of LPS	Loss of LPS	Yes
lpsB [290,291,303]	Mutation	Glycosyltransferase, synthesis of the structural ring of LPS	Upregulation, increased membrane stability	Yes, rare
eptA [295,304,305]	Overexpression, copy number	PmrC-like, lipid A phosphoethanolamine transferase	Reduced negative charge of LPS	ND
lptD [306]	Deletion	LPS translocase	Loss of LPS translocase	ND
vacJ [262]	Mutation	Outer membrane lipoprotein (ABC transporter)	Loss of outer membrane asymmetry	ND
ermAB-like [232]	Overexpression	ABC transporter	Unclear	ND

ND, Not determined

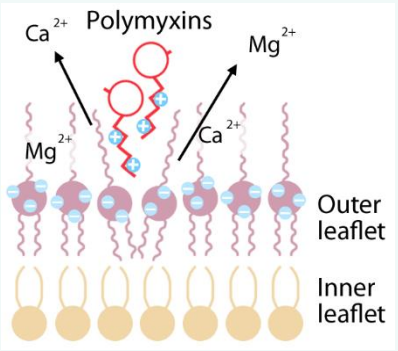
3.2.2. Horizontally acquired mechanisms

Bacteria can become resistant to antibiotics by HGT, which mainly involves the acquisition of integrons, transposons and plasmids that carry the genetic determinants conferring resistance and that help to disseminate resistance mechanisms intra- and interspecies. Remarkably, some *Acinetobacter* species, including *A. baumannii* [307,308], show competence for natural transformation, that is, the ability to uptake foreign DNA and incorporate it into their genomes. This trait is considered one of the characteristics that turned

A. baumannii into such a successful nosocomial pathogen [309,310]. Nevertheless, genomic analyses found that many of the *Acinetobacter* species genomes conserved some of the genetic elements which have been empirically proven to be necessary for DNA uptake in *A. baumannii* and *Acinetobacter baylyi*, such as type IV pili and *com* genes [307,308,312–313]. Thus, it seems that most of the bacteria of the genus might be naturally competent, although the specific conditions for successful transformation need to be further investigated [93].

BOX 7. Polymyxins, proposed mechanism of action and target alteration in *Acinetobacter* spp.

Polymyxins are cationic cyclic peptides with a hydrophobic acyl chain that act by binding to lipid A from the bacterial LPS. Through the competitive displacement of divalent cations and by insertion of the acyl chain, polymyxins disrupt the bacterial membranes [660], leading to altered membrane composition, unbalanced homeostasis and, ultimately, bacterial cell death.



Resistance to these antibiotics is developed by modifications in the LPS that reduce the negative surface charge [289] and, in consequence, lower the binding affinity of polymyxins, avoiding their action. LPS alterations in *Acinetobacter* are mainly caused by the addition of phosphoetanolamine to the lipid A [293-295], a process mediated by PmrC, a lipid A phosphoethanolamine transferase that is regulated by PmrAB [293,295]. Therefore, mutations in *pmrAB* that cause the overexpression of PmrC can lead to colistin resistance [295]. In addition to the *pmrCAB* operon, *A. baumannii* and *A. nosocomialis* isolates can possess one or multiple copies of a *pmrC*-like gene, *eptA*. *eptA* expression and copy number have also been related to colistin resistance in *A. baumannii* [295,304], and have recently been shown to be negatively regulated by histone-like nucleoid-structuring (H-NS) proteins [305].

Carbapenem resistance. In addition to the intrinsic β-lactamases, *Acinetobacter* species can acquire other β-lactamases that expand their MDR profile (**BOX 5**). From a clinical point of view, the most problematic are those that also hydrolyze carbapenems, as these antibiotics are considered one of the last resort treatment options for MDR *A. baumannii* infections. Acquired carbapenem resistance in *Acinetobacter* species can be caused by class A β-lactamases, class B β-lactamases, also known as metallo-β-lactamases (MBLs), and class D carbapenemases, such as those belonging to the OXA-23, OXA-24/OXA-40, OXA-58, OXA-143 and OXA-235 groups [314,315].

The class A β-lactamases are enzymes that comprise a large group of enzymes that confer resistance to penicillins and cephalosporins (narrow spectrum β-lactamases) but sometimes can also hydrolyze cefotaxime, ceftazidime and cefpodoxime (extended spectrum β-lactamases or ESBLs). In particular, NMC/IMI, SME, and KPC enzymes and some GES enzyme variants can also hydrolyze carbapenems. The latter two are plasmid encoded and have been identified in *A. baumannii* [316–320].

Class B β-lactamases, or MBLs, are metal ion (usually zinc) dependent enzymes that are inhibited by EDTA and that can hydrolyze all β-lactams, except monobactams, and confer high levels of resistance to carbapenems. MBLs are typically found as part of class 1 integrons (except for NDM, see below) together with aminoglycoside resistance determinants, and they can be chromosomally or plasmid located. The most common MBLs groups include the VIM, IMP and NDM enzymes (**BOX 8**). Thanks to their association to mobile elements, diverse allelic variants of the MBLs belonging to the IMP [321] and VIM [322] groups are spread across Gram-negative bacteria, including the species of the Ab group [261,323–325]. The SIM enzymes constitute another less frequent group of MBLs, and they were discovered in isolates of *Acinetobacter* spp. not identified to the species level [326]. Further surveillance in the same hospital concluded that SIM was initially detected mostly in *A. pittii* and then shifted to *A. nosocomialis*, but no SIM-positive *A. baumannii* isolates have been identified to date [327]. The GIM enzymes are also MBLs which were originally found in *Pseudomonas aeruginosa* [328] and *Enterobacter cloacae* [329]. In 2014, two studies demonstrated that they had disseminated to *Acinetobacter* species as well, although one of the reports failed to identify the isolates to the species level [330] and, in the other study, GIM positive isolates were characterised as *A. pittii* [331].

In contrast to the other MBLs, the NDM enzymes are usually located in Tn*125*-derived transposons and can be plasmid-borne, but they are not located in integrons [315,332]. In non-*baumannii Acinetobacter* species, such as *A. lwoffii*, *A. bereziniae*, and *A. pittii*, the Tn*125* has only been detected on plasmids, while in *A. baumannii* the Tn*125* has mainly been located in the chromosome and, less frequently, on plasmids [333–338]. Indeed, it is hypothesized that the Tn*125* transposon probably originated in *Acinetobacter*, which acquired it from an environmental species, and then it disseminated to *A. baumannii* and to other species via broad-host-range plasmid transfer [332,335,339]. Furthermore, a second hypothesis suggests that the NDM-1 gene might be the result of a recombination event with *aph6*, an aminoglycoside resistance gene found in *Acinetobacter* [340]. Anyway, the NDM enzyme family has disseminated rapidly worldwide [341], and, beyond *A. baumannii*,

it can be found in several *Acinetobacter* species of the Ab group [332], including *A. nosocomialis* [342] and *A. pittii* [337, 339]. In fact, Bogaerts *et al.* claimed that *A. pittii* could act as a reservoir of the NDM gene [343].

In addition to the intrinsic OXAs, other class D β-lactamases also show low level carbapenem hydrolyzing activity [344], and, thus, are referred to as carbapenem-hydrolyzing class D β-lactamases (CHDLs), and consist mainly of the OXA-23-like, the OXA-24/OXA-40-like, the OXA-58-like, the OXA-143-like and the OXA-235-like groups, which are mostly exclusive of *Acinetobacter* species [345], and the OXA-48-like group, which is prevalent in *Enterobacteriaceae* [226]. CHDL genes can be both chromosomal or plasmid-encoded [226] and, in *Acinetobacter* spp., they are often associated with ISs that facilitate both their mobility and provide strong promoters, leading to overexpression and carbapenem resistance [203], except for OXA-24/OXA-40 and OXA-143 groups that are instead associated with XerC/D specific recombination sequences that can also contribute to their mobilisation [228,345]. Nevertheless, recent publications show that OXA-143 variants might also be coupled with certain IS elements [346,347].

In 1985, the first OXA enzyme with carbapenemase activity was identified in *A. baumannii* and called *Acinetobacter* Resistance Imipenemase, ARI-1 [348], but it was not until the year 2000, after its sequence analysis, that it was determined that it was a novel OXA enzyme and assigned the name of OXA-23 [349]. Nowadays the OXA-23 group is common in *A. baumannii* isolates and has disseminated worldwide [226,345,350] although it is believed that its natural progenitor is *A. radioresistens* [351]. Members of the OXA-23-like group have been isolated in several *Acinetobacter* species, including *A. nosocomialis*, *A. pittii* and *A. seifertii* [350,352–354], and some variants, such as the OXA-134, are naturally occurring in *A. lwoffii* and *A. schindleri* [355,356].

The OXA-24 was first detected in carbapenem resistant *A. baumannii* in Spain [347], however, due to an original error in sequencing it was later revealed that, indeed, its sequence corresponded to the allelic variant OXA-40, which was described afterwards [348,349]. Initially, the OXA-24/OXA-40 group was shown to be highly disseminated in the Iberian Peninsula [360], but now it has been increasingly reported globally [226]. In addition to *A. baumannii*, the OXA-24/OXA-40 group has been detected in several *Acinetobacter* species, including *A. pittii* and *A. nosocomialis* [226,350,352,353].

BOX 8. Naming metallo-β-lactamases (MBLs).

As with organisms, genes and their products need also to be named. Scientist usually do so by trying to describe their functions or some characteristics, such as in the case of the KPC β-lactamases, which stands for "*Klebsiella pneumoniae* carbapenemase" since this carbapenemase was originally detected in this organism [661], or the Ade efflux pumps, for *Acinetobacter* drug efflux, which describes both its function and the organism where these operons can be found [242]. However, genes can also be named after a city or country, and this is the case of some MBLs, such as VIM (Verona imipenemase [322]); SPM (Sao Paulo metallo-β-lactamase [662]), GIM (German imipenemase [328]), and the most controversial, the NDM enzyme, which stands for New Delhi metallo-β-lactamase [664]. The issue with the naming of NDM arose in 2010, when Kumarasamy *et al.* published in a prestigious journal a multicentric study that linked this enzyme to India and Pakistan and warned about the hazards of having surgery in hospitals of these countries [664]. These conclusions were not welcomed in India, where the Indian Health Ministry found that naming the enzyme after the New Delhi city was defaming both the city and the country, as well as giving "malicious propaganda" about the medical tourism industry of India (http://timesofindia.indiatimes.com/india/Lancet-says-sorry-for-Delhi-bug-/articleshow/7261135.cms). The editor of the journal where the study was published apologised for not foreseeing the implications of letting the authors name the enzyme after New Delhi, and many Indian authorities and scientists requested a change of the name under the statement that:

> "The naming of an enzyme, gene, disease, etc. after a city, country or race is convenient but poor science. Naming should rather be based on scientific characteristics or in honour of the scientist/institution who/which pioneered it." [665]

In any case, although their point of view could be seen as fair, their suggestions were in vain and the NDM name stands and it is widely used as the enzyme disseminates worlwide.

Later, in 2005, it was found that MDR *A. baumannii* isolates causing an outbreak in a hospital from France were carrying a novel OXA enzyme that conferred resistance to carbapenems, later identified as OXA-58 [361,362]. The OXA-58-like group is widely associated with outbreaks and has been reported worldwide [345]. Several studies have detected the presence of OXA-58-like genes in *A. pittii* [198,261,349,353,363], *A. nosocomialis* [262,350,353,364,365] *and A. seifertii* [196,197,352,366], in addition to *A. baumannii*, where it is highly prevalent [226,345,367].

There are other minor acquired CHDLs, such as the OXA-143-like enzymes [368], which display weak carbapenemase activity. Mainly detected in *A. baumannii* isolates from Brazil [347,369–371], the OXA-143-like variants have disseminated in the American continent [346,350,372], where they have also been identified in *A. pittii* [350,372]. In addition, they

have also been detected in Korea, both in *A. baumannii* and *A. nosocomialis* [373,374] and have also been reported in *A. baumannii* in Iran [375]. Interestingly, the OXA-143-like enzymes are closely related to the OXA-24/OXA-40-like group, and it has even been suggested that OXA-143-like enzymes might be the progenitors of the OXA-24/OXA-40 enzymes [226].

Aminoglycoside resistance. Resistance to aminoglycosides in *Acinetobacter* can be caused by efflux pumps, as seen in the previous section, but also by the presence of acquired determinants such as aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methyltransferases, which usually confer higher level of resistance to aminoglycosides than the AMEs. The 16S rRNA methyltransferase most frequent in *Acinetobacter* is ArmA, which is mainly reported in *A. baumannii* [376,377], although ArmA has also been detected in *A. calcoaceticus* [378]. The other 16S rRNA methyltransferases (RmtA, RmtB RmtC, RmtD, RmtE, RmtF, RmtG, RmtH and NpmA) seem less frequent or absent in *Acinetobacter* isolates [379–383], in fact, only RmtA and RmtB have been identified in *Acinetobacter* spp. [384–386].

The AMEs are often plasmid-borne and associated with transposable elements, and can be classified in 3 groups, the aminoglycoside acetyltransferases (AAC), the aminoglycoside phosphotransferases (APH) and the aminoglycoside adenylyl or nucleotidyltranferases (AAD or ANT). A great variety of AMEs have been detected in *Acinetobacter* species (*aac*(3)-*I*, *aac*(3)-*II*, *aac*(6')-*I*, *ant*(2"), *ant*(3')-*I*, aph(3')-*I*, *aph*(3')-*VI*, etc. [264]) indicating that they are widely disseminated and not restricted to *A. baumannii*, although most studies focus on this species. In fact, in 2017, a study found that *ant* were widely distributed among the genomes of *Acinetobacter* species [387] and described a novel subclass of ANTs, ANT(3")-II, which was intrinsic to *A. baumannii*, *A. pittii* and *A. gyllenbergii*, and transferable to other *Acinetobacter* species [387]. Other AMEs are also known to be intrinsic to *Acinetobacter* species, like for example APHA6 and AAC(6')-*Ih* which are naturally occurring in *A. guillouiae* and *A. gyllenbergii,* respectively, [388,389]. Thus, *Acinetobacter* species with intrinsic AMEs can act as sources of these enzymes for other species, but essentially for *Acinetobacter* species [388,389]. Furthermore, it should be taken into account that the presence of AMEs is quite widespread thanks to their location in class 1 integrons, which may also harbour an MBL gene, thus conferring resistance to a plethora of different antibiotics.

4. Pathogenesis of the Ab group

Bacterial species belonging to the Ab group comprise the most clinically relevant species of the *Acinetobacter* genus and are widely known because of their ability to cause disease, i. e. pathogenicity. The virulence, or degree of pathogenicity, of the species within the Ab group is a multifactorial trait that can vary between members of the same species, but also depends on the host factors, the interaction between both and the bacterial burden. Unnoticed for many years in the diagnostic laboratories due to identification constraints, the members of the Ab group have been considered as opportunistic pathogens of low grade virulence for a long time [44]. This anonymity that lasted until the late 1980s accounts for our poor understanding of how these pathogens interact with the human host, compared to other pathogens.

Furthermore, studies have mainly focused on *A. baumannii*, although, as said before, correct species identification cannot be granted in all the studies. Nevertheless, some authors consider that due to the genetic and phenotypic similarities between species within the Ab group, results obtained for one species can be extrapolated to all the others [390]. While this might be uncertain, the current status of the literature does not allow for a better interpretation of the existing data, which is mainly *A. baumannii*-centred with few exceptions (**BOX 9**), and underscores the need of proper assessment of the phenotypic differences between the species of the Ab group in order to find the distinctive traits, if any, among the clinically relevant *Acinetobacter* species.

It is generally accepted that the success of *A. baumannii* as a nosocomial pathogen relies on its ability to persist in the hospital environment and its outstanding aptitude to acquire and accumulate antimicrobial resistance mechanisms [310], in what some call a "persist and resist" virulence strategy [390]. However, its capability to cause serious infections should not be taken lightly, since virulence attributes might also be responsible for its spread in the hospitals. In the following sections, some of the virulent determinants that make the species of the Ab group, specially *A. baumannii*, so prosperous in the nosocomial environment are described, as well as the host-pathogen interaction that promote infections caused by these pathogens.

BOX 9. The M2 strain and virulence studies.

A. baumannii is considered the most clinically relevant *Acinetobacter* species, therefore, it is reasonable that most of the virulence studies focused on this pathogen. However, even when considering model strains for such studies, correct identification at the species level can entail a problem when using longstanding strains, and underscores the importance of using appropriate identification methods to distinguish between the species of the Ab group. This is the case of the M2 strain, which was isolated in 1996 from a hip infection at the Cleveland MetroHealth Systems (USA). The M2 strain was initially identified as *A. baumannii* and, since 2008, it has been used in several virulence studies regarding quorum sensing [500,666] (**BOX 11**), motility [464], type IV pilus [307] and type VI secretion system [407] **(BOX 10**), and even a novel fluoroquinolone resistance mechanism was discovered using this strain [667]. However, in 2013, when the genome of the strain was sequenced, the authors realised that the *A. baumannii* M2 strain was indeed *A. nosocomialis* [668]. Therefore, all previous reports are taxonomically inaccurate and can lead to confounding interpretation of the literature. Nevertheless, after this discovery the strain has been used in several other papers, some of great importance in the field of *Acinetobacter* virulence (about type I secretion system [669], type II secretion system [399,400], and type IV pilus [463,670] among others), but, this time, the strain M2 was properly named as *A. nosocomialis*.

4.1. Virulence factors

The pathogenesis of the species of the Ab group relies on several virulence factors that account for some of the most studied virulence-related phenotypes (**TABLE 9**). Unlike other species, no concrete determinant can be used to infer the potential pathogenesis of the isolates of these species. In fact, genomic analyses have revealed that *A. baumannii*, which is considered the most hazardous species within the Ab group, possesses few additional virulence determinants than its counterparts, since most of the virulence-associated genes are also encoded in the core genome of the pathogenic species of the ACB complex [309,391]. However, when the first sequenced genome of an *A. baumannii* strain, ATCC 17978, was compared to the genome of an environmental species, in that case *A. baylyi* [392], it revealed the presence of several putative alien islands in *A. baumannii*, which were possibly acquired by HGT [393]. Within these islands, the coding sequences showed homology to several virulence genes involved in motility, iron uptake, quorum sensing (QS) (**BOX 11**), and protein secretion, but also in drug resistance [393]. Further i*n silico* analyses of the genomes of different *Acinetobacter* species have shown that the species of the ACB complex have a larger core genome than the non-pathogenic species of the genus, a fact that highlights the acquisition, or conservation, of several genes [149].

Of note, most of the virulence factors have been widely studied in *A. baumannii*, while little is known about their role in the other species (**BOX 9**), except for, barely, their presence [149]. Interestingly, some of the most studied virulence factors in *A. baumannii*, like OmpA, the penicillin binding protein (PBP) 7/8 and the phospholipases C and D (**TABLE 9**), have been commonly found in non-pathogenic *Acinetobacter* species [149]. The presence of virulence determinants in non-pathogenic *Acinetobacter* species might be explained by different expression patterns between pathogenic and non-pathogenic species caused by the greater abundance of transcriptional regulators in the ACB species. Hence, it cannot be ruled out that, although encoded in both, some virulence factors may be only active in the pathogenic species [149].

4.1.1. Cell surface and membrane transport

Extracellular bacterial components are the first to get in contact with the host, and thus they can trigger the immune response or protect the bacteria from it. This is the case of exopolysaccharides in *Acinetobacter*, which usually hide bacteria from the recognition systems of the host. In general, exopolysaccharides can be found in the outer membrane leaflet as LPS and glycoproteins, as well as in the capsule. Exopolysaccharide biosynthesis is encoded in the conserved, but sequence-variable, gene cluster called the K locus, which has been found both in *A. baumannii* and *A. nosocomialis* [394,395]. Several virulence factors described in *A. baumannii* are related to exopolysaccharide production and have been involved in different virulence-phenotypes (**TABLE 9**).

In addition to their role in the passive diffusion of small molecules across the membranes, OMPs such as OmpA, Omp33-36 and CarO have been found to be associated with virulence (**TABLE 9**). Particularly, OmpA, previously known as Omp38, is one of the best studied virulence factors of *A. baumannii* and has been shown to display several functions related to the pathogenesis of this species (**FIG 8**) (**TABLE 9**). Furthermore, the role of OmpA in *A. nosocomialis* pathogenesis has also been investigated, in this species OmpA enhances biofilm formation on abiotic surfaces and adherence to A549 cells, but, in contrast to *A. baumannii*, it was not involved in cytotoxicity, suggesting a differential role for OmpA in *A. nosocomialis* infections [396].

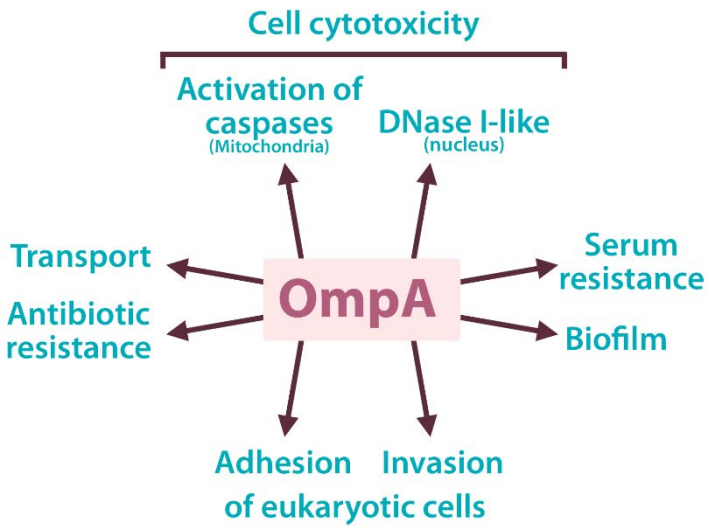


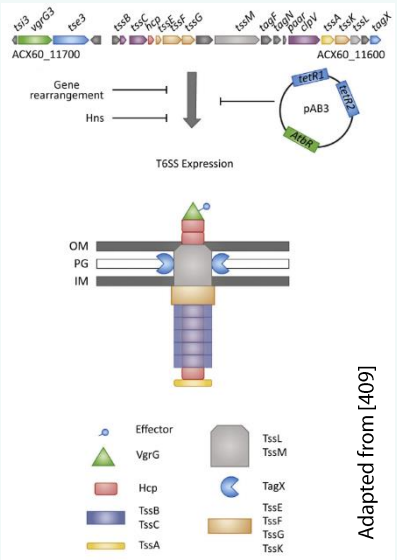
FIG 8. Some of the main functions of *A. baumannii* OmpA (Adapted from [397])

Transport from the inner cell to the extracellular milieu, as well as the presence of the transports systems in the cell surface *per se* have been shown to be involved in *Acinetobacter*'s pathogenesis (**TABLE 9**). The type II secretion system (T2SS) is ubiquitous in Gram-negative bacteria, and it is responsible for the secretion of folded proteins to the extracellular space from the periplasmic space. The T2SS was first detected in *Acinetobacter* by Eijkelkamp *et al.* in 2014 in a comparative study of surface-exposed virulence factors across 8 distinct *A. baumannii* strains [398]. Subsequent studies demonstrated that the T2SS was also present and active in *A. calcoaceticus*, *A. junii*, *A. nosocomialis* and *A. pittii* [399,400] and the presence of this secretion system in *A. nosocomialis* was shown to be essential for virulence in animal models [399,400] (**TABLE 9**). One of the secreted proteins by T2SSs in *Acinetobacter* is the protease CpaA, which deregulates blood coagulation by cleaving factor V and fibrinogen [401] and is thought to have been horizontally acquired in the last 70 years, since it is not present in the ATCC 17978 and ATCC 19606 genomes [399]. The type V secretion system (T5SS) Ata, which stands for *Acinetobacter* Trimeric Transporter [402], has also been shown to be involved in *Acinetobacter* virulence as an adhesin (**TABLE 9**). In contrast, the role of type VI secretion systems (T6SS) (**BOX 10**) in the virulence of *Acinetobacter* is less clear. This system is ubiquitous in both pathogenic and non-pathogenic *Acinetobacter* species, such as *A. baylyi* [403], and it is known to deliver effector proteins both to eukaryotic and bacterial cells [404]. However, in *Acinetobacter* species the current knowledge points towards a main involvement in bacterial killing [405–408]. The expression of T6SS varies among distinct strains of *A. baumannii*, nevertheless, according to the genomic data available in 2013, the absence of several proteins within

the T6SS cluster suggested that T6SS might not be functional among *A. pittii*, *A. nosocomialis*, *A. haemolyticus* and *A. junii* species [409]. However, this same year it was demonstrated that *A. nosocomialis* M2 strain required the T6SS to outcompete other bacteria [407]. Finally, efflux pumps, aside from their involvement in drug transport (see **Section 3.1.2**), have also been shown to participate in some virulence-related phenotypes, especially those of the RND family (**TABLE 9**), however it is unclear if their involvement in *Acinetobacter* pathogenesis is due to the secretion of bacterial effectors or to their presence in the membrane.

BOX 10. The type 6 secretion system (T6SS).

T6SSs are contact-dependent transport systems that deliver proteins both to eukaryotic cells and bacteria. The protein components that constitute T6SSs are conserved among different species [404], and generally consist in 13 structural proteins, which are encoded in the same locus, and accessory factors, which are scattered throughout the genome. Several T6SS clusters can be found in the same organism, although not all of them have to be necessarily functional [404]. Altogether the structural and accessory proteins participate in the translocation of proteins across the membranes to the extracellular milieu.



Adapted from [409]

The structure of the T6SS resembles to an inverted bacteriophage, where the proteins TssB and TssC provide energy for the translocation, Hcp forms a tail-tube structure across which proteins can travel, and VgrG forms a cell-puncturing tip. Interestingly, Hcp and VgrG are not only assembled in the T6SS structure, but are also secreted, and their detection in supernatants has been widely employed for inference of a functionally active T6SS [671]. While many effector molecules have been described for other species, no *bona fide* effector has been found for *Acinetobacter* [672]. Moreover, as one of the functions of T6SS is bacterial competition, it has been observed that some bacteria express immunity proteins to counteract T6SS [673]. The current hypotheses for the regulation of this secretion system in *A. baumannii* involve H-NS proteins [674], permanent inactivation of T6SS by modifying the gene cluster that encodes the system, and plasmid-encoded repressors of the T6SS, which if lost will allow the expression of the T6SS and thus bacterial competition (*killing* mode) [406,410]. Moreover, it is believed that the plasmids harbouring the T6SS repressors may, in addition, accumulate antibiotic resistance genes, a fact that might favour the selection of strains that are repressing their T6SS under antibiotic pressure (*survival* mode) [406,410].

In addition to protein-based secretion systems, *Acinetobacter* species produce Outer Membrane Vesicles (OMVs), which are vesicles that generate from the outer membrane and that contain many different kinds of proteins, but their biogenesis is poorly understood [410]. The OMVs can be found in the supernatants of many *A. baumannii* strains [411–413], *A. baylyi* [414], *A. nosocomialis* [415], and *A. radioresistens* [416], and they constitute one of the major delivery routes of bacterial effectors to host cells. One of the main components of the OMVs of *A. baumannii* is OmpA, thus together with the delivery of other virulence factors, such as CsuA [412] and other OMPs [417] (**TABLE 9**), OMVs have been shown to contribute to the pathogenesis of *A. baumannii* by delivery of toxic effectors and stimulating the pro-inflammatory response [411,412,415,418] but also to participate in horizontal transfer of resistance genes between *Acinetobacter* strains [419].

Metal acquisition systems, especially those systems in charge of iron uptake, play an important role in *Acinetobacter* survival inside the host and pathogenicity. Iron is usually unavailable for bacteria once they are inside the host, thus production of siderophores and other high-affinity iron chelator molecules becomes essential for successful colonisation. *A. baumannii* produces several iron acquisition systems [420], but the most remarkable is acinetobactin [421]. Genomic studies carried in 2014 revealed that acinetobactin is conserved only in *A. baumannii* and *A. pittii*, and that the genes encoding for this iron uptake system might have been acquired by these close species after two independent events of HGT [149]. Nevertheless, an earlier study already pointed out that the acinetobactin gene cluster was absent in *A. nosocomialis* [308]. Interestingly, under iron limiting conditions, the phospholipases C of *A. baumannii* (**TABLE 9**) were overexpressed in comparison to iron rich conditions, leading to higher hemolytic activity of *A. baumannii* [422]. Furthermore, it has been recently shown that, in a murine traumatic wound infection model, iron supplementation to the wound could prevent *A. baumannii* infection [423]. Hence, iron uptake systems are important not only *per se*, but also because iron levels trigger transcriptional regulation, which, in turn, seem to upregulate virulence factors.

4.1.2. Biofilms

Biofilms are ubiquitous in nature and are produced by almost all bacteria [424]. According to Vert *et al.* [425], a biofilm is defined as:

> "[an] Aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface.
>
> *Note 1:* A biofilm is a fixed system that can be adapted internally to environmental conditions by its inhabitants.
>
> *Note 2:* The self-produced matrix of EPS, which is also referred to as slime, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms." [425]

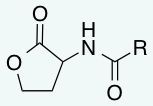
Biofilms are microbial communities, thus, they are the result of the collective behaviour orchestrated by QS of millions of cells (**BOX 11**). The architecture of biofilms enhances cell-to-cell and cell-substrate interactions, and the self-produced extracellular matrix is usually composed of proteins, lipids, saccharides, extracellular DNA, minerals, and water. A part from creating a nutrient-rich environment, during infections, the biofilm provides protection by allowing bacteria to elude the host immune response. Furthermore, tolerance or resistance to certain molecules, such antimicrobials, can develop thanks to the high density of cells, low penetration of molecules to the extracellular matrix, differential expression of targets and resistance genes, efflux of the antibiotics and the presence of persister cells within the biofilm (metabolically inactive and non-proliferating cells that are unchallenged by the antibiotics) [426].

In *Acinetobacter*, biofilm formation is a common trait, however, it is considered a clone specific feature, since, in *A. baumannii*, the amount of biofilm produced can greatly vary from one isolate to another, and presence of the virulence determinants associated with biofilms (**TABLE 9**) is widespread and does not necessarily correlate with biofilm production [427–432]. Furthermore, the *csuA/BABCDE* operon encoding type I pili, which is essential to biofilm formation [433], was likely acquired by HGT [433] and is conserved in *A. baumannii*, *A. nosocomialis* and *A. pittii* [391]. Additionally, the Biofilm Associated Protein (BAP) has also been found in several non-*baumannii Acinetobacter* species, including

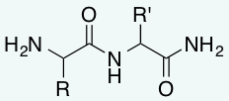
those of the ACB complex [434]. Although biofilm formation has been poorly studied in species other than *A. baumannii*, it has been found that *A. calcoaceticus*, *A. junii*, *A. nosocomialis* and *A. pittii* formed considerable amounts of biofilms [435], and that *A. baumannii*, *A. nosocomialis* and *A. pittii* formed more biofilm than other *Acinetobacter* species when comparing isolates of hospital origin [436]. Interestingly, while some studies did not find significant differences in biofilm formation amongst the former members of the Ab group, i. e. *A. baumannii*, *A. nosocomialis* and *A. pittii* [429,437,438], one report showed that *A. pittii* formed more biofilm than *A. baumannii,* and that *A. nosocomialis* had an intermediate phenotype [439]. Besides, Na *et al.* already described that 5 out of 13 *A. seifertii* isolates were able to produce biofilm, although they also found that none of the *A. pittii* isolates in their study formed biofilm [438], which is in disagreement with other studies [429,436,437,439,440].

BOX 11. Quorum sensing (QS) and *Acinetobacter* spp.

QS is a cell-to-cell communication mechanism that allows bacteria to behave as a collective and, consequently, benefit from cooperative responses. The collective behaviour is achieved by the accumulation in the extracellular milieu of signal molecules, such autoinducers or *N*-acylhomoserine lactones (AHLs), to a threshold concentration where they activate, or repress, the expression of target genes [675]. Gram-negative bacteria usually employ AHLs, 2-alkyl-4-quinolones, long chain fatty acids, and fatty acids methyl esters, while Gram-positive bacteria use linear, modified or cyclic peptides, which are known as autoinducing peptides, and γ-butyrolactones [676]. Besides other phenotypes, QS has been shown to be involved in the virulence of several bacterial species [676].



N- acylhomoserine lactone
Gram-negative bacteria



Autoinducing peptide
Gram-positive bacteria

The production of AHLs in *Acinetobacter* spp. strains of clinical origin as well as in *A. calcoaceticus* has been acknowledged since the beginning of the 2000s [677]. Members of the LuxI protein family, responsible for AHLs synthesis, and of the LuxR family, that interact with AHLs and regulate gene expression, have been found in *Acinetobacter* species [499,500,678]. In *A. baumannii* the LuxI and LuxR homologues are known as AbaI and AbaR, respectively. As in many other bacterial species, QS in *Acinetobacter* species has been shown to be involved in virulence-related phenotypes. In 2011, Clemmer *et al.* showed that QS was required for biofilm formation and motility in *A. nosocomialis* M2 strain [464]. Further studies relate QS to these virulence-associated phenotypes in *Acinetobacter* [475,498,499,666] as well as to regulation of drug resistance in these species [679].

Biofilms become a health problem when they develop on living tissues or medical devices [441], thus helping bacteria to colonise and spread in the clinical settings. *Acinetobacter* medical biofilms are commonly associated with nosocomial infections and usually related to catheters and wound infections [442,443]. In 2008, Rodríguez-Baño *et al.* found that biofilms were involved in the pathogenesis of some device-associated *A. baumannii* infections and that, in their study, all catheter-related urinary and bloodstream infections were caused by *A. baumannii* biofilm-forming strains [427]. On the other hand, recently, Wang *et al.* have shown that the biofilm-forming ability of *A. baumannii* isolates was not associated with worst outcomes in patients with bacteremia pneumonia [444], and Antunes *et al.* already noticed that there was no correlation between the biofilm-forming aptitudes of *A. baumannii* isolates and their clinical impact [445]. Nevertheless, whether considering biofilm formation as an important virulence attribute in *Acinetobacter* species might be controversial, the role of biofilms in persistence of *A. baumannii* in the nosocomial environment cannot be denied because it enables the colonisation of medical devices and environmental surfaces [446–448].

Some studies suggest that biofilm-producing strains tend to be more susceptible to antibiotics [427,439,449–453], although there is also evidence of the contrary [454,455] and of no such relationship between the ability to form biofilm and the susceptibility of a given isolate [433,440,456]. Nevertheless, it makes sense that under antibiotic pressure susceptible strains organise themselves and develop biofilms as a way to overcome the antibiotic challenge, while resistant cells would not need such defence mechanism and can remain planktonic. In this sense, recent studies support that biofilm-embedded *A. baumannii* cells tolerate antibiotic concentrations over 50 folds higher than those tolerated by planktonic cells [457], hence adding further means of resistance to antibiotics and, thereby, hindering the treatment when sessile.

4.1.3. Motility

In general, it is considered that swimming, swarming, twitching, gliding and sliding are the main types of bacterial motility [458]. Swimming and swarming motility require flagella and occur in aqueous and solid media, respectively, while twitching, gliding and sliding are flagella-independent. Twitching depends on type IV pili and occurs upon their extension, surface attachment and retraction; gliding is associated with the secretion of

cellular substances, such as polysaccharides, and adhesion to the substrate; and finally, sliding is promoted by the expansive forces of cell growth, modification of the surfaces and surfactant production.

Initially, *Acinetobacter* spp. were regarded as non-motile rods, as its genus composed name points out (from the Greek [α + κινητο + βακτηρ(ια)]), because of their lack of flagella. However, studies about motility in *Acinetobacter* species date from the 1970s and reported different kinds of motility in *Acinetobacter* isolates [459–461] (**FIG 9**). In these studies, it was already noticed that twitching motility in *Acinetobacter* species was dependent on the presence of polar fimbriae [465], which were later confirmed to be type IV pili [307]. In addition, another form of motility independent of pili was also described, the "ditching" motility, which occurred at the surface of the semi-solid agar plates and, although resembling to swarming or gliding motility, it had the characteristic of forming trenches in the agar [459] (**FIG 9**). Nowadays, this type of motility is known as surface-associated motility.

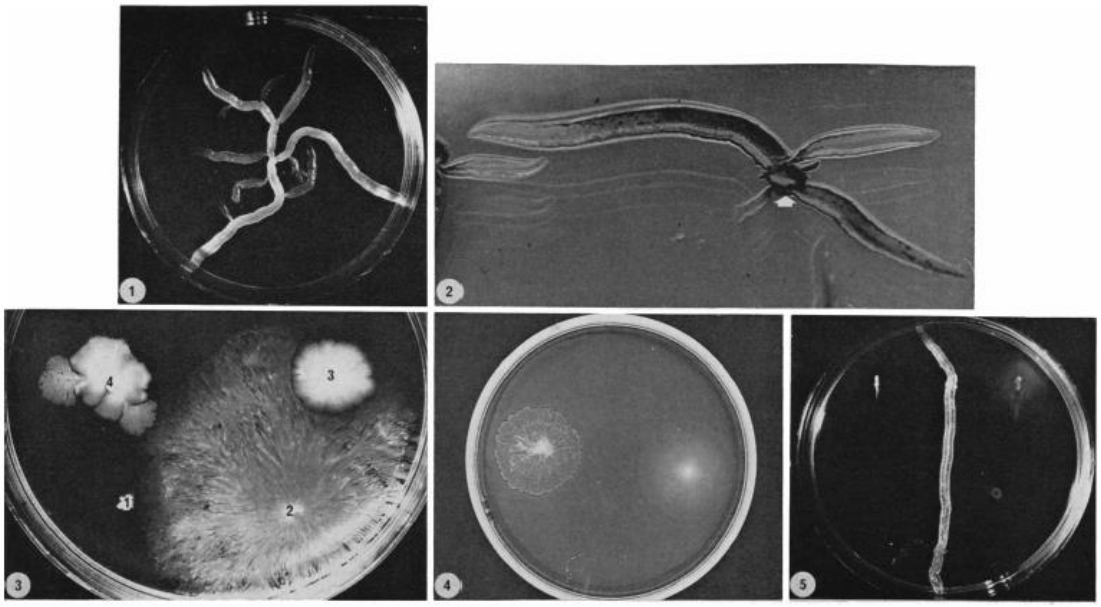


FIG. 1.—Ditching by a strain of *A. anitratus* on a medium containing 0·3% Davis Agar and 1% peptone. The organism was stab-inoculated at the plate centre and during incubation for 18 h it produced the sinuous fissures containing fluid which are referred to as ditches. ×0·5.

FIG. 2.—Ditching by *A. anitratus*; the channels are about 2 mm deep. The organism was stab-inoculated on BBL motility medium at the point indicated and has produced three large ditches and a small one. ×2·25.

FIG. 3.—Four strains of *A. anitratus* growing on 0·3% Oxoid "Ionagar" and 1% peptone; the numbers indicate the points of inoculation of the different strains. Strain no. 1 shows no swarming, whereas nos. 2, 3 and 4 show various types of swarm. Note the inhibition of swarming between strains 2 and 3. ×0·7.

FIG. 4.—*Left*: *A. anitratus* swarming from a stab-inoculum. *Right*: *E. coli* showing typical diffuse growth of motile organisms from a stab inoculum. The medium was 0·3% Oxoid "Ionagar" with 1% peptone. Contact print of the petri dish. ×0·5.

FIG. 5.—Three different organisms were inoculated at the top of three knife-cut faults in BBL motility medium. *Centre*: *A. anitratus* which spread down the cut, extended it at both ends and widened it into a ditch. *Right*: motile *E. coli* which extended only about 1·5 cm down the cut and spread within the medium in the usual way of a motile organism. *Left*: *Klebsiella aerogenes* which shows minimal spread from the inoculation site. ×0·5.

FIG 9. Motility figures from Barker & Maxted (1975) [459]. The surface-associated motility of *Acinetobacter* grown on plates of commercially available semi-solid media can be observed. As seen in the pictures and captions some strains produced channels or ditches in the semi-solid agar. "*A. anitratus*" designation presumably corresponds to *Acinetobacter baumannii* species.

Regarding twitching motility, many *A. baumannii* strains have the genes encoding type IV pili and display this kind of motility [445]. Indeed, the genes necessary to produce the type IV pilus are widespread in *Acinetobacter* species and are highly conserved, except for *pilA*, which has a high degree of sequence divergence [463]. Despite ubiquity, neither the type IV pili nor twitching motility have been associated with *Acinetobacter* virulence in animal models, [31008,390], but it has been demonstrated that type IV pili promote host cell adhesion in *A. nosocomialis* [463]. In the case of surface-associated motility, the mechanisms behind it are unclear. The type IV pili seem to be partially involved because *pilT* inactivation partially eradicates this type of motility [308,464]. Anyway, surface-associated motility has been shown to be influenced by on iron availability [465,466], QS [464] and blue light [467,468 (**BOX 12**). Finally, both twitching and surface-associated motility have been shown to depend on the surfactant 1,3-diaminopropane in clinical isolates of *A. baumannii* [469].

BOX 12. Blue light and *Acinetobacter* spp.

Among the numerous environmental signals that modulate gene expression in bacteria, we often overlook light when we study non-phototrophic organisms. However, photoreceptors are extensively present among chemotrophic microorganisms [680]. There are six well-described families of photoreceptors based on the structure of their light absorbing chromophores, the (bacterio)rhodopsins, the phytochromes, the xanthopsins, the cryptochromes/photolyases, the phototropins and the flavoproteins [681]. Particularly, blue light photoreceptors belong to the flavoproteins family and are characterised by a blue light using Flavin (BLUF) domain, which upon photoactivation suffers a global structural activation that results into gene expression regulation. In 2014, Losi *et al.* found that among 1282 strains from 453 different species, there were 1705 bacterial BLUF proteins, and that many of them were found in pathogenic species such as *Escherichia coli, Klebsiella peumoniae* and also in *Acinetobacter* spp. [681,682]. A deeper search focused on *Acinetobacter* species revealed a total of 198 putative BLUF-domain-encoding genes in 119 fully sequenced *Acinetobacter* genomes from '*A. oleivorans', A. baylyi, A. baumannii, A. nosocomialis, A. calcoaceticus, A. soli, A. pittii, A. venetianus, A. radioresistens, A. gyllenbergii, A. beijerinckii, A. guillouiae, A. bereziniae, A. parvus, A. haemolyticus, A. ursingii, A. tandoi, A. lwoffii, A. indicus, A. harbinensis, A. nectaris, A. gerneri, A. johnsonii and A. schindleri* species [681].

In fact, in 2010, Mussi *et al.* already noticed that *A. baumannii* responded to light trough a temperature dependent process that involved a BLUF-domain-containing protein, the blue light sensing A (BlsA) photoreceptor. Blue light was found to inhibit motility and biofilm formation at 24°C, but not at 37°C, and enhanced killing of *Candida albicans* filaments [467]. Recently, the interconnection between temperature and blue light sensing was found to be related to negligible levels of expression of BlsA at 37°C and conformational changes that inactivate the protein at temperatures above 30°C [683]. In 2013, Golic *et al.* evaluated the effect of blue light in motility and biofilm formation of at least 25 different *Acinetobacter* species, and found that in *A. baylyi, A. calcoaceticus, A. nosocomialis, 'A. oleivorans', A. pittii* and *A. tjernbergiae* blue light inhibited motility, while the rest of species were non-motile, and that blue light enhanced biofilm formation in non-*baumannii Acinetobacter* species [468].

TABLE 9. Main virulence factors described for *Acinetobacter* species and their involvement in virulence-related phenotypes.

Virulence factor	Characteristics	Biofilm	Motility	Serum resistance	Adhesion	Invasion	Cytotoxicity / Apoptosis	Survival in animal model	Others
Cell surface									
Pili									
Type I pili	*csuA/BABCDE*	[433]							
Csu independent pili					To epithelial cells [429]				
Type IV pili	*pil* operon		Twitching and surface-associated (partial) [307,308,470]		To epithelial cells [463]				Competence [307,308]
Lipppolysaccharide (LPS) and exopolysaccharide (EPS)									
LpsB	LPS production		Surface-associated [466]						
PgaABCD	Poly-β-1-6-*N*-acetylglucosamine synthesis	Formation, in dynamic environment [471]							
Ptk and EpsA	Production of capsular polysaccharides			[472]				Rat [472]	
PglC and PglL	Protein *O*-glycosilation	PglC [473]						*Dictyostelium discoideum* (PglL), *Galleria mellonella* (PglL) and mice [473,474]	
Outer membrane proteins (OMP)									
OmpA	Porin	[396,475]	Surface-associated [470]	[476]	To epithelial cells [396,475,477,478]	Of epithelial cells [477]	[475,479–482]	Mice [477]	
Omp33-36	Porin				To epithelial cells [483]	Of epithelial cells [483]	Different cell types [483,484]	Mice [483]	
CarO	Porin						To epithelial cells [485]		

Virulence factor	Characteristics	Biofilm	Motility	Serum resistance	Adhesion	Invasion	Cytotoxicity / Apoptosis	Survival in animal model	Others
Others									
BAP	Biofilm-associated protein	Architecture of mature biofilms [486]			To epithelial cells [487]				
PBP7/8	Biosynthesis of peptidoglycan			[488]				Rat [488]	
Membrane transport									
Secretion systems									
T2SS	Secretion of LipA, LipH and CpaA							Mice, *G. mellonella* [402,403,492]	
T5SS (Ata)	Adhesin	[402]			To abiotic surfaces [490], extracellular matrix [402] and endothelial cells [491]			Mice [402]	
T6SS	Delivery of effector proteins to eukaryotic and bacterial cells								Bacterial killing [405–408]
Efflux pumps									
AdeABC	Active transport outside the cell	[492,493]						Mice [494], *G. mellonella* [493]	
AdeFGH	Active transport outside the cell	[495]							
AdeIJK	Active transport outside the cell	[245,492]	Surface-associated [245]						
Nutrient acquisition									
Acinetobactin	Iron uptake						To epithelial cells [496]	*G. mellonella* [496,497] and mice [496]	

Virulence factor	Characteristics	Biofilm	Motility	Serum resistance	Adhesion	Invasion	Cytotoxicity / Apoptosis	Survival in animal model	Others
Transcriptional regulators									
LuxRI homologues	Quorum sensing (*abaRI*)	[470,498–500]	[470,498,499]						
BfmRS	Two component system	[501]	Surface-associated [470]						Regulation of *csu* operon [501] and K *locus* [502]
BlsA	Blue light photoreceptor	[467,468]	[467,468]					*Candida albicans* [467]	Iron uptake [503]
Others									
Phospholipases C	Degradation of phospholipids						[422,504]	*G. mellonella* [422]	
Phospholipases D	Degradation of phospholipids			[505]		Of epithelial cells [505,506]		Mice [505], *G. mellonella* [506]	

4.2. Host-pathogen interactions

In the last 10 years, researchers have greatly advanced in the knowledge of *Acinetobacter* pathogenesis. Together with the discovery of new virulence factors, the study of how *Acinetobacter* relates with the immune response during infections is slowly helping to uncover the mechanisms by which these organisms have become such great nosocomial pathogens. As mentioned before, *Acinetobacter* has efficient mechanisms, such as siderophores, to avoid one of the main host's defense strategies against bacteria, the restriction of nutrients availability [390]. Another key factor for *Acinetobacter* success, especially of *A. baumannii*, is serum resistance. Recently, in addition to already known virulence factors, such as those related to exopolysaccharide production, several novel genes were shown to be associated with human serum resistance in *A. baumannii* [507], highlighting the complexity of this phenotype but also the great variety of tools that this species has to evade the innate immune response (**TABLE 9**).

Once *A. baumannii* crosses the host barriers, it might be able to evade complement and phagocytosis, likely thanks to alterations in its capsule. If *A. baumannii* can resist the initial innate immune clearance, it can then adhere to different kinds of cells, such as epithelial or endothelial cells [491,508]. Adhesion, which is mainly driven by OmpA [477], BAP [488], Ata [491] and type IV pili [463], can be considered the first step to trigger the immune response in the host (**FIG 10**). Upon adhesion to eukaryotic cells, the innate immune receptors recognise the pathogen-associated molecular patterns (PAMPs) of *A. baumannii*, principally the lipid A of the LPS, which is highly immunostimulatory [509]. In addition, adhesion of *A. baumannii* also activates the secretion of antimicrobial peptides by the host cells [510,511]. It has been observed that adhesion varies greatly depending on the strain [429,508,512] and that the more virulent an *A. baumannii* strains is, the weaker immune response it generates in the host cells [429]. Particularly, the LPS activates the extracellular Toll-like receptor 4 (TLR-4) and CD14, which turn on the nuclear factor κB (NF-κB) signaling cascade [513,514], and together with other activated cascades, like the mitogen activated protein kinase (MAPK) [510], the transcription and secretion of cytokines and chemokines is triggered [513–516]. Furthermore, the TLR-2 has also been found to be involved in the inflammatory response produced by *A. baumannii*. The TLR-2 recognises *A. baumannii* peptidoglycan and lipoproteins, but it is not clear if its effects are pro- or anti-inflammatory [510,513,514]. The secretion of cytokines and chemokines results in sepsis syndrome, but is also essential for the recruitment of immune cells to the

site of infection. The first to arrive are the neutrophils, which are key for bacterial clearance [517]. Aside from neutrophils, other recruited phagocytic cell types are macrophages and natural killer lymphocytes, although to a lesser extent [516,518,519]. Successful clearance of *A. baumannii* cells is primarily mediated by the generation of reactive oxygen species, that kill the phagocytosed bacteria [510,513,520].

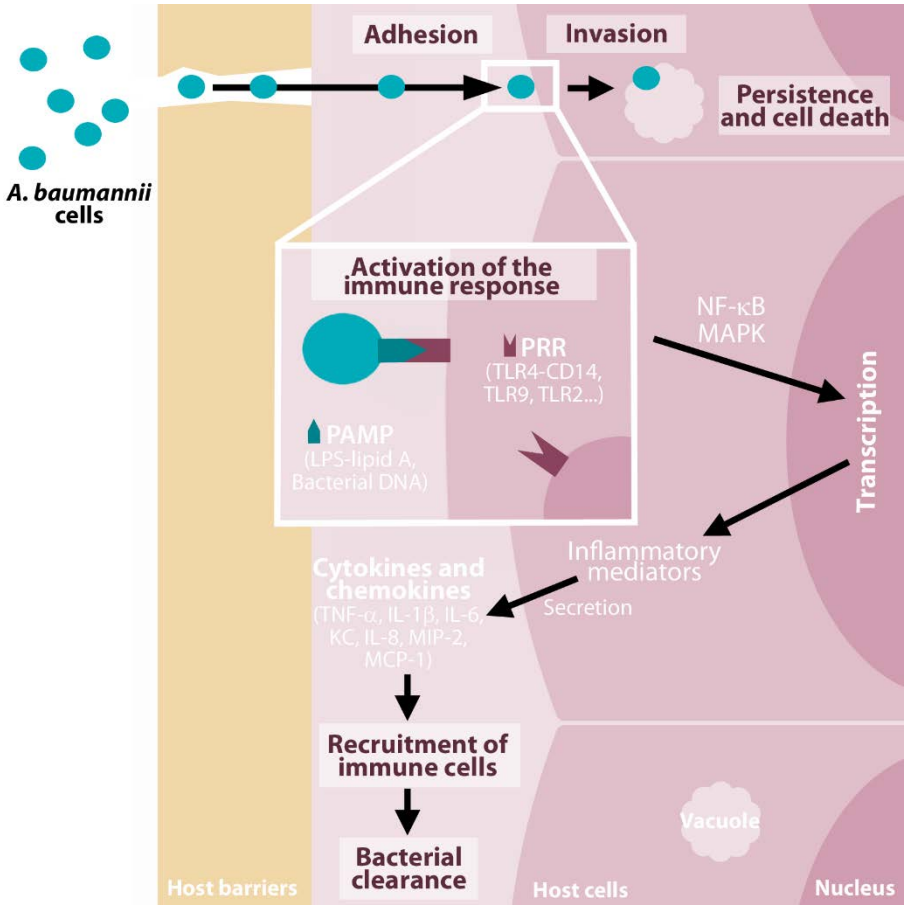


FIG 10. *Acinetobacter baumannii* simplified scheme of host-pathogen interactions. After entrance to the human body and innate immune clearance evasion, *A. baumannii* cells can adhere to eukaryotic cells through OmpA and other adhesines interaction. The pattern recognition receptors (PRRs) of the eukaryotic cells, which can be located on the surface or intracellularly, will trigger the immune response upon contact with the pathogen-associated molecular pattern (PAMPs) of *A. baumannii*. Via signaling cascades (mitogen activated protein kinase (MAPK), nuclear factor κB (NF-κB)…) the production and secretion of chemokines and cytokines will attract immune cells, such as neutrophils, macrophages and natural killer lymphocytes. The activation of phagocytic cells may lead to successful bacterial clearance but may also result in the sepsis syndrome. Nevertheless, *A. baumannii* can also invade eukaryotic cells. OmpA mediates the internalisation of this bacterium, which locates bounded to vacuolar membranes. *A. baumannii* cells can persist intracellularly and upregulate pro-apoptotic factors, such as caspases, which will ultimately lead to eukaryotic cell death.

However, despite *A. baumannii* being generally considered an extracellular pathogen, it can invade epithelial cells through a zipper-like mechanism that depends on microfilaments and microtubules [477]. After invasion *A. baumannii* cells are located in membrane-bound vacuoles, being able to survive intracellularly and cause host cell cytotoxicity, which may ultimately lead to cell death [480,521,522]. The virulence factor OmpA not only participates in the adhesion and invasion processes but also mediates apoptosis, by activating pro-apoptotic factors, like caspase 3 in the mitochondria and exerting DNase I-like activity in the nucleus [479–482]. Interestingly, it has been found that the OmpA-like proteins of the non-ACB complex species have a Lys320Ser or Lys320Asn amino acid substitution in the nuclear localisation signal (NLS) that prevents their entrance to the nucleus of the host cells, in contrast to the NLS sequence of the ACB complex species which allows the nuclear localisation of OmpA [480]. Inside the host cells vacuoles, *A. baumannii* PAMPs are also recognised by intracellular innate immune receptors, such as the endolysosomal TLR-9, which recognises bacterial DNA and has been shown to restrain pneumonia and bacterial dissemination in murine models [523], and the nucleotide-binding oligomerisation domain containing protein 1 and 2 (NOD1 and NOD2) and Rip-2, which ultimately may participate in *A. baumannii* clearance in epithelial cells by activation of NF-κB, and, therefore, production of chemokines and cytokines [515]. Remarkably, it has been demonstrated that *A. baumannii* can disseminate from the infection site to other body locations [521,522], and some authors suggest that *A. baumannii* adheres to neutrophils and uses them for transportation [524]. On the other hand, how *A. baumannii* translocates from the vacuoles to outside the cell is poorly understood. Recently, it has been found that the transcription factor EB, which is known to participate in lysosomal biogenesis, is required for intracellular trafficking of *A. baumannii* ATCC17978 strain in A549 epithelial cells, therefore, the transcription factor EB participates in dissemination and persistence of *A. baumannii* [525].

Immune response studies have been basically performed using *A. baumannii* strains, few studies have provided information about host pathogen interactions of the non-*baumannii Acinetobacter* species. Interestingly, Na *et al.* compared the adherence to A549 cells of *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii* and *A. seifertii*, and observed that, although there was great strain variability, *A. seifertii* and *A. calcoaceticus* showed higher adherence than the other species, however, the study did not deepen on the putative differential underlying mechanisms between species [438]. Intriguingly, another study using *A. baumannii* and *A. pittii* strains found out that both species showed scarce adhesion, no invasion, and failed to produce cytotoxicity on A549 cells [526]. Further studies of this group, using again both *A. baumannii* and *A. pittii* revealed that, *in vitro*, neutrophils phagocytose

these bacteria within 4 hours after bacterial contact and that although macrophages are activated by both species, they did not show phagocytic activity [527], thus reporting a similar behaviour between the two species. Clearly, further studies are necessary to uncover if non-*baumannii Acinetobacter* species, particularly those of the Ab group, interact differentially with host cells and trigger different response mechanisms of those already described for *A. baumannii*.

5. Ab group infections

The *Acinetobacter* species, when regarded as pathogens, are considered opportunistic microorganisms that mainly cause nosocomial infections (**FIG 11**), mostly in immunocompromised patients and in the intensive care units (ICUs). However, community-acquired infections have also been described (**FIG 11**), although to a lesser extent, and are usually accompanied with underlying diseases (chronic obstructive pulmonary disease, diabetes, renal diseases…) and, sometimes, clinical records of heavy smoking and excessive alcohol consumption [45,528]. *Acinetobacter* community-acquired bacteremias and pneumonias seem more severe than those of nosocomial origin, with mortality rates up to 56%, and are typical of tropical or subtropical climate regions, such as China, Taiwan and Australia [528]. Finally, *Acinetobacter* infections have also been related to natural disasters and war infections (**FIG 11**, **BOX 13**), but their acquisition and dissemination are probably healthcare associated rather than originating from environmental sources [529,530].

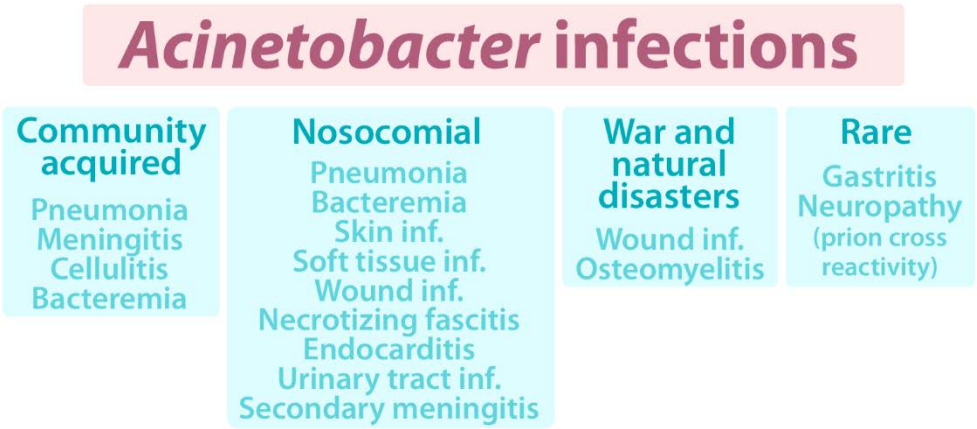


FIG 11. Main types of infections (inf.) caused by *Acinetobacter* spp.

There are several risk factors that promote the spread of *Acinetobacter* infections in the hospitals. These pathogens are able to survive for long periods of time in the environment thanks to their resistance to desiccation and to disinfectants [450,531–533]. This fact, together with resistance to major antimicrobial agents, promotes their survival in the clinical settings and their dissemination among patients. *Acinetobacter* nosocomial infections commonly affect those patients with disrupted anatomic barriers (due to surgery and invasive procedures, among others) and which are under antibiotic selective pressure (thanks to the intrinsic resistance to common antibiotics and abnormal microbiota caused by the use of antibiotics) [153,534]. Commonly, it is assumed that interactions between patients, healthcare staff and contaminated fomites promote the transmission of *Acinetobacter* [529]. Nevertheless, direct contact might not be the only way of transmission, since patients, colonised or infected, can spread *Acinetobacter* in the air [535–538], where it can survive for weeks [538], hindering the *per se* difficult decontamination measures for these pathogens.

BOX 13. The Iraqibacter.

During the United States intervention in Iraq and Afghanistan, *Acinetobacter* earned the nickname of "Iraqibacter" and hit the headlines of several media because of its frequent isolation as the etiological agent of war wound infections, bacteremias and osteomyelitis experienced by many of the injured soldiers deployed to these counties, especially Iraq [684]. One of the reasons for the hype was the fact that many of the infections were caused by multidrug resistant strains, increasing the severity of the injuries due to the lack of effective treatment [530,684]. Furthermore, compared to other war conflicts, the incidence of *Acinetobacter* infections in the troops increased [687], and outbreaks due to the species of the ACB complex were reported [530]. It is quite likely that, in comparison to previous and ongoing conflicts, the desert environment in which the field hospitals were based in Iraq and Afghanistan constituted an excellent habitat for *Acinetobacter*, which is highly tolerant to desiccation [450,532].

Among all the species of the genus, *A. baumannii* constitutes, undoubtedly, the major source of infections, thanks to its great persistence abilities and its intrinsic resistance and capability to acquire antibiotic resistance determinants [310] that, consequently, reduce the treatment options available for this pathogen, especially when considering carbapenem resistant *A. baumannii* (See **Section 5.2**, below). However, we should not disregard the other species of the Ab group. Despite most of the employed identification methods underestimating their prevalence in clinical samples, *A. nosocomialis*, *A. pittii* and

A. seifertii are increasingly being reported as causative agents of infections [192,439,539–542] and some studies show that non-*baumannii* species, especially *A. nosocomialis* and *A. pittii*, can be more prevalent than *A. baumannii* [193,439,541,543,544]. On the other hand, species outside of the Ab group can also be involved in human disease [545] such as *A. junii* [546–548], *A. ursingii* [549–555], *A. schlinederi* [553,556] or *A. lwoffii* [557–562], to name a few, although they are not reported often since they tend to be highly susceptible and are less prone to disseminate and cause outbreaks.

5.1. Clinical impact

The attributable mortality of *Acinetobacter* infections has been a discussed issue for many years [545,563]. Until the publication of studies using multivariate analysis, which mainly focused on *A. baumannii*, it was difficult to discern if the real cause of the outcomes was the underlying diseases usually associated with *Acinetobacter* infections or the infection *per se,* and there were many disparities between studies [45]. In 2007, a review by Falagas & Rafailidis on the current literature concluded that *A. baumannii* infections were, indeed, associated with increased mortality, likely due to inappropriate early treatment [563]. Other authors, however, believed that attributable mortality to *Acinetobacter* species was still a controversial issue [45]. Undeniably, attributable mortality to *Acinetobacter* will always be hard to ascertain due to the fact that the infections caused by this pathogen usually affect immunocompromised patients, thus discerning if the mortality is due to the pathogen or to the underlying diseases will remain a difficult task. Nevertheless, a later study that gathered information from 75 countries, based on 13796 patients in 1265 different ICUs, found that, of the 19 organisms studied, only *Acinetobacter* spp. and *Pseudomonas* spp. were strongly associated with increased mortality by multivariate analysis [564].

For many years, the studies about the clinical impact of *Acinetobacter* infections have focused on *A. baumannii*, however, nowadays, there are several reports that analyse the mortality and risk factors associated with the outcomes of infections caused by the species of the Ab group (**TABLE 9**). It should be stressed out, though, that most of them were conducted in Asian countries, principally Taiwan. Thus, there is a bias in literature and the differences in species prevalence and their associated outcomes between regions need to be further examined. Another common trait among all the studies is that they mainly consider nosocomial bacteremia cases and, therefore, there is little or no information

about the related outcome of other types of *Acinetobacter* infections. In addition, most studies found that *A. baumannii* isolates displayed higher rates of resistance to antimicrobials than *A. nosocomialis* and *A. pittii*, except for colistin, whose rates were sometimes higher in *A. nosocomialis* [151,298,565,566]. Overall, the studies agree on the fact that *A. baumannii* bacteremias are associated with worst outcomes than those where the aetiogical agent is *A. nosocomialis* or *A. pittii* (**FIG 12**). However, there are few exceptions to this general statement. Some reports found similar rates of mortality among the species of the Ab group [151,539,567,568], and the study by Fitzpatrick *et al.*, which compared non-*baumannii* species of the Ab group to susceptible and MDR *A. baumannii*, found that, whether there were differences in mortality between MDR *A. baumannii* and non-*baumannii* species, susceptible *A. baumannii* isolates had similar mortality rates as those of the other species of the Ab group [191].

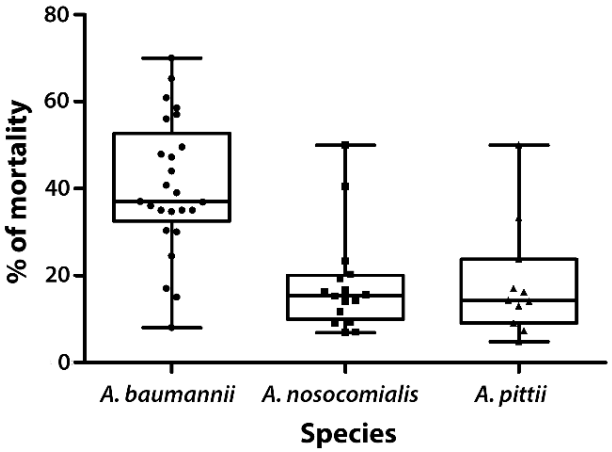


FIG 12. Mortality rates of *A. baumannii*, *A. nosocomialis* and *A. pittii* infections inferred from the studies listed on TABLE 9. All types of mortality (crude, attributable, 14-day, 30-day…) were considered. Overlapping boxplot and dot blot, the boxes span from the first to the third quartile, the median is indicated as a segment inside the box, and the whiskers indicate the minimum and maximum mortality values.

Nevertheless, despite the fact that there are some similarities among the studies, they fail to consistently determine the factors that turn *Acinetobacter* bacteremias into lethal infections (**TABLE 10**). The most common independent risk factors associated with mortality are severity of disease (high Acute Physiology and Chronic Health Evaluation (APACHE) II and Pitt bacteremia scores, **BOX 14**) [190,539,569–572], infection with *A. baumannii* species [151,190,566,570,572], appropriateness of the antimicrobial therapy [190,191,566,573] and multidrug resistance [190,565,573]. However, it is yet unclear whether the association of *A. baumannii* with a less favourable clinical outcome should be attributed to a higher pathogenicity than the other species or, instead, it is related to the frequent MDR phenotype of *A. baumannii*, which, consequently, leads to increased likeli-

hood of treatment failure. In this sense, Yang *et al.* compared the outcomes and risk factors of carbapenem resistant *A. baumannii* and *A. nosocomialis* isolates and found that carbapenem resistant *A. baumannii* was, indeed an independent risk factor for mortality [299]. However, Chusri *et al.* found that carbapenem resistant *A. baumannii*, but not *A. baumannii* species *per se*, was associated with worst prognosis [190]. Anyway, given the scarcity of studies assessing this issue, it is still uncertain if the clinical outcomes of the emergent *A. nosocomialis*, *A. pittii* and *A. seifertii* would be similar to those of *A. baumannii* if all pathogens were carbapenem resistant.

BOX 14. Severity of disease classification indexes: APACHE II and Pitt bacteremia scores

Assessing the degree of severity of an infection is an essential step to provide optimal healthcare because it allows the stratification and treatment of the patients according to the seriousness of their needs. For this purpose, several scoring systems have been proposed, such as the Acute Physiology and Chronic Health Evaluation (APACHE) II and the Pitt scores. The APACHE II index is based on 12 physiologic measurements, that require laboratory data and invasive procedures, age and previous health status; the scoring of these features provides a number which correlates with the likelihood of death [685]. The Pitt score is a simpler, in terms of laboratory requirements, severity of illness index which is used to estimate mortality of bacteremia caused by, mainly, Gram-negative pathogens. Different variables of the patient are initially evaluated (temperature, mental status, hypotension, receipt of mechanical ventilation and cardiac arrest) and scored, and similar to the APACHE II, the higher the score, the higher the risk of death.

TABLE 10. Reports that analyse the mortality and risk factors associated with infections caused by the species of the Ab group.

Report	Country	Species (Prevalence, %)	Antimicrobial resistance	Mortality	Mortality Risk factors (MVA)	Others
Molina *et al.* 2010 [574]	Spain	Abau (92.59), Apit (6.1), *Acinetobacter* spp. (1.4)	ND	*Crude mortality* Abau 18.7% ≈ Apit 9.1%	ND	Abau frequently in respiratory samples; Apit in skin and soft tissues
Chuang *et al.* 2011 [151]	Taiwan	Abau (64.4), Ansc (26.7), Apit (8.9)	Abau higher rates, except for colistin	*Attributable* Apit 33.3% ≈ Abau 58.6% > Ansc 16.7%	Abau species	ICU bacteremia
Lee *et al.* 2011 [565]	Taiwan	Abau (54.4), Ansc (35.8), Apit (9.8)	Abau higher rates, except for colistin	*14-day* Abau 35% > Ansc 14.3%, Apit 4.8% *30-day* Abau 47.2% > Ansc 15.6%, Apit 14.3% *In-hospital* Abau 49.6% > Ansc 23.4%, Apit 23.8%	MDR but not Abau species *per se*	Bacteremia
Wisplinghoff *et al.* 2012 [575]	USA	Abau (63), Ansc (21), Apit (8), *Acinetobacter* spp. (8)	Abau higher rates	*Crude mortality* Abau 36.9% > Ansc 16.4%, Apit 13%, *Acinetobacter* spp. 4.2%	ND	Bacteremia
Chiang *et al.* 2012 [570]	Taiwan	Ansc (47.6), Abau (29.1), Apit (23.3)	Abau higher rates	*14-day* Abau > non-Abau	Abau species and APACHE II score (≥21)	Bacteremia (solid tumors)
Kuo *et al.* 2012 [571]	Taiwan	Ansc		*14-day* Ansc 9.4%	APACHE II score (≥21)	Bacteremia
Lee *et al.* 2012 [566]	Taiwan	Abau vs. Ansc	Abau higher rates, except for colistin	*14-day* Abau 34.7% > Ansc 15.3% *In-hospital* Abau 65.3% ≈ Ansc 40.5%	Abau species, inappropriate therapy [a]	Bacteremic pneumonia

Report	Country	Species (Prevalence, %)	Antimicrobial resistance	Mortality	Mortality Risk factors (MVA)	Others
Wu *et al.* 2012 [569]	Taiwan	Ansc	30.8% MDR	*14-day* Ansc (11.7%)	High Pitt bacteremia score and prior chemotherapy	Bacteremia (solid tumors)
Yin *et al.* 2012 [567]	Taiwan	Abau vs non-Abau (Ansc+Apit)	Abau higher rates	*14-day* Abau 30.4% ≈ non-Abau 21.7% *In-hospital* Abau 60.9% ≈ non-Abau 39.1%	ND	Bacteremia (total parenteral nutrition)
Park *et al.* 2013 [572]	Republic of Korea	Abau (50) vs non-Abau (50: Ansc (33), Asei (9), Apit (6), Acal (1))	MDR: 77% Abau vs. 32% non-Abau	*30-day* Abau 39% > non-Abau 16% *Attributable* Abau 37% > non-Abau 12%	Abau species and high APACHE II score [a]	Bacteremia
Yang *et al.* 2013 [299]	Taiwan	Abau vs. Ansc	All Carb-R. Abau higher rates, except for colistin	*14-day* Abau 40.8% > Ansc 14.1% *28-day* Abau 47.9% > Ansc 20.3%	Abau species [a]	Bacteremia
Chusri *et al.* 2014 [190]	Thailand	Abau (89), Ansc (8), Apit (3)	Carb-R Abau higher rates	*14-day* Abau Carb-R 30%, Carb-S 17% > non-Abau 4% *28-day* Abau Carb-R 56%, Carb-S 35% > non-Abau 8% *In-hospital* Abau Carb-R 57%, Carb-S 35% > non-Abau 12%	High APACHE II score, appropriate therapy, Carb-R Abau infection	Different sites of infection

Report	Country	Species (Prevalence, %)	Antimicrobial resistance	Mortality	Mortality Risk factors (MVA)	Others
Lee *et al.* 2014 [574]	Taiwan	Carb-S: Abau (50.7), Ansc (10.4), Apit (12.4) Carb-R: Abau (22.8), Ansc (0.7), Apit (0.4)	Abau higher rates	*30-day* Carb-S: Abau 24.5%; Ansc 19.4% ≈ Apit 16.2% Carb-R: Abau 70%; Ansc 50% [c]; Apit 50% [c] Carb-R 70% > Carb-S 24.5%	Inappropriate therapy (MDR) [a]	Bacteremia
Fitzpatrick *et al.* 2015 [191]	USA	Abau (78.9), Apit (19), Ansc (2)	MDR: 79% Abau vs. 16% non-Abau	*14-day* non-Abau 13% ≈ Abau susceptible 8% < Abau MDR 36% *30-day* non-Abau 13% ≈ Abau susceptible 8% < Abau MDR 44%	Appropriate therapy [a]	Bacteremia
Fujikura *et al.* 2016 [568]	Japan	Abau (49), Apit (33.3), Ansc (4.5)	Low rates of MDR (common in Japan)	*30-day* and *In-hospital* No differences between species (overall mortality 8.5% and 25.5%, respectively)	ND	Bacteremia
Liu *et al.* 2017 [576]	Taiwan	Apit vs. Ansc	Similar rates	*14-day* Ansc 7% ≈ Apit 14% *28-day* Ansc 9% ≈ Apit 17%	ND	Bacteremia
Chen *et al.* 2018 [539]	China	Abau (51), Apit (28.7), Ansc (20.3)	Abau higher rates	*Crude mortality* Abau 15% ≈ Ansc 6.9% ≈ Apit 7.3%	High APACHE II score	Tracheal secretion (colonisation and infection)

Abau, *A. baumannii*; Acal, *A. calcoaceticus*; Ansc, *A. nosocomialis*; Apit, *A. pittii*; Asei, *A. seifertii*; MVA, Multivariate analysis; ND, not determined; (≈), no statistical significant differences; (<,>), statistical significant differences; ICU, intensive care unit; APACHE II, Acute Physiology and Chronic Health Evaluation II; MDR, multidrug resistant; Carb-R, carbapenem resistant; Carb-S, carbapenem susceptible;

[a] Additional risk factors found in the study; [b] Refers to imipenem only; [c] number of strains too low for statistical analysis

5.2. Therapeutic options

The rise on resistance in *Acinetobacter* species, particularly worrying in *A. baumannii*, limits the number of effective treatment options available for the infections caused by these pathogens. Despite the intrinsic resistance mechanisms, such as the intrinsic OXAs and efflux pumps (see **Section 3.1**), carbapenems have become the antibiotics of choice to confront *Acinetobacter* infections [153]. Unfortunately, since carbapenem resistance is also increasing and it is usually associated with multidrug resistance, the therapeutic options left for carbapenem resistant *Acinetobacter* infections are further reduced, requiring the evaluation of new or once discarded antimicrobials, and the development of alternative approaches to undertake this global health threat. Indeed, in 2017 the WHO placed carbapenem resistant *A. baumannii* at the top of the list of the critical bacteria for which the development of new antimicrobial drugs is considered a priority and urgent public health need (**FIG 13**) [50].

5.2.1. Antimicrobial approaches

Despite the actual menace of carbapenem resistant *A. baumannii* strains, the few antimicrobial choices left hinder the establishment of an optimal and consensus treatment for infections caused by such strains [153]. Fluoroquinolones and aminoglycosides are not recommended given the high rates of resistance associated with concomitant carbapenem resistance, however, whenever a carbapenem resistant strain is susceptible to these antibiotics, they can be potential treatment options [153]. Several studies have evaluated the efficacy of some potentially active antimicrobials, either alone or in combination therapy. Nevertheless, the reports fail to agree on the advantages of combination therapy over monotherapy in terms of improving patients' outcomes [153,577,578].

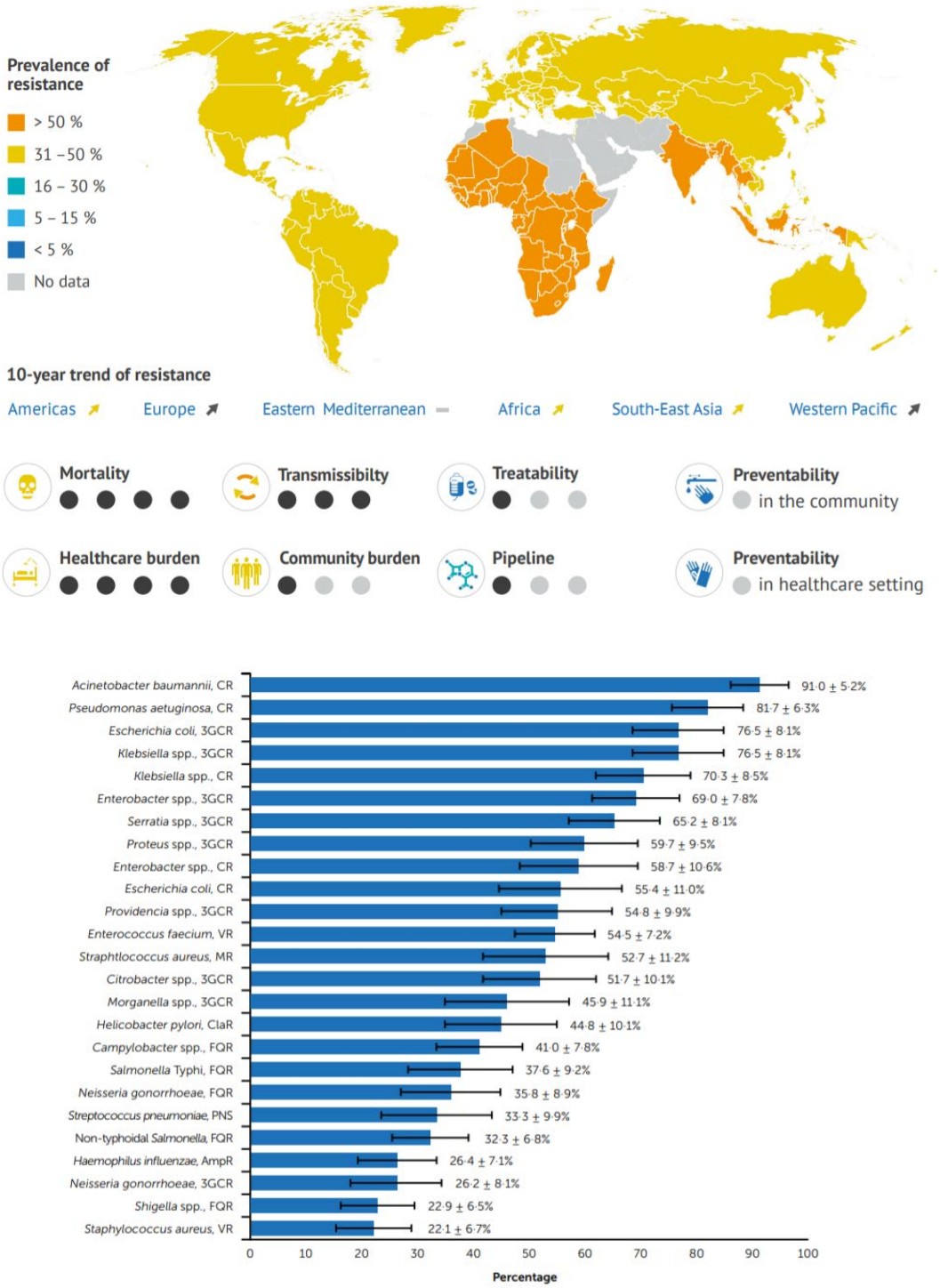


FIG 13. Some of the information presented in the report aimed to prioritize the pathogens that urgently require the development of new antimicrobial drugs developed by the WHO in 2017. [50] A) Infography about the most relevant data about carbapenem resistant (CR) *Acinetobacter baumannii*; B) Final ranking of antibiotic resistant bacteria other than *Mycobacterium tuberculosis*, in which CR *A. baumannii* occupies the first position (mean weight and standard deviation).

Polymyxins. Discovered in the 1940s, polymyxins were used to treat Gram-negative infections, however, in the 1970s their use was restricted due to associated nephrotoxicity and neurotoxicity after long treatments [579]. Nowadays, the use of polymyxins has been recovered and they have become last resort antibiotics to treat *A. baumannii* infections caused by MDR or XDR strains. Nevertheless, their usage is not straightforward since the optimal dose has yet to be established. In fact, the appropriate plasma concentrations might be difficult to reach [577] and may lead to subtherapeutic plasma concentrations, a circumstance which, in addition to toxicity, has prompted researchers to evaluate the potential of local use of these antibiotics, such as nebulisation administration for patients with ventilator associated pneumonia. Unfortunately, the available data about the benefits of nebulised polymyxins administration versus systemic therapy are ambiguous [153]. Furthermore, the use of polymyxins has led to the emergence of resistance (See **Section 3.2.1**) [577] and heteroresistance, thus is, the presence of tolerant subpopulations able to survive polymyxins concentrations above the MIC impeding bacterial clearance [153,577,578,580]. In view of these drawbacks, polymyxins combined therapy is recommended [581], since it might allow the use of lower doses to treat *Acinetobacter* infections, which will, in turn, decrease the side effects of these antibiotics. However, the risks in accelerating the emergence of resistance *in vivo* to the antibiotics employed in combination, like for example carbapenems, rifampin or tigecycline, seems more a threat than an advantage due to the characteristics of this pathogen [153,582]

Tigecycline and minocycline. In 2005, tigecycline, a semisynthetic tetracycline derived from minocycline that acts by inhibiting protein translation, reached the market. This glycylcyline, which is especially active against *Acinetobacter* species [583], has a broad spectrum of action and was designed to address multidrug resistance [584]. However, concerns about associated increased mortality among patients receiving intravenous tigecycline treatment arose in 2010, and were further confirmed in 2013 [584]. Particularly, the use of this antibiotic for treating nosocomial pneumonia was not recommended because of inferior clinical outcomes [585], although, one report pointed out that low dosage might be responsible for the worst performance of tigecycline in treating hospital-acquired pneumonia [586]. Furthermore, the treatment of *Acinetobacter* infections with tigecycline can induce *in vivo* resistance in these pathogens by, for example, overexpression of AdeABC and AdeIJK efflux pumps (see **Section 3.1.2**) [182,245]. Altogether, it was concluded that tigecycline might not be recommended as an optimal treatment for *Acinetobacter* infections [577,578].

In contrast, minocycline, which is a second-generation tetracycline, might seem as a reasonable alternative to tigecycline, since it maintains bacteriostatic activity to some tetracycline resistant *Acinetobacter* strains. However, the lack of clinical data about the outcome of patients treated with this antibiotic make difficult to determine the effectivity of minocycline against *Acinetobacter* infections [153].

Sulbactam. Sulbactam is a β-lactamase inhibitor which has intrinsic bactericidal activity against *Acinetobacter* thanks to its affinity for PBPs, in particular to PBP2 [153,581]. Several studies have shown that sulbactam can be effective against *Acinetobacter* infections, but the rates of cure shown in the reports differ, and the optimal dose is unclear [577]. This antimicrobial agent has been used in combination therapy with carbapenems, either alone or in combination with ampicillin [577]. Resistance to sulbactam, which can be achieved by reduced expression of PBP2, has also been described and seems to be rising [153,577], compromising the potential use of sulbactam as therapy for *Acinetobacter* infections.

Rifampin. This antibiotic cannot be used in monotherapy because of the rapid development of resistance in susceptible strains, thus its use has mainly been explored in combined therapy, especially with colistin, thanks to their *in vitro* synergistic activity, but also with imipenem and sulbactam [581]. However, despite the synergistic activity with colisitin observed *in vitro*, clinical trials have failed to support *in vitro* data and, therefore, this combination therapy is not recommended [577,581].

Fosfomycin. Fosfomycin is a peptidoglycan biosynthesis inhibitor, that, despite not having antimicrobial activity against *Acinetobacter* [587], shows synergistic activity against carbapenem resistant *A. baumannii* with colistin and sulbactam [588]. The usefulness of combination therapy of fosfomycin with other antibiotics needs to be further investigated [578,581].

5.2.2. Non-antimicrobial approaches

Taking into account the threat of antimicrobial resistance and its rising patterns over the years, new approaches need to be investigated in order to find alternative tactics to treat *Acinetobacter* infections. New methods less prone to generate the emergence of bacterial resistance will prove useful if, finally, our worst predictions come true and we run out of effective antibiotics to treat some *Acinetobacter* infections. Nontraditional therapies are far

from being implemented and replacing the use of antibiotics, however, as time passes, it becomes more ostensible the urgent need to develop such strategies. Perez & Bonomo stated that it is now time to think "out of the box" and put our best efforts in developing novel therapies before we exhaust the conventional options [589]. Some of the most promising non-antimicrobial approaches are explained below.

Antimicrobial peptides. Antimicrobial peptides (AMPs) have been isolated from all the multicellular species tested and constitute an innate defense mechanism against bacteria, yeasts, fungi, viruses, and even cancer cells. However, it should not be forgotten that bacteria can also produce AMPs to outcompete other bacteria, like for example vancomycin and polymyxins [590]. Nowadays, the Antimicrobial Peptide Database contains information of more than 3000 AMPs (http://aps.unmc.edu/AP/main.php; last accessed 17/09/2018), however, their mechanisms of action remain uncertain. AMPs can target intracellular components, but they usually act by disrupting negatively charged bacterial membranes thanks to their cationic and amphipathic properties. The efficacy of several natural and synthetic AMPs against *Acinetobacter* has been evaluated both *in vitro* and *in vivo* [591]. Nevertheless, despite showing good antibacterial activities, AMPs are more likely to be used as topical therapy, since their viability as treatment for systemic infections has to overcome their proteolytic degradation and toxicity within the human body.

Bacteriophage therapy. The first two lytic phages specific for *A. baumannii* were isolated in 2010 [592,593], after the initial descriptions, several other *Acinetobacter* specific phages have been described and their therapeutic applicability has been evaluated [591]. One of the most common findings has been that *Acinetobacter* phages have narrow spectrum and that their antibacterial activity is highly strain-dependent [153,591]. On the one hand, in a therapeutic sense, narrow spectrum represents an advantage, since it avoids the depletion of normal microbiota. On the other hand, and given the great diversity of the genus, it reduces the efficacy of phages as therapeutic options, and forces the use of cocktails composed of multiple phages in order to overcome the limited host range. Moreover, the secondary effects of phage administration, such as appearance of resistance, activation of inflammatory response or phage change during treatment, are still unclear.

Metal sequestration. Metal acquisition systems are essential for successful *Acinetobacter* infections (See **Section 4.1.1**). Some studies have demonstrated that outcompeting these bacterial systems and depleting the levels the availability of metals by using metal chelators, such as transferrin [594] or calprotectin [595], can suppress growth and kill *Acinetobacter,* and might be used as therapeutic options in the future [153].

Phototherapy. This therapy combines oxygen, infrared light and a photosensitizer, a non-toxic photoreactive dye that upon light excitement will generate reactive species of oxygen. Some of the most promising photosensitizers with antibacterial activity against *Acinetobacter* are phenothiazinium salts and tetrapyrrole porphyrins [153], although few studies have tested the efficacy of phototherapy against *Acinetobacter* infection *in vivo* [591]. The reactive species of oxygen provoke cell death by damaging DNA and disrupting cellular membranes, this mechanism of action kills bacteria, but can likewise affect host cells, thus the mechanisms of action of phototherapy entails a limitation for this therapy, in addition to the fact that it is restricted to topical use.

Vaccination. Despite the fact that it might seem challenging to define the target population for an *Acinetobacter* vaccine, due to the variety of types of infection caused by this pathogen, Pachón & McConnell identified at least 5 at-risk groups worth to be considered as potential beneficiaries of the development and implementation of *Acinetobacter* vaccines [596]. According to these authors, and taking into account that infections caused by *Acinetobacter* species are mainly hospital-acquired, and thus, subject to local epidemiology, the candidate populations that would benefit from an *Acinetobacter* vaccine are: patients with or likely to require mechanical ventilation; patients at risk of developing nosocomial and soft tissue infections; patients undergoing neurosurgical operations at risk of developing *A. baumannii* meningitis; military personnel who might be exposed to war-related trauma (**BOX 13**); and patients admitted to ICUs in hospitals with ongoing *Acinetobacter* outbreaks or aware of circulating MDR clones [596]. Pachón & McConnell also raise some of the concerns to overcome when developing such a vaccine. Firstly, target populations are usually critically ill, and thus, they might not be able to elicit a good vaccine response. Secondly, the unpredictability of some at-risk situations might require different vaccinations strategies, namely active immunisation for foreseeable populations at risk with enough time to achieve a protective response, such as patients with programmed surgeries or military personnel, and passive immunisation to provide immediate immunisation when the risk of exposure cannot be predicted, such as patients with traumatic injuries and/or in need of urgent surgeries. Finally, they conclude that, given *Acinetobacter* diversity, the development of *Acinetobacter* vaccines should consider the development of polyvalent vaccines rather than single-antigen-based vaccines, this approach would overcome the standardization problems of multicomponent vaccines, such as whole-cell vaccines, and benefit from the variety of antigens in order to increase the efficacy of the vaccine (**TABLE 11**) [596]. Nevertheless, as Perez & Bonomo pointed out, even a partially effective vaccine, with low coverage of the diversity of *A. baumannii* strains, might still be valuable in certain clinical scenarios [589].

TABLE 11. Some of the potential targets evaluated for the development of an *Acinetobacter* vaccine.

Antigen	Reference
Single antigen candidates	
Biofilm associated protein (BAP)	[597]
Poly-*N*-acetyl-β-(1-6)-glucosamine	[598]
Trimeric autotransporter protein (Ata)	[599]
Outer membrane protein A (OmpA)	[600]
K1 capsular polysaccharide	[601]
Outer membrane protein W (OmpW)	[602]
Outer membrane nuclease (NucAb)	[603]
Outer membrane putative pilus assembly protein, FilF	[604]
Outer membrane protein 22-KDa (Omp22)	[605,606]
Capsular polysaccharide	[607]
Outer membrane protein, BamA	[608]
Combination forms candidates	
OMVs+Bap and AbOmpA+Bap	[609]
Multicomponent candidates	
Formalin-inactivated whole cells	[610,611]
Outer membrane complexes (OMCs)	[612]
Outer membrane vesicles (OMVs)	[413]
Lipopolysaccharide-deficient inactivated whole cells	[613]
Antibiotic-exposed inactivated whole cells	[614]
Thioredoxin-deficient live attenuated whole cells	[615]
D-glutamate auxotroph live attenuated whole cells	[616]
Lipopolysaccharide-free OMCs	[617]

Fecal microbiota transplantation (FMT). Many patients harbour *Acinetobacter* in their gut microbiota, especially those in the ICUs, which are also among the most susceptible individuals to develop infections by this pathogen. This is especially problematic when the patient is colonised by MDR or carbapenem resistant strains, thus, decolonising such patients would avoid future infections, and along with vaccination, FMT has also been proposed as an alternative preventive method for eradicating resistant *Acinetobacter* from the intestinal microbiota [618]. As a proof-of-concept, one case report showed that when using FMT to eradicate *Clostridioides difficile* (formerly named *Clostridium difficile* [619,620]) from a patient, FMT was also able to collaterally eliminate the MDR *A. baumannii* that was colonising the patient, in addition to other MDR pathogens [621]. Nevertheless, further investigations on the applicability and effectiveness of this approach are certainly needed.

II. Background and Work Justification

Despite the recent advances [622,623], for many scientists, finding and characterising new cultivable bacterial species can seem odd, since, nowadays many people still tends to believe that there are not so many living beings left to be described. However, the full insight of bacterial diversity is far to be reached, and there is still much to be done in the prokaryotic taxonomy field. Some microbiologists contribute to broadening the knowledge about bacterial diversity by actively searching for new putative bacteria in remote places, and other, like in our case, find them by luck.

In September 2014, we received an *Acinetobacter* spp. isolate (JVAP01) that was recovered in 2009 from a six-year-old female patient, with no history of travelling, admitted to the Adnan Menderes University Hospital (Turkey) with a diagnosis of pyelonephritis. Initial identification by the automated Phoenix system (Becton Dickinson, USA) reported JVAP01 as a member of the ACB complex. Later, JVAP01 was identified as *A. pittii* (identity of 97%) or *A. calcoaceticus* (identity of 99%) by *rec*A and 16S-23S rRNA gene internal transcribed spacer (ITS) sequencing, respectively. In addition, MALDI-TOF MS also identified JVAP01 as *A. pittii* (score value of 2.114, **BOX 4**). The MLST analysis using the Pasteur scheme devised for *A. baumannii* identified a novel *recA* allele (*recA:106)* and JVAP01 was assigned a novel sequence type (ST606) (https://pubmlst.org/bigsdb?db=pubmlst_abaumannii_pasteur_seqdef). MLST analysis against the Pasteur database identified closely related allelic profiles belonging to non-identified *Acinetobacter* spp. included within the ACB complex.

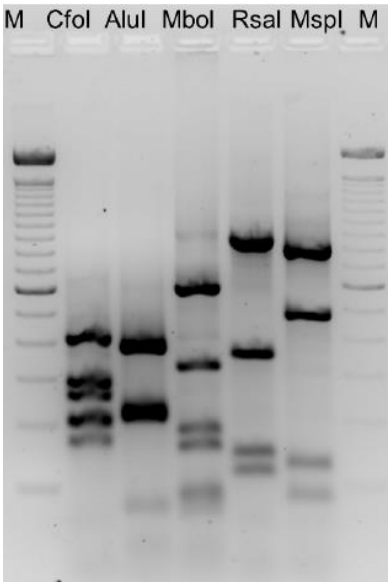


FIG 14. ARDRA profile of the JVAP01 isolate digested by CfoI (2), AluI (5), MboI (1), RsaI (1) and MspI (3).

In view of inconclusive results, ARDRA analysis was performed, and the pattern obtained was 2-5-1-1-3-17 for CfoI, AluI, MboI, RsaI MspI and BfaI, respectively (**FIG 14**). This pattern did not correspond to *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* or *A. pittii* species, instead, it matched the band pattern of a set of isolates from the *Acinetobacter* reference collection of the Leiden University that apparently constituted a novel *Acinetobacter* species, which had been preliminary termed "NB14". These results, together with the characterisation of the genetic platform bearing the NDM carbapenemase present in JVAP01, were published in 2015 [625].

After a retrospective analysis of our own collection of *Acinetobacter* spp. we identified 3 additional isolates with similar ARDRA profiles to JVAP01 and we requested the "NB14" isolates from the Leiden collection in order to gather enough isolates to further define their species status.

Similar to the finding of new bacterial species, the discovery of new antimicrobials drugs is usually achieved by active search, since we cannot wait until we find out an antibiotic by chance, like in old Fleming times. The urgent need to develop new antimicrobial treatments to fight infections has prompted the emergence of alternative approaches. In this sense, we believe that developing narrow-spectrum compounds that do not necessarily kill pathogens, but impair their ability to cause productive infections might be a strategy worth to be considered, because it also reduces the selection pressure and, thereby, the appearance of resistance mechanisms. This strategy might prove useful especially for *A. baumannii*, whose success is highly associated with MDR, usually leaving few options to treat its infections. Furthermore, we think that a better knowledge of the virulence traits and pathogenic potential of *Acinetobacter* spp., and of *A. baumannii* in particular, can provide new targets to develop specific antimicrobial drugs against these pathogens. In line with previous research from our group, which found that *Acinetobacter* spp. overexpressing efflux pumps were significantly more virulent in the *Caenhorabditis elegans* infection model (Dr. P. Espinal, personal communication), we decided to further investigate the dual role of efflux pumps and other transport-related proteins in antimicrobial resistance and virulence to contribute to the general knowledge about the virulence of this pathogen, but also to putatively identify new targets for developing treatments against *A. baumannii*.

III. Hypotheses and Objectives

The awareness and concerns about *Acinetobacter* have grown exponentially as the frequency of MDR isolates has increased in our hospitals and, even when a lot of progress has been achieved in the last decades, there is still much left to learn about this pathogen and how to fight it. In this sense, the aim of this PhD project was to bring furher understanding of the main clinical pathogens within the *Acinetobacter* genus, thus is, about the species of the Ab group. The research performed to achieve this goal is based on two main hypotheses:

Hypothesis 1. The isolates formerly known as *Acinetobacter pittii*-like NB14 group constitute a putative novel *Acinetobacter* species belonging to the Ab group which can be identified by MALDI-TOF MS.

Objective 1. Characterise the genotypic and phenotypic features that support the designation of the isolates of the NB14 group as a tentative novel species, for which the name of *Acinetobacter dijkshoorniae* sp. nov. is proposed, and which might be a new member of the Ab group with putative clinical relevance. (**Papers 1** and **2**)

Aim 1.1. Gather a collection with epidemiologically distinct isolates of the species of the Ab group (*A. baumannii*, *A. nosocomialis*, *A. pittii* and *A. seifertii*) as a framework for the characterisation of the isolates of *A. dijkshoorniae* sp. nov.

Aim 1.2. Perform the genetic characterisation of the isolates, including those of *A. dijkshoorniae* sp. nov., by studying the phylogenetic relatedness of all members of the Ab group by *rpoB* and MLSA cluster analysis.

Aim 1.3. Sequence the whole genome of the type strain JVAP01 of the putative novel species *A. dijkshoorniae* to perform whole genome comparisons (ANIb) analyses.

Aim 1.4. Perform metabolic and physiological assays to identify any putative phenotypic difference of *A. dijkshoorniae* sp. nov. when compared to the other species of the Ab group.

Aim 1.5. As part of the phenotypic characterisation, identify any putative statistically significant *A. dijkshoorniae* sp. nov. species-specific peak by MALDI-TOF MS analyses.

Objective 2. Evaluate the potential of MALDI-TOF MS to rapidly and accurately identify isolates of *A. dijkshoorniae* and *A. seifertii*. (**Paper 3**)

Aim 2.1. Revisit the identification of former members of the Ab group, with particular emphasis on *A. nosocomialis*.

Aim 2.2. Generate *in silico* pattern recognition models to compare the mass spectra of representative isolates from members of the Ab group to identify species-specific protein peaks.

Aim 2.3. Generate *A. seifertii* and *A. dijkshoorniae* reference signatures to allow the correct identification of members of the Ab group by MALDI-TOF MS using either bacterial extracts or direct colonies.

Objective 3. Investigate the presence of *Acinetobacter* spp. in food samples. (**Manuscript 4**)

Objective 4. Identify any differential phenotypic characteristics, in terms of antimicrobial susceptibility and *in vitro* virulence potential, of the members of the Ab group. (**Manuscript 5**)

Aim 4.1. Determine and compare the antimicrobial susceptibility patterns of our collection of isolates representatives of the species of the Ab group.

Aim 4.2. Detect the presence of intrinsic resistance mechanisms (intrinsic OXAs and efflux pumps) in the species of the Ab group.

Aim 4.3. Evaluate and compare the biofilm formation and surface-associated motility abilities of the species of the Ab group

Aim 4.4. Assess the virulence potential of the species of the Ab group in the *Caenhorabditis elegans* infection model.

Hypothesis 2. In addition to their contribution to antibiotic resistance, efflux pumps and transport-related proteins may have an important role in *A. baumannii* virulence's, either in cell-to-cell communication or in the secretion of virulence factors that might be involved in the pathogenesis of this species and could be used as potential targets for antibacterial drug development.

Objective 5. Study different putative active efflux pumps genes and transport-related proteins in order to determine their specific contribution to antibiotic resistance and virulence in *A. baumannii*. (**Manuscript 6**)

Aim 5.1. Obtain a collection of *A. baumannii* mutants with truncated efflux pumps, OMPs and transported-related genes.

Aim 5.2. Determine the role of putative new efflux pumps, OMPs and transport-related proteins in antimicrobial resistance in the mutant collection by determining the changes of MIC to different antibiotics.

Aim 5.3. Evaluate if putative new efflux pumps, OMPs or transport-related proteins are involved in the virulence of *A. baumannii* in the *Galleria mellonella* infection model.

Aim 5.4. Corroborate that the observed altered antimicrobial susceptibility and/or virulence is due to the absence of the bacterial membrane transporter by complementing the mutants (*Not accomplished*)

Objective 6. Characterise the secretome of those mutants showing differences in virulence in order to identify putative virulent factors secreted. (*Not accomplished*)

Objective 7. Identify new additional targets in order to develop antimicrobial drugs by determining the impact of the mutation of the secreted proteins in the virulence of *A. baumannii*. (*Not accomplished*)

IV. Results

This chapter has been divided into two different sections according to the hypotheses proposed (**Hypotheses and Objectives**). Section I, which comprises the results published in **Papers 1-3** and **Manuscripts 4** and **5**, corresponds to the characterisation of the isolates of the NB14 group as a novel species of the Ab group and the evaluation of MALDI-TOF MS as a suitable identification method for the members of the Ab group. Furthermore, in this section, other phenotypes beyond those studied in taxonomy are examined in order to further support the distinctness of the species of the Ab group and provide evidences reinforcing the idea that rather than being reported and studied collectively, they should be considered different entities, with potential different clinical implications. In Section II, which corresponds to the results depicted in **Manuscript 6**, we tried to find new targets to develop specific antimicrobial drugs against *A. baumannii*, while exploring the dual role of transport-related proteins in antimicrobial resistance and virulence in a MDR *A. baumannii* strain.

Section I

Despite the absence of guidelines [4,5] (**Introduction**), bacterial taxonomic descriptions should follow some recommendations. One of them is characterising the maximum number of strains, being 5-10 considered the minimum advisable, and another one is using representative strains of the closest taxa to properly characterise the new taxon [18,36]. Once we received the NB14 group isolates from the Leiden collection, we had enough isolates to further define the species status of the NB14 group isolates, but we also needed a collection of isolates of the current species of the Ab group. We used *A. baumannii*, *A. nosocomialis* and *A. pittii* isolates from our own *Acinetobacter* spp. collection, which contained isolates from several donors (acknowledged in **TABLE S1** from the supplemental material of **Paper 1**), but had to request isolates of *A. seifertii*, since this species had recently been described at that time [105]. Finally, 12 genetically distinct isolates of the NB14 group were compared against 93 reference strains in the phylogenetic studies; the genome of the type strain of the putative novel species (**Paper 2**) was compared to all the ACB complex genomes retrievable from the databases; and four to five isolates of each ACB complex species were used as representatives in the metabolic characterisation of all the NB14 groups isolates (**Paper 1**).

According to the recommendations for an appropriate bacterial taxonomic description, we used the polyphasic approach [13,14], and thus, we performed a phenotypic and genotypic characterisation of the isolates of the NB14 group (**Paper 1**). Both the genotypic data (*rpoB* and MLSA cluster analyses and ANIb genomic comparisons) and the phenotypic traits (metabolic assays and MALDI-TOF MS peak analyses) supported the designation of the isolates of the NB14 group as representing a novel species, for which we proposed the name of *Acinetobacter dijkshoorniae* sp. nov., named after Lenie Dijkshoorn, a relevant Dutch microbiologist in the *Acinetobacter* biology and taxonomy field, who gathered most of the NB14 group isolates (**Paper 1**).

Having found species-specific peaks during the phenotypic characterisation of the NB14 strains using MALDI-TOF MS (**Paper 1**), we presumed that this identification method could potentially be used for the discrimination of the novel species of the Ab group, *A. dijkshoorniae* and *A. seifertii*. Furthermore, despite the fact that the novel species were always misidentified as *A. pittii* and *A. baumannii* by MALDI-TOF MS, respectively, we noticed that the two best identification matches always corresponded to the same *A. pittii* and *A. baumannii* reference spectra, and that the scores for these matches were always above 2, while the others were much lower (**BOX 4**). These observations suggested that the isolates used to create these reference spectra belonged, indeed, to *A. dijkshoorniae* and *A. seifertii*, and, in addition, showed that, even without refinement, MALDI-TOF MS was able to identify the two novel species. Thus, we validated the use of MALDI-TOF MS as a rapid and accurate tool for the identification of the Ab group species, providing reference spectra for the novel species (**Paper 3**). Moreover, we refined the identification of *A. nosocomialis* by selecting more reference spectra to be included into the database, since the analysis of MALDI-TOF MS peak profiles revealed that there are two groups of *A. nosocomialis* isolates and, unfortunately, the Bruker taxonomy database is biased towards one of them, causing low rates of correct identification for this species.

After the inclusion of the reference spectra for *A. dijkshoorniae*, *A. seifertii* and the underrepresented *A. nosocomialis* group in the Bruker taxonomy database, we have successfully used this customised database to identify isolates of the Ab group, and as seen in **Manuscript 4**, MALDI-TOF MS provides accurate species identification for the species of the Ab group. Besides, in this work, we identified for the first time *A. dijkshoorniae* in Peru and reported the first isolation of this species from market meat samples, providing information about the possible sources of *A. dijkshoorniae*, in addition to those described in **Paper 1**, were all isolates studied were from human origin except for one environmental isolate (**TABLE S1**, **Paper 1**).

Finally, in order to further identify any differential characteristics amongst the species of the Ab group we evaluated other phenotypes beyond those used in the taxonomic studies (**Manuscript 5**). The determination of the antimicrobial susceptibility patterns of the species of the Ab group showed that *A. baumannii* is the most resistant species, despite the fact that we detected several RND efflux pumps in non-*baumannii* species and the intrinsic OXAs in all *A. baumannii*, *A. pittii* and *A. dijkshoorniae* isolates (but, as expected [148], not in *A. nosocomialis* and *A. seifertii*).

We also assessed the motility and biofilm capabilities of the different species of the Ab group (**Manuscript 5**), and found that both phenotypes were highly variable among the isolates of all the species. Nevertheless, we could still detect inter-species differences in biofilm formation: *A. baumannii* was the species that formed less biofilm, while *A. dijkshoorniae*, *A. pittii* and *A. seifertii* were good biofilm producers at 28ºC and 37ºC. Remarkably, *A. nosocomialis* biofilm formation was greater at 28ºC, while at 37ºC the amount of biofilm formation was drastically reduced. Similarly, both *A. baumannii* and *A. seifertii* showed reduced biofilm formation upon temperature increase, while *A. dijkshoorniae* and A. *pittii* produced more biofilm at 37ºC. In addition to these phenotypes, we determined the virulence of the isolates using the *C. elegans* model, which showed that *A. dijkshoorniae* was the most virulent species, that *A. pittii* and *A. seifertii* presented two subpopulations, one virulent and other non-virulent, and that *A. baumannii* and *A. nosocomialis* were non-virulent in this model.

Altogether our data supports the study of the members of the Ab group as distinct entities, and we believe that the use of MALDI-TOF MS for their identification in diagnostic laboratories might help to assess the real prevalence and the putative differential outcomes of the emergent species of the Ab group.

Paper 1

Acinetobacter dijkshoorniae sp. nov., a member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex mainly recovered from clinical samples in different countries

Acinetobacter dijkshoorniae sp. nov., a member of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex mainly recovered from clinical samples in different countries

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Hypothesis: A group of 15 *Acinetobacter* strains, mostly from human origin and isolated in different countries over a period of 15 years, could belong to a novel species within the *Acinetobacter calcoaceticus – Acinetobacter baumannii* (ACB) complex.

Objectives: Determine the taxonomic position of the strains by molecular and phenotypic methods.

Material and methods:
ApaI macrorestriction of genomic DNA followed by pulse field gel electrophoresis was used to determine clonal relatedness. 16S rRNA gene sequences, partial *rpoB* gene sequences and multilocus sequence typing (MLST) were used for comparisons against 94 strains representing all species included in the ACB complex by means of cluster analysis. Obtention of the whole genome sequence of *A. dijkshoorniae* sp. nov. strain JVAP01T by next generation sequencing and calculation of the average nucleotide identity based on BLAST (ANIb) using the JSpecies software for pairwise whole genome comparisons. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and carbon utilisation assays were used to evaluate the phenotypic relationship among the species.

Results:
12 clonally unrelated strains of the putative novel species were finally selected for further characterisation. 16S rRNA gene, *rpoB* and MLST cluster analyses confirmed the genetic distinctness of all selected strains and their grouping as a tight monophyletic group, being *Acinetobacter pittii* the closest species, and little horizontal gene transfer between the ACB complex species. ANIb values below 95% when compared to the other ACB complex species proved the species status of *A. dijkshoorniae* sp. nov. The metabolic characteristics of the *A. dijkshoorniae* sp. nov. strains matched those of the ACB complex and the analysis of their protein signatures by MALDI-TOF MS revealed some species specific peaks.

Conclusions: Our results support the designation of these strains as representing a novel species, for which the name *Acinetobacter dijkshoorniae* sp. nov. is proposed.

Acinetobacter dijkshoorniae sp. nov., a member of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex mainly recovered from clinical samples in different countries

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The recent advances in bacterial species identification methods have led to the rapid taxonomic diversification of the genus *Acinetobacter*. In the present study, phenotypic and molecular methods have been used to determine the taxonomic position of a group of 12 genotypically distinct strains belonging to the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex, initially described by Gerner-Smidt and Tjernberg in 1993, that are closely related to *Acinetobacter pittii*. Strains characterized in this study originated mostly from human samples obtained in different countries over a period of 15 years. *rpoB* gene sequences and multilocus sequence typing were used for comparisons against 94 strains representing all species included in the ACB complex. Cluster analysis based on such sequences showed that all 12 strains grouped together in a distinct clade closest to *Acinetobacter pittii*that was supported by bootstrap values of 99 %. Values of average nucleotide identity based on BLAST between the genome sequence of strain JVAP01^T (NCBI accession no. LJPG00000000) and those of other species from the ACB complex were always <91.2 %, supporting the species status of the group. In addition, the metabolic characteristics of the group matched those of the ACB complex and the analysis of their protein signatures by matrix-assisted laser desorption ionization time-of-

†These authors contributed equally to this work.

Abbreviations: ACB complex, *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex; ANIb, average nucleotide identity based on BLAST; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MLSA, multilocus sequence analysis; MLST, multilocus sequence typing; PFGE, pulse-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences determined in this study are: KU167837, KU167840, KU167842, KU167844, KU167846, KU167848–KU167851, KU167853–KU167858, KU167860–KU167865, KU167867–KU167869, KU167871, KU167873–KU167885, KU167888 and KU726606 for the partial sequence of the *rpoB* genes and KX027435 for the complete sequence of the 16S rRNA gene of *Acinetobacter dijkshoorniae* sp. nov. strain JVAP01^T. The partial sequences of the seven genes used for MLSA are available from the PubMLST website (http://pubmlst.org/abaumannii/) under the sequence type codes listed in Table S1.

Six supplementary tables and two supplementary figures are available with the online Supplementary Material.

flight MS identified some specific peaks. Our results support the designation of these strains as representing a novel species, for which the name *Acinetobacter dijkshoorniae* sp. nov. is proposed. The type strain is JVAP01^T (=CECT 9134^T=LMG 29605^T).

The genus *Acinetobacter* comprises, at the time of writing, 49 validly named species (http://www.bacterio.cict.fr/a/acinetobacter.html), including two recently described in this journal (Li *et al.*, 2015; Poppel *et al.*, 2015). In addition, there are a number of putative novel species without validly published names, several of which were identified as a result of recent advances in DNA-based methods. Since 2014, 17 new *Acinetobacter* species have been validly described, notably *Acinetobacter seifertii*, a novel human pathogen included within the '*Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex' (Du *et al.*, 2016; Feng *et al.*, 2014a, b; Kim *et al.*, 2014b; Krizova *et al.*, 2014, 2015a, b; Li *et al.*, 2014a, b, 2015; Nemec *et al.*, 2015, 2016; Poppel *et al.*, 2015; Smet *et al.*, 2014). The ACB complex includes five different species, the environmental *A. calcoaceticus* and the human pathogens *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *A. seifertii* (Nemec *et al.*, 2015). Members of this complex are difficult to distinguish phenotypically and are also closely related to each other based on DNA–DNA hybridization, to the point that accurate differentiation of the four clinically relevant members of the group can only be accomplished by means of molecular methods (Gundi *et al.*, 2009; Sousa *et al.*, 2014).

The introduction of molecular typing methods in the routine characterization of *Acinetobacter* clinical specimens recently allowed us to identify a group of strains highly related to other members of the ACB complex that nevertheless seemed to constitute a novel *Acinetobacter* species (Espinal *et al.*, 2015). In the present work we have used phenotypic and molecular methods relevant to the genus *Acinetobacter* to assess the taxonomic position of these strains and support the designation of another member of the ACB complex with putative clinical relevance.

The strains characterized in this work were mainly obtained from clinical samples of patients in different geographical locations over a period of 15 years and the results shown below clearly support the taxonomic distinctness of the group at the species level. We propose the name *Acinetobacter dijkshoorniae* sp. nov. to accommodate these strains, which will be used hereafter.

Fifteen *A. dijkshoorniae* sp. nov. isolates were initially included in the study. Their genetic relatedness was evaluated by *Apa*I macrorestriction of genomic DNA and subsequent analysis of the pulse-field gel electrophoresis (PFGE) fingerprints (Seifert *et al.*, 2005). As shown in Fig. 1, 12 genetically distinct fingerprints were identified, with three pairs of isolates presenting identical PFGE patterns. For the purpose of the present study, 12 genetically unique strains were selected and subsequently studied following a polyphasic approach using both genomic and phenotypic techniques.

The partial *rpoB gene* sequences (zones 1 and 2) of all 12 *A. dijkshoorniae* sp. nov. isolates (La Scola *et al.*, 2006) were compared to those of 93 reference strains belonging to other members of the ACB complex, according to Nemec *et al.* (2011). Data on the origin and characteristics of the strains used for sequence analysis are shown in Table S1, available in the online Supplementary Material. Cluster analysis was performed using the MEGA 5.1 software (Tamura *et al.*, 2011). The program MUSCLE was used for sequence alignment (Edgar, 2004) and phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood methods, with genetic distances computed by Kimura's two-parameter model (Kimura, 1980), but also using the maximum-parsimony method, all three with bootstrap values based on 1000 replicates.

Results of the *rpoB*-gene-based sequence analysis confirmed the genetic distinctness of all selected *A. dijkshoorniae* sp. nov. isolates and showed their grouping together in a tight monophyletic group within the ACB complex that was closest to *A. pittii*. Representative strains from all other species of the ACB complex also formed respective clusters that were supported by high bootstrap values, as shown in Fig. 2(a). The intraspecies *rpoB* gene sequence similarity values of *A. dijkshoorniae* sp. nov. strains ranged between 98.61 and 100 %, while the interspecies similarity values between *A. dijkshoorniae* sp. nov. and the other ACB complex strains ranged from 91.29 % (*A. seifertii* ANC 4233) to 98.03 % (*A. pittii* JVAP02) (Table S2). Intraspecies and interspecies *rpoB* gene sequence similarity values for all other taxa were also in good agreement with similarity values previously published (Nemec *et al.*, 2015).

The distinct grouping of all strains in well-defined phylogenetic taxa was further evaluated using multilocus sequence analysis (MLSA) of the concatenated partial sequences of all housekeeping genes used for multilocus sequence typing (MLST) according to the Pasteur scheme (Diancourt *et al.*, 2010).

The partial sequences of the *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB* genes for all strains were either obtained by PCR amplification and subsequent DNA sequencing, as described by Diancourt *et al.* (2010), or retrieved from nucleotide sequence repositories whenever available (Table S1). New allele sequences as well as unique allele combinations were submitted to the *Acinetobacter* MLST database (http://pubmlst.org/abaumannii/) and were used to assign novel sequence types (Table S1).

As shown in Fig. 2(b), MLSA was in very good agreement with results from *rpoB* gene sequence comparisons, with all

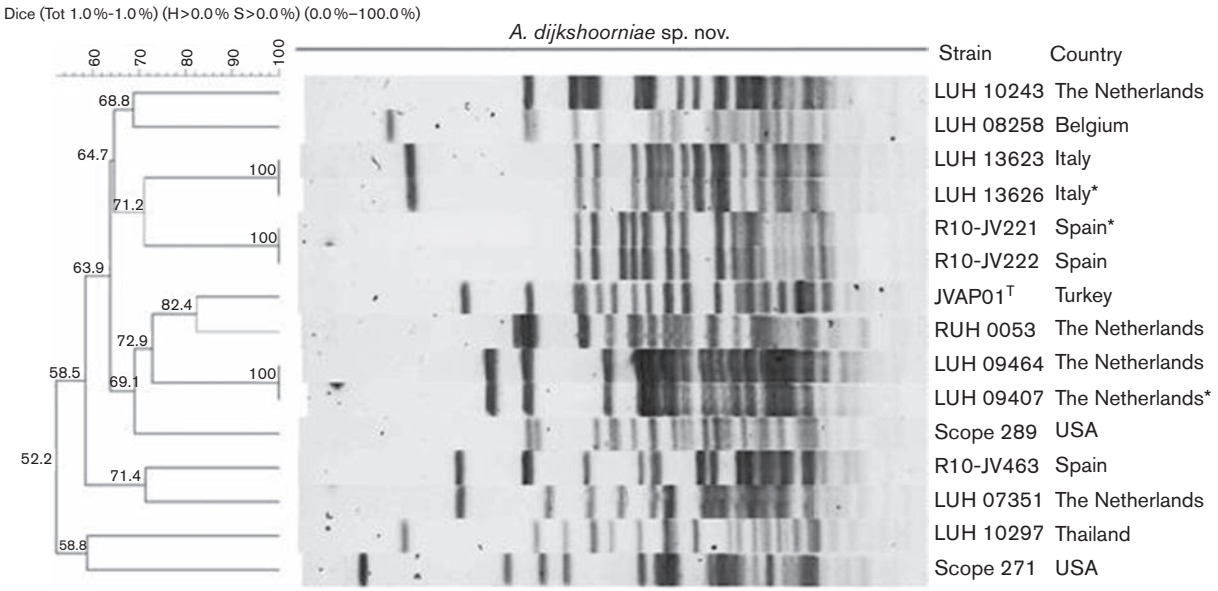

Fig. 1. PFGE profiles of *Apa*I-digested genomic DNA of 15 *A. dijkshoorniae* sp. nov. strains. DNA digestion and fragment separation were performed as previously described (Seifert *et al.*, 2005). PFGE patterns were calculated by the Dice coefficient method (with an optimization of 1 % and a position tolerance of 1 %) using BioNumerics software (version 7.1; Applied Maths). Asterisks indicate strains with an identical PFGE pair that have been excluded from the study. Identical PFGE pairs also shared sequence type profiles and *rpoB* gene sequences. The x-axis shows percent similarity distance and numbers above branches indicate distance values (%).

A. dijkshoorniae sp. nov. strains forming again a separate monophyletic group that was closer to *A. pittii*. MLSA intraspecies similarity values for *A. dijkshoorniae* sp. nov. were 98.82–100 % and the MLSA interspecies similarity values between *A. dijkshoorniae* sp. nov. and all other ACB complex species ranged from 91.4 % (*A. baumannii* NIPH 1734) to 98.89 % (*A. pittii* JV-2628), suggesting a high degree of genetic relatedness between *A. dijkshoorniae* sp. nov. and *A. pittii* (Table S3).

Cluster analysis based on the independent partial sequences of each of the seven MLST genes showed little horizontal transfer between the other species of the ACB complex and strains of *A. dijkshoorniae* sp. nov., with only the *fusA* allele of strain LUH 08258, the *recA* allele of strain RUH 14531 and the *rpoB* allele of strain LUH 10297 clustering together with the respective sequences of *A. pittii* (Fig. S1). Limited horizontal transfer between closely related species of the ACB complex has been previously reported (Nemec *et al.*, 2015), thus reinforcing the use of MLSA instead of that of individual genes for species differentiation.

The whole genome sequence of the *A. dijkshoorniae* sp. nov. strain JVAP01^T was obtained using next generation sequencing. Briefly, DNA was extracted and DNA libraries were generated with the Illumina Nextera XT sample preparation kit (Illumina) following the manufacturer's recommendations. DNA libraries were then sequenced using an Illumina MiSeq system (Illumina) and 150 bp paired-end reads were generated. The assembled genome was used to calculate the average nucleotide identity based on BLAST (ANIb) of strain JVAP01^T against the genome sequences of 33 strains representing all *Acinetobacter* species of the ACB complex, including that of the *A. dijkshoorniae* sp. nov. strain SCOPE 172 which was previously available at NCBI (NCBI accession no. APQO00000000). ANIb was calculated with the JSpecies software (http://imedea.uib-csic.es/jspecies) with default settings (Richter & Rosselló-Móra, 2009). The NCBI accession numbers of all genomes used in the study are listed in Table S1.

Results from the ANIb analyses (Table S4) proved the species status of *A. dijkshoorniae* sp. nov. since identity values between the JVAP01^T genome and other ACB complex species genomes were below the proposed species demarcation level of 95 % (Kim *et al.*, 2014a), ranging from 86.74 % (*A. nosocomialis* ATCC 17903) to 93.13 % (*A. pittii* RUH 2206^T). Interestingly, the ANIb value of the JVAP01^T genome against that of strain SCOPE 172, which clustered together with *A. dijkshoorniae* sp. nov. strains in both the *rpoB* gene- and MLSA-based sequence analysis, was 96.64 %, further supporting the distinctness of this novel species from other members of the ACB complex (Table S5).

The genome sequence of *A. dijkshoorniae* sp. nov. JVAP01^T contains three complete and identical sequences of the 16S rRNA gene (1526 bp) and two partial sequences, and that of strain SCOPE 271 contains six complete and identical 16S rRNA gene sequences. The nearly complete 16S rRNA gene sequences (positions 48–1450) from all other *A. dijkshoorniae* sp. nov. strains included in the study were obtained by Sanger

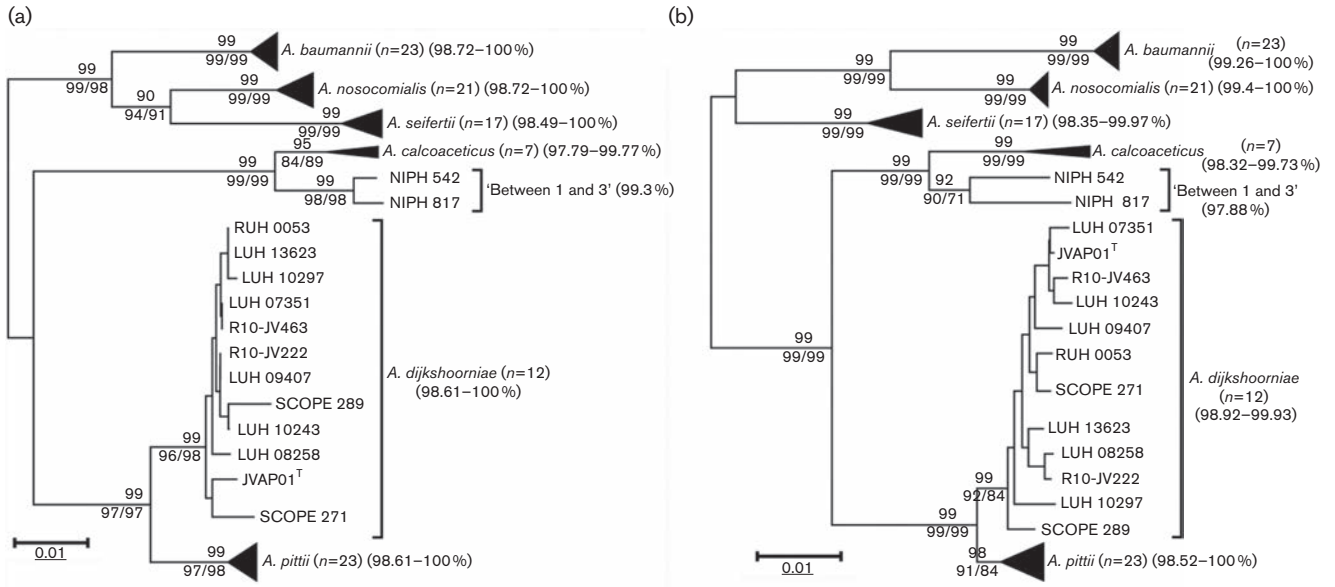

Fig. 2. Cluster analysis of all 105 ACB complex strains included in the study based on (a) the partial sequences of the *rpoB* gene (861 bp) and (b) the concatenated partial sequences of the *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB* genes used for multilocus sequence typing under the Pasteur scheme (http://pubmlst.org/abaumannii/). Unrooted phylogenetic trees were reconstructed using the neighbour-joining method with genetic distances computed by Kimura's two-parameter model (Kimura, 1980) with bootstrap values based on 1000 replicates. Boostrap values (%) are indicated above branches. Bootstrap values obtained by maximum-likelihood/maximum-parsimony analyses (1000 replicates) are given below branches, respectively. The range of intraspecies similarity values for each species is shown in parentheses. Bar, 0.01 sequence divergence. The GenBank/EMBL/DDBJ accession numbers for all *rpoB* gene sequences used in the study are listed in Table S1. The partial sequences of the individual genes used for multilocus sequence analysis can be retrieved from the PubMLST website (http://pubmlst.org/abaumannii/) under the sequence type codes listed in Table S1.

sequencing. The intraspecies 16S rRNA gene sequence similarity values of *A. dijkshoorniae* sp. nov. strains ranged between 99.36 and 100 %, while the interspecies similarity values between *A. dijkshoorniae* sp. nov. and the other ACB complex strains ranged from 96.15 % (*A. baumannii*) to 99.57 % (*Acinetobacter* sp. 'Between 1 and 3'). Similarity values to *A. calcoaceticus* ranged between 98.57 and 99.22 % and to *A. pittii* between 98.29 and 99.14 %. Cluster analysis also grouped *A. dijkshoorniae* sp. nov. strains closer to *Acinetobacter* sp. 'Between 1 and 3' than to *A. pittii*, although all *A. dijkshoorniae* sp. nov. strains formed a single monophyletic group clearly separated from the other species (Fig. S2).

Likewise, the four different 16S rRNA gene sequences from *A. seifertii* NIPH 973^T could not be clearly allocated as a unique taxon. This result supports previous studies showing that differentiation of species within the ACB complex based on 16S rRNA gene sequence analysis is of little value, the *rpoB* gene being much more reliable for differentiation of this group of highly related species (Gundi *et al.*, 2009; Nemec *et al.*, 2015).

To further evaluate the phenotypic relationships among strains clustering together as *A. dijkshoorniae* sp. nov., bacterial extracts were obtained using the ethanol/formic acid extraction method as previously described (Espinal *et al.*, 2012) and analysed by matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS). Spectra profiles were acquired with a Microflex LT (Bruker Daltonik) benchtop instrument in linear positive mode at a laser frequency of 20 Hz. The resulting raw spectra were loaded into the ClinProTools software (version 2.2; Bruker Daltonics), grouped according to each bacterial species within the ACB complex, and used to calculate the average spectra for each species. Mass to charge values (*m*/*z*) from the average spectra of each species were extracted and we identified three peaks at 4429, 5788 and 8856 *m*/*z* values that were present in all *A. dijkshoorniae* sp. nov. strains but absent in all the spectra from other ACB species (Fig. 3). The presence/absence of such *A. dijkshoorniae* sp. nov. specific peaks could potentially be used for MALDI-TOF MS direct discrimination of strains of *A. dijkshoorniae* sp. nov.

Carbon utilization assays were performed to assess the phenotypic traits of the different species as previously described (Bouvet & Grimont, 1987; Krizova *et al.*, 2014; Nemec *et al.*, 2010, 2011) with minor modifications. Briefly, inocula were prepared by growing strains on tryptic soy agar (TSA; Oxoid) at 30 °C for 24 h and subcultured once under the same conditions before being used. Cells were removed with a cotton swab and suspended in a carbonless mineral broth medium until a transmittance of 90 % (turbidimeter;

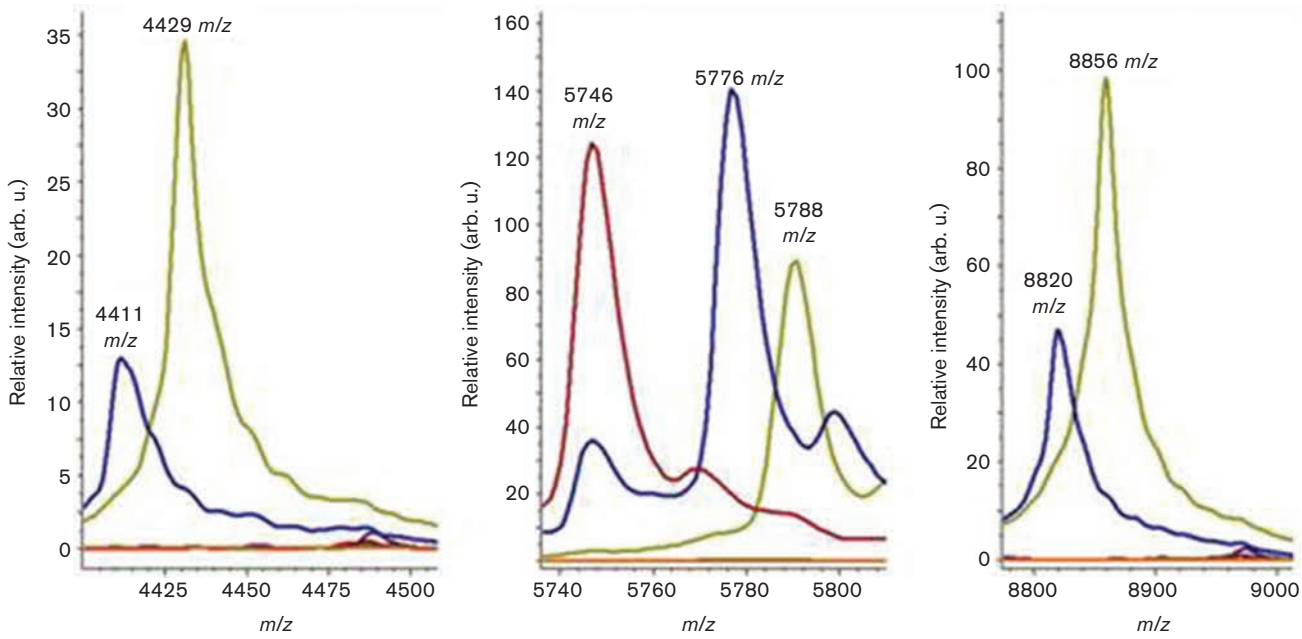

Fig. 3. MALDI-TOF MS averaged spectra plots from strains of the ACB complex showing specific peaks for *A. dijkshoorniae* sp. nov., *A. baumannii* (red), *A. nosocomialis* (green), *A. pittii* (blue), *A. dijkshoorniae* (yellow) and *A. seifertii* (purple). The background noise signal is shown in orange. The *x*-axis shows the mass per charge ratio values (*m/z*) and *y*-axis indicates the intensities of peaks expressed in arbitrary intensity units.

Biolog) was reached. Microtitre plates containing 180 µl mineral broth medium supplemented with 0.11 % (w/v) carbon sources were inoculated with 20 µl of cell suspension. Plates were covered with breathe-easy membranes (Diversified Biotech) to prevent evaporation. Plates were read by visual inspection after 2, 6 and 10 days of incubation at 30 °C. *Acinetobacter guillouiae* LUH 13183 was used as a positive control for histamine and tryptamine utilization as a carbon source and *Acinetobacter baylyi* LUH 09341 for D-glucose and D-gluconate (Nemec *et al.*, 2009).

Overall, the metabolic traits of the *A. dijkshoorniae* sp. nov. strains were in good agreement with those shown by Nemec *et al.* (2015) using the extended Bouvet and Grimont system for members of the ACB complex. Our data confirm that a clear phenotypic differentiation between all members of the ACB complex based on this panel of carbon utilization assays is not possible (Table 1). Nevertheless, some *A. dijkshoorniae* sp. nov. strains could be differentiated by their ability to use tryptamine as a sole carbon source. Table S6 provides a comprehensive comparison between the phenotypic traits described by (Nemec *et al.*, 2015) and the results obtained in the present study.

Description of *Acinetobacter dijkshoorniae* sp. nov.

Acinetobacter dijkshoorniae (dijks.hoorn′i.ae. N.L. gen. fem. n. *dijkshoorniae* of Dijkshoorn, named after Lenie Dijkshoorn, a Dutch microbiologist, for her life-long dedication to the taxonomy of the genus *Acinetobacter*).

Phenotypic characteristics correspond to those of the genus, i.e. Gram-stain-negative, strictly aerobic, oxidase-negative, catalase-positive coccobacilli, incapable of swimming motility, capable of growing in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen, and incapable of dissimilative denitrification. Colonies on TSA after 24 h of incubation at 30 °C are 1.0–2.0 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs in brain heart infusion broth (BD Difco) at temperatures ranging from 25 to 44 °C in 1 day. All strains produce acid from D-glucose and fail to hydrolyse gelatin. Haemolysis of erythrocytes on sheep blood agar medium is not observed. All strains are able to use acetate, *trans*-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, azelate, benzoate, 2,3-butanediol, citrate, gentisate, L-glutamate, glutarate, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, D-malate, malonate, L-phenylalanine, putrescine, tricarballylate and L-tyrosine as a sole carbon source. None of the tested strains are able to utilize citraconate, D-gluconate, D-glucose or histamine for growth. Some strains are able to grow on ethanol, levulinate, L-ornithine, phenylacetate, D-ribose, L-tartrate, trigonelline and triptamine.

The type strain is JVAP01^T (=CECT 9134^T=LMG 29605^T), isolated from a urine sample of a human patient in Turkey in August 2009 (Espinal *et al.*, 2015). In addition to the

Table 1. Metabolic characteristics of *A. dijkshoorniae* sp. nov. and other species of the ACB complex

Species: 1, *A. dijkshoorniae* (*n*=15); 2, *A. baumannii* (*n*=5); 3, *A. calcoaceticus* (*n*=5); 4, *A. nosocomialis* (*n*=4); 5, *A. pittii* (*n*=5); 6, *A. seifertii* (*n*=5). Carbon utilization tests were evaluated after 2, 6 and 10 days of incubation. +, All strains were positive; −, all strains were negative; D, mostly doubtful carbon utilization; W, mostly weak positive carbon utilization. Numbers indicate the percentage of strains with a positive reaction. All strains were able to use acetate, *trans*-aconitate, 4-aminobutyrate, L-arginine, L-aspartate, citrate, L-glutamate, glutarate, L-histidine, 4-hydroxybenzoate and DL-lactate as a sole carbon source. None of the tested strains were able to utilize D-gluconate, D-glucose or histamine for growth.

	1	2	3	4	5	6
Utilization of:						
Adipate	+	+	+	+	+	60
β-Alanine	+	+	80	+	+	80
L-Arabinose	+	+	40	+	+	−
Azelate	+	+	+	+	+	60
Benzoate	+	+	+	75	+	80
2,3-Butanediol	+	+	+	75	+	+
Citraconate	−	60	−	−	−	−
Ethanol	43 (D,W)	+	80	+	+	+
Gentisate	+	40	−	−	40	80
L-Leucine	+	+	80	+	+	80
Levulinate	80	40	80	−	−	20
D-Malate	+	+	60	+	+	+
Malonate	+	80	+	25	+	80
L-Ornithine	93	60	80	75	+	80
Phenylacetate	63 (W)	+	+	+	80	+
L-Phenylalanine	+	+	+	+	80	+
Putrescine	+	80	+	75	+	80
D-Ribose	93	+	20	+	80	20
L-Tartrate	67	40	−	−	80	+
Tricarballylate	+	+	+	75	+	+
Trigonelline	80	80	−	25	60	20
Tryptamine	53	−	−	−	−	−
L-Tyrosine	+	+	+	75	+	80

above-mentioned carbon sources, the type strain is also able to utilize L-ornithine, phenylacetate, D-ribose and trigonelline as a carbon source. Growth on tryptamine is also observed although weakly. No growth is observed with citraconate, ethanol, D-gluconate, D-glucose, levulinate or L-tartrate. MALDI-TOF MS analysis of JVAP01^T showed the presence of the *A. dijkshoorniae* sp. nov. specific peaks centred at 4429, 5788 and 8856 *m*/*z* values. The whole genome sequence of the type strain is available from NCBI under accession number LJPG00000000 (size 3.85 Mb, number of contigs 92, DNA G+C content 38.8 mol%). The GenBank/EMBL/DDBJ accession numbers for the partial sequence of the *rpoB* gene and the complete 16S rRNA gene sequence of strain JVAP01^T are KJ600793 and KX027435, respectively.

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Acinetobacter dijkshoorniae sp. nov., a new member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex mainly recovered from clinical samples in different countries

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Table S1

Source and characteristics of *Acinetobacter* sp. strains used for sequence analysis.

Clinical specimens were of human and environmental origin. Underlined *rpoB* accession numbers and STs indicate sequences obtained in the present study. The concatenated sequences of the seven genes used in MLSA are available under the Pasteur scheme ST codes at http://pubmlst.org/abaumannii/. Collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection, University of Göteborg, Sweden; CECT, Spanish Type Culture Collection, Universidad de Valencia, Valencia Spain; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris; CUHK, The Chinese University of Hong Kong; LMD, Laboratory Microbiology Delft collection, now hosted by Centraal Bureau Schimmelcultures, CBS, Utrecht, The Netherlands; LMG, Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium. ANC and NIPH are strain designations used by A. Nemec; LUH and RUH are strain designations used by L. Dijkshoorn; R10, R00 and JV are strain designations used by J. Vila; SCOPE, SH and HS are strain designations used by H. Seifert; TH are strain designations used by G. Huys.

Strain	Country and year or period of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
***Acinetobacter dijkshoorniae* sp. nov. (n=15)**						
JVAP01^T (CECT 9134^T // LMG 29605^T)	Turkey, 2009	Urine	ST606	KJ600793	LJPG00000000	(Espinal *et al.*, 2015)
LUH 07351	The Netherlands, 2001	Nephrology drain	ST565	KJ600787		(Espinal *et al.*, 2015)
LUH 08258 (ACI 749*)	Belgium, 2002	Catheter	ST566	KU167880		L. Dijkshoorn <<M. Vaneechoutte
LUH 09407	The Netherlands, 2004	Wound	ST801	KJ600791		(Espinal *et al.*, 2015)
LUH 09464[a]	The Netherlands, 2004	Wound	ST801	KU167881		L. Dijkshoorn
LUH 10243	The Netherlands, 2005	Sputum	ST802	KJ600790		L. Dijkshoorn (Espinal *et al.*, 2015)
LUH 10297 (TH344)	Thailand, 2005	Freshwater fishpond farm	ST798	KU167882		This study
LUH 13623 (AN-26*)	Italy, 2004	Sputum	ST800	KU167883		L. Dijkshoorn<<APSI-group (Carretto *et al.*, 2011)
LUH 13626[a] (MOD-3*)	Italy, 2004	Wound	ST800	KJ600788		L. Dijkshoorn<<APSI-group (Carretto *et al.*, 2011)
SCOPE 271 (ANC 4052 // LUH 14530)	USA, 1995	Blood	ST568	KU167884	APQO00000000	(Wisplinghoff *et al.*, 2004)
R10-JV221[a]	Spain, 2010	Human sample[b]	ST796	KU167877		This study
R10-JV222	Spain, 2010	Human sample[b]	ST796	KU167878		This study
R10-JV463	Spain, 2010	Human sample[b]	ST797	KU167879		This study
RUH 0053 (LMD 71.43)	The Netherlands,	Not Known	ST564	KJ600789		(Espinal *et al.*, 2015)

Strain	Country and year or period of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
SCOPE 289 (RUH 14531)	USA, 1995	Blood	ST799	KU167885		(Wisplinghoff *et al.*, 2004)
***Acinetobacter baumannii* (n=23)**						
ATCC 19606^{T} (LMG 1041^{T} // NIPH 501^{T} // CIP 70.34^{T})	USA, 1948	Urine	ST52	EU477108	APRG00000000	(Roca *et al.*, 2009)
ATCC 17978 (CIP 53.77//LMG 1025)	USA, 1967	Cerebrospinal fluid	ST437	CP000521	CP000521	(Roca *et al.*, 2014)
JV-Ab30	Spain, 2000	Human sample[b]	ST273	KU167844		This study
LUH 05875 (LMG 25784 // NIPH 1669)	The Netherlands, 1997	Blood	ST3	HQ123411	APOQ00000000	(Nemec *et al.*, 2011)
LUH 4631 (CIP 110424 // NIPH 60)	Czech Republic, 1992	Sputum	ST34	HQ123412	APPM00000000	(Nemec *et al.*, 2011)
LUH 4633 (CIP 110429 // NIPH 190)	Czech Republic, 1993	Tracheal secretion	ST9	HQ123414	APPL00000000	(Nemec *et al.*, 2011)
LUH 4708 (CIP 110426 //NIPH 70)	Czech Republic, 1992	Tracheal secretion	ST36	HQ123413	APRC00000000	(Nemec *et al.*, 2011)
LUH 4722 (CIP 110434 // NIPH 410)	Czech Republic, 1996	Cannula	ST39	HQ123415	ATGJ00000000	(Nemec *et al.*, 2011)
NIPH 1362 (CIP 110463)	Czech Republic, 2000	Tracheal aspirate	ST47	KJ956459	APOR00000000	(Nemec *et al.*, 2011)
NIPH 146 (CIP 110428)	Czech Republic, 1993	Wound	ST25	KJ956449	APOU00000000	(Nemec *et al.*, 2011)
NIPH 1734 (CIP 110466)	Czech Republic, 2001	Sputum	ST15	KJ956460	APOX00000000	(Nemec *et al.*, 2011)
NIPH 201 (CIP 110430)	Czech Republic, 1992	Nasal swab	ST38	KJ956450	APQV00000000	(Nemec *et al.*, 2011)
NIPH 329 (CIP 110432)	Czech Republic, 1994	Tracheal secretion	ST11	KJ956451	APQY00000000	(Nemec *et al.*, 2011)
NIPH 335 (CIP 110433)	Czech Republic, 1994	Sputum	ST10	KJ956452	APQX00000000	(Nemec *et al.*, 2011)
NIPH 601 (CIP 110437)	Czech Republic, 1993	Urine	ST40	KJ956454	APQZ00000000	(Nemec *et al.*, 2011)
NIPH 615 (CIP 110438)	Czech Republic, 1994	Tracheal secretion	ST12	KJ956455	APOV00000000	(Nemec *et al.*, 2011)
NIPH 67 (CIP 110425)	Czech Republic, 1992	Tracheal secretion	ST35	KJ956447	APRA00000000	(Nemec *et al.*, 2011)
NIPH 80 (CIP 110427)	Czech Republic, 1993	Intravenous cannula	ST37	KJ956448	APRE00000000	(Nemec *et al.*, 2011)
R10-JV226	Spain, 2010	Human sample	ST181	KU167837		This study
R10-JV295	Spain, 2010	Human sample	ST2	KU167840		This study
R10-JV80	Spain, 2010	Human sample	ST632	KU167842		This study
RUH 134 (CCM 7290 // LMG 10541 //NIPH 528)	The Netherlands, 1982	Urine	ST2	HQ123410	APRB00000000	L. Dijkshoorn (Nemec *et al.*, 2011)
RUH 875 (CCM 7289 // LMG 10543 // NIPH 527)	The Netherlands, 1984	Urine	ST1	EU477108	APQW00000000	(Nemec *et al.*, 2011)

Strain	Country and year or period of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
***Acinetobacter* gen. sp. 'Between 1 and 3' (n=2)**						
NIPH 542 (CIP 110441 // CCUG 34788)	Denmark, 1990	Sputum	ST538	NZ_KB850209	APSC00000000	(Nemec *et al.*, 2015)
NIPH 817 (CIP 110472 // CCUG 34786)	Denmark, 1990	Abscess	ST88	NZ_KB849432	APPF00000000	(Nemec *et al.*, 2015)
***Acinetobacter calcoaceticus* (n=7)**						
NIPH 2245^T (ATCC 23055^T // CIP 81.8^T // LMG 1046^T)	The Netherlands, 1900-1910	Soil	ST62	EU477149	APQI00000000	(Nemec *et al.*, 2011)
ANC 3680 (CIP 110488)	Czech Republic, 2008	Soil	ST539	HQ123424	APQH00000000	(Nemec *et al.*, 2011)
ANC 4104	Czech Republic, 2011	Well water	ST541	KJ956437		(Nemec *et al.*, 2011)
NIPH 13 (CIP 110439 // CCM4665)	Czech Republic, 1991	Burn	ST484	HQ123422	APOE00000000	(Nemec *et al.*, 2011)
NIPH 2254 (LMG 10517 // MGH 42*)	Sweden, 1980	Wound	ST92	HQ123418	ACPK00000000	L. Dijkshoorn<<I. Tjernberg (Nemec *et al.*, 2011)
NIPH 2706 (LUH 12679)	Czech Republic, 2006	Sputum	ST540	HQ123423		(Nemec *et al.*, 2011)
NIPH 2814 (LUH 9144)	The Netherlands, 2004	Urinary catheter	ST61	HQ123421		L. Dijkshoorn (Nemec *et al.*, 2011)
***Acinetobacter nosocomialis* (n=21)**						
RUH 2376^T (CCUG 61663^T // LMG 10619^T // NIPH 2119^T)	The Netherlands, 1987	Sputum	ST76	HQ123395	APOP00000000	L. Dijkshoorn (Nemec *et al.*, 2011)
ATCC17903 (LMG 993 // NIPH 523)	Before 1950	Not known	ST74	EU477118	AIEJ00000000	(Nemec *et al.*, 2011)
LUH 14363 (NIPH 97)	Czech Republic, 1993	Bronchial secretion	ST543	HQ123404		(Nemec *et al.*, 2011)
LUH 14367 (CIP 110440 // NIPH 386)	Czech Republic, 1996	Sputum	ST410	HQ123406	APPP00000000	(Nemec *et al.*, 2011)
RUH 2041 (LMG 10621)	The Netherlands, 1990	Autopsy	ST410	HQ123392		L. Dijkshoorn
RUH 2211 (LMG 10625 // MGH 100*)	Sweden, 1990	Gastric fistula	ST359	HQ123393		L. Dijkshoorn<<I. Tjernberg
RUH 2624 (NIPH 2120)	The Netherlands, 1990	Skin	410	KJ956466	ACQF00000000	L. Dijkshoorn (Nemec *et al.*, 2011)
RUH 2627 (LMG 10623)	The Netherlands, 1990	Rectum	ST410	HQ123396		L. Dijkshoorn (Nemec *et al.*, 2011)
RUH 412 (LMG 10624)	The Netherlands, 1990	Blood	ST787	HQ123390		L. Dijkshoorn (Nemec *et al.*, 2011)
RUH 503	The Netherlands, 1990	Urine	ST68	HQ123391		L. Dijkshoorn (Nemec *et al.*, 2011)
SCOPE 150	USA, 1995	Blood	ST501	KU167846		(Wisplinghoff *et al.*, 2004)
SCOPE 192	USA, 1995	Blood	ST71	KU167848		(Wisplinghoff *et al.*, 2004)
SCOPE 212	USA, 1995	Blood	ST786	KU167849		(Wisplinghoff *et al.*, 2004)

Strain	Country and year or period of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
SCOPE 249	USA, 1995	Blood	ST279	KU167850		(Wisplinghoff *et al.*, 2004)
SCOPE 54	Akron, OH, USA, 1995	Blood	ST784	KU167851		(Wisplinghoff *et al.*, 2004)
SCOPE 73	USA, 1995	Blood	ST530	KU167853		(Wisplinghoff *et al.*, 2004)
SCOPE 76	USA, 1995	Blood	ST785	KU167854		(Wisplinghoff *et al.*, 2004)
SCOPE 79	Richmond, VA, USA, 1995	Blood	ST781	KU167855		(Wisplinghoff *et al.*, 2004)
SCOPE 80	USA, 1995-1996	Blood	ST781	KU167856		(Wisplinghoff *et al.*, 2004)
SCOPE 81	Richmond, VA, USA, 1995	Blood	ST782	KU167857		(Wisplinghoff *et al.*, 2004)
SCOPE 95	USA, 1995	Blood	ST395	KU167858		(Wisplinghoff *et al.*, 2004)
Acinetobacter pittii **(n=23)**						
RUH 2206^{T} (ATCC19004^{T} // CIP 70.29^{T} // LMG 1035^{T} // NIPH 519^{T})	Before 1967	Cerebrospinal fluid	ST63	EU477114	APQP00000000	(Nemec *et al.*, 2011)
ANC 3678 (CIP 110468)	Czech Republic, 2008	Water pond	ST457	HQ123388	APQN00000000	(Nemec *et al.*, 2011)
ANC 3870 (CCUG 57818 // SH024)	Germany, 1993	Axilla	ST93	KJ956425	ADCH00000000	(Nemec *et al.*, 2011)
JV-2628	Colombia, 2010	Catheter	ST209	KU167874		(Montealegre *et al.*, 2012)
JV-Ab41	Spain, 2000	Blood	ST788	KU167873		This study
JV-IRS_Fr	France, 2010	Blood	ST64	KU167875		This study
JVAP02	Turkey, 2006	Blood	ST457	KU167876		(Roca *et al.*, 2014)
LUH 14362 (NIPH 95)	Czech Republic, 1993	Urine	ST205	HQ123386		(Nemec *et al.*, 2011)
LUH 14366 (NIPH 336)	Czech Republic, 1993	Urine	ST542	HQ123387		(Nemec *et al.*, 2011)
R00-JV14	Spain, 2000	Human sample[b]	ST789	KU167861		This study
R00-JV195	Spain, 2000	Human sample[b]	ST64	KU167871		This study
R00-JV20	Spain, 2000	Human sample[b]	ST805	KU167862		This study
R00-JV21	Spain, 2000	Human sample[b]	ST667	KU167863		This study
R00-JV60	Spain, 2000	Human sample[b]	ST783	KU167867		This study
R00-JV65	Spain, 2000	Human sample[b]	ST790	KU167868		This study
R00-JV90	Spain, 2000	Human sample[b]	ST791	KU167869		This study
R10-JV2	Spain, 2010	Human sample[b]	ST794	KU167860		This study
R10-JV23	Spain, 2010	Human sample[b]	ST214	KU167864		This study
R10-JV28	Spain, 2010	Human sample[b]	ST795	KU167865		This study
RUH 1163 (LMG 10555 //NIPH 2256)	The Netherlands, 1985	Toe web	ST75	HQ123378		(Nemec *et al.*, 2011)
RUH 1944 (NIPH 2805)	The Netherlands, 1986	Urine	ST70	HQ123381		(Nemec *et al.*, 2011)
RUH 2204 (LMG 10553 // MGH 102* // NIPH 2258)	Sweden, 1980	Wound	ST73	HQ123379		L. Dijkshoorn<<I. Tjernberg (Nemec *et al.*, 2011)
RUH 509 (LMG 10559)	The Netherlands, 1984	Bronchus	ST72	HQ123375		L. Dijkshoorn
Acinetobacter seifertii **(n=17)**						

Strain	Country and year or period of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
NIPH 973^{T} (CIP 110471^{T} // CCM 8535^{T} // CCUG 34785^{T} // SSI 1090^{T}*)	Denmark, 1990	Ulcer	ST91	EU477126	APOO00000000	L. Dijkshoorn<<P. Gerner-Smidt (Nemec *et al.*, 2015)
SCOPE 006 (ANC 4045)	USA, 1995	Blood	ST483	KJ956427		(Nemec *et al.*, 2015)
SCOPE 012 (ANC 4046)	USA, 1995	Blood	ST546	KJ956428		(Nemec *et al.*, 2015)
SCOPE 035 (ANC 4049)	USA, 1995	Blood	ST548	KJ956430		(Nemec *et al.*, 2015)
ANC 4225	Norway, 2007	Blood	ST553	KJ956440		(Nemec *et al.*, 2015)
ANC 4232	Norway, 2006	Blood	ST554	KJ956442		(Nemec *et al.*, 2015)
ANC 4233	Norway, 2007	Blood	ST555	KJ956443		(Nemec *et al.*, 2015)
LUH 01471 (CCUG 34787 //NIPH 826 // SSI 5804*)	Sweden, 1990	Blood	ST90	HQ123426		L. Dijkshoorn<<P. Gerner-Smidt (Nemec *et al.*, 2015)
LUH 05789 (CUHK 3088 // NIPH 1777)	Hong Kong, 1997	Blood	ST549	KJ956461		L. Dijkshoorn<<E. Houang (Nemec *et al.*, 2015)
LUH 05793 (CUHK 7611 // NIPH 1781)	Hong Kong, 1998	Soil	ST551	KJ956464		L. Dijkshoorn<<E. Houang (Nemec *et al.*, 2015)
LUH 08128	The Netherlands, 2002	Catheter	ST803	KU167888		L. Dijkshoorn (Nemec *et al.*, 2015)
NIPH 1780 (CUHK 7009)	Hong Kong, 1997	Hospital environment	ST550	KJ956463		
NIPH 1782 (LUH 858)	The Netherlands, 1993	Blood	ST552	KJ956465		(Nemec *et al.*, 2015)
NIPH 417	Czech Republic, 1996	Tracheal secretion	ST148	KJ956453		(Nemec *et al.*, 2011)
NIPH 744 (HS A23-2)	Germany, 1993	Lake Water	ST544	KJ956456		(Nemec *et al.*, 2015)
R00-JV54	Spain, 2000	Human sample[b]	ST834	KU726606		This study
RUH 1139 (NIPH 806)	The Netherlands, 1985	Throat swab	ST545	KJ956457		L. Dijkshoorn (Nemec *et al.*, 2015)

[a] Strains excluded from the sequence-based analysis

[b] Strains recovered from human patients but the specimen is unknown

*Strain designation used by the donor

Table S2

Percentage of identity between the partial *rpoB* sequences (zones 1+2) of the 105 ACB complex strains included in the study.
Interval values equal or higher than 97% are shown in bold.

Species	*A. dijkshoorniae*	*A. baumannii*	'Between 1 and 3'	*A. calcoaceticus*	*A. nosocomialis*	*A. pittii*	*A. seifertii*
A. dijkshoorniae (12)	**98.61-100**						
A. baumannii (23)	92.8-94.31	**98.72-100**					
'Between 1 and 3' (2)	92.45-93.26	91.06-91.99	**99.3-100**				
A. calcoaceticus (7)	91.99-93.38	90.94-92.22	**96.4-97.56**	**97.79-99.77**			
A. nosocomialis (21)	92.22-93.15	94.31-95.59	91.17-91.75	90.36-91.52	**98.72-100**		
A. pittii (23)	**96.4-98.03**	92.45-93.35	92.22-92.92	91.75-93.26	92.57-93.5	**98.61-100**	
A. seifertii (17)	91.29-92.45	93.96-95.12	90.48-91.41	90.13-91.52	94.66-96.05	91.06-92.68	**98.49-100**

Numbers in parentheses indicate the number of strains included

Table S3

Percentage of identity between the concatenated MLST sequences of the 105 ACB complex strains included in the study.
Interval values equal or higher than 97% are shown in bold.

Species	*A. dijkshoorniae*	*A. baumannii*	'Between 1 and 3'	*A. calcoaceticus*	*A. nosocomialis*	*A. pittii*	*A. seifertii*
A. dijkshoorniae (12)	**98.82-99.83**						
A. baumannii (23)	91.4-92.1	**99.26-100**					
'Between 1 and 3' (2)	94.39-95.56	91.57-92.2	**97.88**				
A. calcoaceticus (7)	94.19-95.36	90.52-91.6	96.17-96.98	**98.32-99.73**			
A. nosocomialis (21)	92.1-92.51	95.3-95.77	92.24-92.61	91.4-92.2	**99.4-100**		
A. pittii (23)	**97.68-98.89**	91.23-92.04	94.32-95.46	93.88-95.13	92.07-92.67	**98.52-100**	
A. seifertii (17)	93.21-94.15	92.98-93.78	93.11-94.09	92.04-93.28	94.02-94.72	93.11-94.22	**98.35-99.97**

Numbers in parentheses indicate the number of strains included

Table S4

ANIb values between the genome sequence of the *A. dijkshoorniae* sp. nov. JVAP01^{T} strain and the genome sequences of representative strains of the ACB complex. Interval values equal or higher than 95% are shown in bold.

Species	*A. dijkshoorniae* JVAP01^{T}	*A. baumannii*	'Between 1 and 3'	*A. calcoaceticus*	*A. nosocomialis*	*A. pittii*	*A. seifertii*
A. dijkshoorniae SCOPE 271	**96.64**						
A. baumannii (18)	87.20-87.35	**97.25-99.73**					
'Between 1 and 3' (2)	90.74-90.87	86.71-87.16	94.87				
A. calcoaceticus (4)	89.65-89.67	86.07-86.39	90.93-91.62	**96.14-97.06**			
A. nosocomialis (4)	86.74-86.83	91.15-91.6	86.62-86.87	85.95-86.12	**97.23-98.78**		
A. pittii (3)	93.12-93.13	87.34-87.95	90.77-90.88	89.34-89.64	86.99-87.43	**96.71-97.52**	
A. seifertii NIPH 973^{T}	87.05	89.57-90.18	87.47-87.55	86.23-86.49	91.49-91.69	87.54-87.78	-

Numbers in parentheses indicate the number of strains included

Table S5

Average nucleotide identity based on BLAST (ANIb) values between the genome sequence of *A. dijkshoorniae* sp. nov. JVAP01^{T} and those of other species of the ACB complex.

ANIb values (means of reciprocal values) were calculated using the JSpecies web program with default settings (http://www.imedea.uib.es/jspecies). Values equal or higher than 95% are shown in bold.

Strain	**Genome NCBI Accession Nº**	**JVAP01 LJPG00000000**
A. dijkshoorniae SCOPE 271	APQO00000000	**96.64**
A. baumannii ATCC 19606^{T}	APRG00000000	87.3
A. baumannii LUH-05875	APOQ00000000	87.32
A. baumannii LUH-4631	APPM00000000	87.32
A. baumannii LUH-4633	APPL00000000	87.31
A. baumannii LUH-4708	APRC00000000	87.28
A. baumannii LUH-4722	ATGJ00000000	87.30
A. baumannii NIPH 1362	APOR00000000	87.24
A. baumannii NIPH 146	APOU00000000	87.28
A. baumannii NIPH 1734	APOX00000000	87.25
A. baumannii NIPH 201	APQV00000000	87.22
A. baumannii NIPH 329	APQY00000000	87.28
A. baumannii NIPH 335	APQX00000000	87.24
A. baumannii NIPH 601	APQZ00000000	87.20
A. baumannii NIPH 615	APOV00000000	87.25
A. baumannii NIPH 67	APRA00000000	87.30
A. baumannii NIPH 80	APRE00000000	87.35
A. baumannii RUH-134	APRB00000000	87.29
A. baumannii RUH-875	APQW00000000	87.31
A. calcoaceticus NIPH 2245^{T}	APQI00000000	89.67
A. calcoaceticus ANC 3680	APQH00000000	89.66
A. calcoaceticus NIPH 13	APOE00000000	89.67
A. calcoaceticus NIPH 2254	ACPK00000000	89.65
A. nosocomialis RUH-2376^{T}	APOP00000000	86.79
A. nosocomialis ATCC17903	AIEJ00000000	86.77
A. nosocomialis LUH-14367	APPP00000000	86.74
A. nosocomialis RUH-2624	ACQF00000000	86.83
A. pittii RUH-2206^{T}	APQP00000000	93.12
A. pittii ANC 3678	APQN00000000	93.12
A. pittii ANC 3870	ADCH00000000	93.13
A. seifertii NIPH 973^{T}	APOO00000000	87.05
Genomic sp. between 1 & 3 NIPH 542	APSC00000000	90.74
Genomic sp. between 1 & 3 NIPH 817	APPF00000000	90.87

Table S6

Metabolic characteristics of *A. dijkshoorniae* sp. nov. and other species of the ACB complex.

1= Values correspond to data obtained from the present study. 2= Values correspond to data taken from Nemec *et al.* (2015).

Carbon utilization tests were evaluated after 2, 6 and 10 days of incubation. +, All strains were positive; -, all strains were negative; D, mostly doubtful carbon utilization; W, mostly weak positive carbon utilization. Numbers indicate the percentage of strains with a positive reaction.

All strains were able to use acetate, 4-aminobutyrate, L-arginine, L-aspartate, L-glutamate, and D,L-lactate as a sole carbon source. None of the tested strains were able to utilize D-gluconate, D-glucose, and histamine for growth.

	Strains										
	A. dijkshoorniae	***A. baumannii***		***A. calcoaceticus***		***A. nosocomialis***		***A. pittii***		***A. seifertii***	
	1	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Nº of strains	n=15	n=5	n=25	n=5	n=11	n=4	n=20	n=5	n=20	n=5	n=16
Utilization of:											
Acetate	+	+	+	+	+	+	+	+	+	+	+
trans-Aconitate	+	+	92	+	+	+	60	+	+	+	+
Adipate	+	+	88	+	+	+	95	+	+	60	63
β-Alanine	+	+	+	80	91	+	85	+	90	80	88
4-Aminobutyrate	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	84	40	27	+	+	+	85	-	-
L-Arginine	+	+	+	+	+	+	+	+	+	+	+
L-Aspartate	+	+	+	+	+	+	+	+	+	+	+
Azelate	+	+	88	+	+	+	95	+	+	60	63
Benzoate	+	+	84	+	+	75	90	+	90	80	94
2,3-Butanediol	+	+	+	+	+	75	90	+	85	+	+
Citraconate	-	60	40	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	91 (D)	+	+	+	+	+	+
Ethanol	43 (D,W)	+	96	80	91	+	+	+	+	+	+
Gentisate	+	40	4	-	-	-	10	40	25	80	75
D-Gluconate	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	-	-	-	-	-	-	-	-	-	-	-
L-Glutamate	+	+	+	+	+	+	+	+	+	+	+
Glutarate	+	+	96	+	91	+	95	+	90	+	+

	Strains										
	A. dijkshoorniae	***A. baumannii***		***A. calcoaceticus***		***A. nosocomialis***		***A. pittii***		***A. seifertii***	
Nº of strains	**1** n=15	**1** n=5	**2** n=25	**1** n=5	**2** n=11	**1** n=4	**2** n=20	**1** n=5	**2** n=20	**1** n=5	**2** n=16
Histamine	-	-	-	-	-	-	-	-	-	-	-
L-Histidine	+	+	96	+	+	+	+	+	+	+	94
4-Hydroxybenzoate	+	+	92	+	91	+	80	+	+	+	94
D,L-Lactate	+	+	+	+	+	+	+	+	+	+	+
L-Leucine	+	+	88	80	91	+	95	+	95	80	94
Levulinate	80	40	24	80	91	-	5	-	5	20	6
D-Malate	+	+	92	60	D	+	+	+	95	+	88
Malonate	+	80	88	+	+	25	20	+	95	80	75
L-Ornithine	93	60	76	80	+	75	95	+	95	80	81
Phenylacetate	63 (W)	+	84	+	+	+	85	80	75	+	88
L-Phenylalanine	+	+	84	+	+	+	85	80	75	+	88
Putrescine	+	80	96	+	+	75	95	+	+	80	81
D-Ribose	93	+	76	20	45	+	80	80	35	20	-
L-Tartrate	67	40	32	-	9	-	-	80	85	+	31
Tricarballylate	+	+	92	+	+	75	95	+	+	+	+
Trigonelline	80	80	60	-	9	25	20	60	20	20	-
Tryptamine	53	-		-		-		-		-	
L-Tyrosine	+	+		+		75		+		80	

Fig. S1

Cluster analysis of all 105 ACB complex strains included in the study based on the independent partial sequences of each of the seven genes used for MLSA. Unrooted phylogenetic trees were constructed using the neighbour-joining method with genetic distances computed by Kimura's two-parameter model (Kimura, 1980) with a bootstrap value of 1000 replicates. Boostrap values (%) are indicated below branches. The scale bar indicates sequence divergence. Red dots indicate alleles of *A. dijkshoorniae* sp. nov. strains showing recombination with the respective alleles from *A. pittii.*

Fig. S2

Cluster analysis of *A. dijkshoorniae* sp. nov. strains and strains from all other species of the ACB complex with an available genome sequence (see Table S5) based on the partial sequences (positions 48-1450) of the 16SrRNA gene. Non-identical 16SrRNA gene sequences originated from the same strain were also included. Unrooted phylogenetic tree was constructed using the neighbour-joining method with genetic distances computed by Kimura's two-parameter model (Kimura, 1980) with a bootstrap value of 1000 replicates. The range of intraspecies similarity values for each species is shown in brackets. The scale bar indicates sequence divergence.

Figure S1

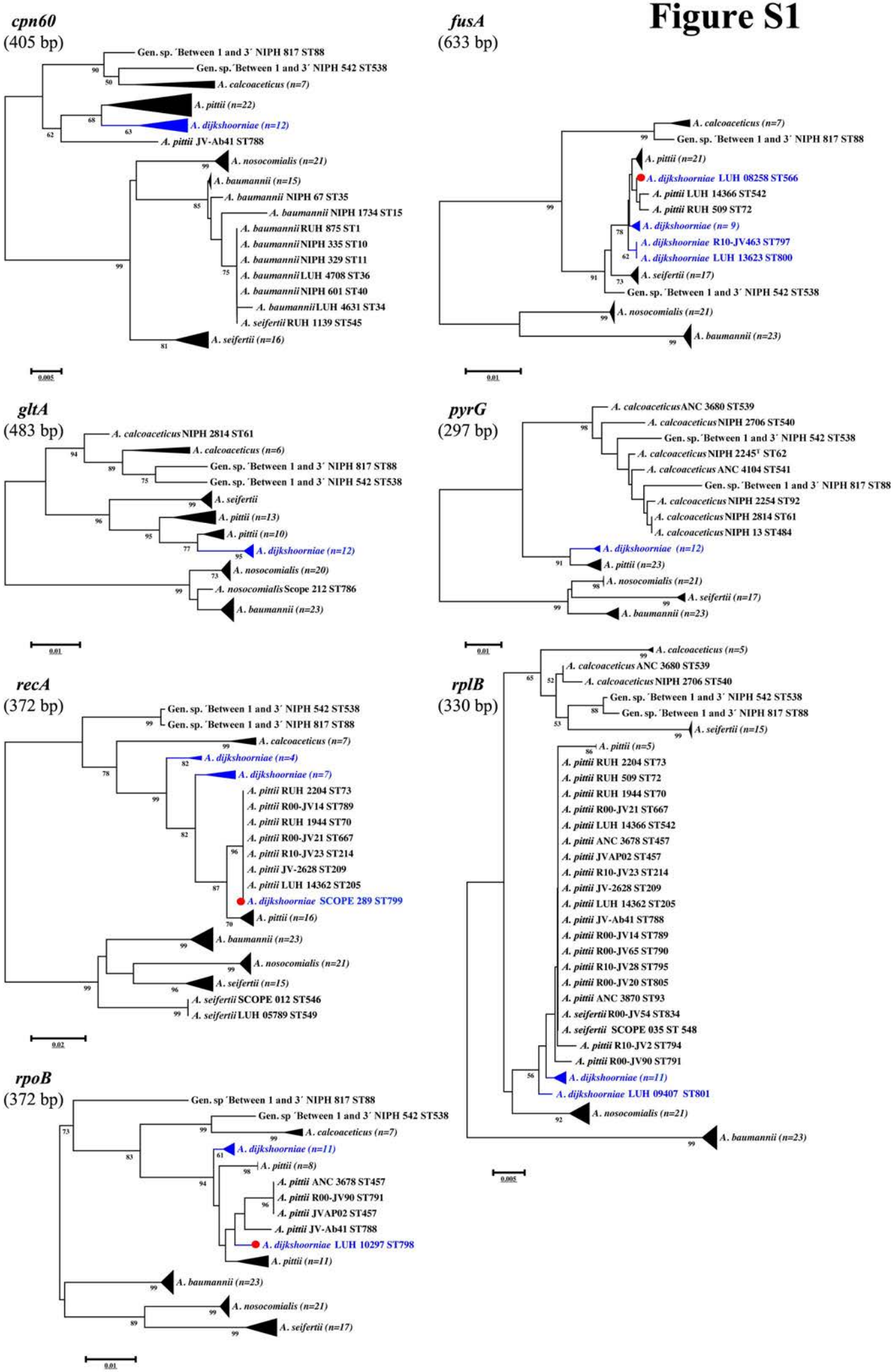
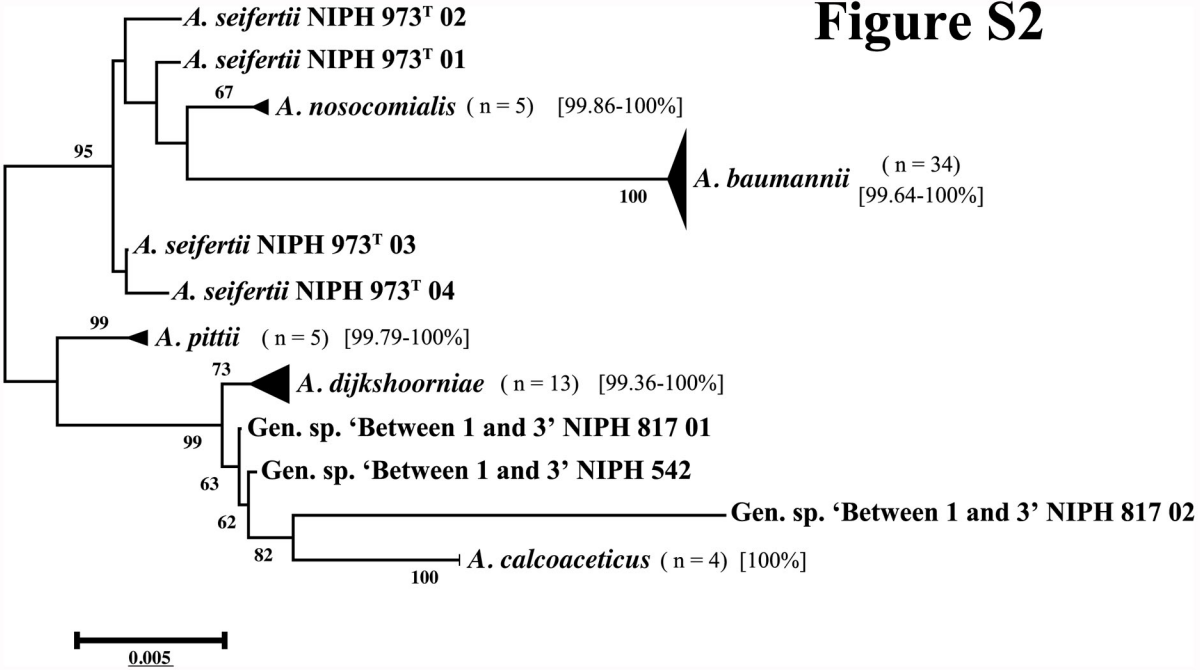

cpn60
(405 bp)
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
Gen. sp.´Between 1 and 3´ NIPH 542 ST538
A. calcoaceticus (n=7)
A. pittii (n=22)
A. dijkshoorniae (n=12)
A. pittii JV-Ab41 ST788
A. nosocomialis (n=21)
A. baumannii (n=15)
A. baumannii NIPH 67 ST35
A. baumannii NIPH 1734 ST15
A. baumannii RUH 875 ST1
A. baumannii NIPH 335 ST10
A. baumannii NIPH 329 ST11
A. baumannii LUH 4708 ST36
A. baumannii NIPH 601 ST40
A. baumannii LUH 4631 ST34
A. seifertii RUH 1139 ST545
A. seifertii (n=16)
0.005
fusA
(633 bp)
A. calcoaceticus (n=7)
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
A. pittii (n=21)
A. dijkshoorniae LUH 08258 ST566
A. pittii LUH 14366 ST542
A. pittii RUH 509 ST72
A. dijkshoorniae (n= 9)
A. dijkshoorniae R10-JV463 ST797
A. dijkshoorniae LUH 13623 ST800
A. seifertii (n=17)
Gen. sp. ´Between 1 and 3´ NIPH 542 ST538
A. nosocomialis (n=21)
A. baumannii (n=23)
0.01
gltA
(483 bp)
A. calcoaceticus NIPH 2814 ST61
A. calcoaceticus (n=6)
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
Gen. sp. ´Between 1 and 3´ NIPH 542 ST538
A. seifertii
A. pittii (n=13)
A. pittii (n=10)
A. dijkshoorniae (n=12)
A. nosocomialis (n=20)
A. nosocomialis Scope 212 ST786
A. baumannii (n=23)
0.01
pyrG
(297 bp)
A. calcoaceticus ANC 3680 ST539
A. calcoaceticus NIPH 2706 ST540
Gen. sp. ´Between 1 and 3´ NIPH 542 ST538
A. calcoaceticus NIPH 2245T ST62
A. calcoaceticus ANC 4104 ST541
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
A. calcoaceticus NIPH 2254 ST92
A. calcoaceticus NIPH 2814 ST61
A. calcoaceticus NIPH 13 ST484
A. dijkshoorniae (n=12)
A. pittii (n=23)
A. nosocomialis (n=21)
A. seifertii (n=17)
A. baumannii (n=23)
0.01
recA
(372 bp)
Gen. sp. ´Between 1 and 3´ NIPH 542 ST538
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
A. calcoaceticus (n=7)
A. dijkshoorniae (n=4)
A. dijkshoorniae (n=7)
A. pittii RUH 2204 ST73
A. pittii R00-JV14 ST789
A. pittii RUH 1944 ST70
A. pittii R00-JV21 ST667
A. pittii R10-JV23 ST214
A. pittii JV-2628 ST209
A. pittii LUH 14362 ST205
A. dijkshoorniae SCOPE 289 ST799
A. pittii (n=16)
A. baumannii (n=23)
A. nosocomialis (n=21)
A. seifertii (n=15)
A. seifertii SCOPE 012 ST546
A. seifertii LUH 05789 ST549
0.02
rplB
(330 bp)
A. calcoaceticus (n=5)
A. calcoaceticus ANC 3680 ST539
A. calcoaceticus NIPH 2706 ST540
Gen. sp. ´Between 1 and 3´ NIPH 542 ST538
Gen. sp. ´Between 1 and 3´ NIPH 817 ST88
A. seifertii (n=15)
A. pittii (n=5)
A. pittii RUH 2204 ST73
A. pittii RUH 509 ST72
A. pittii RUH 1944 ST70
A. pittii R00-JV21 ST667
A. pittii LUH 14366 ST542
A. pittii ANC 3678 ST457
A. pittii JVAP02 ST457
A. pittii R10-JV23 ST214
A. pittii JV-2628 ST209
A. pittii LUH 14362 ST205
A. pittii JV-Ab41 ST788
A. pittii R00-JV14 ST789
A. pittii R00-JV65 ST790
A. pittii R10-JV28 ST795
A. pittii R00-JV20 ST805
A. pittii ANC 3870 ST93
A. seifertii R00-JV54 ST834
A. seifertii SCOPE 035 ST 548
A. pittii R10-JV2 ST794
A. pittii R00-JV90 ST791
A. dijkshoorniae (n=11)
A. dijkshoorniae LUH 09407 ST801
A. nosocomialis (n=21)
A. baumannii (n=23)
0.005
rpoB
(372 bp)
Gen. sp ´Between 1 and 3´ NIPH 817 ST88
Gen. sp ´Between 1 and 3´ NIPH 542 ST538
A. calcoaceticus (n=7)
A. dijkshoorniae (n=11)
A. pittii (n=8)
A. pittii ANC 3678 ST457
A. pittii R00-JV90 ST791
A. pittii JVAP02 ST457
A. pittii JV-Ab41 ST788
A. dijkshoorniae LUH 10297 ST798
A. pittii (n=11)
A. baumannii (n=23)
A. nosocomialis (n=21)
A. seifertii (n=17)
0.01

Figure S2


A. seifertii NIPH 973T 02
A. seifertii NIPH 973T 01
67
A. nosocomialis (n = 5) [99.86-100%]
95
100
A. baumannii (n = 34) [99.64-100%]
A. seifertii NIPH 973T 03
A. seifertii NIPH 973T 04
99
A. pittii (n = 5) [99.79-100%]
73
A. dijkshoorniae (n = 13) [99.36-100%]
99
Gen. sp. 'Between 1 and 3' NIPH 817 01
63
Gen. sp. 'Between 1 and 3' NIPH 542
62
Gen. sp. 'Between 1 and 3' NIPH 817 02
82
100
A. calcoaceticus (n = 4) [100%]
0.005

Paper 2

Draft Genome Sequence of JVAP01T, the Type Strain of the Novel Species *Acinetobacter dijkshoorniae*

Draft Genome Sequence of JVAP01T, the Type Strain of the Novel Species *Acinetobacter dijkshoorniae*

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Hypothesis: -

Objectives: Report the draft genome sequence of the type strain of *Acinetobacter dijkshoorniae*, a novel human pathogen within the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex.

Material and methods:
Genomic DNA was extracted from cultured bacteria and an Illumina library was generated following Nextera XT (Illumina, Inc., San Diego, CA, USA) manufacturer's protocol with paired-end libraries (2 150). Sequencing was performed in an Illumina MiSeq system. De novo assembly was performed using Velvet version 1.2.10 in conjunction with the Velvet optimizer (http://bioinformatics.net.au/software.velvetoptimiser.shtml), ABySS v1.5.2 and Spades v3.5.0 (8–10). Contigs for all assemblers were joined using CISA v1.3 (11). CISA contigs below 200 nucleotides were discarded.

Results:
The draft genome comprised a total assembly length of 3,858,459 bp and the GC content was in accordance with that of *Acinetobacter* spp., at 38.8%. The RAST server predicted 3599 coding sequences, 26 rRNAs, and 134 rRNAs in the 92 contigs obtained. Resfinder v2.1 showed the presence of plasmidic bla_{NDM-1} and *aphA6* genes, conferring resistance to most β-lactams and aminoglycosides, and also chromosomal bla_{ADC}- and $bla_{OXA-213}$-group genes. PathogenFinder v1.1 predicted that this species had 83.3% probability of being a human pathogen.

Conclusions: According to its genome *Acinetobacter dijkshoorniae* can be considered a human pathogen belonging to the ACB complex and can carry plasmids with genes conferring resistance to last resort antibiotics such as carbapenems.

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PROKARYOTES

Draft Genome Sequence of JVAP01^T, the Type Strain of the Novel Species *Acinetobacter dijkshoorniae*

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ABSTRACT Here, we report the draft genome sequence of the type strain of *Acinetobacter dijkshoorniae*, a novel human pathogen within the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex. Strain JVAP01^T has an estimated genome size of 3.9 Mb, exhibits a 38.8% G+C content, and carries a plasmid with the bla_{NDM-1} carbapenemase gene.

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D.F.-O. and C.C. contributed equally to this article.

The *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex currently comprises six different *Acinetobacter* species, the environmental *A. calcoaceticus* and five *Acinetobacter* species that are potential human pathogens, that is, *A. baumannii*, *A. pittii*, *A. nosocomialis*, *A. seifertii*, and *A. dijkshoorniae*, the latter two only recently discovered (1). Members of the ACB complex are virtually undistinguishable from a biochemical standpoint and can only be differentiated by means of molecular methods (2, 3). Hospital outbreaks are mostly attributed to *A. baumannii*, whose innate ability to accumulate multiple antibiotic resistance mechanisms is greatly feared (4). The advent of more reliable identification methodologies, however, has shown an alarming abundance of all other species in the clinical setting, as well as their potential to bear resistance mechanisms to last resort antibiotics (5, 6). Here we report the draft genome sequence of strain JVAP01^T (CECT 9134^T, LMG 29605^T), the type strain of *Acinetobacter dijkshoorniae* that was recovered in 2009 from a urine sample in Turkey. JVAP01^T produces the NDM-1 metallo-β-lactamase and is resistant to β-lactam antibiotics and kanamycin (7).

Genomic DNA was extracted from cultured bacteria and an Illumina library was generated following Nextera XT (Illumina, Inc., San Diego, CA, USA) manufacturer's protocol with paired-end libraries (2 × 150). Sequencing was performed in an Illumina MiSeq system. *De novo* assembly was performed using Velvet version 1.2.10 in conjunction with the Velvet optimizer (http://bioinformatics.net.au/software.velvetoptimiser.shtml), ABySS v1.5.2 and Spades v3.5.0 (8–10). Contigs for all assemblers were joined using CISA v1.3 (11). CISA contigs below 200 nucleotides were discarded to yield a total of 92 contigs with a 90-fold coverage. The draft genome comprised a total assembly length of 3,858,459 bp and the G+C content was in accordance with that of *Acinetobacter* spp., at 38.8%.

The sequence of the 47 kilobase plasmid (pNDM-JVAP01) containing the bla_{NDM-1} gene and a type VI secretion system was previously published (7).

All 92 contigs and the plasmid were further annotated using the RAST server (12), which predicted 3,599 coding sequences (CDS), 26 rRNAs, and 134 tRNAs in the genome. In order to classify the antibiotic resistance gene pools, Resfinder v2.1 with a threshold of 85% identity and a minimum length of 40% was used (13). Results showed the presence of the bla_{NDM-1} and *aphA6* genes, described previously and conferring

resistance to most β-lactams and aminoglycosides, respectively (7), but also of the bla_{ADC}- and $bla_{OXA-213}$-family genes, respectively encoding an *Acinetobacter*-derived cephalosporinase and a class D oxacillinase, both of chromosomal location and conferring resistance to β-lactams. PathogenFinder v1.1 was used for the prediction of bacterial pathogenicity (14). Results revealed this species as human pathogen (83.8% probability) matching common sequences with 24 pathogenic families. Those, among others, were from *A. baumannii* ATCC 17978, AB0057, ACICU, and AYE strains.

Accession number(s). This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. LJPG00000000 and KM923969 for its associated plasmid. The version described in this paper is LJPG00000000.1.

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Paper 3

MALDI-TOF/MS identification of species from the *Acinetobacter baumannii* (Ab) group revisited: inclusion of the novel *A. seifertii* and *A. dijkshoorniae* species

MALDI-TOF/MS identification of species from the *Acinetobacter baumannii* (Ab) group revisited: inclusion of the novel *A. seifertii* and *A. dijkshoorniae* species

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Hypothesis: The novel species *A. seifertii* and *A. dijkshoorniae* can be identified by Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF/MS).

Objectives: Evaluate the use of MALDI-TOF/MS to identify isolates of *A. seifertii* and *A. dijkshoorniae* and revisit the identification of *A. nosocomialis* to update the Bruker taxonomy database.

Material and methods:
Species characterisation was performed by *rpoB*-clustering and MLSA. MALDI-TOF/MS spectra were recovered from formic acid/acetonitrile bacterial extracts overlaid with α-cyano-4-hydroxy-cinnamic acid matrix on a MicroflexLT in linear positive mode and 2000-20000 *m/z* range mass. Spectra were examined with the ClinProTools v2.2 software. Mean spectra (MSP) were created with the BioTyper software.

Results:
Seventy-eight *Acinetobacter* isolates representative of the Ab group were used to calculate the average spectra/species and generate pattern recognition models. Species-specific peaks were identified for all species. MSPs derived from three *A. seifertii*, two *A. dijkshoorniae*, and two *A. nosocomialis* strains were added to the Bruker taxonomy database, allowing successful identification of all isolates using spectra from either bacterial extracts or direct colonies, resulting in a positive predictive value (PPV) of 99.6% (777/780) and 96.8% (302/312), respectively.

Conclusions: The use of post-processing data software identified statistically significant species-specific peaks to generate reference signatures for rapid accurate identification of species within the Ab group, including the novel species *A. seifertii* and *A. dijkshoorniae*, providing relevant information for the clinical management of *Acinetobacter* infections.

Clinical Microbiology and Infection 23 (2017) 210.e1–210.e9

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Original article

MALDI-TOF/MS identification of species from the *Acinetobacter baumannii* (Ab) group revisited: inclusion of the novel *A. seifertii* and *A. dijkshoorniae* species

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ABSTRACT

Objectives: Rapid identification of *Acinetobacter* species is critical as members of the *A. baumannii* (Ab) group differ in antibiotic susceptibility and clinical outcomes. *A. baumannii*, *A. pittii*, and *A. nosocomialis* can be identified by MALDI-TOF/MS, while the novel species *A. seifertii* and *A. dijkshoorniae* cannot. Low identification rates for *A. nosocomialis* also have been reported. We evaluated the use of MALDI-TOF/MS to identify isolates of *A. seifertii* and *A. dijkshoorniae* and revisited the identification of *A. nosocomialis* to update the Bruker taxonomy database.

Methods: Species characterization was performed by *rpoB*-clustering and MLSA. MALDI-TOF/MS spectra were recovered from formic acid/acetonitrile bacterial extracts overlaid with α-cyano-4-hydroxy-cinnamic acid matrix on a MicroflexLT in linear positive mode and 2000–20 000 *m/z* range mass. Spectra were examined with the ClinProTools v2.2 software. Mean spectra (MSP) were created with the BioTyper software.

Results: Seventy-eight *Acinetobacter* isolates representative of the Ab group were used to calculate the average spectra/species and generate pattern recognition models. Species-specific peaks were identified for all species, and MSPs derived from three *A. seifertii*, two *A. dijkshoorniae*, and two *A. nosocomialis* strains were added to the Bruker taxonomy database, allowing successful identification of all isolates using spectra from either bacterial extracts or direct colonies, resulting in a positive predictive value (PPV) of 99.6% (777/780) and 96.8% (302/312), respectively.

Conclusions: The use of post-processing data software identified statistically significant species-specific peaks to generate reference signatures for rapid accurate identification of species within the Ab group, providing relevant information for the clinical management of *Acinetobacter* infections. **M. Marí-Almirall, CMI 2017;23:210.e1–210.e9**

Introduction

The use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) for identification of bacterial species has been a major breakthrough in clinical microbiology. MALDI-TOF/MS has proven to be a rapid and accurate methodology highly relevant for differentiation of closely related

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bacterial species that are otherwise indistinguishable by conventional phenotypic methods, providing an inexpensive alternative to the laborious and time-consuming molecular identification methods [1].

Former members of the *Acinetobacter baumannii* (Ab) group (*A. baumannii*, *A. nosocomialis* and *A. pittii*) are virtually indistinguishable using conventional phenotypic tests while accurate species differentiation is achieved by sequencing of the RNA polymerase β-subunit (*rpoB*) gene, the DNA gyrase B (*gyrB*) gene, and/or by multilocus sequence analysis (MLSA), all of which most likely constitute the current reference standard for molecular identification [2–4].

In a previous work, we evaluated and optimized the use of MALDI-TOF/MS for species identification of the former members of the Ab group and demonstrated that it was an accurate and reliable method [5]. Subsequent MALDI-TOF/MS studies by several other groups together with the recent technological advances in molecular methods (such as whole genome sequencing) have revealed a relative abundance of non-*baumannii Acinetobacter* species of the Ab group in clinical specimens, mostly involving *A. nosocomialis* and *A. pittii* isolates [6–11].

In the last few years the taxonomy of the genus *Acinetobacter* has undergone major modifications, with more than 18 new species having been described since 2014 [3]. In particular, two novel pathogenic species, *A. seifertii* and *A. dijkshoorniae*, have recently been included within the Ab group and, like the former members of the group, they can be best differentiated by molecular methods [3,12]. Identification of these novel species by MALDI-TOF/MS is not yet possible, as a thorough study that evaluates the distinctness of spectral signatures of all the species within the Ab group and provides reference spectra for the novel species is still lacking. In addition, several studies have shown that while the Bruker MALDI-TOF BioTyper system correctly identifies almost all *A. baumannii* and *A. pittii* isolates, identification rates for *A. nosocomialis* range at about 70%, suggesting that the Bruker database should be updated and further improved to allow efficient identification of all *Acinetobacter* species [8,13,14].

The aim of the present study was to perform an in-depth analysis of the spectrum profiles of all the *Acinetobacter* species currently included in the Ab group, and generate reference spectra to allow accurate and reliable identification to the species level by MALDI-TOF/MS.

Materials and methods

Bacterial isolates

The present study included 78 isolates belonging to the five *Acinetobacter* species within the Ab group, *A. baumannii* (n=16), *A. nosocomialis* (n=24), *A. pittii* (n=15), *A. dijkshoorniae* (n=12), and *A. seifertii* (n=11), mainly obtained from clinical samples in different geographical locations over a period of 15 years (Table S1). Isolates were identified at the species level by sequencing of the RNA polymerase β-subunit (*rpoB*) gene and multilocus sequence analysis (MLSA), as described previously [3]. Isolates were preserved at -80°C in 10% skimmed milk until use.

Sample preparation and MALDI-TOF/MS data acquisition

Bacterial cultures were grown overnight on Columbia sheep blood agar (Becton Dickinson, Heidelberg, Germany) at 37°C and subjected to ethanol-formic acid extraction according to [5].

One microlitre of each bacterial extract was spotted onto a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. Each spotted sample was then overlaid with 1 μL of a saturated matrix solution (α-cyano-4-hydroxy-cinnamic acid; Bruker Daltonics) in 50% acetonitrile-2.5% trifluoroacetic acid (Sigma-Aldrich chemical Co., Madrid, Spain) and air-dried.

For MALDI-TOF/MS analysis performed directly from grown bacterial colonies, a small fraction of a single colony was spotted onto the MALDI target plate, carefully spread and subsequently overlaid with 1 μL of matrix.

MALDI-TOF/MS was conducted in a Microflex LT (Bruker Daltonics) benchtop instrument as described previously [5]. Bacterial extracts from all isolates were spotted five times onto a MALDI target plate and each spot was measured twice, resulting in 10 mass spectra for each individual isolate. Direct colony samples were spotted twice, and each spot was also measured twice, resulting in four mass spectra for each individual isolate.

MALDI-TOF/MS data analysis

Spectra from bacterial extracts were loaded into the ClinProTools software (version 2.2; Bruker Daltonics) and prepared for analysis with the following parameters: 800 resolution, Top Hat baseline subtraction with a 10% minimal baseline width and no data reduction. Null spectra and noise spectra exclusion with a noise threshold of 2.00 were both enabled and spectra grouping was also supported. Peak selection and average peak list calculation ranged from 2000 to 10 000 mass to charge ratio values (m/z), and recalibration was performed with a 1000 parts per million (ppm) maximal peak shift and 30% match to calibrant peaks. Non-recalibrated spectra were excluded.

m/z values from average spectra were identified according to their statistical significance, as determined by the different statistical tests supported by ClinProTools: Anderson-Darling test, t-/ANOVA test and Wilcoxon/Kruskal-Wallis test. Informative peaks were those showing a significant difference among all species as described previously [15].

For the generation and validation of pattern recognition models, the 78 isolates were divided into two sets: (i) a reference set containing 40 isolates: *A. baumannii* (n=7), *A. nosocomialis* (n=13), *A. pittii* (n=8), *A. dijkshoorniae* (n=6), and *A. seifertii* (n=6); and (ii) a validation set containing 38 isolates: *A. baumannii* (n=9), *A. nosocomialis* (n=11), *A. pittii* (n=7), *A. dijkshoorniae* (n=6), and *A. seifertii* (n=5). Selection was performed on the grounds of the spectral analysis to include as much diversity as possible within both sets, prioritizing the reference set whenever an equitable distribution was not possible. Classification models were generated using the genetic algorithm (GA), supervised neural network (SNN), and QuickClassifier (QC) algorithms with default settings. The recognition capability and cross validation values were calculated to demonstrate the reliability and accuracy of the model.

Bacterial identification

Spectra were analysed with the MALDI BioTyper software (version 3.1; Bruker Daltonics) using the pre-processing and BioTyper main spectrum (MSP) identification standard methods (mass range 2000-20 000 m/z) against either the default Bruker database or the association of the Bruker database and our own reference spectra. Accuracy of the identification was determined by a logarithmic score value resulting from the alignment of peaks to the best matching reference spectrum [5].

Bacterial extracts of isolates selected for MSP creation were re-spotted 10 times onto a ground steel target and each spot was measured three times. The resulting 30 mass spectra were carefully analysed using the FlexAnalysis software (version 3.4; Bruker Daltonics) to yield a minimum of 20 spectra per isolate with a *m/z* shift of less than 0.05%. Selected spectra were then uploaded onto the MALDI BioTyper to create a single MSP for each isolate with the BioTyper MSP creation standard method.

The MSP dendrogram was constructed using the correlation distance measure with the weighted linkage algorithm settings of the MALDI BioTyper software.

rpoB-based cluster analysis as well as MLSA cluster analysis were performed as described elsewhere [3].

Results

Spectral analysis

Seventy-eight *Acinetobacter* isolates representative of *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. dijkshoorniae*, and *A. seifertii* were used to identify species-specific biomarker peaks using the Bruker ClinProTools software. Acquired spectra were loaded into ClinProTools and grouped into five different classes, one for each *Acinetobacter* species, and the average spectrum for each class was calculated. A detailed spectra analysis of each species was performed in the region between 2000 and 10 000 *m/z* that concentrated the bulk of mass peaks, and several species-specific peaks ranging from 2876 to 8857 *m/z* values were identified (Table 1), as were six peaks (4265, 4661, 5175, 6090, 6948, and 9319 *m/z*) that were present in all isolates.

For *A. baumannii*, a biomarker peak unique to this species and present in all isolates was located at 5747 *m/z* (Fig. 1(e)), as described previously [5,14,16,17]. Two additional peaks at 4244 and 8485 *m/z*, likely representing the single and double protonation states of the same protein, were also identified in all *A. baumannii* isolates but these were also shared with some isolates of *A. nosocomialis*, as shown below. Spectra from all *A. baumannii* isolates were correctly identified as *A. baumannii* (100%) by the Bruker BioTyper using the default taxonomy database.

For *A. nosocomialis*, several peaks unique to this species were identified. A pair of peaks at 4069 and 8135 *m/z* (Fig. 1(b) and 1(h)), also likely corresponding to different protonation states of a single protein, were present in all isolates but one, with the latter isolate displaying a shifted version of the pair located at 4084 and 8165 *m/z*, respectively (data not shown), in good agreement with previous reports [14,17]. A second pair of unique peaks was located at 4180 and 8358 *m/z* but it was present in only 12 out of 24 isolates (Fig. 1(c) and 1(i)).

Bacterial identification using the default Bruker taxonomy database was able to identify as *A. nosocomialis* only 13 out of 24 isolates (54%), while the remaining isolates were misidentified as *A. baumannii* (46%). Similar inconsistent results regarding identification of *A. nosocomialis* also have been reported by other authors [8,13,14]. The *A. nosocomialis* isolates that were correctly classified by the Bruker BioTyper software were clustered together (group I) and compared with those that were misidentified (group II). Spectra within each group showed very similar peak profiles but there were some significant differences between both groups (Fig. 2(d)).

As shown in Fig. 2(a) and 2(c), isolates in *A. nosocomialis* group I and II shared the *A. nosocomialis* species-specific peaks centred around 4069 and 8135 *m/z*. All but one isolate in group I also presented the other two species-specific peaks centred around 4180 and 8358 *m/z*, which were absent among group II profiles. Instead, isolates in group II presented the two peaks at 4244 and 8485 *m/z* that were also present in all *A. baumannii* isolates, as mentioned before.

Despite the clear splitting of all *A. nosocomialis* isolates into two separate groups according to their peak signatures, this clear distinction was not observed by *rpoB*-based clustering (data not shown) or MLSA analysis (Fig. 3).

For *A. pittii*, the analysis of spectra recognized a higher degree of variability with only a few species-specific peaks shared by a majority of isolates. A major peak at 5777 *m/z* (Fig. 1(e)) was identified in all isolates but one, in good agreement with previous reports [14,16–18], and a second unique peak at 6692 *m/z* was present in 10 out of 15 isolates (Fig. 1(f)). Eight out of 15 isolates also showed a pair of peaks located at 4411 and 8821 *m/z*, respectively, again likely representing the differently charged states of a single protein (Fig. 1(d) and 1(j)). These two peaks were also identified in two additional isolates, although they were shifted to 4347 and 8691 *m/z*, respectively (data not shown). Similarities among *A. pittii* isolates

Table 1
ClinProTools peak statistics for all the species-specific peaks

Peak number	Mass	DAve	PTTA	PWKW	PAD	*Abau*	*Anos*	*Apit*	*Adij*	*Asei*
21	3948.53	13.65	0.00344	0.0000533	< 0.000001					
23	4069.73	4.81	0.000539	< 0.000001	< 0.000001					
26	4180.56	9.36	< 0.000001	< 0.000001	< 0.000001					
27	4194.14	13.42	< 0.000001	< 0.000001	< 0.000001					
34	4411.95	11.99	< 0.000001	< 0.000001	< 0.000001					
35	4430.15	37.02	< 0.000001	< 0.000001	< 0.000001					
58	5747.48	137.56	< 0.000001	< 0.000001	< 0.000001					
59	5777.3	157.06	< 0.000001	< 0.000001	< 0.000001					
60	5788.92	101.76	< 0.000001	< 0.000001	< 0.000001					
78	6692.55	5.97	0.00000267	< 0.000001	< 0.000001					
80	6729.52	3.94	0.0000755	< 0.000001	< 0.000001					
88	7446.28	8.73	0.000984	0.0000173	< 0.000001					
89	7893.14	61.67	0.000187	< 0.000001	< 0.000001					
92	8135.43	33.8	< 0.000001	< 0.000001	< 0.000001					
96	8358.1	50.16	< 0.000001	< 0.000001	< 0.000001					
97	8385.13	53.54	< 0.000001	< 0.000001	< 0.000001					
101	8821.13	43.15	< 0.000001	< 0.000001	< 0.000001					
102	8857.63	103.26	< 0.000001	< 0.000001	< 0.000001					

Peak number, correlative numbering of the peak in the average spectra; Mass, *m/z* value; DAve, difference between the maximal and the minimal average peak area/intensity of all the species; PTTA, p value of t-/analysis of variance test; PWKW, p value of Wilcoxon/Kruskal–Wallis test (preferable for non-normally distributed data); PAD, p value of Anderson–Darling test, which gives information about normal distribution (p-value AD <0.05, non-normally distributed; p-value AD >0.05, normally distributed); *Abau*, *A. baumannii*; *Anos*, *A. nosocomialis*; *Apit*, *A. pittii*; *Adij*, *A. dijkshoorniae*; *Asei*, *A. seifertii*. Shaded boxes indicate species specificity.

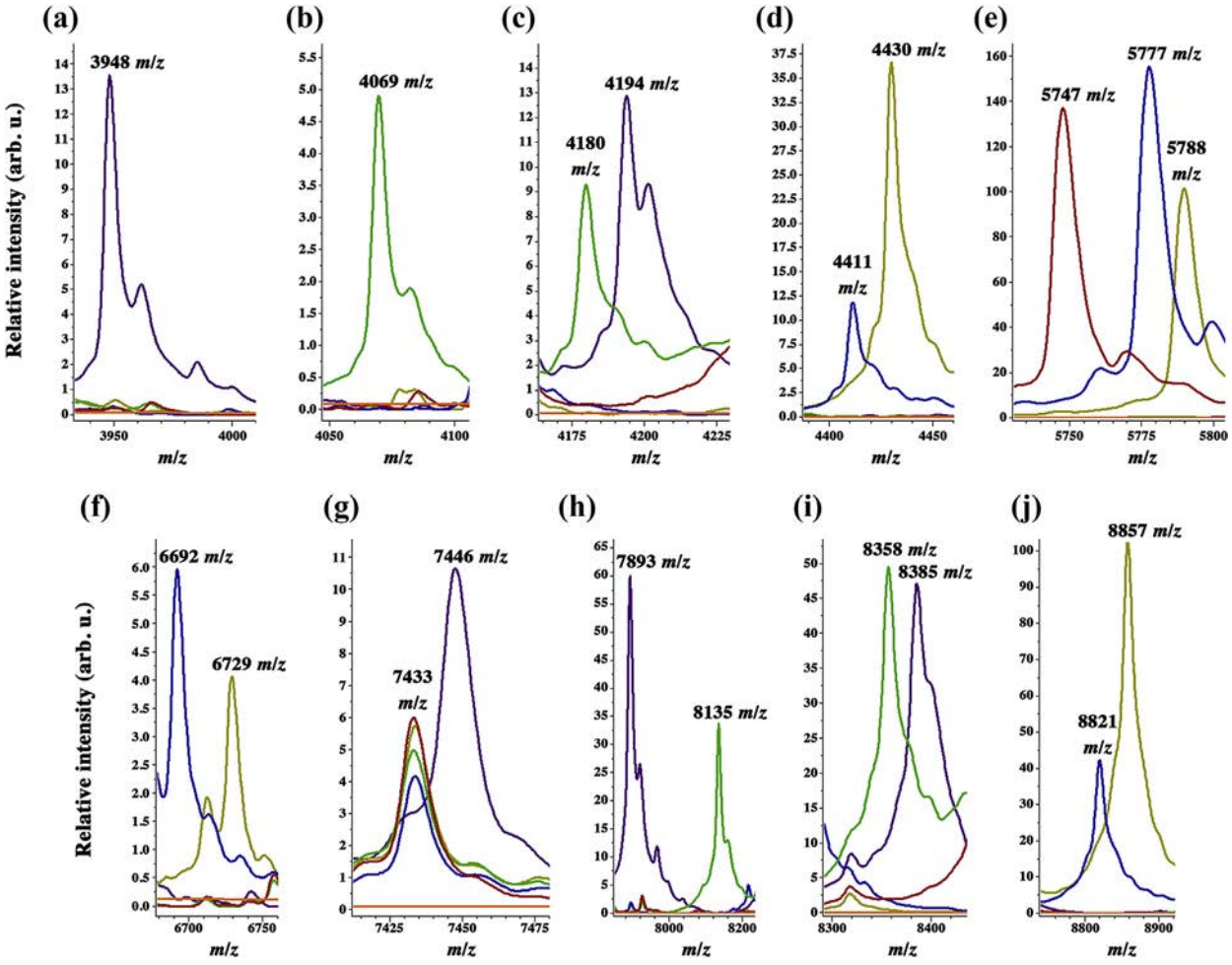

Fig. 1. MALDI-TOF/MS averaged spectra plots from isolates of the Ab group showing specific peaks for: *A. baumannii* (red), *A. nosocomialis* (green), *A. pittii* (blue), A. *dijkshoorniae* (yellow), and A. *seifertii* (purple). The background noise signal is shown in orange. The x-axis shows the *m/z* values and the y-axis indicates the intensities of the peaks expressed in arbitrary intensity units. Peaks are ordered from left to right as A-J according to their ascending *m/z* values.

at the spectra level did not correlate with either *rpoB* or MLSA clustering either (data not shown). Species identification using the default Bruker taxonomy database correctly identified all spectra as *A. pittii*, in agreement with previous reports [13,14].

For *A. dijkshoorniae*, the analysis of spectra identified four masses that were present in all isolates and were also unique to this species: three major peaks located at 4430, 5788, and 8857 *m/z* values (Fig. 1(d), 1(e), and 1(j)), as described previously [3], as well as a smaller peak at 6729 *m/z* (Fig. 1(f)). As there were no reference spectra for this novel *Acinetobacter* species in the Bruker taxonomy database, the best identification matches of spectra from *A. dijkshoorniae* isolates were to *A. pittii* reference spectra. Interestingly, the first two best matches were always to the same *A. pittii* reference spectra (*A. pittii* serovar 18 DSM9341 and serovar 22 DSM9318), with log scores >2.0, while the subsequent best matches against *A. pittii* isolates showed log scores <2.0. It is plausible that the two isolates originally used to create these MSPs belonged to the novel *A. dijkshoorniae* species. Of note, the MSPs for these isolates were used to screen our *Acinetobacter* collection and led to the identification of three isolates that turned out to be *A. dijkshoorniae* by molecular methods.

For *A. seifertii*, a unique peak was present at 7446 *m/z* (Fig. 1(g)) in all isolates and several additional species-specific peaks were also identified, albeit with differences depending on the isolates. A pair of peaks located at 4194 and 8385 *m/z* (Fig. 1(c) and 1(i)) was identified in all but two isolates that, nevertheless, presented a similar pair but shifted at 4122 and 8244 *m/z* (data not shown). Likewise, another pair of peaks located at 3948 and 7893 *m/z* (Fig. 1(a) and 1(h)) was present in all but three isolates, the latter showing a shifted pair at 3985 and 7968 *m/z* (two isolates) or 3961 and 7920 *m/z* (one isolate) (data not shown).

As occurred with *A. dijkshoorniae*, there were no reference spectra for *A. seifertii* in the Bruker taxonomy database, and the best identification matches were to *A. baumannii* reference spectra. However, the first best match was always to the same *A. baumannii* reference spectra (*A. baumannii* CS_62_1 BRB) with log scores >2.0, while the subsequent best matches showed log scores <2.0. The MSP for *A. baumannii* CS_62_1 BRB was also used to screen our *Acinetobacter* collection and it led to the identification of one isolate that was confirmed as *A. seifertii* by molecular methods, again suggesting that the isolate used to create the MSP *A. baumannii* CS_62_1 BRB most likely belonged to the novel *A. seifertii* species.

Generation and validation of pattern recognition models

Spectra from a reference set of isolates (see Materials and methods) were uploaded to the ClinProTools software and grouped again into five different classes according to each *Acinetobacter* species. The average spectra from each *Acinetobacter* species were used to generate classification models based on the

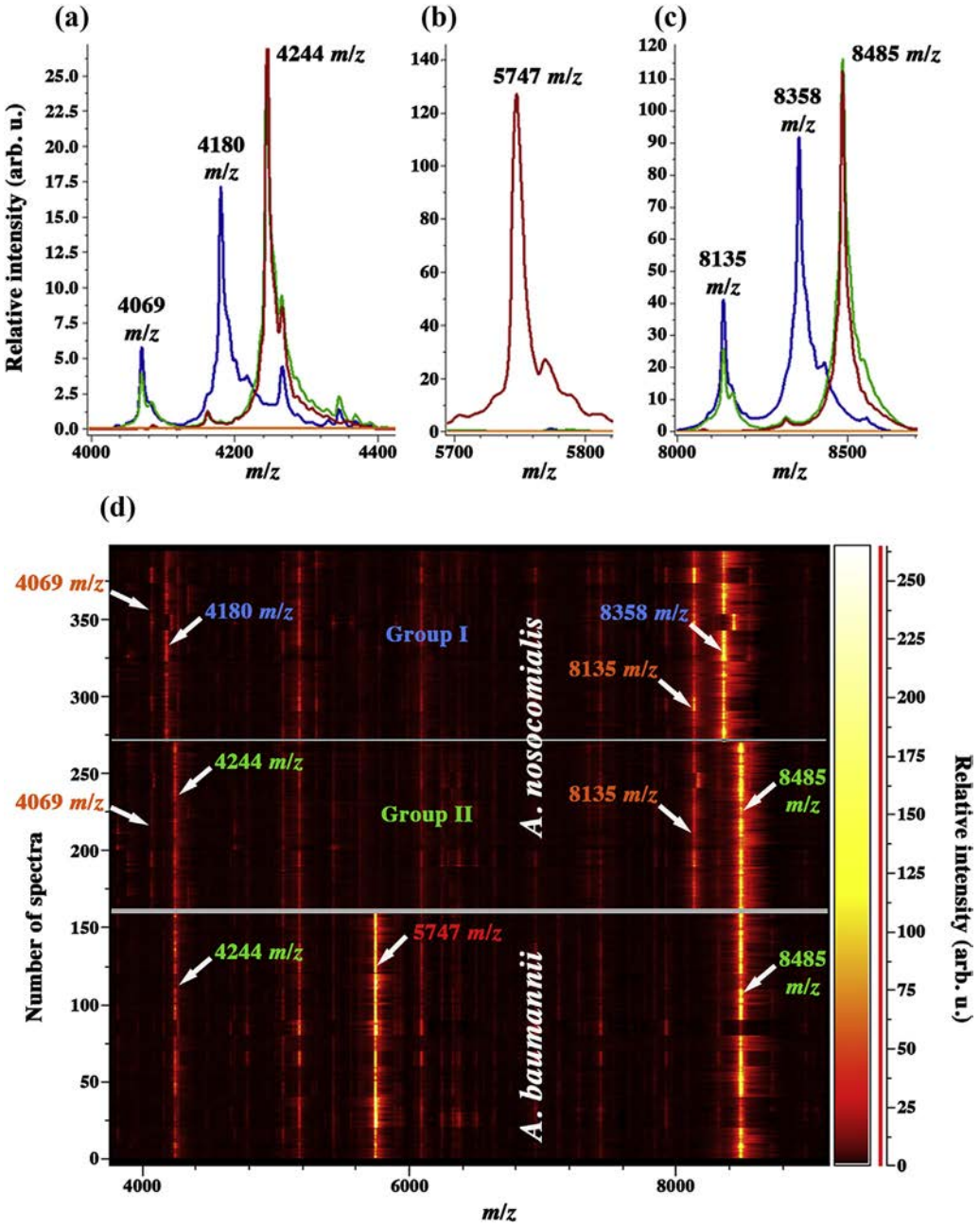

Fig. 2. Spectral analysis of *A. nosocomialis* and *A. baumannii* isolates. *A. nosocomialis* isolates are clustered into groups I and II according to BioTyper results. (a, b, and c) Averaged spectra plots for all the spectra included within each group. *A. baumannii* (red), *A. nosocomialis* group I (blue), *A. nosocomialis* group II (green), background noise signal (orange). The x-axis shows the *m/z* values and the y-axis indicates the intensities of the peaks expressed in arbitrary intensity units. (d) Gel view representation in quadratic mode and chromatic scale of all independent spectra within the 4000–9000 *m/z* mass range. Each isolate is represented by 10 independent spectra. The x-axis shows the *m/z* values and the y-axis indicates the number of spectra (left) as well as intensities of the peaks expressed in arbitrary intensity units (right). Grey lines are used to separate spectra from different groups. Arrows indicate *m/z* values that are present in: *A. nosocomialis* group I and II (orange labels); only in *A. nosocomialis* group I (blue labels); in both *A. nosocomialis* group II and in *A. baumannii* (green labels); and only in *A. baumannii* (red label).

Genetic Algorithm, SNN, and Quick Classifier algorithms to select an optimal set of peaks that allowed correct species allocation of the spectra used for model generation. All three algorithms provided recognition and cross-validation values above 95% and 87%, respectively, suggesting that successful differentiation of all five *Acinetobacter* species was possible. Of the three algorithms, the SNN model yielded the highest recognition and cross-validation values (100% and 92.6%, respectively) and was therefore selected to evaluate its ability to classify spectra from isolates not included in the generation of the model (external validation). The SNN model was able to allocate most of the spectra from the 38 isolates of the validation set to their corresponding *Acinetobacter* species, resulting in a positive predictive value (PPV) of 96.8% (Table 2).

BioTyper database update and automated identification

As described in Materials and methods, new BioTyper MSPs were created from representative isolates to account for the intra- and inter-species variability observed. MSPs for *A. seifertii* derived

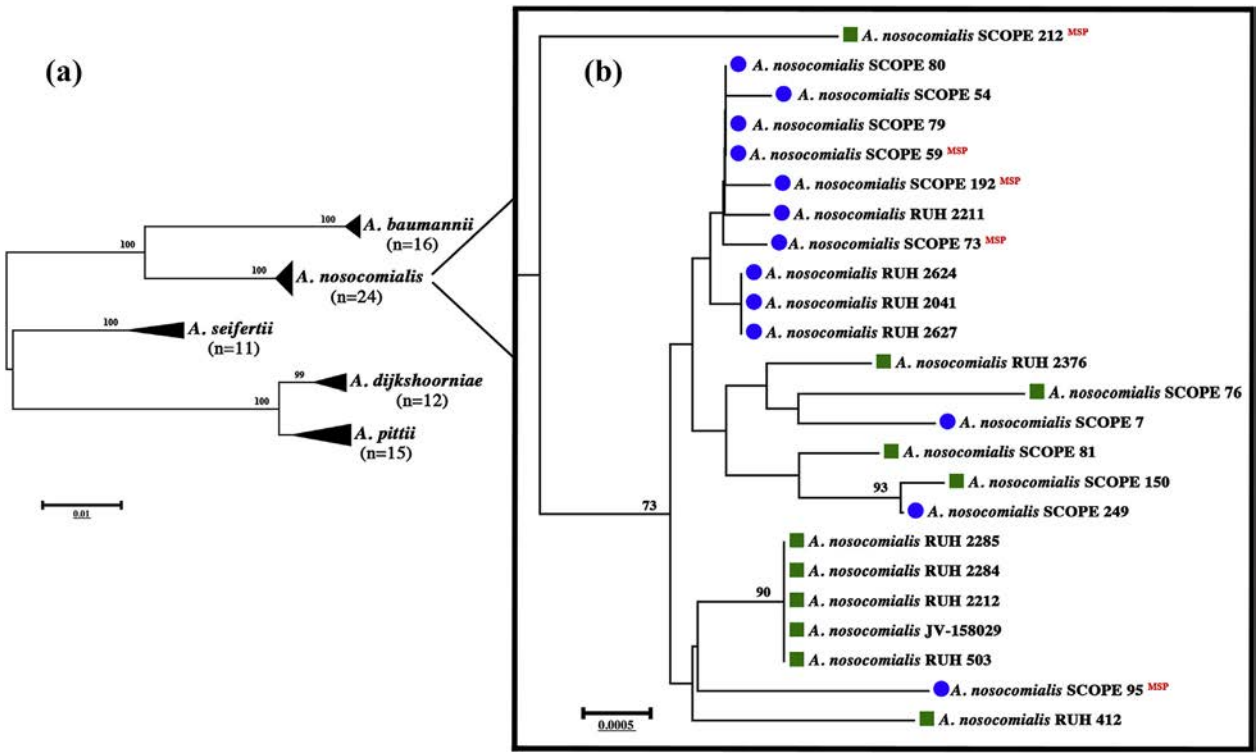

Fig. 3. Cluster analysis of all the 78 Ab group isolates included in the study based on the concatenated partial sequences of the *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* genes used for MLST under the Pasteur scheme. The partial sequences of the individual genes used for MLSA can be retrieved from the PubMLST website (http://pubmlst.org/abaumannii/) under the sequence type codes listed in Table S1. Phylogenetic trees were constructed using the neighbour-joining method with genetic distances computed by Kimura's two-parameter model (Kimura, 1980) with a bootstrap value of 1000 replicates. Bootstrap values (%) are indicated above the branches. The scale bar indicates sequence divergence. (a) Collapsed phylogenetic tree showing the monophyletic clustering of isolates from each *Acinetobacter* species within the Ab group. (b) Expanded phylogenetic tree showing the clustering of all the *A. nosocomialis* isolates. Circles (in blue) and squares (in green) indicate *A. nosocomialis* isolates classified as belonging to group I (correct BioTyper identification) or group II (BioTyper misidentification), respectively, using the default Bruker taxonomy database. The MSP label indicates isolates that originated the five reference spectra (MSPs) for *A. nosocomialis* currently included in the default Bruker taxonomy database.

from isolates NIPH 973^T (type strain), R00-JV54 and LUH 05789. MSPs for *A. dijkshoorniae* originated from isolates JVAP01^T (type strain) and R10-JV222. In addition, we included new MSPs for *A. nosocomialis* that derived from isolates SCOPE 150 and RUH 503, to account for the identification of *A. nosocomialis* isolates belonging to *A. nosocomialis* group II (Fig. 2).

Cluster analysis of MSPs from all the *Acinetobacter* species within the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (which includes the Ab group) grouped MSPs from each *Acinetobacter* species into separate monophyletic clusters (Fig. 4). Interestingly, the two MSPs from representative isolates of *A. nosocomialis* group II were grouped more closely to *A. baumannii* MSPs than to those of *A. nosocomialis* group I, while still forming a separate clade, also in good agreement with results from the spectral analysis.

Spectra from all 78 isolates were then analysed against a custom database that included the MSPs from all the *Acinetobacter* species within the default Bruker taxonomy database plus the novel reference signatures for *A. seifertii*, *A. dijkshoorniae*, and *A. nosocomialis*. As shown in Table 2, the allocation of spectra obtained from bacterial extracts to their corresponding *Acinetobacter* species provided sensitivity and specificity values ranging from 98.8% to 100% and 99.6% to 100%, respectively, resulting in a PPV of 99.6%. In addition, strains RUH 204 (*A. junii*), RUH 44 (*A. haemolyticus*), RUH 45 (*A. lwoffii*), RUH 3517 (*A. radioresistens*), and RUH 584 (*A. calcoaceticus*), representing a set of reference *Acinetobacter* strains belonging to *Acinetobacter* species other than those included within the Ab group [5], were also correctly identified (data not shown). These results showed the absence of cross-identification between the novel MSPs and other *Acinetobacter* spp.

Likewise, the identification of spectra from direct colonies instead of bacterial extracts yielded sensitivity and specificity values ranging from 91.7% to 100% and 98.0% to 100%, respectively, with a PPV of 96.8% (Table 2).

Discussion

In the present study we have compared for the first time the spectral profiles of the current members of the Ab group, including the novel *A. seifertii* and *A. dijkshoorniae* species. Spectral analysis allowed identification of a conserved set of peaks that are present in all isolates and, therefore, are linked at least to the Ab group. Four of these peaks correspond to four out of the five peaks described by Sousa *et al.* as being specific to the *Acinetobacter* genus (4662, 5176, 6949, and 9323 *m/z*) [17]. We have found, however, that the peak described by Sousa *et al.* at 7435 *m/z* is present in all species except *A. seifertii*, which instead presents a unique peak at 7446 *m/z*.

The thorough analysis of all spectra has also led to identification of several peaks that are unique to each *Acinetobacter* species and might serve for identification purposes. The majority of such peaks corroborate previous findings but, nevertheless, remarkable differences have also been found. For instance, previous studies have reported a peak at 2875 *m/z* as specific to *A. baumannii* [5,19], although Sousa *et al.* reported such a peak in *A. nosocomialis* [17]. In the present study, we have identified a peak at 2876 *m/z* in all

Table 2
External validation of the supervised neural network (SNN) model and the novel mean spectra (MSPs) using the ClinProTools and the MALDI BioTyper software, respectively

Method	*Acinetobacter* species	Nº of Isolates	Nº of spectra	Spectra classification					Sen (%)	Spe (%)	PPV (%)
				A. baumannii	*A. nosocomialis*	*A. pittii*	*A. dijkshoorniae*	*A. seifertii*			
ClinProTools	*A. baumannii*	9	90	89	1	0	0	0	98.9	100	96.8
	A. nosocomialis	11	110	0	110	0	0	0	100	96.7	
	A. pittii	7	70	0	7	61	0	2	87.1	99.7	
	A. dijkshoorniae	6	60	0	0	1	59	0	98.3	100	
	A. seifertii	5	50	0	1	0	0	49	98.0	99.4	
BioTyper (Bacterial extracts)	*A. baumannii*	16	160	158	2	0	0	0	98.8	99.8	99.6
	A. nosocomialis	24	240	1	239	0	0	0	99.6	99.6	
	A. pittii	15	150	0	0	150	0	0	100	100	
	A. dijkshoorniae	12	120	0	0	0	120	0	100	100	
	A. seifertii	11	110	0	0	0	0	110	100	100	
BioTyper (Direct colonies)	*A. baumannii*	16	64	63	1	0	0	0	98.4	98.0	96.8
	A. nosocomialis	24	96	5	91	0	0	0	94.8	99.5	
	A. pittii	15	60	0	0	60	0	0	100	98.4	
	A. dijkshoorniae	12	48	0	0	4	44	0	91.7	100	
	A. seifertii	11	44	0	0	0	0	44	100	100	

Sen (%), sensitivity (%); Spe (%), specificity (%); PPV, positive predictive value.

A. baumannii isolates that overlaps with a small intensity peak centred around 2869 *m/z* present in some *A. nosocomialis* isolates. The presence of such a peak might be misleading for identification purposes (data not shown). Likewise, Hsueh *et al.* identified a peak at 2889 *m/z* that was unique to *A. pittii* [14] and in our study this peak is indeed present in all *A. pittii* isolates but it is also identified in several isolates of *A. nosocomialis* and *A. dijkshoorniae*; and a peak at 9542 *m/z* considered to be unique to *A. seifertii* by Sousa *et al.* [17]

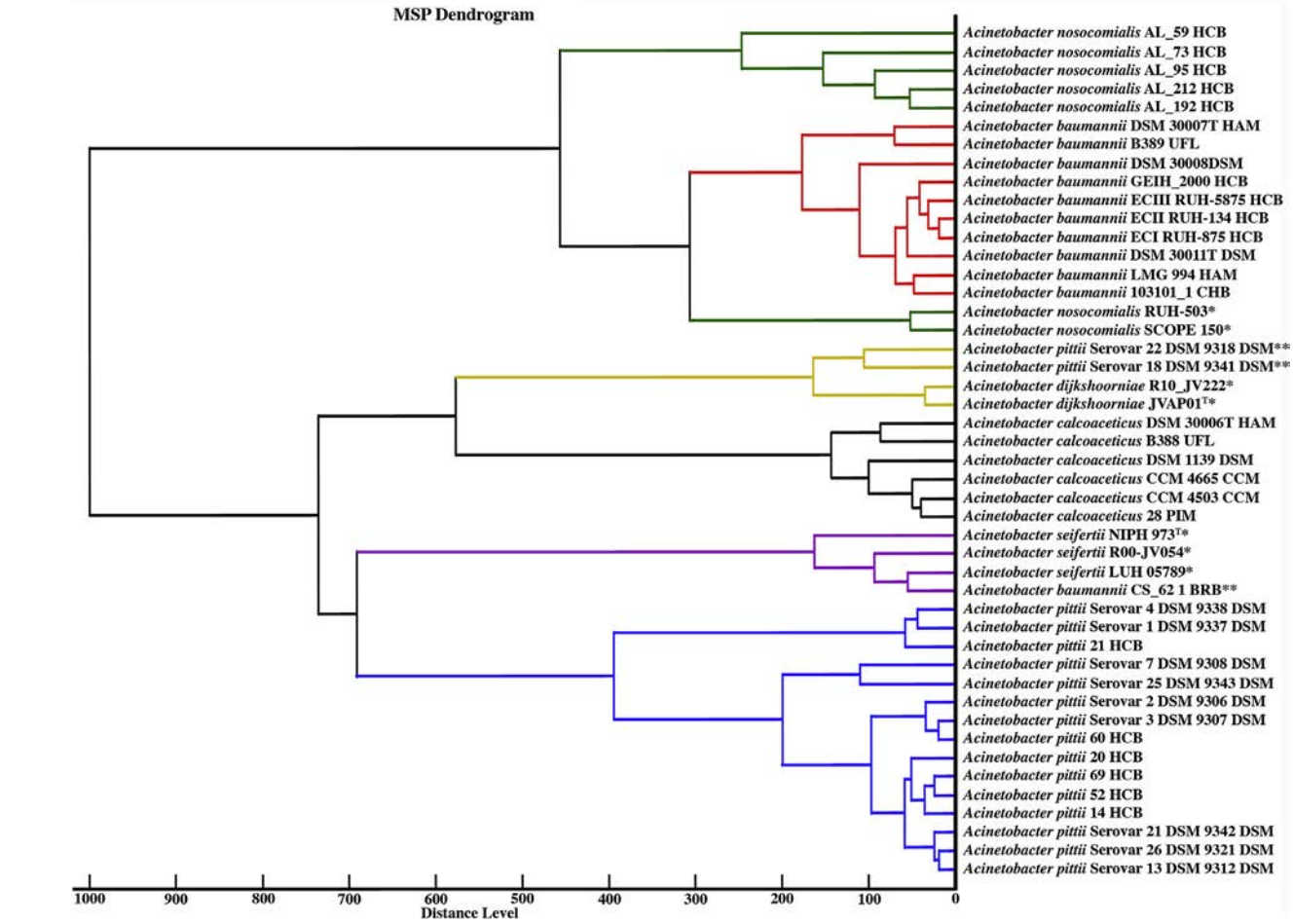

Fig. 4. MSP dendrogram containing all the MALDI-TOF/MS specific signatures of isolates from the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex included within the default Bruker taxonomy database as well as specific signatures for *A. dijkshoorniae*, *A. seifertii*, and *A. nosocomialis* created in this study. Distance values are relative and normalized to a maximal value of 1000. The novel MSPs created in this study are labelled with a single asterisk (*). MSPs from isolates that failed to cluster with their corresponding *Acinetobacter* species are labelled with a double asterisk (**). T, Type strain.

is clearly present in several *A. pittii* and *A. dijkshoorniae* isolates in our study.

In addition, the comprehensive examination of the *A. nosocomialis* isolates has led to their differentiation into two groups according to their spectra profiles. Isolates included in group I contain four *A. nosocomialis*-specific peaks while isolates in group II show only two of these peaks but share two additional peaks with *A. baumannii*. Interestingly, of the five reference spectra (MSPs) for *A. nosocomialis* included in the Bruker taxonomy database (Fig. 3), four originated from isolates belonging to group I and only one was representative of group II. These differences together with the underrepresentation of group II MSPs in the default BioTyper software might account for the low rates of successful identification of *A. nosocomialis* isolates reported by several authors [8,13,14].

So far only two studies have confronted the ambiguous identification of certain isolates from the Ab group, either using alternative sample preparation protocols [16] or by coupling MALDI-TOF/MS with chemometric methods [17]. These novel approaches have certainly improved the differentiation of the former members of the Ab group, but they have failed to provide automated spectra acquisition linked to automated species identification and, therefore, cannot be successfully implemented in routine clinical laboratories. In addition, none of these studies have thoroughly evaluated the identification of the novel members of the Ab group, *A. dijkshoorniae* and *A. seifertii*.

The results from the spectral analysis and the validation of the SNN pattern recognition model in our study suggest that conventional and automated MALDI-TOF/MS identification of all the current members of the Ab group is possible with an updated reference taxonomy database. We have created novel reference signatures (MSPs) to improve the identification of group II *A. nosocomialis* isolates as well as of the novel species *A. dijkshoorniae* and *A. seifertii*. Cluster analysis of the novel MSPs together with those already present in the default Bruker taxonomy database also support the unambiguous identification of all species using this technology. Of note, the MSPs from isolates *A. pittii* serovar 18 DSM9341 and *A. pittii* serovar 22 DSM9318 are clustered together with those of *A. dijkshoorniae*, and the MSP from *A. baumannii* CS_62_1 BRB is clustered together with the MSPs from *A. seifertii* isolates (Fig. 4), emphasizing that the species identification of these isolates should be revisited since the characterization of novel *Acinetobacter* species.

Bacterial identification by MALDI-TOF/MS using our custom taxonomy database has shown correct identification of all *Acinetobacter* species within the Ab group with sensitivity and specificity values well above 98% when using spectra from bacterial extracts, and above 91% and 98%, respectively, when using spectra directly from bacterial colonies. It should be noted, however, that the quality of spectra with the use of direct colonies highly depends on technician expertise during sample loading, with identification rates varying greatly.

Inclusion of these novel MSPs into the Bruker taxonomy database should allow rapid automated identification of all the *Acinetobacter* species within the group, contributing to the assessment of the clinical and epidemiological relevance of the different species in the Ab group and, eventually, improving the treatment and management of *Acinetobacter* infections [20].

We acknowledge that the small number of isolates included might have been a limitation in our study, in particular for *A. pittii*. *A. pittii* isolates show the largest variability, both regarding spectra profiles and genetic sequences, and although there is no cross-identification between *A. pittii* and other *Acinetobacter* spp., the inclusion of additional strains may contribute to further delineate this species.

It is also clear from this study that achieving correct identification of bacterial species by MALDI-TOF/MS relies strongly on the accuracy and robustness of the reference database, which needs to be constantly refined and validated on a par with an evolving taxonomic classification.

Transparency declaration

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Appendix A. Supporting information

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MALDI-TOF/MS identification of species from the *Acinetobacter baumannii* (Ab) group revisited: inclusion of the novel *A. seifertii* and *A. dijkshoorniae* species

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Running head: MALDI-TOF/MS identification of *Acinetobacter* spp. revisited

Keywords: *Acinetobacter*, MALDI-TOF/MS, *rpoB*, MLSA, ClinProTools.

Supplementary Material

TABLE S1. Source and characteristics of *Acinetobacter* strains of the Ab group used in this study.

Clinical specimens were of human and environmental origin. The concatenated sequences of the seven genes used in MLSA are available under the Pasteur scheme ST codes at http://pubmlst.org/abaumannii/. Collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection, University of Göteborg, Sweden; CECT, Spanish Type Culture Collection, Universidad de Valencia, Valencia Spain; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris; CUHK, The Chinese University of Hong Kong; LMD, Laboratory Microbiology Delft collection, now hosted by Centraal Bureau Schimmelcultures, CBS, Utrecht, The Netherlands; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium. ANC and NIPH are strain designations used by A. Nemec; LUH and RUH are strain designations used by L. Dijkshoorn; R10, R00 and JV are strain designations used by J. Vila; SCOPE, SH and HS are strain designations used by H. Seifert; TH are strain designations used by G. Huys.

Strain	Country and year of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
***Acinetobacter baumannii* (n=16)**						
ATCC 19606^T (LMG 1041^T // NIPH 501^T // CIP 70.34^T)	USA, 1948	Urine	ST52	EU477108	APRG00000000	[1]
ATCC 17978 (CIP 53.77//LMG 1025)	USA, 1967	Cerebrospinal fluid	ST437	CP000521	CP000521	[2]
JV-Ab30	Spain, 2000	Human sample[a]	ST273	KU167844		[3]
R10-JV08	Spain, 2010	Human sample[a]	ST2	KU167841		This study
R10-JV108	Spain, 2010	Human sample[a]	ST2	KU167843		This study
R10-JV137	Spain, 2010	Human sample[a]	ST2	KU167834		This study
R10-JV174	Spain, 2010	Human sample[a]	ST2	KU167835		This study
R10-JV210	Spain, 2010	Human sample[a]	ST2	KU167836		This study
R10-JV226	Spain, 2010	Human sample[a]	ST181	KU167837		[3]
R10-JV247	Spain, 2010	Human sample[a]	ST2	KU167838		This study
R10-JV260	Spain, 2010	Human sample[a]	ST181	KU167839		This study
R10-JV295	Spain, 2010	Human sample[a]	ST2	KU167840		[3]
R10-JV80	Spain, 2010	Human sample[a]	ST632	KU167842		[3]
R10-JV83	Spain, 2010	Human sample[a]	ST632	KU167843		This study
RUH 134 (CCM 7290 // LMG 10541 //NIPH 528)	The Netherlands, 1982	Urine	ST2	HQ123410	APRB00000000	L. Dijkshoorn [4]
RUH 875 (CCM 7289 // LMG 10543 // NIPH 527)	The Netherlands, 1984	Urine	ST1	EU477108	APQW00000000	[4]
***Acinetobacter dijkshoorniae* (n=12)**						
JVAP01^T (CECT 9134^T // LMG 29605^T)	Turkey, 2009	Urine	ST606	KJ600793	LJPG00000000	[5]
LUH 07351	The Netherlands, 2001	Nephrology drain	ST565	KJ600787		[5]
LUH 08258 (ACI 749*)	Belgium, 2002	Catheter	ST566	KU167880		L. Dijkshoorn <<M. Vaneechoutte
LUH 09407	The Netherlands, 2004	Wound	ST801	KJ600791		[5]
LUH 10243	The Netherlands, 2005	Sputum	ST802	KJ600790		L. Dijkshoorn [5]
LUH 10297 (TH344)	Thailand, 2005	Freshwater fishpond farm	ST798	KU167882		[3]
LUH 13623 (AN-26*)	Italy, 2004	Sputum	ST800	KU167883		L. Dijkshoorn<<APSI-group [6]
SCOPE 271 (ANC 4052 // LUH 14530)	USA, 1995	Blood	ST568	KU167884	APQO00000000	[7]
R10-JV222	Spain, 2010	Human sample[a]	ST796	KU167878		[3]
R10-JV463	Spain, 2010	Human sample[a]	ST797	KU167879		[3]
RUH 0053 (LMD 71.43)	The Netherlands	Not Known	ST564	KJ600789		[5]
SCOPE 289 (RUH 14531)	USA, 1995	Blood	ST799	KU167885		[7]
***Acinetobacter nosocomialis* (n=24)**						
RUH 2376^T (CCUG 61663^T // LMG 10619^T // NIPH 2119^T)	The Netherlands, 1987	Sputum	ST76	HQ123395	APOP00000000	L. Dijkshoorn [4]

Strain	Country and year of isolation	Specimen	ST	NCBI accession nº		Donor and/or (Reference)
				rpoB gene	Whole genome	
JV-158029	Spain, 2011	Urine	ST68	KU167847		[8]
RUH 2041 (LMG 10621)	The Netherlands, 1990	Autopsy	ST410	HQ123392		L. Dijkshoorn
RUH 2211 (LMG 10625 // MGH 100*)	Sweden, 1990	Gastric fistula	ST359	HQ123393		L. Dijkshoorn<<I. Tjernberg
RUH-2212 (LMG 10626 // MGH165*)	Sweden, 1990	Urine	ST68	HQ123394		L. Dijkshoorn<<I. Tjernberg
RUH-2284 (LMG 10622)	The Netherlands, 1990	Bronchus	ST68	HQ123395		L. Dijkshoorn
RUH-2285 (LMG 10618)	The Netherlands, 1990	Bronchus	ST68	KU167859		L. Dijkshoorn
RUH 2624 (NIPH 2120)	The Netherlands, 1990	Skin	410	KJ956466	ACQF00000000	L. Dijkshoorn [4]
RUH 2627 (LMG 10623)	The Netherlands, 1990	Rectum	ST410	HQ123396		L. Dijkshoorn [4]
RUH 412 (LMG 10624)	The Netherlands, 1990	Blood	ST787	HQ123390		L. Dijkshoorn [4]
RUH 503	The Netherlands, 1990	Urine	ST68	HQ123391		L. Dijkshoorn [4]
SCOPE 150	USA, 1995	Blood	ST501	KU167846		[7]
SCOPE 192	USA, 1995	Blood	ST71	KU167848		[7]
SCOPE 212	USA, 1995	Blood	ST786	KU167849		[7]
SCOPE 249	USA, 1995	Blood	ST279	KU167850		[7]
SCOPE 54	Akron, OH, USA, 1995	Blood	ST784	KU167851		[7]
SCOPE 59	USA, 1995	Blood	ST781	KU167852		[7]
SCOPE 7	USA, 1995	Blood	ST543	KU167845		[7]
SCOPE 73	USA, 1995	Blood	ST530	KU167853		[7]
SCOPE 76	USA, 1995	Blood	ST785	KU167854		[7]
SCOPE 79	Richmond, VA, USA, 1995	Blood	ST781	KU167855		[7]
SCOPE 80	USA, 1995	Blood	ST781	KU167856		[7]
SCOPE 81	Richmond, VA, USA, 1995	Blood	ST782	KU167857		[7]
SCOPE 95	USA, 1995	Blood	ST395	KU167858		[7]
Acinetobacter pittii **(n=15)**						
JV-2628	Colombia, 2010	Catheter	ST209	KU167874		[9]
JV-Ab41	Spain, 2000	Blood	ST788	KU167873		[3]
JV-Gr0527H10	Spain, 2000	Blood	ST793	KU167866		This study
JV-IRS_Fr	France, 2010	Blood	ST64	KU167875		[3]
JVAP02	Turkey, 2006	Blood	ST457	KU167876		[2]
R00-JV14	Spain, 2000	Human sample[a]	ST789	KU167861		[3]
R00-JV21	Spain, 2000	Human sample[a]	ST667	KU167863		[3]
R00-JV243	Spain, 2000	Human sample[a]	ST792	KU167872		This study
R00-JV60	Spain, 2000	Human sample[a]	ST783	KU167867		[3]
R00-JV65	Spain, 2000	Human sample[a]	ST790	KU167868		[3]
R00-JV90	Spain, 2000	Human sample[a]	ST791	KU167869		[3]
R10-JV2	Spain, 2010	Human sample[a]	ST794	KU167860		[3]
R10-JV23	Spain, 2010	Human sample[a]	ST214	KU167864		[3]

Strain	Country and year of isolation	Specimen	ST	NCBI accession n°		Donor and/or (Reference)
				rpoB gene	Whole genome	
R10-JV28	Spain, 2010	Human sample[a]	ST795	KU167865		[3]
RUH 509 (LMG 10559)	The Netherlands, 1984	Bronchus	ST72	HQ123375		L. Dijkshoorn
***Acinetobacter seifertii* (n=11)**						
NIPH 973^T (CIP 110471^T // CCM 8535^T // CCUG 34785^T // SSI 1090^T*)	Denmark, 1990	Ulcer	ST91	EU477126	APOO00000000	L. Dijkshoorn<<P. Gerner-Smidt [10]
SCOPE 006 (ANC 4045)	USA, 1995	Blood	ST483	KJ956427		[10]
SCOPE 012 (ANC 4046)	USA, 1995	Blood	ST546	KJ956428		[10]
SCOPE 035 (ANC 4049)	USA, 1995	Blood	ST548	KJ956430		[10]
LUH 01471 (CCUG 34787 //NIPH 826 // SSI 5804*)	Sweden, 1990	Blood	ST90	HQ123426		L. Dijkshoorn<<P. Gerner-Smidt [10]
LUH 05789 (CUHK 3088 // NIPH 1777)	Hong Kong, 1997	Blood	ST549	KJ956461		L. Dijkshoorn<<E. Houang [10]
LUH 05793 (CUHK 7611 // NIPH 1781)	Hong Kong, 1998	Soil	ST551	KJ956464		L. Dijkshoorn<<E. Houang [10]
LUH 08128	The Netherlands, 2002	Catheter	ST803	KU167888		L. Dijkshoorn
R00-JV54	Spain, 2000	Human sample[a]	ST834	KU726606		[3]
RUH 1139 (NIPH 806)	The Netherlands, 1985	Throat swab	ST545	KJ956457		L. Dijkshoorn [10]
RUH-3243						

[a] Strains recovered from human patients but the specimen is unknown

*Strain designation used by the donor

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Manuscript 4

Isolation of different *Acinetobacter* species from market meat: first identification of *Acinetobacter dijkshoorniae* in Peru

Isolation of different *Acinetobacter* species from market meat: first identification of *Acinetobacter dijkshoorniae* in Peru

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Objectives: The aim of the present study was to analyse the phenotypic and genotypic characteristics of *Acinetobacter* spp. recovered from meat samples in Peru.

Material and methods:
Meat samples were obtained by random sampling of traditional markets. Samples were homogenised and 2 g each were used to enrich the bacterial burden in overnight LB cultures. Bacterial colonies were isolated in different selective media agar plates. Identification at the species level was performed by MALDI-TOF MS, *rpoB* sequencing and multilocus sequene analysis (MLSA). Clonal relatedness was assessed by multi-locus sequence typing (MLST) and pulse-field gel electrophoresis (PFGE). Antimicrobial susceptibility was determined by gradient difusion and broth microdilution.

Results:
Twelve strains of *Acinetobacter* spp. were isolated from five different calves out of 138 meat samples obtained from two independent central markets in Lima, Peru. Species identification studies recognised 9 strains as *A. pittii*, 1 strain as *A. baumannii*, 1 strain as the recently described novel species *A. dijkshoorniae,* and 1 strain which was identified as *A. beriziniae*. Six strains were further selected according to either their unique PFGE profiles or if recovered from different calves. All strains were susceptible to all antibiotics tested and some presented novel MLST alleles and ST types.

Conclusions: The isolation of pathogenic *Acinetobacter* species from human consumption meat may represent a risk to public health, and environmental sources such as animals and food should not be disregarded as potential reservoirs for the spread of these pathogens into community and healthcare settings. This is the first time that the novel *Acinetobacter dijkshoorniae* species is identified in Peru and from meat samples.

Isolation of different *Acinetobacter* species from market meat: first identification of *Acinetobacter dijkshoorniae* in Peru

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The genus *Acinetobacter* currently comprises up to 60 species with validly published species names and 6 species with tentative species designations (https://apps.szu.cz/anemec/Classification.pdf, last accessed December 2018), some of them environmental and some considered human pathogens. Among the latter, species within the *Acinetobacter baumannii* (Ab) group are of particular clinical relevance, often associated with nosocomial infections and outbreaks [1–3]. *A. baumannii* is clearly the most prevalent pathogen of the Ab group, probably due to its inherent ability to persist and survive in the hospital environment as well as to acquire resistance to multiple antimicrobial drugs and disinfectants [4]. Despite the clinical relevance of members of the Ab group, pathogenic *Acinetobacter* spp. have also been reported from food and food-producing animals, which might constitute an overlooked reservoir and source of bacterial pathogens to the human population [5–8].

The aim of the present study was to analyse the phenotypic and genotypic characteristics of *Acinetobacter* spp. recovered from market meat samples in Peru.

From July through August 2012, 138 meat samples from poultry (n=64), swine (n=30) and beef (n=44) were obtained by random sampling from six traditional markets scattered throughout the city of Lima, Peru. Meat samples were transported in sterile bags to the laboratory, homogenised in a paddle blender (Stomacher® 400 circulator, Seward, UK) and 2 g of each were used to enrich the bacterial burden in overnight lysogeny broth (LB) cultures. Liquid cultures were plated in different agar mediums such as Xylose Lysine Deoxycholate (XLD) agar, Salmonella-Shigella (SS) agar, MacConkey agar and Hektoen agar from Oxoid (Oxoid®, Cambridge, UK) to select for different Enterobacteria and other Gram-negative bacilli. Twelve strains of *Acinetobacter* spp. were isolated in MacConkey agar plates from the meat samples of five different calves obtained in two independent markets in Lima, Peru.

Species identification of the twelve isolates was initially performed by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) in a Microflex LT benchtop instrument (Bruker Daltonics, Bremen, Germany) by comparing the spectra profiles against the updated Bruker taxonomy database, which included reference spectra for *Acinetobacter dijkshoorniae* and *Acinetobacter seifertii*, the novel members of the Ab group [9].

Three isolates were identified as *A. baumannii*, *A. guillouiae* and *A. dijkshoorniae*, respectively, and the remaining 9 isolates were identified as *A. pittii*. Of note, the log score values for the identification of *A. guillouiae* as well as 5 out of the 9 *A. pittii* isolates ranged between 1.9 and 2.01, which constitutes a poor probable species identification, according to the manufacturer's specifications.

Pulsed-field gel electrophoresis (PFGE) analysis of ApaI-digested genomic DNA revealed five distinct fingerprints (A-E) (**TABLE 1**). *A. pittii* isolates recovered from meat samples from the same calf shared identical fingerprints (B and D), except for one *A. pittii* isolate that was isolated from samples derived from a third calf (together with *A. guillouiae*) and also presented the D fingerprint.

Six isolates were selected for further characterisation, one representative isolate from each fingerprint plus the *A. pittii* isolate that derived from a different calf. Identification at the species level for these isolates was re-evaluated using two different molecular methods; sequencing of the partial *rpoB* gene sequences (zones 1 and 2) according to La Scola *et al.* [10], and by multilocus sequence analysis (MLSA) of the concatenated partial sequences of all house-keeping genes used for multilocus sequence typing (MLST), according to the Pasteur scheme [11]. Sequence alignment was done with Clustal Ω and phylogenetic trees were constructed using the neighbour-joining method, with genetic distances computed by Kimura's two-parameter model [12]. Sequences were compared with those of reference strains belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex as well as other *Acinetobacter* species that were retrieved from public repositories [3].

Molecular methods confirmed the identification of *A. baumannii*, *A. dijkshoorniae* and *A. pittii* isolates from fingerprints A-C, as expected. Isolates from fingerprint D, showed closest similarity to *A. pittii* (97.3-98.1% and 98.4-99.0% for *rpoB* and MLSA, respectively) and were considered as such, even though the *A. pittii* group often embraces strains that are somehow related to each other but do not always meet the technical requirements used for species circumscription. Finally, the isolate corresponding to fingerprint E, designated as *A. guillouiae* by MALDI-TOF MS albeit with an ambiguous identification score, clustered together with *A. bereziniae* isolates with 96.4-96.9% and 96.6-96.7% similarity for *rpoB* and MLSA, respectively. According to these data, this isolate was designated as *A. bereziniae*-like although whole-genome sequencing could aid to clarify the taxonomic nature of this isolate. Nevertheless, we acknowledge that additional isolates with similar characteristics are needed before proposing the existence of new taxa. It is worth noticing though that, at the time of the study, there were no reference spectra for *A. bereziniae* in the Bruker taxonomy database (there was for *A. guillouiae*), but during the writing of the

present report the Bruker database was updated to include two reference spectra for *A. bereziniae*. Re-identification of this isolate by MALDI-TOF MS currently matches with *A. bereziniae* (**TABLE 1**).

Antimicrobial susceptibility testing of the selected isolates by gradient diffusion and broth microdilution (colistin) showed low minimum inhibitory concentration (MIC) values to all the antimicrobial agents tested except for chloramphenicol, to which MIC values below 8 mg/L were only observed in *A. pittii* isolates representative from fingerprint D (**TABLE 1**). MLST studies identified the *A. baumannii* isolate as belonging to sequence type (ST) ST273, which had been previously reported in 2000 from a hospitalised patient in Spain and, interestingly, had also been identified from a calf in Switzerland in 2013 [5]. ST273 is clustered in to clonal complex (CC) CC33, which contains STs related to clinical isolates. The *A. pittii* isolates from fingerprint D were assigned to ST312, an ST previously described from a patient in Belgium in 2009 but to our knowledge, never reported in animals and not included in any CC. The *A. dijkshoorniae*, *A. bereziniae* and the *A. pittii* isolate from fingerprint B all presented novel MLST alleles and were assigned new STs (ST1256, ST1258 and ST1257, respectively) by the curators at https://pubmlst.org/. None of them clustered in any CC either.

The identification of *Acinetobacter* isolates belonging to several species of the Ab group in raw meat samples is of particular concern, since these species are usually associated with the clinical setting. Previous studies had already reported the presence of species belonging to this group from diverse meat samples, including poultry, swine and beef, although members of the Ab group were not necessarily the most prevalent species [8]. In our study, *Acinetobacter* isolates were only recovered from beef samples and the majority of isolates were identified as *A. pittii*. Interestingly, another study from Lebanon also reported the presence of *A. baumannii, A. pittii* and *A. bereziniae* in cow meat samples, with a prevalence similar to that observed in our investigation (28% and 27%, respectively) and *A. pittii* was again the predominant *Acinetobacter* species [14]. It is worth mentioning, however, that the present study was not initially designed to identify *Acinetobacter* spp. from food samples and, therefore, the lack of specific enrichment media or pre-enrichment steps might have underestimated the actual burden of *Acinetobacter* spp. isolates.

Multidrug resistant *Acinetobacter* spp. from the Ab group currently represent a serious threat to public health and, despite the fact that the *Acinetobacter* isolates recovered in this study were generally susceptible to clinically relevant antibiotics, contaminated meat should not be neglected as a source for the transmission of *Acinetobacter* spp. into domestic and hospital settings where it may also contribute to the evolution of clinical lineages [15].

Of note, we have also reported here the first identification of the recently described *A. dijkshoorniae* in meat samples of animal origin as well as its first identification in Peru.

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TABLE 1. Characteristics of the *Acinetobacter* spp. isolates recovered from market meat samples in Lima, Peru.

Rows display the genotypic and phenotypic information under the same pulsed-field gel electrophoresis (PFGE) cluster and/or origin of the samples. Data was obtained from a representative isolate each. Isolate, designation of the representative isolate for each pulsotype; n, number of isolates; Calf, animal from which they were recovered; Area, the location of the markets in Lima; Species, identification to the species level; ST, sequence type; MIC, minimum inhibitory concentration; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; CIP, ciprofloxacin; LVX, levofloxacin; COL, colistin; FEP, cefepime; CAZ, ceftazidime; MEM, meropenem; IMI, imipenem; CHL, chloramphenicol; TGC, tigecycline.

							MIC (μg/mL)											
PFGE	**n**	**Calf**	**Area**	**Isolate**	**Species**	**ST**	**AMK**	**TOB**	**KAN**	**CIP**	**LVX**	**COL**	**FEP**	**CAZ**	**MEM**	**IPM**	**CHL**	**TGC**
A	1	I	North	APT-1	*A. dijkshoorniae*	1256	2	0,19	1,5	0,5	0,38	0,38	4	4	0,25	0,25	256	0,5
B	4	II	North	APT-5	*A. pittii*	1257	3	0,19	1,5	0,25	0,38	0,38	4	6	0,38	0,38	96	0,38
C	1	III	Center	APT-6	*A. baumannii*	273	2	0,25	1	0,25	0,25	0,5	2	3	0,25	0,25	96	0,38
D	1	IV	Center	APT-7B	*A. pittii*	312	1,5	0,125	1	0,25	0,25	0,125	3	3	0,19	0,25	4	0,38
E	1	IV	Center	APT-7T	*A. bereziniae*-like	1258	0,75	0,047	0,047	0,25	0,25	1,5	1	4	0,38	0,25	16	0,38
D	4	V	Center	APT-8	*A. pittii*	312	1,5	0,125	1	0,38	0,25	0,125	3	3	0,25	0,25	8	0,19

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Manuscript 5

In vitro and *in vivo* virulence potential of the emergent species of the Ab group

In vitro and *in vivo* virulence potential of the emergent species of the Ab group

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Hypothesis: Species of the *A. baumannii* (Ab) group other than *A. baumannii* may pose a health challenge and further studies evaluating their distinct traits are needed.

Objectives: Identify any differential phenotypic characteristics, in terms of antimicrobial susceptibility and *in vitro* virulence potential, of the members of the Ab group.

Material and methods:
The antimicrobial susceptibility was determined by broth microdilution or gradient diffusion. The presence of genes encoding RND efflux pumps and intrinsic OXA groups was evaluated by PCR. Biofilm formation was assessed in 96-well plates using the crystal violet quantification method after 44 h of incubation at 28ºC or 37ºC. The surface-associated motility was measured after 18 h of growth at 37ºC. The virulence was studied as the ability to kill nematode worms using a *Caenorhabditis elegans* infection assay.

Results:
Seventy-six isolates of the 5 species within the Ab group (16 *A. baumannii*, 12 *A. dijkshoorniae*, 16 *A. nosocomialis*, 20 *A. pittii*, and 12 *A. seifertii*) were compared. In good agreement with previous studies, antimicrobial resistance was common among *A. baumannii* isolates while isolates of all other species were generally more susceptible. Concomitant carriage of genes encoding different efflux pumps was frequent in all species and the presence of intrinsic class D β-lactamases was reported in *A. baumannii*, *A. dijkshoorniae* and *A. pittii* but not in *A. nosocomialis* and *A. seifertii*, although susceptibility profiles did not correlate with the presence of particular efflux systems or intrinsic class D β-lactamases. Overall, isolates of *A. baumannii* and *A. nosocomialis* presented a weaker pathogenic potential than those of *A. seifertii*, *A. pittii* and, especially, *A. dijkshoorniae*, meaning that isolates from the former species showed decreased biofilm formation and required a longer time to kill *C. elegans* in our model.

Conclusions: Although their clinical impact remains to be determined, these results suggest a distinct degree of adaptation to the nosocomial setting of the *Acinetobacter* species within the Ab group, but also highlight the potential clinical relevance of the emerging species of the group.

In vitro and *in vivo* virulence potential of the emergent species of the Ab group

Running title:

Virulence and resistance of *Acinetobacter* spp.

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Keywords:

Acinetobacter, multidrug-resistance, virulence, biofilm formation, motility

Abstract

The introduction into the clinical practice of molecular identification methods as well as mass spectrometry has revealed that *Acinetobacter* spp. of the *A. baumannii* (Ab) group other than *A. baumannii* are increasingly being recovered from human samples and may pose a health challenge if neglected. In the present study 76 isolates of 5 species within the Ab group, including 16 *A. baumannii*, 12 *A. dijkshoorniae*, 16 *A. nosocomialis*, 20 *A. pittii*, and 12 *A. seifertii*, were compared in terms of antimicrobial susceptibility, carriage of intrinsic resistance genes, biofilm formation, and the ability to kill nematode worms using a *Caenorhabditis elegans* infection assay. In good agreement with previous studies, antimicrobial resistance was common among *A. baumannii* isolates while isolates of all other species were generally more susceptible. Concomitant carriage of genes encoding different efflux pumps was frequent in all species and the presence of intrinsic class D β-lactamases was reported in *A. baumannii*, *A. dijkshoorniae* and *A. pittii* but not in *A. nosocomialis* and *A. seifertii,* although susceptibility profiles did not correlate with the presence of particular efflux systems or intrinsic class D β-lactamases. Overall, isolates of *A. baumannii* and *A. nosocomialis* presented a weaker pathogenic potential than those of *A. seifertii*, *A. pittii* and, especially*, A. dijkshoorniae*, meaning that isolates from the former species showed decreased biofilm formation and required a longer time to kill *C. elegans* in our model. Although their clinical impact remains to be determined, these results suggest a distinct degree of adaptation to the nosocomial setting of the *Acinetobacter* species within the Ab group, but also highlight the potential clinical relevance of the emerging species of the group.

Introduction

Among the 60 validly named *Acinetobacter* species (https://apps.szu.cz/anemec/Classification.pdf, last accessed December 2018), *Acinetobacter baumannii* has, for many years, monopolised our attention, as shown by its growing importance as a nosocomial pathogen since the late 1990s. Capable of causing bloodstream infections, ventilator-associated pneumonia, wound infections, and meningitis, among other infections, *A. baumannii* excels in its ability to persist and survive in the nosocomial settings[1]. In comparison, however, little is known regarding the closely related members of the so-called *A. baumannii* (Ab) group, such as *Acinetobacter nosocomialis* and *Acinetobacter pittii*, previously known members of the group, but also *Acinetobacter dijkshoorniae* and *Acinetobacter seifertii*, whose species

status has recently been acknowledged[2,3]. Species other than *A. baumannii* among the Ab group are increasingly being isolated from human specimens, albeit not as frequently as *A. baumannii,* and they also possess an undeniable ability to cause disease[4-9]. Unfortunately, they are often erroneously identified as *A. baumannii* due to the difficulty of distinguishing them using common phenotypic methods such as biochemical test and semi-automated identification systems, thus underestimating their true prevalence and clinical relevance. Accurate species identification can only be achieved by molecular methods, such as AR-DRA, *rpoB* and *gyrB* sequence analysis[10-12] and, more recently, mass spectrometry[13]. However, species-level identification using phenotypic tests is still widely employed, leading to incorrect premises in the literature regarding the biology of the Ab group.

Antimicrobial resistance surveillance studies containing information about *A. dijkshoorniae*, *A. nosocomialis*, *A. pittii* and *A. seifertii* are scarce, although existing data support their general susceptibility to most antibiotics[5,7,14,15]. In contrast, *A. baumannii* is feared in the clinical settings because of its frequently reported multidrug resistant (MDR) phenotype. This pathogen has developed resistance to all antibiotics, including last resort carbapenems, tigecycline and colistin[16,17]. Antimicrobial resistance represents one of the best assets of *A. baumannii* and may account for its success in the hospital setting; nonetheless other pathogenic attributes may as well impact its ability to infect the host.

Likewise, there is a good amount of information on the virulence traits of *A. baumannii*[18] but little is known regarding the other members of the Ab group. Comparison studies regarding the virulence attributes of different *Acinetobacter* species are rare, and usually include only a few isolates[19-22] or are limited to bloodstream isolates from a unique region[23]. Noticeably, due to its recent classification as a novel species, none of the abovementioned studies include information about *A. dijkshoorniae* and there is just one report (published prior to its species designation) that highlights its ability to cause disease and carry genetic determinants conferring carbapenem resistance[24].

A few reports, however, have suggested relevant differences in the clinical outcomes of infections caused by former members of the Ab group[14,25-27], suggesting that, in order to understand the clinical relevance and the virulence potential of closely related *Acinetobacter* spp., further studies on susceptibility patterns and pathogenesis need to address them as distinct entities rather than as a homogeneous group. In this work we have gathered a collection of representative isolates from all 5 known *Acinetobacter* species of the Ab group and compared their antibiotic susceptibility profiles and carriage of resistance determinants as well as their pathogenic potential using both *in vitro* and *in vivo* assays.

Results

Antimicrobial susceptibility profiles. In this study we have characterised the antimicrobial susceptibility patterns of 76 bacterial isolates from 5 different *Acinetobacter* species within the Ab group, as shown in **TABLE 1**. Overall, isolates other than *A. baumannii* were usually susceptible to almost all antibiotics tested, with only a few exceptions. The *A. seifertii* isolates from our collection were susceptible to all the antimicrobial agents tested, thus constituting the species with the lowest rates of resistance. Likewise, isolates of *A. nosocomialis* were susceptible to all agents except the aminoglycosides, for which rates of resistance were 25% for gentamicin and 5% for amikacin.

Carbapenem-resistance was identified in three *A. pittii* isolates and one *A. dijkshoorniae* isolate, and was associated with the presence of genes encoding NDM or OXA-type enzymes (**TABLE S1**). *A. pittii* and *A. dijkshoorniae* isolates, however, tested susceptible to almost all other antimicrobial agents but ceftazidime, with rates of resistance of 5% and 25%, respectively. One *A. pittii* isolate was resistant to ciprofloxacin and another isolate of the same species was resistant to gentamicin. In contrast, *A. baumannii* isolates presented higher rates of resistance (75% for ceftazidime, 68.8% for ciprofloxacin and gentamicin, 62.5% for imipenem, 56.3% for tobramycin, 43.8% for meropenem and 31.5% for amikacin), and resistance to carbapenems was usually associated with the production of OXA-type carbapenemases (**TABLE S1**). Furthermore, resistance to 3 or more antimicrobials agents from different categories, i.e., multidrug resistance[28], was determined in 62.5% of *A. baumannii* isolates, while no MDR isolates were found in species other than *A. baumannii*. Finally, all the species of the Ab group showed low minimum inhibitory concentration (MIC) values for tigecycline (**TABLE 1**) and, among all the isolates of the Ab group, only one *A. baumannii* isolate, which was already considered MDR, was resistant to colistin with an MIC of 8 mg/mL.

Distribution of resistance nodulation cell division (RND) efflux pumps and presence of intrinsic OXAs. The occurrence of the different RND efflux pumps in the species of the Ab group is shown in **FIG 1A-D**. All isolates encoded at least one of the RND efflux systems known to be associated with antimicrobial resistance, and it was common to detect three or even four different efflux pumps in isolates from all *Acinetobacter* species (**TABLE S1**). *adeG*-like and *adeJ*-like genes were identified in all species and were commonly present in most isolates (80 to 100% and 50 to 100%, respectively). *adeE*-like was highly disseminated within *A. nosocomialis* (100%) and *A. pittii* (90%), and was also present in 75% of *A. seifertii* isolates. However, *adeE* was only found in one isolate

of *A. dijkshoorniae* and it was missing in *A. baumannii*. The *adeB*-like gene, on the other hand, was absent in *A. seifertii*, was infrequently present in *A. baumannii* (37.5%), but was ubiquitous in *A. nosocomialis* (100%). Interestingly, isolates from *A. pittii* and *A. dijkshoorniae* carried a distinct *adeB*-like gene with 75% and 86% similarity to the *adeB* gene from *A. baumannii*, respectively. This gene was present in 90% and 66.7% of *A. pittii* and *A. dijkshoorniae* isolates, respectively.

In addition to the presence of different RND efflux pumps, we also evaluated the carriage of genes encoding intrinsic class D enzymes (OXA-type). Intrinsic OXA genes were reported in all *A. baumannii, A. dijkshoorniae* and *A. pittii* isolates but we were not able to detect them in any of the *A. nosocomialis* and *A. seifertii* isolates.

Cluster analysis of all sequences revealed that the OXA variants harboured by members of each of the three *Acinetobacter* species clustered together with high bootstrap values (**FIG 2**), the only exception being the intrinsic OXA β-lactamase of the *A. dijkshoorniae* RUH-14531 isolate that clustered together with OXA sequences from *A. pittii*.

Biofilm formation at 28ºC and 37ºC. Biofilm formation values at both 28°C and 37°C showed a high degree of variability among isolates of each of the five different *Acinetobacter* species included in the study (**FIG 3A**). Nevertheless, statistically significant differences were observed between some species at each temperature; *A. dijkshoorniae* formed significantly more biofilm than *A. baumannii* at both temperatures and than *A. nosocomialis* at 37ºC (Kruskal-Wallis test, *post hoc P*-value of 0.009, 0.001 and 0.004, respectively); and *A. pittii* and *A. seifertii* produced significantly more biofilm than *A. baumannii* at 37ºC (Kruskal-Wallis test, *post hoc P*-values of 0.024 and 0.035, respectively). Despite the high dispersion of the data, and thus the lack of statistical significant differences for most pairwise comparisons, there were a few observations worth mentioning. *A. baumannii* isolates tended to form less biofilm than those of the other species, and biofilm formation was similar between *A. dijkshoorniae*, *A. pittii* and *A. seifertii*. Likewise, biofilm formation in *A. nosocomialis* resembled that of other Ab group species other than *A. baumannii* at 28ºC but at 37ºC it was comparable to that of *A. baumannii*.

Similar trends were observed when classifying isolates as biofilm producers and non-producers (**FIG 3B**). Our results showed that all *A. dijkshoorniae* isolates produced biofilm at both temperatures, and that the genetically related *A. pittii* species also presented a high proportion of biofilm-producing isolates (90%). In contrast, *A. baumannii* was the species with the least amount of biofilm-producing isolates, 50% or less, depending on the temperature. Also, upon shifting the temperature from 28ºC to 37ºC, a reduction in the

number of biofilm-producing isolates was observed in *A. baumannii*, *A. nosocomialis* and *A. seifertii,* while the number of biofilm-producing isolates remained unchanged in *A. dijkshoorniae* and *A. pittii*. While such reduction was not statistically significant for *A. baumannii* and *A. seifertii* (from 50% to 43.8%, and from 83.3% to 75%, respectively), the effect of the temperature was greater in *A. nosocomialis*, in which shifting the temperature from 28°C to 37°C decreased the percentage of biofilm-producing isolates from 93.8% to 43.8% (**FIG 3B**, McNemar test, *P*-value of 0.008). Thus, and under our experimental conditions, half of the isolates of this species were able to develop biofilm at 28°C but not at 37°C, and even in those isolates that did not lose the ability to produce biofilm at 37ºC, a net reduction in the biofilm formation values was observed (Wilcoxon rank sum test, *P*-value of 0.01). On the other hand, the biofilm formation values of *A. dijkshoorniae* and *A. pittii* isolates were higher at 37°C than at 28°C (Wilcoxon rank sum test, *P*-value of 0.002 and 0.005, respectively; up to 2.3 fold changes, data not shown), although we did not detect biofilm non-producing isolates at 28ºC turning into biofilm-producers at 37ºC.

Surface-associated motility across species of the Ab group. In our collection, surface-associated motility was observed in isolates of all the species studied, with greater intra-species variability for *A. baumannii*, *A. nosocomialis* and *A. seifertii* isolates (mean values ± SD (cm) of 2.315 ± 1.722, 2.078 ± 1.775 and 3.188 ± 1.755, respectively), than for *A. pittii* and *A. dijkshoorniae* (2.717 ± 0.829 and 1.653 ± 0.844, respectively) (**FIG 4**). Overall, differences in the distribution of surface-associated motility between species were not detected when taking into account the extent of motility. However, classification of the isolates into motile vs. non-motile according to the individual degree of motility showed that higher rates of motility were found in *A. pittii* (95%) and *A. dijkshoorniae* (83.3%), followed by *A. seifertii* and *A. baumannii* (75% and 62.5%, respectively). *A. nosocomialis* was found to be the less motile species of the group under the conditions tested with only half of the isolates being considered as motile. Interestingly, rates of motility resembled those described above regarding biofilm formation at 37°C for *A. nosocomialis*. There was a positive correlation between motility and biofilm formation at 37°C in all species except for *A. pittii*, (Spearman's correlation coefficients between 0.622 and 0.7128, and *P*-values of 0.006 to 0.033), meaning that, in general, the more motile an isolate was, the more biofilm it tended to produce.

Infection assays using the *C. elegans* infection model. Isolates from the different species studied were used to feed Fer-15 *C. elegans* line as a model of infection. The daily survival of the nematodes was scored during 15 days in order to obtain the lethal time 50% (LT50) values of each isolate. Determination of the LT50 values presented, once

more, ample intra-species variability (**FIG 5A**). Nevertheless, all isolates could be easily grouped into two distinct clusters according to their LT50 values. Those considered as virulent presented LT50 values ranging from 0.48 to 1.90 days and those included in the non-virulent group showed LT50 values ranging from 4.10 to 8.19 days. In view of these results, an LT50 cut-off value of 3 days was selected to differentiate virulent (LT50 < 3 days) from non-virulent isolates (LT50 > 3 days). Interestingly, all *A. baumannii* isolates were included within the non-virulent cluster, presenting LT50 values ranging from 4.85 to 8.04 days. *A. nosocomialis* was also shown to be an overall non-virulent species under this model, with 87.5% of isolates included in the non-virulent cluster and a median LT50 value of 4.65 days. On the other hand, according to this model, *A. dijkshoorniae* turned out to be the most virulent species (median LT50 of 0.871 days), with only one isolate included within the non-virulent group (**FIG 5A**). Of note, this was the only isolate of *A. dijkshoorniae* that carried a carbapenemase gene. In terms of virulence, *A. dijkshoorniae* presented significant differences in virulence only with *A. baumannii* (Kruskal-Wallis test, *post hoc P*-value <0.001).

Interestingly, *A. pittii* and *A. seifertii*, when considered as a whole, showed very large dispersion of the data and presented no significant differences compared to either *A. baumannii* or *A. dijkshoorniae* (**FIG 5A**). However, *A. pittii* and *A. seifertii* isolates could be clearly divided into virulent (with LT50 values comparable to those of *A. dijkshoorniae*) and non-virulent subgroups (with LT50 values similar to those of *A. baumannii* and *A. nosocomialis*). When considered as independent groups, the virulent isolates of both *A. pittii* and *A. seifertii* presented significant differences in terms of virulence when compared to *A. baumannii* (Kruskal-Wallis test, *post hoc P*-value <0.001 and 0.011, respectively), and the non-virulent isolates also presented significant differences when compared to *A. dijkshoorniae* (Kruskal-Wallis test, *post hoc P*-value <0.001 and 0.001, respectively) (**FIG 5B**).

In view of these results, we wondered if there were intra-species differences between the virulent and non-virulent subgroups of *A. pittii* and *A. seifertii* in regard to the other virulence-related phenotypes studied above, *i.e.* biofilm formation and motility, instead of considering these species as a whole. In *A. pittii*, isolates included within the virulent group were also shown to produce more biofilm at 28ºC as well as to be more motile than those isolates included in the non-virulent group (**FIG 5C** and **5D**, Mann-Whitney U test, *P*-value of 0.038 and 0.002, respectively). In *A. seifertii,* however, it was not possible to associate virulent and non-virulent isolates with any other phenotype as there were no significant differences between both groups.

Discussion

Despite the phenotypic similarities that hinder an accurate identification at the species level of the members of the Ab group using conventional identification procedures, here we have sought any relevant differences among them in terms of antibiotic susceptibility patterns and virulence potential. Our efforts are aimed at reinforcing the idea that reporting and studying them collectively may be misleading in the clinical setting, as infections caused by different members of the Ab group might have substantially different clinical implications, as it has been noted for *A. pittii* and *A. nosocomialis* but has not yet been thoroughly examined for *A. dijkshoorniae* and *A. seifertii*[14,25].

In terms of antimicrobial resistance, results from our study are in good agreement with those of previous investigations reporting overall less susceptibility to most antimicrobial agents in *A. baumannii,* while *A. pittii* and *A. nosocomialis* tended to be more susceptible[5,7,14,15]. Studies that report on the antimicrobial susceptibility of *A. seifertii* or *A. dijkshoorniae*, on the other hand, are almost non-existent, since both species have only recently been described. Nevertheless, a study by Karah *et al.* in 2011 included 3 isolates that, at the time, were classified as "genomic species *close to* 13TU", the former designation for *A. seifertii*[29]. In good agreement with our results, those isolates were susceptible to all antibiotics tested. Another report that identified 28/287 bloodstream isolates as "genomic species *close to* 13TU", detected two carbepenem-resistant *A. seifertii* isolates with a MDR phenotype, including resistance to colistin[30]. Although some authors have claimed that *A. seifertii* might be intrinsically resistant to polymyxins[31,32], we did not find colistin-resistant isolates of this species among our collection. Indeed, our results suggest that the novel members of the Ab group might be susceptible to most antibiotics, resembling the other non-*baumannii Acinetobacter* species.

Another characteristic of many *Acinetobacter* species is the carriage of naturally occurring oxacillinases (OXA enzymes), such as OXA-51 in *A. baumannii*, and it is widely accepted that members of the same *Acinetobacter* species seem to harbour OXA allelic variants that belong to the same OXA group[33]. Such intrinsic OXA groups may account for β-lactam resistance, especially when insertion sequences are found upstream from the *bla*$_{OXA}$ gene[34]. Nevertheless, bacterial strains belonging to *A. nosocomialis* and *A. seifertii* might be an exception since no intrinsic OXAs have yet been found in the genome sequences of *A. nosocomialis* and *A. seifertii*[33], except for the presence of plasmid-borne intrinsic OXA from *A. baumannii*[35]. In our study, also in good agreement with data from Périchon *et al.*[33], we confirmed the presence of species-specific intrinsic OXA genes in *A. baumannii*, *A. dijkshoorniae* and *A. pittii,* and the lack of them in *A. nosocomialis* and *A. seifertii* (**FIG 2**).

It is also worth mentioning that the prevalence of *adeB* in *A. baumannii* from our collection was lower (37.5%) than previously reported (53 – 97%)[36-41] but we acknowledge that isolates of *A. baumannii* in our study are the least polyclonal of all five species, although it is not absolutely clear what was the degree of clonality in previous studies either. Interestingly, the *adeB*-like gene was present in all the *A. nosocomialis* isolates as well as in many *A. dijkshoorniae* and *A. pittii* isolates, clearly confirming that this efflux system is not restricted to *A. baumannii,* as already noticed by Espinal *et al.*[4]. Likewise, *adeIJK* and *adeFGH* were previously considered as being highly specific of *A. baumannii*, and *adeDE* as being specific of A. *pittii*. Here we have shown that both *adeJ*-like and *adeG*-like genes are present in all species and are rather ubiquitous, and that *adeDE* was also present in all *A. nosocomialis* isolates and was detected among *A. seifertii* and *A. dijkshoorniae,* although it was missing in *A. baumannii*. In 2012 Hou *et al* reported a small number of *A. baumannii* isolates harbouring *adeE* together with *adeB,* albeit species identification was performed with phenotypic methods which we now know may lead to misidentification[42].

The study of virulence-related phenotypes revealed that biofilm formation and surface-associated motility remained highly variable among isolates from the same species. Indeed, many authors have pointed out that the variability observed in biofilm formation might be due to this phenotype being clone-specific[20,43-47]. Moreover, as previously described for *A. baumannii*[48], we observed that motile isolates displayed different surface-associated morphotypes, which were not species-dependent but, again, strain-specific (**Annex I**). Nonetheless, despite the intra-species variability observed, we were still able to find overall inter-species differences in the virulence phenotypes. In fact, the ability to produce biofilm not only differed between species but it was also temperature-dependent. Interestingly, temperature modulation of biofilm formation, as well as motility and antibiotic resistance, have been previously observed in *A. baumannii* ATCC 17978, although the underlying regulatory mechanisms remain unclear[49]. Our results suggest different thermoregulation strategies of biofilm formation between isolates of *A. baumannii*, *A. nosocomialis* and *A. seifertii* and those of *A. dijkshoorniae* and *A. pittii*.

Similarly, infection assays using the *C. elegans* animal model also revealed overall differences between isolates of *A. baumannii* and *A. nosocomialis,* being mostly non-virulent, and those of *A. dijkshoorniae,* being highly virulent, although statistical significance was only achieved between *A. baumannii* and *A. dijkshoorniae*. The infection assays also highlight the existence of two subpopulations within our collection of *A. pittii* and *A. seifertii* isolates although it remains unclear whether this observation represents the intrinsic heterogeneity within each species or it is an indication that the taxonomic

delineation for these two species needs to be further investigated. In this regard, it is worth mentioning that *A. dijkshoorniae* isolates were previously classified as "*A. pittii*-like"[24], since both species are closely related, and the *A. pittii* taxon seems to be taxonomically difficult as it contains several related ("-like") strains with no clear phenotypic/genotypic discontinuities[13,33]. It is also plausible that our results reflect a process of adaptation to a particular ecological niche, such as the human host.

To summarise, we observed that species which are more related to the nosocomial environment, i. e. *A. baumannii* and *A. nosocomialis,* had non-virulent phenotypes according to the *C. elegans* infection model and tended to form less biofilm than the other species. In contrast, *A. pittii,* which has been shown to be ecologically diverse[50], showed greater virulence, as well as the two recently described species *A. dijkshoorniae* and *A. seifertii* which, when compared to *A. baumannii* and *A. nosocomialis*, have only seldom been recovered from the human host[2,3]. Interestingly, the only study that so far attempted to compare the virulence traits of different *Acinetobacter* spp. of members of the Ab group causing bloodstream infection (although *A. dijkshoorniae* was not included at the time) also reported that *A. seifertii* isolates showed enhanced virulence capabilities[23].

These findings however, do not seem to correlate with clinical studies, where *A. nosocomialis* and *A. baumannii* in particular seem to be associated with a less favourable outcome than that of patients with infections caused by the other members of the Ab group[25,51]. Nevertheless, there are a few studies that suggest that such observation might not be due to a higher pathogenicity of the former species and rather be the consequence of inappropriate early empirical treatment, since a higher proportion of *A. baumannii* infections are caused by MDR strains. The underlying question here is whether the novel *Acinetobacter* species present a higher pathogenicity potential than *A. baumannii* that is currently masked by their overall antimicrobial susceptibility, and whether we should expect worse clinical outcomes upon the acquisition of resistance mechanisms by these *Acinetobacter* species.

A. nosocomialis and *A. baumannii* might have suffered a reduction of intra-species diversity (population bottleneck) resulting from adaptation to the human host and/or antibiotic selection, while the variability observed among isolates of *A. pittii*, *A. seifertii* and *A. dijkshoorniae* might reflect their emergence and distinct degrees of adaptation as human nosocomial pathogens[5]. With the recent advances in bacterial identification methods the number of reported infections caused by the novel members of the group is likely to increase, and the clinical characteristics of such infections will have to be evaluated to answer this question.

Materials and Methods

Bacterial strains and culture conditions. Isolates used in this study belong to the five species within the Ab group, namely *A. baumannii* (n=16), *A. dijkshoorniae* (n=12), *A. nosocomialis* (n=16), *A. pittii* (n=20) and *A. seifertii* (n=12). The isolates were mainly recovered from human clinical samples in different countries (**TABLE S1**). The identification to the species level was performed by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) according to our updated Bruker taxonomy database[13]. The clonal relatedness was established either by REP-PCR or pulsed-field gel electrophoresis of ApaI-digested DNA[54]. Furthermore, the sequence type (ST) was determined following the Pasteur MLST scheme[55]. Clonally-related isolates were excluded from the study, except for *A. baumannii* isolates R10-JV32 and R10-JV80, and R10-JV317 and R10-JV319; both pairs presented one single locus variant in their STs and were included because they harboured different carbapenem resistance mechanisms (**TABLE S1**). Bacterial cultures were routinely grown on Columbia sheep blood agar (Becton Dickinson, Heidelberg, Germany) at 37°C, unless stated otherwise.

Antimicrobial susceptibility testing and presence of acquired resistance genes. The MICs were determined by broth microdilution or gradient diffusion (AB bioMérieux, Solna, Sweeden) in Mueller Hinton II broth (CONDA, Madrid, Spain) or agar plates (Becton Dickinson, Heidelberg, Germany), respectively, and interpreted according to EUCAST or CLSI guidelines when appropriate[56,57]. The antibiotics tested were amikacin, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, tigecycline and tobramycin. Presence of metallo-β-lactamases, bla_{KPC}, bla_{OXA-23}, $bla_{OXA-24/40}$ and bla_{OXA-58} carbapenemase genes was determined by multiplex PCR[57] or characterised in previous studies (**TABLE S1**).

Detection of genes potentially associated with intrinsic resistance. The presence of genes encoding efflux pumps belonging to the RND family (*adeABC*, *adeDE*, *adeFGH* and *adeIJK*), and intrinsic OXA groups from the class D β-lactamases was evaluated by PCR using the primers listed in **TABLE S2**. *In silico* analysis of available genomic sequences at NCBI were performed in order to ensure amplification in all the Ab group species, otherwise, species-specific primers were designed. Please note that, at the time of the analysis, only one *A. seifertii* genome and only two *A. dijkshoorniae* genomes were available at the NCBI databases. Intrinsic OXA β-lactamases were further analysed by DNA sequencing, followed by cluster analysis of partial amino acid sequences (residues 9 to 267) using the MEGA version 6 software[59]. Briefly, sequences were aligned using

MUSCLE[60] and unrooted phylogenetic trees were constructed using the neighbour-joining method with bootstrap values based on 1000 replicates, with software default settings. When necessary, additional sequences were retrieved from public databases.

Quantitative biofilm formation assay. Biofilm formation was assessed in non-treated 96-well polystyrene microtiter plates. Briefly, bacteria were grown overnight at 37°C with shaking (180 rpm) in 5 mL of modified M63 medium consisting of KH_2PO_4 (12 g/l), K_2HPO_4 (7 g/l), $(NH_4)_2SO_4$ (2 g/l), adjusted to pH 7 with KOH prior to autoclaving, and supplemented with glucose (0.2% w/v), $MgSO_4$ (1 mM) and casaminoacids (0.5% w/v). Overnight cultures were adjusted to an OD_{600} of 0.1 and diluted 1:10 in modified M63 medium. For each isolate four wells were inoculated with 150 μL of the former suspension. Afterwards, plates were sealed with parafilm and incubated statically at 28°C or 37°C. After 44 h, the bacterial content of one of the wells was thoroughly resuspended and used to measure the OD_{600}. The supernatant of the remaining wells was carefully removed with a 20 G needle, wells were washed once with PBS, and biofilm was fixed with methanol 99% for 10 min and evaporated at 65°C for 20 min. Biofilm was stained with crystal violet 2% for 20 min. After two washes with sterile distilled water, glacial acetic acid 33% was used to solubilise the dye and absorbance was recorded at 580 nm. In order to measure both the biofilm formed in the liquid-solid interface and that growing upwards onto the walls, a volume of 200 μl (instead of 150 μl) was used in all washing, fixing, staining and solubilisation steps[48,61]. Those isolates showing OD_{580} covariance values greater than 30% were repeated. In order to normalise for bacterial growth differences, the OD_{580}/OD_{600} ratio was used to quantify biofilm formation. At least three biological replicas with a covariance for the OD_{580}/OD_{600} ratio less than 45% were performed for each isolate. Isolates were considered biofilm producers when the OD_{580}/OD_{600} ratio was greater than 1. Appropriate controls were added for all assays and all plates, and consisted of bacteria-free media and the inclusion of *A. baumannii* ATCC 19606 and *A. baumannii* ATCC 17978 bacterial strains as strong and weak biofilm producers, respectively.

Surface-associated motility assay. Motility plates consisted of tryptone (5 g/L), NaCl (2.5 g/L) and agarose (0.3% w/v) (CONDA, Madrid, Spain). Prior to pouring, media was cooled down to 50°C in a water bath. Twenty mL were dispensed in 90 mm diameter petri dishes and allowed to solidify for 45 min plus an additional 15 min for air drying in a biosafety cabinet. Then, 1 μL of the same bacterial suspension used for biofilm assays was inoculated onto the centre of the surface of the media, plates were sealed with parafilm and incubated at 37°C for 18 h. Isolates tended to grow concentrically from the

inoculation point, thus surface-associated motility was recorded as the mean value (cm) of three diameter measurements per plate. Isolates were considered motile when the diameter exceeded 1 cm. In every assay, isolates were tested in duplicate, and at least four biological replicates were performed for all the isolates. In all the assays, *A. baumannii* ATCC 19606 and *A. baumannii* ATCC 17978 were used as non-motile and motile control strains, respectively.

***C. elegans* killing assays**. The Fer-15 line of *C. elegans* was provided by the Caenorhabditis Genetics Center and was used for the infection model due to being fertile at 15°C but not at 25°C. Nematodes were routinely maintained at 15°C and fed with the non-virulent *E. coli* OP50 strain in Nematode Growth Medium (NGM) agar consisting of NaCl (3 g/L), tryptone (2.5 g/L) and Eurobio agar 2% (w/v) (CONDA, Madrid, Spain), supplemented with pH 6 KPO_4 buffer (1mM), $CaCl_2$ (1mM), $MgSO_4$ (1mM) and cholesterol (5 mg/L). Assays were performed using L4 stage worms, which were obtained after collecting the eggs by the hypochlorite method[62], which allows a synchronised growth of the worms. Bacteria for the killing assay were freshly grown in Luria-Bertani broth to an OD_{600} of 0.6, then 50 μL were used to inoculate the centre of the NGM plates and incubated at 37°C for 2 h. Meanwhile, L4 stage worms were washed four times with M9 medium with gentamicin (100 mg/L) in order to eliminate *E. coli* OP50 and then three times with M9 medium only, to remove the antibiotic. After cooling down the NGM plates with bacteria to room temperature, 30 ± 2 L4 stage worms were seeded and plates were incubated at 25°C. Live worms were scored every day for 15 days using a stereomicroscope. Worms were considered dead when unresponsive to touch and immotile for more than 20 s. LT50, that is, the time (days) needed to kill 50% of the worms, of each isolate in each assay was obtained by extrapolation from the sigmoidal regression of the killing curves ($R^2 > 0.95$) using GraphPad Prism version 5 for Windows, (GrapPad Software, La Jolla, California, USA). At least three biological replicates were performed for each isolate. In all the assays, *E. coli* OP50 was used as a non-virulent control strain.

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). Kolmogorov-Smirnov test or Shapiro-Wilk test was used to assess normal distribution among the variables studied when $n > 30$ or $n < 30$, respectively. For the regression models, the Pearson correlation coefficient was determined when the variables followed a normal distribution. Whenever non-normally distributed variables or ordinal variables were analysed, the Spearman rank correlation coefficient or the Kendall's τ correlation coefficient were calculated,

respectively. The Kruskal-Wallis test was performed in order to compare the distribution of the variables studied among the different species; *post-hoc* analyses were automatically performed by the software. Pairwise differences were compared using Mann-Whitney U test. The Wilcoxon signed rank test was used to compare biofilm formation at 28°C and 37°C using quantitative data, whereas the McNemar test was used to compare the proportions of biofilm-producing isolates at both temperatures. For all tests performed, *P*-values < 0.05 were considered to be statistically significant.

Data availability. Table S1 provides information regarding strain source and their characteristics as well as the raw data used for statistical analyses.

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Conflicts of interests

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflicts of interest.

Author's Contributions

Collected the strains: C.C., L.R., P.H., H.S., I.R., J.V. Conceived and designed the experiments: C.C., H.S., I.R., J.V. Performed the experiments: C.C., C.R., M.M.-A., L.R., P.H. Analyzed and interpreted the data: C.C., C.R., M.M.-A., P.H., H.S., I.R., J.V. Wrote the manuscript: C.C., P.H., H.S., I.R., J.V. All authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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Figures

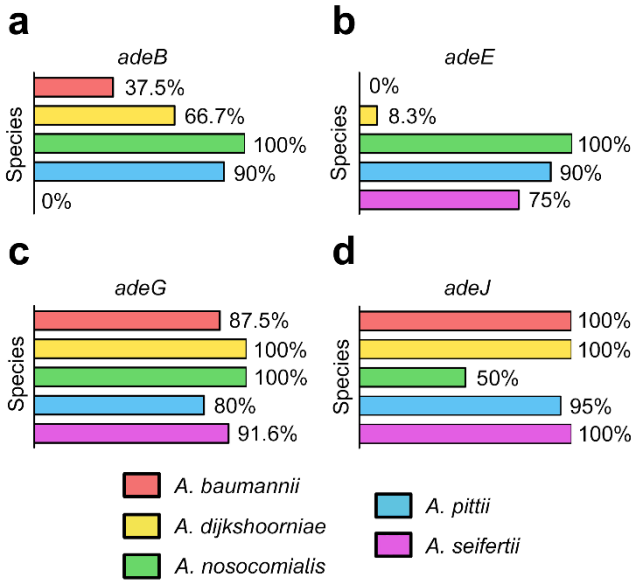

FIG 1. Occurrence of the genes encoding efflux pumps in the Ab group species **A**) *adeABC*, **B**) *adeDE*, **C**) *adeFGH*, and **D**) *adeIJK*. The presence of the genes was evaluated by PCR. Those isolates that yielded PCR amplicons of the expected size were considered positive and the results were used to calculate the percentage of isolates of each species harbouring the efflux pump.

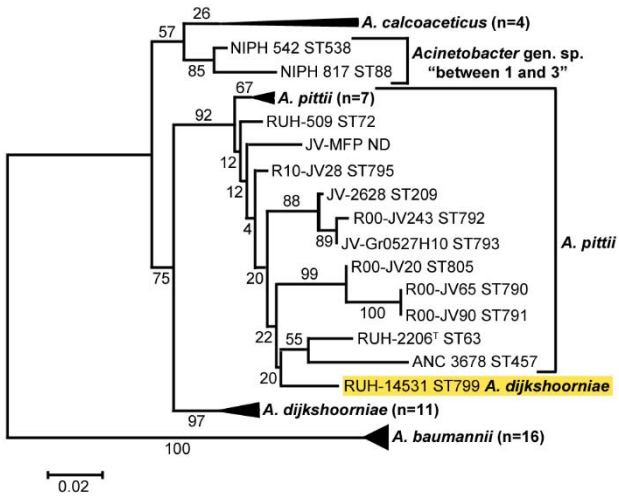

FIG 2. Cluster analysis of *A. baumannii*, *A. dijkshoorniae* and *A. pittii* based on the partial amino acid sequence of their intrinsic OXA β-lactamases. Unrooted phylogenetic trees were constructed using the neighbour-joining method with a bootstrap value of 1000 replicates. Bootstrap values (%) are indicated in the branches. The scale bar indicates sequence divergence. Highlighted in yellow is indicated the allele of *A. dijkshoorniae* showing recombination with the respective alleles from *A. pittii*. (ST) sequence type; (ND) sequence type not determined.

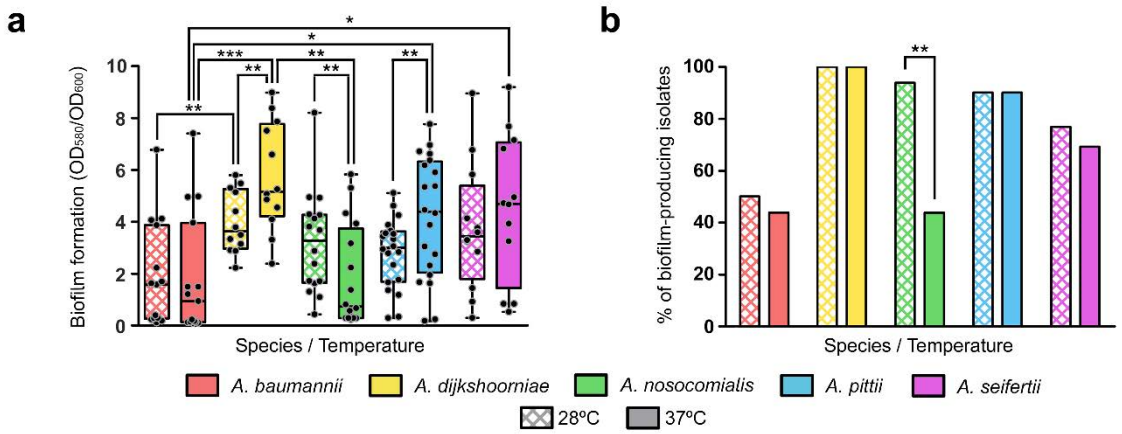

FIG 3. Biofilm formation at 28°C and 37°C according to the Ab group species. Biofilm formation was measured as the OD_{580}/OD_{600} ratio after crystal violet staining of biofilms formed in polystyrene microtiter plates in M63 medium after 44 h of static growth at 28°C or 37°C. **A**) Overlapping dot plot and box plot of the biofilm formation values of each species at 28°C and 37°C. The boxes span from the first to the third quartile, the median is indicated as a segment inside the box, and the whiskers indicate the minimum and maximum biofilm formation values. Each dot corresponds to the average biofilm formation value of an isolate after at least three biological replicates. Statistically significant differences upon temperature shift (Wilcoxon signed rank test) and between species (Kruskal-Wallis and *post hoc* tests) are highlighted with asterisks: (*) if the *P*-value < 0.05, (**) if the *P*-value ≤ 0.01 and (***) when the *P*-value ≤ 0.001. **B**) Percentage of biofilm-producing isolates of each species at 28°C and 37°C. Isolates were considered as biofilm producers when the biofilm formation value was greater than 1. Statistically significant differences upon temperature shift (McNemar test) are highlighted with asterisks: (*) if the *P*-value < 0.05 and (**) when the *P*-value ≤ 0.01.

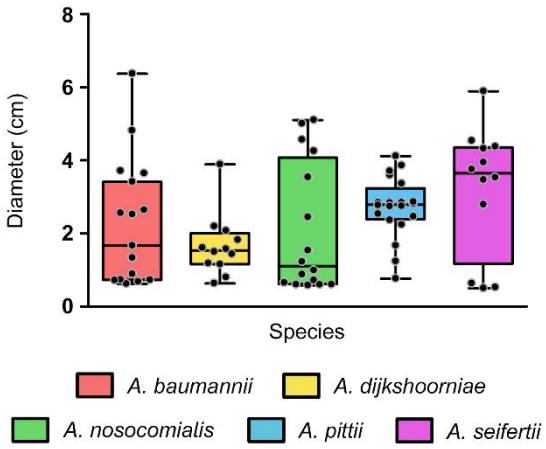

FIG 4. Surface associated motility in the Ab group species. Overlapping dot plot and box plot of the surface-associated motility of each species. Surface-associated motility was recorded as the diameter of growth after 18 h of incubation at 37°C using tryptone (5 g/L), NaCl (2.5 g/L) and agarose (0.3% w/v) motility media. The boxes span from the first to the third quartile, the median is indicated as a segment inside the box, and the whiskers indicate the minimum and maximum diameters measured. Each dot corresponds to the average diameter measured for an isolate after at least four biological replicates. Statistically significant differences were not found (Kruskal-Wallis test, *P*-value > 0.05).

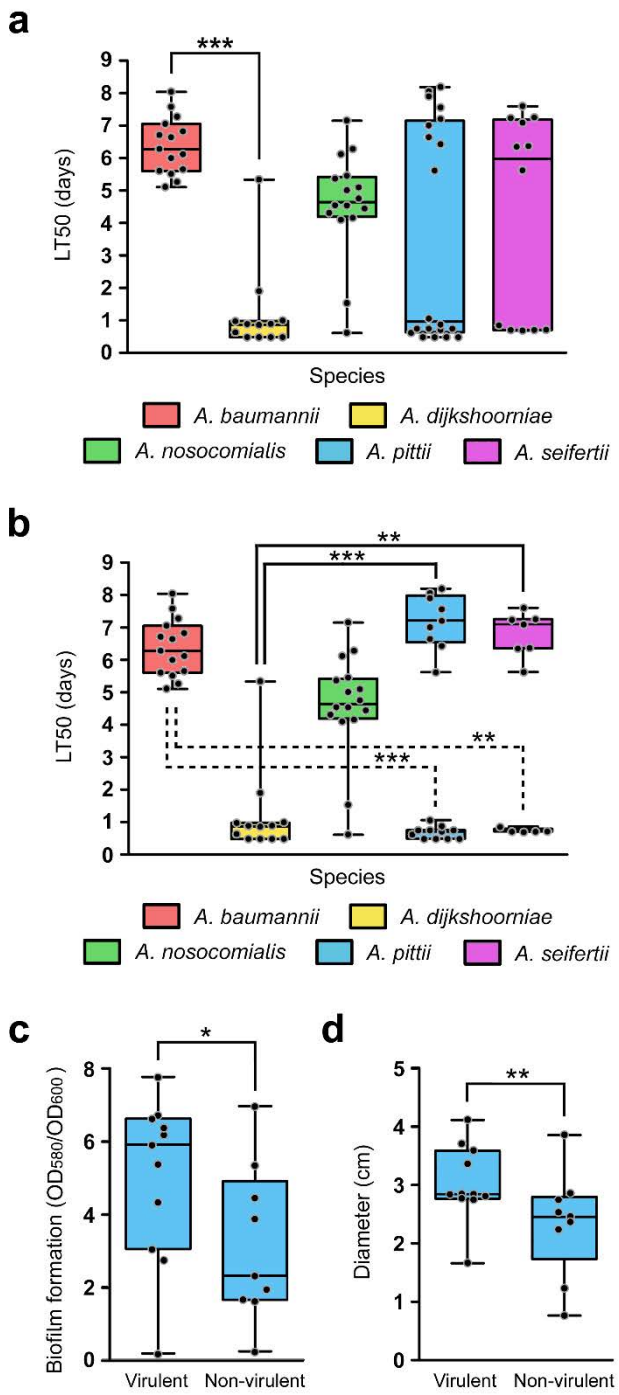

FIG 5. *In vivo* virulence of the Ab group species using the *C. elegans* infection model. Isolates were used to feed 30±2 nematodes and their daily survival was scored during 15 days, lethal time 50% (LT50) was calculated and used as the virulence measure. Overlapping dot plot and box plot of **A**) the overall LT50 values of each species; **B**) the LT50 values of each species splitting *A. pittii* and *A. seifertii* in virulent (LT50 < 3 days) and non-virulent (LT50 > 3 days) subgroups; **C**) the biofilm formation values at 28ºC; and **D**) the surface-associated motility values of *A. pittii* isolates divided into virulent and non-virulent subgroups. The boxes span from the first to the third quartile, the median is indicated as a segment inside the box, and the whiskers indicate the minimum and maximum LT50 (**A, B**), biofilm formation value (**C**) and diameter (**D**). Each dot corresponds to the average phenotype value for an isolate after at least three biological replicates. Statistically significant differences between species (Kruskal Wallis test) and subgroup species (Mann-Whitney U test) are highlighted with asterisks: (*) if the *P*-value < 0.05, (**) if the *P*-value < 0.01 and (***) when the *P*-value < 0.001.

Tables

TABLE 1. Antimicrobial susceptibility profiles of the Ab group species for the antibiotics amikacin, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, tigecycline and tobramycin. For each species range of the minimal inhibitory concentration (MIC), MIC_{50}, MIC_{90}, and percentage of resistant isolates (%R) is provided. MICs are expressed in µg/mL.

Antimicrobial		*A. baumannii* (n=16)	*A. dijkshoorniae* (n=12)	*A. nosocomialis* (n=16)	*A. pittii* (n=20)	*A. seifertii* (n=12)
Amikacin	MIC_{50}	4	0.75	1.5	1.5	1
	MIC_{90}	128	1.5	6	4	2
	Range	0.38 - >256	0.38 - 3	1 - 12	0.75 - 24	0.5 - 12
	%R	31.3	0	5	0	0
Ceftazidime	MIC_{50}	128	3	3	3	4
	MIC_{90}	>128	32	4	16	6
	Range	2 - >128	2 - >256	1.5 - 4	1.5 - >256	2 - 12
	%R [a]	75	25	0	5	0
Ciprofloxacin	MIC_{50}	64	0.25	0.0125	0.19	0.25
	MIC_{90}	>64	0.38	0.5	0.5	0.38
	Range	0.094 - >64	0.19 - 0.38	0.064 - 0.75	0.125 - >32	0.064 - 0.75
	%R	68.85	0	0	5	0
Colistin	MIC_{50}	0.38	0.25	0.19	0.19	0.125
	MIC_{90}	1	0.38	0.38	0.5	0.25
	Range	0.38 - 8	0.19 - 0.38	0.38 - 0.5	0.19 - 1	0.125 - 0.38
	%R	6.3	0	0	0	0
Gentamicin	MIC_{50}	32	0.38	0.75	0.38	0.25
	MIC_{90}	>64	0.5	8	4	1
	Range	0.25 - >256	0.25 - 0.75	0.38 - 12	0.19 - 6	0.094 - 3
	%R	68.8	0	25	5	0
Imipenem	MIC_{50}	16	0.25	0.25	0.38	0.25
	MIC_{90}	64	0.38	0.25	>32	0.25
	Range	0.38 - >64	0.094 - >32	0.19 - 0.38	0.25 - >32	0.125 - 1.5
	%R	62.5	8.3	0	15	0
Meropenem	MIC_{50}	8	0.25	0.25	0.75	0.5
	MIC_{90}	>64	0.38	0.5	>32	0.75
	Range	0.25 - >64	0.125 - >32	0.19 - 0.5	0.25 - >32	0.25 - 1.5
	%R	43.8	8.3	0	15	0
Tigecycline	MIC_{50}	1	0.19	0.19	0.19	0.19
	MIC_{90}	2	0.25	1.5	0.75	0.25
	Range	0.19 - 2	0.125 - 0.75	0.125 - 2	0.094 - 2	0.094 - 1
	%R [b]	ND	ND	ND	ND	ND
Tobramycin	MIC_{50}	8	0.38	0.38	0.38	0.25
	MIC_{90}	>64	0.38	2	1.5	0.38
	Range	0.38 - 96	0.19 - 0.38	0.25 - 3	0.19 - 2	0.094 - 1.5
	%R	56.3	0	0	0	0

ND, not determined; [a] Clinical breakpoint according to CLSI guidelines[57], [b] No clinical breakpoints available

Supplemental material

TABLE S1. Characteristics of *Acinetobacter* isolates of the Ab group used in this study per species.

A. baumannii

						MICs								
Isolate	**Country**	**Year**	**Specimen**	**ST**	**AcqCar**	**AMK**	**CAZ**	**CIP**	**CST**	**GEN**	**IPM**	**MEM**	**TGC**	**TOB**
R10-JV08	Spain	2010	H.S.	ST2	N	8	>128	>64	<0.5	32	16	8	2	8
R10-JV19	Spain	2010	H.S.	ND	N	16	>128	64	<0.5	32	16	8	0.5	64
R10-JV32	Spain	2010	H.S.	ST2	OXA-24	<2	>128	>64	1	>64	64	>64	1	8
R10-JV80	Spain	2010	H.S.	ST632	OXA-58	8	>128	>64	1	64	16	4	2	2
R10-JV177	Spain	2010	H.S.	ST2	OXA-58	<2	>128	>64	<0.5	>64	64	>64	1	32
R10-JV210	Spain	2010	H.S.	ST2	OXA-24	32	128	64	8	8	>64	>64	<0.25	1
R10-JV226	Spain	2010	H.S.	ST181	OXA-24	128	128	64	<0.5	>64	64	>64	1	>64
R10-JV247	Spain	2010	H.S.	ST2	OXA-58	4	>128	64	<0.5	<0.5	32	32	2	32
R10-JV295	Spain	2010	H.S.	ST2	N	<2	>128	>64	2	2	8	4	2	<0.5
R10-JV308	Spain	2010	H.S.	ST2	OXA-23	>256	>128	64	<0.5	>64	16	16	0.5	>64
R10-JV317	Spain	2010	H.S.	ST169	N	<2	128	<0.5	<0.5	<0.5	1	<0.5	0.5	1
R10-JV319	Spain	2010	H.S.	ST79	OXA-24	256	64	>64	<0.5	>64	64	>64	1	16
JV-Ab30	Spain	2000	H.S.	ST273	N	2	2	0.094	0.38	0.5	0.38	0.25	0.19	0.38
RUH-134	T. N	1982	Urine	ST2	N	3	3	0.5	0.5	64	0.5	0.75	1.5	1
RUH-875	T. N	1984	Urine	ST1	N	4	4	0.75	0.38	>256	0.75	1.5	0.5	96
ATCC17978	France	1951	Meninges	ST437	N	0.38	3	0.19	0.5	0.25	0.5	0.25	0.25	0.38

	RND efflux pumps					*C. elegans*			Biofilm 28°C			Biofilm 37°C			Motility		
Isolate	***adeB***	***adeJ***	***adeG***	***adeE***	**#**	**Mean[a]**	**SD**	**0/1**	**Mean[b]**	**SD**	**0/1**	**Mean[b]**	**SD**	**0/1**	**Meanc**	**SD**	**0/1**
R10-JV08	0	1	0	0	1	7.58	1.07	0	0.27	0.04	0	0.13	0.04	0	0.62	0.04	0
R10-JV19	0	1	1	0	2	6.28	0.93	0	0.12	0.04	0	0.10	0.04	0	0.73	0.05	0
R10-JV32	0	1	1	0	2	5.26	0.29	0	4.07	1.60	1	4.98	1.21	1	3.65	1.78	1
R10-JV80	0	1	1	0	2	6.82	0.33	0	4.12	0.98	1	4.96	1.02	1	3.71	1.42	1
R10-JV177	1	1	1	0	3	5.11	0.67	0	0.19	0.07	0	0.11	0.04	0	0.73	0.04	0
R10-JV210	0	1	0	0	1	6.00	0.55	0	1.58	0.48	1	1.22	0.45	1	2.64	1.32	1
R10-JV226	0	1	1	0	2	6.71	0.12	0	2.23	0.63	1	0.95	0.25	0	0.68	0.08	0
R10-JV247	0	1	1	0	2	7.27	0.48	0	0.24	0.08	0	0.14	0.05	0	1.67	2.19	1
R10-JV295	1	1	1	0	3	5.65	0.75	0	0.29	0.10	0	0.15	0.05	0	3.41	2.88	1
R10-JV308	0	1	1	0	2	5.60	0.72	0	0.30	0.09	0	0.17	0.05	0	0.89	0.31	0
R10-JV317	0	1	1	0	2	7.05	0.87	0	0.40	0.12	0	0.23	0.08	0	0.71	0.06	0
R10-JV319	0	1	1	0	2	6.12	0.62	0	1.70	0.66	1	1.50	0.49	1	1.33	1.61	1
JV-Ab30	1	1	1	0	3	5.51	0.75	0	1.64	0.26	1	1.50	0.19	1	2.52	2.02	1
RUH-134	1	1	1	0	3	8.04	0.38	0	6.78	0.71	1	7.41	1.50	1	6.37	2.16	1
RUH-875	1	1	1	0	3	6.64	0.98	0	3.88	0.94	1	3.96	0.84	1	2.55	1.63	1
ATCC17978	1	1	1	0	3	4.85	0.41	0	0.58	0.24	0	0.37	0.17	0	4.85	0.28	1

MICs, minimal inhibitory concentration; ST, sequence type; AcqCar, Acquired carbapenemases; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TOB, tobramycin; T.N., The Netherlands; H.S., Human sample; ND, not determined; N, negative; RND, Resistance-Nodulation-cell Division; #, number; SD, standard deviation; 0/1, phenotype considered: *C. elegans* (0, non-virulent; 1, virulent), Biofilm (0, non-producer; 1, producer), Motility (0, non-motile; 1, motile);
[a] LT50, days; [b] OD_{580}/OD_{600} ratio, a. u.; [c] diameter, cm

A. dijkshoorniae

Isolate	Country	Year	Specimen	ST	AcqCar	MICs								
						AMK	CAZ	CIP	CST	GEN	IPM	MEM	TGC	TOB
LUH-10297	Thailand	2005	Freshwater	ST798	N	0.75	2	0.19	0.38	0.38	0.19	0.19	0.19	0.38
SCOPE 289	USA	1995	Blood	ST799	N	1	3	0.25	0.38	0.38	0.094	0.38	0.19	0.38
LUH-07351	T. N	2001	Nephrology drain	ST565	N	1	8	0.38	0.38	0.75	0.25	0.5	0.75	0.38
LUH-13623	Italy	2004	Sputum	ST800	N	0.75	3	0.25	0.38	0.5	0.38	0.19	0.25	0.38
RUH-0053	T. N	NK	NK	ST564	N	0.75	2	0.19	0.19	0.38	0.38	0.19	0.38	0.25
SCOPE 271	USA	1995	Blood	ST568	N	0.75	3	0.19	0.19	0.38	0.25	0.125	0.19	0.38
LUH-10243	T. N	2005	Sputum	ST802	N	0.5	4	0.25	0.19	0.38	0.25	0.19	0.19	0.25
LUH-08258	Belgium	2002	Catheter	ST566	N	0.38	3	0.19	0.19	0.25	0.19	0.25	0.25	0.19
JVAP01	Turkey	2009	Urine	ST606	NDM-1	3	>256	0.25	0.19	0.5	>32	>32	0.25	0.38
R10-JV222	Spain	2010	H.S.	ST796	N	<2	32	<0.5	<0.5	<0.5	<0.5	<0.5	<0.25	<0.5
R10-JV463	Spain	2010	H.S.	ST797	N	<2	32	<0.5	<0.5	<0.5	<0.5	<0.5	<0.25	<0.5
LUH-09407	T. N	2004	Wound	ST801	N	1.5	4	0.25	0.25	0.38	0.38	0.38	0.125	0.25

Isolate	RND efflux pumps					*C. elegans*			Biofilm 28°C			Biofilm 37°C			Motility		
	adeB	*adeJ*	*adeG*	*adeE*	#	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1
LUH-10297	1	1	1	0	3	0.99	0.11	1	5.81	2.07	1	8.99	1.67	1	1.57	0.56	1
SCOPE 289	1	1	1	0	3	1.90	0.38	1	2.91	0.29	1	5.08	1.23	1	2.07	0.42	1
LUH-07351	0	1	1	0	2	0.97	0.16	1	3.34	0.30	1	4.86	0.95	1	1.43	0.22	1
LUH-13623	1	1	1	0	3	0.63	0.25	1	5.15	2.05	1	5.26	0.18	1	1.82	0.22	1
RUH-0053	1	1	1	0	3	0.48	0.00	1	3.79	1.01	1	4.56	1.59	1	1.50	0.31	1
SCOPE 271	1	1	1	0	3	0.48	0.00	1	3.50	0.58	1	7.87	0.92	1	1.15	0.15	1
LUH-10243	0	1	1	1	3	0.88	0.11	1	5.31	0.53	1	7.51	0.46	1	3.89	0.66	1
LUH-08258	1	1	1	0	3	0.86	0.03	1	3.15	1.13	1	6.60	2.51	1	1.61	0.42	1
JVAP01	1	1	1	0	3	5.34	0.78	0	2.88	0.74	1	3.31	1.11	1	0.80	0.04	0
R10-JV222	1	1	1	0	3	0.48	0.00	1	5.48	0.81	1	8.38	1.67	1	2.19	1.25	1
R10-JV463	0	1	1	0	2	0.89	0.02	1	4.41	0.27	1	4.10	0.91	1	1.18	0.63	1
LUH-09407	0	1	1	0	2	0.48	0.00	1	2.23	0.86	1	2.39	0.55	1	0.63	0.07	0

MICs, minimal inhibitory concentration; ST, sequence type; AcqCar, Acquired carbapenemases; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TOB, tobramycin; T.N., The Netherlands; H.S., Human sample; NK, not known; N, negative; RND, Resistance-Nodulation-cell Division; #, number; SD, standard deviation; 0/1, phenotype considered: *C. elegans* (0, non-virulent; 1, virulent), Biofilm (0, non-producer; 1, producer), Motility (0, non-motile; 1, motile);
[a] LT50, days; [b] OD_{580}/OD_{600} ratio, a. u.; [c] diameter, cm

A. nosocomialis

Isolate	Country	Year	Specimen	ST	AcqCar	MICs								
						AMK	CAZ	CIP	CST	GEN	IPM	MEM	TGC	TOB
RUH-503	T. N	1990	Urine	ST68	N	6	3	0.5	0.38	8	0.25	0.38	1.5	2
JV-158029	Spain	2011	Urine	ST68	N	8	4	0.5	0.25	12	0.38	0.5	1.5	3
SCOPE 7	USA	1995	Blood	ST543	N	1.5	2	0.094	0.38	0.38	0.19	0.25	0.19	0.25
SCOPE 54	USA	1995	Blood	ST784	N	1.5	2	0.064	.125	0.75	0.25	0.19	0.125	0.38
SCOPE 59	USA	1995	Blood	ST781	N	1.5	1.5	0.094	0.19	0.75	0.25	0.19	0.19	0.38
SCOPE 73	USA	1995	Blood	ST530	N	2	2	0.125	0.19	1	0.25	0.25	0.19	0.5
SCOPE 76	USA	1995	Blood	ST785	N	6	4	0.75	0.19	6	0.25	0.5	1.5	2
SCOPE 79	USA	1995	Blood	ST781	N	1.5	3	0.125	0.25	0.38	0.19	0.38	0.38	0.38
SCOPE 81	USA	1995	Blood	ST782	N	12	4	0.75	0.19	12	0.38	0.5	2	3
SCOPE 95	USA	1995	Blood	ST395	N	1.5	2	0.125	0.19	0.75	0.25	0.25	0.19	0.38
SCOPE 150	USA	1995	Blood	ST501	N	1.5	3	0.094	0.25	0.5	0.25	0.38	0.19	0.38
SCOPE 192	USA	1995	Blood	ST71	N	1	1.5	0.125	0.19	0.5	0.25	0.19	0.19	0.38
SCOPE 212	USA	1995	Blood	ST786	N	1.5	3	0.19	0.25	1	0.25	0.25	0.19	0.75
SCOPE 249	USA	1995	Blood	ST279	N	2	3	0.25	0.19	0.75	0.19	0.38	0.25	1
RUH-2376	T. N	1987	Sputum	ST76	N	2	3	0.25	0.38	0.75	0.19	0.25	0.19	0.5
RUH-2284	T. N	1990	Bronchial secretion	ST68	N	1.5	3	0.25	0.5	1.5	0.19	0.38	0.19	0.38

Isolate	RND efflux pumps					*C. elegans*			Biofilm 28°C			Biofilm 37°C			Motility		
	adeB	*adeJ*	*adeG*	*adeE*	#	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1
RUH-503	1	0	1	1	3	5.01	0.46	0	3.68	0.21	1	5.31	1.45	1	4.25	2.17	1
JV-158029	1	1	1	1	4	4.44	0.48	0	4.26	0.45	1	2.24	0.38	1	4.56	2.65	1
SCOPE 7	1	1	1	1	4	4.16	0.56	0	1.31	0.34	1	0.30	0.11	0	0.65	0.10	0
SCOPE 54	1	0	1	1	3	4.31	0.43	0	8.21	2.51	1	5.84	2.01	1	1.53	1.21	1
SCOPE 59	1	0	1	1	3	5.36	0.25	0	4.29	1.51	1	0.24	0.08	0	0.87	0.17	0
SCOPE 73	1	1	1	1	4	5.45	0.83	0	4.93	1.91	1	1.38	0.53	1	0.71	0.06	0
SCOPE 76	1	1	1	1	4	6.12	0.55	0	1.63	0.51	1	0.57	0.18	0	0.59	0.02	0
SCOPE 79	1	0	1	1	3	5.09	0.56	0	1.72	0.49	1	0.33	0.06	0	0.60	0.04	0
SCOPE 81	1	1	1	1	4	6.28	1.17	0	4.13	0.83	1	4.33	0.61	1	5.10	1.93	1
SCOPE 95	1	0	1	1	3	4.54	0.32	0	2.39	0.91	1	0.29	0.04	0	0.99	0.35	0
SCOPE 150	1	0	1	1	3	4.75	0.69	0	1.73	0.63	1	0.29	0.11	0	0.58	0.05	0
SCOPE 192	1	0	1	1	3	4.10	0.73	0	4.95	1.35	1	0.68	0.28	0	0.60	0.00	0
SCOPE 212	1	0	1	1	3	0.61	0.23	1	3.82	0.26	1	3.18	0.33	1	5.01	0.95	1
SCOPE 249	1	1	1	1	4	4.53	0.24	0	1.10	0.24	1	0.82	0.19	0	2.44	1.17	1
RUH-2376	1	1	1	1	4	1.53	0.35	1	0.44	0.17	0	0.29	0.05	0	1.22	0.96	1
RUH-2284	1	1	1	1	4	7.16	0.82	0	2.88	0.37	1	3.94	1.05	1	3.54	1.03	1

MICs, minimal inhibitory concentration; ST, sequence type; AcqCar, Acquired carbapenemases; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TOB, tobramycin; T.N., The Netherlands; N, negative; RND, Resistance-Nodulation-cell Division; #, number; SD, standard deviation; 0/1, phenotype considered: *C. elegans* (0, non-virulent; 1, virulent), Biofilm (0, non-producer; 1, producer), Motility (0, non-motile; 1, motile);

[a] LT50, days; [b] OD_{580}/OD_{600} ratio, a. u.; [c] diameter, cm

A. pittii

Isolate	Country	Year	Specimen	ST	AcqCar	MICs								
						AMK	CAZ	CIP	CST	GEN	IPM	MEM	TGC	TOB
JV-MFP	Spain	2014	H.S.	ND	N	0.75	2	0.125	0.19	0.38	1.5	1.5	0.25	0.38
JV-Ab41	Spain	2000	Blood	ST788	N	1.5	2	0.125	0.19	0.75	0.38	0.25	0.19	0.75
JV-2628	Colombia	2010	Catheter	ST209	OXA-72	1	4	0.125	0.19	0.5	>32	>32	0.25	0.38
JVAP02	Turkey	2006	Blood	ST457	NDM-1	24	>256	0.25	0.19	4	>32	>32	0.5	2
JV-IRS-Fr	France	2010	Blood	ST64	OXA-58	3	3	>32	0.38	0.75	>32	>32	0.38	0.5
RUH-509	T. N	1984	Bronchus	ST72	N	1.5	2	0.125	0.19	3	1.5	1.5	0.38	1.5
R00-JV14	Spain	2000	H.S.	ST789	N	1	2	0.125	0.5	0.38	0.25	0.25	0.125	0.25
R00-JV20	Spain	2000	H.S.	ST805	N	4	4	0.125	0.5	0.5	>32	>32	0.38	0.38
R00-JV21	Spain	2000	H.S.	ST667	N	1.5	4	0.5	0.19	4	0.5	1.5	2	1.5
R00-JV65	Spain	2000	H.S.	ST790	N	1	3	0.125	0.38	0.5	0.38	0.38	0.094	0.25
R00-JV90	Spain	2000	H.S.	ST791	N	12	4	0.5	0.125	6	0.5	1	2	2
R00-JV195	Spain	2000	H.S.	ST64	N	1	3	0.19	0.5	0.38	0.38	0.75	0.125	0.25
R00-JV243	Spain	2000	H.S.	ST792	N	1.5	3	0.25	0.125	0.25	0.38	0.38	0.19	0.19
JV-Gr1115O15	Spain	2012	Urine	ST804	N	1	2	0.19	0.5	0.25	0.38	0.75	0.125	0.25
JV-Gr0527H10	Spain	2014	Blood	ST793	N	1	2	0.19	0.19	0.19	0.38	0.5	0.19	0.19
R10-JV02	Spain	2010	H.S.	ST794	N	<2	16	<0.5	1	<0.5	<0.5	8	≤0.25	≤0.5
R10-JV23	Spain	2010	H.S.	ST214	N	<2	16	<0.5	<0.5	<0.5	<0.5	<0.5	≤0.25	≤0.5
R10-JV28	Spain	2010	H.S.	ST795	N	<2	16	<0.5	<0.5	<0.5	<0.5	<0.5	≤0.25	<0.5
JV-2663673	Spain	2015	Urine	ND	N	1	2	0.19	0.125	0.38	0.38	0.75	0.19	0.19
JV-CROIN20	Croatia	NK	NK	ND	N	1	1.5	0.38	0.19	0.25	0.38	0.75	0.75	0.25

Isolate	RND efflux pumps					*C. elegans*			Biofilm 28°C			Biofilm 37°C			Motility		
	adeB	*adeJ*	*adeG*	*adeE*	#	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1
JV-MFP	1	1	1	1	4	8.19	0.50	0	1.36	0.31	1	1.63	0.52	1	2.24	1.33	1
JV-Ab41	1	1	1	1	4	1.05	0.06	1	5.12	1.47	1	4.34	1.68	1	2.84	1.63	1
JV-2628	1	1	1	1	4	0.48	0.00	1	4.62	1.29	1	7.77	2.97	1	3.59	1.42	1
JVAP02	1	1	1	1	4	7.00	0.31	0	1.18	0.44	1	1.95	0.23	1	0.76	0.16	0
JV-IRS-Fr	1	1	0	1	3	8.05	1.00	0	2.95	0.76	1	5.35	1.35	1	2.74	0.95	1
RUH-509	1	1	0	1	3	0.48	0.00	1	3.34	0.46	1	5.91	1.30	1	2.84	1.36	1
R00-JV14	1	1	1	1	4	7.20	0.48	0	3.06	0.20	1	6.96	1.32	1	3.86	1.70	1
R00-JV20	0	0	0	1	1	0.48	0.00	1	3.88	0.99	1	6.19	0.72	1	2.81	1.50	1
R00-JV21	1	1	1	1	4	6.43	0.52	0	1.67	0.25	1	2.32	1.01	1	2.46	1.06	1
R00-JV65	0	1	0	1	2	0.48	0.00	1	3.66	0.43	1	5.38	0.26	1	4.12	2.23	1
R00-JV90	1	1	1	0	3	0.74	0.23	1	3.44	0.56	1	6.72	0.89	1	2.84	1.23	1
R00-JV195	1	1	1	1	4	6.64	0.36	0	2.35	0.59	1	4.46	1.49	1	2.53	0.68	1
R00-JV243	1	1	1	1	4	0.70	0.20	1	2.84	0.33	1	3.05	0.44	1	1.66	0.58	1
JV-Gr1115O15	1	1	1	0	3	0.61	0.23	1	0.30	0.07	0	0.18	0.06	0	3.36	0.56	1
JV-Gr0527H10	1	1	1	1	4	0.74	0.23	1	3.56	0.69	1	6.62	0.90	1	2.75	0.50	1
R10-JV02	1	1	1	1	4	0.87	0.06	1	3.54	0.62	1	6.38	1.88	1	2.77	0.86	1
R10-JV23	1	1	1	1	4	7.56	0.90	0	1.77	0.66	1	1.68	0.58	1	2.86	0.70	1
R10-JV28	1	1	1	1	4	0.75	0.23	1	4.25	0.91	1	2.75	0.70	1	3.70	0.89	1
JV-2663673	1	1	1	1	4	5.61	0.33	0	0.34	0.06	0	0.24	0.03	0	2.37	0.33	1
JV-CROIN20	1	1	1	1	4	7.90	0.86	0	2.79	1.10	1	3.88	1.01	1	1.23	0.42	1

MICs, minimal inhibitory concentration; ST, sequence type; AcqCar, Acquired carbapenemases; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TOB, tobramycin; T.N., The Netherlands; H.S., Human sample; NK, not known; ND, not determined; N, negative; RND, Resistance-Nodulation-cell Division; #, number; SD, standard deviation; 0/1, phenotype considered: *C. elegans* (0, non-virulent; 1, virulent), Biofilm (0, non-producer; 1, producer), Motility (0, non-motile; 1, motile);

[a] LT50, days; [b] OD_{580}/OD_{600} ratio, a. u.; [c] diameter, cm

A. seifertii

Isolate	Country	Year	Specimen	ST	AcqCar	MICs								
						AMK	CAZ	CIP	CST	GEN	IPM	MEM	TGC	TOB
RUH-3243	Japan	NK	Blood	ND	N	1	2	0.125	0.125	0.19	0.19	0.5	0.19	0.19
RUH-1139	T. N	1985	Throat swab	ST545	N	2	4	0.38	0.19	1.5	0.25	0.75	0.25	0.5
LUH-08128	T. N	2002	Catheter	ST803	N	2	12	0.75	0.38	1	0.38	1.5	0.75	0.38
LUH-05793	Hong Kong	1998	Soil	ST551	N	1.5	3	0.25	0.125	0.75	0.25	0.38	0.19	0.38
LUH-01472	Sweeden	1990	Trachea	ST91	N	1	2	0.064	0.25	0.125	0.19	0.38	0.125	0.125
LUH-01471	Sweeden	1990	Ulcer	ST90	N	6	6	0.38	0.125	3	0.25	0.5	1	1.5
LUH-05789	Hong Kong	1997	Blood	ST549	N	12	3	0.125	0.125	0.25	0.125	0.25	0.094	0.125
R00-JV54	Spain	2000	H.S.	ST834	N	1	4	0.125	0.38	0.38	1.5	0.75	0.19	0.38
SCOPE 6	USA	1995	Blood	ST483	N	1	4	0.25	0.125	0.25	0.25	0.5	0.19	0.25
SCOPE 12	USA	1995	Blood	ST546	N	0.75	8	0.25	0.125	0.38	0.25	0.5	0.25	0.38
SCOPE 35	USA	1995	Blood	ST548	N	1	6	0.25	0.19	0.19	0.19	0.38	0.125	0.25
NIPH 973T-I	Denmark	1990	Ulcer	ST91	N	0.5	2	0.094	0.25	0.094	0.25	0.25	0.125	0.094

Isolate	RND efflux pumps					*C. elegans*			Biofilm 28°C			Biofilm 37°C			Motility		
	adeB	*adeJ*	*adeG*	*adeE*	#	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1	Mean	SD	0/1
RUH-3243	0	1	1	1	3	6.37	0.64	0	8.96	3.19	1	4.72	0.49	1	0.63	0.06	0
RUH-1139	0	1	1	1	3	0.84	0.09	1	6.78	1.79	1	9.20	3.24	1	5.89	1.65	1
LUH-08128	0	1	1	0	2	7.60	0.86	0	3.25	0.53	1	3.94	1.52	1	3.95	1.72	1
LUH-05793	0	1	0	0	1	0.69	0.29	1	3.77	0.90	1	4.95	1.99	1	4.37	1.32	1
LUH-01472	0	1	1	1	3	0.70	0.20	1	0.30	0.06	0	0.54	0.16	0	0.53	0.05	0
LUH-01471	0	1	1	1	3	5.62	0.80	0	3.60	0.80	1	6.82	2.62	1	4.32	3.19	1
LUH-05789	0	1	1	1	3	7.25	0.22	0	5.83	1.80	1	4.67	0.89	1	3.75	1.31	1
R00-JV54	0	1	1	1	3	6.34	0.41	0	1.45	0.33	1	0.84	0.29	0	3.47	0.49	1
SCOPE 6	0	1	1	0	2	0.71	0.21	1	4.14	0.44	1	7.15	2.44	1	2.79	0.94	1
SCOPE 12	0	1	1	1	3	7.09	1.11	0	3.30	0.33	1	7.68	2.61	1	4.53	0.46	1
SCOPE 35	0	1	1	1	3	0.70	0.20	1	0.92	0.18	0	0.84	0.21	0	3.53	0.81	1
NIPH 973T-I	0	1	1	1	3	7.23	0.60	0	2.85	0.12	1	3.25	0.25	1	0.50	0.08	0

MICs, minimal inhibitory concentration; ST, sequence type; AcqCar, Acquired carbapenemases; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC, tigecycline; TOB, tobramycin; T.N., The Netherlands; H.S., Human sample; ND, not determined; N, negative; RND, Resistance-Nodulation-cell Division; #, number; SD, standard deviation; 0/1, phenotype considered: *C. elegans* (0, non-virulent; 1, virulent), Biofilm (0, non-producer; 1, producer), Motility (0, non-motile; 1, motile);
[a] LT50, days; [b] OD_{580}/OD_{600} ratio, a. u.; [c] diameter, cm

TABLE S2. Primers used in this study. Boxes in dark grey indicate the primer combination used to obtain the results shown in this report for each species; boxes in light grey indicate that the primers were employed, but that amplification was inefficient or absent. Abau, *A. baumannii*; Adij, *A. dijkshoorniae*; Ansc, *A. nosocomialis*; Apit, *A. pittii*; Asei, *A. seifertii*.

Gene, Name	Sequence (5' - 3')	Species				
adeB		Abau	Adij	Ansc	Apit	Asei
adeBUp	ATGTCACAATTTTTTATTCGTCGTC					
adeBLw	TTAGGATGAGATTTTTTTCTTAGAGG					
adeB-pitLw	GTGCAGTATCGTAAGGAACGC					
adeB_dijUp	CGTGTTGAAGGTGTTGGTAAAGT					
adeB_dijLw	ATGCAGGCATACCTAAGTTCGC					
adeE						
adeEUp	GATGACGCGATTGTCGTTG					
adeELw	GGATCCGCACGAGCAATCACAGCTTC					
adeELw2	CCTGCTCCTGTAGATACAGC					
adeG						
adeGUp	GTTGCTCGTGTCGAACTTGC					
adeGLw	AGGAACGAAACCACCTGGAAC					
adeJ						
adeJUp	CTGGCTTATGACACGACTC					
adeJLw	GGATCCCCATACCACGCTGAATCAATG					
Intrinsic OXA						
OXA69-F	GATCTACTCAAGTTACATTAATTAGC					
OXA69-R	AGTGAACTGGGCTATAAAC					
OXA51nF	TAATGCTTTGATCGGCCTTG					
OXA51nR	TGGATTGCACTTCATCTTGG					
OXA_G3_Up	GCTCTTTTCCTTGCTATTAGTAC					
OXA_G3_Lw	TACCTAGCTGTTCTAATCC					

Section II

A. baumannii infections pose a serious health threat due to the high incidence of MDR of this pathogen, but especially when bacteria are carbapenem resistant, since the treatment options left are drastically reduced. To face this situation, the WHO has recently positioned carbapenem-resistant *A. baumannii* at the top of the list of the critical bacteria for which the development of new antimicrobial drugs is considered a priority and an urgent public health need [4]. Part of this PhD project wanted to address this issue by investigating the putative dual role of transport-related proteins in the antimicrobial resistance and virulence of *A. baumannii*. This approach would enlarge our knowledge about known and novel transport proteins in this pathogen, and, ultimately, it would also lead to the identification of putative new targets for the development of novel antibacterial drugs directed specifically against *A. baumannii*.

After several failed attempts to build our own in-house transport protein deletion mutant collection, we decided to use the transposon mutant collection developed in 2015 by Gallagher *et al.* using the *A. baumannii* AB5075 strain [625] (**BOX 6**). This strain had previously been described as a suitable type strain thanks to its recent isolation, high virulence in invertebrate and mammalian infection models, and its resistance to carbapenems due to the presence of an OXA-23 carbapenemase [626]. We selected 83 mutant strains from the transposon mutant library mainly based on their putative function description, and, after verification, 65 mutant strains were selected for further analyses. Of those, the truncated genes putatively belonged to: the ABC family (n=12), the MATE family (n=3); the MFS family (n=28); OMPs (n=8), the RND family (n=9), and other transport proteins/permeases (n=5).

Comparison of their antimicrobial susceptibility against the *A. baumannii* AB5075 strain revealed that at least one member of each transport family seemed to be involved in antibiotic resistance (**Manuscript 6**). Overall, 10 mutant strains had altered antimicrobial susceptibility and aminoglycosides were the antibiotics most commonly affected. Altered susceptibility to meropenem, levofloxacin and moxifloxacin was detected in mutant strains defective for OmpA-like and an uncharacterised transporter of the autoinducer-2 exporter (AI-2E) superfamily, despite the presence of multiple mechanisms of antibiotic

resistance. Other mutant strains with altered susceptibility presented the transposon insertion at genes codifying known transporter proteins involved in antimicrobial translocation, such as AbeM, CraA, AdeJ and AdeB. The remaining mutant strains were defective for transport proteins not previously reported to participate in antimicrobial resistance from the ABC (n=1) and MFS (n=3) families, and, therefore, require further characterisation.

One of the reasons we chose *A. baumannii* AB5075 strain for our study was that, in addition to the availability of the mutant collection, this strain displayed highly virulent phenotypes in the *G. mellonella* waxworm and the mice pulmonary infection models [626]. Thus, despite our previous experience with the *C. elegans* infection model (**Manuscript 5**), we decided that the most suitable animal model to test the virulence of our transport mutant collection would be *G. mellonella*, since working with mice would be unethical in this part of the project due to the high number of strains to be tested. As we were unfamiliar with this model, this part of the project was performed under the supervision of Dr. David W. Wareham (Blizard Institute – Barts and The London, Queen Mary University of London, London, UK) during two internships at his facilities. Firstly, in order to determine the putative role in virulence of active transport-related proteins, we corroborated that heat inactivated bacteria were not able to cause infection in the *G. mellonella* infection model. Next, we used efflux pumps inhibitors (EPI) to preliminary evaluate the role of efflux in *A. baumannii* AB5075 strain pathogenesis, and found that only 1-(1-naphthylmethyl)-piperazine (NMP) reduced significantly the virulence in the *G. mellonella* model. Finally, we performed the assays using the mutant strains. Our results show that most of the transport-related proteins putatively involved in the virulence of *A. baumannii* AB5075 strain in the *G. mellonella* infection model are uncharacterised. Overall, six mutant strains from different families (MATE (n=2), OMP (n=2), MFS (n=1) and ABC (n=1)) showed decreased virulence in the *G. mellonella* infection model, and only three of them displayed sequence similarity to known proteins (OmpA-, MacB- and TolC-like proteins).

Unfortunately, we have not been able to complement the mutant strains (**Manuscript 6**), in spite of several attempts and approaches, and thus, the data presented in this section is still preliminar. Nevertheless, the identification of novel membrane transporters that might contribute to antimicrobial resistance and/or virulence in the *A. baumannii* AB5075 strain, suggests that transport protein families less studied than RND, such as ABC and MATE families, might have key roles in resistance and pathogenesis, and that further studies are needed to corroborate their involvement and identify any putative substrate that could be used as a target for novel drug development.

Manuscript 6

Unravelling the role of bacterial membrane translocation in the virulence and resistance of MDR *Acinetobacter baumannii*

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Hypothesis: Transport-related proteins may have an important role in *A. baumannii* virulence's and could be used as potential targets for antibacterial drug development.

Objectives: Study different putative transport-related proteins in order to determine their specific contribution to antibiotic resistance and virulence in *A. baumannii*.

Material and methods:
A. baumannii AB5075 knock-out mutants for different transport-related proteins located on the chromosome were acquired from a transposon mutant library. T26 insertion at the desired *loci* was verified by PCR in those strains growing in LB agar plates with 5 mg/L tetracycline. The minimum inhibitory concentrations were determined by broth microdilution or gradient diffusion. The *Galleria mellonella* infection model was used to assess differential virulent capabilities. Fitness assays were done in 96 well-plates using antibiotic-free LB broth at 37ºC, OD_{600} was measured every 15 min during 24 h.

Results:
Using a collection of 65 *A. baumannii* AB5075 transport protein mutant strains we have identified 10 mutant strains with altered antimicrobial susceptibility and 6 mutant strains with decreased virulence in the *Galleria mellonella* infection model. Aminoglycosides were the antibiotics most commonly affected, but we also observed altered susceptibility to meropenem, levofloxacin and moxifloxacin despite the presence of multiple mechanisms of antibiotic resistance. Five mutant strains with altered susceptibility presented transposon insertions in genes encoding for the AbeM, CraA, OmpA, AdeJ and AdeB efflux systems while in the remaining strains the transposon interrupted genes encoding efflux pumps not previously reported to be involved in antimicrobial resistance. The mutant strains with reduced virulence were defective for novel efflux pumps from different families, three of them showing sequence similarity to OmpA-, MacB- and TolC-like proteins.

Conclusions: We need to solve some technical issues or seek alternative methods in order to complement the mutants and corroborate these findings. Further studies are needed to characterise the novel target genes that seem to contribute to the resistant and virulent phenotype of the *A. baumannii* AB5075 strain.

Unravelling the role of bacterial membrane translocation in the virulence and resistance of MDR *Acinetobacter baumannii*

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Abstract

The rise on antimicrobial resistance in the nosocomial pathogen *Acinetobacter baumannii* requires the finding of new antimicrobials to treat the infections caused by this bacterium. The interplay between low membrane permeability and increased efflux is responsible for intrinsic resistance to certain antibiotics, but few transporters other than those of the RND family have been studied. Furthermore, efflux pumps and other transport-related proteins might modulate the pathogenesis of *A. baumannii* through membrane translocation of virulence factors. Using a collection of 65 *A. baumannii* AB5075 transport protein mutant strains we have identified 10 mutant strains with altered antimicrobial susceptibility and 6 mutant strains with decreased virulence in the *Galleria mellonella* infection model. Aminoglycosides were the antibiotics most commonly affected, but we also observed altered susceptibility to meropenem, levofloxacin and moxifloxacin despite the presence of multiple mechanisms of antibiotic resistance. Five mutant strains with altered susceptibility presented transposon insertions in genes encoding for the AbeM, CraA, OmpA, AdeJ and AdeB efflux systems while in the remaining strains the transposon interrupted genes encoding efflux pumps not previously reported to be involved in antimicrobial resistance. The mutant strains with reduced virulence were defective for novel efflux pumps from different families, three of them showing sequence similarity to OmpA-, MacB- and TolC-like proteins. Further studies are needed to characterise the novel target genes that seem to contribute to the resistant and virulent phenotype of the *A. baumannii* AB5075 strain.

Introduction

Acinetobacter baumannii is a frequent cause of nosocomial infections, such as bacteraemia, pneumonia and wound infections [1], whose success relies on its great capability to persist in the clinical setting and to acquire and accumulate different antimicrobial resistance mechanisms [2]. This species presents broad intrinsic resistance, mainly provided by intrinsic β-lactamases, low permeability and constitutive expression of efflux pumps, but it can also become resistance to antibiotics through the horizontal transfer of genetic elements carrying resistance determinants or mutations in endogenous genes [2]. Resistance to several antibiotics reduces the treatment options available for this pathogen, inevitably

leading to worst outcomes [3]. The clinical relevance of this species and the high incidence of multidrug resistant (MDR) isolates in the clinical settings has positioned carbapenem-resistant *A. baumannii* at the top of the WHO list of the critical bacteria for which the development of new antimicrobial drugs is considered a priority and an urgent public health need [4].

Nevertheless, *A. baumannii* has generally been considered to have low virulence potential and to affect mainly immunocompromised hosts [1] and, thus, most studies have mainly focused on the mechanisms of antimicrobial resistance of this species and only a few have investigated its pathogenesis. A better knowledge of the virulence traits and pathogenic potential of *A. baumannii* could provide new targets to allow the development of specific antimicrobial drugs against this pathogen. In this regard, efflux pumps contribute to antimicrobial resistance but also participate in the extrusion of other substrates such as siderophores, quorum sensing molecules and, interestingly, virulence factors [5]. Overall the studies regarding efflux pumps in *A. baumannii* have mainly focused on the contribution of proteins from the Resistance Nodulation Division (RND) family to the resistant phenotype [6–9], while similar studies on members from other transport proteins, such as ATP-Binding Cassette (ABC), Multidrug And Toxic compound Extrusion (MATE), Major Facilitator Superfamily (MFS) and Small Multidrug Resistance (SMR) families, remain scarce [2], and even less is known about the involvement of all of them in the pathogenesis of *A. baumannii*, except perhaps for OmpA [2].

A. baumannii AB5075 is a well-characterised strain showing MDR and an exceptional genome plasticity [10]. Furthermore, it is highly virulent both in the invertebrate *Galleria mellonella* infection model and the mice pulmonary infection model [11]. Aside from all this characteristics, the fact that it was recently isolated from a tibia osteomyelitis in 2008 and that harbours an OXA-23 carbapenemase [11], hence being highly resistant to carbapenems, make of *A. baumannii* AB5075 an excellent type strain to search for new targets to develop novel antimicrobial drugs according to WHO recommendations, since other widely used strains are susceptible to most antibiotics and were isolated long time ago, and thus, they are no longer representatives of the current clones circulating in our hospitals. In this study, we have assessed the contribution of different putative transport proteins to the MDR phenotype and the virulence in the *Galleria mellonella* infection model using a collection of *A. baumannii* AB5075 transport protein mutants with the long-term goal of identifying new targets for antimicrobial drug development.

Materials and Methods

Bacterial strains. *A. baumannii* AB5075 knock-out mutants for different efflux pump genes and transport-related proteins located on the chromosome were acquired from a transposon (T26, conferring tetracycline resistance) mutant library (http://www.gs.washington.edu/labs/manoil/baumannii.htm; [10]). Upon arrival, mutants were assigned a number and this internal nomenclature is used throughout the manuscript instead of the original strain name (For example mutant strain 1 was originally designated tnab1_kr121204p08q153 by the supplier and corresponded to item AB00015, check **TABLE 1** for equivalences). Afterwards, growth in Luria-Bertani (LB) agar plates supplemented with 5 mg/L tetracycline was examined. T26 insertion at the desired *loci* was verified by PCR using two sets of primers (**TABLE 1**): in-house-designed external primers (specific for each mutant), and a transposon-specific primer (Pgro-172) in combination with the suitable external primer (differs for each mutant depending on the strand where the transposon was inserted and the orientation relative to the replicon of the transposon).

Antimicrobial susceptibility testing. The minimum inhibitory concentrations (MICs) were determined by broth microdilution (amikacin, gentamicin, moxifloxacin and tobramycin) or gradient diffusion (cefepime, ceftazidime, ciprofloxacin, kanamycin, levofloxacin, meropenem, norfloxacin and tigecycline) (AB bioMérieux, Solna, Sweeden) on Mueller Hinton (MH) II broth (CONDA, Madrid, Spain) or MH agar plates (Becton Dickinson, Heidelberg, Germany), respectively. Experiments were performed in triplicate if a change in the MIC was noticed. Significant differences between the MICs of the wild type (WT) and the mutant strains were considered when ≥3 dilution differences in at least two replicates were observed.

***Galleria mellonella* infection model.** The *G. mellonella* waxworm caterpillars were purchased from Livefood, UK and stored in the dark with wood chips at 15ºC for no more than a week prior to their use. Briefly, bacteria were grown overnight (ON) at 37ºC with shaking (180-200 rpm) in LB broth, supplemented with 5 mg/L tetracycline in the case of the mutants. For each strain, 1 mL of the ON culture was washed twice and serially diluted in Phosphate Buffered Saline (PBS) to determine the colony forming units (CFU)/mL of each dilution, which were maintained at 4ºC until used. The next day, 10 µl of the dilution at 10^7 CFU/ml were injected into the haemolymph of the larvae using a Hamilton syringe to achieve an inoculum of 10^5 CFU/larvae. Then, the larvae were incubated at 37ºC for up to 3 days and survival was assessed every 24 h. Bacterial strains

were analysed on at least three separate occasions, whenever the statistical analyses (see below) showed differences with the WT strain or inconclusive results, the assay was repeated twice more to corroborate the results. Sixteen larvae were randomly selected for each group, and for each replica, one negative control group was inoculated with PBS, as well as a positive control group was inoculated with the WT AB5075 strain (n=16). Only those experiments in which the PBS-injected larvae had a survival greater than 90% and the WT-injected larvae had a survival lesser than 50% were taken into consideration. The GraphPad Prism software version 5 for Windows, (GraphPad Software, La Jolla, California, USA) was employed for the statistical analysis of bacterial virulence. The Log-rank (Mantel-Cox) test was used to evaluate differences between survival curves. Moreover, the percentage of survival at 24 hours was analysed using paired Student t test. For these analyses, statistical significant differences between the WT and mutant strains were considered if the p-values of both tests were < 0.01.

Effect of efflux pumps inhibitors (EPIs) on *A. baumannii* AB5075 WT virulence on the *G. mellonella* infection model. The EPIs used in these assays were: carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), phenylalanine-β-naphthylamide (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP) and omeprazole (OPZ). Prior to performing the virulence experiments, standalone injections with increasing EPI concentrations were used to evaluate the toxicity of the EPIs. Once the non-toxic concentrations of the EPIs were determined, the virulence assays were performed by inoculating PBS-washed bacteria at 10^5 CFU/larva (weight 250 ± 25 mg) followed by inoculation of either PBS or the EPI 30 min after the inoculation of the bacteria. PBS+PBS and PBS+EPI combinations were used as controls. At least 3 replicas (n=16) were performed per condition. The GraphPad Prism software version 5 for Windows (GraphPad Software, La Jolla, California, USA) was employed for the statistical analysis of bacterial virulence. The Log-rank (Mantel-Cox) test was used to evaluate differences between survival curves. For these analyses, statistical significant differences between untreated and EPI-treated AB5075 WT strain were considered if the p-values < 0.01.

Fitness assessment. Liquid bacterial cultures were grown ON at 37ºC in LB with no antibiotic pressure. The next day, the OD_{600} of all cultures was adjusted to $OD_{600} = 1$ with LB medium, and subsequently diluted 1:100. For each strain, 100 μL/well of the former bacterial suspension were inoculated in four wells of a 96-well microtiter polystyrene plate. Then, the plates were incubated at 37ºC with orbital shaking in an EPOCH 2 Microplate Reader (BioTek; Winooski, USA) instrument and the OD_{600} was automatically measured every 15 min for 20 h. The growth rate (μ, h^{-1}) of the AB5075 WT strain and

the mutant strains was determined after three biological replicas. Pairwise differences were compared using the paired Student's t test with GraphPad Prism software version 5 for Windows, (GraphPad Software, La Jolla, California, USA). p-values < 0.05 were considered to be statistically significant.

Construction of the complementation plasmids. The defective loci of the mutants showing altered phenotypes were amplified from AB5075 WT strain using the primers listed in **TABLE 2**. All additional primers used to construct the complementation plasmids are also included in **TABLE 3**. Amplicons from PCR were usually ligated to the ColE1 cloning vector (pGEM®-T, Promega) by A/T cloning according to the supplier's recommendations. The resulting fragments were heat-shocked into competent *Escherichia coli* DH5α and transformants were selected in 50 μg/ml ampicillin LB agar plates, since the pGEM®-T vector harbours an ampicillin resistance gene (bla_{TEM}), and the insertion of the DNA fragment was corroborated by PCR using the M13 universal primers. Then, when convenient, the constructs were digested with the appropriate restriction enzymes (New England Biolabs®) according to the supplier's guidelines, and the purified products were ligated to the backbone of the corresponding complementation plasmid (pCSG005 or pCSG005::prom, discussed below) using T4 DNA ligase (New England Biolabs®) at 16ºC ON, and subsequently transformed into competent *E. coli* DH5α. Once the final plasmid construct for complementing a mutant strain was obtained it was electroporated into electrocompetent cells of the corresponding mutant. Transformants were usually selected in 30 μg/ml potassium tellurite LB agar plates. After incubation, the colonies growing in the supplemented media were screened by PCR for the presence of the plasmid.

TABLE 1. Mutant strains verification primers list and results obtained. The strain name corresponds to the item number in the original transposon mutant collection database. Primer sequences are read 5′ to 3′.

#	Strain name	Direction	Strand	Forward Primer	Tm	Reverse Primer	Tm	WT size	Mut size	Verification
1	AB00015	F	-	GCATCAGTTCAACCACCG	54.1	GCATCCCTAACTCACCTCG	55	2404	4204	+
2	AB00076	F	+	CGTTCGCAATACGATACGG	53.6	CGCATCATCCACAACAGC	54.5	4107	5907	+
3	AB00165	R	+	GCTGATTTTGCGAACTTGGC	55.6	GGTGTTGGGATTACCACG	53.3	2039	3839	+
5	AB00304	F	-	GGCTCAACTGTAGAAGGC	53	CCTAATACGGTTCTGCTTTGC	53.9	2149	3949	+
7	AB00454	R	-	CGTCCAACGATTGTGTTTGC	55.1	CAGGGTCTTCTAACTCACG	52.2	1914	3714	+
10	AB00545	F	+	GTCATTTGTTAGGTGGTGAGC	53.9	GTTTCAGGATCACGACCC	52.9	2067	3867	+
11	AB00590	R	-	CGTCTTTCTATGACCTCACG	52.6	CAGCAATGAGTGGTTCAGC	54.2	2372	4172	+
13	AB00738	F	+	TGTAGCCTGCGTGAATGG	55.2	CAACCAGTAAGTTGCGTAGC	53.9	2246	4046	+
14	AB00812	R	+	CTGTTCAGCCGAAATAGGAG	53	GCAAGCACTAATGCCTTACG	54.3	3072	4872	+
15	AB00941	F	+	GAGTGAGGATGCTGTTTCG	53.3	CCTTGCCTGAACTCATAAGC	53.6	1837	3637	+
16	AB01344	F	-	CCAGACTTAGTAGTTGTGGC	52.6	CTCTGAACATCACCTGATTGC	53.7	2060	3860	+
18	AB01774	F	+	CAGTCGGCTAGAATACTGG	52	GTCACCATAACTATGTGAGTC	50.2	2014	3814	+
19	AB01842	F	-	CTTCCATAGCACCTGAACGG	55.6	GCTCAACAAGCCAATGTGG	54.8	4001	5801	-
21	AB01921	F	+	CTCGCAGGAGTCATATCG	52.2	GCAGCAGATGATGAAGAGC	52.6	2085	3885	+
22	AB02233	R	+	CCCATCAGACCAGATTCG	52.5	CTGTTGTTGCTGTACTTGACG	54.1	2759	4559	+
24	AB02295	R	-	GCTACGACCTGAAATAGACC	52.5	GCTGGTGCTTTGGTTACTGC	57.1	3884	5684	+
25	AB02468	R	-	GAGGCGTAGTCTGACATGC	55.5	CCATACAGCACTTTGAGTTGC	54.4	2082	4238	+
26	AB02501	F	+	GCTTACATTGGTTGTTCCACC	54.4	GCAAGATCCCGTTCTAGG	52.5	3775	5575	+
27	AB02621	R	-	CAATAGAGGTGGCAAGACC	52.9	TAGACCGAGGCTCTCAACAG	55.9	2240	4040	+
28	AB02656	F	+	CCTTCATACATGCCAGGC	53.5	CACTCACTGCTTGTGTAAGTC	53.4	2314	4114	+
29	AB02711	F	+	GAAGCACGTAGCGTAAACG	54	AGCACATTGTTCATAGTTCCC	52.9	2177	3977	+
30	AB02744	R	+	GAGGAACAACACGAACACC	53.7	CTTTATGCTCACCTGATGC	51.1	1507	3307	+
31	AB02915	F	-	CAACAACGGCATTTGCTGG	55.5	CGACACCTAATTCCACACAG	53.2	2869	4669	-
32	AB03081	F	+	GCTGTTCATCACCAGTTTCC	54	GAGTGAGTTCAAGCACGG	53.4	3297	5097	+
33	AB03226	F	+	CATACCGTCTTGCTGATGTGG	55.8	AAACGCACACCAGTAAGG	52.8	2430	4230	+
34	AB03340	R	-	CTGTTACCACAACTTTACTGCC	53.9	GCACCTATCGTTACCGTAGG	54.7	2381	4181	+
35	AB03344	F	-	CCAAGTAAGAGAAGGGTGG	52.3	GGTGAAACCTCTATTCTGGC	53	2475	4275	+
36	AB03380	R	-	ACGCATTCCTGAACCACG	55.7	ACACTTATCTCGGCTCGC	54.6	2048	3848	+
37	AB03598	F	-	CCATGAGTGAGGACAACAACC	55.8	CAGCGAATGCTAACCTACAAGC	56.3	3990	5790	+

#	Strain name	Direction	Strand	Forward Primer	Tm	Reverse Primer	Tm	WT size	Mut size	Verification
38	AB04058	R	-	CGTGTTTCACCAGTTGCTG	54.5	GGTTCCATATCAGGTTTGAGTTC	53.5	2190	3990	-
42	AB04074	R	-	CCAAGTCACCAATACCACC	53.1	CGTGAATCAGCCAATCAGC	54.3	1714	3514	+
43	AB04177	F	-	GCAACTGTATTGGTGTAGG	50.7	GCATTGAGTGTTGTTATTCGG	52.2	2045	3845	+
45	AB04230	F	-	TCAATCGCTGCCTGTTGC	56.3	CACTCACCACCATTTCTGG	53.4	2243	4043	-
46	AB04245	F	-	GAGTTACTTCAAGTGCGTGG	53.6	CCTATTGAGGCAACATCTGC	53.6	1935	3735	+
47	AB04386	F	+	GCACTTGTAAACCTTATGGC	51.8	AGTATCATTCTCACCTTGGC	52	1847	3647	+
48	AB04394	F	-	GTATGTGCCAGTTGGTTTCC	54	GAAGAGTTGGGTTCAGACC	52.7	2757	4557	+
49	AB04481	F	+	GGACCGTAGAGAGTATCAACC	54	CACTACATCGTTACTACTGCG	52.8	2247	4047	+
50	AB04849	R	-	TCTAGCCAGAGTTCACCG	53.6	CCAGTATCACTGACATTGCC	53.3	2602	4402	-
51	AB05065	F	+	GAGGTAGGTCTGGTTCAGG	54.2	GACATACTTTGCTGGTGTCTC	53.3	1995	3795	+
52	AB05078	R	-	CTTGGAGCGAAGGTTGGTG	56.3	TGGTTGGCTGCTTAGGTG	55.2	2122	3922	+
53	AB05082	F	-	GGTTGTCTTTCTACTGCCAGC	56	GGAACCTTACAGCCTCTGC	55.6	2478	4278	+
55	AB05142	F	+	GGTTGAGTTTGCCGATTGG	54.5	GATCAGGAACATCCAGTGCC	55.7	2438	4238	-
56	AB05156	F	+	CTTCGGGTAATGGCTTACTGC	55.9	CGTGTTAGCAAGTGGTGG	53.7	1830	3630	+
57	AB05228	F	+	CCGTTCGTATTACCAATAGCC	53.2	GTCATTCCTAAACCCACCTG	53	4063	5863	+
58	AB05252	F	+	TGTCTGGGAACGAAATGG	52.3	CACAATGCTGTTTACTGCC	52.1	1713	3513	+
59	AB05504	R	-	TGGTTTGTTAGCAGGACG	52.5	TCTGAGCTTCTGATGTGCC	54.7	2263	4063	-
60	AB05735	F	-	GTGCGTAGAGATTGTTGGG	53.4	GCTGGACATCATCAACTGC	54	2898	4698	+
61	AB05825	F	-	CGTTCCTAATGTGACTGCC	53.4	GGTGACAACCTGAACAACC	53.8	1708	3508	-
62	AB05975	R	+	CGTAAATCCTTCAACTGCGAGC	56.4	GCTTTGACCAGTACGCTGC	56.8	1179	2979	+
63	AB06254	R	+	CATCCGACTCTTGTTCTACC	52.4	CAATCCCAATCACAGGTGC	54.1	2543	4343	+
64	AB06328	F	+	GCAGCAGGTTCAATAAGTGC	54.6	CAGACAAGTACCGACGTG	52.7	3124	4924	+
66	AB06490	F	-	CGTAGATGAGAACGGTAAGGTG	54.8	CAGATGTCGCAACTGCCAC	56.8	1823	3623	-
67	AB06527	R	+	CCTAAACCGACCATTGCACG	56.5	CCGACATGCGATGAACTGG	56.3	2004	3804	-
68	AB06557	F	-	ACGAACCTGACAACTTCC	52.1	CTTTGACGAACAAGGGTACG	53.6	2402	4202	+
69	AB06623	R	-	GCT TTG AGC ATG AGT GAA TCG	54.4	GTGAAAGGGAAGTGTTTAGCC	53.8	1928	3728	+
72	AB06816	R	-	GTATCCACATCAACTTTCTGG	51	GAAGCGTATGCCATTCAGG	53.8	1636	3436	+
74	AB06960	F	-	CAAAGGCTCTGTCATGGC	53.9	CAACAACGGTTCGTACACG	54.2	2909	4709	+
75	AB07143	F	-	GATGACTTGAATGTCTGTGC	51.5	GTGGGTGTTGGTAGATCC	52.4	2136	3936	-
76	AB07029	R	+	CCATCTGCTGTGACTGGTG	55.8	CCTTGGTTGATGAATCCTTAGC	53.6	2419	4219	+
77	AB07775	R	+	CATTGTACTTGCCTCGATTACC	53.6	ACTGTGTCAGCGACATGC	55.6	2587	4387	+
78	AB07248	F	-	CGACTCGCTACCAAAGTGG	55.7	CATCTCAAGTCAGCATTGC	51.6	2347	4147	-

#	Strain name	Direction	Strand	Forward Primer	Tm	Reverse Primer	Tm	WT size	Mut size	Verification
79	AB07431	R	-	CCACAAGTACAGTCCAGC	52.9	CCAAAGACCGTGAACAGC	54	1954	3754	-
81	AB07615	F	-	CTGGAGTGGACATTAGTGACC	54.8	GACAGTACAGGGTGGTTAGAC	54.5	3181	4981	-
82	AB09431	R	-	GTCCACCCATTGGTAGTGC	55.9	GGTTTCTCACCGTTAGCG	53.4	1507	3307	-
83	AB07674	R	-	CTTCCCATTCAAGGTTTCCTG	53.7	GTCTTGCTGCCAATGTTGC	55.3	2171	3971	+
84	AB07704	F	+	CCACAGCATTCATTTCAGGC	54.7	GCAACCAACTCAACAGGG	54.1	1935	3735	+
85	AB07727	F	-	CCTTCACACTGTGGTGGTAGC	57.9	GATTGTGTTGTAGTGGGTGC	54	2071	3871	+
87	AB07857	F	+	TGGTGTGCGTAACATGCG	56.2	GGTGCCTTCATCGTTCCAG	56.1	2203	4003	+
88	AB07942	F	-	CAAACGCTGCCAGTAGTAAACG	56.5	GATAGCATTGGTGGAATTGTGG	54.2	1749	3549	+
90	AB08175	F	-	CTCCGACATTCACAAAGTCC	53.4	GCAAGAAGAAGCGTCTCACG	56.3	1945	3745	+
91	AB08251	R	+	CAGGTGTAGGTAGAGCGAC	54.3	CTTTGGCAACTAGAACATTGGC	54.7	2772	4572	+
93	AB08557	R	+	CACGGTAAGCGTATTGACC	53.3	CCTATTACTTTGGTTGCTGGC	54	2009	3809	+
94	AB08647	F	+	CTGTCTTACCTACCGTTATGC	52.6	CCCAAGGAAACTCTGAATGG	53.3	2500	4300	+
96	AB08797	R	+	CCACAAGTTCAACAACAAGC	52.6	GTGAGGAGAAGTGCTACGC	55.4	2566	4366	+
97	AB09043	R	+	CGAAGTTCAGCACGAAGG	53.7	GGACCATTGAAACTCACCG	53.6	1756	3556	+
98	AB09160	R	+	CATTTACGCACGCACCAAGG	57.2	GACCGTCATGTGATTGCTGC	56.8	2775	4575	+
99	AB09863	R	-	GGAACCTTACTTGGTGTAGC	52.9	GGAAGCATACACGCTCATCC	55.9	1858	3658	-
100	AB09515	F	-	CATTGGCAACCTGTTGTTCG	55	GGTGTTGTGGCAGTTGGG	57	2331	4131	+
103	AB09723	F	-	GTACATCACCCACTGTACGC	55.5	GGAGTATGGACTGGTTCGG	54.8	2350	4150	+
104	AB09814	R	-	CCACATCCAGAGGGTTTGG	55.7	GCTATGAACTGTTCTGATGCC	53.7	1615	3415	+
105	AB09822	F	+	CCAACATACAACCTGCTGGC	56.7	CGAGGATGTAAACGAATGC	51.3	2020	3820	+
107	AB09951	R	-	CAACTTCACAAACTCCAGC	51.7	CTATGATTGCCACTAAACTGC	51.6	1164	2964	-
110	AB06964	F	+	TACACGATCCGACACACG	54.4	GTTGACCCAATTTCAGTAGG	50.9	2667	4467	-

TABLE 2. Complementation primers used to amplify the genes putatively involved on antimicrobial resistance and virulence. Restriction sequences are showed in lowercase. F, forward; R, reverse; Tm, primer annealing temperature (°C). Primer sequences are read 5′ to 3′.

#	Strategy	Restriction site Used in primer F	Forward Primer[a]	Restriction site Used in primer R	Reverse Primer[a]	Size	Tm F/Tm R
5	Normal	SpeI	actagtGGCTCAACTGTAGAAGGC	SpeI	actagtCTGGTTAGAACTGATCGCAC	2692	53/53,2
18	Normal	SpeI	actagtCGATGGGTAATCCAGAACTACC	SpeI	actagtGAGCAGGTTTAGATGAGTTAGG	2454	54,7/52,6
64	Normal	SpeI	actagtGCAGCAGGTTCAATAAGTGC	SpeI	actagtCAGACAAGTACCGACGTGG	3124	54,6/55,3
3	Operon	StuI	aggcctAAGGAGATTTTTTGATGAACCAGATTTTTA	NsiI	atgcatGGTGTTGGGATTACCACG	1460	47,9/53,3
28	Operon	StuI	aggcctAAGGAGAGATTTTATGCAAGATCG	NsiI	atgcatCCAGTTGGAGTTGACGC	1242	44,5/55,2
56	Operon	HindIII	aagcttAAGGAGAACAACTATGAAAGGGATGAAG	PstI	ctgcagCGTGTTAGCAAGTGGTGG	1362	51,5/53,7
72	Operon	HindIII	aagcttAAGGAGTTTCTATATGATTAAAAAAGCG	PstI	ctgcagCACACCTGAGTTTAAACAGC	1212	46,3/51,6
96	Operon	StuI	aggcctAAGGAGTGTAAGCATGACAAAACAAGC	PstI	ctgcagGTGAGGAGAAGTGCTACGC	2056	51,9/55,4

[a] Lower case nucleotides indicate restriction sequences. Underlined nucleotides indicate Shine-Dalgarno sequences and those highlighted in red indicate transcription initiation codons.

TABLE 3. Additional primers used for the construction of the complementation plasmids. Restriction sequences are underlined.

Primer name	Sequence (5′-3′)	Purpose
M13 F	GTTTTCCCAGTCACGAC	Universals Primers /M13-40FOR
M13 R	CAGGAAACAGCTATGAC	Universals Primers /M13-26REV
OriUp	GATCGTAGAAATATCTATG	Amplification of the origin of replication of pWH1266
Ori _Lw_SmaI	CCCGGGATTTTAACATTTTGCGTTG	Amplification of the origin of replication of pWH1266
pBADpromF	ACTAGTCTGTCAAATGGACGAAGCAGG	Amplification of the arabinose-inducible promoter from pBAD18
pBADpromR	ACTAGTAGGCCTAAGCTTCCAAAAAAACGGGTATGGAGAAACAG	Amplification of the arabinose-inducible promoter from pBAD18
TelF	GGATGACATGCTCAATACGC	Parcial amplification of the tellurite resistance cassette from pMO130Tel
TelR	CAACCTGGGTCAGTTTCTCG	Parcial amplification of the tellurite resistance cassette from pMO130Tel
TpmF	CCCGGGCAACACTATTCATGGATGCG	Amplification of the tellurite resistance gene *tpm*
TpmR	CCCGGGTACCTCATGTACCATACACC	Amplification of the tellurite resistance gene *tpm*

Results and Discussion

Selection and verification of transport-related mutant strains from the AB5075 transposon mutant library. Mutants of efflux pumps, OMPs and other transport-related proteins from the transposon mutant library were selected based on their putative function description in the library database (http://www.gs.washington.edu/labs/manoil/baumannii.htm; [10]), and T26's preferable central position relative to the Open Reading Frame (ORF), whenever possible. A total of 83 mutants were selected and acquired for analysis (**TABLE 1** and **Annex II**). Of those, only 65 grew under tetracycline pressure and had the T26 inserted at the right locus. According to the putative function description and sequence similarities, the T26 insertion truncated genes putatively belonging to: the ABC family (n=12), the MATE family (n=3); the MFS family (n=28); OMPs (n=8), the RND family (n=9), and other transport proteins/permeases (n=5).

Alteration of antimicrobial susceptibility in the transport-related mutant collection. Antimicrobial susceptibility testing of mutant strains compared to the WT revealed that all the mutants showed MIC values similar to those of the WT strain for the β-lactams ceftazidime and cefepime; the fluoroquinolones ciprofloxacin and norfloxacin; and the glycylcycline tigecycline. In contrast, we observed altered MICs for meropenem, levofloxacin, moxifloxacin, kanamycin, amikacin, tobramycin, and gentamicin in 10 out of the 65 mutant strains when compared to the WT strain, although 3 of them also showed impaired fitness (**TABLE 4**, **TABLE 5**). Overall, 7/10 mutant strains showed altered susceptibility to more than one antibiotic, although in only 2 of them different antibiotic families were affected.

Aminoglycosides were the antibiotics most commonly affected, especially gentamicin, for which the MICs decreased in 8/10 mutant strains that showed altered antimicrobial susceptibility. It should be noted, though, that when we tested the MICs for aminoglycosides using gradient diffusion methods, the AB5075 strain showed heteroresistance to aminoglycosides, particularly to amikacin, gentamicin and tobramycin, and also to the fluoroquinolone moxifloxacin. Heteroresistance to aminoglycosides in *A. baumannii* has been recently described for the first time in the AB5075 strain, and it was shown to be an unstable phenotype caused by the extensive amplification of a plasmidic region containing antimicrobial resistance genes, including *aadB*, which encodes an aminoglycoside modifying enzyme [12]. This phenomenon, added to the phase variant phenotype observed in the *A. baumannii* AB5075 strain, in which cells interconvert between opaque colonies (which are more resistant to aminoglycosides) and translucent colonies (which are more

susceptible to aminoglycosides), hindered the accurate assessment of the MICs of these four antibiotics. The changes in antimicrobial susceptibility were therefore re-evaluated using broth microdilution. The MIC values for these antibiotics still oscillated between different ranges of values in the WT (**TABLE 4**) and many mutant strains (data not shown), in good agreement with the current literature, in which the reported MIC values for amikacin, gentamicin and tobramycin are of 64 to >256 μg/mL, 32 to >256 μg/mL, and 16 to >256 μg/mL, respectively [12,13]. Despite these drawbacks we were still able to assess repeatable and reproducible differences between the mutant strains listed in **TABLE 4** and the WT strain, showing that active efflux plays and important role on aminoglycoside resistance of AB5075, despite the presence of other mechanisms in this strain such as 5 different aminoglycoside resistance genes encoded in a plasmid-borne resistance island [12].

TABLE 4. *A. baumannii* AB5075 transport-related mutant strains showing altered susceptibility (MICs, μg/mL) and fitness compared to the wild type (WT) strain. "↑" and "↓" indicate significant increased and decreased phenotype when compared to the WT strain, respectively. "*" indicates the phenomenon of heteroresistance and oscillating MIC values, for which the most common values of the WT are provided. MEM, meropenem; LVX, levofloxacin; MOX, moxifloxacin; KAN, kanamycin; AMK, amikacin; TOB, tobramycin; GEN, gentamicin; ABC, ATP binding cassette; MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; OMP, outer membrane protein; AI-2E, autoinducer 2 exporter; RND, resistance-nodulation-cell division.

Mutant	Gene Name	Efflux family	MEM	LVX	MOX	KAN	AMK	TOB	GEN
72	-	ABC					↓	↓	↓
98	*abeM*	MATE					↓	↓	↓
15	*craA*	MFS						↓	↓
21	-	MFS						↓	↓
53	-	MFS							↓
94	-	MFS							↓
18	*ompA*-like	OMP	↓	↓	↓	↓	↓	↓	↓
28		AI-2E	↓	↓					
24	*adeJ*	RND			↑				
57	*adeB*	RND						↓	↓
WT			>32	>32	8-12*	>256	32*	32-64*	>256*

TABLE 5. Growth rates (μ) for the selected mutants displaying altered antimicrobial susceptibility (AMS) or virulence. Mutant strains with impaired fitness (paired Student t test, p-value < 0.05) when compared to the WT strain are highlighted in grey. SD, standard deviation; ABC, ATP binding cassette; MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; OMP, outer membrane protein; AI-2E, autoinducer 2 exporter; RND, resistance-nodulation-cell division.

#	Gene Name	Efflux type	AMS	Virulence	Fitness	μ (h^{-1})	SD (±)	p-value
72	-	ABC	↓			2.07	0.26	0.36
96	*macB*	ABC		↓		2.07	0.14	0.59
3	-	MATE		↓		2.17	0.10	0.40
5	-	MATE		↓	↓	1.51	0.11	0.00
98	*abeM*	MATE	↓		↓	1.90	0.06	0.01
15	*craA*	MFS	↓			2.13	0.31	0.32
21	-	MFS	↓			2.19	0.27	0.42
53	-	MFS	↓		↓	1.98	0.14	0.05
64	-	MFS		↓		2.14	0.24	0.85
94	-	MFS	↓			2.20	0.28	0.96
24	*adeJ*	RND	↑			2.36	0.13	0.13
57	*adeB*	RND	↓			2.02	0.13	0.06
18	*ompA*-like	OMP	↓	↓	↓	1.40	0.40	0.04
56	-	OMP		↓		2.00	0.11	0.23
28	-	AI-2E	↓			2.00	0.35	0.52

Our results show that all transport protein families studied might contribute to antimicrobial resistance in the MDR *A. baumannii* AB5075 strain, since at least one mutant from each family presented altered susceptibility. Of note, the involvement on the MDR phenotype of some transport proteins was still noticeable in spite of the multiple additional resistance mechanisms that are present in the *A. baumannii* AB5075 strain. For instance, decreased MICs for meropenem were observed in two mutant strains (**18** and **28**, MICs of 8-16 and 16 μg/mL, respectively, *vs.* WT MIC of >32 μg/mL, **TABLE 4**) even though the AB5075 strain carries the bla_{OXA-23} gene. Interestingly, none of the truncated genes in these mutant strains belongs to the RND family of efflux pumps, some of whose members are known to be able to extrude meropenem, such as the AdeABC and the AdeDE systems [7,14]. Likewise, MIC values of fluoroquinolones were reduced in the mutant strains 18 (levofloxacin, MICs of 4-6 *vs.* WT MIC of >32 μg/mL, and moxifloxacin 2-3 μg/mL *vs.* WT MIC of 8 μg/mL) and 28 (only levofloxacin, MIC of 4-6 μg/mL *vs.* WT MIC of >32 μg/mL), despite the WT strain being highly resistant to fluoroquinolones due to *gyrA*/*parC* mutations [15,16].

The mutant strain 28, in which we observed a decrease in meropenem and levofloxacin MIC values when compared to the WT strain, presents the T26 inserted in a permease gene, which, according to BLASTX searches and conserved protein domain presence, putatively belongs to the autoinducer-2 exporter (AI-2E) superfamily (Pfam family PF01594). The AI-2E superfamily is large, but so far only TqsA homologues (transport of quorum sensing signal A, previously named YdgG) have been characterised [17]. The export of the autoinducer 2 (AI-2) molecule by the TqsA transporter has been empirically demonstrated in *E. coli* strain K12 and *Klebsiella pneumoniae* [18,19]. In those species, the deletion of *tqsA* altered the intracellular levels of AI-2, playing a role in biofilm formation in both *E. coli* and *K. pneumonia*e and in motility in *E. coli* [18,19]. Furthermore, Herzberg *et al.* also showed that the absence of TqsA in *E. coli* increased drug susceptibility to spectinomycin, streptomycin, chloramphenicol and amoxicillin, while susceptibility to penicillin G, erythromycin, ampicillin and tetracycline remained unaltered [18]. Recently, a study using the *A. baumannii* AB5075 strain and likewise transposon mutants from the collection developed by Gallagher *et al.* [10], showed that the locus truncated in the mutant strain 28 was the primary determinant of chloramphenicol intrinsic resistance, and that the mutation of the locus did not affect the susceptibility to rifampin, ceftazidime, carbecillin, amikacin, gentamicin, trimethoprim, imipenem or kanamycin, in good agreement with our findings [20]. Overall, our results indicate that transporters of the AI-2E family might have a wider substrate profile and might also be involved in the export of other antibiotics, such as meropenem and levofloxacin.

The mutant strain 18 has knocked-out a gene that encodes a putative OmpA-like protein (93.93% sequence identity to the OmpA encoding locus AIS_2840 from *A. baumannii* strain ATCC17978). OmpA is known for its role in *A. baumannii* pathogenesis [21], but also in antimicrobial resistance. Smani *et al.* already showed that, in the susceptible *A. baumannii* strain ATCC17978, OmpA was involved in chloramphenicol, aztreonam and nalidixic acid resistance [22]. Despite the fact that mutant strain 18 had impaired fitness, our results suggest a relevant role of OmpA in the MDR phenotype of *A. baumannii*, since we found that it might be also involved in resistance to meropenem, some fluoroquinolones and aminoglycosides (**TABLE 4**). However, given the abundance of this OMP in the outer membrane of *A. baumannii* [23], we cannot rule out that the alterations in susceptibility to several antibiotics might be due to the cumulative effect of the absence of the protein and membrane composition rearrangements.

Interestingly, an increase rather than a decrease in the MIC value of moxifloxacin was detected in the mutant strain 24, in which the T26 is truncating *adeJ*, the inner membrane transporter protein of a well-studied tripartite efflux pump belonging to the AdeIJK RND family [8]. Damier-Piolle *et al.* showed that defective strains for *adeJ* extruded fluoroquinolones [8], resulting in higher susceptibility to these antibiotics, as opposed to our results. Nevertheless, when this mutant strain was assayed under the presence of the EPI CCCP, no significant changes in the MIC values were observed (from 32 to 16 mg/L), while in the presence of PAβN, an eight-fold decrease in the MIC of moxifloxacin was observed in the mutant strain (from 32 to 4 mg/L), restoring its moxifloxacin MIC values to the levels of the WT strain. These results suggest that the higher resistance observed in the mutant strain might be due to a compensative overexpression of other redundant efflux pumps. Additionally, our results corroborate previous findings regarding the inability of this pump to extrude aminoglycosides [8,14].

Some of the transport-related proteins truncated in the remaining mutants with altered MIC values have already been described as antibiotic extruders. For example, the locus truncated in the mutant strain 57 encodes AdeB, the inner membrane transporter protein of AdeABC, a well-characterised RND efflux pump [6]. Our findings are in good agreement with those of Magnet *et al.,* who found that aminoglycosides were AdeABC substrates, being tobramycin and gentamicin more effectively transported by this pump than the more hydrophilic kanamycin and amikacin [6]. Interestingly, we only detected one MATE gene involved in antimicrobial resistance in *A. baumannii* strain AB5075, this gene corresponds to *abeM* (mutant strain 98, with impaired fitness, **TABLE 4**, **TABLE 5**), which, as previously described, seems to participate in the extrusion of aminoglycosides [24]. Nevertheless, fluoroquinolones and some β-lactams have also been shown as substrates for both AbeM and AdeB [6,24] but we did not detect any differences between the WT and our mutant strains, thus suggesting that the additional resistance mechanisms found in *A. baumannii* strain AB5075, such as the *bla*$_{\text{OXA-23}}$ gene, *gyrA*/*parC* mutations and other transporters might be masking these phenotypes. In contrast, we found additional substrates for the gene knocked-out in mutant strain 15, *craA*, an MFS pump with substrate specificity for chloramphenicol in *A. baumannii* [25], which, according to our results, might be also involved in aminoglycosides extrusion (tobramycin and gentamicin). Interestingly, CraA shows sequence similarities to the MdfA transport protein of *E. coli* [25], which has already been shown to confer resistance to some aminoglycosides (kanamycin and neomycin) [26].

There is, however, little information regarding the transporters encoded in the genes of the other mutant strains from the ABC (n=1) and MFS (n=3) families that seem to be involved in aminoglycoside resistance. The mutant strain 72 encodes, according to the conserved protein domains present, a putative periplasmic protein, that binds the substrates and interacts with the membrane ACB transport complexes triggering the translocation of substrates across the membrane. Putatively, periplasmic binding proteins are involved in the transport of inorganic ions, such as bicarbonates and nitrates, taurine and aliphatic sulfonates. However, it is quite likely that the gene truncated in our mutant strain would be involved in the transport of urea, since the allophanate hydrolase and the urea amidolyase and carboxylases (alternative enzymes to urease for urea degradation) are encoded really close in the genome (truncated locus ABUW_2585, urea degradation enzymes loci ABUW_2586 and ABUW_2591-2593). Similarly, the protein encoded in the gene knocked-out in the mutant strain 21 seems yet uncharacterised, and according to the conserved protein domains found *in silico*, it is an MFS transporter of the Bcr/CflA subfamily. This subfamily includes antimicrobial drug transporters such as Bcr (bicyclomycin resistance protein), Flor (chloramphenicol and florfenicol resistance), and CmlA (chloramphenicol resistance), however, sequence similarity between the protein sequence of the gene knocked-out in the mutant strain 21 and these proteins is low (similarity values range from 44 to 51%) and suggest that it might be a novel transporter of this subfamily. Even less information is available about the putative MFS transporters truncated by T26 in the mutant strains 53 and 94. Further studies are still needed to fully characterise their ability to translocate aminoglycosides through bacterial membranes as well as their specific contribution to the susceptibility profile of *A. baumannii*.

Involvement of efflux pumps and OMPs in the virulence of AB5075 strain in the *G. mellonella* infection model. Some capsule and envelope components, such as LPS, are able to trigger the immune response of bacterial host. Since proteins from the inner and the outer membrane are missing in the mutant strain collection evaluated, we first wanted to confirm that infection of *G. mellonella* by the AB5075 WT strain needed alive bacteria to successfully infect and kill the larvae. Assays using heat-inactivated AB5075 WT strain, thus is bacteria incubated for 1 h at 80ºC, demonstrated that active bacteria were required to kill the caterpillars, as all *G. mellonella* larvae inoculated with heat inactivated bacteria survived (data not shown). For our study, these results indicate that the mere presence of transporter proteins in the membrane is not enough to develop a successful infection and/or immune response, and thus, it suggests that any difference that we might observed between the mutant and WT strains would be due to the lack of an active and functional protein rather than to its absence in the bacterial envelopes.

Next, in order to preliminary evaluate the putative role of efflux pumps, OMPs and other transport-related proteins, we assessed the virulence of the AB5075 WT strain in the *G. mellonella* infection model in the presence of EPIs. No previous data regarding the toxicity of CCCP, PAβN, NMP and OPZ in *G. mellonella* was available in the literature, so we first determined the individual ability of these EPIs to kill *G. mellonella* caterpillars at different doses. The doses used to evaluate the toxicity of the EPIs were as follows (mg EPI/kg larvae, average larvae weight: 250 ± 50 mg): CCCP 2.5, 5, 8, 10 and 20; PAβN 10, 20, 25 and 50; NMP 10, 20, 25 and 50; and OPZ 10, 20 and 40. CCCP was the most toxic compound, killing all the larvae at 20 mg/kg. All the other EPIs did not compromise the survival of the larvae at any of the doses tested and, thus, the higher dose evaluated was selected for the virulence assays, except for CCCP, for which we used 8 mg/kg. Survival curves between the EPI-treated and non-treated larvae infected with the AB5075 WT strain showed a significant increase in surviving larvae only for NMP (Log-rank test, *P*-value < 0.0001) (**FIG 1**, **A**). No significant differences between EPI-treated or non-treated larvae were observed for all the other EPIs tested (**FIG 1**, **B-D**). NMP reduction of the highly virulent phenotype of AB5075 points to the involvement of efflux pumps in the virulence of this strain, although, due to efflux pump nonspecific inhibitory effect of this EPI, the exact mechanism involved is difficult to determine. Nevertheless, these results should be interpreted carefully since the loss of virulence in the presence of NMP might also be due to mechanisms other than inhibition of efflux pumps, such bacterial fitness decrease or enhancement of the immune system of the larvae.

Similar to aminoglycoside resistance, the virulence of the AB5075 strain in the *G. mellonella* infection model is greatly affected by the phase variation phenotype. It has been found that opaque cells are more virulent than the translucent ones, and that inside the *G. mellonella* caterpillars there is a strong shift to the opaque-virulent variant [27]. Since the interconversion between the two phases is unstable and difficult to control in every step of the infection protocol (overnight liquid culture growth, injection and infection of the larvae, growth in agar plates, etc.), especially when working with mutant strains, we included more larvae and replicates than usual for this kind of assay. We used 16 larvae instead of 10, which is the most common number employed per replica, and we did 5 replicates when assays gave inconclusive or promising results, instead of the usual 3. In addition, we analysed the results obtained adding a second statistical analysis, the paired Student t test of the percentage of survival at 24 h, time at which the most striking differences were observed, and we lowered the p-value at which we considered statistical significant differences between the WT and mutant strains to below 0.01 in both statistical analyses employed, the Log-rank test and the Student t test. By doing so, we avoided

selecting for further analysis mutant strains with random behaviours and ensured to choose those where the differences were patent and reproducible (**Annex III**).

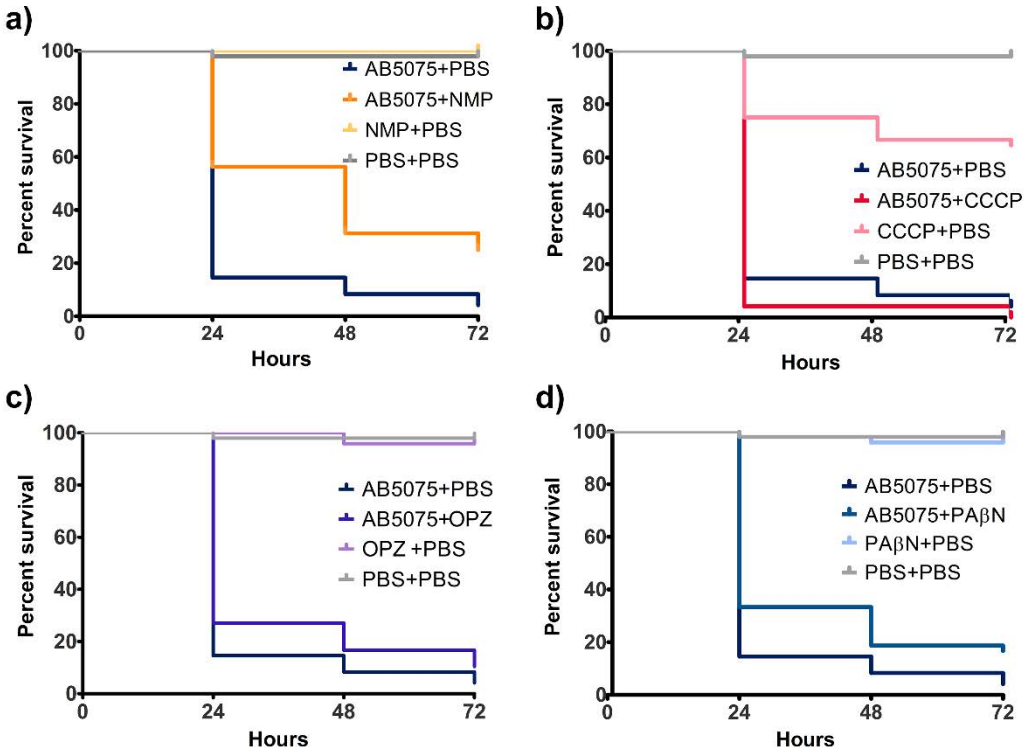


FIG 1. Survival curves of *Galleria mellonella* larvae inoculated with *A. baumannii* AB5075 WT strain and different efflux pump inhibitors: **A)** 1-(1-naphthylmethyl)-piperazine (NMP, 50 mg/kg), **B)** carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 20 mg/kg), **C)** phenylalanine-β-naphthylamide (PaβN, 50 mg/kg), and **D)** omeprazole (OPZ, 40 mg/kg).

After the analyses, only 6 out of 65 mutant strains analysed showed statistically significant attenuated virulence in the *G. mellonella* infection model when compared to the AB5075 WT strain (**FIG 2**). The genes truncated on these mutants corresponded to the MATE family (n=2, mutant strains **3** and **5**), OMPs (n=2, mutant strains **18** and **56**), the MFS (n=1, mutant strain **64**) and the ABC family (n=1, mutant strain **96**). Unfortunately, the mutant strains 5 (MATE family) and 18 (OmpA-like) had impaired fitness (**TABLE 5**), and thus it seems quite likely that this defect, rather than the truncation of the gene, might be causing the attenuation of virulence observed in these mutants.

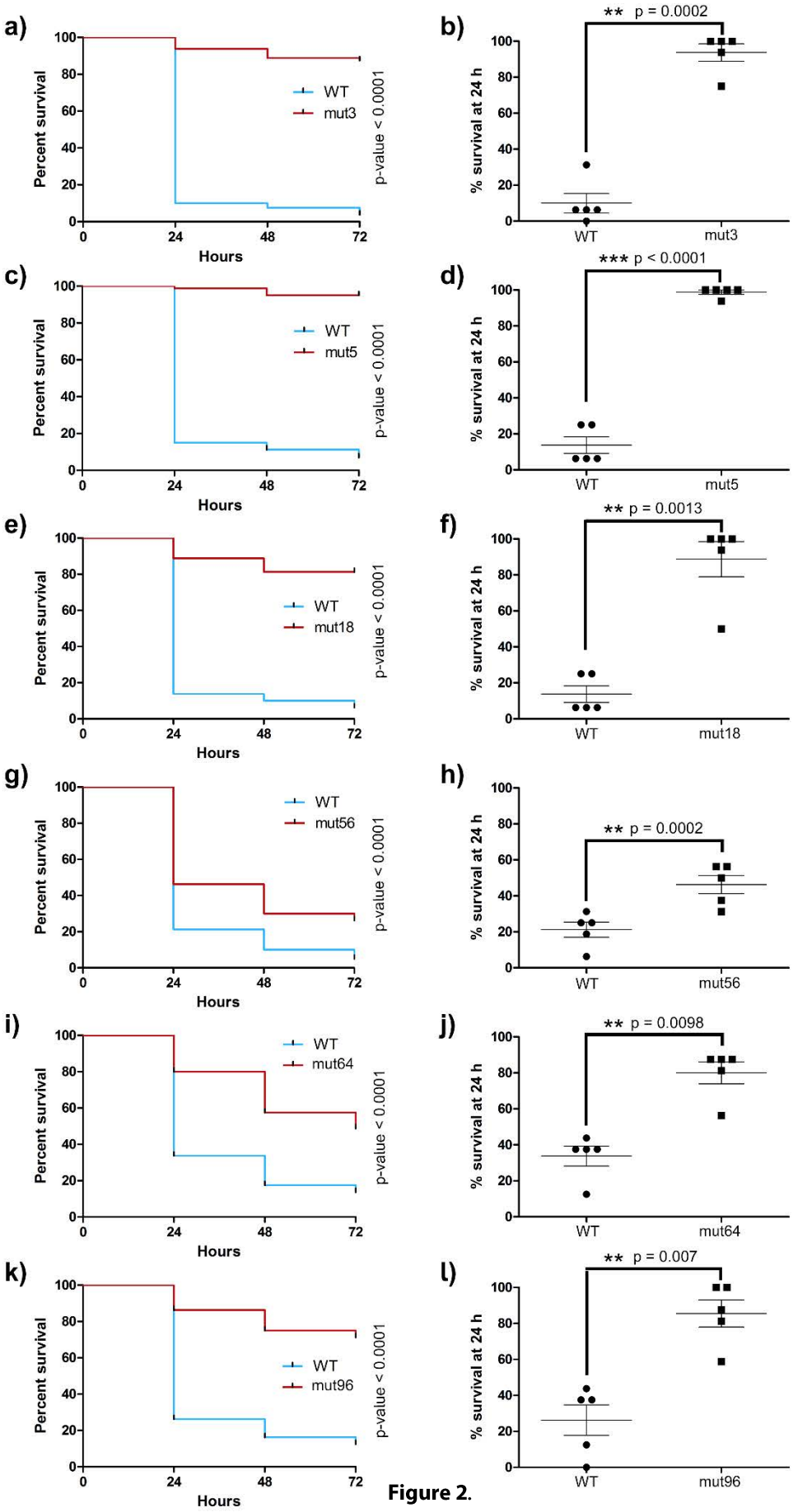

a)
Percent survival
Hours
WT
mut3
p-value < 0.0001
b)
** p = 0.0002
% survival at 24 h
WT
mut3
c)
Percent survival
Hours
WT
mut5
p-value < 0.0001
d)
*** p < 0.0001
% survival at 24 h
WT
mut5
e)
Percent survival
Hours
WT
mut18
p-value < 0.0001
f)
** p = 0.0013
% survival at 24 h
WT
mut18
g)
Percent survival
Hours
WT
mut56
p-value < 0.0001
h)
** p = 0.0002
% survival at 24 h
WT
mut56
i)
Percent survival
Hours
WT
mut64
p-value < 0.0001
j)
** p = 0.0098
% survival at 24 h
WT
mut64
k)
Percent survival
Hours
WT
mut96
p-value < 0.0001
l)
** p = 0.007
% survival at 24 h
WT
mut96

Figure 2.

FIG 2. *Acinetobacter baumannii* transport-related mutant strains (mut) showing attenuated virulence in the *Galleria mellonella* infection model when compared to the wild type (WT) AB5075 strain (mut3, mut5, mut18, mut56, mut64 and mut96). The results are represented as Kaplan-Meier survival curves (**A**, **C**, **E**, **G**, **I**, **K**) and dot blots of the percentages of survival at 24 h (**B**, **D**, **F**, **H**, **J**, **L**), where each dot corresponds to one replicate. Statistical significant differences in virulence between the WT and mutant strains were considered when both the Log-rank test analysis and the paired Student t test comparison of the percentage of survival at 24 h gave p-values < 0.01.

According to BLAST searches and protein domain presence, the protein encoded in the locus truncated in the mutant strain 3 is a putative MATE efflux pump that belongs to the NorM superfamily. The NorM efflux pump was amongst the first transporters categorised in the MATE family [28]. The NorM-like efflux pumps from *Vibrio parahaemolyticus*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* and the YdhE efflux pump from *E. coli* have been involved in antimicrobial drug efflux, although with different substrate specificities [29–31]. In *Erwinia amylovora*, a plant pathogen, the NorM efflux pump has been associated with bacterial competition [32], however there is little evidence of MATE transporters involved in bacterial virulence [33], except for DinF and ClbM [34,35]. DinF is a MATE efflux pump of the phytopathogen *Ralstonia solanacearum* that if inactivated attenuates the infection symptoms caused in tomatoes, hypothetically, DinF protects bacteria of the toxic effects of host antimicrobial compounds [34]. In contrast, the MATE transporter from *E. coli*, ClbM, is involved in the active transport of precolibactin to the periplasm, the precursor of the toxin colibactin, a molecule causing double strand DNA breaks [35]. Thus, these evidences show that the bacterial MATE efflux pumps can, indeed, transport toxins across the membrane, although in this case the molecule transported was the pretoxin. Interestingly, ClbM was not involved in the transport of antibiotics [35]. We neither observed changes in the susceptibility to antimicrobials in the mutant strain 3 when compared to the WT strain, however, further studies are needed in order to determine if the mutated MATE efflux pump is involved in the extrusion of *G. mellonella* antimicrobial compounds or in the transport of virulence effectors of *A. baumannii* AB5075.

The mutant strain 96 also showed attenuated virulence in the *G. mellonella* infection model. The gene truncated in this mutant strain putatively encodes a transporter of the MacB family (macrolide-specific ABC-type efflux carrier [36]) which belongs to the ABC family. In *E. coli*, the MacB transporter is located at the inner membrane and needs the membrane fusion protein MacA and the OMP TolC in order to successfully transport compounds across the bacterial envelope [36]. MacB-like proteins can confer resistance to antibiotics,

however, the MacAB-TolC tripartite system differs from other ABC transporters in the sense that ATP consumption does not promote the translocation of substrates from the cytoplasm to the periplasm, since the channel of MacB is not big enough to allow the transport of substrates [37]. Instead, ATP consumption triggers a conformational change, helping MacB to bind to periplasmic substrates and initiate their export through MacA-TolC to the extracellular milieu [37]. MacB does not only mediate the extrusion of antibiotics such as macrolides, but it can be involved in the transport of substrates related to virulence in Gram-negative bacteria, such as peptidic toxins and virulence factors [38,39]. The crystal structure of MacB from *A. baumannii* has recently been elucidated and, in 2015, it was found that its expression was regulated by the two component system BaeSR [40,41]. However, little is known about the substrates that are transported by the MacAB-TolC ABC efflux pump in *A. baumannii*. Interestingly, the mutant strain 56, which also showed attenuated virulence in the *G. mellonella* infection model, has the T26 inserted in a locus which putatively encodes, according to BLAST searches and protein domains present, an OMP of the TolC superfamily. Thus, we could speculate that the loss of virulence observed in these two mutants could be, somehow, connected. In addition, we observed that, in *A. baumannii* AB5075, virulence attenuation on the MacB mutant was greater than that of the strain defective of the TolC-like OMP, similar to what has been reported in mice infected with *Salmonella* [39] (**FIG 2**). This fact might reflect the promiscuity of both proteins since the putative virulence-related substrate binding to MacB might specifically require this protein, while its expulsion through the outer membrane might not be restricted to TolC.

Finally, the locus truncated in the mutant strain 64, which is a putative MFS efflux pump, might correspond to a novel transporter of this family. BLAST searches did not give any characterised hit, but the membrane protein topology prediction program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) predicted 12 transmembrane helices, which corresponds to the topology found in almost all MFS proteins [42].

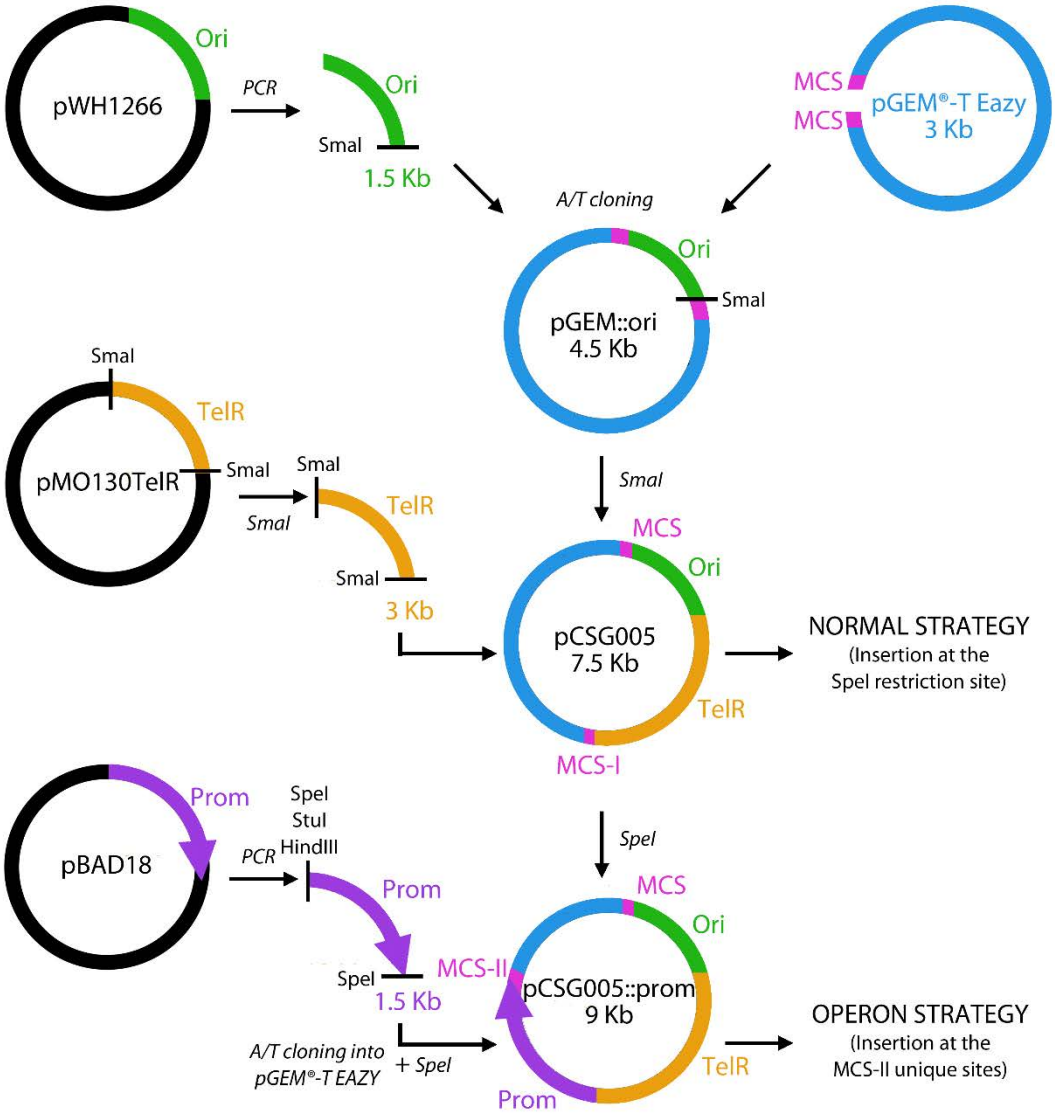
Complementation strategies. In order to corroborate that the truncation of the genes and, thus, the absence of the encoded protein, are the cause of the differences in antimicrobial susceptibility and virulence observed, it is necessary to provide a functional copy of the gene in *trans* to see if it restores the WT phenotype. The *A. baumannii* AB5075 WT strain is resistant to almost all antibiotics with few exceptions as tetracycline, colistin and tigecycline. In addition, all mutant strains are resistant to tetracycline due to the insertion of the T26 transposon. Consequently, the pool of selection markers for complementation

studies with these mutant strains is rather limited. To overcome this issue, we built a plasmid containing a heavy metal resistance cassette and an origin of replication for *Acinetobacter* spp. In order to do so, we used the commercial vector pGEM®-T Eazy, from Promega, as scaffold, and the plasmids pMO130TelR and pWH1266 as the donors of the tellurite resistance cassette and a functional origin of replication in *A. baumannii*, respectively (**FIG 3**).

The origin of replication from pWH1266 was amplified by PCR with primers designed to introduce an SmaI restriction site (≈1.5 Kb fragment) and ligated to pGEM®-T Eazy (≈3 Kb) by A/T cloning. The resulting plasmid was called pGEM::ori (≈4.5 Kb). Subsequently, the tellurite resistance cassette was extracted from pMO130TelR by SmaI digestion (≈3 Kb) and introduced into pGEM::ori in the SmaI restriction site, this plasmid was designated pCSG005 (≈7.5 Kb). Then we chose the unique restriction site SpeI from the multiple cloning site (MCS) of pGEM®-T, absent in all the truncated genes, to introduce those transport-related genes which were not located in an operon.

The primers used to amplify the genes that we wanted to complement introduced the SpeI restriction site at both ends of the amplicon and, in addition, they amplified around 500 bp upstream of the initiation codon to ensure including the native promoter of the gene (**TABLE 2**). The resulting amplicons were subsequently ligated to pGEM®-T Eazy by A/T cloning, and posteriorly digested and ligated to pCSG005 at the SpeI site (normal strategy), generating the complementation plasmids.

Alternatively, in order to complement those mutants in which the truncated gene was putatively encoded by an operon, we followed a different approach. We introduced the arabinose-inducible promoter from pBAD18 (≈1.5 Kb fragment) into pCSG005 at the SpeI unique site. The plasmid obtained was called pCSG005::prom (≈9 Kb). The primers used to amplify the promoter from pBAD18 were designed in order to provide additional unique restriction sites, HindIII and StuI, which combined with those already available in the MCS of pGEM®-T Eazy (PstI, BstXI and NsiI), allowed us to use different enzyme combinations to guarantee the orientated insertion of the genes downstream of the promoter (the restriction enzymes selected to introduce the gene in each mutant are described in **TABLE 2** and **Annex IV**). In addition to the corresponding restriction sites, the forward primers used to amplify the genes were designed to include a Shine-Dalgarno sequence 7 or 8 nucleotides upstream of the initiation codon (**TABLE 2**). The resulting amplicon was subsequently ligated to pGEM®-T Eazy by A/T cloning, and posteriorly digested and ligated to pCSG005::prom at the corresponding restriction sites (operon strategy), producing the complementation plasmids of the genes putatively encoded on operons.



Unique sites of interest (Ori>TelR direction, marked in bold and with "*"):

MCS-I: **SpeI***>EcoRI>NotI>BstZI>**PstI***>SalI>NdeI>SacI>**BstXI***>**NsiI***

MCS-II: **HindIII***>**StuI***>SpeI>EcoRI>NotI>BstZI>**PstI***>SalI>NdeI>SacI>**BstXI***>**NsiI***

FIG 3. Plasmid construction for the normal and operon complementation strategies. The commercial vector pGEM®-T Eazy, from Promega, was used as scaffold, and the plasmids pWH1266, pMO130TelR and pBAD18 as the donors of a functional origin of replication in *A. baumannii* (Ori, in green), the tellurite resistance cassette (TelR, in orange), and an arabinose-inducible promoter (Prom, in purple), respectively. MCS, multiple cloning site. Drawings are not to scale.

Following this approach, we first focused on obtaining the complementation plasmids of the mutant strains with decreased virulence, including those with impaired fitness (**TABLE 5**) and also the complementation plasmids of the mutant strains with altered antimicrobial susceptibility of the ABC and the AI-E2 families. We designated the complementation plasmids as pM#C, where # corresponds to the mutant strain number. Unfortunately, we were unable to introduce the complementation plasmids into the selected mutant strains. We were only able to successfully introduce pCSG005 in the AB5075 WT strain and the mutant strain 5 (MATE), and the pCSG005::prom in the mutant strain 56 (OMP). Of note, we introduced the pCSG005 and the respective complementation plasmids (normal strategy) into mutant strains 35 and 47, which were initially selected for further analyses, but posteriorly discarded when we decided to lower the p-value at which we considered statistical significant differences between the WT and mutant strains (**Annex III**).

Different transformation protocols were tested, such as using competent cells directly from ON liquid cultures or adjusting the OD_{600} to 0.6; washing the cells with sucrose 300 mM or with distilled water; adapting protocols for other species such as the 10-min method for *Pseudomonas aeruginosa* proposed by Choi *et al.* [43], and we also tested different electroporation and growth conditions (temperature, potassium tellurite concentration, incubation time…). Previous passage of complementing plasmids into "lab-friendly" *Acinetobacter* strains (plasmids were otherwise maintained in *E. coli*), such as ATCC19606 and ATCC17878, in order to deceive the innate modification and restriction systems was also fruitless. We speculate that, since the mutant strains have lost an envelope protein, in some of them the membranes might be altered in such a way that undermines the introduction of DNA by electroporation, suggesting that using a conjugative plasmid instead might be a good option. In addition, the tellurite resistance cassette was spontaneously lost in some constructs, which prevented further selection of transformants. The size of the tellurite resistance cassette was relatively large (3kb) and we hypothesized that its loss might be caused by a recombination event between the cassette and some sequence in the plasmid backbone. A much shorter tellurite cassette (*tpm* gene, 0.5 kb long) from *A. baylyi* ADP1 was then selected and it is currently being introduced back into the complementation plasmids. Another set of plasmids using a hygromicine resistance cassette is also under development as a parallel strategy to the use of tellurite resistance.

Conclusions

In summary, antimicrobial susceptibility and virulence assays with a collection of mutant strains defective in several membrane transporters have identified several novel target genes that seem to contribute to the resistant and virulent phenotypes of the *A. baumannii* AB5075 strain. It is of particular relevance the identification of target genes from the MATE and ABC families of efflux proteins, since efflux studies in *Acinetobacter* so far have always centred in the characterisation of efflux proteins from the RND family, given their predominant role in antimicrobial resistance, and have neglected additional efflux systems that might have a pivotal role in pathogenicity. It is clear, however, that we need to solve some technical issues or seek alternative methods in order to perform the additional studies that are needed to corroborate these findings. Further studies to identify the particular effectors that constitute the actual substrates for these efflux systems are also needed.

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V. Discussion

In the last decades, the rise of antimicrobial resistance in nosocomial pathogens has placed *Acinetobacter* spp. in the spotlight of clinical microbiologists. Among all the species from the genus, *A. baumannii* stands out due to a higher clinical significance, mainly achieved by its ability to persist in the hospital environment and competence to acquire and accumulate antibiotic resistance determinants [310]. However, other species of the genus, especially those belonging to the Ab group, have emerged during the past years, and are increasingly being reported as aetiogical agents of nosocomial infections [151,191,439,539,565,568,570,575]. This incremented recovery of *A. nosocomialis* and *A. pittii* has been deeply facilitated by their taxonomical valid naming in 2011 [94], which increased the awareness of these two species in the scientific community. The achievement of the species status of *A. nosocomialis* and *A. pittii* has prompted many clinical microbiologists to go beyond the "ACB complex" level of identification and has led to the development of identification methodologies able to accurately and rapidly identify the otherwise phenotypically undistinguishable species of the Ab group [161,182]. Nevertheless, the use of techniques which do not allow the identification of the members of the Ab group to the species level still jeopardises the accuracy of the current clinical and epidemiological studies, since species of the Ab group other than *A. baumannii* are still underestimated because of their frequent misidentification as *A. baumannii* by several automated systems used in routine clinical microbiology laboratories [627].

In that sense, not only using up-to-date techniques ensures proper identification, but also being able to detect idiosyncratic isolates, which, if recovered repeatedly, may indicate the emergence of a new group and the likely need of further taxonomical delineation. One of the main findings of this thesis was the demonstration that the isolates formerly known as "NB14" group constitute, indeed, a novel species of the Ab group, for which we proposed the name of *Acinetobacter dijkshoorniae* (**Paper 1** and **2**). We have to acknowledge, though, that previous efforts made by microbiologists at the Leiden University facilitated that achievement. Firstly, they realised and recorded that some *Acinetobacter* strains had a different ARDRA pattern (2-5-1-1-3-17 for CfoI, AluI, MboI, RsaI MspI and BfaI, respectively), gathered most of the isolates used in our studies, and willingly shared them with us (see **Background and Work Justification**). Secondly, their caution on not describing them until having collected enough strains (5 – 10 strains are the minimum recommended to characterise a new species, but the use of as many as possible is encouraged [18,36]), allowed us to participate into such an extraordinary event for non-taxonomist microbiologists: the description and naming of a new bacterial species with putative clinical significance. After all, once the genetic relatedness between the isolates was established (**Paper 1**), 9 out of the 11

isolates of the Leiden collection and 3 out of the 4 isolates from our own collection were included in the species characterisation, adding together a final number of 12 isolates, and guaranteeing enough coverage of the intrinsic variations that may exist within the group.

Both the phenotypic and genotypic approaches employed in **Paper 1** to characterise the *A. dijkshoorniae* isolates supported their species status and inclusion within the Ab group. Not only the characteristics of the isolates sustained their close relatedness to *A. baumannii*, *A. nosocomialis*, *A. seifertii* and, especially, *A. pittii*, but the predominant isolation of *A. dijkshoorniae* from human samples, as well as the 83.3% probability of constituting a human pathogen according to PathogenFinder prediction (**Paper 2**), reinforce their inclusion into the Ab group together with the clinically relevant species of the ACB complex [45]. However, we cannot neglect the environment as a potential source of *A. dijkshoorniae*, since one *A. dijkshoorniae* isolate was recovered from water samples in a fishpond farm (**Paper 1**), and another isolate was reported in calf meat samples from a Peruvian market (**Manuscript 4**). It is, however, difficult to assess whether the presence of *A. dijkshoorniae* in these two examples is representative of their natural habitat or it is due to human activities. Future surveillance studies using updated identification methods might shed some light into this matter. Nevertheless, the finding of *A. dijkshoorniae* isolates in different continents reflects that, although probably misrepresented in current studies, this species is spread all over the world.

The recent taxonomic description of the members of the Ab group other than *A. baumannii* clearly reflects the current state of *Acinetobacter*'s taxonomy which has recently experienced an extensive progress; more than 30 new species have been validly published in the last five years. Such increase in the description of new *Acinetobacter* species is mainly due to the implementation of molecular and genomic techniques, and also to the active search for novel species. Unavoidably, the description of new species outpaces the development of bacterial identification methods, and it is difficult to find up-to-date methodologies with enough discriminatory power to discern amongst the recently described species. Regarding the Ab group, ARDRA is powerful enough to distinguish the novel species, i.e. *A. dijkshoorniae* and *A. seifertii* [172,624]. However, the library of ARDRA profiles has not been updated since 2009, and has not included the recent changes in nomenclature of *A. dijkshoorniae*, *A. nosocomialis*, *A. pittii* and *A. seifertii* [94,105,628], although the profiles for these species are referred in the library as phenon 5, gen. sp. 13TU, gen. sp. 3, and gen. sp. ct13TU ('close to 13TU'), respectively (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html). It should be noted that, despite ARDRA being considered as a relatively easy

to perform and economical method, it has not been implemented in clinical microbiology routine laboratories due to not being fast enough for clinical purposes.

Our results show that sequence-based identification methods, such as *rpoB* and MLSA phylogenetic analyses, accurately identify the species of the Ab group (**Paper 1**) and are of great value in taxonomic studies. Both approaches gave congruent results and supported the monophyly of the species of the Ab group, as well as provided high percentages of intraspecies similarity values. The individual phylogenetic analyses of the partial sequences of the 7 genes used to perform the MLSA showed that there is little horizontal transfer between the species of ACB complex, as already reported by Nemec *et al.* [105] and also corroborated by our cluster analyses of the intrinsic OXAs of the Ab group (**Manuscript 5**). However, the finding of some alleles of *A. dijkshoorniae* that clustered with *A. pittii*, reinforces the use of MLSA instead of individual genes for the identification of the species of the Ab group. Nevertheless, despite their accuracy, the use of *rpoB* or MLSA cluster analyses in diagnostic laboratories seems unlikely, since the cost of these techniques is high, especially for MLSA that requires the sequencing of 7 genes per isolate, an unaffordable expense for many laboratories. In addition to the costs, these methodologies involve several steps (bacterial purification, sequencing, phylogenetic analyses…) that require highly qualified staff as well as increase the time needed to obtain the identification results, making them unsuitable for routine diagnostic laboratories.

Once again, our study showed the limitations of the 16S rRNA cluster analysis for *Acinetobacter* species in comparison to *rpoB*-based and MLSA phylogenetic analyses. In contrast to the two latter analyses, which showed that *A. dijkshoorniae* was closest to *A. pittii*, the 16S rRNA analyses grouped the *A. dijkshoorniae* isolates closer to *Acinetobacter* gen. sp. "Between 1 & 3" (**Paper 1**). Of note, the number of 16S rRNA genes differed between *A. dijkshoorniae* isolates, we detected 3 complete and 2 partial genes in the genome of JVAP01, which was sequenced as part of this thesis (**Paper 1** and **2**), while the SCOPE 271 isolate had 6 complete 16S rRNA genes. Similarly, 16S rRNA analyses were inconsistent for *A. seifertii*, since 16S rRNA cluster analyses did not support their monophyly, while *rpoB*-based and MLSA phylogenetic analyses did (**Paper 1**), in good agreement with previous studies [105,179]. These results suggest that 16S rRNA cluster analyses should not be required in *Acinetobacter* spp. taxonomic studies and that more efforts should be made in order to adapt valid publication requirements to the taxa under study, since 16S rRNA analyses are still compulsory regardless of the species described [11].

In contrast, genomic analyses proved really useful, especially if we take into account that the species of the Ab group are mainly indistinguishable by traditional phenotypic methods [67,106,147,105] (**Paper 1**), and thus, the more genotypic analyses prove their species status, the better sustained is their designation as a novel species. A fact in favour of the genomic analyses for taxonomic purposes, instead of other outdated methods such as DDH, is the availability of genomic sequences from public repositories, which allowed the inclusion of additional isolates in our genotypic studies, despite not "physically" owning the isolates. In addition, the analyses of whole genome sequences provide useful information that reaches beyond the taxonomy level. For instance, the use in our study of prediction software, such as ResFinder and PathogenFinder, enabled us to identify the presence of several antimicrobial resistance genes as well as to infer pathogenicity, recognising *A. dijkshoorniae* as a potential human pathogen (**Paper 2**). Nevertheless, whole genome sequencing and analyses such as ANIb, despite providing precise identification of the species of the Ab group, are far from being integrated into routine identification laboratories for similar reasons as those exposed for *rpoB* and MLSA analyses: the costs in money and time.

Anyway, it should be noted that, aside from the techniques employed, it is of utter importance to include closely related species in any analysis aimed at describing a new bacterial species, using the maximum number of strains possible [4]. In that sense, we gathered a collection of 93 reference strains from the species of the ACB complex (**Paper 1**), and included them in the molecular analyses (*rpoB*-based and MLSA phylogenetic analyses), while genome analyses were performed using all the genomes of the species of the ACB complex available in the public databases at the time of the study, in addition to the genome sequence of the *A. dijkshoorniae* type strain (**Paper 2**). The identification of species-specific peaks by MALDI-TOF MS was also carried out with all *A. dijkshoorniae* isolates and a large number of reference strains of the ACB collection (**Paper 1** and **Paper 3**). In addition, we performed the metabolic analyses with all the *A. dijkshoorniae* isolates available at that time (n=15) and compared them against the species of the ACB complex (*A. baumannii* (n=5), *A. calcoaceticus* (n=5), *A. nosocomialis* (n=4), *A. pittii* (n=5) and *A. seifertii* (n=5) (**Paper 1**), and also examined the coherence of our results with previous data from other authors (**Paper 1**, **Supplemental material**).

All these efforts were made to detect any variations among the *A. dijkshoorniae* isolates and determine if the differences were shared with some of the closest species, but also to provide a better characterisation and contextualisation of the new bacterial species. Only by including several isolates of the closest species as references, the

characterisation of a group of isolates putatively constituting a new bacterial species would achieve its real purpose: to properly allocate the organisms in the context of its closest relatives, following the hierarchic classification schemes.

Rapid identification of the species of the Ab group is critical in the clinical settings. Inappropriate therapy has been detected as one of the main drivers of the clinical outcomes of infections caused by the Ab group [190,191,566,573]. Taking into account that nowadays *A. baumannii* is overall less susceptible to most antimicrobial agents than the other members of the Ab group (**Manuscript 5**), identifying the species within this group as soon as possible could help to initiate early appropriate treatment. Furthermore, despite the inherent role of MDR in the treatment of infections caused by *A. baumannii* is evident but difficult to ascertain individually, some studies have pointed to this species as an increased risk factor for mortality [151,190,222,570,572], usually together with an increased severity of the infection [190,5730,572]. Consequently, avoiding the misidentification of the more susceptible *A. dijkshoorniae*, *A. nosocomialis*, *A. pittii* and *A. seifertii* as *A. baumannii*, which is frequently MDR, might be decisive for proper clinical management of infections caused by pathogens from the Ab group.

Many routine clinical microbiology laboratories still rely on manual and semi-automated commercial identification systems based on phenotypic tests, although traditional phenotypic tests are unable to distinguish the species of the Ab group [152,157]. For instance, when we performed the phenotypic characterisation of *A. dijkshoorniae* we found no major differences in the use of sole carbon sources among the species of the Ab group, and we only detected that 53% of the *A. dijkshoorniae* isolates could use tryptamine while none of the other members of the Ab group could (**Paper 1**). We did find, however, species-specific peaks for *A. dijkshoorniae* isolates when analysing the MALDI-TOF MS spectra of the Ab group species for the characterisation of the novel species (4429, 5788 and 8856 m/z values, **Paper 1**), and so did Nemec *et al.* for *A. seifertii* [105]. MALDI-TOF MS has already been shown to be a simple and fast method able to identify the former members of the Ab group (*A. baumannii*, *A. nosocomialis*, and *A. pittii*) [161], although there were some concerns regarding the accuracy of the method to identify *A. nosocomialis* [162–164]. This user-friendly technique requires tiny amounts of sample, but yet it provides precise results within minutes after bacterial growth. Thus, MALDI-TOF MS stands as an alternative to conventional identification methods that can be successfully implemented in routine diagnostics. In fact, several studies report the reduction in the turn-around time of identification when using MALDI-TOF MS instead of other conventional methods [629], and many

laboratories are further reducing this time by identifying bacteria directly from positive blood cultures [630–633]. However, MALDI-TOF MS accurate identification deeply relies on the databases, which need to be regularly updated so as to adjust to the current state of taxonomy. For instance, in **Manuscript 4** due to the lack of reference spectra for *A. bereziniae* in the Bruker taxonomy database (v.5.0.0.0) at the time of the study, we had to further verify by molecular methods the MALDI-TOF MS results of one isolate which was ambiguously identified as *A. guillouiae*, but indeed belonged to the closely-related *A. bereziniae* [73] according to cluster analyses. Nowadays, the database has been updated (v.8.0.0.0), and re-identification of this isolate currently matches with *A. bereziniae*. Recently, Šedo *et al.* pointed out that, in addition to extending the database, the use of an alternative matrix solution (**BOX 1**) might also improve the identification of problematic *Acinetobacter* spp. [634].

Prior to our study published in **Paper 3**, bacterial identification using the default Bruker taxonomy database misidentified *A. dijkshoorniae* as *A. pittii*, and *A. seifertii* as *A. baumannii*, and thus, unless the database was updated, this would lead to underestimating the real prevalence of these two novel species. Interestingly, despite the incorrect identification using the default database, in both species the best matches always corresponded to the same reference spectra, respectively, suggesting that the isolates originally used to create these reference spectra may belong to the novel species, which further supported the discriminatory power of MALDI-TOF MS even without refinement. In **Paper 3** we demonstrated the distinctness of spectral signatures for all the species of the Ab group and provided reference spectra for the novel species (*A. dijkshoorniae* and *A. seifertii*) that avoided cross identification between them and with other *Acinetobacter* spp. These results proved the suitability of MALDI-TOF MS for the reliable identification of the species of the Ab group, including *A. seifertii*, for which some authors claimed that identification by MALDI-TOF MS alone was not possible [168]. In fact, by screening our *Acinetobacter* spp. collection using MALDI-TOF MS we were able to detect some additional isolates of *A. dijkshoorniae* and *A. seifertii* that were included in our studies (**Paper 1**, **Paper 3** and **Manuscript 5**), and, in addition, the use of our custom MALDI-TOF MS database allowed us to successfully identify isolates from the Ab group in **Manuscript 4**.

Furthermore, the presence of reference spectra in the Bruker taxonomy database generated from isolates incorrectly identified highlights the importance of properly validating the species identity of those isolates used to create reference spectra, as well as it emphasizes the relevance of selecting appropriate representatives of each species for the generation of the taxonomy database. In this sense, it is also worth mentioning

that we found out one of the main reasons why MALDI-TOF MS bacterial identification using the default Bruker taxonomy database was misidentifying *A. nosocomialis* as *A. baumannii* (**Paper 3**), an issue that some authors had already reported [162–164]. We observed that, according to their peak signatures, although not to *rpoB* or MLSA cluster analyses, there are two groups of *A. nosocomialis* isolates, and that one of these groups, which shares some peaks with *A. baumannii*, was underrepresented in the default Bruker taxonomy database, leading to the correspondent misidentification. Consequently, we have created additional reference spectra of the underrepresented group to improve their identification by MALDI-TOF MS and confirmed that after their introduction in the database, the misidentification problems subsided (**Paper 3**).

In **Manuscript 4**, we successfully employed MALDI-TOF MS to identify the *Acinetobacter* spp. isolates of the Ab group that were recovered from market meat samples in Peru using our custom database. Molecular methods corroborated the identification results as well as MLST studies allowed us to connect some of the STs of the isolates from meat samples to clinical isolates, such as *A. baumannii* ST273, which has been described both in the nosocomial environment and a calf [135], and *A. pittii* ST312, which, to our knowledge, has only been detected in patients so far. The isolation of species of the Ab group and some STs previously related to the clinical setting from market meat samples point to these sources as potential transmission routes of the species of the Ab group. Despite the fact that the isolates recovered were susceptible to all antibiotics tested (**Manuscript 4**), the isolation of clinically relevant *Acinetobacter* species from meat highlights the importance of the One Health approach to tackle the spread of antibiotic resistance, since other studies have already reported higher rates of antimicrobial resistance in *Acinetobacter* spp. recovered from food samples [127,134]. Of note, this study also reported the first identification of *A. dijkshoorniae* in meat samples of animal origin as well as its first identification in Peru and South America.

It is worth mentioning that during the development of this thesis, a US-based group isolated an *Acinetobacter* strain from lettuce that resembled the isolates of the NB14 group [82]. In their study Rooney *et al.* defined a novel species, *Acinetobacter lactucae*, isolated from iceberg lettuce in the USA. In this report, 4 *Acinetobacter* isolates, which putatively belonged to a novel species, were recovered from the same lettuce sample and, since the clonal-relatedness between the isolates was not defined, it can be assumed that the description is based on a single isolate (**TABLE 3**, https://apps.szu.cz/anemec/Classification.pdf). Rooney *et al.* used 16S rRNA anal-

yses (n=4) to infer that the isolates belonged to the *Acinetobacter* genus, and the genomic analyses performed (n=1) corroborated the species status of isolate NRRL B-41902 (the type strain), as well as it relatedness to *A. pittii*, *Acinetobacter* gen. sp. "Between 1 & 3" and '*Acinetobacter oleivorans*', an effectively but not validly published *Acinetobacter* species [116].

Phenotypic characterisation was performed using only the NRRL B-41902 type strain and just one isolate of the above-mentioned species, which falls short when compared to the standard recommendation to use a larger number of non-related isolates for each bacterial species. Nevertheless, the data generated by Rooney *et al.* provided enough evidence to recognise a new species that was validly published with the proposed name of *A. lactucae*. For their sequence analyses, Rooney *et al.* used the sequenced genome of our type strain (**Paper 2**, unpublished at that time but uploaded to the databases for publication purposes) and that of strain SCOPE 271 in their genetic analyses and found that JVAP01 and SCOPE 271 were conspecific strains of *A. lactucae* [82], but they did not refer to the publication by Espinal *et al.* where the putative status of the "NB14" group as a novel *Acinetobacter* species was suggested [624]. The study by Rooney *et al.* was published in the same volume of the International Journal of Systematic and Evolutionary Microbiology (2016), volume 66, than our study, but it was submitted and accepted at an earlier date, and thus it appears in prior pages (*A. lactucae* description in pages 3566-3572 vs. *A. dijkshoorniae* description in pages 4105-4111, **Paper 1**). Consequently, in accordance to the rule 24b(2) (Chapter 3, section 5 of the International Code of Nomenclature of Prokaryotes, latest version [35]), *A. dijkshoorniae* should be considered a later heterotypic synonym of *A. lactucae*, as Dunlap & Rooney already pointed out in a recent publication [635].

One of our purposes in **Paper 3** was to bring closer to routine diagnostics our taxonomical findings from **Paper 1**, so that we made clinical microbiologists aware of the existence of a new potential pathogenic species within the Ab group and that the taxonomic description reached other microbiology fields beyond taxonomy. Hence, the importance of providing an identification method appropriate for the clinical management of infections caused by the Ab group that is reliable, fast and implementable. From our point of view, the use of MALDI-TOF MS could help to reduce the underestimation of the presence of the novel and emergent species of the Ab group in the clinical settings, and, consequently, contribute to our better understanding of their clinical characteristics.

Bacterial taxonomic studies describing new taxa usually lack applicable information regarding the nature of the organisms. While the current bacterial taxonomy focuses on introducing all sort of techniques to better characterise a group of organisms, earlier taxonomists' practices have been overlooked, and aspects such as the ability to cause disease are no longer included in the taxonomic studies (See **TABLE 1**). Unarguably, the phenotypic and genotypic techniques employed nowadays help to establish objective boundaries between taxa, but, many times, these methodologies relegate the taxonomic description to a batch of tests with no further applicability in other microbiological fields. We wanted to investigate further the differences between all the members of the Ab group, since these species are not usually identified to the species level, are incorrectly referred as *A. baumannii* due to identification limitations, or are misidentified as other members of the Ab group, like *A. dijkshoorniae*, which was misidentified as *A. pittii* by some molecular techniques [624] and MALDI-TOF MS (**Paper 3**). In **Manuscript 5**, our efforts aimed at reinforcing the idea that reporting and studying the species of the Ab group collectively may be misleading.

In order to provide additional information sustaining the study of the species of the Ab group as different entities, we evaluated the antimicrobial resistance patterns and characterised the presence of intrinsic resistance mechanisms, as well as examined some *in vitro* phenotypes that have been associated with pathogenicity and the virulence of the different species in the *C. elegans* nematode infection model (**Manuscript 5**). Unfortunately, due to not having clinical data for all the isolates other than the specimen from which they were recovered, we could not evaluate which isolates were mere colonisers and which ones were causing infections, and even less what were the patients' outcomes. Although this is a controversial topic (see **Introduction**, **Section 5.1** and **TABLE 10**), some studies suggest that the different species of the Ab group might have different mortality and clinical outcomes [151,190,299,566,570,572], and thus, further emphasizes the need for studying the infections caused by the different species of the Ab group separately so that we could better determine the differences in the prognosis of their infections. Despite lacking the clinical information, we assessed some differences among the species of the Ab group that support their study as distinct entities.

As already mentioned, we observed overall less susceptibility to most antibacterial agents in *A. baumannii*, and that the novel species *A. dijkshoorniae* and *A. seifertii* had susceptibility profiles resembling those of *A. nosocomialis* and *A. pittii*, which are frequently susceptible to most antibiotics (**Manuscript 5**). Nevertheless, we detected re-

sistant isolates to certain antibiotics in species of the Ab group other than *A. baumannii*, which raised some questions such as: Do the emergent species of the Ab group display a higher pathogenicity potential than *A. baumannii* that is currently masked by their overall antimicrobial susceptibility? Should we expect worse clinical outcomes if these species reached similar rates of resistance as *A. baumannii* and became frequent MDR pathogens? Do they have the inherent capacity to evolve into such direction? Certainly, this is an intriguing panorama in the clinical setting, since high levels of resistance in species of the Ab group are still not so commonly found in environmental or food samples, as we observed in **Manuscript 4**. While we might expect an increase in the identification of non-*baumannii* isolates ought to their emergence and improved identification techniques, we cannot predict if there will be a shift in the susceptibility profiles of these pathogens, but their close relationship to the successful *A. baumannii* suggests that such progression is not unlikely if antibiotic pressure keeps at the current levels. As evidenced in **Manuscript 5**, species of the Ab group encode in their genomes several intrinsic mechanisms known to be able to confer resistance if overexpressed, but which have been mainly studied in *A. baumannii*, such as the intrinsic ADC and OXA enzymes (although we could not detect the latter in *A. nosocomialis* and *A. seifertii* (**Manuscript 5**) [148]) and efflux pumps, whose overexpression has already been shown to lead to carbapenem resistance in species of the Ab group other than *A. baumannii* [636]. Furthermore, several papers have reported the acquisition of carbapenemases by species of the Ab group other than *A. baumannii*, even in the recently described *A. dijkshoorniae* and *A. seifertii* [196,624], further supporting the potential drift to a more common MDR phenotype in these species if intrinsic and acquired mechanisms are expressed in the same isolate.

The other phenotypes studied in **Manuscript 5**, i. e. motility, biofilm and virulence in the *C. elegans* model, presented a high degree of intraspecies variability, pointing to an elevated strain-specific component in these phenotypes. Despite the variations, we could still assess differences among the species of the Ab group, except for motility. The role of motility in *Acinetobacter* species is not as obvious as in other pathogens with flagella-dependent motility, where the flagellin itself can trigger the immune response [637]. Nevertheless, surface-associated motility has been linked to DNA uptake [308], and thus the ability to move along wet surfaces may play an important role in HGT and in the acquisition of antimicrobial resistance determinants. Moreover, surface-associated motility has been found to be a common feature in clinical *A. baumannii* isolates (82/83 motile isolates) [470]. However, in our collection of isolates of the Ab group the prevalence of surface-associated motility in *A. baumannii* and *A.*

nosocomialis was much lower (62.5% and 50%, respectively), while in *A. pittii*, *A. dijkshoorniae* and *A. seifertii* most of the isolates were considered motile (95%, 83.3% and 75%, respectively) (**Manuscript 5**). This data suggests that, despite not being as predominant as previously found, the surface-associated motility in the Ab group is frequent. Furthermore, we found that, although the association between motility and biofilm formation in *A. baumannii* is ambiguous [466,512,638], under our experimental conditions there was a positive correlation between motility and biofilm formation at 37°C in all species except for *A. pittii* (**Manuscript 5**). Whether it is a stochastic event due to our isolate collection or if there is a real connection between these two phenotypes in the species of the Ab group is unknown and requires further studies.

Bacterial biofilms are considered one of the persistence mechanisms responsible for the success of *A. baumannii* as a nosocomial pathogen [310], however, little is known about the other species of the Ab group. A few studies comparing the biofilm formation abilities of *A. baumannii*, *A. nosocomialis* and *A. pittii* found that they displayed a similar behaviour [429,437,438], but Jain *et al.* found that *A. pittii* formed more biofilm than *A. baumannii*, and that *A. nosocomialis* displayed an intermediate phenotype [439]. Our results show that biofilm formation both at 28ºC and 37ºC was frequent among *A. dijkshoorniae*, *A. pittii* and *A. seifertii* while *A. baumannii* was the species with least biofilm forming ability (**Manuscript 5**). Furthermore, *A. nosocomialis* clearly presented a different extent of biofilm formation depending on the temperature, at 28ºC it resembled the non-*baumannii* species of the Ab group and thus, most of the strains were able to produce biofilms, while at 37ºC biofilm formation decreased to levels comparable to those of *A. baumannii* (**Manuscript 5**). Interestingly, some authors have also detected a reduction of biofilm formation in *A. nosocomialis* when temperature shifted from 25-28ºC to 37ºC [429,436], thus our results seem to confirm that a rise on temperature reduces biofilm formation in this species. *A. baumannii* isolates also showed a moderate decrease on biofilm formation upon temperature rise and similar results were also found by Martí *et al.* [436]. Noteworthy, many studies perform biofilm assays at 37ºC, although Tomaras *et al.* reported in 2003 that *A. baumannii* attachment to polystyrene is more efficient at 30ºC [433] and other authors have shown that biofilm production was more abundant at 25-30ºC than at 35 or 37ºC [436,443,639]. These observations suggest that the ability to produce biofilm not only differs amongst species of the Ab group but it is also temperature-dependent. Our findings indicate that *A. baumannii*, *A. nosocomialis* and *A. seifertii*, in which we observed a decrease in biofilm formation upon temperature rise, might have different thermoregulation strategies of biofilm production than *A. dijkshoorniae* and *A. pittii*, which formed

more biofilm when temperature increased. Although this hypothesis needs to be further investigated so as to find the putative underlying mechanisms that control biofilm formation at different temperatures in these species, these findings imply that results obtained in one species of the Ab group cannot be extrapolated to another.

Likewise, infection assays using the *C. elegans* model revealed differences among the species of the Ab group. *A. baumannii* and *A. nosocomialis* were overall non-virulent, while *A. dijkshoorniae* isolates were all virulent, except for just one isolate (**Manuscript 5**). Interestingly, we could identify a clear cut-off between isolates considered virulent and those considered non-virulent due to the fact that the LT50 values of all isolates were either below 2 days (virulent) or above 4 days (non-virulent). Whereas in *A. baumannii*, *A. nosocomialis* and *A. dijkshoorniae* almost all isolates within the same species presented the same behaviour, in *A. pittii* and *A. seifertii* we could distinguish two subpopulations according to their virulent or non-virulent phenotype in the *C. elegans* model (**Manuscript 5**). It remains unclear whether these subpopulations just reflect the intrinsic heterogeneity within *A. seifertii* and *A. pittii* or if they are indicative of the need to delineate these species further from a taxonomic point of view. Due to its recent characterisation [105] and the low number of *A. seifertii* isolates used in our study, it might be too soon to infer speciation in this taxon. In contrast, the *A. pittii* species has already been shown to contain several groups of strains with no clear phenotypic/genotypic discontinuities [148,640], called "*A. pittii*-like" strains, among which were classified the *A. dijkshoorniae* isolates prior to their taxonomic description, and thus, further delineation in *A. pittii* should not be disregarded. Nevertheless, we examined the MALDI-TOF MS peak profiles used in **Paper 3** of *A. pittii* and *A. seifertii* isolates grouped into virulent and non-virulent, and we could not detect any distinctive peak allowing the demarcation of the groups using MALDI-TOF MS (data not shown).

In the clinical setting, *A. baumannii* and *A. nosocomialis* are more frequently reported as aetiogical agents of infections and seem to have less favourable outcomes [190,191,565,573], however, the results obtained in the *C. elegans* model showed completely the opposite: the most pathogenic species in humans, *A. baumannii* and *A. nosocomialis*, were the least virulent (**Manuscript 5**). These discrepancies between clinical studies and our findings show that there might be some limitations in this model and that it might not be appropriate for inferring the virulence characteristics of *Acinetobacter* infections in the human host, and thus that the literature regarding the virulence of *Acinetobacter* spp. using this model should be interpreted with caution. Nevertheless,

the higher virulence of *A. dijkshoorniae*, the mixt population of virulent and non-virulent isolates of *A. pittii* and *A. seifertii*, and the lower virulence of *A. baumannii* and *A. nosocomialis* in the *C. elegans* model (**Manuscript 5**) might reflect a shift in the infection capabilities towards the human host, showing different degrees of adaptation and the narrowed abilities to infect other hosts, such as *C. elegans*, of the clinical isolates of the Ab group. This hypothesis is in line with the findings of Diancourt *et al.*, who reported that clinical *A. baumannii* might have suffered a reduction of intra-species diversity (population bottleneck) resulting from adaptation to a restricted ecological niche [641].

In addition to having adapted to the human host, the clinical lineages of *A. baumannii* have disseminated through the hospitals thanks to the selective advantages of antimicrobial resistance [641]. As seen in **Manuscript 4** and **Manuscript 5**, antimicrobial resistance in the non-*baumannii* species of the Ab group is still less frequent than in clinical *A. baumannii*, which has become virtually resistant to almost all commonly used antibiotics [310]. Nevertheless, many studies still use laboratory-friendly but susceptible *A. baumannii* strains in order to find new targets for the development of novel antibacterial drugs, despite the current recommendations encouraging to focusing on carbapenem resistant *A. baumannii* for this purpose [50]. In contrast, in **Manuscript 6**, we addressed this issue using the MDR *A. baumannii* AB5075 strain, which was isolated from a tibia osteomyelitis in 2008 and that harbours an OXA-23 carbapenemase, and thus, is carbapenem resistant [626].

In our attempt to find novel therapeutic targets, we wanted to characterise the role of known and putatively novel efflux pumps and porins in the transport of antibiotics across membranes, but, most importantly, we explored their role beyond resistance and tried to determine their putative involvement in the pathogenesis of *A. baumannii* (**Manuscript 6**), so that in future studies we could investigate if they participate in the translocation of virulence effectors that could be used as drug targets. In this study, we identified several novel target genes that seem to contribute to the resistant and virulent phenotypes of the *A. baumannii* AB5075 strain, and we found additional substrates for known transport proteins.

In **Manuscript 6**, antimicrobial susceptibility testing of mutant strains of transport-related genes compared to the WT *A. baumannii* AB5075 revealed that the MICs for meropenem, levofloxacin, moxifloxacin, kanamycin, amikacin, tobramycin, and gentamicin were altered in 10 out of the 65 mutant strains, being aminoglycosides the

most commonly affected antibacterial agents. Five mutant strains with altered susceptibility presented transposon insertions in genes encoding for the AbeM, CraA, OmpA, AdeJ and AdeB efflux systems while in the remaining strains the transposon interrupted genes encoding efflux pumps not previously reported to be involved in antimicrobial resistance (**Manuscript 6**), suggesting that the role of many novel transport proteins is yet to be determined.

Remarkably, the involvement on the MDR phenotype of some transport proteins was still noticeable in spite of the multiple additional resistance mechanisms that are present in the *A. baumannii* AB5075 strain (**Manuscript 6**). Although this strain harbours an OXA-23 and *gyrA*/*parC* mutations [280,281], we could detect a decrease in the MICs for meropenem and some fluoroquinolones in the mutant strains of an OmpA-like orthologue and a putative permease of the AI-2E superfamily, but not of RND efflux pumps [243,247]. Smani *et al.* already showed that, in the susceptible *A. baumannii* ATCC17978 strain, OmpA was involved in chloramphenicol, aztreonam and nalidixic acid resistance [231], and our results suggest a relevant role of OmpA in the MDR phenotype of *A. baumannii*, since we found that it might be also involved in resistance to meropenem, some fluoroquinolones and aminoglycosides (**Manuscript 6**). Interestingly, one study analysing the protein interactions in *A. baumannii* AB5075 strain found that OXA-23 was interacting with OmpA and other porins in their periplasmic side, and suggested that the binding of the carbapenemase at the exit of the porin channel might allow the hydrolysis of the antibacterial agents immediately after entering the cells [642]. Our observations might support this hypothesis, since in the absence of OmpA, not only less meropenem would diffuse into the cell, but also, according to Wu *et al.,* the localisation of OXA-23 might be less effective, and for this reason we might have observed an increased susceptibility in the mutant strain for the gene encoding OmpA-like (**Manuscript 6**). Recently, a study using the *A. baumannii* AB5075 strain and likewise transposon mutants from the collection developed by Gallagher *et al.* [625], showed that the putative permease of the AI-2E superfamily was the primary determinant of chloramphenicol intrinsic resistance, but did not affect the susceptibility to rifampin, ceftazidime, carbecillin, amikacin, gentamicin, trimethoprim, imipenem or kanamycin, in good agreement with our findings [643]. Overall, our results indicate that transporters of the AI-2E family may have a wider substrate profile and might also be involved in the export of other antibacterial agents, such as meropenem and levofloxacin.

In addition, we found that, although fluoroquinolones and some β-lactams have been shown to be substrates of both AbeM (MATE) and AdeB (RND) [233,242], the contribution to fluoroquinolone and β-lactam resistance of these efflux pumps in the *A. baumannii* AB5075 strain might be masked by other transporters and additional resistance mechanisms, while their participation in aminoglycoside extrusion was still measurable (**Manuscript 6**). Strikingly, we noticed an increase rather than a decrease in the MIC value of moxifloxacin in the mutant strain defective for *adeJ*, in contrast to previous findings by Damier-Piolle *et al.* [248], but further experiments using EPIs suggested that our observations might be due to a compensative overexpression of other redundant efflux pumps, since the presence of PAβN restored moxifloxacin MIC values of the mutant strain to the levels of the WT strain (**Manuscript 6**). Furthermore, and according to our results, tobramycin and gentamicin might be additional substrates of CraA (MFS) (**Manuscript 6**), which was known to extrude chloramphenicol in *A. baumannii* [238]. The additional transporters that seem to be involved in aminoglycoside resistance belong to the ABC (n=1) and MFS (n=3) families and are yet uncharacterised (**Manuscript 6**), and thus their contribution to the susceptibility profile of *A. baumannii* AB5075 needs to be further studied.

The *A. baumannii* AB5075 strain is highly virulent both in the invertebrate *G. mellonella* infection model and the mice pulmonary infection model [626], hence we decided to use the *G. mellonella* infection model to screen for virulence attenuation in our transport protein mutant strain collection (**Manuscript 6**). The mutant strains with attenuated virulence in the *G. mellonella* infection model were defective for efflux pumps from different transport protein families (MATE (n=2), OMP (n=2), MFS (n=1) and ABC (n=1). Half of them corresponded to putatively novel transport systems, while the remaining three showed sequence similarity to OmpA- MacB- and TolC-like proteins, respectively (**Manuscript 6**). One MATE mutant strain and the *ompA*-like mutant strain showed decreased fitness, and because this could affect their ability to infect *G. mellonella*, they were discarded for further analyses. The identification of target genes from the MATE and ABC families of efflux proteins is remarkable, since efflux studies in *Acinetobacter* so far have mainly focused on the characterisation of efflux proteins from the RND family, given their predominant role in antimicrobial resistance, and have neglected additional efflux systems that might have a pivotal role in pathogenicity. Interestingly, our mutant strain for *adeB* did not showed attenuated virulence in *G. mellonella* (**Manuscript 6**), reinforcing the findings of Richmond *et al.*, who found that *adeB* affected biofilm formation and the virulence in *G. mellonella* in a strain-dependent manner [493], which, at the same time, is congruent with our results from **Manuscript 5**: we did not find any association between the presence or absence of *adeB* in the

isolates of the Ab group and their ability to produce biofilm, a phenotype which seems rather clonespecific, or their virulence in the *C. elegans* model.

Although further studies are needed to determine if the mutated transport-related proteins are involved in the extrusion of virulence effectors of *A. baumannii* AB5075 strain, for those proteins showing sequence similarity to known efflux pumps or porins we found in the literature some promising functions in virulence. Some MATE proteins of the NorM family have been linked to bacterial virulence. For instance, the DinF of *Ralstonia solanacearum* seems to be involved in infection success in tomatoes [644] and ClbM has been shown to transport a pretoxin across the membranes of *E. coli* [645]. Likewise, MacB (ABC) and TolC (OMP) constitute, together with MacA, the tripartite system MacAB-TolC [646], which in addition to the extrusion of macrolides, can transport peptidic toxins and virulence factors in other species [647,648]. Little is known about the substrates that are transported by the MacAB-TolC ABC efflux pump in *A. baumannii*, but our results show that, similar to what has been reported in mice infected with *Salmonella* [648], the virulence attenuation on the MacB mutant was greater than that of the strain defective for the TolC-like OMP, suggesting that the extrusion of the substrates through this system might not be restricted to the OMP TolC.

Nevertheless, we need to overcome the technical issues found to complement the mutant strains with altered phenotype in order to corroborate our findings and to identify the particular effectors that constitute the actual substrates for these efflux systems. Despite recent advances [649], there is a clear need for molecular vectors to work with MDR *Acinetobacter*, since the introduction of additional antibiotic resistance cassettes for the selection of transformants is limited by the resistance profile of the strain being used. For example, we could not use the recently engineered plasmid from Lucidi *et al.* because *A. baumannii* AB5075 strain is resistant both to gentamicin and zeocin [649]. However, expanding the resistance profile of MDR clinical strains seems rather questionable from an ethical point of view, and for this reason the use of heavy metal resistance cassettes seems a good alternative, despite the technical issues we encountered (**Manuscript 6**).

In summary, it is clear that beyond their distinct taxonomical characteristics (**Paper 1** and **2**) the species of the Ab group behave differently (**Manuscript 5**) and thus, might show distinct degrees of adaptation to the hospital environment and the human host. Therefore, as proven distinct biological entities, they have to be studied separately so that we could properly assess if their clinical characteristics and outcomes converge

or diverge. The use of accurate bacterial identification methods, such as MALDI-TOF MS (**Paper 3**), is likely going to increase the number of reported infections caused by the novel members of the group, helping to gain insight about their clinical characteristics, but will also provide valuable information regarding the sources and potential routes of transmission of the species of the Ab group (**Manuscript 4**). Altogether, a better knowledge of the biology of the novel species, surveillance of their epidemiology and monitoring of the evolution of their antimicrobial resistance patterns, might help us to contain their global spread and the acquisition of resistance mechanisms that have succeeded in *A. baumannii*. In addition, we also need to focus our efforts in finding new therapeutic targets and develop novel treatment strategies (**Manuscript 6**), a recognised worldwide priority [50].

VI. Conclusions

1. According to *rpoB* and MLSA cluster analyses, the *Acinetobacter* isolates of the NB14 group clearly group together in a monophyletic group closest to *A. pittii*, and ANIb analyses prove the species status of the NB14 group isolates

2. A clear differentiation between NB14 group isolates and other members of the ACB complex is not possible by metabolic and physiological assays, in good agreement with previous studies. However, MALDI-TOF MS analyses identify statistically significant species-specific peaks for this group of isolates.

3. Our data support the designation of the isolates formerly known as *Acinetobacter pittii*-like NB14 group as representing a novel species, for which the name *Acinetobacter dijkshoorniae* sp. nov. is proposed.

4. *A. dijkshoorniae* sp. nov. is frequently isolated from clinical specimens, however the environment and food cannot be disregarded as sources of this pathogen.

5. According to genomic analyses, the *A. dijkshoorniae* sp. nov. has a high probability of being a human pathogen. This fact and the frequent isolation from human samples of *A. dijkshoorniae* sp. nov. points to its likely clinical relevance and to the need for accurate species identification.

6. *A. nosocomialis* isolates show two differential groups according to their MALDI-TOF MS spectra profiles, the underrepresentation of one of these groups in the Bruker taxonomy database accounts for its current low rates of successful identification.

7. Inclusion of novel reference spectra into the Bruker taxonomy database for the underrepresented *A. nosocomialis* group and for the novel *A. dijkshoorniae* and *A. seifertii* species shows that MALDI-TOF MS can be used as a rapid and accurate method for the identification of the species of the Ab group, contributing to the assessment of their clinical and epidemiological relevance.

8. Overall, *A. baumannii* is less susceptible to antimicrobials than the other species of the Ab group, which still remain susceptible to most antimicrobials.

9. Motility and biofilm formation phenotypes are highly strain-specific.

10. Surface-associated motility in the Ab group is a common feature shared by all the species.

11. The ability to produce biofilm differs amongst the species of the Ab group and is temperature-dependent. There seems to be different thermoregulation strategies for *A. dijkshoorniae* and *A. pittii* than for *A. baumannii*, *A. nosocomialis* and *A. seifertii*.

12. The higher virulence of *A. dijkshoorniae*, the mixt population of virulent and non-virulent isolates of *A. pittii* and *A. seifertii*, and the lower virulence of *A. baumannii* and *A. nosocomialis* in the *C. elegans* model might reflect a shift in the infection capabilities towards the human host and different degrees of adaptation.

13. Cluster analyses based on the independent partial sequences of each of the seven MLST genes and of the intrinsic OXA of *Acinetobacter* spp. show little horizontal transfer between the species of the Ab group.

14. *A. pittii* isolates show the largest variability, both regarding MALDI-TOF MS spectra profiles and genetic sequences, which together with the clear splitting into virulent and non-virulent isolates in the *C. elegans* infection model, might suggest that this species can be further delineated.

15. Beyond their distinct taxonomical characteristics, the species of the Ab group behave differently, results obtained for one species of the Ab group cannot be extrapolated to another, and thus, they should be studied separately so that we could properly assess their clinical characteristics and outcomes.

16. Some known *A. baumannii* transport-related proteins might have additional antimicrobial substrates than those previously described (OmpA, AI-2E family of transporters and CraA).

17. MATE and ABC families of efflux proteins might have a pivotal role in the pathogenicity of the *A. baumannii* AB5075 strain.

18. Although the role of many novel transport-related proteins in *A. baumannii* is yet to be determined, several known and novel transport-related proteins seem to contribute to the resistant and virulent phenotypes of the *A. baumannii* AB5075 strain.

VII. Resumen

I. Introducción

1. Taxonomía bacteriana

La taxonomía es la disciplina encargada de asignar nombres a la biodiversidad que nos rodea, clasificando los organismos de acuerdo a sus propiedades únicas que los caracterizan como un grupo o taxón independiente. Por lo tanto, la caracterización de los organismos, su clasificación y posterior nomenclatura constituyen campos interrelacionados y dependientes que, de manera conjunta, conforman la taxonomía.

En procariotas, la falta de una definición satisfactoria y consensuada del término *especie* [3] y la ausencia de un sistema oficial de caracterización y clasificación [4,5] dificultan el establecimiento de los límites entre taxones. Habitualmente las caracterizaciones de éstos se basan en lo que se conoce como taxonomía *polifásica* [13,14], en la que a la hora de caracterizar un grupo de individuos como un taxón independiente se utilizan rasgos tanto fenotípicos como basados en métodos moleculares.

1.1. Caracterización

Para caracterizar un nuevo taxón, uno de los mayores objetivos de la taxonomía es proporcionar una definición precisa de los rasgos distintivos del grupo de organismos con el fin de respaldar su correcta clasificación. De acuerdo a la taxonomía polifásica, la caracterización debe efectuarse a nivel fenotípico y genotípico.

La caracterización fenotípica es de carácter observacional, y está conformada por aquellas características que son consecuencia de la expresión de los genes de un organismo. Los rasgos a considerar incluyen desde la morfología de la colonia, condiciones de crecimiento, etc. [4,15,17] a rasgos fisiológicos o químicos [4,15]. Con motivo de poder determinar la variabilidad de los rasgos fenotípicos se aconseja utilizar más de una cepa representativa del nuevo taxón y también cepas de taxones cercanos [4]. Debido a la poca reproducibilidad de algunas de las metodologías empleadas tradicionalmente para definir los rasgos fenotípicos de un nuevo taxón, se han propuesto métodos alternativos como la espectrometría de masas "matrix-assisted laser desorption/ionisation time-of-flight" (MALDI-TOF MS).

Los rasgos genotípicos son las características que podemos encontrar en el ADN, como el contenido de guaninas y citosinas del ADN o la hibridación ADN-ADN (DDH), que consiste en la cuantificación de la hibridación del ADN genómico fragmentado de dos organismos. La DDH ha sido empleada en muchos estudios taxonómicos, a pesar de que se trata de una técnica compleja y poco reproducible [3], pues ofrece un umbral de separación entre especies: valores de DDH < 70% señalan que dos organismos pertenecen a especies diferentes [14]. Hoy en día, con la generalización de la secuenciación genómica, las técnicas *in silico* como el "average nucleotide identity" (ANI) han reemplazado el uso de la DDH. Los valores de la DDH y el ANI correlacionan, y el umbral de especies de la primera (70%) corresponde a un valor de ANI de ≈94% [23,24].

1.2. Clasificación

La aparición de las técnicas moleculares supuso un avance para el campo de la taxonomía bacteriana debido a que, al poder conocer las secuencias del ADN, se pudo inferir el proceso evolutivo [27] y ajustar la clasificación jerárquica de los microorganismos en base a sus relaciones genéticas y similitudes. En 1987, el gen que codifica el 16S rRNA fue propuesto como un marcador molecular apropiado para llevar a cabo análisis filogenéticos, dadas su ubicuidad y funciones conservadas [28]. Su uso para establecer las relaciones filogenéticas está ampliamente extendido, y es obligatorio proveer dicha información para describir un nuevo taxón [11]. Cabe destacar, que otros genes pueden ser más adecuados a la hora de inferir las relaciones filogenéticas entre determinados taxones, y que, en filogenia, es aconsejable el estudio de varias secuencias de genes concatenados, con un mínimo de 5 [11], lo que se conoce como "multilocus sequence analysis" (MLSA). A la hora de definir especies el MLSA constituye una alternativa de características intermedias al DDH y al 16S rRNA [11,33], sin embargo, al no poder proponerse un conjunto de genes universal a analizar [2], otras metodologías, como el ANI, parecen más prometedoras.

1.3. Nomenclatura

Una vez hay suficientes evidencias de que un grupo de organismos es monofilético y tiene características únicas y distintivas, se puede proponer un nombre, de acuerdo al sistema binomial y siguiendo las reglas del International Code of Nomenclature of Prokaryotes (última versión [35]). El International Code of Nomenclature of Prokaryotes sólo reconocerá un nombre de especie como válido si ha sido *publicado válidamente*, lo que significa que tiene que haberse publicado de manera efectiva a toda la comunidad científica e ir acompañado de una descripción o hacer referencia a ella [35]. Además, para que una publicación sea válida la cepa tipo tiene que ser depositada en dos colecciones de acceso

público en dos países diferentes. También es aconsejable que la descripción se base en el mayor número de cepas posible, siendo el mínimo entre 5 y 10 [18,36], aunque se permite describir especies nuevas basadas en una sola cepa [18].

1.4. Estado actual de la taxonomía bacteriana

La generalización de la secuenciación genómica está revolucionando el campo de la taxonomía. Algunos autores reclaman una modernización de la taxonomía e integrar la genómica en detrimento de la aproximación polifásica, que consideran obsoleta [39,40]. Incluso, se ha propuesto que las secuencias genómicas deberían sustituir a las cepas tipo [38,42]. Si bien esto puede ser irrealizable de momento [2,19], se han sugerido otros cambios más factibles: la generación de bases de datos, el uso del MALDI-TOF MS como técnica de caracterización fenotípica y nuevos formatos de publicación [2,5,19,38].

2. El género *Acinetobacter*

Los organismos que hoy en día conocemos como *Acinetobacter,* a lo largo de los años han sido clasificados en al menos 15 especies diferentes, lo cual les ha llevado a pasar desapercibidos hasta que su posición taxonómica se asentó a finales de los 80. Desde 2017, la especie de más relevancia clínica, *Acinetobacter baumannii*, encabeza la lista de patógenos para los cuales el desarrollo de nuevos antimicrobianos es prioritario según la Organización Mundial de la Salud (OMS) [50].

El género *Acinetobacter* está compuesto por un grupo heterogéneo de bacterias Gram-negativas, estrictamente aerobias, no fermentadoras, no móviles, catalasa positivas y oxidasa negativas, que presentan morfología cocobacilar y un contenido de guanina y citosina que oscila del 39 al 47% [44,45]. La temperatura óptima de crecimiento suele hallarse entre los 33-35ºC, sin embargo, suelen crecer bien entre los 20 y 37ºC. Debido a sus características heterogéneas eran, y siguen siendo, difíciles de caracterizar a nivel fenotípico.

El uso de la DDH fue fundamental para delinear las especies del género, y en 1989 se definieron 12 grupos de acuerdo a la DDH (especies genómicas, gen. sp.) [65]. Posteriormente se han ido describiendo nuevas especies, hasta llegar al número actual de 60, habiéndose descrito más de 30 nuevas especies en los últimos 5 años gracias a las técnicas moleculares y genómicas (https://apps.szu.cz/anemec/Classification.pdf último acceso diciembre de 2018).

2.1. Hábitats

Las especies del género *Acinetobacter* se caracterizan por su ubicuidad, sin embargo, cada especie suele estar adaptada a un nicho diferente, de modo que algunas son principalmente ambientales como *Acinetobacter calcoaceticus*, mientras otras son de origen humano, como el patógeno *A. baumannii*, cuyo reservorio y hábitat natural son desconocidos [45]. Las ambientales pueden hallarse tanto en suelos, agua, océanos como en plantas [75,123,124]. En muchas ocasiones también encontramos especies del género *Acinetobacter* en productos para el consumo humano, como vegetales [125–127] y productos lácteos y cárnicos [103,128–134]. Su presencia en ganado [84,136–140] y animales de compañía [131,141,142] es también frecuente, e incluso algunos autores sugieren que las cigüeñas o el alimento de éstas podrían constituir el reservorio de *A. baumannii* [143]. Además de *A. baumannii*, otras especies del género *Acinetobacter* pueden provocar también infecciones en humanos [104], y algunas forman parte de la microbiota de la piel [45]. Junto a *A. baumannii* las especies con más relevancia clínica son *Acinetobacter nosocomialis y Acinetobacter pittii.*

2.3. El grupo Ab

En 1989, los experimentos de DDH demostraron que las especies *A. baumannii, A. calcoaceticus, A. nosocomialis* y *A. pittii* (conocidas en aquel entonces como gen. sp. 2, 1, 13TU y 3, respectivamente) estaban altamente relacionadas, pues sus valores de DDH oscilan entre 65-75% [67]. Posteriormente, se decidió agruparlas bajo el nombre de "*A. calcoaceticus-A. baumannii* (ACB) complex" [147], pues si bien existen diferencias a nivel genotípico entre ellas, a nivel fenotípico resultan indistinguibles [93,94,149,149]. De hecho, aún hoy en día, su identificación a nivel de especie puede suponer un reto según las técnicas empleadas, y en muchas ocasiones se reportan las bacterias del ACB complex como *A. baumannii* [152], una generalización que puede tener relevancia a nivel clínico, pues la gravedad de las infecciones suele diferir entre las especies del ACB complex [151]. Desde un punto de vista hospitalario, parece más apropiado agrupar a las especies clínicas y referirse a ellas como "grupo *A. baumannii* (Ab)" [45], dejando de lado a *A. calcoaceticus*, que es mayoritariamente ambiental. Dentro del grupo Ab también estarían incluidas la gen. sp. "Between 1 & 3" [106], muy cercana a *A. calcoaceticus* y *A. pittii*, y la especie *Acinetobacter seifertii*, previamente designada gen. sp. "close to 13TU" por su relación con *A. nosocomialis* [106], y que adquirió el status de especie en 2015 [105].

2.4. Identificación de las especies

Los miembros del género *Acinetobacter* son muy difíciles de distinguir mediante métodos fenotípicos, de modo que el uso de técnicas moleculares ha sido esencial tanto en su taxonomía como en el desarrollo de técnicas de identificación. El uso de la espectrometría de masas, gracias a su fácil implementación en laboratorios de rutina, también supone una alternativa a las tradicionales pruebas fenotípicas.

A finales de los años 80, se propusieron diversos esquemas de pruebas fenotípicas, que si bien presentaban problemas para diferenciar entre *A. baumannii*/*A. nosocomialis,* y *A. calcoaceticus*/*A. pittii* [144,147]*,* fueron usados ampliamente en estudios taxonómicos. Hoy en día estas pruebas han quedado obsoletas dado el elevado número de especies descritas desde entonces. Los métodos comerciales de identificación, como API 20NE o Vitek 2, tampoco tienen suficiente poder discriminatorio para identificar de manera fiable a las especies del género *Acinetobacter* [152,157], aunque su uso en el ámbito hospitalario es común. Finalmente, diversos estudios demuestran que el uso del MALDI-TOF MS para la identificación de especies de *Acinetobacter* supone una gran ventaja a otras metodologías más laboriosas, permitiendo obtener resultados fiables en un menor rango de tiempo, con la ventaja de poder diferenciar entre *A. baumannii*, *A. nosocomialis* y *A. pittii* [161].

Por el contrario, existe una gran variedad de métodos moleculares para la identificación de las especies del género *Acinetobacter*, entre ellos destacan el ARDRA (Amplified rRNA Restriction Analysis), que se basa en la amplificación por PCR y posterior digestión con varios enzimas del gen del 16S rRNA, generando un patrón de bandas que permite distinguir entre diversas especies del género *Acinetobacter*, incluyendo las del grupo Ab [171,172]. Otras técnicas moleculares se basan en la amplificación y secuenciación de diversos genes que han demostrado ser útiles para la identificación de especies del género *Acinetobacter*, como el gen *rpoB* [178,179] o el gen *gyrB* [180].

3. Resistencia a los antibióticos en el grupo Ab

La resistencia a antibióticos es un rasgo característico del género *Acinetobacter* y, en concreto, *A. baumannii* destaca por la alta frecuencia de cepas multirresistentes que se detectan a nivel hospitalario.

3.1. Resistencia intrínseca

La resistencia en las especies de *Acinetobacter* puede ser de origen intrínseco, como sería la ocasionada por β-lactamasas o bombas de flujo presentes en los cromosomas de las especies del género de forma habitual, y que si son sobreexpresadas pueden provocar resistencia a ciertos antibióticos.

Las β-lactamasas intrínsecas del género *Acinetobacter* son la β-lactamasa de clase C del tipo AmpC, llamada *Acinetobacter*-Derived Cephalosporinases (ADCs) [199] que proporciona resistencia a penicilinas y cefalosporinas, y una β-lactamasa de clase D, también conocida como oxacillinasa intrínseca. En general, cada especie posee sus propias variantes alélicas, que en *A. baumannii* son del grupo OXA-51. La inserción del IS*Aba1* delante del gen de la oxacilinasa intrínseca [203,217] y algunas de sus variantes alélicas [216] pueden otorgar resistencia a imipenem, además de degradar antibióticos como las penicilinas, oxicilina, cloxacilina y algunas cefalosporinas [215].

Los cambios en la permeabilidad de la envuelta bacteriana también pueden provocar la aparición de resistencia a antibióticos. En el caso de las porinas u OMPs (outer membrane proteins), su ausencia o expresión reducida disminuye la entrada del antibiótico al interior celular, mientras que la sobreexpresión de las bombas de flujo, ocasionada generalmente por alteraciones en sus reguladores, lleva a la expulsión de los antibióticos del interior celular, evitando así en ambos casos que el antibiótico pueda llegar a su diana intracelular. Dentro de las porinas, destacan por su papel en resistencia OmpA, AbuO y CarO [231,252–255]. En el caso de las bombas de flujo, existen diversas familias implicadas en la expulsión activa de antibióticos: ABC, ATP-binding cassette family; RND, resistance-nodulation-cell division superfamily; MFS, major facilitator superfamily; MATE, multidrug and toxic compound extrusion family; SMR, small multidrug resistance family; PACE, proteobacterial antimicrobial compound efflux family, sin embargo la más estudiada es la superfamilia de las RND [242,263,264].

3.2. Resistencia adquirida

El género *Acinetobacter*, y en concreto *A. baumannii*, son conocidos por su gran capacidad de adquirir mecanismos de resistencia. La resistencia adquirida puede deberse a la acumulación de mutaciones en un gen diana, como en el caso de las mutaciones en *gyrA* y *parC* que provocan resistencia a fluoroquinolonas [280–286] o la resistencia a polymyxinas ocasionada por mutaciones en *pmrAB* y *lpxACD* que alteran la composición del lipopolisacárido [288–296]. Asimismo, la resistencia adquirida también puede producirse por transferencia horizontal de genes de resistencia mediante integrones, transposones y plásmidos.

La resistencia a carbapenems en bacterias del género *Acinetobacter* en muchas ocasiones es debida a la adquisición de carbapenemasas del tipo metallo-β-lactamasas (NDM, VIM…) o de clase D (OXAs de los grupos 23, 24/40, 58, 143 y 235), que junto con los mecanismos de resistencia intrínsecos y la presencia de genes de resistencia a otros antibióticos en las plataformas que utilizan para su movilización de bacteria en bacteria, ocasionan el temido fenotipo de multirresistencia. Una de las clases de antibióticos para la cual también suelen encontrarse genes de resistencia en elementos móbiles son los aminoglicósidos, que ven reducida su efectividad debido a enzimas que modifican el antibiótico en sí o la diana de éste, reduciendo así su efectividad.

4. Patogenicidad del grupo Ab

Las especies del grupo Ab son las de más relevancia clínica dentro del género, sin embargo, son consideradas como patógenos de baja virulencia. Aun así, cabe destacar que los mecanismos mediante los cuales *Acinetobacter* causa infecciones son poco conocidos, y se centran principalmente en *A. baumannii*, cuya capacidad de acumular mecanismos de resistencia y sobrevivir en el ámbito hospitalario, son las mayores causas de su elevada incidencia como patógeno nosocomial [310].

4.1. Factores de virulencia

A diferencia de otros patógenos, la virulencia de *Acinetobacter* es multifactorial, pues no se ha encontrado aún ningún determinante concreto que por sí solo pueda explicar su patogenicidad. Numerosos estudios demuestran que elementos de superficie y de transporte a través de las membranas están implicados en la virulencia de este patógeno. De este modo la producción de exopolisacáridos, así como diversos sistemas de secreción y OMPs, entre las cuales destaca notablemente OmpA [397], están relacionados con diversos fenotipos de virulencia [310]. La formación de biofilms es también un rasgo común en *Acinetobacter* [427–432], siendo el pili de tipo I, codificado por el operón *csuA/BABCDE,* esencial para este fenotipo [433]. El desarrollo de biofilms es de gran relevancia en *Acinetobacter*, pues es uno de los factores que favorecen su persistencia en el ámbito médico [446–448]. A pesar de su nombre de origen griego que significa "bacteria no motil" ([α + κινητο + βακτηρ(ια)]), se han descrito diferentes tipos de motilidad no mediada por flagelo en *Acinetobacter*, el "twitching", que está mediado por pili de tipo IV [307,445,4663] y la motilidad asociada a superficie, ambas dependientes de la secreción de surfactantes [469].

4.2. Interacciones huésped patógeno

La investigación acerca del proceso infeccioso de *Acinetobacter* una vez dentro del huésped se centra, mayoritariamente, en *A. baumannii*. Después de entrar en el cuerpo humano y evadir al sistema inmune innato, las células de *A. baumannii* pueden adherirse a las células eucariotas [491,508] y lo hacen mediante adhesinas como OmpA [477]. Las células eucariotas tienen receptores extra e intracelulares que reconocen las bacterias y que en contacto con estas desencadenan la respuesta inmune, activando la producción y secreción de citoquinas que atraen a las células del sistema inmune [513–516], como neutrófilos, macrófagos y linfocitos "natural killers". La activación de células fagocíticas puede resultar en la eliminación de las bacterias [510,513,520] o, si fracasa, conducir a una infección generalizada. Además, a pesar de que *A. baumannii* es considerado un patógeno extracelular, también puede invadir las células eucariotas, este proceso de internalización está mediado por OmpA [477]. Una vez dentro de las vacuolas, *A. baumannii* puede persistir intracelularmente y activar factores pro-apoptóticos, como las caspasas, que conducen a la muerte de las células eucariotas huésped [480,521,522].

5. Infecciones causadas por el grupo Ab

El género *Acinetobacter* causa principalmente infecciones de tipo hospitalario (neumonías, bacteriemias, infecciones de tejidos blandos y heridas, infecciones urinarias…), pero también se dan casos en la comunidad, y las infecciones por *Acinetobacter* pueden adquirir una gran relevancia en heridos de guerra y por desastres naturales, causando infecciones en heridas y en huesos, probablemente transmitidas en los centros de atención hospitalaria [529,530]. El éxito de *Acinetobacter* en los hospitales recae en su gran resistencia a la desecación y a los desinfectantes [450,531–533], que, junto a su gran capacidad de adquirir y acumular mecanismos de resistencia a antibióticos, dificultan su eliminación tanto del ambiente como de los pacientes.

5.1. Impacto clínico

A. baumannii suele ser la especie más común y que causa más mortalidad, sin embargo, el motivo de su éxito sobre las otras especies del grupo Ab no está claro, y se cree que más que deberse a una mayor patogenicidad, se debe a su habitual fenotipo de multirresistencia [190,565,573], que dificulta proporcionar el tratamiento empírico adecuado a los pacientes [190,191,566,573]. De todos modos, muy pocos estudios se han centrado en evaluar si, ante un fenotipo de resistencia parecido, la mortalidad de *A. baumannii* y las otras especies

del grupo Ab sería similar, y aquellos que lo han hecho han encontrado una estrecha relación entre resistencia y mortalidad, más que en la especie causante de la infección en sí [190,299].

5.2. Opciones terapéuticas

Debido al aumento de la resistencia a antibióticos en las especies de *Acinetobacter*, y en especial en *A. baumannii*, ha surgido la necesidad de buscar nuevos tratamientos para estas bacterias. Algunos de estos tratamientos alternativos se basan en el uso de antibióticos considerados activos frente a *Acinetobacter*, o la combinación de éstos. Dentro de esta aproximación cabe destacar el uso de las polimixinas, glicilciclinas, sulbactam, fosfomicina o rifampina, sin embargo, la aparición de mecanismos de resistencia a estos antibióticos y la necesidad de realizar más estudios que demuestren su efectividad en combinación, han llevado a enfocar el desarrollo de nuevos fármacos desde diferentes perspectivas. Algunas de las terapias alternativas propuestas se basan en el uso de péptidos antimicrobianos, la terapia fágica, el uso de secuestradores de metales, la fototerapia, la vacunación e, incluso, el trasplante de microbiota faecal. Estos enfoques para el desarrollo de fármacos contra *Acinetobacter*, si bien resultan prometedores, aún están en etapas preliminares de desarrollo.

II. Antecedentes, Hipótesis y Objetivos

En septiembre de 2014 recibimos un aislado (JVAP01) procediente de Turquía cuya identificación a nivel de especie no estaba clara tras ser identificado mediante varias metodologías, que lo situaban muy cercano a *A. pitiii*. El análisis mediante ARDRA reveló que el aislado no pertenecía a ninguna de las especies del grupo Ab, pero coincidía con un conjunto de aislados llamados grupo NB14 de la colección de la Leiden University. Un análisis retrospectivo de nuestra colección de cepas de *Acinetobacter* identificó 3 aislados adicionales, por lo que junto con los de la colección de la Leiden University se decidió llevar a cabo la descripción taxonómica de la posible nueva especie.

Además, de acuerdo con resultados previos obtenidos en nuestro grupo, parecía que cepas de *Acinetobacter* spp. que sobreexpresaban bombas de flujo presentaban un fenotipo más virulento en el modelo de infección animal de *Caenhorabditis elegans*. Siguiendo esta línea de investigación, nos planteamos profundizar en el conocimiento sobre el papel de las proteínas implicadas en el transporte a través de las membranas de *A. baumannii*, pues un mayor conocimiento sobre éstas puede aportar información relevante sobre la virulencia de este patógeno, así como ayudar a identificar nuevas dianas para el desarrollo de fármacos.

De acuerdo a estos antecedentes se plantearon dos hipótesis, con sus correspondientes objetivos:

Hipótesis 1. Los aislados previamente conocidos como *Acinetobacter pittii*-like del grupo NB14 constituyen una posible nueva especie de *Acinetobacter* perteneciente al grupo Ab que puede ser identificada mediante MALDI-TOF MS.

Objetivo 1. Caracterizar los rasgos fenotípicos y genotípicos que apoyan la designación de los aislados del grupo NB14 como una posible nueva especie, para la cual se propone el nombre de *Acinetobacter dijkshoorniae* sp. nov., y que podría ser un nuevo miembro del grupo Ab con potencial relevancia clínical. (**Paper 1** y **2**).

Objetivo 2. Evaluar el potencial del MALDI-TOF MS para identificar de manera rápida y precisa los aislados de *A. dijkshoorniae* y *A. seifertii*. (**Paper 3**)

Objetivo 3. Investigar la presencia de *Acinetobacter* spp. en muestras de comida (**Manuscrito 4**)

Objetivo 4. Identificar características fenotípicas de los miembros del grupo Ab, en términos de susceptibilidad antimicrobiana y potencial de virulencia *in vitro*. (**Manuscrito 5**)

Hipótesis 2. Además de su contribución a la resistencia a antibióticos, las bombas de flujo y otras proteínas implicadas en transporte pueden tener un papel relevante en la virulencia de *A. baumannii*, ya sea en comunicación celular o en la secreción de factores de virulencia que puedan estar involucrados en la patogénesis de esta especie y puedan ser utilizados como potenciales dianas para el desarrollo de fármacos antimicrobianos.

Objetivo 5. Estudiar diferentes bombas de flujo y proteínas relacionadas con el transporte para determinar su contribución específica en la resistencia a antibióticos y virulencia de *A. baumannii*. (**Manuscrito 6**)

Objetivo 6. Caracterizar el secretoma de aquellos mutantes que presenten diferencias en virulencia para identificar posibles factores de virulencia secretados. (*No conseguido*)

Objetivo 7. Identificar nuevas dianas adicionales para desarrollar fármacos antimicrobianos mediante la determinación del impacto en la virulencia de *A. baumannii* de las mutaciones en las proteínas secretadas. (*No conseguido*)

III. Resultados

Los resultados de esta tesis se han estructurado en dos secciones, de acuerdo a las dos hipótesis, y sus correspondientes objetivos, establecidos en el capítulo anterior. La sección I comprende los resultados publicados en los **Papers 1-3** y los **Manuscritos 4** y **5**, que corresponden a la caracterización de los aislados del grupo NB14 como una nueva especie y a la evaluación del MALDI-TOF MS como una herramienta adecuada para la identificación de las especies del grupo Ab. Además, en esta sección se han evaluado otros fenotipos que van más allá de los que se estudian habitualmente en taxonomía, para respaldar que las especies del grupo Ab corresponden a entidades biológicas distintas que deben ser reportadas y estudiadas independientemente debido a que pueden tener diferentes implicaciones a nivel clínico. La sección II, corresponde al **Manuscrito 6**, en el que intentamos identificar nuevas dianas para el desarrollo de fármacos antimicrobianos contra *A. baumannii* mediante la determinación del papel dual de las proteínas de transporte en resistencia a antibióticos y virulencia en una cepa de *A. baumannii* MDR.

Sección I

A pesar de la ausencia de directrices [4,5], la descripción taxonómica de una nueva especie bacteriana debe seguir una serie de recomendaciones, como que deben caracterizarse un mínimo número de cepas (entre 5-10) y que la caracterización debe realizarse junto con cepas representativas de las especies más cercanas [18,36]. Una vez recibimos las cepas del grupo NB14 de la colección de la Leiden University, el número de cepas del grupo Ab era suficiente como para proceder a definir su status de especie, sin embargo, también necesitábamos cepas del resto de especies del grupo Ab. Los representantes de *A. baumannii*, *A. nosocomialis* y *A. pittii* los obtuvimos de nuestra propia colección, mientras que tuvimos que solicitar las cepas de *A. seifertii*, que en aquel entonces acababa de ser descrita [105].

Siguiendo de nuevo las recomendaciones, utilizamos un enfoque polifásico [13,14] para describir la nueva especie. Para ellos realizamos una caracterización de las cepas del grupo NB14 y las representantes de las especies del grupo Ab tanto a nivel fenotípico como genotípico. Tanto los datos genotípicos (análisis filogenéticos de *rpoB* y MLSA, y comparaciones genómicas *in silico*) como los fenotípicos (ensayos metabólicos y análisis de los

picos generados por MALDI-TOF MS) respaldaron la designación de los aislados del grupo Ab como una nueva especie, para la cual propusimos el nombre de *Acinetobacter dijkshoorniae* sp. nov., en honor a Lenie Dijkshoorn, una microbióloga holandesa de gran relevancia en el campo de la biología y taxonomía de *Acinetobacter* (**Paper 1**).

Debido a que encontramos picos específicos de especie mediante MALDI-TOF MS cuando caracterizábamos los rasgos fenotípicos de *A. dijkshoorniae* (**Paper 1**), consideramos que esta metodología podía ser empleada para la identificación de las nuevas especies del grupo Ab: *A. dijkshoorniae* y *A. seifertii*. Además, a pesar de que esta metodología identificaba estas especies como *A. pittii* y *A. baumannii*, respectivamente, nos dimos cuenta de que los dos mejores resultados siempre coincidían con los mismos espectros de referencia de la base de datos. Estas observaciones sugerían que las cepas utilizadas para crear esos espectros de referencia deben pertenecer a las nuevas especies, pero además indicaban que, aún sin optimizar, la técnica de MALDI-TOF MS es capaz de identificar a *A. dijkshoorniae* y *A. seifertii*. En consecuencia, decidimos evaluar el uso del MALDI-TOF MS como herramienta de identificación de las especies del grupo Ab rápida y precisa (**Paper 3**). Para ello, además de proporcionar espectros de referencia de las nuevas especies, también optimizamos la identificación de *A. nosocomialis*, pues el análisis de los perfiles de picos generados por MALDI-TOF MS reveló que hay dos grupos de perfiles para *A. nosocomialis*, y uno de ellos esta sub-representado en la base de datos taxonómica de Bruker, causando errores en la identificación de esta especie.

Tras actualizar la base de datos taxonómica de Bruker, el uso de MALDI-TOF MS ha demostrado ser de gran utilidad en la identificación de especies del grupo Ab (**Manuscrito 4**). Además, en este estudio identificamos por primera vez la especie A. *dijkshoorniae* en Perú, así como también consiste en la primera vez que esta especie es aislada a partir de muestras de carne de ternera. Estos datos proporcionan información sobre otras posibles fuentes de *A. dijkshoorniae*, ya que en su descripción taxonómica todos los aislados, a excepción de uno ambiental, provenían de muestras de origen humano.

Finalmente, quisimos identificar características fenotípicas diferenciales entre las especies del grupo Ab más allá de las evaluadas en su descripción taxonómica (**Manuscrito 5**). Para ello determinamos los perfiles de resistencia a antibióticos de las especies del grupo Ab, y los resultados indican que *A. baumannii* es la especie que presenta mayores tasas de resistencia, a pesar de que en las otras especies podemos encontrar también mecanismos de resistencia intrínseca como las bombas de flujo de la familia RND o las oxacilinasas intrínsecas, que a excepción de *A. nosocomialis* y *A. seifertii*, fueron detectadas en todos los aislados.

También determinamos la motilidad y capacidad de formación de biofilm de las diferentes especies del grupo Ab (**Manuscrito 5**), y los resultados indicaron que ambos fenotipos presentan una gran variabilidad en los aislados de todas las especies. No obstante, pudimos determinar diferencias en la formación de biofilm de las distintas especies del grupo Ab: *A. baumannii* es la especie con menor formación de biofilm, mientras que *A. dijkshoorniae*, *A. pittii* y *A. seifertii* son buenas productoras de biofilm tanto a 28ºC como 37ºC. En cuanto a *A. nosocomialis*, esta especie forma más biofilm a 28ºC que a 37ºC, temperatura a la cual la formación de biofilm de los aislados se reduce drásticamente. Además de estos dos fenotipos, también evaluamos la virulencia de los aislados en el modelo de infección de *C. elegans*. En este modelo, *A. dijkshoorniae* resultó ser la especie más virulenta, mientras que *A. pittii* y *A. seifertii* presentaron dos subpoblaciones, una virulenta y otra no virulenta, y *A. baumannii* y *A. nosocomialis* no eran virulentas en este modelo.

En conjunto, nuestros resultados respaldan el estudio de las especies del grupo Ab como entidades biológicas diferentes, y creemos que el uso de MALDI-TOF MS como método de identificación en los laboratorios de diagnóstico puede ayudar enormemente a la determinación de la prevalencia real e implicaciones clínicas diferenciales de las especies emergentes del grupo Ab.

Paper 1- *Acinetobacter dijkshoorniae* sp. nov. un miembro del *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex recuperado principalmente de muestras clínicas en diferentes países

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International Journal of Systematic and Evolutionary Microbiology, 66(10): 4105–4111, 2016 Octubre

Factor de impacto: 2.134 – Q3 (2016)

Hipótesis: Un grupo de 15 cepas de *Acinetobacter*, de origen mayoritariamente humano y aisladas en diferentes países durante un período de 15 años, podría pertenecer a una especie nueva dentro del *Acinetobacter calcoaceticus – Acinetobacter baumannii* (ACB) complex.

Objetivos: Determinar la posición taxonómica de las cepas mediante métodos moleculares y fenotípicos.

Metodología:
La relación clonal se determinó mediante macrorrestricción del DNA genómico con el enzima ApaI, seguido por electroforesis de campo pulsado. Las secuencias del gen 16S rRNA, *rpoB* y el multilocus sequence typing (MLST) fueron comparadas con 94 cepas representativas de todas las especies del ACB complex mediante análisis filogenético. Se obtuvo el genoma completo de la cepa JVAP01T de *A. dijkshoorniae* sp. nov. mediante "next generation sequencing" y se calculó el "average nucleotide identity based on BLAST" (ANIb) utilizando el programa JSpecies. La relación fenotípica entre las especies se determinó mediante Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) y ensayos de utilización de carbono.

Resultados:
Se seleccionaron 12 cepas de la posible nueva especie que no estaban relacionadas clonalmente para ser caracterizadas. Los análisis filogenéticos del 16S rRNA, *rpoB* y el MLST confirmaron que a nivel genético las 12 cepas son diferentes a las de otras especies y se agrupan monofiléticamente, siendo *A. pittii* la especie más cercana y observándose poca transferencia horizontal de genes entre las especies del ACB complex. Los valores de ANIb inferiores al 95% también demostraron el status de especie de *A. dijkshoorniae* sp. nov. Las características metabólicas de *A. dijkshoorniae* sp. nov. concordaron con las del ACB complex, y el análisis de los perfiles proteicos generados por MALDI-TOF MS revelaron picos específicos de especie.

Conclusiones: Nuestros resultados respaldan que las cepas estudiadas pertenecen a una especie novel, para la cual proponemos el nombre de *Acinetobacter dijkshoorniae* sp. nov.

Paper 2 - Secuencia genómica preliminar de la cepa tipo JVAP01T de la nueva especie *Acinetobacter dijkshoorniae*

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Factor de impacto: -

Hipótesis: -

Objetivos: Publicar la secuencia del genoma de la cepa tipo de *Acinetobacter dijkshoorniae*, un patógeno humano novel del *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex.

Metodología:
El DNA genómico fue extraído a partir de un cultivo bacteriano y se generó una librería genómica de acuerdo al protocolo de Nextera XT (Illumina, Inc., San Diego, CA, USA). La secuenciación se llevó a cabo en un Sistema Illumina MiSeq. El ensamblado *de novo* se realizó mediante el programa Velvet version 1.2.10 junto con el optimizador Velvet (http://bioinformatics.net.au/software.velvetoptimiser.shtml), ABySS v1.5.2 y Spades v3.5.0 (8–10). Los contigs generados durante el ensamblaje se unieron utilizando CISA v1.3 (11). Los contigs menores a 200 nucleótidos fueron descartados.

Resultados:
El genoma de la cepa JVAP01T de *A. dijkshoorniae* tiene una longitud de 3 858 459 pares de bases y el contenido de guaninas y citosinas, de un 38.8%, está dentro de los umbrales de las especies de *Acinetobacter*. El servidor RAST predijo 3599 secuencias codificantes, 26 rRNAs, y 134 rRNAs en los 92 contigs obtenidos. El programa Resfinder v2.1 detectó la presencia de los genes bla_{NDM-1} y *aphA6* localizados en un plásmido, que confieren resistencia a la mayoría de β-lactámicos y aminoglicósidos, y también de los genes bla_{ADC} y $bla_{OXA-213}$ localizados en el cromosoma. El programa PathogenFinder v1.1 predijo que *A. dijkshoorniae* tiene una probabilidad del 83.3% de ser un patógeno humano.

Conclusiones: De acuerdo a su genoma *Acinetobacter dijkshoorniae* puede ser considerado un patógeno humano del ACB complex, y la cepa JVAP01 contiene plásmidos que confieren resistencia a antibióticos de último recurso como los carbapenems.

Paper 3 - Revisión de la identificación mediante MALDI-TOF/MS de las especies del grupo *Acinetobacter baumannii* (Ab): inclusión de las nuevas especies *A. seifertii* y *A. dijkshoorniae*

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Factor de impacto: 5.394 – D1 (2017)

Hipótesis: Las especies recientemente descritas *A. seifertii* y *A. dijkshoorniae* pueden ser identificadas mediante espectrometría de masas (Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry, MALDI-TOF/MS).

Objetivos: Evaluar el uso del MALDI-TOF/MS para identificar *A. seifertii* y *A. dijkshoorniae*, y revisar la identificación de *A. nosocomialis* para actualizar la base de datos taxonómica

Metodología:
La caracterización a nivel de especie se realizó mediante el análisis filogenético de *rpoB* y MLSA. Los espectros de MALDI-TOF/MS se obtuvieron a partir del análisis de los extractos bacterianos realizados con ácido fórmic/acetonitrilo cubiertos por una matriz de ácido α-cyano-4-hydroxy-cinnámico en un MicroflexLT en modo lineal positivo y en un rango de masa de 2000-20000 *m/z*. Los espectros se examinaron con el programa ClinProTools v2.2. Los espectros medios (MSP) se crearon con el programa BioTyper.

Resultados:
Se utilizaron 88 aislados de *Acinetobacter* representativos del grupo Ab para calcular el MSP de cada especie y generar los modelos de reconocimiento de patrones. Se identificaron picos específicos de especie para todas las especies analizadas. Los MSPs derivados de 3 cepas de *A. seifertii*, 2 cepas de *A. dijkshoorniae*, y 2 cepas de *A. nosocomialis* se añadieron a la base de datos taxonómica de Bruker, permitiendo una correcta identificación de todas las cepas utilizando tanto extractos bacterianos como directamente de colonia bacteriana, con valores predictivos positivos del 99.6% (777/780) y 96.8% (302/312), respectivamente.

Conclusiones: El uso de programas de procesado de datos identificó picos específicos de especie estadísticamente significativos que permitieron generar perfiles proteicos de referencia para la rápida y exacta identificación de las especies del grupo Ab, incluidas las nuevas especies *A. seifertii* y *A. dijkshoorniae,* proporcionando de este modo herramientas para obtener información relevante para el manejo de las infecciones clínicas causadas por *Acinetobacter.*

Manuscrito 4 - Aislamiento de diferentes especies de *Acinetobacter* en carne de mercados: primera identificación de *Acinetobacter dijkshoorniae* en Perú

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Objetivos: Analizar las características fenotípicas y genotípicas de *Acinetobacter* spp. recuperados en muestras de carne de Perú.

Metodología:
Las muestras de carne se obtuvieron mediante muestreo aleatorio de mercados tradicionales; éstas fueron homogeneizadas y se utilizaron 2 g para enriquecer la carga bacteriana en cultivos de LB. Las colonias bacterianas fueron aisladas utilizando diferentes medios selectivos. La identificación a nivel de especie se realizó mediante MALDI-TOF MS, secuenciación de *rpoB*, y MLSA. La relación clonal se estableció por MLST y electroforesis de campo pulsado (PFGE). La susceptibilidad a antimicrobianos se determinó por gradiente de difusión y microdilución en caldo.

Resultados:
Doce cepas de *Acinetobacter* spp. fueron aisladas de 5 muestras de ternera diferentes entre un total de 138 muestras de carne obtenidas en 2 mercados independientes en Lima, Perú. Se identificaron a nivel de especie 9 cepas de *A. pittii*, 1 cepa de *A. baumannii*, 1 cepa de la especie recientemente descrita *A. dijkshoorniae,* y 1 cepa que fue identificada como *A. guillouiae* mediante MALDI-TOF MS, pero que los métodos moleculares demostraron que pertenecía a *A. beriziniae*. Se seleccionaron 6 cepas para estudios adicionales de acuerdo a su perfil de PFGE único o procedencia de diferentes muestras. Todas las cepas fueron susceptibles a todos los antibióticos testados y algunas presentaban alelos de MLST y STs nuevos.

Conclusiones: El aislamiento de especies de *Acinetobacter* patógenicas en carne para el consume humano puede representar un gran riesgo para la salud pública, fuentes ambientales como los animales y la comida no deben ser descartados como potenciales reservorios para la propagación de estos patógenos en la comunidad y centros de salud. Ésta es la primera vez que la especie *Acinetobacter dijkshoorniae* se identifica en Perú y en muestras de carne para consumo humano.

Manuscrito 5 - Potencial de virulencia *in vitro* e *in vivo* de las especies emergentes del grupo Ab

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Hipótesis: Las especies del grupo *A. baumannii* (Ab) diferentes a *A. baumannii* pueden representar un riesgo sanitario y se necesitan más estudios relativos a sus rasgos distintivos.

Objetivos: Identificar rasgos fenotípicos diferenciales, en términos de susceptibilidad antimicrobiana y potencial de virulencia de los miembros del grupo Ab.

Metodología:
La susceptibilidad antimicrobiana se determinó por microdilución en caldo o difusión en gradiente. Los genes de bombas de flujo del tipo RND y de OXAs intrínsicas se detectaron por PCR. La formación de biofilm se realizó en placas de 96 pocillos y se cuantificó mediante el método del cristal violeta después de 44 h de incubación a 28ºC o 37ºC. La motilidad en superficie se midió tras 18 h de crecimiento a 37ºC. La virulencia se evaluó como la capacidad de matar nemátodos en el modelo de infección de *Caenorhabditis elegans*.

Resultados:
Se compararon 76 aislados de las 5 especies del grupo Ab (16 *A. baumannii*, 12 *A. dijkshoorniae*, 16 *A. nosocomialis*, 20 *A. pittii*, y 12 *A. seifertii*). De acuerdo a estudios previos, la resistencia a antibióticos fue frecuente en los aislados de *A. baumannii*, mientras que las otras especies presentaron, en general, mayores tasas de susceptibilidad. La presencia de bombas de flujo del tipo RND fue frecuente en todas las especies del grupo Ab, las OXAs intrínsecas solo se detectaron en *A. baumannii*, *A. dijkshoorniae* y *A. pittii*, sin embargo, los perfiles de susceptibilidad no correlacionaron con la presencia de ninguna bomba o β-lactamasa de clase D intrínseca. En general, los aislados de *A. baumannii* y *A. nosocomialis* presentan un potencial patógenico menor que los de *A. seifertii*, *A. pittii* y, especialmente, que los de *A. dijkshoorniae*: los aislados de *A. baumannii* y *A. nosocomialis* forman menos cantidad de biofilm y requieren mayor tiempo para matar a *C. elegans* que las otras especies.

Conclusiones: A pesar de que su impacto a nivel clínico aún está por determinar, estos resultados sugieren un grado distinto de adaptación al ambiente hospitalario de las especies de *Acinetobacter* del grupo Ab, y a la vez enfatizan la potencial relevancia clínica de las especies emergentes del grupo Ab.

Sección II

Las infecciones causadas por *A. baumannii* suponen una gran amenaza sanitaria debido a la alta incidencia de la multirresistencia en este patógeno, especialmente cuando las bacterias son carbapenem resistentes, ya que las opciones de tratamiento se ven drásticamente reducidas. Para confrontar esta situación, la OMS ha colocado recientemente a *A. baumannii* carbapenem-resistente encabezando el listado de las bacterias para las cuales el desarrollo de nuevos fármacos antimicrobianos se considera una prioridad y una necesidad urgente en la salud pública [4]. Parte de este proyecto de doctorado quería abordar este problema mediante la investigación de la doble función de las proteínas relacionadas con el transporte en la resistencia antimicrobiana y la virulencia de *A. baumannii*. Este enfoque ampliará nuestro conocimiento sobre proteínas de transporte nuevas y también conocidas en este patógeno y, en última instancia, también podría acercarnos a la identificación de nuevas dianas para el desarrollo de nuevos fármacos antibacterianos dirigidos específicamente contra *A. baumannii*.

Después de varios intentos fallidos de construir nuestra propia colección de mutantes por deleción en genes codificantes de proteínas relacionadas con el transporte, decidimos utilizar la colección de mutantes por transposición desarrollada en 2015 por Gallagher *et al.* utilizando la cepa *A. baumannii* AB5075 [625]. Esta cepa había sido descrita previamente como una cepa tipo recomendable debido a su reciente aislamiento, su alta virulencia tanto en modelos de infección en invertebrados como en mamíferos, y su resistencia a carbapenems causada por una carbapenemasa del tipo OXA-23 [626]. Seleccionamos 83 mutantes de la colección basándonos en la descripción de sus supuestas funciones, y tras ser verificados, se seleccionaron 65 para llevar a cabo los análisis posteriores. De éstos, los genes truncados pertenecían, putativamente, a: la familia ABC (n=12), la familia MATE (n=3); la familia MFS (n=28); OMPs (n=8), la familia RND (n=9), y otras proteínas relacionadas con el transporte y permeasas (n=5).

La comparación de la susceptibilidad antimicrobiana entre la cepa salvaje y los mutantes, reveló que al menos un miembro de cada familia de transportadores evaluada parecía estar involucrado en la resistencia a antibióticos (**Manuscrito 6**). En conjunto, 10 mutantes presentaron variaciones en su susceptibilidad antimicrobiana, y los aminoglicósidos fueron los antibióticos más afectados. A pesar de la presencia de múltiples mecanismos de resistencia, se pudieron detectar diferencias en susceptibilidad respecto a la cepa salvaje para meropenem, levofloxacino y moxifloxacino en los mutantes para una OMP similar a OmpA y un transportador sin caracterizar de la familia autoinducer-2 exporter (AI-2E).

Otros mutantes con variaciones en su susceptibilidad tenían el transposón insertado en genes codificantes para proteínas cuya función en el transporte de antibióticos ya había sido descrita anteriormente, como AbeM, CraA, AdeJ y AdeB. El resto de mutantes tenían afectadas proteínas de transporte cuya función en resistencia a antibióticos aún no se ha descrito de las familias de bombas de flujo ABC (n=1) y MFS (n=3), y que, por lo tanto, requieren caracterización adicional.

Uno de los motivos por los cuales seleccionamos la cepa de *A. baumannii* AB5075 para nuestro estudio fue que, además de la disponibilidad de la colección de mutantes, esta cepa presentaba un fenotipo altamente virulento en las larvas de la polilla *Galleria mellonella* y en un modelo de infección de pulmón en ratón [626]. En consecuencia, a pesar de haber trabajado con el modelo de infección en *C. elegans* (**Manuscrito 5**), decidimos que *G. mellonella* era más adecuado para analizar nuestra colección de mutantes de proteínas de transporte, ya que, debido al gran número de cepas a analizar, trabajar con ratones sería poco ético. Como no teníamos experiencia con el modelo, esta parte del proyecto se realizó bajo la supervisión del Dr. David W. Wareham (Blizard Institute – Barts and The London, Queen Mary University of London, London, UK), durante dos estancias en su laboratorio. En primer lugar, para determinar que las proteínas de transporte funcionalmente activas participan en la virulencia de *A. baumannii* AB5075, corroboramos que *A. baumannii* AB5075 inactivada por calor no podía causar una infección en el modelo de infección por *G. mellonella.* A continuación, utilizamos inhibidores de bombas de flujo (EPI) para evaluar preliminarmente el papel del transporte de membrana en la patogénesis de la cepa de *A. baumannii* AB5075, y encontramos que solo la 1-(1-naftilmetil)-piperazina (NMP) redujo significativamente la virulencia en el modelo de *G. mellonella*. Finalmente, realizamos los ensayos utilizando los mutantes. Nuestros resultados muestran que la mayoría de las proteínas relacionadas con el transporte de la cepa *A. baumannii* AB5075 implicadas en la virulencia en el modelo de infección por *G. mellonella* no están caracterizadas. En resumen, seis mutantes de diferentes familias (MATE (n=2), OMP (n=2), MFS (n=1) y ABC (n=1)) mostraron una menor virulencia en el modelo de infección por *G. mellonella*, y solo tres de ellos presentaron similitud de secuencia con proteínas conocidas (proteínas similares a OmpA, MacB y TolC).

Desafortunadamente, no hemos podido complementar los mutantes (**Manuscrito 6**), a pesar de varios intentos y enfoques, y, por lo tanto, los datos presentados en esta sección aún son preliminares. Sin embargo, la identificación de nuevos transportadores de membrana que podrían contribuir a la resistencia antimicrobiana y/o virulencia en la cepa *A. baumannii* AB5075, sugiere que las familias de proteínas de transporte menos estudiadas

que la de RND, como las familias ABC y MATE, podrían tener un papel clave en la resistencia y patogénesis, y que se necesitan más estudios para corroborar su participación e identificar posibles sustratos que puedan utilizarse como una diana para el desarrollo de nuevos fármacos.

Manuscrito 6 - Caracterización del papel del transporte de membrana en la virulencia y resistencia de *Acinetobacter baumannii* multirresistente

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Hipótesis: Las proteínas de transporte pueden tener un papel importante en la virulencia de *A. baumannii* y pueden ser utilizadas como dianas para el desarrollo de fármacos antibacterianos.

Objectivos: Estudiar proteínas de transporte para determinar su contribución específica a la virulencia y resistencia de *A. baumannii*.

Metodología:
Se adquirieron mutantes por deleción de *A. baumannii* AB5075 de diferentes proteínas de transporte de membrana codificadas en el cromosoma de una librería de mutantes por transposición. La inserción del T26 en los *loci* adecuados se verificó por PCR en aquellos mutantes que crecieron en presencia de tetraciclina (5 mg/L). Las concentraciones mínimas inhibitorias se determinaron por microdilución en caldo o difusión en gradiente. El modelo de infección de *Galleria mellonella* se utilizó para determinar las diferencias en virulencia. Los ensayos de fitness se realizaron en placas de 96 pocillos utilizando medio LB sin antibiótico a 37ºC, la DO_{600} se midió cada 15 min durante 24 h.

Resultados:
De una colección de 65 mutantes para proteínas de transporte en *A. baumannii* AB5075 se identificaron 10 mutantes que presentan alteraciones en su susceptibilidad antimicrobiana y 6 que tienen la virulencia atenuada en el modelo de infección de *G. mellonella*. Los aminoglicósidos fueron los antibióticos afectados más frecuentemente, pero también se observaron, cambios en susceptibilidad a meropenem, levofloxacino y moxifloxacino a pesar de la presencia de múltiples mecanismos de resistencia a antibióticos. Los genes afectados en cinco de los mutantes con susceptibilidad alterada corresponden a AbeM, CraA, OmpA, AdeJ y AdeB, mientras que en el resto de cepas las proteínas implicadas no se habían relacionado con resistencia a antibióticos con anterioridad. Los mutantes con la virulencia atenuada tenían interrumpidos genes que codifican proteínas de transporte nuevas y de diferentes familias, tres de los cuales muestran similitud de secuencia con OmpA, MacB y TolC.

Conclusiones: Debemos resolver algunos problemas técnicos que nos impidieron complementar los mutantes para poder corroborar nuestros resultados Se necesitan estudios adicionales para caracterizar las posibles nuevas proteínas de transporte que contribuyen a la resistencia y virulencia en *A. baumannii* AB5075.

IV. Conclusiones

1. De acuerdo con los análisis filogenéticos de *rpoB* y MLSA, los aislados de *Acinetobacter* del grupo NB14 forman un grupo monofilético cercano a *A. pittii*, y los análisis de ANIb demuestran su status de especie.

2. No es posible diferenciar mediante ensayos metabólicos ni fisiológicos los aislados del grupo NB14 de los otros miembros del ACB complex, de acuerdo con estudios previos. Sin embargo, los análisis mediante MALDI-TOF MS identifican picos específicos de especie para este grupo de aislados.

3. Nuestros datos respaldan la descripción de los aislados previamente conocidos como *Acinetobacter pittii*-like NB14 como una nueva especie, para la cual proponemos el nombre de *Acinetobacter dijkshoorniae* sp. nov.

4. *A. dijkshoorniae* sp. nov. es aislada frecuentemente de muestras clínicas, sin embargo, el medio ambiente y la comida no deben ser descartados como fuentes de este patógeno.

5. De acuerdo a los análisis genómicos, *A. dijkshoorniae* sp. nov. tiene una alta probabilidad de ser patógeno humano. Este hecho, así como su frecuente aislamiento de muestras humanas, apuntan a su probable relevancia clínica y a la necesidad de una correcta identificación a nivel de especie.

6. Los aislados de *A. nosocomialis* presentan dos grupos diferenciados de acuerdo a los perfiles de los espectros generados por MALDI-TOF MS. Las bajas tasas de correcta identificación de *A. nosocomialis* se deben a la subrepresentación de uno de estos grupos en la base de datos taxonómica de Bruker.

7. La inclusión de nuevos espectros de referencia en la base de datos taxonómica de Bruker para el grupo subrepresentado de *A. nosocomialis* y las nuevas especies *A. dijkshoorniae* y *A. seifertii* permite la correcta identificación mediante MALDI-TOF MS de las especies del grupo Ab con una alta sensibilidad y especificidad tanto a partir de extractos como colonias bacterianas.

8. En general, *A. baumannii* es menos susceptible a los antibióticos que otras especies del grupo Ab, las cuales siguen siendo susceptibles a la mayoría de los antibióticos.

9. Los fenotipos de motilidad y formación de biofilm son altamente específicos de cepa.

10. La motilidad asociada a superficie en el grupo Ab es una característica común compartida por todas las especies.

11. La habilidad de producir biofilm difiere entre las especies del grupo Ab y es temperatura dependiente. Parece que existen diferentes estrategias de termorregulación para *A. dijkshoorniae* y *A. pittii* que para *A. baumannii*, *A. nosocomialis* y *A. seifertii*.

12. La mayor virulencia de *A. dijkshoorniae*, la población mixta de aislados virulentos y no-virulentos de *A. pittii* y *A. seifertii*, y la menor virulencia de *A. baumannii* y *A. nosocomialis* en el modelo de *C. elegans* podría reflejar un cambio en las capacidades de infección hacia el huésped humano y diferentes grados de adaptación.

13. Los análisis filogenéticos basados en las secuencias parciales de cada uno de los siete genes de MLST y de las OXAs intrínsecas de *Acinetobacter* muestran poca transferencia horizontal entre las otras especies del ACB complex y los aislados del grupo NB14.

14. Los aislados de *A. pittii* muestran una variabilidad mayor, tanto a nivel de espectros de MALDI-TOF MS como en sus secuencias genéticas, lo cual sumado a su clara separación en aislados virulentos y no-virulentos en el modelo de infección de *C. elegans* podría sugerir que pueden ser redefinidos a nivel taxonómico.

15. Más allá de sus características distintivas a nivel taxonómico, las especies del grupo Ab se comportan diferencialmente, los resultados obtenidos para una especie no pueden ser extrapolados a otra, y en consecuencia, deben estudiarse por separado para que podamos evaluar adecuadamente sus características clínicas.

16. Algunas proteínas de transporte ya conocidas podrían tener sustratos antimicrobianos adicionales a los descritos previamente (OmpA, familia de transportadores AI-E2 y CraA).

17. Las familias MATE y ABC de proteínas de flujo pueden tener un papel esencial en la patogénesis de la cepa *A. baumannii* AB5075.

18. A pesar de que el papel de muchas proteínas de transporte de *A. baumannii* aún está por determinar, varias proteínas relacionados con el transporte, tanto ya descritas como desconocidas, parecen contribuir a los fenotipos de resistencia y virulencia de la cepa *A. baumannii* AB5075.

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IX. Annexes

Annex I

Surface-associated motility in the species of the Ab group

When we evaluated the surface-associated motility abilities of the species of the Ab group (**Manuscript 5**) we observed that the isolates displayed different surface-associated morphotypes, as previously described for *A. baumannii* [501] in a rather strain-specific than species-dependent manner. A part from non-motile isolates (morphotype A), 4 additional different morphotypes (B, C, D and E, in order of abundance,) were defined: morphotype B presented striated radiations from the inoculation point with well-defined edges; morphotype C radiated uniformly from the inoculation point and presented soft edges; morphotype D displayed densely-grown stria with thick edges; and morphotype E included the less abundant or unique morphotypes that could not be included in the any of the other groups.

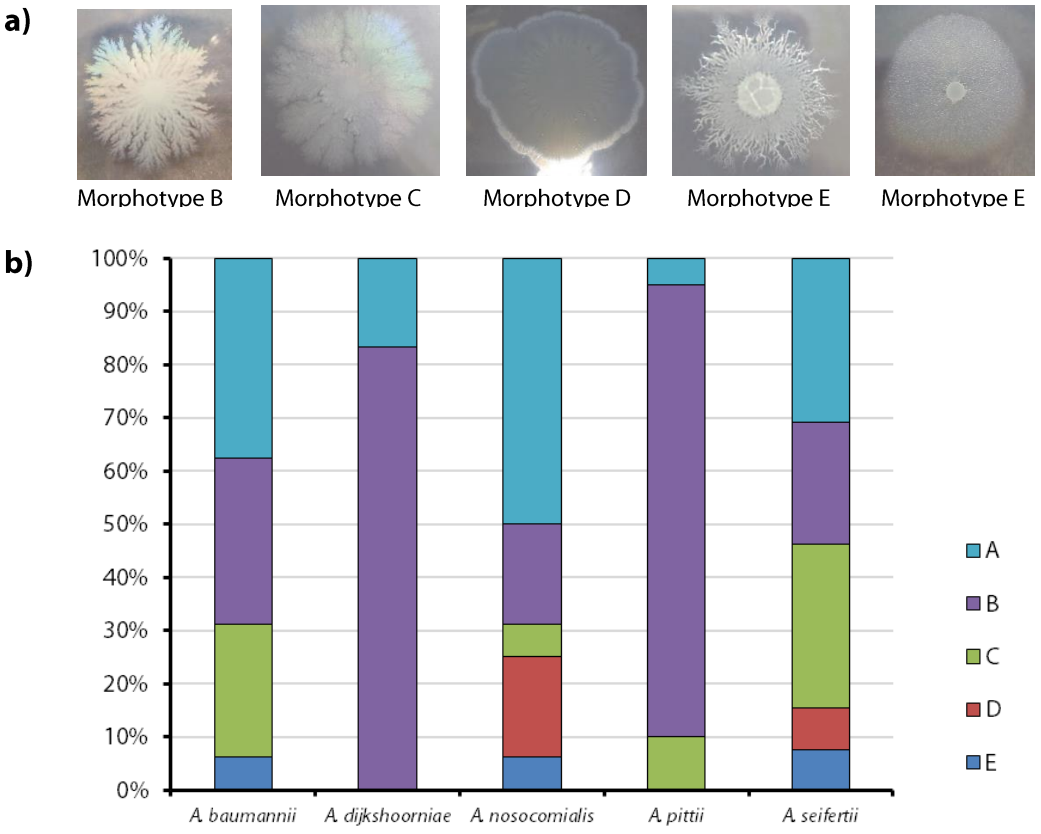


FIG 1. Surface-associated morphotypes observed in our collection of isolates of the Ab group. **A)** Representative images for each morphotype observed (B-E), and **B)** Distribution of the morphotypes (A-E) among the different species.

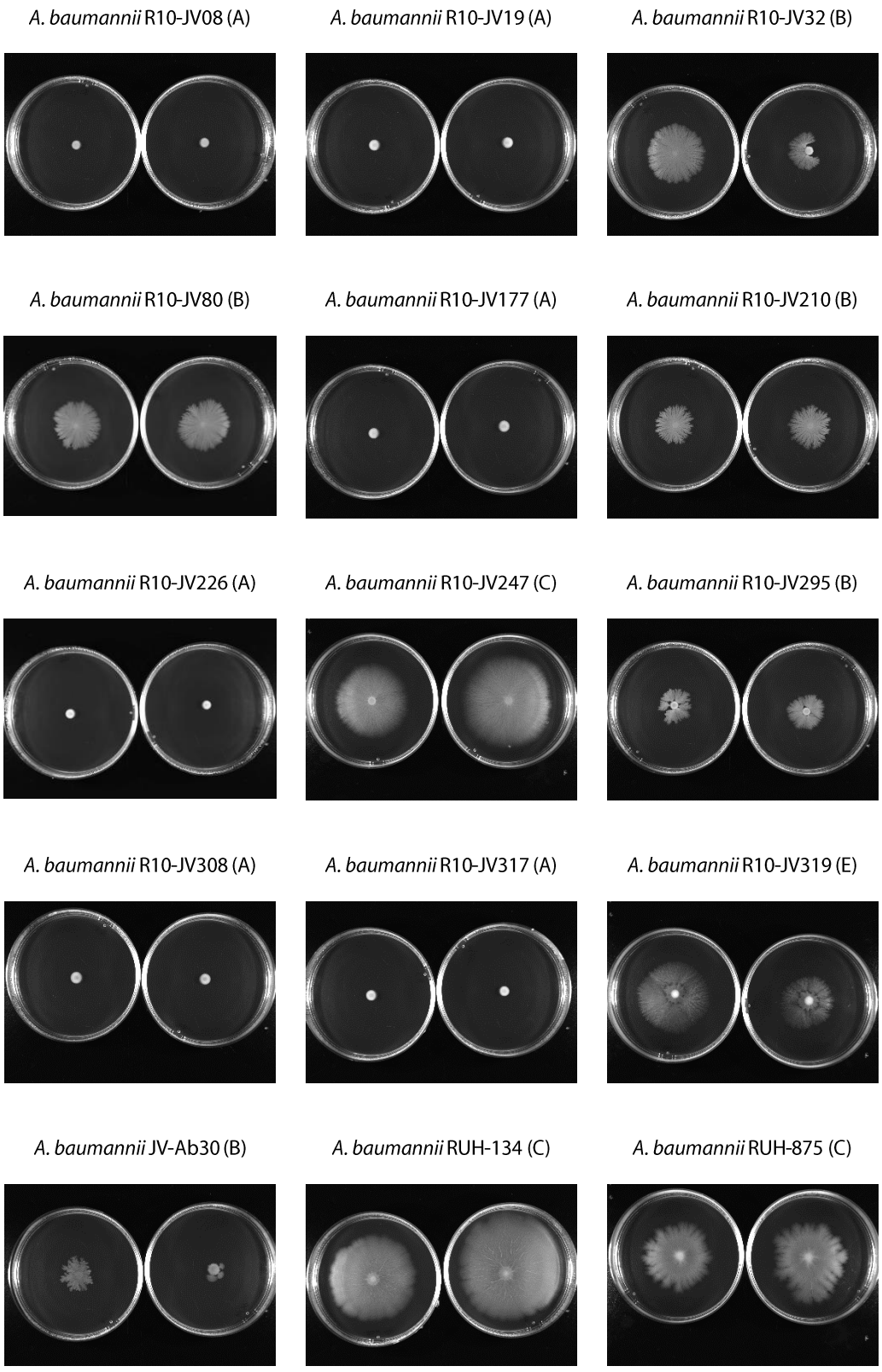


FIG 2. Representative pictures of the surface-associated motility of each isolate studied Pictures correspond to one of the four replicates that were performed for each strain, the morphotype assigned (A-E) is shown in parentheses. *(Continues in the next pages)*

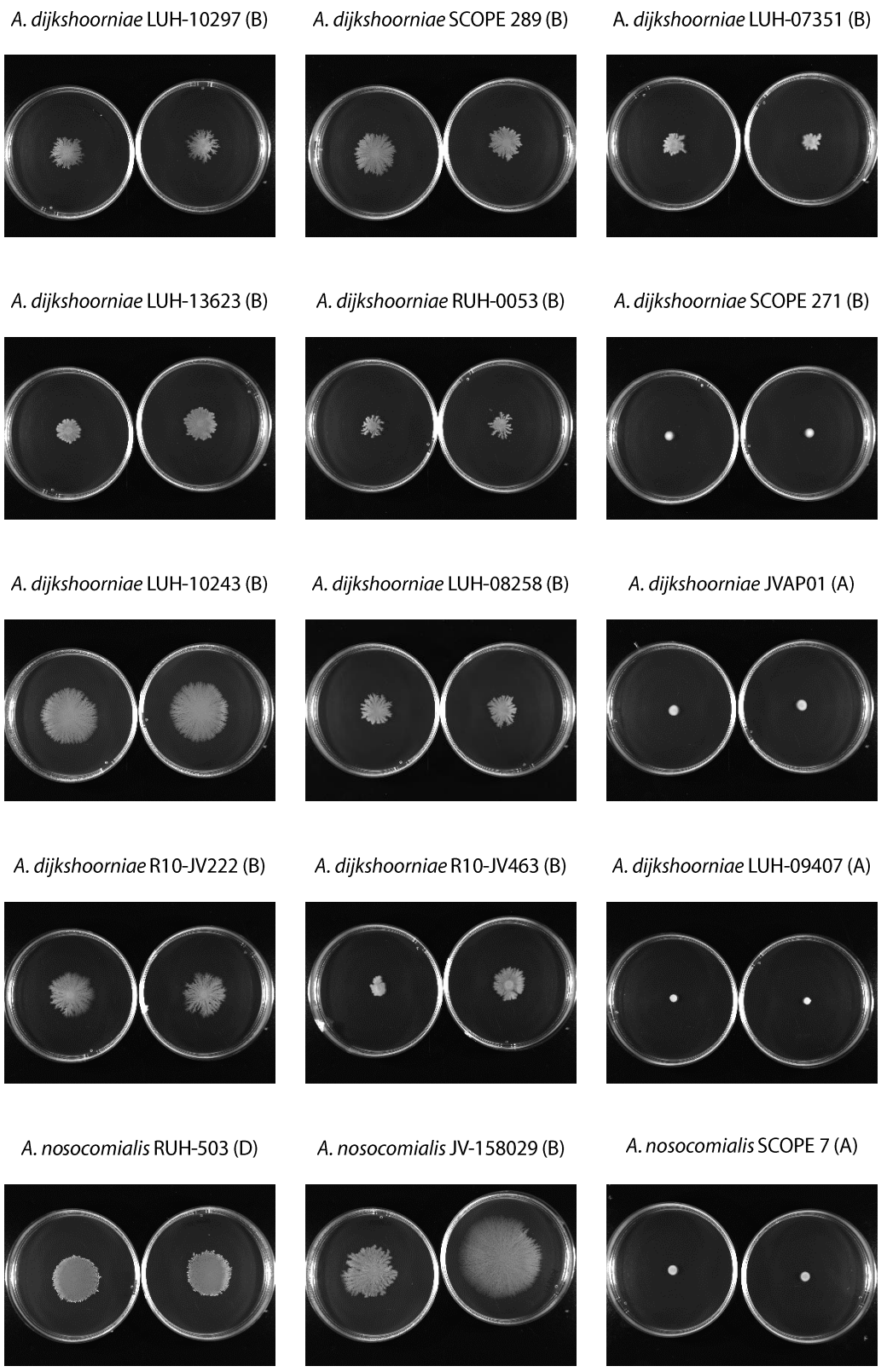


FIG 2. Representative pictures of the surface-associated motility of each isolate studied Pictures correspond to one of the four replicates that were performed for each strain, the morphotype assigned (A-E) is shown in parentheses. *(Continues in the next pages)*

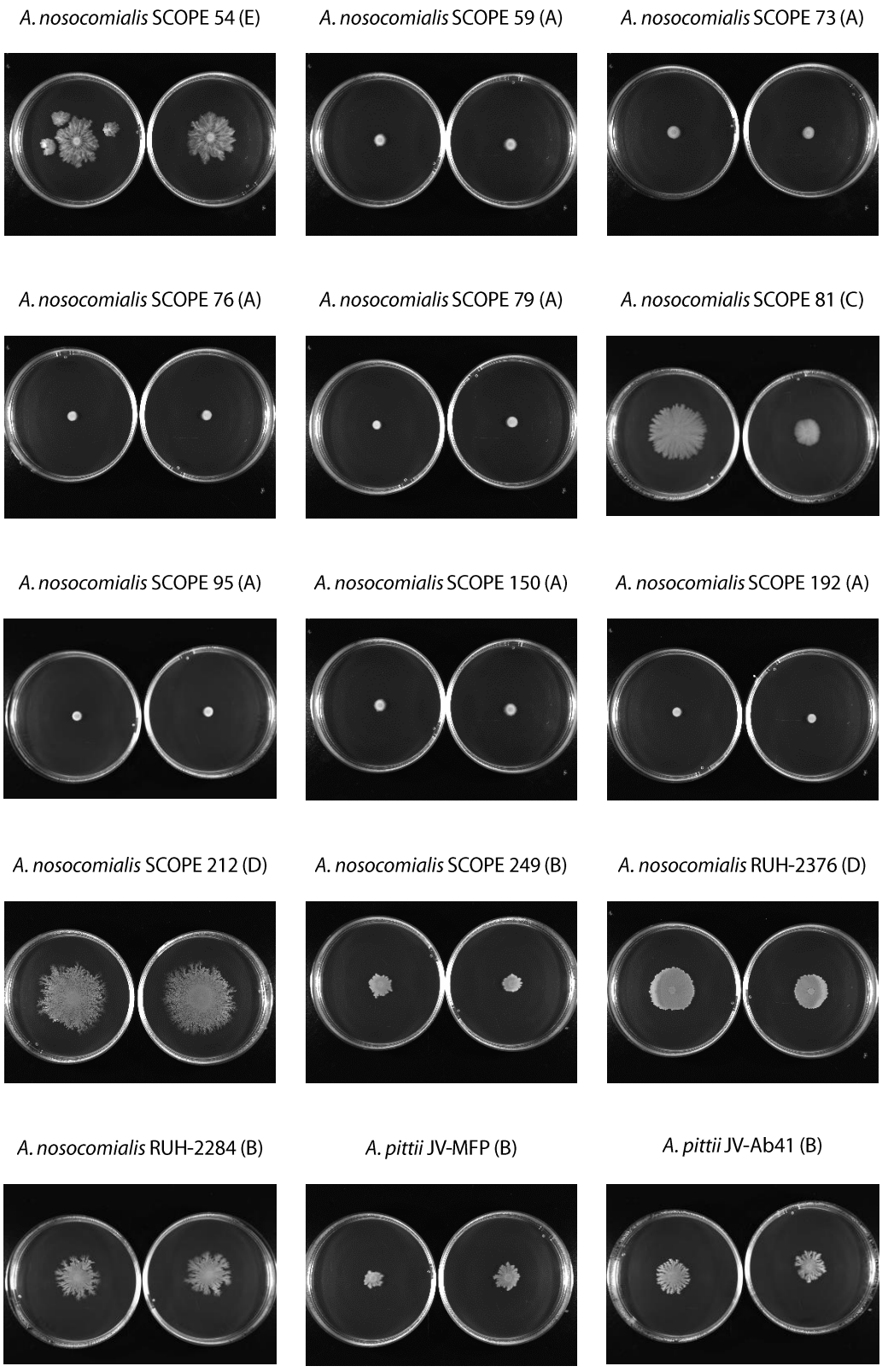


FIG 2. Representative pictures of the surface-associated motility of each isolate studied Pictures correspond to one of the four replicates that were performed for each strain, the morphotype assigned (A-E) is shown in parentheses. *(Continues in the next pages)*

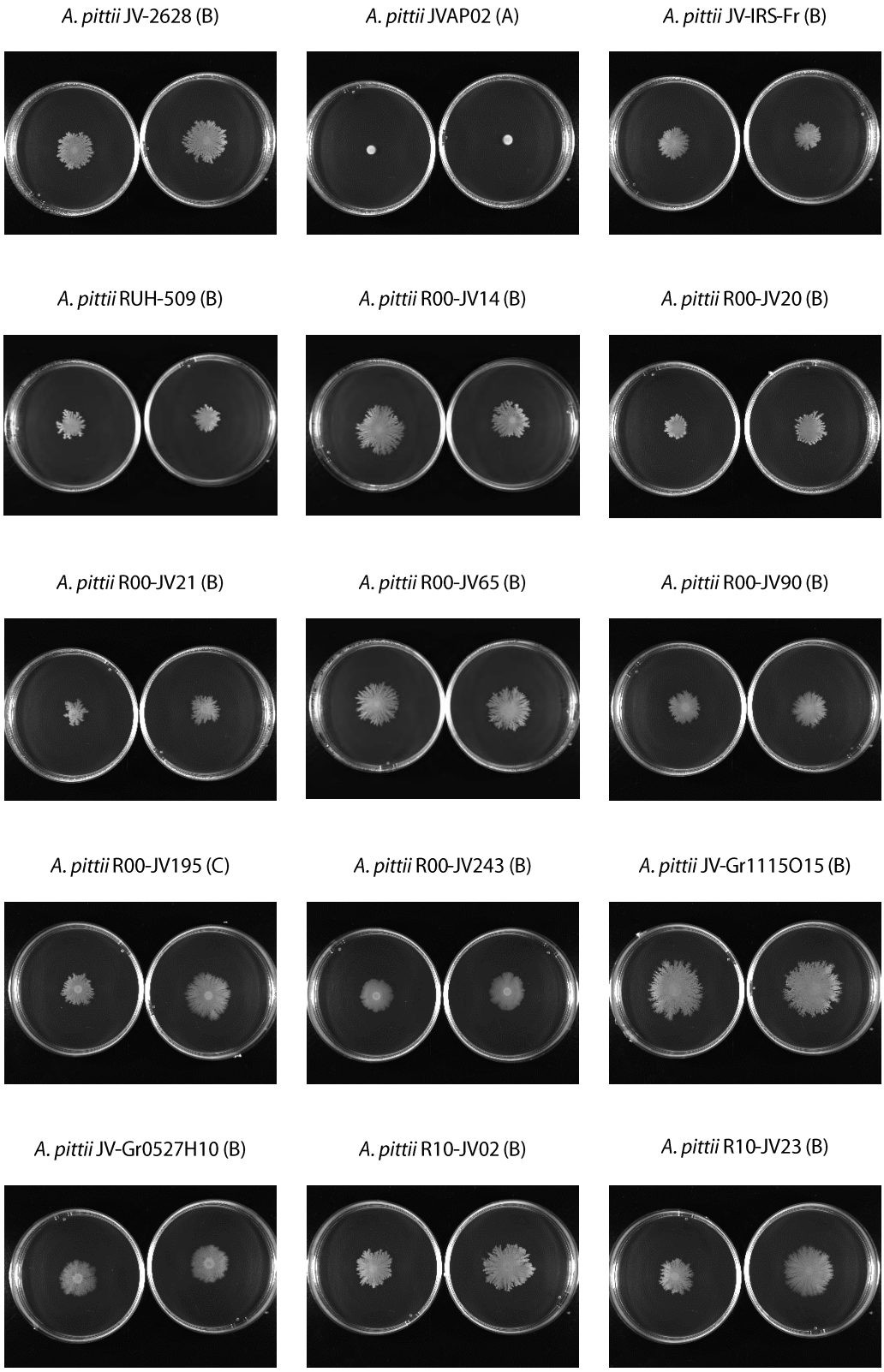


FIG 2. Representative pictures of the surface-associated motility of each isolate studied Pictures correspond to one of the four replicates that were performed for each strain, the morphotype assigned (A-E) is shown in parentheses. *(Continues in the next page)*

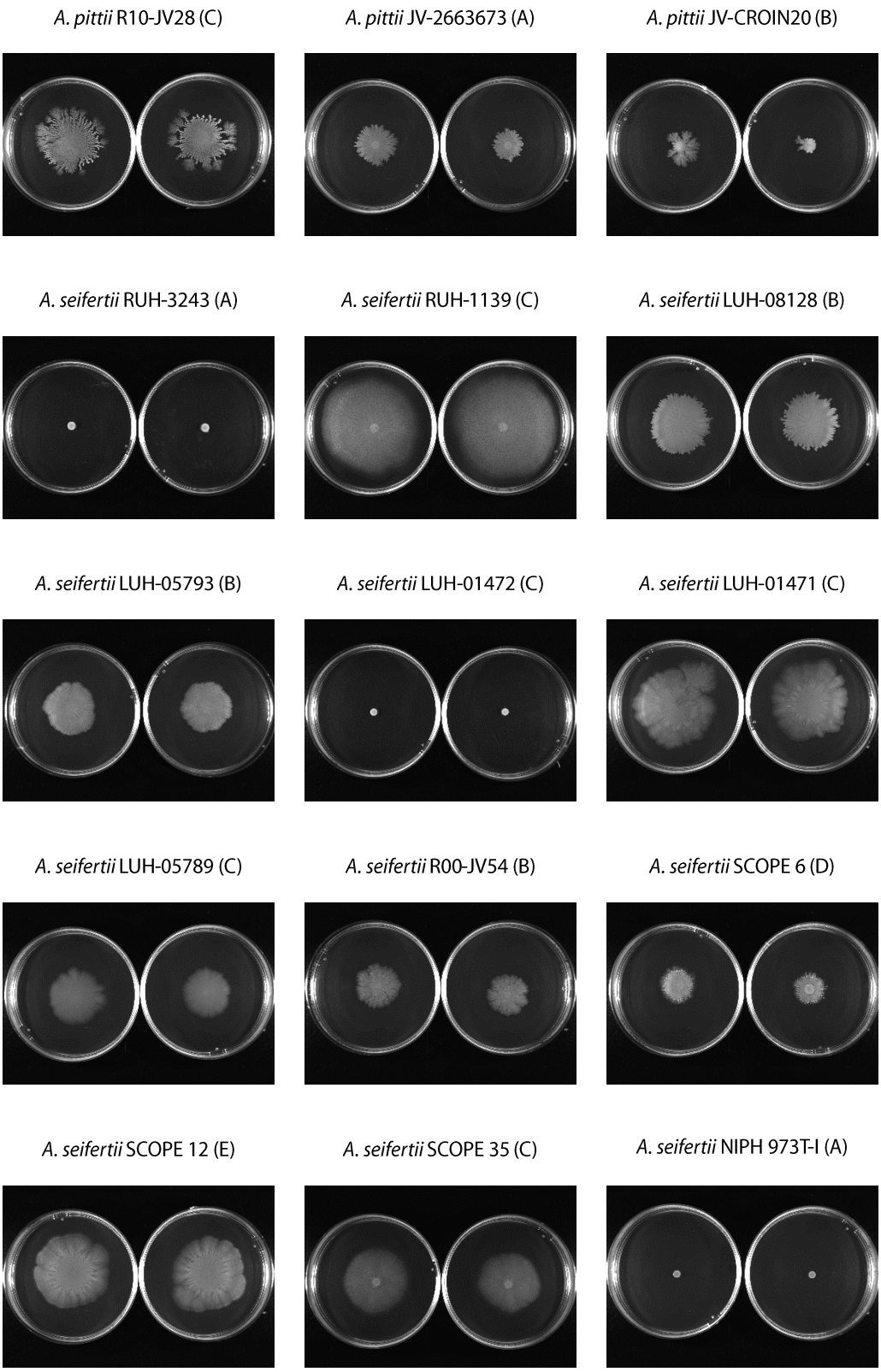


FIG 2. Representative pictures of the surface-associated motility of each isolate studied Pictures correspond to one of the four replicates that were performed for each strain, the morphotype assigned (A-E) is shown in parentheses.

Annex II

A. baumannii AB5075 transport-related mutant strains list

#	Item #	Strain Name	Ab Locus	Position in ORF (bp)	Gene Name	Putative function + Blast searches	Efflux type	Observations
1	AB00015	tnab1_kr121204p08q153	ABUW_0007	966(1932)	-	ABC transporter, ATP-binding protein	ABC	-
2	AB00076	tnab1_kr121119p04q172	ABUW_0035	1370(3126)	*arpB*	RND EFFLUX transporter -acrB	RND	Phase variant
3	AB00165	tnab1_kr130913p01q177	ABUW_0064	580(1344)	-	MATE family drug transporter	MATE	-
5	AB00304	tnab1_kr130913p07q152	ABUW_0109	608(1368)	-	MATE EFFLUX family protein	MATE	-
7	AB00454	tnab1_kr121205p01q148	ABUW_0166	511(768)	-	outer membrane protein	OMP	-
10	AB00545	tnab1_kr121119p02q129	ABUW_0196	599(1362)	-	major facilitator family transporter	MFS	-
11	AB00590	tnab1_kr121119p03q101	ABUW_0213	570(1245)	-	major facilitator family transporter	MFS	Phase variant
13	AB00738	tnab1_kr121205p05q136	ABUW_0262	774(1662)	-	ABC transporter, ATP-binding protein - YjiK [Acinetobacter baumannii UH9907]	ABC	-
14	AB00812	tnab1_kr121203p06q147	ABUW_0294	975(2166)	*comQ*	fimbrial assembly protein PilQ, putative OMP (ComQ),type IV pilus secretin PilQ, T2SS, component HofQ	Other	-
15	AB00941	tnab1_kr130913p07q157	ABUW_0337	750(1230)	*craA*	chloramphenicol resistance pump cmr	MFS	Phase variant
16	AB01344	tnab1_kr121203p07q119	ABUW_0505	284(480)	-	OmpA/MotB	OMP	
18	AB01774	tnab1_kr121213p04q112	ABUW_0649	517(1062)	*OmpA-like*	outer membrane protein A	OMP	Mucose
19	AB01842	tnab1_kr130903p03q179	ABUW_0675	1164(3099)	*nolG*	RND multidrug efflux transporter, heavy metal efflux pump, CzcA family, HAE1 family, Acriflavin resistance protein, nodulation protein NolG, Multidrug resistance protein MdtC, AcrB/AcrD/AcrF family protein	RND	-
21	AB01910	tnab1_kr121127p05q161	ABUW_0701	718(1215)	-	drug resistance transporter, Bcr/CflA subfamily	MFS	Phase variant
22	AB02233	tnab1_kr121128p04q109	ABUW_0825	615(1248)	-	major facilitator superfamily MFS_1/MFS_PMR	MFS	-

#	Item #	Strain Name	Ab Locus	Position in ORF (bp)	Gene Name	Putative function + Blast searches	Efflux type	Observations
24	AB02295	tnab1_kr130913p04q121	ABUW_0843	1711(3177)	*adeJ*	multidrug EFFLUX protein AdeJ	RND	Mucose (LB Tet)/Low growth
25	AB02468	tnab1_kr121205p08q107	ABUW_0910	411(1320)	*kgtP*	MFS permease	MFS	-
26	AB02501	tnab1_kr130913p05q152	ABUW_0922	1866(3096)	-	multidrug efflux RND transporter permease subunit, HAE1 family, multidrug resistance protein mexB (multidrug-efflux transporter mexB), inner membrane transporter CmeB, putative ATP synthase F0, A subunit, hydrophobe/amphiphile efflux-1 family protein, aminoglycoside/multidrug transporter subunit AcrD	RND	-
27	AB02621	tnab1_kr130916p05q144	ABUW_0966	425(1071)	-	multidrug ABC transporter permease	ABC	Phase variant
28	AB02656	tnab1_kr130909p02q143	ABUW_0982	421(1194)	-	permease/autoinducer transporter	Other	Phase variant
29	AB02711	tnab1_kr121203p06q152	ABUW_1003	463(1206)	-	MFS family drug transporter	MFS	-
30	AB02744	tnab1_kr121128p05q139	ABUW_1015	282(741)	*carO*	outer membrane protein CarO	OMP	
31	AB02915	tnab1_kr121210p02q192	ABUW_1078	709(1179)	-	major facilitator family transporter	MFS	-
32	AB03081	tnab1_kr121203p02q144	ABUW_1134	1011(2190)	-	major facilitator superfamily MFS_1/AmpG	MFS	Mucose
33	AB03226	tnab1_kr121204p08q194	ABUW_1184	955(1611)	-	ABC transporter, ATP-binding protein	ABC	
34	AB03340	tnab1_kr130913p08q180	ABUW_1232	679(1347)	-	MFS transporter, NNP family	MFS	-
35	AB03344	tnab1_kr121119p04q105	ABUW_1233	426(1119)	-	OmpW family protein	OMP	
36	AB03380	tnab1_kr121211p04q108	ABUW_1248	427(936)	-	ABC transporter ATP-binding protein	ABC	
37	AB03598	tnab1_kr130916p04q117	ABUW_1335	1668(3180)	-	EFFLUX transporter, RND family	RND	-
38	AB04058	tnab1_kr121127p06q145	ABUW_1523	434(1203)	-	transporter, major facilitator family	MFS	-
42	AB04074	tnab1_kr121128p06q118	ABUW_1529	647(1026)	-	ABC transporter, ATP-binding protein	ABC	Phase variant
43	AB04177	tnab1_kr121204p02q152	ABUW_1570	711(975)	-	aliphatic sulfonates family ABC transporter, periplasmic ligand-binding protein	ABC	
45	AB04230	tnab1_kr130903p01q162	ABUW_1589	495(1323)	-	major facilitator superfamily MFS_1	MFS	-
46	AB04245	tnab1_kr121127p07q108	ABUW_1596	486(1200)	-	MFS superfamily permease	MFS	-

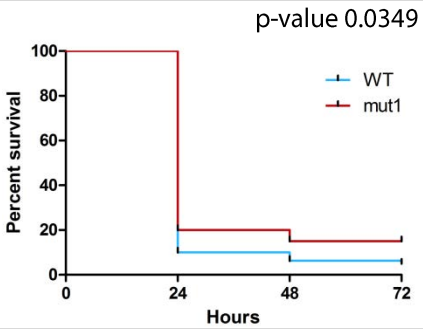
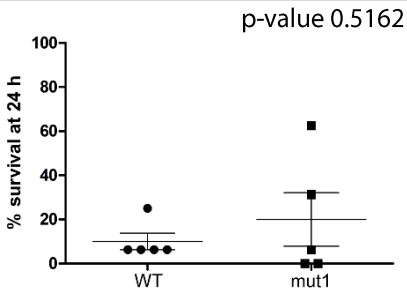
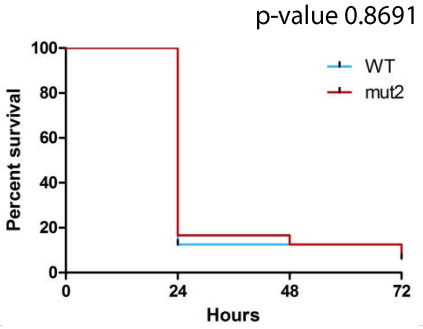
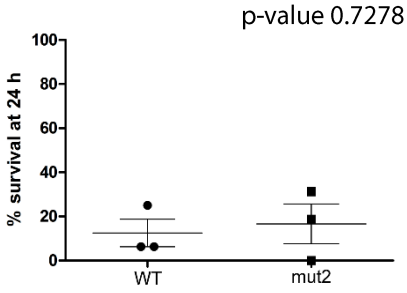
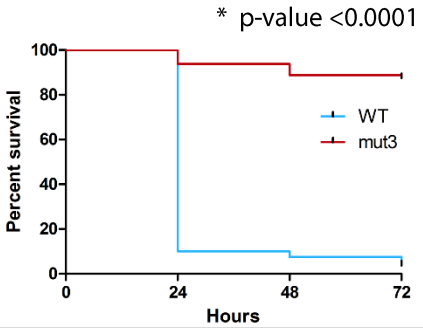
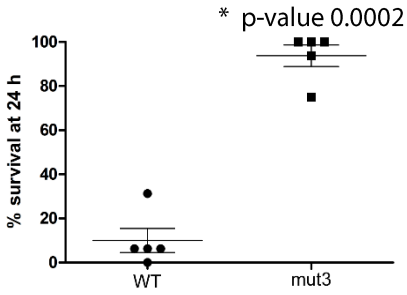
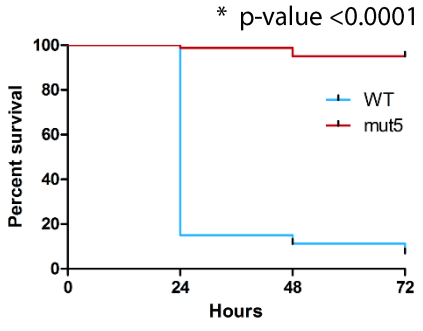
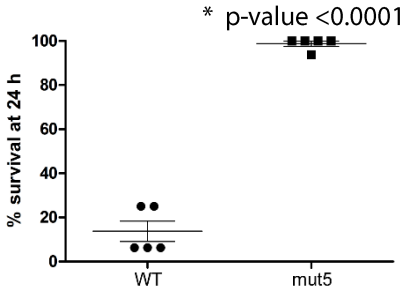
#	Item #	Strain Name	Ab Locus	Position in ORF (bp)	Gene Name	Putative function + Blast searches	Efflux type	Observations
47	AB04386	tnab1_kr121210p02q103	ABUW_1656	753(1113)	-	OmpW family protein	OMP	-
48	AB04394	tnab1_kr121119p03q187	ABUW_1660	1211(1584)	-	ABC transporter, ATP-binding protein	ABC	-
49	AB04481	tnab1_kr130916p01q141	ABUW_1694	545(1167)	-	metabolite transporter, MFS superfamily	MFS	-
50	AB04849	tnab1_kr130904p04q175	ABUW_1855	513(1350)	-	general substrate transporter:Major facilitator superfamily	MFS	Phase variant
51	AB05065	tnab1_kr130913p10q161	ABUW_1926	674(1344)	-	transporter, major facilitator family	MFS	-
52	AB05078	tnab1_kr121210p04q140	ABUW_1930	476(1086)	-	multidrug resistance EFFLUX pump/hemolysin/EmrA, HlyD family secretion protein, hemolysin D, efflux transporter, RND family, MFP subunit; auxiliary transport protein	RND	-
53	AB05082	tnab1_kr121212p01q111	ABUW_1931	864(1578)	-	transporter, major facilitator family	MFS	-
55	AB05142	tnab1_kr130917p02q128	ABUW_1949	515(1062)	-	RND family drug transporter/hemolysin/EmrA	RND	-
56	AB05156	tnab1_kr121127p02q160	ABUW_1953	626(1329)	-	outer membrane EFFLUX protein/TolC	OMP	
57	AB05228	tnab1_kr121210p02q150	ABUW_1975	1030(3108)	*adeB*	multidrug EFFLUX protein AdeB	RND	-
58	AB05252	tnab1_kr130917p01q120	ABUW_1982	387(1260)	-	outer membrane protein E	OMP	
59	AB05504	tnab1_kr121210p03q167	ABUW_2109	258(840)	-	aliphatic sulfonates import ATP-binding protein SsuB, sulfonate ABC transporter ATP-binding lipoprotein; ATP-binding protein, Taurine import ATP-binding protein TauB; putative transport protein (ABC superfamily, atp_bind); putative nitrate ABC transporter, ATP-binding protein	ABC	-
60	AB05735	tnab1_kr130916p04q115	ABUW_2187	509(1440)	-	transporter, major facilitator family	MFS	-
61	AB05825	tnab1_kr121204p06q112	ABUW_2223	289(939)	-	ABC-type dipeptide/oligopeptide/nickel transport system permease component	ABC	
62	AB05975	tnab1_kr121203p02q184	ABUW_2278	122(681)	-	permease; multidrug efflux RND transporter permease subunit; Putative RND family drug transporter; Multidrug resistance protein mexB (Multidrug-efflux transporter mexB); HAE1 family; inner membrane transporter CmeB, putative ATP synthase F0, A subunit	RND	-
63	AB06254	tnab1_kr121127p07q180	ABUW_2377	625(1470)	-	permease; putative permease; nitrate reductase; Permease for cytosine/purines, uracil, thiamine, allantoin family protein, NCS1 nucleoside transporter family protein; putative allantoin permease; Hydantoin permease	Other	-

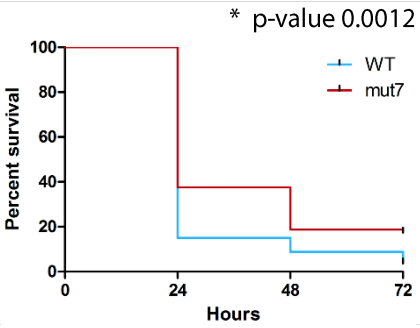
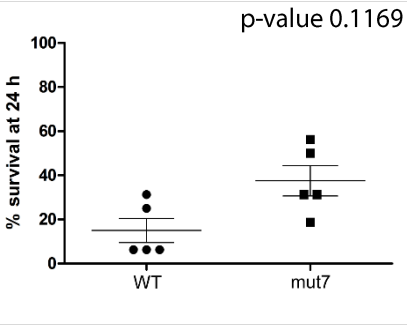
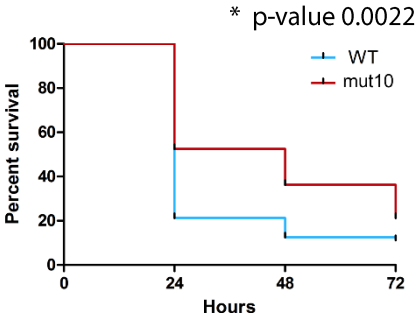
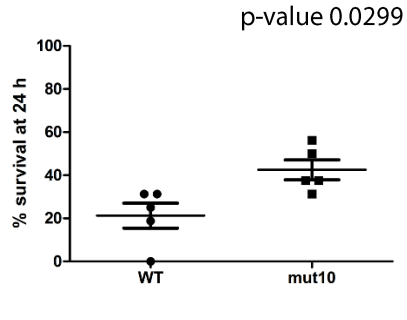
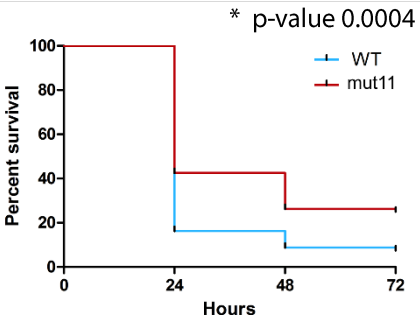
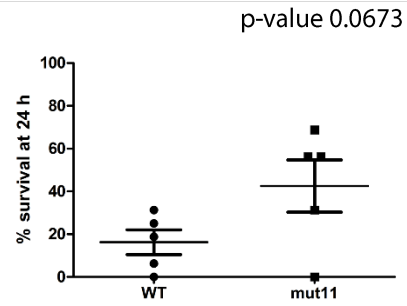
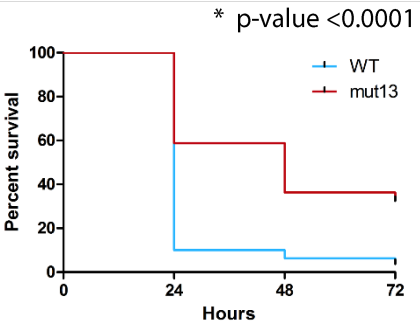
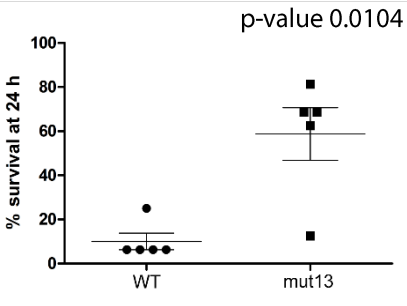
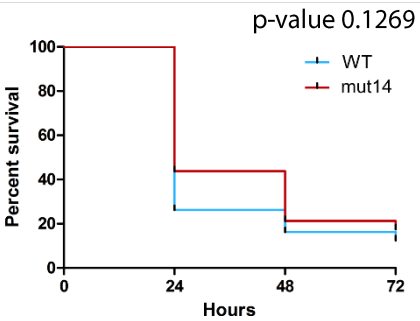
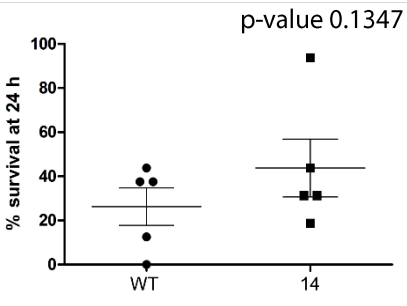
#	Item #	Strain Name	Ab Locus	Position in ORF (bp)	Gene Name	Putative function + Blast searches	Efflux type	Observations
64	AB06328	tnab1_kr121203p06q159	ABUW_2403	769(1188)	-	MFS permease	MFS	-
66	AB06490	tnab1_kr121204p06q115	ABUW_2462	436(1218)	-	major facilitator superfamily MFS_1	MFS	-
67	AB06527	tnab1_kr140806p01q151	ABUW_2473	136(1170)	-	major facilitator superfamily MFS_1	MFS	-
68	AB06557	tnab1_kr130916p06q130	ABUW_2483	714(1710)	-	ABC transporter, permease	ABC	
69	AB06623	tnab1_kr130917p06q176	ABUW_2505	534(1317)	-	major facilitator superfamily MFS_1	MFS	Phase variant
71	AB06771	tnab1_kr121127p03q125	ABUW_2571	284(768)	-	OmpA/MotB domain protein	OMP	No growth on LB Tet plates
72	AB06816	tnab1_kr121128p04q142	ABUW_2585	758(1023)	-	ABC transport system substrate-binding protein	ABC	-
74	AB06960	tnab1_kr121211p04q113	ABUW_2643	923(2142)	-	protein secretion ABC EFFLUX system, permease and ATP-binding protein	ABC	-
75	AB06964	tnab1_kr121204p07q153	ABUW_2644	478(1467)	-	putative RND family drug transporter/ABC-transporter?; type I secretion OMP, TolC family; outer membrane efflux family protein; putative RND family drug transporter, Type I secretion system, outer membrane component LapE	RND/ABC/ OMP	-
76	AB07029	tnab1_kr130917p01q175	ABUW_2683	492(1215)	-	transporter, major facilitator family	MFS	-
77	AB07105	tnab1_kr121205p02q156	ABUW_2715	721(1359)	*benK*	major facilitator superfamily MFS_1	MFS	-
78	AB07143	tnab1_kr121210p01q145	ABUW_2730	326(1416)	-	OmpA/MotB	OMP	-
79	AB07248	tnab1_kr121128p05q148	ABUW_2771	632(1281)	-	porin	OMP	-
81	AB07431	tnab1_kr121128p01q196	ABUW_2838	495(1053)	-	peptide ABC transporter, permease protein	ABC	-
82	AB07615	tnab1_kr121203p01q161	ABUW_2910	1138(2460)	-	EFFLUX ABC transporter, permease protein	ABC	-
83	AB07674	tnab1_kr121204p06q160	ABUW_2929	598(1329)	-	major facilitator superfamily MFS_1	MFS	-
84	AB07704	tnab1_kr130917p10q113	ABUW_2939	593(1299)	-	major facilitator family transporter	MFS	-
85	AB07727	tnab1_kr121128p08q125	ABUW_2947	544(1224)	-	major facilitator superfamily MFS_1	MFS	-
87	AB07857	tnab1_kr121203p02q189	ABUW_2990	517(1386)	-	major facilitator superfamily MFS_1	MFS	-
88	AB07942	tnab1_kr121210p03q154	ABUW_3020	640(1152)	*emrA*	RND family multidrug resistance secretion protein, TWITCHING	RND	-
90	AB08175	tnab1_kr130917p08q173	ABUW_3124	536(1248)	-	major facilitator superfamily MFS_1	MFS	-
91	AB08251	tnab1_kr130917p03q193	ABUW_3152	766(1644)	-	major facilitator superfamily	MFS	Phase variant

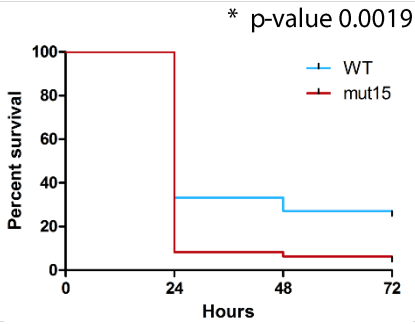
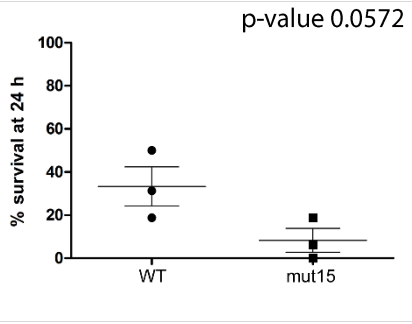
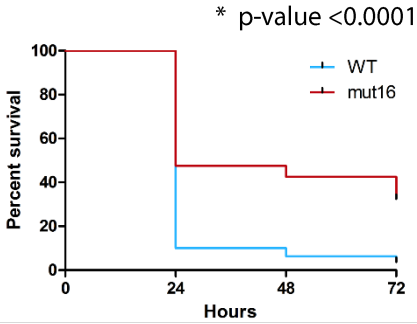
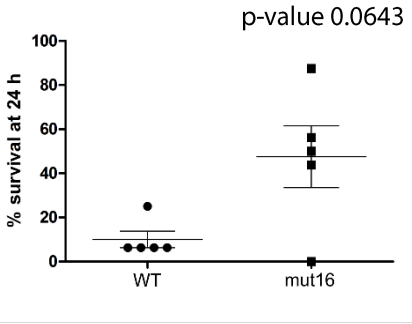
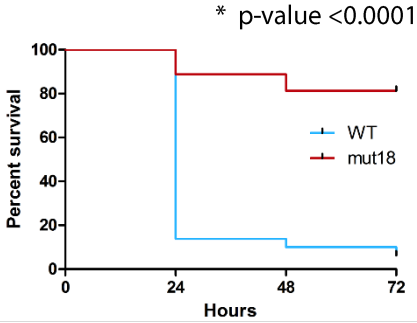
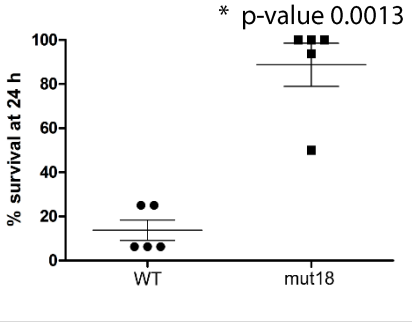
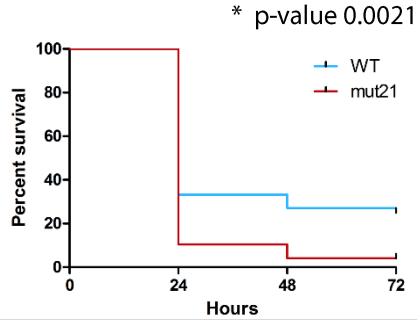
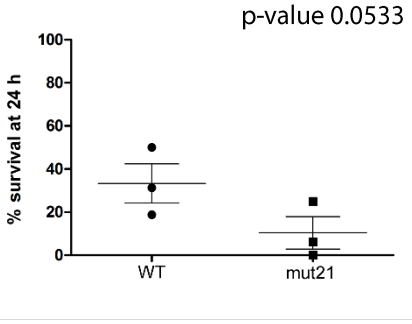
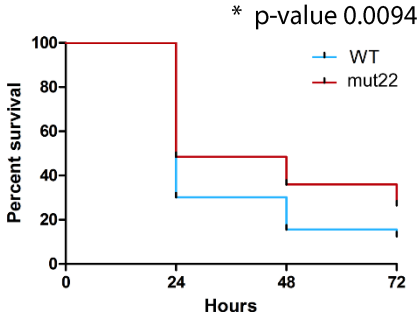
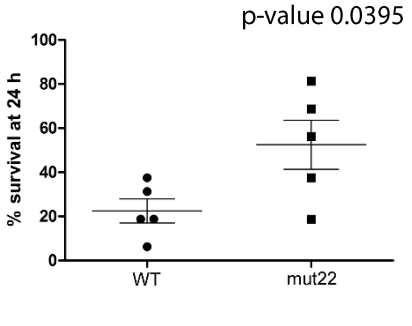
#	Item #	Strain Name	Ab Locus	Position in ORF (bp)	Gene Name	Putative function + Blast searches	Efflux type	Observations
93	AB08557	tnab1_kr130913p04q137	ABUW_3286	583(1251)	-	major facilitator superfamily MFS_1	MFS	-
94	AB08647	tnab1_kr130916p05q173	ABUW_3317	455(1185)	-	major facilitator superfamily MFS_1	MFS	Phase variant
96	AB08797	tnab1_kr130913p07q179	ABUW_3363	852(1995)	*macB*	ABC transporter family protein, macrolide export ATP-binding/permease protein MacB, macrolide ABC transporter permease/ATP-binding protein MacB; macrolide transport protein (ABC superfamily, atp_bind (N-terminal), membrane (C-terminal)) ; MacB-like periplasmic core domain protein	ABC	-
97	AB09043	tnab1_kr121212p01q192	ABUW_3446	205(633)	-	RhtB family transporter; LysE family translocator; lysE type translocator family protein;	Other	-
98	AB09160	tnab1_kr121210p03q184	ABUW_3486	320(1347)	*abeM*	multidrug EFFLUX pump	MATE	-
99	AB09431	tnab1_kr121128p05q132	ABUW_3583	328(582)	-	putative outer membrane protein W	OMP	-
100	AB09515	tnab1_kr130916p02q102	ABUW_3620	796(1332)	*tolC*	type I secretion OMP; RND transporter; OMP tolC precursor; type I secretion outer membrane protein, TolC family; channel-tunnel spanning the outer membrane; putative RND family drug transporter; channel-tunnel spanning the outer membrane and periplasm segregation of daughter chromosomes;	RND/OMP	-
103	AB09723	tnab1_kr121213p04q156	ABUW_3691	659(1320)	-	major facilitator superfamily MFS_1	MFS	-
104	AB09814	tnab1_kr121212p01q101	ABUW_3725	435(921)	-	transporter, drug/metabolite exporter family, EamA/RhaT family transporter; putative membrane protein ; EamA-like transporter family protein; Permease of the drug/metabolite transporter (DMT) superfamily; DMT family permease	Other	-
105	AB09822	tnab1_kr121203p07q167	ABUW_3727	494(1215)	-	major facilitator superfamily MFS_1	MFS	-
107	AB09863	tnab1_kr121210p04q125	ABUW_3744	298(627)	*rhtB*	homoserine/homoserine lactone EFFLUX protein	QS	Phase variant
110	AB09951	tnab1_kr121204p06q185	ABUW_3776	218(552)	*cepI*	homoserine lactone synthase	QS	-

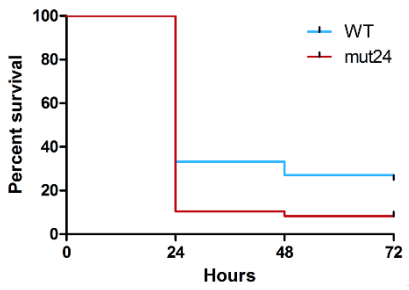
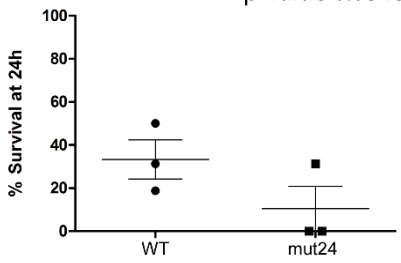
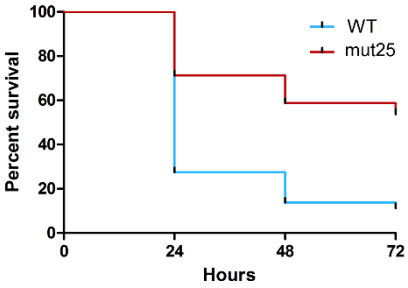
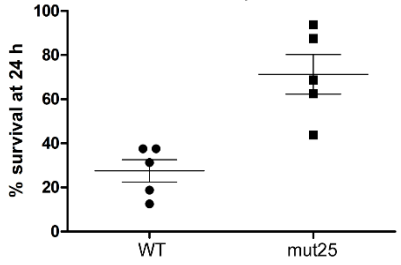
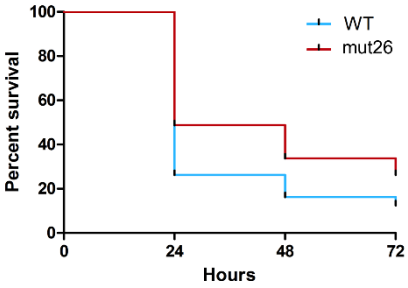
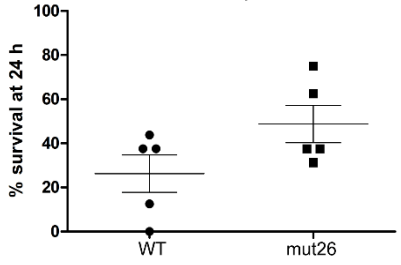
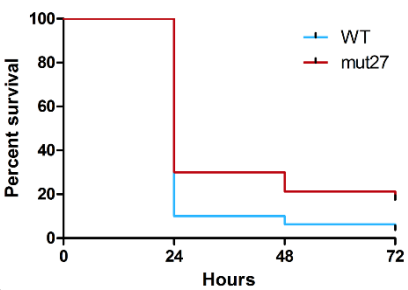
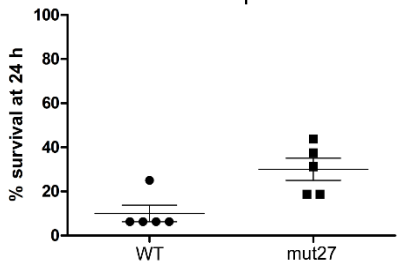
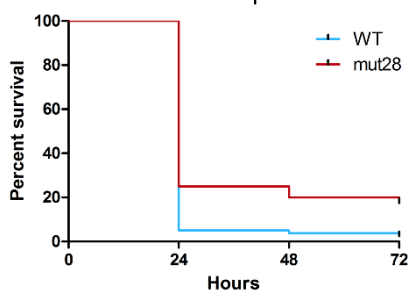
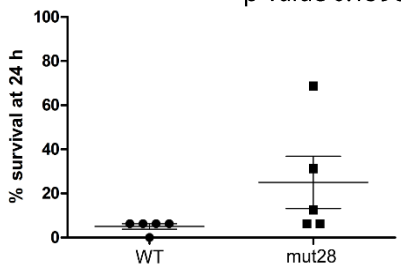
Annex III

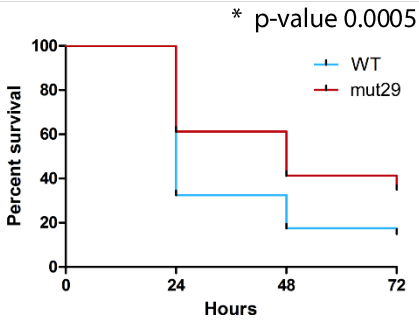
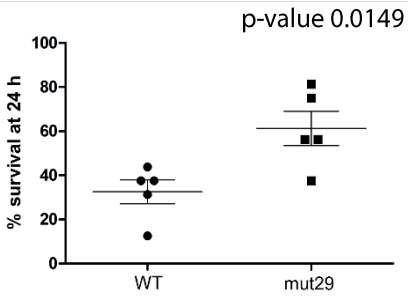
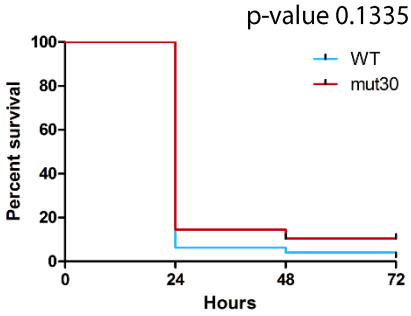
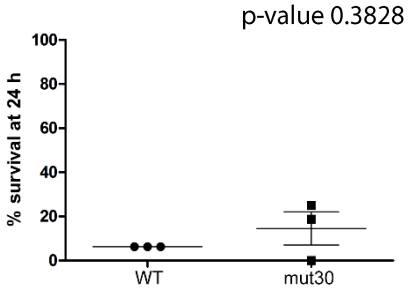
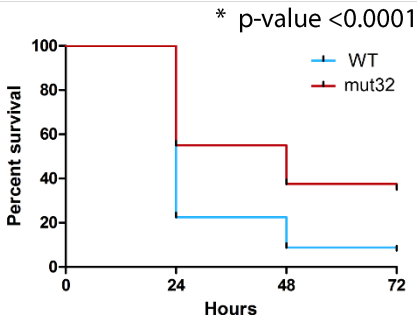
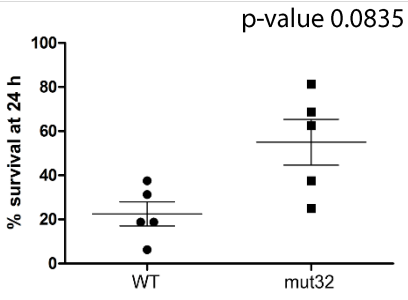
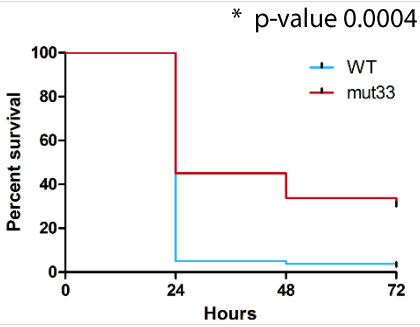
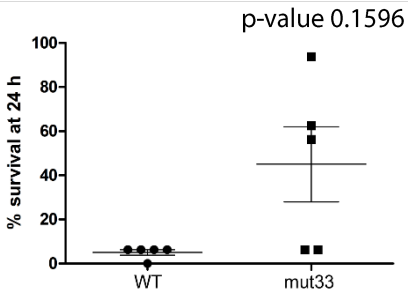
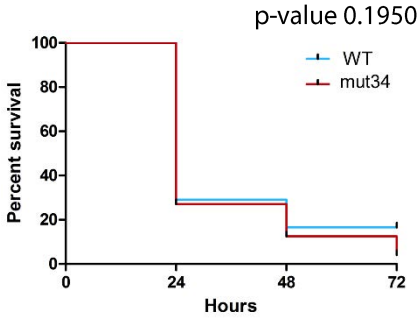
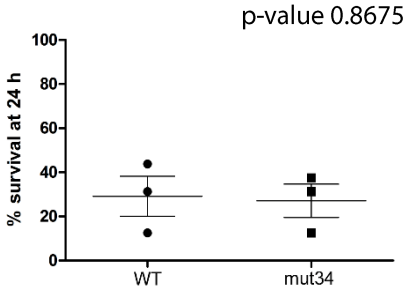
Virulence analyses of the mutant strains in the *Galleria mellonella* infection model

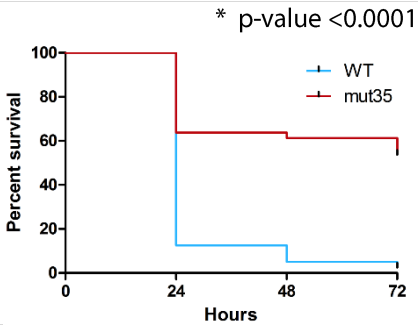
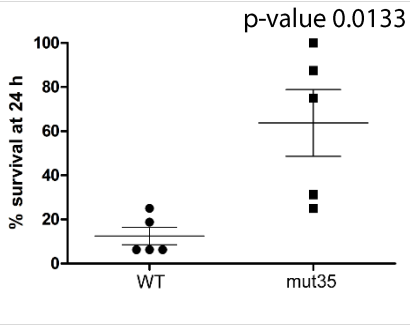
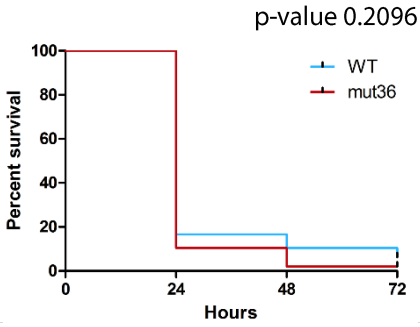
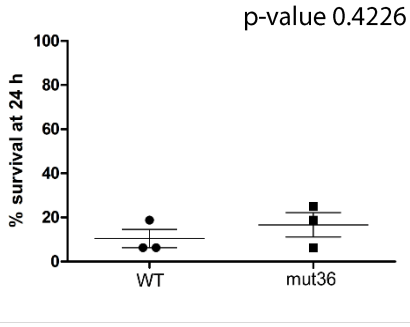
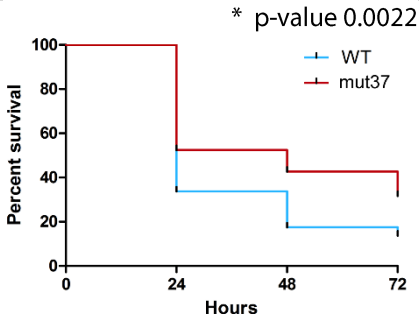
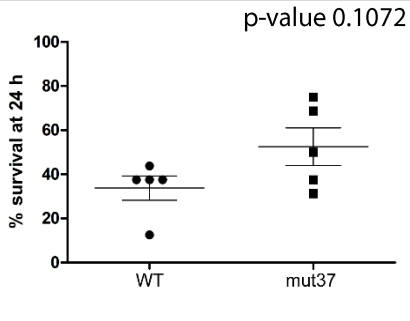
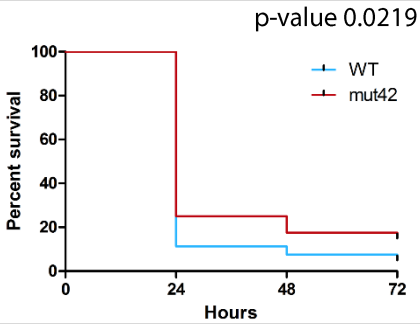
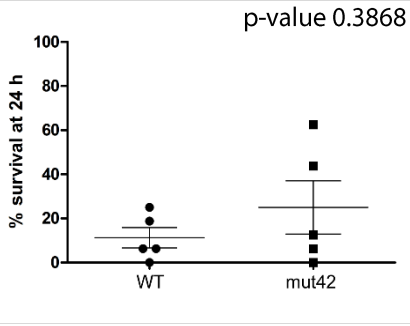
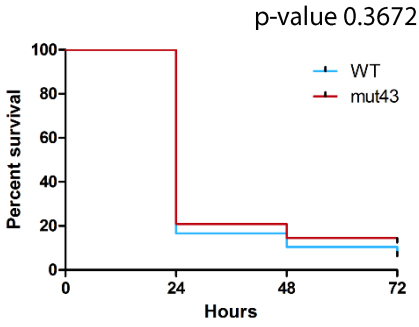
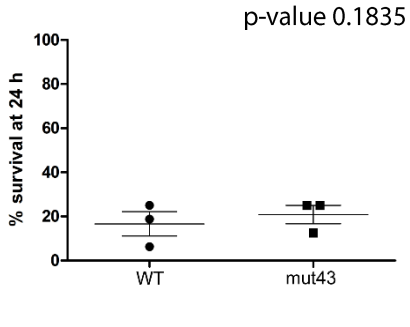
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut1 ABC		
Mut2 RND (ArpB)		
Mut3 ** MATE		
Mut5 ** MATE		

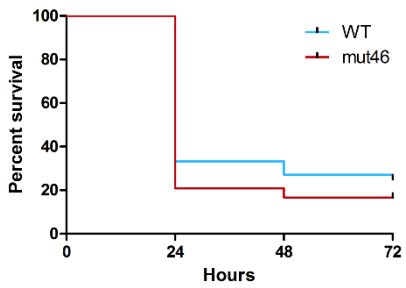
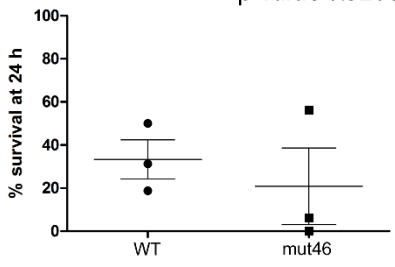
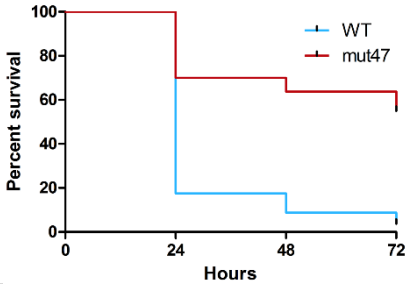
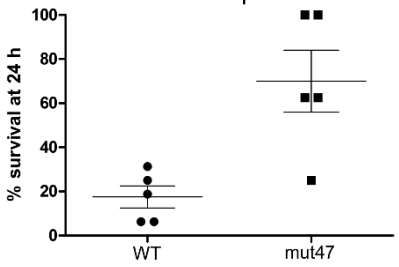
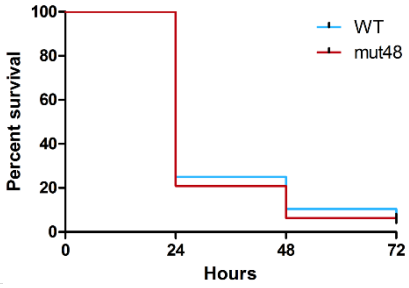
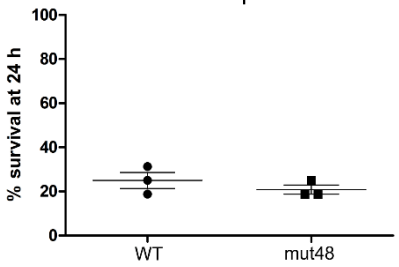
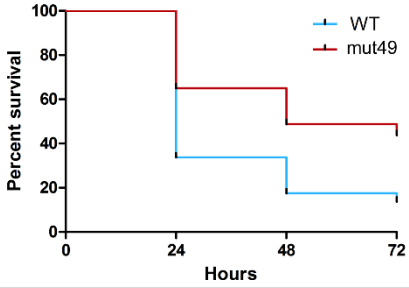
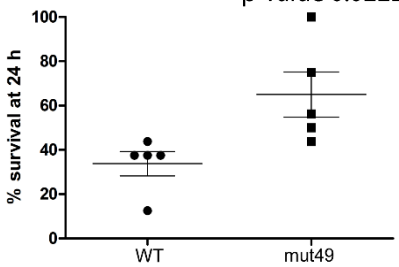
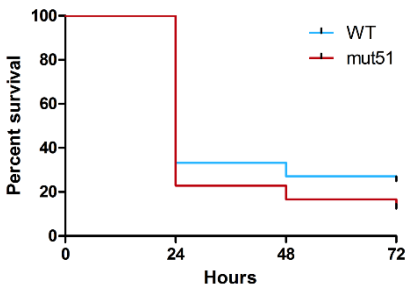
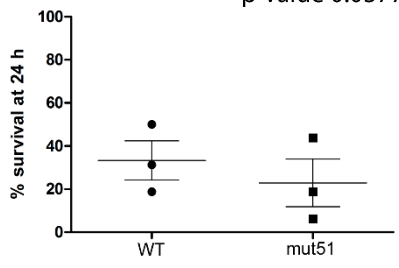
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut7 OMP		
Mut10 MFS		
Mut11 MFS		
Mut13 ABC		
Mut14 Other transport re-lated proteins		

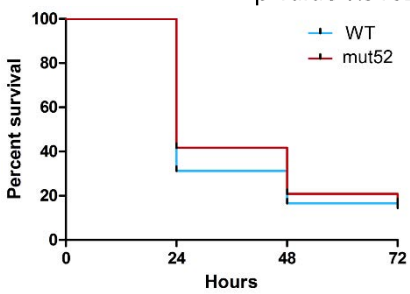
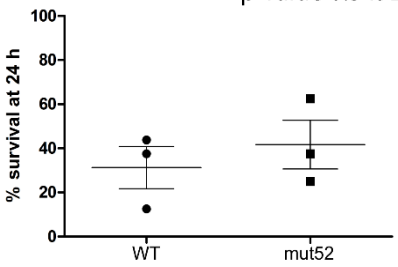
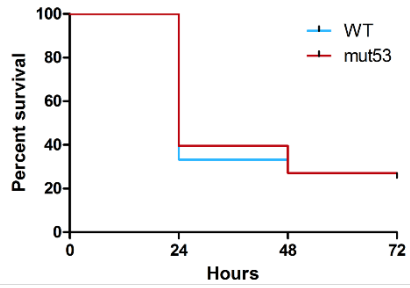
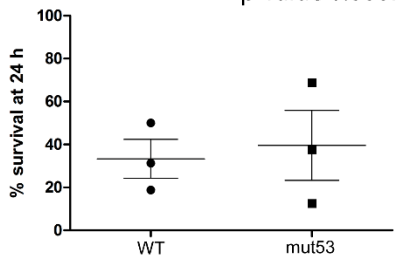
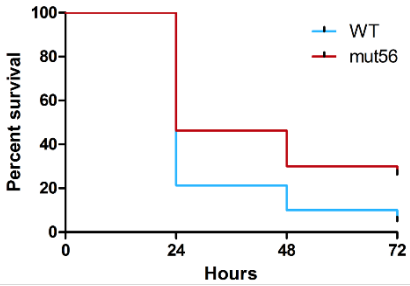
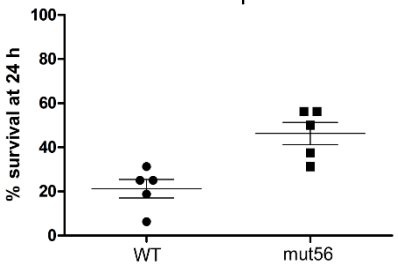
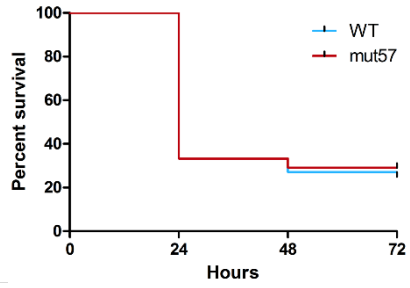
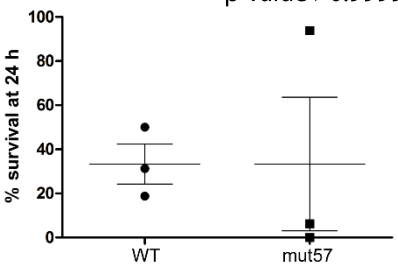
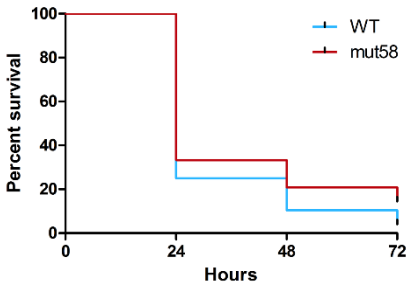
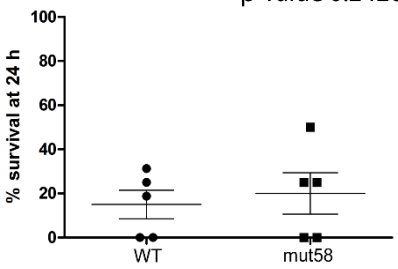
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut15 MFS		
Mut16 OMP		
Mut18 ** OMP (OmpA-like)		
Mut21 MFS		
Mut22 MFS		

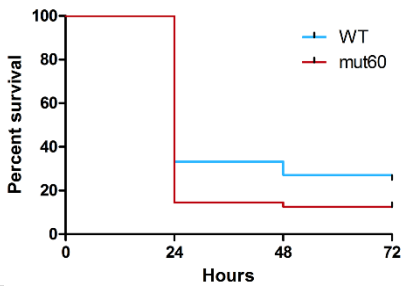
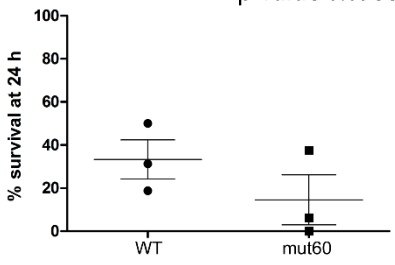
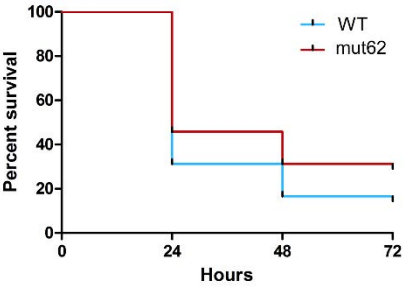
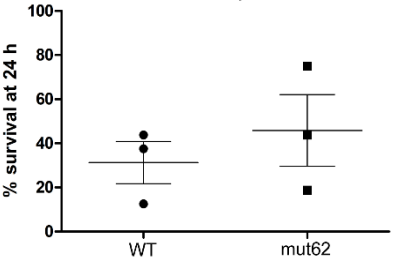
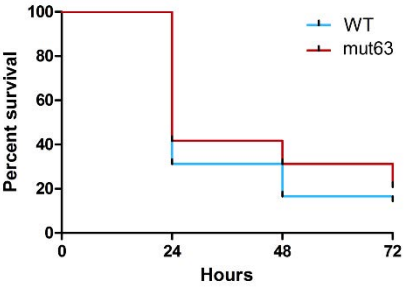
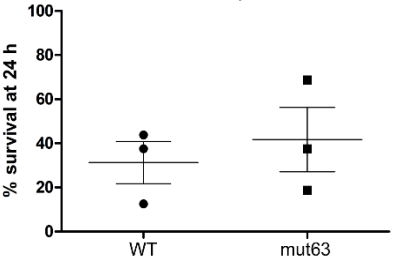
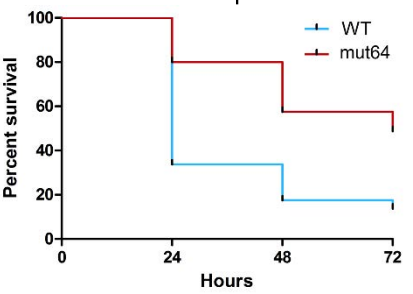
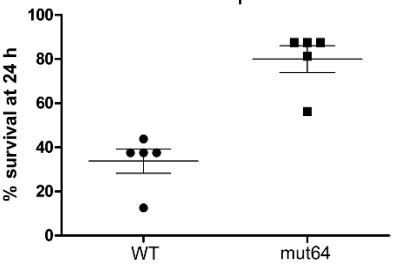
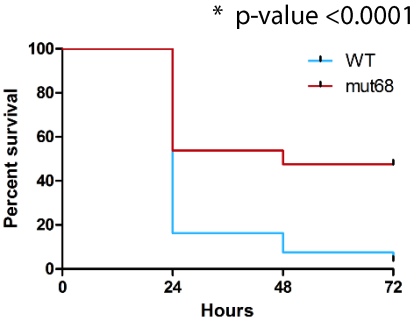
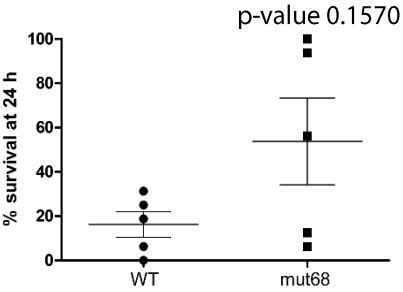
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut24 RND (AdeJ)	p-value 0.0169 	p-value 0.0315 
Mut25 MFS	* p-value <0.0001 	p-value 0.0147 
Mut26 RND	* p-value 0.0073 	p-value 0.0491 
Mut27 ABC	* p-value 0.0013 	p-value 0.0299 
Mut28 Other transport re-lated proteins	* p-value 0.0004 	p-value 0.1596 

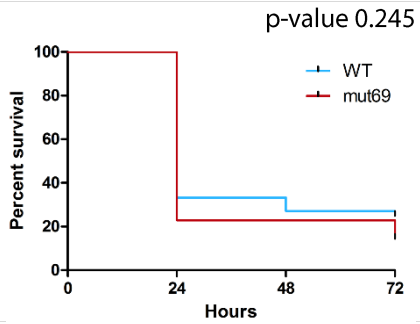
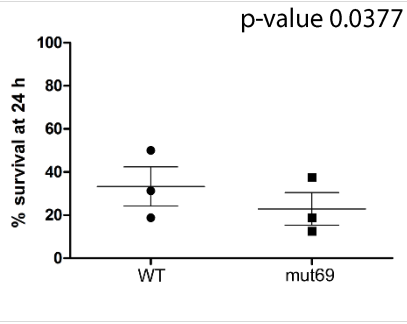
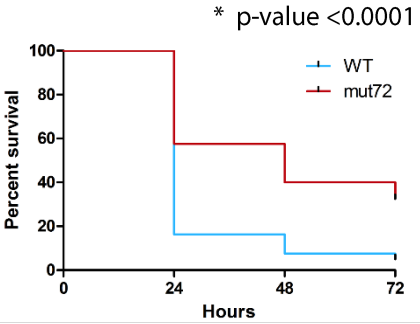
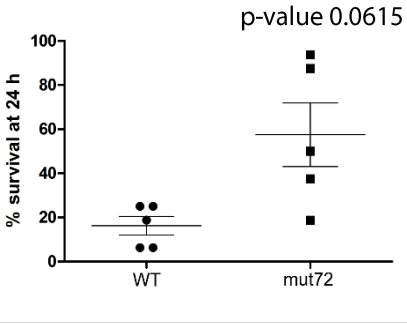
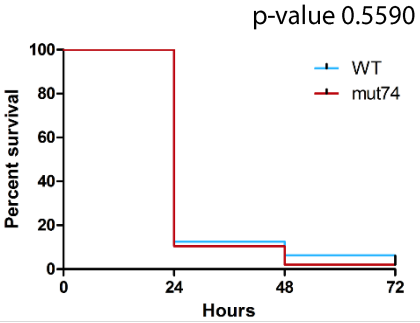
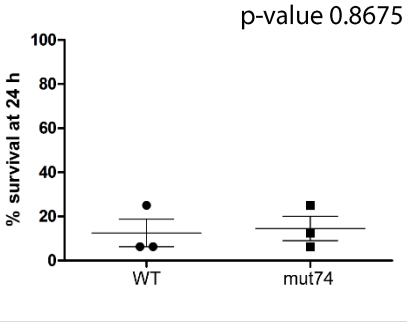
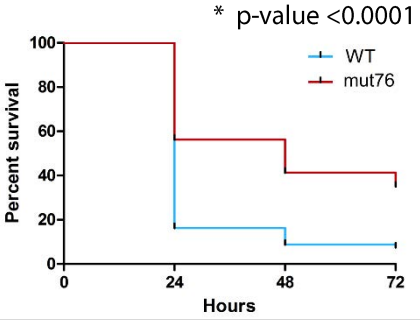
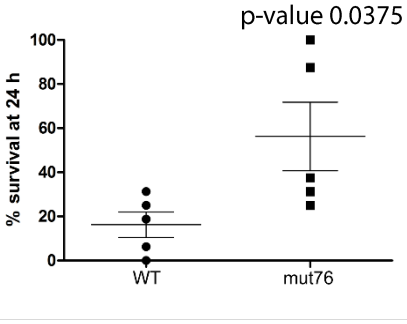
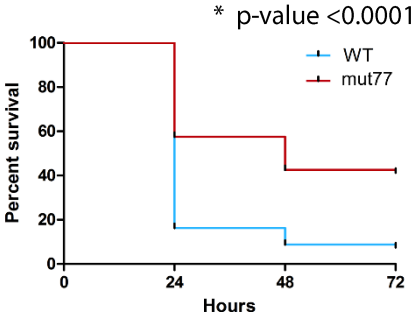
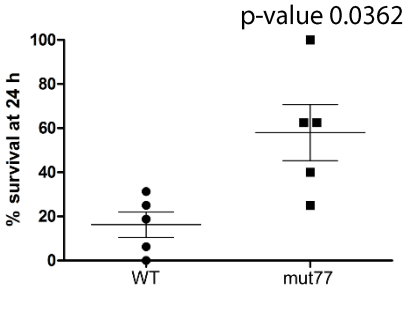
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut29 MFS		
Mut30 OMP		
Mut32 MFS		
Mut33 ABC		
Mut34 MFS		

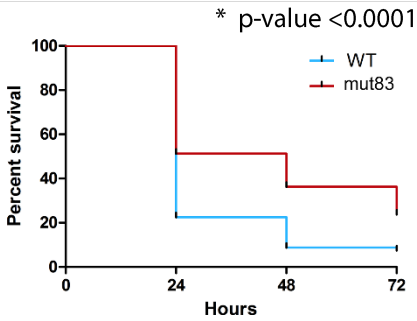
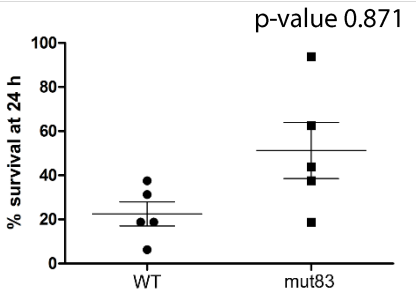
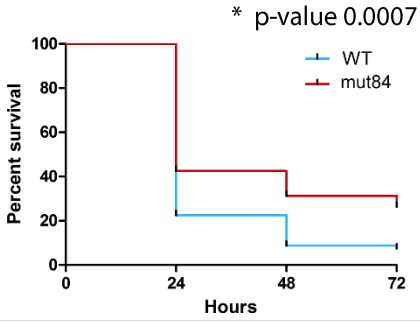
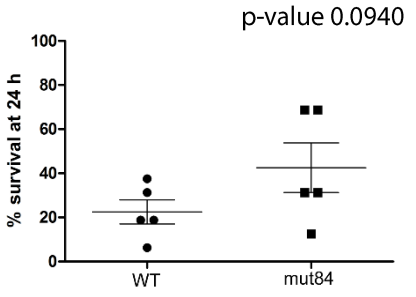
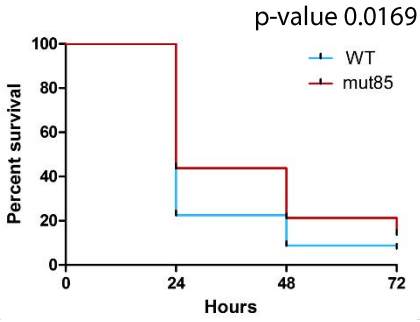
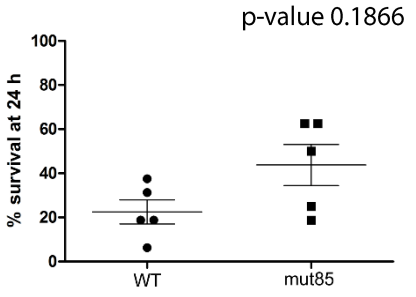
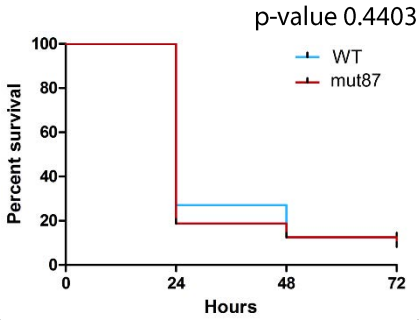
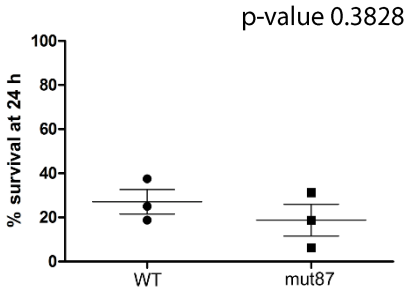
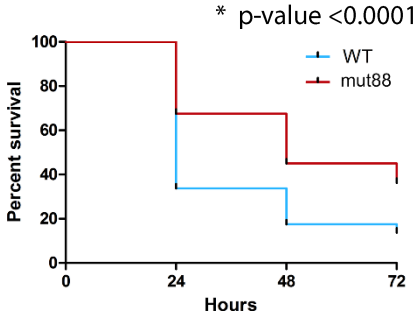
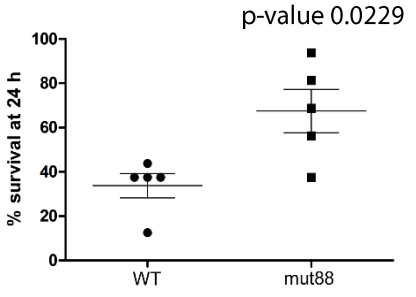
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut35 OMP		
Mut36 ABC		
Mut37 RND		
Mut42 ABC		
Mut43 ABC		

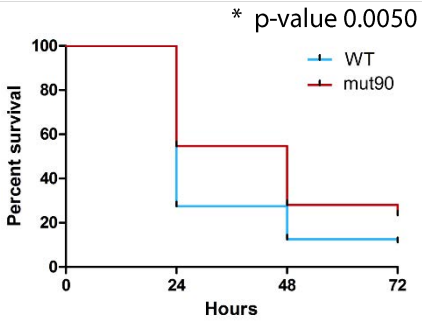
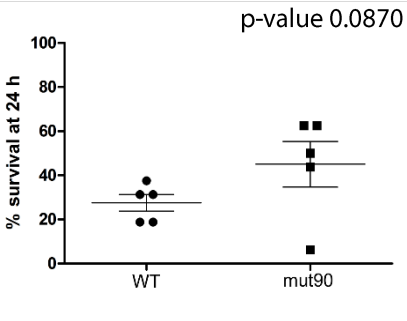
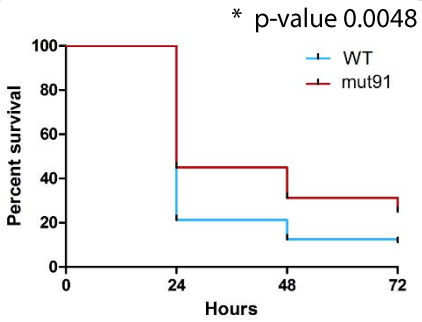
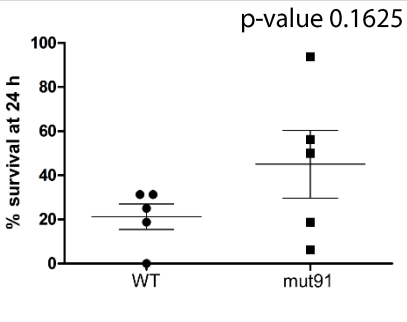
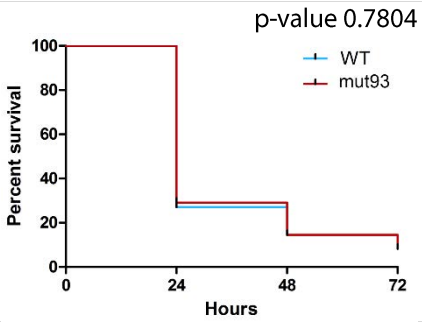
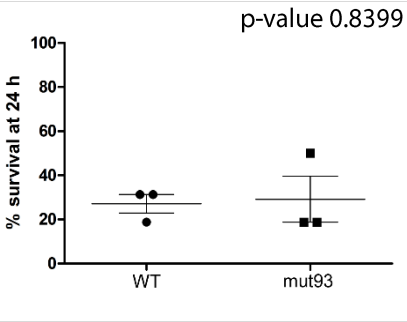
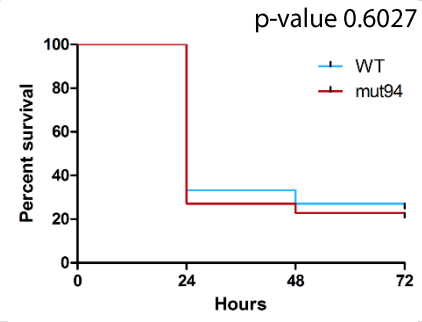
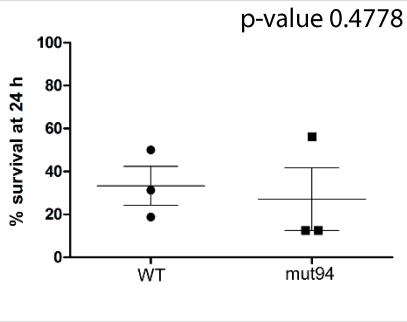
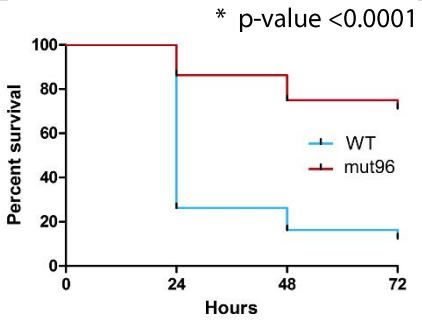
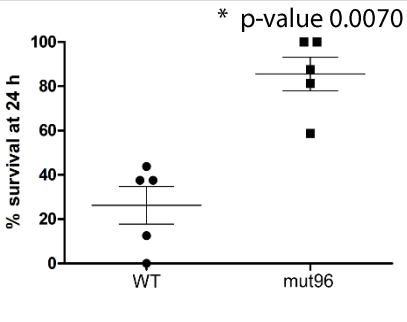
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut46 MFS	p-value 0.2718 	p-value 0.3206 
Mut47 OMP	* p-value <0.0001 	p-value 0.0277 
Mut48 ABC	p-value 0.8361 	p-value 0.1835 
Mut49 MFS	p-value <0.0001 	p-value 0.0222 
Mut51 MFS	p-value 0.1309 	p-value 0.0377 

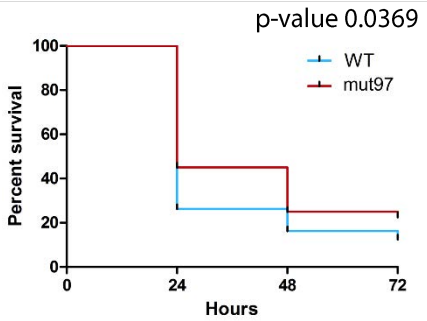
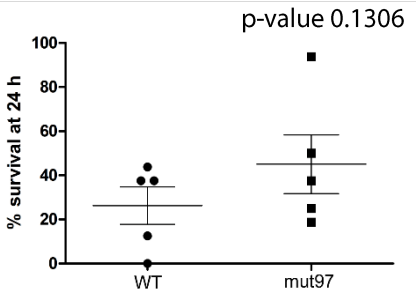
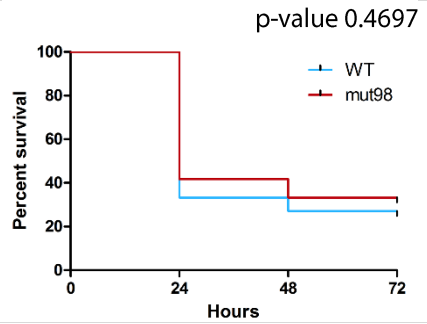
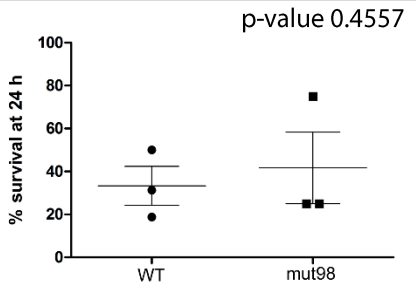
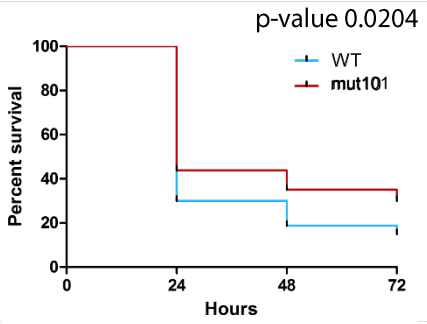
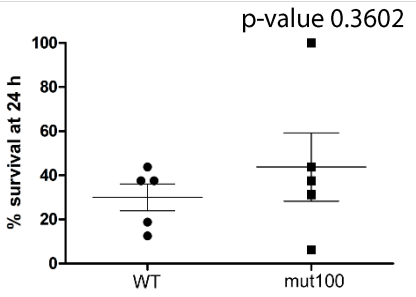
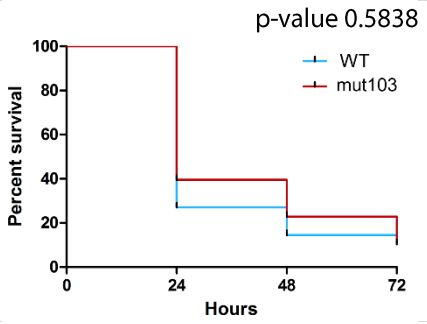
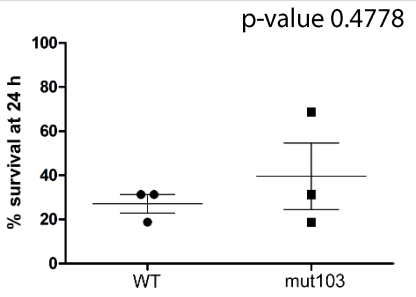
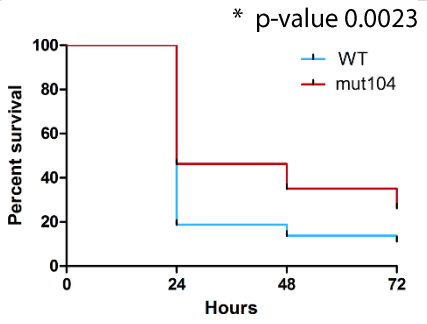
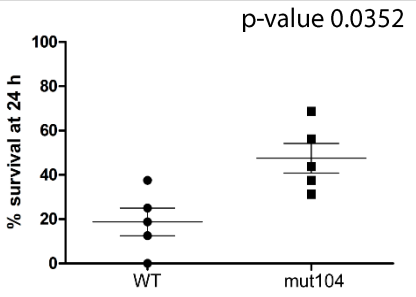
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut52 RND	p-value 0.5182 	p-value 0.5492 
Mut53 MFS	p-value 0.8902 	p-value 0.6667 
Mut56 ** OMP	* p-value <0.0001 	* p-value 0.0002 
Mut57 RND (AdeB)	p-value 0.6946 	p-value >0.9999 
Mut58 OMP	p-value 0.1090 	p-value 0.2420 

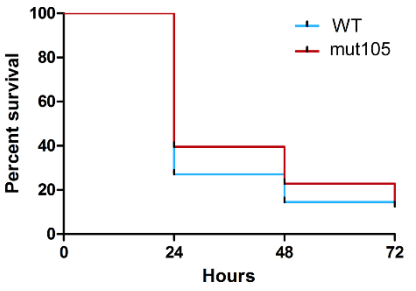
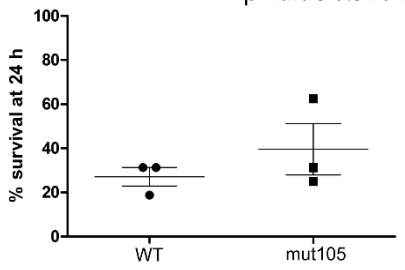
	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut60 MFS	p-value 0.0868 	p-value 0.0955 
Mut62 RND	p-value 0.0756 	p-value 0.3356 
Mut63 Other transport re-lated proteins	p-value 0.2664 	p-value 0.4444 
Mut64 ** MFS	* p-value <0.0001 	* p-value 0.0098 
Mut68 ABC	* p-value <0.0001 	p-value 0.1570 

	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut69 MFS		
Mut72 ABC		
Mut74 ABC		
Mut76 MFS		
Mut77 MFS		

	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut83 MFS		
Mut84 MFS		
Mut85 MFS		
Mut87 MFS		
Mut88 RND		

	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut90 MFS		
Mut91 MFS		
Mut93 MFS		
Mut94 MFS		
Mut96 ** ABC		

	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut97 Other transport re-lated proteins		
Mut98 MATE (AbeM)		
Mut100 RND		
Mut103 MFS		
Mut104 Other transport re-lated proteins		

	Kaplan-Meier curves – Log-Rank (Mantel-Cox) test	Comparison of the percentage of survival at 24 h – Paired t test
Mut105 MFS	p-value 0.4936 	p-value 0.5101 

Annex IV

Restriction sites evaluated for complementation primers

TABLE 1. Complementation strategy used for the mutants selected for complementation, presence and chosen restriction sites according to the strategy. F, forward primer; R, reverse primer.

#	Locus	Strategy	ApaI	SacII	SalI	SpeI	HindIII	StuI	PstI	BstXI	NsiI	Restriction site used F	Restriction site used R
5	ABUW_0109	Normal	NO	NO	NO	NO			YES			SpeI	SpeI
18	ABUW_0649	Normal	NO	NO	NO	NO			YES			SpeI	SpeI
64	ABUW_2403	Normal			NO	NO			NO			SpeI	SpeI
3	ABUW_0064	Operon					YES	NO	YES	NO	NO	StuI	NsiI
28	ABUW_0982	Operon					YES	NO	YES	YES	NO	StuI	NsiI
56	ABUW_1953	Operon					NO	NO	NO	NO	YES	HindIII	PstI
72	ABUW_2585	Operon					NO	NO	NO	NO	NO	HindIII	PstI
96	ABUW_3363	Operon					YES	NO	NO*	YES	YES	StuI	PstI

*This restriction site is located close to the transcription initiation codon ATG

TABLE 2. Characteristics of the features added in the 5' end of the complementation primers.

Feature	Sequence	Type of ends		
SpeI	ACTAGT	5'-A	CTAGT-3'	3' protuding
HindIII	AAGCTT	5'-A	AGCTT-3'	3' protuding
StuI	AGGCCT	5'-AGG	CCT-3'	blunt
PstI	CTGCAG	5'-CTGCA	G-3'	5' protuding
NsiI	ATGCAT	5'-ATGCA	T-3'	5' protuding
Shine-Dalgarno (SD)	AAGGAG	-		-

X. Acknowledgements

> La vida es una unión simbiótica y cooperativa
> que permite triunfar a los que se asocian."
>
> **Lynn Margulis**

Indudablemente, no habría llegado hasta aquí sin el apoyo y ayuda de muchas personas que han hecho que todo el esfuerzo de estos últimos años culmine en esta tesis, la cual firmo a mi nombre, pero contiene pedacitos de todos los que me habéis acompañado estos años y me habéis hecho crecer profesionalmente, pero sobretodo a nivel personal.

Jordi e Ignasi, agradeceros haber sido mi guía durante este periodo. No os imagináis lo afortunada que me siento de haberos tenido como directores. Jordi, por estar ahí cuando se te necesita, a pesar de lo liado que vas siempre… tu tranquilidad y *savoir faire* hace que todo parezca más fácil y asequible. Gracias también por ponerme bajo la tutela de Ignasi. Qué dirte, Ignasi… se'm queden curtes les paraules. Echaré de menos nuestras charlas, discutirme contigo y que me trates como a una igual, aunque me des mil vueltas. Me has enseñado tanto… y me has tenido que aguantar tantas otras veces… que la palabra director se queda corta.

Si he tenido suerte con mis directores, ya ni os digo con el grupo. Trabajar en un ambiente así es impagable, y sois, sin duda, lo mejor que me llevo. Marta, no podria haver tingut una millor companya al Acineto team que tu, la teva generositat no té límits, ni tampoc la teva dedicació… a la feina, al bàsquet, però sobretot als amics, gràcies per entendre'm tan bé… Segueix així, sé que arribarás lluny i que tindré la sort de veure-ho de ben a prop. Las vacas no llegaron a engordar nunca lo suficiente, pero las risas que compartimos las tres juntas no nos las quitará nadie, porque aunque no estés en el lab, Anna Elena, tu paso dejó huella y agradezco haberte tenido de "alumna" y haber hecho tantos CHEFs de las provis contigo, pues ello ha permitido que formes parte de mi vida. Por muchas más quedadas *mensuales* de las tres Marías…

Eli, gràcies per aquests anys de consells, i per haver-me acompanyat en aquest tram final demostrant un cop més que sempre estàs disposada a donar un cop de mà. Lauri, gracias por acogerme como a una más desde el primer día, por preocuparte por mi (y por todos) y por tu derroche de alegría. Claretaaa! Ets un model a seguir, sempre alegre, enérgica, treballadora… i amb temps per passar-ho bé! Amb tu tot s'omple d'energia positiva. Javi y Andrea, habéis llegado más tarde pero me habéis robado un trocito de corazón… Javi, que

nuestro mundo freaky nos mantenga en contacto por mucho tiempo. Andrea, me encanta el buen rollo que transmites, ojalá hubiésemos coincidido en el lab más tiempo, pero me llevo Viena como recuerdo ;).

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GRACIAS

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