DOCTORAL SCHOOL IN BIOLOGY

Biology applied to Human Health

XXVIII DOCTORAL PROGRAM

New insights into the virulence potential of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii has emerged as an important opportunistic nosocomial pathogen which can cause a wide range of infections among patients in intensive care and burns units. Species of the genus *Acinetobacter* colonise a wide range of habitats, but the infection reservoir(s) of *A. baumannii* outside the hospital is(are) yet unknown. First, we addressed the question of whether the colonization of different environments and/or ecological niches has led to a differentiation of multiple intra-specific *A. baumannii* populations with diversified phenotypic traits, or whether *A. baumannii* is a phenotypically homogeneous species endowed with broad adaptability. To address this point, the genotypic and phenotypic diversity of a wide collection of *A. baumannii* strains isolated from environmental, veterinary and human clinical sources was evaluated.

Given the crucial role of iron for *A. baumannii* growth and infectivity, in the second part of the thesis iron metabolism has been assessed as a possible target for chelation-based antibacterial chemotherapy. The effect of iron restriction on *A. baumannii* growth and biofilm formation using different iron chelators and culture conditions was determined. Finally, in the third part of this thesis the contribution of individual iron uptake systems to survival and pathogenicity of *A. baumannii* in the human host was investigated. To this purpose, single and multiple knock-outs of *A. baumannii* genes implicated in iron uptake were generated. The iron uptake mutants were compared with the wild type strain for growth capability under different conditions of iron availability. To assess the uptake specificity of the mutated systems, growth promotion assays were performed in the presence of exogenously added iron sources. Lastly, the pathogenicity of the iron uptake mutants was also performed in the *Galleria mellonella* insect model of infection.

Riassunto

Acinetobacter baumannii è emerso come un importante patogeno opportunista in ambito nosocomiale, capace di provocare una vasta gamma di infezioni in pazienti critici ricoverati nelle unità per terapia intensiva o per grandi ustionati. Le specie del genere *Acinetobacter* colonizzano una vasta gamma di habitat, ma il serbatoio di infezione di *A. baumannii* è ancora sconosciuto. Innanzitutto, abbiamo affrontato il quesito se la colonizzazione di diverse nicchie abbia portato ad una differenziazione di popolazioni multiple intra-specifiche dotate di diversi tratti fenotipici, o se *A. baumannii* sia una specie fenotipicamente omogenea capace di adattarsi a diversi ambienti. A tal fine è stata studiata la diversità genotipica e fenotipica di una vasta collezione di ceppi di *A. baumannii* isolati di diverse origini, in particolare l'ambiente naturale, animali e pazienti infetti.

In seguito, considerato il ruolo cruciale del ferro per la crescita e la patogenicità di *A. baumannii*, sono stati condotti degli studi mirati a verificare se il metabolismo del ferro possa costituire un bersaglio per la chemioterapia antibatterica basata sull'impiego di chelanti già ad uso clinico. È stato valutato l'effetto della carenza di ferro sulla crescita e sulla formazione del biofilm di *A. baumannii* utilizzando diversi chelanti del ferro e diverse condizioni di coltura. Infine, l'ultimo obiettivo di questa tesi di dottorato è stato quello di generare una combinazione di mutanti di *A. baumannii* per delezione sito-specifica di geni implicati nell'acquisizione del ferro, al fine di determinare il contributo dei singoli sistemi di acquisizione del ferro ai fini della sopravvivenza e della patogenicità di questo batterio. Questi mutanti sono stati confrontati con il ceppo selvatico per la capacità di crescere in diverse condizioni di disponibilità di ferro. Per valutare la specificità di acquisizione dei sistemi mutati, sono stati effettuati saggi di stimolazione della crescita in presenza di varie fonti esogene di ferro. La patogenicità dei vari mutanti nei sistemi di acquisizione del ferro è stata analizzata in un semplice modello d'infezione dell'insetto *Galleria mellonella*.

Contents

Chapter 1

Introduction and aims

1.1 *Acinetobacter* genus

The genus *Acinetobacter* (from the Greek [akinetos], i.e. nonmotile) was first described in 1954 (Brisou and Prevot, 1954). This genus has undergone a series of revisions, initially based on its morphological and metabolic characteristics, and followed, more recently, by detailed molecular and genomic analysis. Since most of the species could not be accurately distinguished by phenotypic methods, novel techniques were incorporated into the identification schemes of the different species of the genus, including phenotyping (Hansen *et al*., 1977), serotyping (Das *et al*., 1984), the characterization of membrane proteins (Alexander *et al*., 1984, Dijkshoorn *et al*., 1996), the study of bacteriophages o phagetyping (Bouvet *et al*., 1990), ribotyping (Garcia *et al*., 1996, Vila *et al*., 1994), ribosomal DNA restriction analysis (Koeleman *et al*., 1998) and, particularly, homology studies on chromosomal DNA using different techniques (Dijkshoorn *et al*., 1996, Janssen *et al*., 1997, Tjenberg and Ursing, 1989).

Today, researchers continue to propose names for the genomospecies not assigned (Nemec *et al*., 2009), and include new species within the genus *Acinetobacter* (Nemec *et al*., 2010).

By 2012, over 43 genomic species in the genus *Acinetobacter* had been identified, of which 27 species have been given valid names (http://www.bacterio.net/acinetobacter.html#r).

The genus *Acinetobacter* is currently defined as Gram-negative, non-motile, oxidase-negative, non-glucose-fermenting, strictly aerobic, catalase-positive bacteria with a G+C content of 39–47% (Bouvet and Grimont, 1986). *Acinetobacter* spp. are considered ubiquitous in nature, with most being distributed widely throughout many environments, including soil, water, vegetables, animals, sewage, and humans (Fournier *et al*., 2006). In addition, they are known to be effective degraders of alkanes and aromatic hydrocarbons, but only a minority of the genus is pathogenic.

The most clinically successful species of the genus are *A. baumannii* (aka genospecies 2), *A. pittii* (aka genospecies 3) and *A. nosocomialis* (aka genospecies 13TU). Strains belonging to these species and to *A. calcoaceticus* (aka genospecies 1) are impossible to distinguish phenotypically and thus are often grouped under the umbrella name of '*A. calcoaceticus-A. baumannii* (Acb) complex', which confuses strain differentiation in the clinical practice. These problems have led to the development of genotypic methods for *Acinetobacter* species identification. In the past, the most frequently used methods were amplified fragment length polymorphism (AFLP), amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) and DNA-sequence analysis (Dijkshoorn *et al*., 2007). More recently, MALDI-TOF mass

spectra provided sufficient discrimination of most clinically relevant *Acinetobacter* species (Toh *et al*., 2015).

1.2 *Acinetobacter baumannii*

A. baumannii (Figures 1 and 2) is an important opportunistic bacterial pathogen responsible for 2-10% of all Gram-negative hospital infections (Joly-Guillou, 2005). It is classified by the Infectious Diseases Society of America as one of the six most important multidrug resistant (MDR) bacteria in hospitals worldwide (Talbot *et al*., 2006). Contrary to the other species of the genus *Acinetobacter*, it is almost exclusively found in the nosocomial environment with sporadic strains isolated from non-human sources. It can be responsible for severe infections, in particular ventilator-associated pneumonia and bloodstream infections. The clinical relevance of *A. baumannii* has dramatically increased since the 1980s, with the emergence and spread of three predominant clones capable of causing hospital outbreaks worldwide (aka 'international clonal lineages', ICLs), of which ICL-I and ICL-II are MDR.

More than 400 MLST sequence types (STs) are currently listed in the *A. baumannii* MLST database (http://pubmlst.org/abaumannii/), and a recent analysis has evidenced the existence of at least six major ICLs distributed through continents (Karah *et al*., 2012). These six ICLs include the previously identified three major outbreak clones, and demonstrate the worrisome emergence of new epidemic clones. It has been suggested that this species distribution is the result of the low phylogenetic diversity of *A. baumannii* and rapid spread following a selective bottleneck, probably corresponding to the antibiotic era (Diancourt *et al*., 2010).

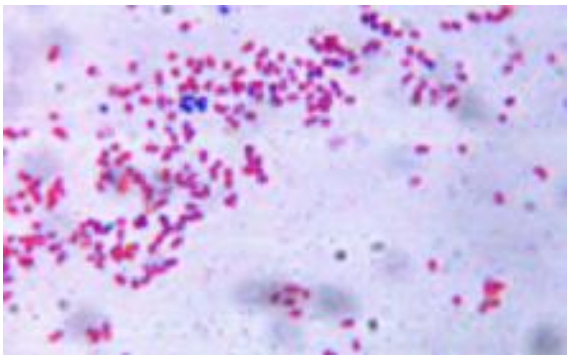

Figure 1. Gram stain of *A. baumannii* cells: notice that most cells appear Gram-negative (red) while few ones show the typical feature of retaining the crystal violet dye appearing as Gram-positive (blue) (candidate's picture).

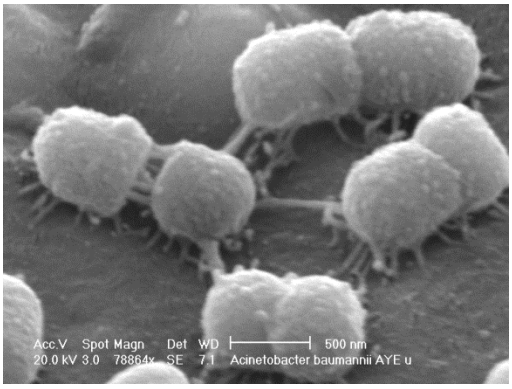

Figure 2. Scanning electron microscope (SEM) micrograph of *A. baumannii* cells taken at in the Interdepartment Electron Microscopy Facility (LIME) of Roma Tre University (courtesy of P. Visca).

1.3 *A. baumannii* in the hospital setting

A. baumannii is the *Acinetobacter* species most commonly involved in hospital infection, causing a wide range of diseases, most often pneumonia and blood-stream infections. The Intensive Care Units (ICUs) of hospital harbour critically ill patients who are extremely vulnerable to *A. baumannii* infections. The environment of these units, and their critical patients, provide a niche for opportunistic microorganisms as *A. baumannii* that are generally harmless for healthy individuals but that are often highly resistant to antibiotics and can spread epidemically among susceptible patients. As a general consideration, infections by such organisms are difficult to treat and can lead to an increase in morbidity and mortality. Furthermore, their eradication from the hospital environment can require targeted measures, such as the isolation of patients and temporary closure or even reconstruction of wards. The presence of these organisms, therefore, poses both a medical and an organizational burden to health-care facilities (Dijkshorn *et al*., 2007).

Numerous studies have reported the occurrence of MDR *A. baumannii* in hospitals and, at some locations, pandrug-resistant strains have been identified.

The rank of *A. baumannii* among dangerous nosocomial pathogens is continuously rising. Additionally, the number of reports of community-acquired *A. baumannii* infection has been steadily increasing, although this type of infection remains rare. Despite the numerous publications that have commented on the epidemic spread of *A. baumannii*, little is known about the mechanisms that have favoured the evolution of this organism towards multidrug resistance and epidemicity.

The success of *A. baumannii* as nosocomial pathogen is probably based on the capability to acquire resistant mechanisms during the spread of a hospital outbreak, or national and international clonal dissemination (Dijkshoorn *et al*., 2007). The problem with dissemination of this pathogen is the difficulty involved in the treatment of the infections caused and the eradication of this pathogen from the hospital environment (Karah *et al*., 2012; Perez *et al*., 2007). Several factors are involved in the spread and dissemination of *A. baumannii*: its adaptability to adverse environmental conditions, its ability to develop multidrug resistance, and the extensive use of broad-spectrum antimicrobials (Cisneros *et al*., 2003).

A. baumanni can survive on dry abiotic surfaces, and during outbreaks has been recovered from various sites in the hospital ward environment (bed curtains, furniture and hospital equipment). These observations emphasize the hospital environment as a reservoir for *A. baumannii* during outbreaks (Figure 3).

The most common modes of transmission are: direct contact with the patient infected by *A. baumannii*, from the hands of hospital staff (Bernards *et al*., 1998) and by indirect contact (e.g. through abiotic surfaces) (Figure 3).

Moreover, it is possible isolating *A. baumannii* by various furnishing elements (curtains, pillows and beds), water present in humidifiers and all medical devices (catheters, respiratory equipment, etc.) (Villegas *et al.*, 2003).

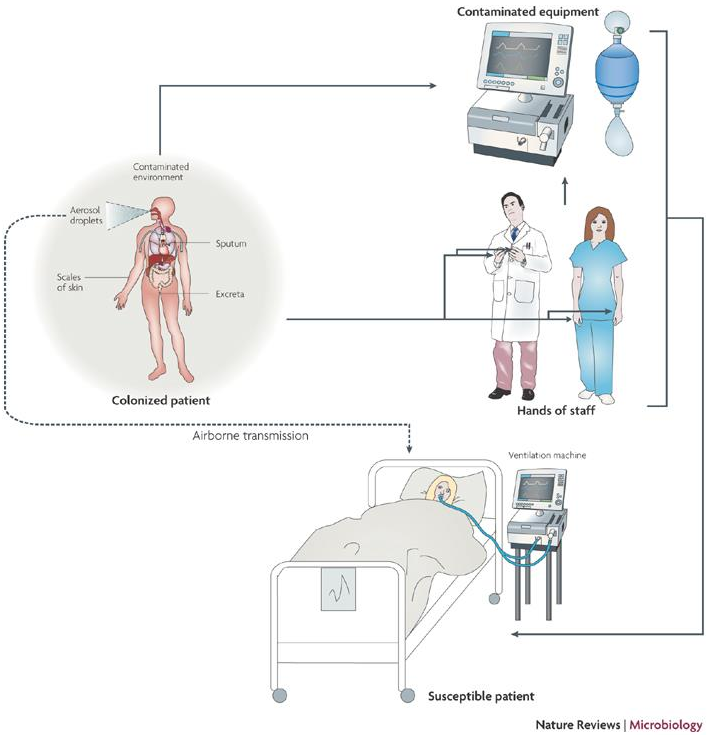

Figure 3. The possible modes of *A. baumannii* entry into a ward are shown: colonized or susceptible patient, hands of hospital staff and contaminated equipment (Dijkshoorn *et al*., 2007)

A. baumannii can form biofilm, which is a community of many bacterial cells associated with a surface (either biotic or abiotic), arranged in a tridimentional structure in intimate contact with each other and encased in an extracellular matrix that can be comprised of carbohydrates, nucleic acids, proteins and other biological macromolecules (Costerton *et al*., 1999; Sauer *et al*., 2004, Vidal *et al*., 1996). *A. baumannii* can attach and generate thin biofilm layers on glass, metal, ceramic and other abiotic surfaces (Dijkshoorn *et al*., 2007; Gaddy and Actis, 2009). The biofilm formation is an ideal growth condition for bacteria and allows them protection against the penetration of antibiotics and other toxic agents. Moreover, the lazy metabolic state of biofilm-encased cells protects them from the action of antibiotics targeting active metabolic functions. Thus, the ability to adhere and form biofilm on biotic and abiotic surfaces guarantees to *A. baumannii* survival even in the most hostile conditions (Piéchaud *et al*., 1956; Vidal *et al*., 1996).

1.4 Clinical importance and antibiotic resistance

As anticipated, in addition to asymptomatic colonisation and carriage on skin and in the respiratory tract, *A. baumannii* can cause a broad range of severe nosocomial infections, including pneumonia, sepsis, skin and soft-tissue infections, wound infections, urinary tract infections and secondary meningitis (Figure 4). Indeed, the most important infections, with the highest mortality rates, are ventilator-associated pneumonia and bloodstream infections (Dijkshoorn *et al*., 2007). The fact that *A. baumannii* is able to colonise the skin and respiratory tract without causing infection (Doughari *et al*., 2011), with skin carriage being 10-20% in healthy individuals, and as high as 50-75% in hospitalized patients, is a factor to consider regarding its transmission within ICUs (Zeana *et al*., 2003). Infections are more common in patients suffering from an underlying disease or who have undergone intensive surgical procedures. *A. baumannii* can easily enter the body through open wounds, intravascular catheters and mechanical ventilators, and infections caused by *A. baumannii* are associated with long periods of hospitalisation (Peleg *et al*., 2008), the male gender, and elderly (Wisplinghoff *et al*., 1999).

A. baumannii can also cause community-acquired infections. The most common is pneumonia (accounting for 85% of reports of community infections caused by *A. baumannii*), followed by bacteraemia. Other possible community-acquired infections include skin, soft-tissue and ocular infections, secondary meningitis and endocarditis (Chang *et al*., 2000,

Falagas *et al*., 2007). These infections are more common in the male gender and are associated with old age, alcoholism, heavy smoking, diabetes mellitus, chronic obstructive pulmonary disease and renal disease. Pneumonia often occurs in the summer months in tropical and subtropical climates (Southeast Asia and North Australia). Community-acquired *A. baumannii* pneumonia is more serious than nosocomial pneumonia, is generally fulminant (death within eight days of diagnosis) and rates of mortality can be as high as 60% (Falagas *et al*., 2007).

The predominance of *A. baumannii* has also been reported to be rising in infections associated with injuries in war zones. Whereas no descriptions of this bacterium existed up to the Vietnam conflict (1955-75), in the last thirty years several studies have reported *A. baumannii* infections associated with the Iran-Iraq war (1980-88), the Gulf war (1990-91) and the Iraq war (2003-11). *A. baumannii* infections have also been associated with natural catastrophes such as the Turkey Marmara Earthquake and with the tsunami in Thailand (Centers for Disease Control and Prevention, 2004; Joly-Guillou, 2005; Murray *et al*., 2006).

The predominance and severity of *A. baumannii* infections seems to be related to the MDR phenotype of infecting strains (Diancourt *et al*., 2010). *A. baumannii* antimicrobial resistance has progressively increased. By 2007 up to 70% of isolates in certain settings (depending on country, hospital, medical department and clinical sample) were MDR, including resistance to carbapenems, which were once used as last resort against MDR-*A. baumannii* infections (Kempf and Rolain, 2012).

Currently, colistin (polymyxin E) seems to be the most reliably effective drug against MDR *A. baumannii*, but it has nephrotoxic effects (Falagas and Rafailidis, 2009) and there are recent reports of resistance (Cai *et al*., 2012), resulting in the emergence of strains resistant to all known antibiotics (Al-Sweih *et al*., 2011).

Worryingly, resistance appears to be spreading to the community, with reports of carbapenem-resistant *Acinetobacter* spp. in cattle (Poirel *et al*., 2012) and in fresh water (Girlich *et al*., 2010).

A. baumannii has the remarkable ability to acquire and accumulate antibiotic resistance determinants (Kempf and Rolain, 2012). While *A. baumannii* possesses intrinsic resistance determinants, it can also acquire resistance determinants from other species (Bonomo and Szabo, 2006). In general, the main genetic structures associated with acquired antibiotic resistance genes in *A. baumannii* are integrons and transposons. Multidrug-resistance in *A. baumannii*, *i.e.* resistance to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012), seriously compromises treatment of infected patients and poses a

serious risk to patient's life (Bonomo and Szabo, 2006; Kempf and Rolain, 2012). Since the 1970s *A. baumannii* antimicrobial resistance has increased, and nowadays the resistance to broad-spectrum antibiotics such as amikacin, expanded-spectrum cephalosporins, carbapenems, and tigecycline is rather common, especially in some regions, leaving very few effective therapeutic choices (Roca *et al*., 2012). In particular, carbapenem-resistance has become a significant health problem, since carbapenem-resistance is invariably associated with the MDR phenotype. Treatment options for carbapenem-resistant *A. baumannii* are very limited, and include colistin, tigecycline, and in some cases the combination ampicillin/sulbactam (Kempf and Rolain, 2012). It is worrisome that pandrug-resistant strains, *i.e.* strains resistant also to these last resort drugs, have increasingly been reported (D'Andrea *et al*., 2009; Lopez-Rojas *et al*., 2011; Rolain *et al*., 2011). Several mechanisms of resistance to carbapenems have been described in *A. baumannii*, including production of β-lactamases, decreased permeability, altered penicillin binding proteins and overexpression of efflux pumps (Brown *et al*., 2005; Poirel and Nordmann, 2006; Roca *et al*., 2012). The most common carbapenem-hydrolyzing enzymes in *A. baumannii* are the class D (OXA-type) and, less frequently, the class B (metallo) β-lactamases (Brown and Amyes, 2006; Cornaglia *et al*., 1999; Poirel and Nordmann, 2006). *A. baumannii* possesses two chromosomally-encoded intrinsic β-lactamases, the AmpC-type cephalosporinase and the OXA-51 carbapenemase, generally expressed at low levels (Poirel and Nordmann, 2006).

However, expression of these enzymes can be enhanced by the presence of different type of Insertion Sequences (ISs) located upstream or downstream of the resistance genes, and may subsequently impact on susceptibility to cephalosporins or carbapenems (Turton *et al*., 2006a; Poirel and Nordmann, 2006). Five phylogenetic groups of clavulanic-acid resistant carbapenem-hydrolyzing class D β-lactamases (CHDLs) have been described in *A. baumannii*, including enzymes of the OXA-23, OXA-24/40, OXA-58 and OXA-143 families, encoded by acquired genetic elements which can be located either on plasmids or in the chromosome, and of the OXA-51 group, which is chromosomally encoded and intrinsic of the species (Brown and Amyes, 2006; Higgins *et al*., 2009; Kempf and Rolain, 2012; Poirel and Nordmann, 2006). The OXA-23 (originally named ARI-1) was the first CHDL to be identified. The *bla*OXA-23 gene has been identified as part of different transposon structures, namely *Tn*2006, *Tn*2007, and *Tn*2008 (Mugnier *et al*., 2010). OXA-23 is the most widespread CHDL in *A. baumannii* and has been identified worldwide (Zarrilli *et al*., 2013). A second group of CHDLs that can be present either on the chromosome or on plasmids is the so-called OXA-24/40 group, reported worldwide but found particularly in *A. baumannii* from the USA,

Spain, and Portugal (Acosta *et al*., 2011; Bou *et al*., 2000; Grosso *et al*., 2011). A third group of CHDLs comprises OXA-58, which is preferentially plasmid encoded and associated with specific ISs, reported in worldwide isolates of *A. baumannii* (reviewed in Zarrilli *et al*., 2013). Moreover, carbapenems resistance can be mediated by the acquisition of narrow-spectrum clavulanic acid inhibited penicillinases (including TEM-1 and TEM-2, CARB-5) and MBLs (Roca *et al*., 2012). Four groups of MBLs have been described in *A. baumannii*, namely IMP-like, VIM-like, SIM-1, and NDM-type enzymes (Zarrilli *et al*., 2013). Resistance to broad-spectrum cephalosporins in *A. baumannii* can also depend on from the acquisition of different extended-spectrum β-lactamases (ESBLs) (Kempf and Rolain, 2012).

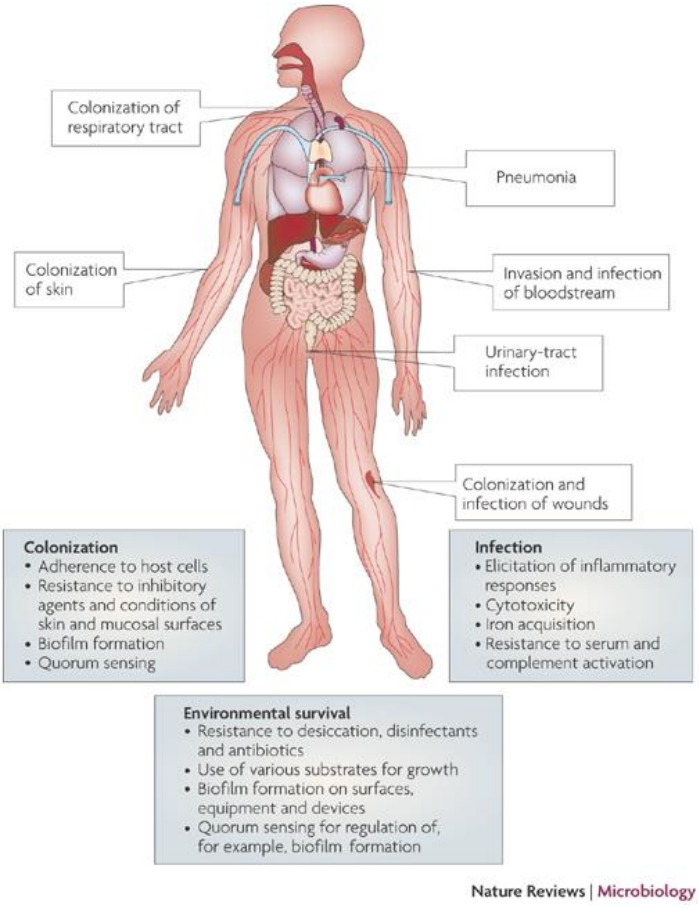

Figure 4. Factors contributing to the environmental persistence, host colonization and infection by *A. baumannii* (Dijkshoorn *et al*., 2007).

1.5 Therapeutic strategies against *A. baumannii*

The global emergence of MDR *A. baumannii* isolates and the impressive resistance to antibiotics of this species has propelled a number of studies aimed at the development of alternative antimicrobial strategies, especially those for which resistance is less probably developed. Several studies have started to explore combinatorial therapies with different antibiotics or non-antibiotic approaches to achieve antibacterial activity. The emerging therapies for multidrug resistant *A. baumannii* have recently been reviewed (Garcia-Quintanilla *et al*., 2013).

Therapeutic options usually retaining activity against *A. baumannii* include sulbactam, carbapenems, aminoglicosides, polymyxins and tigecycline. Sulbactam is a β-lactamase inhibitor endowed with bactericidal activity on *A. baumannii* (Peleg *et al*., 2008), whose efficacy has been proven in mild to severe infections (Fishbain and Peleg, 2010). Carbapenems, including imipenem, meropenem, ertapenem and doripenem, when active (*i.e.* in strains lacking acquired carbapenemases) are considered one of the most effective therapeutic options for treating serious infections caused by MDR *A. baumannii*, with good bactericidal activity and resistance to hydrolysis by the majority of β-lactamases. Amikacin and tobramycin still retain activity against some *A. baumannii* isolates, and can be used in combination therapy (Roca *et al*., 2012). Polymyxins, which include colistin and polymyxin B, are considered the last resort treatment for extensively drug-resistant Gram negative bacteria, including *A. baumannii* (Roca *et al*., 2012). Tigecycline is a new generation tetracycline (glycylcycline) that has the advantage to be scarcely recognized by the tetracycline-specific efflux pumps (the *tet* systems), therefore providing a broader spectrum of activity (Fishbain and Peleg, 2010). Besides their use in monotherapy (*i.e.* provided alone), several efforts have been also addressed to test the efficacy, both *in vitro* and *in vivo*, of combination of these antibiotics. Encouraging results for some antibiotic combinations have been reported in several studies (Bassetti *et al*., 2008; Falagas *et al*., 2005; Lee *et al*., 2007; Petrosillo *et al*., 2008).

Phage therapy is a cheap and very specific, though still problematic, antibacterial strategy. Two *A. baumannii*-specific lytic phages, named phages AB1 and AB2, have been isolated (Lin *et al*., 2010; Yang *et al*., 2010), and proven to be effective *in vitro* and *in vivo* against *A. baumannii* clinical isolates (Jin *et al*., 2012; Lai *et al*.,2011; Lee *et al*., 2011; Popova *et al*., 2012; Thawal *et al*., 2012; Yele *et al*., 2012). *A. baumannii*-specific phages showed also good inhibitory activity on biofilm formation, and demonstrated bactericidal effect of preformed

biofilms, raising expectation on the use of phages for removing *A. baumannii* from colonized surfaces (Garcia-Quintanilla *et al*., 2013). Disadvantages of phage therapy include the possibility that resistance can emergence at high frequency, the high strain-specificity of the phages, and the likelihood to generate immune response and allergy in humans (Matsuzaki *et al*., 2005).

Antimicrobial peptides are able to modulate the innate immune response of higher organisms making them potential candidates for use as antibacterial agents, and resistance to these compounds has rarely been observed (Garcia-Quintanilla *et al*., 2013). Polymyxins (e.g. colistin) are examples of such bioactive compounds already used in therapy. Additional antibacterial peptides have been studied *in vitro* and *in vivo* against *A. baumannii*. Among these, P1, magainin II and K6L9 peptides are among the most active, even at very low concentrations, with K6L9 proven to be effective also *in vivo* (Alarcon *et al*., 2001; Braunstein *et al*., 2004; Conlon *et al*., 2010; Routsias *et al*., 2010). The efficacy of all these peptides for the treatment of MDR *A. baumannii* infections in humans remains to be determined. In particular, it must be considered that antimicrobial peptides can be inactivated by proteolytic degradation in human serum, and therefore may need structural modifications in order to be effective (Garcia-Quintanilla *et al*., 2013). Peptide nucleic acids targeting essential *A. baumannii* genes would circumvent the problem of enzymatic degradation, but so their development for *A. baumannii* is still at an initial stage (Hatamoto *et al*., 2010).

Iron is an essential nutrient for all living organism and acts as cofactor of many enzymes involved in vital processes. The essential role of iron in bacterial physiology has raised the possibility of exploiting iron metabolism as an antibacterial strategy. *A. baumannii* possesses a multiplicity of iron uptake systems, including the production of high affinity iron-chelating compounds (siderophores), and uptake systems for heme and ferrous iron (Antunes *et al*., 2011; Dorsey *et al*., 2003). Higgins and coworkers (2012) explored a “Trojan-horse strategy” targeting *A. baumannii* iron metabolism that involved the administration of BAL30072, *i.e.* a siderophore-monosulfactam antibiotic, endowed with good antimicrobial activity and that moved into phase-1 clinical trials. Targeting iron metabolism can be achieved also by the use of iron chelators. Recently, the activity of some iron chelators, including deferoxamine, deferiprone and 2,2-dipyridyl, has been tested on the growth of *A. baumannii* (de Leseleuc *et al*., 2012; Thompson *et al*., 2012). Deferoxamine and deferiprone showed weak or no activity on *A. baumannii*, while 2,2- dipyridyl retained the highest antimicrobial activity (de Leseleuc *et al*., 2012; Thompson *et al*., 2012). Notably, the ability of *A. baumannii* to acquire iron bound to deferoxamine or deferiprone was not assessed. Gallium (Ga^{3+}) is a transition metal

showing extensive chemical similarity with iron (Fe^{3+}), thus being able of competing with iron for binding to essential iron requiring enzymes (Chitambar, 2010). The antimicrobial activity of gallium has been demonstrated for several bacterial species (Baldoni *et al*., 2010; Banin *et al*., 2008; Bonchi *et al*., DeLeon *et al*., 2009; Kaneko *et al*., 2007; Minandri *et al*., 2014; Nerren *et al*., 2011; Olakanmi *et al*., 2000; Rzhepishevska *et al*., 2011), and preliminary results have also been obtained with the reference *A. baumannii* strain ATCC 17978 (Antunes *et al*., 2012; de Leseleuc *et al*., 2012).

1.6 *A baumannii* in the extra-hospital environments

A. baumannii is genotypically well separated from other *Acinetobacter* spp., and its scarce gene polymorphism indicates that it probably suffered a recent and severe evolutionary constriction (probably the antibiotic pressure) which could be related to adaptation to a restricted niche (probably the hospital setting) followed by a rapid expansion of multi-drug resistance clones (Diancourt *et al.*, 2010).

However, since few strains have been recovered from non-human sources, there is the possibility that clinical isolates represent only a small portion of the diversity of the species.

Although with low recovery rates, *A. baumannii* strains from non-human sources have been isolated (Eveillard *et al.*, 2013; Dhakephalkar and Chopade, 1994), in particular: from soil and aquaculture (Guardabassi *et al.*, 1999; Hoa *et al.*, 2011; Huys *et al.*, 2007; Rokhbakhsh-Zamin *et al.*, 2011), slaughterhouse meat (Ercolini *et al.*, 2009), poultry (Hamouda *et al.*, 2011), fish (Houang *et al.*, 2001; Huys *et al.*, 2007), vegetables (Berlau, 1999; Gennari and Lombardi, 1993), milk (Nam *et al.*, 2011), body and head lice (Kempf *et al.*, 2012; La Scola and Raoult, 2004¸ Eveillard *et al.*, 2013), and animals as cats, dogs and horses from veterinary clinics (Endimiani *et al.*, 2011; Zordan *et al.*, 2011). Recently, carbapenemase-producing *A. baumannii* isolates in Lebanon were collected from livestock animals (pig, fowl and cattle) (Al Bayssari *et al.*, 2014) as seen also in France in previous years.

Moreover extra-hospital *A. baumannii* isolates have been recovered from sites contaminated with petroleum hydrocarbons both in India e in France (Sarma PM *et al.*, 2004; Bordenave S *et al.*, 2007). A recent study of Pailhoriès *et al*. (2015) showed the great diversity of some *A. baumannii* strains outside the hospital setting in Reunion Island. The best-characterised non-human strain is SDF, a strain isolated from a human body louse (La Scola and Raoult, 2004) whose genome has been sequenced and annotated. This strain differs from human isolates in having a reduced genome (3.2 Mb instead of 3.9 Mb) that is riddled with putative prophage regions. Unlike clinical *A. baumannii* isolates, SDF is susceptible to desiccation and to most

antimicrobials, shows limited metabolic capacity, including lack of growth at fever-associated temperatures, is impaired in the production of many common *A. baumannii* virulence factors and is non-virulent in a non-mammalian model of infection (Antunes *et al.*, 2011). All of these factors indicate a plausible evolution of SDF strain to a non-human restricted niche.

1.6.1 *A. baumannii* in animals

The scientific papers of *A. baumannii* strains isolated from infected animals have increased in the last decades. Francey and colleagues (Francey *et al*., 2000) found *A. baumannii* in 17 dogs and two cats recovered at the Companion Animal Hospital at the University of Bern, Switzerland between August 1995 and December 1997. Seven dogs were presented for neurological problems (intervertebral disk protrusions, vertebral fracture, poisoning, and otitis interna). Six dogs had other no neurological traumatic conditions (multiple bite wounds, fractures, and luxations), and 6 animals, including both cats, were presented for a medical problem (urolithiasis, chylothorax, pyothorax, sinusitis frontalis, and abdominal fibrosarcoma). One of the main common features of the isolates was their resistance to a wide range of antibiotics. All isolates were resistant against first- and third-generation cephalosporins, amoxicillin–clavulanic acid, chloramphenicol, and nitrofurantoin and also to antibiotics commonly used to treat infections with gram-negative bacteria, including aminoglycosides (gentamicin, kanamycin). Clinical and epidemiological evidence indicated that this bacterium is also an opportunistic pathogen in dogs and cats and was responsible for an increase in the morbidity and the mortality of the affected patients. Another interesting study was published in the same year by Vannechoutte and colleagues in The Netherlands (Vannechoutte *et al*., 2000). *A. baumannii* was isolated from tips clipped from seven intravenous jugular catheters collected from horses in the Ghent University equine clinic. They originated from 7 different horses. Three of the seven isolates showed evidence of local infection. All strains were resistant to the antibiotics used in equine practice (Table 1).

Strain designation	Resistance of isolates to drugs:								API 20NE code
	A	A + C	C	T	S + T	G	N	E	
4982	R	R	R	R	R	X	S	S	0001073
5007	R	R	R	R	R	X	S	S	0041473
5014	R	R	R	R	R	R	S	R	0041473
5017	R	R	R	R	R	X	S	S	0041473
5022	R	R	R	R	R	R	S	R	0041473
5412	R	R	R	R	R	X	S	S	0041073
5440	R	R	R	R	R	R	S	S	0001473

[a] A, amoxicillin; A + C, amoxicillin plus clavulanic acid; C, ceftiofur; T, tetracyclines; S + T, sulfonamides plus trimethoprim; G, gentamicin; N, neomycin; E, enrofloxacin; R, resistant; X, intermediate; S, susceptible.

Table 1. Susceptibility patterns and API codes for the seven *A. baumannii* horse isolates (Vannechoutte *et al*., 2000).

Another work by Boerlin *et al*. (2001) reported high antibiotic resistance among *A. baumannii* isolates from horses and companion animals in veterinary clinics. The transmission of *A. baumannii* strains from companion animal's to horses demonstrates the potential spread of multi-resistant *A. baumannii* strains between veterinary clinics, similar to the spread between human hospitals (Aubert *et al*., 1995; Dijkshoorn *et al*., 1996). Transmission could have occurred through the hands of staff personal and students working simultaneously in the companion animals and horse clinics.

Other two studies on *A. baumannii* isolation from veterinary clinics were conducted by Endimiani *et al.* (2011) and Zordan *et al.* (2011). The first work analyzed 19 *A. baumannii* isolates collected in pet and horse clinics. Also in this case, isolates were antibiotic-resitant, and 3 were non-susceptible to carbapenems. MLST typing revealed that the isolates belonged to ST10/ST 12 (ICL-II) and ST15 (ICL-I). The spread of these *A. baumannii* isolates in companion animals is concerning, because both ICL I and II are highly prevalent in humans and are associated with multiple mechanisms of resistance, including those against "last-resort" antibiotics. The second work (Zordan *et al*. 2011) focused on 52 *A. baumannii* isolates from 137 hospitalized animals. Again, isolates revealed a high resistance to all antibiotics tested and belonged to the major clonal lineages.

1.6.2 *A. baumannii* in the environment

There are a few studies focusing on *A. baumannii* isolation from natural environments. For example, Guardabassi *et al.* (2001) isolated 99 *Acinetobacter* strains from different aquatic samples (sewage, aquaculture freshwater, intestinal contents of rainbow trout and frozen shrimps). Sewage isolates were collected in a previous study from sites situated upstream and downstream of the discharge point of a pharmaceutical plant (Guardabassi *et al*. 1998). Some of the *Acinetobacter* isolates from sewage and frozen shrimps belonged to the *A. baumannii* species.

Most *Acinetobacter* strains derived from sewage belonged to genomic species able to grow at 37 °C and characterized by high nutritional versatility (*Acb* complex, genomic species 10, 14BJ and 16BJ). These genomic species were not recovered from unpolluted aquatic habitats, suggesting that they are not autochthonous in the aquatic environment and their presence in sewage could be a result of contamination by human activities. Moreover, *Acinetobacter* isolates from sewage showed higher levels of antibiotic resistance compared with isolates from less polluted aquatic habitats, such as those situated in the vicinity of the two trout farms. Independent from their source of isolation, isolates belonging to the *Acb* complex,

genomic species 10, 14BJ and 16 BJ were resistant to amoxicillin and chloramphenicol, confirming that some *Acinetobacter* species could be inherently resistant to these classes of antibiotics (Gerner Smidt and Frederiksen, 1993).

Another study is by Huys *et al.* (2007) was conducted on 23 *A. baumannii* isolates of samples of pond water, sediment and farmed species at different fish and shrimps farms in Malaysia, Thailand and Vietnam. The aim of this work was to determine the biodiversity and environmental distribution of chloramphenicol-resistant mesophilic heterotrophic bacteria in Southeast Asian aquaculture sites. The results demonstrate that the majority of the chloramphenicol-resistant isolates recovered in this study belonged to a range of species that have been implicated in various opportunistic human infections, *i.e. Escherichia coli*, *Klebsiella pneumoniae* and *A. baumannii*. This finding indicated a potential health risk (e.g. wound or respiratory infection) to staff of aquaculture farms or processing units if exposed to multidrug resistant members of these species.

1.6.3 *A. baumannii* in food

A preliminary study by Gennari and Lombardi (1993) compared 170 *Acinetobacter* isolates from various food sources (including fresh and spoiled meat and fish, vegetables, raw milk and cheese) with 83 isolates from clinical sources. The results demonstrated clear difference in the distribution of genomic species between the food and clinical isolates, with *A. johnsonii* (sp.7) and *A. lwoffii* (spp. 8/9) predominating in foods.

Some years later, Berlau *et al.* (1999) used fresh vegetables as an indicator of the natural environment and questioned whether *Acinetobacter* could be isolated from them and which species was the most prevalent. They also ascertained the degree of resistance to antimicrobials among these natural isolates to gauge their potential significance as hospital pathogens. They purchased vegetables (apple, carrot, cauliflower, potato, onion and so on) from greengrocers or supermarkets, and noticed that 17% of vegetables examined harboured *Acinetobacter*, usually in small numbers. Worryingly, the commonest species were *A. baumannii* and genospecies 11. As expected, members of the *A. calcoaceticus-baummannii* complex were generally more resistant to selected antimicrobials than other genospecies. Thus, vegetables could represent a source of *A. baumannii*, and this potential risk should be recognized in the preparation of food served to predisposed patients in hospitals.

Nam *et al*. (2010) isolated *A. baumannii* from raw milk and mastitis in dairy cattle i. The 17 *A. baumannii* isolates analyzed in this study showed much lower susceptibility to several

antimicrobials than *Acinetobacter* spp., confirming that multiple antimicrobial resistance is prevalent among *A. baumannii* isolates from mastitis milk.

To understand the scale of resistance problem and predict future trends in resistance development, antimicrobial resistance patterns of the environmental pathogens are important. The data obtained from all above studies provide useful information on the status of antimicrobial resistance among non-hospital *A baummannii* isolates.

1.7 Iron and *A. baumannii* infection

The mechanisms which enable *A. baumannii* to colonize the human host and establish infection are not yet fully established. One of the major challenges for pathogenic bacteria in their hosts is the paucity of freely available iron. Iron is an essential nutrient for bacteria, and plays a role in differential gene expression. Although iron is one of the most abundant elements on the earth, the concentration of biologically useful iron is generally extremely low. In aerobic inorganic environment, iron is mainly present in the oxidized ferric form Fe(III) which aggregates into insoluble oxy-hydroxide polymers and, consequently, it is not easily available to microorganisms. Once in the human host, pathogens such as *A. baumannii* are forced with the problem of iron acquisition. Bacterial pathogens respond to iron limitation imposed by the human host by expressing different high-affinity uptake systems (Noinaj *et al*., 2010; Zimbler *et al*., 2013) including siderophore-dependent and siderophore-independent systems, as well as systems that remove iron from host compounds, such as heme (Wandersman *et al*., 2012), by either direct contact or by producing scavengers known as hemophores. Transport of ferric siderophores and heme across outer membrane requires a specific receptor, while translocation through the inner membrane requires a periplasmic binding protein and an ABC transporter. The TonB energy transducing machinery, encoded by *tonB-exbB-exbD* genes, is needed for translocation of ferric siderophore and heme through the outer membrane receptor of Gram negative bacteria (Proschak *et al*., 2013; Noinaj *et al*., 2010). In the case of *A. baumannii*, experimental data show that clinical isolates have evolved multiple iron-uptake systems (Antunes *et al*., 2011; Dorsey *et al*., 2004; Eijkelkamp *et al*., 2011; Zimbler *et al*., 2009; Proschak *et al*., 2013). Currently, the best-characterized system is that expressed by the, which is based on the production and utilization of the acinetobactin siderphore in the ATCC 19606^{T} type strain (Dorsey *et al*., 2004). Acinetobactin is a mixed catechol-hydroxamate siderophore composed of a non-cyclic derivative of 2, 3-dihydroxybenzoic acid (DHBA) linked to threonine and *N*-hydroxyhistamine. The acinetobactin-mediated system is the best characterized high-affinity iron acquisition system

expressed by ATCC 19606^{T}. Novel mixed catechol-hydroxamate siderophores (named "fimsbactins") were identified in *A. baumannii* ATCC 17978 and in *Acinetobacter baylyi* ADP1 (Proschak *et al*., 2013).

1.8 A multiplicityof high-affinity iron uptake systems in *A. baumannii*

Evidence has emerged tha *A. baumannii* strains carry multiple iron uptake systems, whose repertoire can vary among different isolates. Proteins involved in ferrous iron uptake are encoded by the *feoABC* operon. Transport through the Feo system is not dependent of the TonB energy transducing machinery. All the siderophore and heme iron uptake systems of *A. baumannii* are instead predicted to require the TonB system, encoded by *tonB-exbB-exbD* genes, as further discussed in this thesis (§ 1.8.2).

The ferrous iron uptake system, encoded by the operon *feoABC* (**1** in Fig. 1), is composed of three proteins: FeoA, a small hydrophilic cytoplasmic protein, FeoB, a large protein with an integral membrane domain and a G-protein like domain, and FeoC, another hydrophilic cytoplasmic protein probably acting as transcriptional regulator. The *feo*B (permease) mutation eliminates Fe^{2+} transport in *E. coli* (Cartron *et al*., 2006). Transport through the Feo system is independent of the TonB energy transducing machinery.

All siderophore and heme iron uptake systems of *A. baumannii* are instead predicted to require the TonB system, encoded by *tonB-exbB-exbD* genes. Note that *A. baumanni* has three of such gene clusters (as discussed in § 1.8.2), one of which is adjacent to the iron uptake system **2** (Figure 5). Two heme uptake systems have been identified in *A. baumannii* (**2** and **3** in Figure 5). In addition to acinetobactin (**4** in Figure 5), another putative hydroxamate siderophore cluster (**5** in Figure 5) is present.

The last system (**6** in Figure 5) corresponds to the fimsbactin gene cluster, which is present only in *A. baumannii* ATCC 17978 and *A. baylyi* ADP1 (Antunes *et al*., 2011; Proschak *et al*., 2013). The acinetobactin and fimsbactin systems (systems **4** and **6**, respectively) have been characterized in some detail, but ferrous iron (system **1**), heme (systems **2** and **3**) and the hydroxamate (system **5**) iron uptake clusters deserve more in-depth genetic and functional characterization.

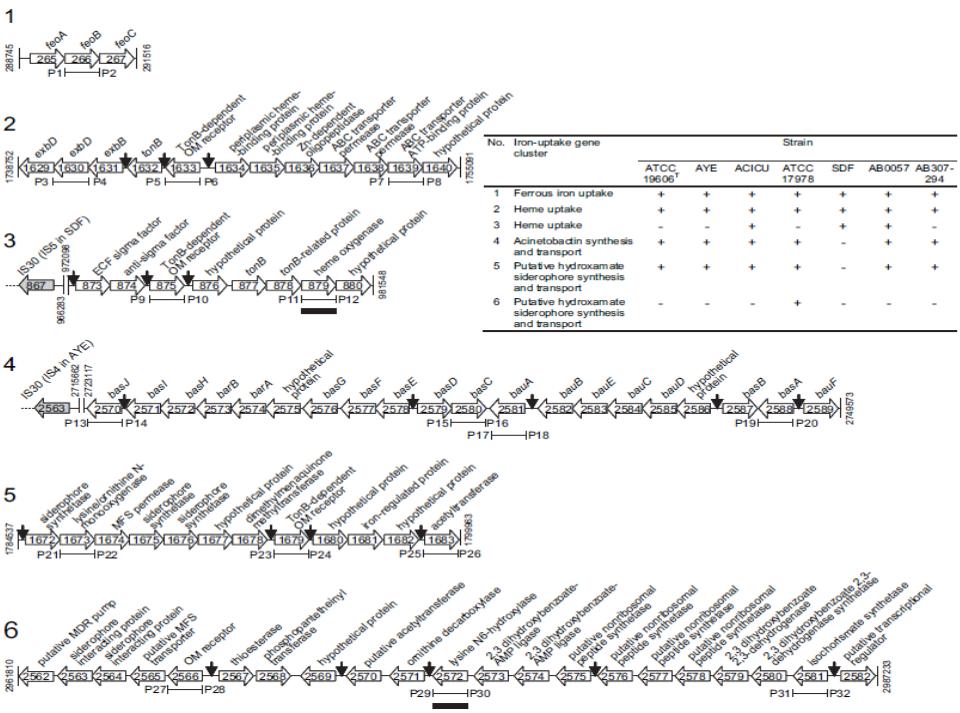

Figure 5. **Schematic representation of the iron-acquisition gene clusters identified in the whole genome sequence of six *A. baumannii* strains. The table shows the presence (+) or the absence (-) of each cluster in the reference strains. Adapted from Antunes *et al.* (2011).**

1.8.1 Siderophores: acinetobactin, fimsbactin, and other putative siderophores

Currently, the best-characterized system is that expressed by the ATCC 19606^{T} type strain, which is based on the production and utilization of acinetobactin (Dorsey *et al*., 2004; Mihara *et al*., 2004; Yamamoto *et al*., 1994).

Genetic and functional analyses indicate that the acinetobactin-mediated system is the only high-affinity iron acquisition system expressed by the ATCC 19606^{T} type strain (Dorsey *et al*., 2004). The *bas*, *bau* and *bar* genes needed for the production, transport and secretion of acinetobactin, respectively, are located in a 26.5-kb chromosomal region containing seven operons (Dorsey *et al*., 2004; Mihara *et al*., 2004). However, this locus does not include an *entA* ortholog, coding for the biosinthetic enzyme 2,3-dihydro-2,3-dihydroxy-benzoate dehydrogenase. This enzyme is involved in the last step of the conversion of chorismate into DHBA, which is essential for the biosynthesis of the catechol moiety of catecholate siderophores such as enterobactin (Earhart, 1996; Walsh *et al*., 1990).

This observation indicates that at least two chromosomal regions are involved in the biosynthesis of acinetobactin in the ATCC 19606^{T}; one containing the *bas*, *bau* and *bar* genes

and another harboring at least the *entA* gene (Penwell *et al*., 2012). The expression of an active *entA* gene is needed for the full virulence of the ATCC 19606^{T} strain when tested using A549 human alveolar epithelial cells and *Galleria mellonella* caterpillars as experimental infection models (Penwell *et al*., 2012).

Recent in silico comparative analysis of sequenced genomes from 50 different clinical isolates showed that the acinetobactin gene cluster is highly conserved among different *A. baumannii* isolates (Antunes *et al*., 2011). However, this study also identified two additional gene clusters, 5 and 6, both of which could code for the production and utilization of hydroxamate siderophores. Interestingly, cluster 6 was found only in the genome of *A. baumannii* ATCC 17978, whereas cluster 5 was found ubiquitously among the *A. baumannii* isolates examined, including the AYE clinical isolate, whose iron acquisition ability seems to be mostly related to the production of hydroxamates when cultured under free-iron-limiting conditions. In a study that investigated *A. baumannii* ATCC 17978 under iron-limiting conditions, Eijkelkamp *et al*. (2011) determined that all components of gene cluster 5 were up regulated under iron-chelated conditions, suggesting that this siderophore system could play a role in iron acquisition. Despite these studies, no experimental data have been reported demonstrating the production and chemical nature of the products of these two *A. baumannii* siderophore gene clusters.

The *A. baumannii* clinical isolate AYE possesses a natural mutation in the *entA* orthologue and cannot synthesize 2,3-dihydroxybenzoic acid (DHBA), a precursor required for the production of acinetobactin. However, *A. baumannii* AYE can grow under iron-limiting conditions, and culture supernatants from this strain are still positive for siderophore activity, indicating that this clinical isolate produces a siderophore in spite of a natural *entA* mutation (Penwell *et al*., 2015).

These observations, together with the comparative genomic studies by Antunes *et al*. (2011) showing the presence of gene cluster 5, which could code for the synthesis and transport of a hydroxamate siderophore, suggest that *A. baumannii* AYE might use this uncharacterized siderophore-mediated system to acquire iron under limiting conditions. Thus, the objective of the study of Pennel *et al.* (2015) was to characterize the molecular nature of the siderophore that is predicted to be encoded by genes located in cluster 5.

These studies showed that *A. baumannii* AYE produces a hydroxamate siderophore named baumannoferrin, which has siderophore activity when tested in colorimetric, biochemical, and biological assays. In silico analysis of the components of gene cluster 5, which will be referred to as the baumannoferrin cluster/locus, and their products resulted in a predicted

biosynthetic pathway that uses diaminopropane, decenoic acid, citrate, diaminobutyrate, and ketoglutarate as precursors.

The last siderophore, named fimsbactin, is encoded by cluster **6** (Figure 1), now renamed *fbs* locus (Proschak *et al*., 2013). As for acinetobactin, also fimsbactin is a mixed chatechol/hydroxamate siderophores, but its distribution appears to be limited to *A. baumannii* ATCC 17978 and *A. baylyi*.

These data highlight the complexity and the multiplicity of *A. baumannii* siderophore-mediated iron-acquisition systems, and probably denote the versatility of this species with respect to the acquisition of different iron sources available in the environment and in the infected host.

1.8.2 The TonB system

The ExbB-ExbD-TonB system provides Gram-negative bacteria the energy needed to transport various iron carriers (siderophores, heme etc.) into the periplasm once these complexes are bound to cognate TonB-dependent outer membrane receptors (Figure 6; Wandersman, 2004; Postle, 2007). This system transduces the proton motive force (PMF) to facilitate the active transport of substrates through the outer membrane. Much work has been done to understand the working mode as well as the components of the TonB system in *E. coli* K-12. ExbB and ExbD are inner membrane proteins, with homology to flagellar motor proteins MotA and MotB, which use the PMF to generate an energized form of TonB (Cascales *et al*., 2001). TonB is a periplasmic protein anchored to the inner membrane by its hydrophobic N-terminal domain, and associated with both ExbB and ExbD. A rigid proline-rich spacer/periplasmic domain spans the periplasmic space and a structurally conserved C-terminal domain (CTD) interacts with specific regions of TonB-dependent receptors located in the outer membrane (Wandersman, 2004; Chu *et al*., 2007). The energized CTD of TonB mediates a conformational change of the TonB-dependent receptors by interacting directly with a conserved hydrophobic five-aminoacid region, termed the TonB box, located in the N-terminal plug domain of the receptor (Krewulak, 2011). This leads to the transport of the receptor-bound ligand into the periplasm and the de-energization of TonB by a poorly understood mechanism. No reports on the features of TonB energy-transducing systems in *A. baumannii* are present in the literature, even though three highly conserved putative *tonB* genes are predicted in all available *A. baumannii* genome sequences: two occur as components of *exbB-exbD-tonB* operons and one as a monocistronic copy. All are actively transcribed in strain ATCC 19606^{T} (Zimbler *et al*., 2013). Phylogenetic analysis indicates

that these *tonB* genes, which are present in the genomes of all sequenced *A. baumannii* strains, were acquired from different sources.

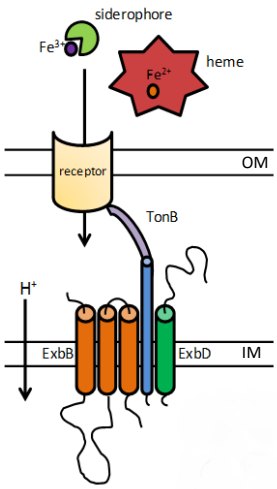

Figure 6. Schematic representation of the TonB energy transducing machinery, which is encoded by *tonB-exbB-exbD* genes.

1.8.3 The Feo system for ferrous iron uptake

The major route for bacterial ferrous iron uptake would appear to be, in many cases, via Feo (Ferrous iron transport). The Feo system appears to differ from any other bacterial transporter. The first bacterial ferrous iron transport (Feo) system discovered was that of *E. coli* K-12 (Hantke, 1987), a facultative anaerobic gut commensal. Ferrous iron is only likely to predominate over ferric iron under reducing (anaerobic) conditions, or at low pH, conditions that favour ferrous iron stability. So, unsurprisingly, ferrous iron transport is generally associated with bacteria growing under anaerobic–microaerophilic conditions, and possibly low pH. While these are not the optimal conditions for *A. baumannii*, a Feo systems appears to be invariably present in all sequenced genomes of this species.

In addition, some bacteria are capable of actively reducing extracellular ferric iron in order to solubilize it for the purpose of uptake, although little is known about the mechanism or components of extracellular-ferric iron reduction in bacteria (Cowart, 2002). Fe^{2+} transport in *E. coli* occurs both aerobically and anaerobically, but is anaerobically induced. In *A. baumannii* it is possible that the ferrous iron uptake is expressed aerobically, because this species is known for being strictly aerobic.

The nucleotide sequence of the *feo* locus of *E. coli* initially suggested the presence of two genes (*feoA* and *feoB*; Kammler *et al*., 1993) although it is now known to constitute three

closely associated, copular (clockwise orientation) genes likely to form an operon, *feoABC* (Figure 5; Hantke, 2003). The operon thus encodes three predicted proteins: FeoA, a small, soluble SH3-domain protein probably located in the cytosol; FeoB, a large protein with a cytosolic N-terminal G-protein domain and a C-terminal integral inner-membrane domain containing two 'Gate' motifs which likely function as the Fe^{2+} permease; and FeoC, a small protein apparently functioning as an [Fe–S]-dependent transcriptional repressor (Figure 7).

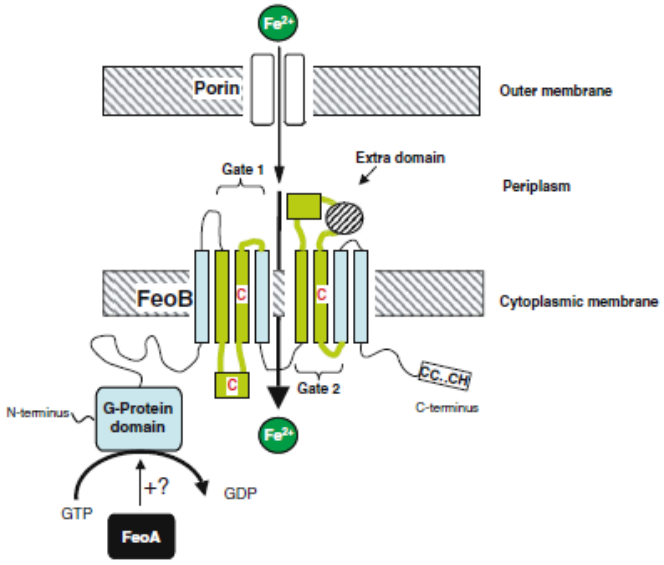

Figure 7. Schematic representation of ferrous iron uptake by Feo of *E. coli* adapted from Cartron *et al*. (2006). Fe^{2+} diffuses into the periplasm via porins and it is transported across the cytoplasmic membrane into the cytoplasm by FeoB by an ATP/GTP active transport. FeoB has a G-protein domain and two motif regions in the cytoplasmic membrane. FeoA is shown activating the GTPase function of FeoB.

However, $^{55}Fe^{2+}$ uptake experiments showed a clear distinction between the *feoA* and *feoB* mutants: the *feoB* mutation virtually eliminated Fe^{2+} transport activity; whereas the *feoA* mutation only reduced uptake by about 25% (Kammler *et al*. 1993) possibly due to polar effects. In addition, expression of *feoB* from a plasmid resulted in enhanced rates of Fe^{2+} uptake both aerobically and anaerobically, confirming that Feo activity is not inhibited by O_2, at least in *E. coli*.

1.9 Aims of the thesis

Acinetobacter baumannii has emerged as an opportunistic nosocomial pathogen that causes a wide range of severe infections, especially among intensive care unit and burn patients. Species of the genus *Acinetobacter* colonise a wide range of habitats, including soil, surface water, vegetables, animals and humans (Baumann, 1968; Towner, 2009). Except the hospital, natural reservoirs of *A. baumannii* remain ill-defined. During the first part of this PhD thesis we addressed the question of whether the colonization of different environments and/or ecological niches has led to a differentiation of multiple intra-specific *A. baumannii* populations with diverse phenotypic traits, or whether *A. baumannii* is a phenotypically homogeneous species endowed with broad adaptability. For this purpose, the genotypic and phenotypic diversity of a wide collection of *A. baumannii* strains isolated from environmental (non clinical, NCL), veterinary (VET) and human clinical (HCL) sources was assessed. These investigations provided a clearer understanding of *A. baumannii* diversity, and of the adaptive mechanisms that allowed this species to become a successful nosocomial pathogen.

Given the crucial role of iron in *A. baumannii*-host interactions (Zimbler *et al*., 2009; Mortensen *et al*., 2013), the iron metabolism has been exploited as a possible target for chelation-based antibacterial chemotherapy (Gentile *et al*., 2014). Then, the effect of iron restriction on *A. baumannii* growth and biofilm formation using different iron chelators and culture conditions was assessed. Strains and optimal growth conditions for the generation *A. baumannii* biofilms were preliminarily established (Gentile *et al*., 2014).

Lastly, the contribution of iron uptake systems to the virulence of *A. baumannii* ATCC 19606$^{T.}$was assessed. Siderophore-dependent and independent systems, as well as systems that remove iron from host's compounds, such as the heme uptake system, were investigated. Single and multiple knock-outs of *A. baumannii* genes implicated in iron uptake were generated, in order to gain insight into the contribution of individual iron uptake systems to survival and pathogenicity of *A. baumannii*. The iron uptake mutants were compared with the wild type strain for growth capability under different conditions of iron availability (e.g. in human serum, or in the presence of exogenous chelators). To assess the uptake specificity of the mutated systems, growth promotion assays were also performed with specific iron carriers. Lastly, the lethality in the *Galleria mellonella* insect infection model, was compared between the various mutants and the wild type.

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Chapter 2

Genotypic and phenotypic diversity of *A. baumannii* isolates from different sources

As outlined in Chapter 1, there are only sporadic isolates of *A. baumannii* obtained from the extra-hospital environment. Strain SDF, a body louse isolate, is the only well characterized environmental isolate until now, and showed substantial diversity from human isolates.

Thus, the the actual reservoir of *A. baumannii* species outside the hospital remains ill-defined, and it is not clear if extra-hospital (non human) isolates have specific adaptive traits that allow their differentiation from hospital isolates, or whether both hospital and non-hospital isolates share a common set of adaptive determinants.

To address these questions, in this Chapter, a representative collection of *A. baumannii* composed of isolates from non-human (environmental, veterinary) sources and isolates of human (clinical) origin was characterized by genotypic and phenotypic methods.

The genotypic assays were sequence group typing, that determines the belonging of a given isolate to a specific sequence group, including those representative of the major ICLs (Karah *et al*., 2012; Turton *et al*., 2006), and Random Amplified Polymorphic DNA (RAPD)-PCR (Grundmann *et al*., 1997), a fine typing method that evidences even minor genotypic differences between isolates. After having ascertained the extent of genetic diversity within the *A. baumannii* collection, a number of phenotypic assays were performed in order to differentiate isolates from different origins. Phenotyping included growth assays in different media, analysis of the production of virulence-associated exoproducts, the ability to form biofilm and resist to desiccation and, lastly, the study of antimicrobial and heavy metal resistance.

Manuscript in preparation

Genotypic and phenotypic diversity among *Acinetobacter baumannii* isolates from human, veterinary and environmental sources

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Introduction

Acinetobacter baumannii has emerged as an opportunistic nosocomial pathogen that causes a wide range of nosocomial infections, especially among patients in intensive care units (ICUs) and burn centers. Infections caused by *A. baumannii* include ventilator-associated pneumonia, bloodstream infections, secondary meningitis, wound, skin, soft tissue and urinary tract infections (Bergogne-Berezin & Towner 1996; McConnell *et al*., 2013; Roca *et al*., 2012). While most *Acinetobacter* species are free-living saprophytes, *A. baumannii* and closely-related species *A. nosocomialis* and *A. pittii* are almost exclusively found in the nosocomial setting (Tjernberg and Ursing, 1989; Towner, 2009). Three major groups of *A. baumannii* isolates, named International Clonal Lineages (ICL) I, II and III, are associated with infection and epidemic spread in hospitals worldwide (Visca *et al.*, 2011). The extraordinary ability of *A. baumannii* to survive on inanimate surfaces for long periods of time and to resist to antimicrobials and disinfectants could make the nosocomial environment a main reservoir for this species (Peleg *et al.*, 2008). Notably, a natural *A. baumannii* reservoir has not been identified yet.

A. baumannii is genotypically well separated from other *Acinetobacter* spp., and its low amounts of gene polymorphism indicates that it probably suffered a recent and severe evolutionary constriction, which could be related to adaptation to a restricted niche followed by a rapid expansion of multi-drug resistance clones (Diancourt *et al.*, 2010). This phase of clonal expansion could be driven by the major selective advantage associated with the widespread antibiotic resistance. However, since few strains have been recovered from non-

human sources, there is the possibility that clinical isolates represent only a small portion of the diversity of the species.

Although with low recovery rates, *A. baumannii* strains from non-human sources have been isolated (Dhakephalkar and Chopade, 1994; Eveillard *et al.*, 2013), in particular: from soil and aquaculture (Guardabassi *et al.*, 1999; Hoa *et al.*, 2011; Huys *et al.*, 2007; Rokhbakhsh-Zamin *et al.*, 2011), slaughterhouse meat (Ercolini *et al.*, 2009), poultry (Hamouda *et al.*, 2011), fish (Houang *et al.*, 2001; Huys *et al.*, 2007), vegetables (Berlau, 1999; Gennari and Lombardi, 1993), milk (Nam *et al.*, 2011), body and head lice (Eveillard *et al.*, 2013; Kempf *et al.*, 2012; La Scola and Raoult, 2004), and animals as cats, dogs and horses from veterinary clinics (Endimiani *et al.*, 2011; Zordan *et al.*, 2011). Recently, carbapenemase-producing *A. baumannii* isolates were collected from livestock animals (pig, fowl and cattle) in Lebanon (Al Bayssari *et al.*, 2014). Extra-hospital *A. baumannii* isolates have also been recovered from sites contaminated with hydrocarbons (Bordenave S *et al.*, 2007; Sarma PM *et al.*, 2004). A recent study showed the great diversity of some *A. baumannii* strains outside the hospital setting in Reunion Island (Pailhoriès *et al*., 2015). The best-characterised non human *A. baumannii* strain is SDF, originating from a human body louse (La Scola and Raoult, 2004). Unlike clinical *A. baumannii* isolates, SDF is susceptible to desiccation and to most antimicrobials, shows limited metabolic capacity, is impaired in the production of many virulence factors, and is non-virulent in an insect model of infection (Antunes *et al.*, 2011a). Combined with gene decay, these findings suggest an evolution of SDF to a restricted niche.

Understanding of the virulence mechanisms of *A. baumannii* is still at an initial stage (for a review, see Antunes *et al.*, 2014). There is a significant gap in our knowledge and understanding of the factors involved in *A. baumannii* pathogenesis. It is unknown whether other non-human *A. baumannii* strains are as specialised to environment(s) other than the hospital, or whether all *A. baumannii* strains strains share a common set of adaptive traits. Indeed, it is not clear whether now we are seeing the worldwide spread of multi-resistant *A. baumannii* lineages selected primarily on the basis of the resistance genes that they carry and/or if there is something special (eg. an adaptive/pathogenic trait) that confers epidemic potential.

To addess these questions, we investigate if there are evident differences between clinical *A. baumannii* strains and isolates found outside the hospital setting. To this purpose, the genetic fingerprint and the phenotypes related to growth and pathogenicity characteristics were investigated in a collection of 81 *A. baumannii* isolates from a environmental, veterinary and clinical sources.

Materials and methods

A. baumannii strains and species identification

Eighty-one *A. baumannii* isolates from a variety of sources throughout the world were studied (Table S1 of the Supplementary Materials). Strains were divided into 3 groups of 27 isolates each: non-clinical (NCL) strains from a variety of environmental sources (raw vegetables, raw milk, cheese, pre-cooked food, cow faeces, and water); veterinary strains (VET) from different animals (dogs, cats, horses and cows); human clinical strains (HCL) from blood, respiratory secretions, wounds, urine and cerebrospinal fluid of infected patients. Moreover, four representative and reference strains are inserted in this collection: AYE, ACICU, RUH 5875 and ATCC 17978. AYE is an epidemic multidrug resistant (MDR) clinical isolate responsible for an outbreak in France belonging to the ICL-I (Fournier *et al*., 2006). ACICU is another epidemic MDR isolate responsible for an outbreak in Rome (Italy) belonging to ICL-II (Iacono *et al*., 2008). The reference strain ATCC 17978 was isolated in 1951 from a 4-month old infant with fatal meningitis and it is different from AYE and ACICU for being susceptible to most antibiotics. RUH 5875, isolated from blood, is a reference strain of the north western ICL-III. For all isolates, identification as *A. baumannii* was confirmed by PCR detection of the $bla_{\text{OXA-51-like}}$ carbapenemase gene intrinsic to this species (Turton *et al*., 2006).

Isolates were preliminarily selected from a larger collection considering their geographical distribution (Table S1, in Supplementary material) so to maximize diversity, as inferred by Random Amplified Polymorphic DNA fingerprints with primer DAF4 (Antunes *et al*., 2011b; D'Arezzo *et al*., 2011; Gentile *et al*., 2014), and represent the most widespread ICLs and some emerging lineages.

Culture conditions

Bacterial strains were cultured in Luria Broth (LB) and in Chelex 100 resin-treated trypticase soy broth dialysate (TSBD) supplemented with 2.5 mM $MgSO_4$ and 0.5 mM $CaCl_2$ (Ohman *et al.*, 1980). The production of virulence-associated exoproducts (except for the release of lipopolysaccharides and the siderophore activity assay) was performed on the supernatants of the strains grown in TSBD for 18h. LB was used to determine the temperatures growth range (4°C to 47°C; 48-h incubation).

Mueller-Hinton (MH) broth (Becton Dickinson) was used to determine antibiotic and heavy metal minimum inhibitory concentrations (MICs).

The M9 iron-poor culture medium (Guterman, 1973) supplemented with 20 mM sodium succinate as the carbon source was used to determine siderophore production and lipopolysaccharides (LPS) release.

The Leeds Acinetobacter Medium (LAM; Jawad *et al.*, 1984) and Sodium Acetate Medium (SAM; Baumann, 1968) plates were used as elective media for the isolation of *Acinetobacter* species.

Polysaccharolytic activity assay

Cellulolytic, xylanolytic, pectinolytic, amylolytic activities were assessed on SAM agar plates supplemented with 5 g carboxymethyl cellulose (CMC) L^{-1} (Sigma), 5 g xylan L^{-1} (Sigma), 5 g apple pectin L^{-1} (Sigma) and 5 g starch L^{-1} (Sigma), respectively. For visualisation of β-D-glucan and xylan hydrolysis, an aqueous solution of 1 g congo red L^{-1} was added for 15 min, followed by addition of a 1M NaCl destaining solution for 15 min and then 1M HCl (Teather and Wood, 1982). Pectinolytic activity was revealed adding an aqueous 1% solution of hexadecyl trimethyl ammonium bromide (CTAB) for 10 min (Hankin *et al*., 1971). Amylolytic activity was detected by Lugol (iodine) staining for 10 min (Pascon RC *et al*., 2011). Enzymatic activities were identified by the development of a zone of clearing (halo) around the bacterial colonies. A positive control was used for all the polysaccharolytic activities.

Molecular typing and clonal diversity

DNA extraction from NCL, VET and HCL strains was obtained by the Wizard Genomic DNA Purification kit (Promega, USA). *A. baumannii* identification was confirmed by the detection of $bla_{\text{OXA-51-like}}$ carbapenemase gene intrinsic in this species (Turton *et al.*, 2006).

Major *A. baumannii* sequence groups (SGs) were assigned by PCR using primers for *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes (Turton *et al.*, 2007, Karah *et al.*, 2012). PCR products were checked by agarose gel electrophoresis. Interpretation of amplicon profiles was performed according to Karah *et al.* (2012).

NCL, VET and HCL strains were typed by RAPD-analysis with the DAF-4 primer (5'-CGGCAGCGCC). PCR conditions were: 94°C for 2 min, followed by 45 cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min (Grundmann *et al.*, 1997). ACICU replicates were used as internal controls of the RAPD-PCR profiles. Electropherograms were analyzed using Bionumerics 5.1 version (Applied

Maths), the Dice coefficient and UPGMA method. Similarity threshold ≥72% was used to define isolates belonging to the same RAPD cluster (Grundmann *et al.*, 1997).

Growth in human serum

The growth of the isolates in complement-free human serum (cfHS) was monitored for 48 h at 37°C, upon inoculation (OD_{600}=0.01) with an overnight M9 succinate culture.

The bactericidal activity of normal human serum (HS) was expressed as the Log_{10} of the ratio of forming units (CFU) before and after 2 h of incubation in HS.

Total extracellular proteins

The release of extracellular proteins was assessed in culture supernatants of *A. baumannii* strains grown in TSBD for 18h at 37°C using the Bradford (1976) assay.

Release of lipopolysaccharide (LPS)

Strains were cultured in M9 succinate medium for 18 h at 37°C. The OD_{600} was checked and the supernatants of the cultures were obtained and stored at -20°C. Samples were tested for the quantitative detection of bacterial endotoxin according the specifications of the Toxin Sensor™ Chromogenic LAL Endotoxin assay Kit (GenScript). The endotoxin concentration was measured as Endotoxin Unit per mL (EU/mL), and finally normalized to the optical density of each culture.

Phospholipase C activity assay

Extracellular phospholipase C activity was determined using the chromogenic substrate p-nitrophenylphosphorylcholine (PNPC) as described (Berka, 1981). Briefly, 900 µl of a solution of 10 mM PNPC in 250 mM Tris-HCl, pH 7.2, 1 µM $ZnCl_2$, 60% glycerol was added to 100 µl of filter-sterilized supernatants from overnight cultures. The reaction mixture was incubated at 37ºC for 24 h and the OD_{405} was measured. Activity was expressed in U/L of culture supernatants, and normalized to the OD_{600} of the cultures.

Phospholipase D activity assay

Phospholipase D production was assessed using Amplex Red Phospholipase D Assay Kit (Molecular Probes, Life Technologies) according to the manufacturer's specifications. The activity was expressed in U/L normalized to the total the OD_{600} of the cultures.

Proteolytic activity assay

Extracellular proteolytic activity was assessed using the azoalbumin assay as previously described (Philips *et al*., 1984). Briefly, 250 µl of a 1 mg/ml azoalbumin solution in Tris-HCl, pH 7.7, were added to 250 µl of supernatants from cultures cultivated in TSBD for 16 h, and then incubated in static at 37°C for 24 h. 80 µl of trichloroacetic acid was added at 13% final concentration to precipitate the non-degraded protein. Samples were incubated at -20°C for 20 min, centrifuged at 15,000 g for 10 min, and the OD_{440} of the resulting supernatants was measured at the spectrophotometer (BioSpectrometer basic, Eppendorf). Activity was expressed in U/L of culture supernatants and normalized to the OD_{600} of the cultures.

Hemolytic activity assay

Horse defibrinated blood was washed several times with sterile ice-cold phosphate buffered saline (PBS), pH 7.4, and 100 µl were incubated with 0.9 ml of supernatants from overnight cultures. After 3 h of incubation at 37ºC with gentle agitation, intact erythrocytes were harvested by centrifugation (1,000 g for 20 min, 4ºC). The amount of haemoglobin released in supernatants was measuring spectrophotometrically at 545 nm. The percentage of haemolysis (P) was calculated using the equation $P=(X-B)/(T-B)\times 100$, and then normalized to the OD_{600} of the bacterial cultures. X is the OD_{545} of the sample, while B and T represent the baseline and total haemolysis, i.e. the OD_{545} obtained with sterile LB and deionized water, respectively (Blocker *et al*., 1999).

Siderophore activity assay

The total iron-chelating activity in culture supernatants was determined by the chromoazurol S (CAS) liquid assay (Schwyn and Neilands, 1987). The Abs_{600} was read in a Wallac 1420 Victor3 V multilabel plate reader (Perkin Elmer). Iron-chelating activity was expressed as siderophore units (U) normalized to the cell density of the bacterial culture. Siderophore units are defined as $[(Abs_{600}\text{ reference} - Abs_{600}\text{ strain})/Abs_{600}\text{ reference}] \times 100$ (Payne, 1994).

Biofilm formation, surface pellicle and resistance to desiccation assays

Biofilm formation was performed in 96-wells-microtiter plates (O'Toole *et al*., 1999). OD_{600} of all the strains grown in 100 µl of TSBD at 37°C for 48h was measured with Wallac 1420 Victor 3V multilabel plate reader. Planktonic cells were removed, and the attached cells were gently washed with PBS. The plates were finally soaked on wet paper and air-dried. Then, the

plates were stained with 0.1% crystal violet solution for 20 minutes and gently washed with water. The surface-associated dye was solubilised with 96% ethanol. The microtiter plates were closed with parafilm and stored 20 minutes at –20°C. The biofilm biomass was quantified as OD_{600} of the released dye normalized to the OD_{600} of the culture.

To detect the surface pellicle, air-liquid interfaces were generated in 5mL glass tubes with a diameter of 12.8 mm. Pellicle formation was determined in MH broth (Oxoid, France) inoculated at initial OD_{600} = 0.01 following 72 h incubation at 37°C under static conditions. Pellicles were visually detected, and isolates were considered positive when a pellicle was covering the whole liquid surface (Marti *et al*., 2011).

Resistance to desiccation was tested in microtiter plates, according to a modification of a previous procedure (Jawad *et al*., 1998). Briefly, bacterial cells were grown in LB at 37°C for 16 h, then washed and diluted in water to an OD_{600} of 1.0 (about 10^9 viable cells). Three dilutions of the bacterial suspension were then performed in water (10^{-2}, 10^{-4} and 10^{-6}), and 10 µL of the undiluted and diluted samples (corresponding to about 10^7, 10^5, 10^3 and 10 cells) were poured onto the flat bottom of microtiter plates and air-dried. Then, plates were kept at constant 31% relative humidity (RH) obtained by the presence of a saturated solution of $CaCl_2$. The RH was constantly measured by digital hygrometer (Oregon Scientific). At different times, fresh LB medium was added and microtiter plates were incubated with shaking for 48 h at 37°C. Resistance to desiccation was assessed after 7, 15, 21 and 42 days through the determination of the Log_{10} reduction of viable cells. Resistance was expressed as the percentage of isolates for each group (NCL, VET and HCL) surviving after 42 days desiccation (*i.e.*, rescued following LB addition) at any of the tested dilution (most often the undiluted sample).

Susceptibility tests to heavy- and semi-metals and to antibiotics

Susceptibility to heavy- and semi-metals was determined by the agar-diluition method in MH agar (Riley and Taylor, 1989). Heavy metal salts and concentration ranges were: $K_2Cr_2O_7$ and $CdSO_4$ 0.03-2 mM; $NaAsO_2$ and $NiSO_4$ 0.3-20.0 mM; $HgCl_2$ 3.1-200 µM, Na_2TeO_3 0.8-50 µM. Isolates (ca. 10^4 cells) were spotted onto the agar surface the replica-plating method, then incubated at 37℃ for 72 hours. The MIC was calculated as the lowest metal concentrations which did not allow visible growth on the agar surface.

Antibiotic susceptibility was determined by the disc-diffusion method (Bauer *et al.*, 1966). The following antibiotics were tested: amikacin (AKN), aztreonam (ATM), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (COL), gentamicin (GMN), imipenem (IPM), ampicillin-

sulbactam (SAM), sulphametoxazole-trimethoprim (SXT), tigecyclin (TIG). Susceptibility data were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2007), European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2013) and British society for antimicrobial chemotherapy (BSAC), Methods for Antimicrobial Susceptibility Testing (2013), and expressed as susceptible (S), intermediate (I) and resistant (R).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5 software. The one-way Anova test (with Post test analysis, Turkey's multiple comparison) was used to compare quantitative differences in phenotypic assays between NCL, VET and HCL strains. Differences were considered statistically significant when the p value was <0.05.

Ethic statement

Human serum was obtained from healthy volunteers who gave their written informed consent to the study, which was approved by the Review Board of the Department of Science of the University Roma Tre.

Results and Discussion

Molecular typing and clonal diversity

The *bla*$_{\text{OXA-51-like}}$ carbapenemase gene was amplified from all isolates, confiming their identification as *A. baumanni*.

The distribution of SGs among NCL, VET and HCL *A. baumannii* isolates is shown in Figure 1A. SGs 1, 2 and 3 (ICL-II, I and III, respectively) prevailed among VET and HCL isolates, whereas NCL isolates were distributed among less common SGs, including 4 new SG variants (represented by 5 NCL isolates; Figure S1 in Supplementary material). RAPD typing with primer DAF4 (Grundmann *et al*., 1997) evidenced 17 clusters (A-Q) with a similarity threshold ≥ 72% (Figure 1B). All RAPD clusters included ≥2 isolates belonging to the same sequence group and/or having the same origin. Four of the five NCL isolates showing the new SG variant, belonged to the same cluster.

By combining SG and RAPD results, it can be concluded that both VET and HCL isolates, although being unrelated in time and place of origin, show much higher genetic homogeneity than NCL isolates. This observation is in line with the notion that only a few *A. baumannii* lineages are responsible for the majority of human and animal infections.

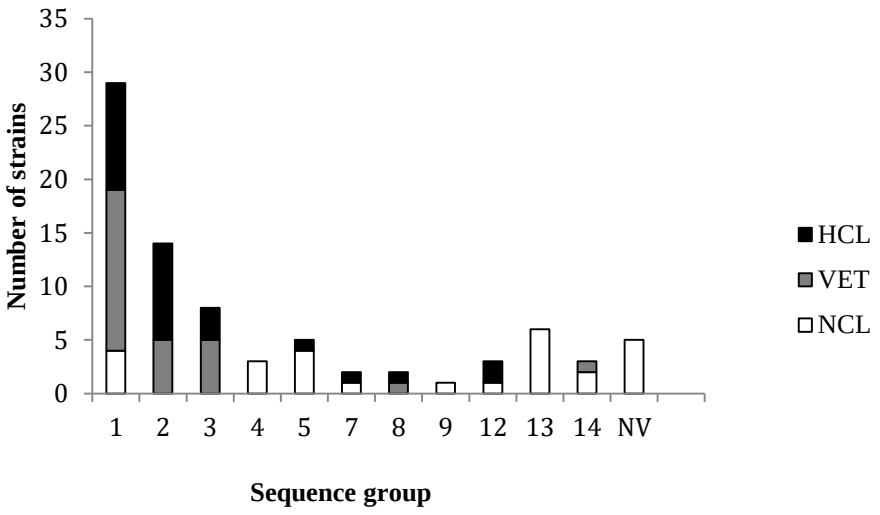

Figure 1 A . Sequence group distibution of non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* strains. NV, new sequence group variant.

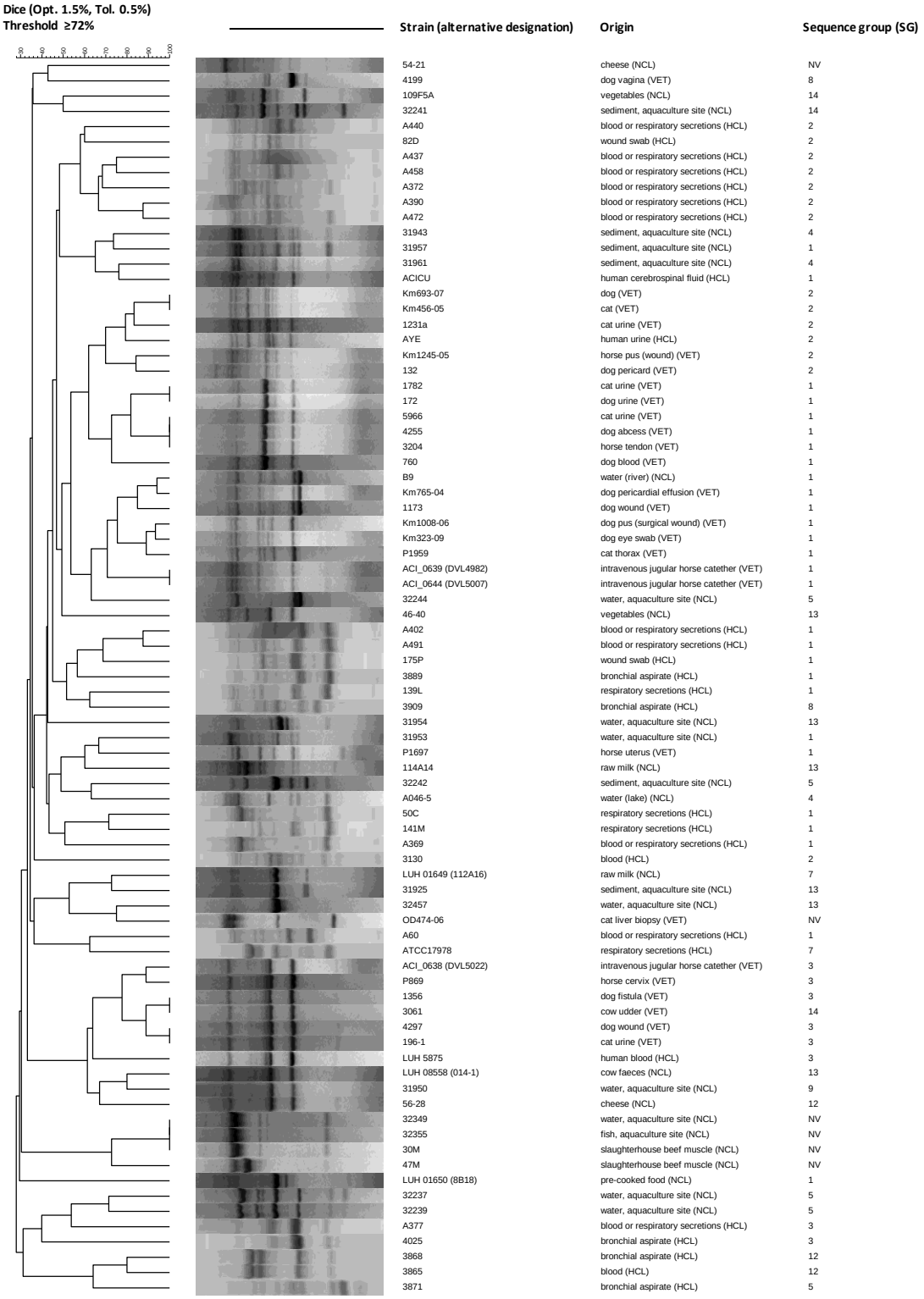

Figure 1 B. Clustering relationship of 81 *A. baumannii* strains from non-clinical (NCL), veterinary (VET) and human clinical (HCL) sources analyzed by RAPD with primer DAF-4. The dendrogram was generated using the Dice coefficient and UPGMA clustering with a 1.5 % optimization and 0.5 % tolerance limit (Spence *et al*., 2002). Similarity threshold of ≥72 % was used to define isolates belonging to the same RAPD type (Spence *et al*., 2002). The sequence groups (SG) and the origin of each strain are shown. NV, new SG variant. Isolates are also labelled according to their origin: NCL, non-clinical; VET, veterinary origin; HCL, human clinical.

Growth characteristics

The temperature range (4°C-47°C interval) for *A. baumannii* growth was compared between NCL, VET and HCL isolates. All isolates grew in LB medium at temperatures ranging from 18°C to 44°C, and 55 of them grew also at the highest temperature tested (47°C, Table S2). However, only 12 (44%) of NCL isolates grew at 47°C, as opposed the vast majority of VET and HCL isolates (21 and 22 isolates, accounting for 78 and 81%, respectively). Irrespective of the source, it can be noticed that all *A. baumannii* strains tested could grow at febrile temperatures for both humans and animals.

Whereas all *A. baumannii* isolates grew on LAM, it was noted that 7 isolates (6 NCL and 1 HCL) did not grow on SAM mineral medium (Table S2 in Supplementary Material), in spite of the fact that this medium is commonly used in enrichment cultures for *A. baumannii*.

Polysaccharolytic activity

The ability to degrade polysaccharides is a hallmark of bacteria living in soil or in association with plants (Lynch, 1976). Thus, we wondered whether environmental (NCL) *A. baumannii* isolates could be differentiated from VET and HCL counterparts based on their polysaccharolytic activity. To this purpose, the ability of hydrolysing different vegetal polysaccharides was tested in mineral medium (SAM agar plates) supplemented with xylan, starch, cellulose and pectin. Only 10 isolates, including 1 NCL and 9 VET isolate, had weak amylolytic activity, while no isolate had xylanolytic, cellulolytic or pectinolytic activities (Table S2 in Supplementary Materials), indicating that polysaccharide hydrolysis is uncommon in *A. baumannii*, irrespective of the isolate source.

Resistance to human serum

With few exceptions, *A baumannii* isolates showed an overall resistance to the killing activity of HS, and some could even multiply in this medium. Althoug no statistically significant differences in bacterial killing were observed between the three groups (Figure 2), it is interesting to note that more sensitive isolates (*i.e.*, the isolates showing ≥ 1 Log_{10} reduction of viability) were more prevalent among NCL (30%) than VET and HCL isolates (7% and 14%, respectively).

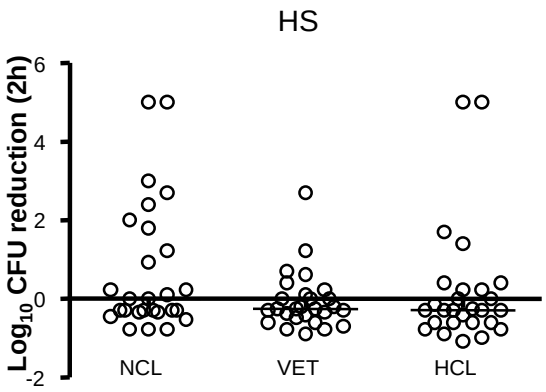

Figure 2. Bactericidal activity of human serum (HS), calculated as Log_{10} of the ratio between viable cells (CFU) before and after 2 h incubation in HS.

The serum bactericidal activity is primarily ascribed to the bacteriolytic properties of complement. However, even upon complement inactivation, HS retains antibacterial properties and provides a hostile environment for bacterial growth due to the presence of heat-resistant innate immunity factors (Stratton, 1988). For this reason, the growth levels of *A baumannii* isolates in cfHS were determined after 48 h at 37°C. Coherent with HS resistance results, NCL isolates showed significantly lower capability to multiply in cfHS than VET and HCL isolates (Figure 3).

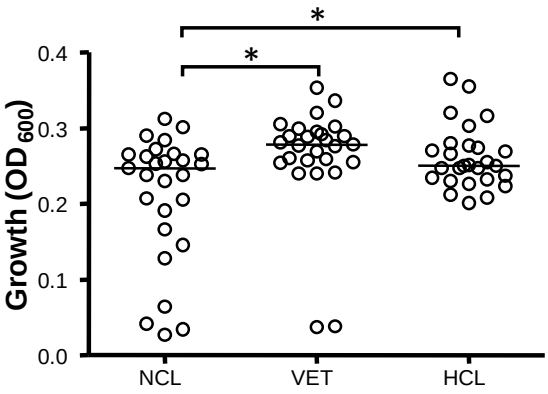

Figure 3. Growth of *A. baumannii* isolates from non-clinical (NCL), veterinary (VET) and human clinical (HCL) sources in complement-free human serum for 48 h at 37°C. One way Anova with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups. Asterisks indicate statistically significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

The above results denote that VET and HCL *A. baumannii* isolates differ from NCL isolates for their capability to resist to and multiply in human serum, a milieu which closely mimics

the environment encountered by bacteria during hematogenous dissemination. Thus *A. baumannii* isolates from VET and HCL sources could be endowed with greater propensity to systemic infection**.**

Iron uptake capabilities

The observation that nearly all *A. baumannii* isolates can multiply in cfHS implies that this species can efficiently counteract the iron withholding properties of HS (i.e. transferrin), so to obtain iron during *in vivo* growth. Siderophores are the main bacterial iron carriers, and many siderophore systems have so far been described in *A. baumannii* (§ 1-8 in this thesis). Therefore, siderophore activity levels were compared between NCL, VET and HCL *A. baumannii* isolates. Siderophore levels were higher in VET isolates than in the other two groups (Figure 4A).

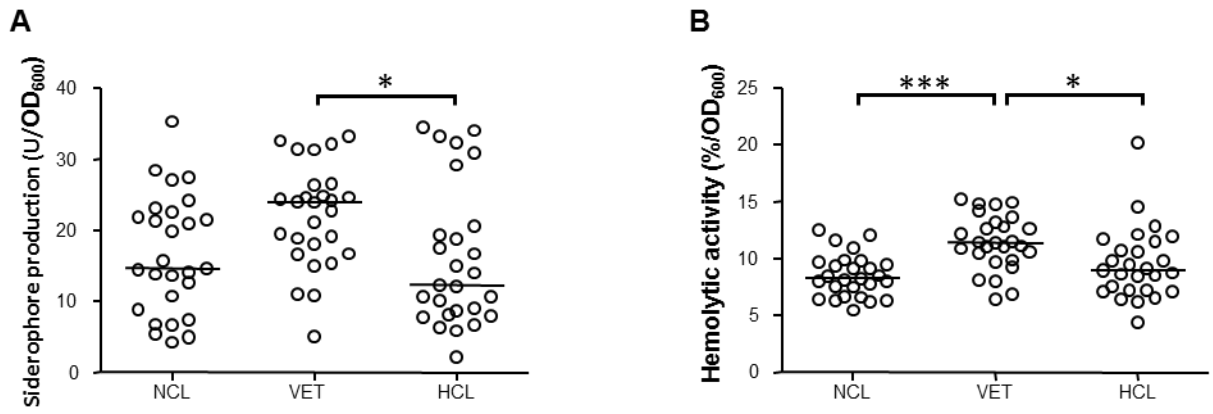

Figure 4. Siderophore production (A) and hemolytic activity on horse erythrocytes (B) in culture supernatants of non-clinical (NCL), veterinary (VET) or human clinical (HCL) *A. baumannii* isolates. Bacteria were grown in TSBD (A) or M9 (B) at 37°C for 18 h. Values were normalized to the cell density. Data points represent the median values from three independent experiments for each isolate tested. The line bar represents the mean value for each group. One way Anova with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups. Asterisks indicate statistically significant differences (* p<0.05; ** p<0.01; *** p<0.001).

In addition to siderophores, *A. baumannii* possess heme uptake systems (§ 1.8), and some strains also hemolytic properties (Antunes *et al.*, 2011a; Modarresi *et al.*, 2015). Since hemolysis can facilitate heme iron acquisition during infection, we compared the haemolytic activity between the three groups of *A. baumannii* isolates. While no differences in hemolysis

were observed between NCL and HCL isolates, VET isolates were significantly more haemolytic than the other two groups (Figure 4B).

Thus, VET isolates appear to be endowed with more pronounced iron uptake capabilities than NCL and VET isolates.

Release of extracellular proteins and proteolytic activity

Preliminary to the investigation of virulence-associated exoproducts, the amount of total extracellular proteins was compared between *A. baumannii* groups from different sources. Interestingly, VET isolates released significantly higher protein levels than the other two groups (Figure 5A).

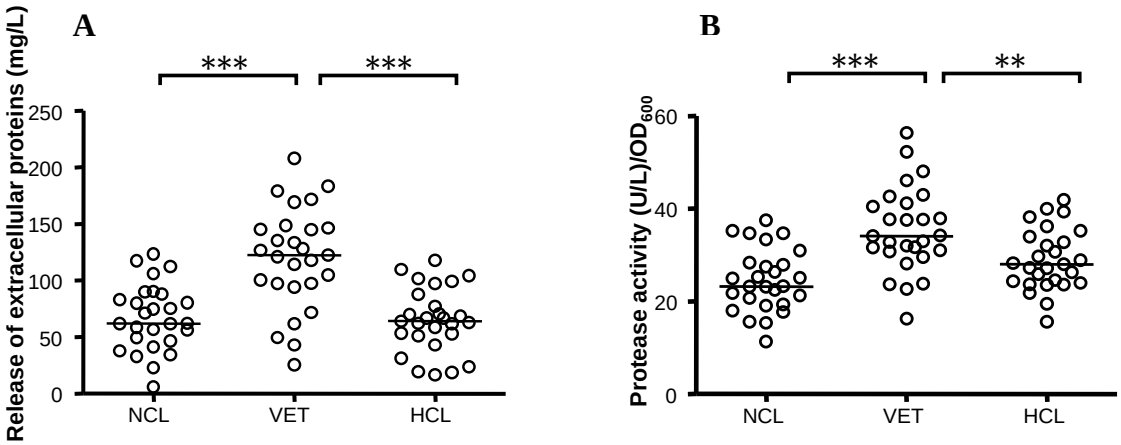

Figure 5. Release of extracellular proteins (A) and protease activity (B) in culture supernatants of non-clinical (NCL), veterinary (VET) or human clinical (HCL) *A. baumannii* was grown in TSBD at 37°C for 18 h. Extracellular protein concentrations are expressed in mg of protein per L of supernatant. Data points of A and B represent the median values from three independent experiments for each isolate tested. The line bar represents the mean value for each group. One way Anova with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups. Asterisks indicate statistically significant differences (* p<0.05; ** p<0.01; *** p<0.001).

Proteases are extracellular enzymes commonly implicated in bacterial pathogenesis. Recenty, a zinc-dependent, metallo-endopeptidase (CpaA) was identified in *A. baumannii*, and found to deregulate human blood coagulation *in vitro*, thereby contributing to *A. baumannii* virulence (Tilley *et al.*, 2014). CpaA is exported extracellularly by a type II secretion apparatus (Harding *et al.*, 2016). Moreover, a proteomic analysis of *A. baumannii* outer membrane vesicles identified putative proteases and a putative hemolysin as potential

virulence factors (Jin *et al.*, 2011). These findings led us to investigate the proteolytic activity released by the three groups of isolates, in search for distinctive features. As observed for total released proteins, also protease activity was higher for VET than NCL or HCL isolates (Figure 5B).

Phospholipase C and D production

Phospholipases catalyze the cleavage of phospholipids. *A. baumannii* produces phospholipases C and D which contribute to bacterial pathogenesis by aiding in the lysis of host cells, and by degrading phospholipids present at mucosal barriers to facilitate bacterial invasion. These enzymes probably act in a concerted manner as virulence factors, thus playing an important role in host cell invasion (Antunes *et al.*, 2011a; Stahl *et al.*, 2015). On this basis, we attempted to differentiate NCL *A. baumannii* isolates from VET and HCL counterparts according to phospholipases production levels. Surprisingly, phospholipase C production was higher in NCL than in VET or HCL isolates (Figure 6A), while there were no statistically significant differences for phospholipase D activity between the three groups (Figure 6B).

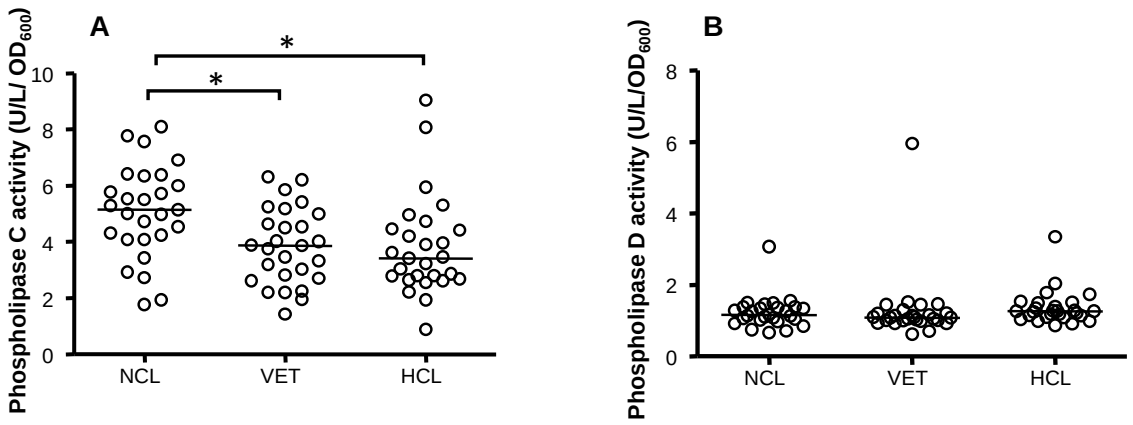

Figure 6. Phospholipase C (A) and phospholipase D production (B) in culture supernatants of non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* strains grown in TSBD at 37°C for 18 h. The activity was expressed as U/L of culture supernatants normalized to the cell density (OD_{600}). Data points represent the median values from three independent experiments for each isolate tested. The line bar represents the mean value for each group. One way Anova with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups. Asterisks indicate statistically significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Lipopolysaccharide shedding

LPS typically consists of a hydrophobic outer membrane anchor domain, termed lipid A or endotoxin, a nonrepeated core oligosaccharide, and a O distal antigen polysaccharide comprised of repeat-unit structures having variable lengths. Lipid A is the most toxic and pro-inflammatory region of LPS, capable of mounting an overwhelming host inflammatory response that correlates with the morbidity and mortality of infected patients (Pier, 2007). *A. baumannii* LPS is also important for serum resistance and *in vivo* survival (Luke *et al.*, 2010). LPS-mediated activation of TLR4 induces the septic shock cascade during infection and, interestingly, more-virulent *A. baumannii* strains shed more LPS during growth than less-virulent strains, resulting in enhanced TLR4 activation (Lin *et al.*, 2012). Since LPS shedding is a major pathogenicity signature of *A. baumannii*, we searched for differences in the amount of shed LPS through whole *A. baumannii* collection. To this purpose, isolates were grown in M9 minimal medium to the mid-exponential phase (18 h) to avoid LPS release due to bacterial death and lysis. Although no significant differences were observed among the three groups, it was interesting to notice that NCL isolates released median LPS levels almost twice the other groups, and showed greater intra-group variability (Figure 7).

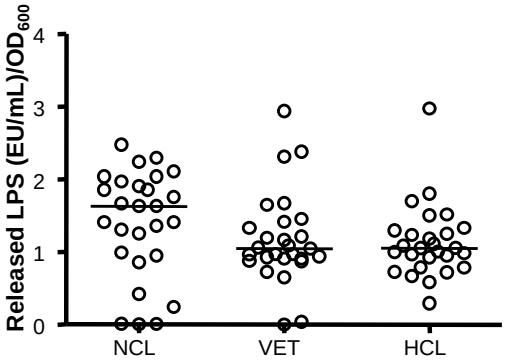

Figure 7. Release of lypopolysaccharide in culture supernatants of non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* strains grown in M9 at 37°C for 18 h. Data points represent single value for each strain. The line bar represents the median value for each group. One way Anova analysis with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups (* p<0.05; ** p<0.01; *** p<0.001).

Biofilm, surface pellicle formation, and resistance to desiccation

A. baumannii can form multicellular communities encased in an extracellular matrix, consisting of secreted proteins, polysaccharides, nucleic acids, and cellular debris, attached to abiotic or biotic surfaces, called biofilm. These sessile microbial communities are composed of by cells that are reversibly attached to a solid matrix, and that are embedded in a layer of self-produced extracellular polymeric substances (reviewed by Longo *et al.*, 2014). The clinical relevance of *A. baumannii* biofilms has increased with the increasing prevalence of biofilm-related infections (Navon Venezia *et al.*, 2005). These infections are extremely difficult to cure due to the inherent resistance of biofilms to antibiotics and host's immune defences. However, the biofilm mode of growth is also beneficial to bacteria living in natural environments, since it allows the attachment to solid substrates, and provides a protected mode of growth that facilitates survival in hostile environments (Hall-Stoodley *et al.*, 2004). Therefore, it is not surprising that biofilm levels did not differ significantly between NCL, VET and HCL *A. baumannii* isolates tested in this study (Figure 8).

Different from biofilm, the ability to form air-surface pellicles was more frequent among HCL isolates (48%) than among NCL and VET isolates (26% and 22%, respectively; see Table S2 in Supplementary Material). This observation suggests that biofilm and surface pellicle formation are independent characteristics, the latter being significantly associated with isolates responsible for human infection.

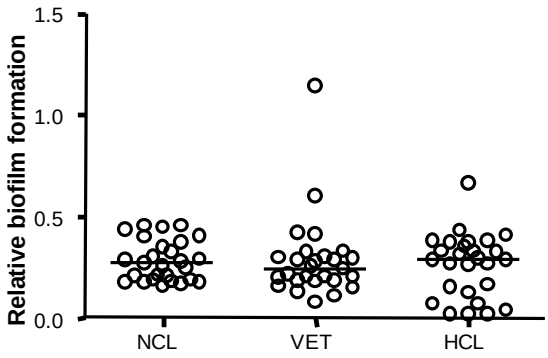

Figure 8. Biofilm formation onto the surface of 96-well polystyrene microtiter plates by non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* isolates grown statically for 48 h in TSBD at 37ºC. Biofilm levels were normalized by the bacterial growth. Data points represent the mean values from two independent experiments for each isolate tested. The line bar represents the median value for each group. One way Anova analysis with Post test (Tukey's multiple comparison) was used to determine statistical differences between the three groups (* p<0.05; ** p<0.01; *** p<0.001).

Resistance to desiccation is an intrinsic feature of *A. baumannii* and has been proposed to contribute to the success of epidemic lineages because of their long persistence in the hospital environment (Jawad *et al.*, 1998). Therefore, the survival of the different *A. baumannii* isolates onto a dry surface was weekly monitored over a period of 6 weeks. Results (Table S3 in Supplementary Material) denote no significant differences in survival rates between NCL, VET and HCL isolates, although VET isolates showed stronger tendency to 6-weeks survival (93% survivors) compared with NCL and HCL isolates (75% survivors each).

Heavy metal and antimicrobial resistance

Some heavy and semi-metal salts, including chromium, mercury, cadmium, tellurium, arsenic and nickel, are major environmental pollutants. These elements can be present in the environment at high levels as the result of man-made activities, and can exert a selective pressure on the environmental microflora, resulting in the selection of resistant strains. Some of these elements, however, have also been used until the 20th century as components of medicines. In particular, chromium, mercury and arsenic have been incorporated as active components of various disinfectant or chemotherapeutic formulations. Therefore, we conceived to determine the resistance to such compounds in the three *A. baumannii* groups, looking for source-dependent differences. While groups could not be differentiated based on a univocal resistance pattern to any of the tested compounds, some significant differences were observed (Figure 9). The most intriguing differences were those observed for mercury and arsenic resistance in VET and HCL groups, respectively, compared with the NCL group. On the other hand, the NCL group was significantly more resistant to nickel ad cadmium compared with the HCL group. It is therefore tempting to speculate that previous exposure to mercury and arsenic in the veterinary and clinical practice may have selected for higher resistance to these elements, as opposite to what observed for nickel ad cadmium, two typical environmental pollutants, to which HCL isolates showed relatively low resistance. No significant differences in resistance to Cr and Te were observed between NCL, VET and HCL isolates (Figure 9).

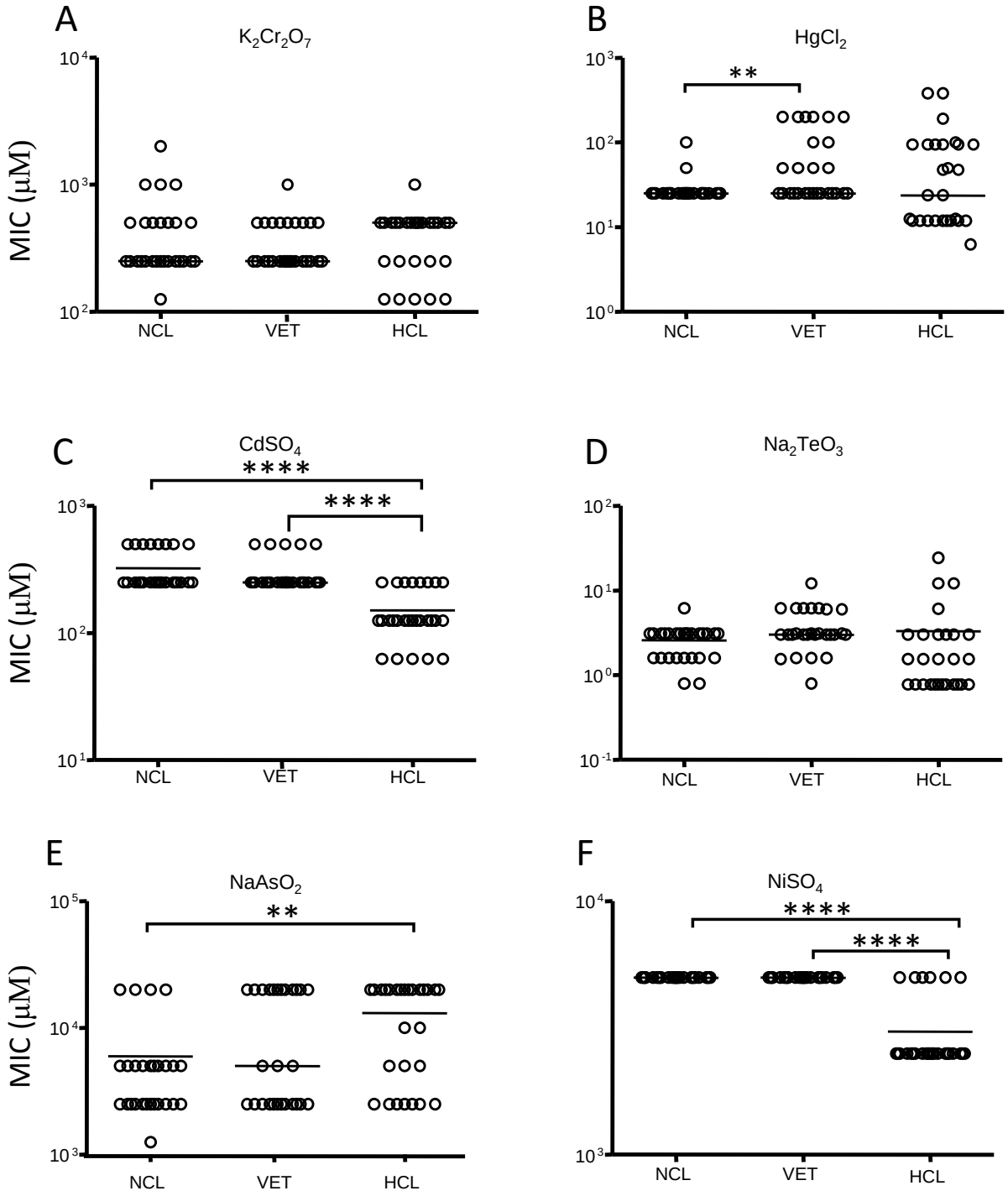

Figure 9. Distribution of the MIC (µM) values for $K_2Cr_2O_7$, $HgCl_2$, $CdSO_4$, Na_2TeO_3, $NaAsO_2$ and $NiSO_4$ in non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* isolates. The concentration ranges were: 0.8-50 µM for Na_2TeO_3 (D); 3.125-200 µM for $HgCl_2$ (B); 31.2-2,000 µM for $K_2Cr_2O_7$, (A) and $CdSO_4$ (C); 312.5-200,000 µM for $NaAsO_2$ (E) and $NiSO_4$, (F). One way Anova with Post test (Tukey's multiple comparison test) was used to determine statistical differences between the three groups. Asterisks indicate statistically significant differences (*p value< 0.05; ** p<0.01; *** p<0.001).

Lastly, antibiotic susceptibility testing was performed using a panel of 10 antibiotics (Figure 10). The vast majority of isolates were resistant to aztreonam and susceptible to tigecycline, irrespective of their source. These results were expected, since aztreonam resistance is conferred by the AmpC-type β-lactamase which is widespread in *A. baumannii* (Rodríguez-Martínez *et al.*, 2010) whereas tigecycline is a novel glycilglycine antibiotic which reached the market after the isolation of almost all the strains used in this work. Also sulbactam (in combination with ampicillin) and colistin were active on most or all isolates of the three groups, in line with the efficacy of these drugs on recent clinical isolates (Garnacho-Montero *et al.*, 2015). Striking differences between HCL and the other two groups were indeed observed for amikacin, ceftazidime, and imipenem; in fact, resistance to these drugs was very frequent among clinical isolates only (70% to 100% non-susceptible isolates), while it was absent or very infrequent among veterinary and environmental isolates. Finally, resistance to ciprofloxacin, gentamycin, and sulphamethoxazole/trimethoprim appeared very common in both VET and HCL groups, while it was nearly absent in the NCL group (Figure 10). This is because these drugs, or their congeners, have broadly been used in both animal and human therapy.

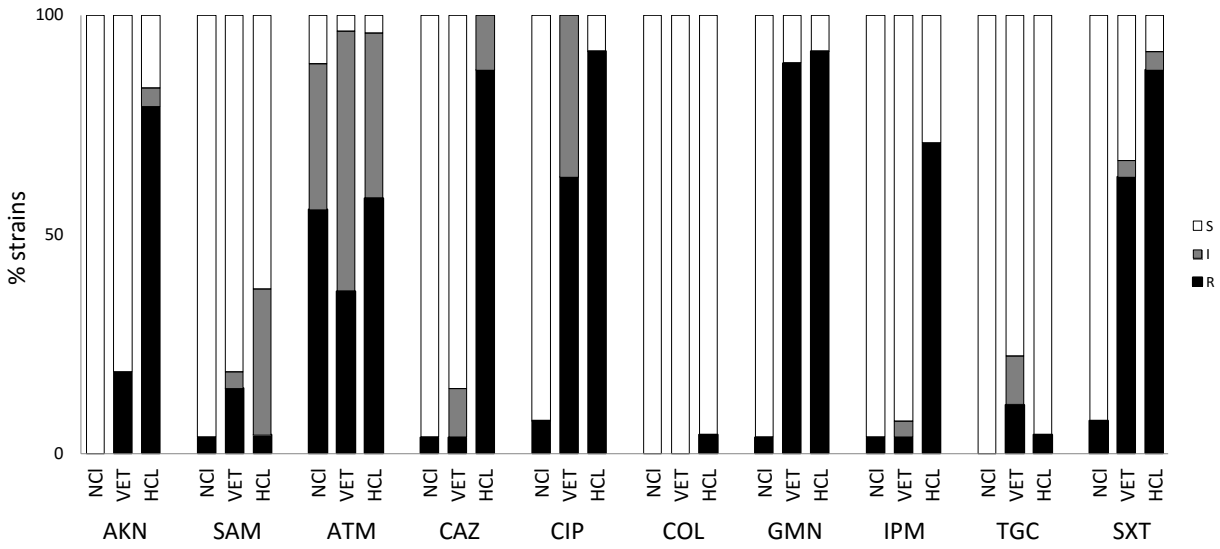

Figure 10. Antibiotic susceptibility of non-clinical (NCL), veterinary (VET) and human clinical (HCL) *A. baumannii* isolates. Each histogram reports the percentage of sensitive (S, white), intermediate (I, grey) and resistant (R, resistant) for each group. AKN, amikacin, SAM ampicillin-sulbactam, ATM, aztreonam, CAZ, ceftazidime, CIP, ciprofloxacin, COL, colistin, GMN, gentamycin, IPM, imipenem, TGC, tigecycline and SXT, sulphamethoxazole/trimethoprim.

Concluding remarks

The extra-hospital reservoir of *A. baumannii*, if any, remains still elusive. Intensive searches conducted over many years have led to the isolation of a limited number strains from non-hospital sources, and few of such strains originate from natural environments or sources certainly unlinked to human infection. Furthermore, *A. baumannii* is increasingly reported in association with animal infections, raising the possibility that animals could become infected from still unknown environmental sources. However, the origin(s) and transmission route(s) is(are) difficult to trace for veterinary infections. Indeed, it is unclear if different populations of *A. baumannii* exist in natural habitats, as opposed to those found in the clinics (either human or veterinary). MLST studies have clearly delineated a few major lineages responsible for most of the infections worldwide, but a number of minor types represented by one or few isolates also exist, and most often these isolates originate from sporadic cases or from the extra-hospital environment. In addition, while virulence properties have been delineated for clinical and veterinary *A. baumannii* isolates and their association with human and animal pathology established, very few data are available on the virulence potential of isolates from environmental sources.

Based on the above considerations, we conducted an in-depth genetic and phenotypic characherization of NCL, VET and HCL *A. baumannii* isolates, focusing on traits related to environmental fitness and virulence in animal hosts. To ensure diversity inside the three groups, representative isolates from several collections and different regions were selected. When available, typing data were preliminarily reviewed for isolates from similar sources or from the same country, so to avoid the selection of potentially related isolates. A total of 81 isolates, equally distributed in the three groups, was analysed.

RAPD fingerprinting substantiated remarkable diversity between isolates, since identical fingerprints were obtained for only 7 groups composed of 2 or 3 isolates each, whereas unique fingerprints were observed by 65 out of 81 isolates. Notably, sequence grouping revealed that isolates belonging to epidemic ICL-I, -II and -III lineages were more frequent in VET (88.9%) and HCL (81.5%) than in NCL (14.8%) isolates. Moreover, rare or new SG profiles (*i.e.*, additional to those defined by Karah *et al*., 2012), were characteristic of NCL isolates only, indicating greater genetic diversity within this group.

A number of phenotypic traits were then analysed with the aim of identifying distinctive features that could allow differentiation between groups.

All isolates grew on LAM and in M9 mineral medium supplemented with succinate, but 7 failed to grow on SAM, indicating that: *i*) auxotrophy can be excluded as cause of growth

failure and, *ii*) acetate is not invariably used as carbon source by *A. baumannii*. The possibility that SAM does not support the growth of all *A.baumannii* strains should be taken into account, since SAM is indicated as an elective medium for *A. baumannii* enrichment.

Polysaccharolytic activity was infrequently found in all three groups and observed only using starch as the substrate, denoting the limited potential of *A. baumanni* to obtain sugars from hydrolysis of plant polysaccharides, which represent the main carbon sources in soil.

Among several phenotypes tested, no differences in serum resistance, phospholipase D production, biofilm formation and LPS shedding were observed between the three groups. Assuming that these phenotypes can influence (either directly or indirectly) bacterial virulence, NCL isolates would predictably have the same potential as VET and HCL counterparts to cause disease through these pathogenicity traits. It is also possible that these characterisics are equally important for the success both in the host and in the environment. However, remarkable differences between NCL and either VET or HCL isolates were observed for other traits related to *in vivo* fitness and virulence, and these differences would provide some clues to understanding *A. baumannii* pathogenicity in animals. For instance, NCL isolates showed a narrower growth temperature range and reduced ability to multiply in serum and form surface pellicle, suggesting limited growth capabilities *in vivo*. Compared with the VET group, NCL isolates produced less siderophores and proteases, though the importance of these characteristics in determining virulence in humans is questionable, since not evident for the HCL group.

Furthermore, it was interesting to observe a different pattern of resistance to some toxic metals between NCL and VET or HCL isolates. The NCL group was more sensitive to mercury and arsenic, two metals with long history as anti-infectives, but more resistant to nickel ad cadmium, two typical environmental pollutants. However, the most striking differences were observed at the level of antibiotic resistance, which clearly delineated two groups; one composed of the overall susceptible NCL population, and the other including both VET and HCL resistant isolates. Thus, it appears that the *A. baumannii* population living in the extra-hospital environment is genetically distinct from epidemic ICLs and invariably susceptible to antibiotic since not previously exposed to these drugs. Conversely, VET and HCL strains belong to the epidemic ICLs and are endowed with much higher resistance to antimicrobials, irrespective of whether they originate from animals of humans.

In conclusion, this study suggests that *A. baumannii* pathogenicity is both multifactorial and combinatorial, and indicates that antibiotic resistance rather than virulence could have played a crucial role for the success of *A. baumannii* as a pathogen of both humans and animals.

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Supplementary material

Table S1. Strains used in this study

Strain (alternative designation)	Origin (sample)[a]	Year of isolation	Country	R-profile[b]	Sequence group (International clonal lineages, ICL)	Reference
46-40	NCL (raw vegetables)	1984	Italy	ATM	13	Gennari *et al.*, 1993
109F5A	NCL (raw vegetables)	1984	Italy	ATM	14	Gennari *et al.*, 1993
54-21	NCL (cheese)	1984	Italy	ATM	NV1	Gennari *et al.*, 1993
56-28	NCL (cheese)	1984	Italy	ATM	12	Gennari *et al.*, 1993
114A14	NCL (raw milk)	1984	Italy	ATM	13	Gennari *et al.*, 1993
LUH01649 (112A16)	NCL (raw milk)	1984	Italy	ATM	7	Gennari *et al.*, 1993
LUH01650 (8B18)	NCL (pre-cooked food)	1984	Italy	ATM	1 (ICL-II)	Gennari *et al.*, 1993
LUH08558 (014-1)	NCL (cow faeces)	1996	The Netherlands	ATM	13	Dijkshoorn, 1996;
31925	NCL (sediment, aquaculture site)	1982	Malaysia	ATM	13	Huys *et al.*, 2007
31943	NCL (sediment, aquaculture site)	1988	Malaysia	ATM	4	Huys *et al.*, 2007
31950	NCL (water, aquaculture site)	2006	Malaysia	ATM	9	Huys *et al.*, 2007
31953	NCL (water, aquaculture site)	2006	Malaysia	ATM	1 (ICL-II)	Huys *et al.*, 2007
31954	NCL (water, aquaculture site)	2006	Malaysia	ATM, CIP, SXT	13	Huys *et al.*, 2007
31957	NCL (sediment, aquaculture site)	2006	Malaysia	ATM	1 (ICL-II)	Huys *et al.*, 2007
31961	NCL (sediment, aquaculture site)	2006	Malaysia	ATM	4	Huys *et al.*, 2007
32237	NCL (water, aquaculture site)	2006	Thailand	ATM	5	Huys *et al.*, 2007
32239	NCL (water, aquaculture site)	2006	Thailand	ATM	5	Huys *et al.*, 2007
32241	NCL (sediment, aquaculture site)	2006	Thailand	ATM	14	Huys *et al.*, 2007
32242	NCL (sediment, aquaculture site)	2006	Thailand	ATM	5	Huys *et al.*, 2007
32244	NCL (water, aquaculture site)	2006	Thailand	ATM	5	Huys *et al.*, 2007
32349	NCL (water, aquaculture site)	2006	Vietnam		NV2	Huys *et al.*, 2007
32355	NCL (fish, aquaculture site)	2006	Vietnam	ATM	NV2	Huys *et al.*, 2007
32457	NCL (water, aquaculture site)	2006	Vietnam		13	Huys *et al.*, 2007
B9	NCL (water, river)	2009	France	SAM, ATM, CAZ, CIP, GMN, IPM, SXT	1 (ICL-II)	Girlich *et al.*, 2010
30M	NCL (slaughterhouse beef muscle)	2008	Italy		NV3	Ercolini *et al.*, 2009
47M	NCL (slaughterhouse beef muscle)	2008	Italy	ATM	NV4	Ercolini *et al.*, 2009
A046-5	NCL (water, lake)	2008	Italy	ATM	4	Seifert & Dijkshoorn, 2008

Km1008-06	VET (dog pus surgical wound)	2004-2009	Switzerland	ATM, CIP, GMN, TGC	1 (ICL-II)	Endimiani *et al.*, 2011
OD474-06	VET (cat liver biopsy)	2004-2009	Switzerland	CIP, GMN, SXT	1 (ICL-II)	Endimiani *et al.*, 2011
Km693-07	VET (dog bronchial aspirate)	2004-2009	Switzerland	SAM, ATM, GMN, SXT	2 (ICL-1)	Endimiani *et al.*, 2011
Km323-09	VET (dog eye swab)	2004-2009	Switzerland	SAM, ATM, CIP, GMN, IPM, TGC, SXT	1 (ICL-II)	Endimiani *et al.*, 2011
Km456-05	VET (cat urine)	2004-2009	Switzerland	GMN, SXT	2 (ICL-1)	Endimiani *et al.*, 2011
Km1245-05	VET (horse pus wound)	2004-2009	Switzerland	GMN, SXT	2 (ICL-1)	Endimiani *et al.*, 2011
Km765-04	VET (dog pericardial effusion)	2004-2009	Switzerland	CIP, GMN	1 (ICL-II)	Endimiani *et al.*, 2011
ACI_0638 (DVL5022)	VET (intravenous horse catheter)	2000-2008	Belgium	AKN, ATM, CIP, GMN, SXT	3 (ICL-III)	Vaneechoutte *et al.*, 2000
ACI_0639 (DVL4982)	VET (intravenous horse catheter)	2000-2008	Belgium	ATM, GMN, SXT	1 (ICL-II)	Vaneechoutte *et al.*, 2000
ACI_0644 (DVL5007)	VET (intravenous horse catheter)	2000-2008	Belgium	GMN, SXT	1 (ICL-II)	Vaneechoutte *et al.*, 2000
5966	VET (cat urine)	2000-2008	Germany	CIP, GMN	1 (ICL-II)	Zordan *et al.*, 2011
132	VET (dog pericardium)	2000-2008	Germany	SAM, ATM, GMN, SXT	2 (ICL-1)	Zordan *et al.*, 2011
4255	VET (dog abscess)	2000-2008	Germany	ATM, CIP, GMN, SXT	1 (ICL-II)	Zordan *et al.*, 2011
3204	VET (horse tendon)	2000-2008	Germany	CIP, GMN, SXT	1 (ICL-II)	Zordan *et al.*, 2011
P1697	VET (cat urine)	2000-2008	Germany		1 (ICL-II)	Zordan *et al.*, 2011
1782	VET (dog urine)	2000-2008	Germany	CIP, GMN, SXT	1 (ICL-II)	Zordan *et al.*, 2011
172	VET (dog urine)	2000-2008	Germany	CIP, GMN, SXT	1 (ICL-II)	Zordan *et al.*, 2011
P1959	VET (cat thorax)	2000-2008	Germany	ATM, CIP, GMN, TGC, SXT	1 (ICL-II)	Zordan *et al.*, 2011
4297	VET (dog wound)	2000-2008	Germany	AKN, CIP, GMN, TGC	3 (ICL-III)	Zordan *et al.*, 2011
196-1	VET (cat urine)	2000-2008	Germany	AKN, ATM, CIP, GMN, SXT	3 (ICL-III)	Zordan *et al.*, 2011
1231	VET (cat urine)	2000-2008	Germany	CIP, GMN, SXT	2 (ICL-1)	Zordan *et al.*, 2011
P869	VET (horse cervix)	2000-2008	Germany	AKN, ATM, CIP, GMN, IPM, SXT	3 (ICL-III)	Zordan *et al.*, 2011
760	VET (dog blood)	2000-2008	Germany	ATM, CIP, GMN, TGC	1 (ICL-II)	Zordan *et al.*, 2011
1173	VET (dog wound)	2000-2008	Germany	CAZ, CIP, GMN	1 (ICL-II)	Zordan *et al.*, 2011
1356	VET (dog fistula)	2000-2008	Germany	AKN, ATM, CIP, GMN, SXT	3 (ICL-III)	Zordan *et al.*, 2011
3061	VET (cow udder)	2000-2008	Germany	ATM	14	Zordan *et al.*, 2011
4199	VET (dog vagina)	2000-2008	Germany		8	Zordan *et al.*, 2011

A60	HCL[c] (blood or respiratory secretions)	2001-2004	Argentina	IPM, MEM, AKN, CAZ, GMN, SXT	1 (ICL-II)	Towner, 2008; Antunes *et al*., 2011
A369	HCL[c] (blood or respiratory secretions)	2001-2004	Spain	IPM, MEM, AKN, SAM, ATM, CAZ, CIP, GMN, SXT	1 (ICL-II)	Towner, 2008; Antunes *et al*., 2011
A372	HCL[c] (blood or respiratory secretions)	2001-2004	Greece	AKN, SAM, ATM, CAZ, CIP, GMN, IPM, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A377	HCL[c] (blood or respiratory secretions)	2001-2004	Germany	IPM, MEM, AKN, CAZ, CIP, GMN, IPM, SXT	3 (ICL-III)	Towner, 2008; Antunes *et al*., 2011
A390	HCL[c] (blood or respiratory secretions)	2001-2004	Bulgaria	IPM, MEM, AKN, ATM, CAZ, CIP, GMN, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A402	HCL[c] (blood or respiratory secretions)	2001-2004	Taiwan	AKN, SAM, ATM, CAZ, CIP, GMN, SXT	1 (ICL-II)	Towner, 2008; Antunes *et al*., 2011
A437	HCL[c] (blood or respiratory secretions)	2001-2004	Croatia	CAZ, CIP, GMN, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A440	HCL[c] (blood or respiratory secretions)	2001-2004	Bulgaria	AKN, CIP, GMN, IPM, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A458	HCL[c] (blood or respiratory secretions)	2001-2004	Estonia	AKN, CAZ, CIP, GMN, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A472	HCL[c] (blood or respiratory secretions)	2001-2004	Poland	MEM, SAM, CIP, GMN, SXT	2 (ICL-1)	Towner, 2008; Antunes *et al*., 2011
A491	HCL[c] (blood or respiratory secretions)	2001-2004	India	AKN, SAM, ATM, CAZ, CIP, GMN, SXT, IPM	1 (ICL-II)	Towner, 2008; Antunes *et al*., 2011
50C	HCL (respiratory secretions)	2004	Italy	CAZ, ATM, CIP, IPM, MEM, AKN, GMN, SAM, AMP, COL, SXT	1 (ICL-II)	D'Arezzo. *et al*., 2009
139L	HCL (respiratory secretions)	2007	Italy	CAZ, ATM, CIP, IPM, MEM, AKN, GMN, SXT	1 (ICL-II)	D'Arezzo *et al*.,2009
175P	HCL (wound swab)	2008	Italy	CAZ, ATM, CIP, IPM, SAM, AKN, GMN, SXT	1 (ICL-II)	D'Arezzo *et al*.,2009
141M	HCL (respiratory secretions)	2007	Italy	CAZ, ATM, CIP, IPM, AKN, GMN, SXT	1 (ICL-II)	D'Arezzo *et al*.,2009
82D	HCL (wound swab)	2004	Italy	TIG, ATM, CAZ, CIP, GMN, SXT	2 (ICL-1)	D'Arezzo *et al*.,2009
3130	HCL (blood)	2004	Lebanon	IPM, AKN, ATM, CAZ, CIP, GMN, SXT	2 (ICL-1)	Zarrilli *et al*., 2008
3889	HCL (bronchial aspirate)	2005	Greece	IPM, AKN, ATM, CAZ, CIP	1 (ICL-II)	Pournaras *et al*., 2006
4025	HCL (bronchial aspirate)	2005	Lebanon	IPM, AKN, ATM, CAZ, CIP	3 (ICL-III)	Di Popolo *et al*., 2011
3868	HCL (bronchial aspirate)	2003	Turkey	IPM, AKN, SAM, ATM, CAZ, CIP, GMN, SXT	12	Pournaras *et al*., 2006
3871	HCL (bronchial aspirate)	2003	Turkey	CAZ, CIP, SXT, GMN	5	Vahaboglu *et al*., 2006
3865	HCL (blood)	2005	Turkey	IPM, AKN, SAM, CAZ, CIP, GMN, SXT	12	Vahaboglu *et al*., 2006
3909	HCL (bronchial aspirate)	2007	Italy	IPM, AKN, ATM, CAZ, CIP, GMN, SXT	8	Giannouli *et al*., 2010

ATCC 17978	HCL (respiratory secretions)	1951	France	SXT	7	Baumanni *et al*, 1968
AYE	HCL (urine)	2001	France	AKN, ATM, CAZ, CIP, GMN, TGC, SXT	2 (ICL-1)	Fournier *et al*., 2006
ACICU	HCL (cerebrospinal fluid)	2005	Italy	AKN, ATM, CAZ, CIP, GMN, IPM, SXT	1 (ICL-II)	Iacono *et al*., 2008
RUH 5875	HCL (blood)	1997	The Netherlands	AKN, CIP, GMN,	3 (ICL-III)	Diancourt *et al*., 2010 Van Dessel *et al.*, 2004

[a]NCL, non clinical; VET, veterinary; HCL, human clinical.
[b]Isolates showing an intermediate level of susceptibility were classified as resistant. For some isolates an extended antibiotic resistance profile is available in the corresponding reference
AKN, amikacin, ATM, aztreonam, CIP, ciprofloxacin, CAZ, ceftazidime, COT, cotrimoxazole, COL, colistin, CTR, ceftriaxone FEP, cefepime, GMN, gentamicin, IPM, imipenem, LVX, levofloxacin, MEM, meropenem, PIP, piperacillin, P/T, , SAM, ampicillin-sulbactam, SXT, trimethoprim-sulfamethoxazole, TGC, tigecyclin, TZP, piperacillin-tazobactam
[c]human clinical isolates obtained from ARPAC project (A60-A491) derived from either respiratory secretion or blood, without specification from the sender laboratory

Table S2. Growth characteristics of NCL, VET and HCL *A. baumannii* isolates.

Source	Strain	Growth on Luria agar at					Growth on LAM	Growth on SAM	Polysaccharolytic activity	Surface pellicle formation
		4°C	18°C	37°C	44°C	47°C				
NCL	46-40	-	+	+	+	-	+	+	-	+
	109F5A	-	+	+	+	-	+	-	-	+
	54-21	-	+	+	+	-	+	+	-	-
	56-28	-	+	+	+	-	+	+	-	-
	114A14	-	+	+	+	-	+	-	-	-
	LUH01649	-	+	+	+	-	+	+	-	-
	LUH01650	-	+	+	+	-	+	+	-	-
	LUH08558	-	+	+	+	-	+	+	-	-
	31925	-	+	+	+	+	+	+	-	+
	31943	-	+	+	+	-	+	+	-	-
	31950	-	+	+	+	+	+	+	-	-
	31953	-	+	+	+	+	+	+	-	-
	31954	-	+	+	+	-	+	+	-	-
	31957	-	+	+	+	-	+	+	-	-
	31961	-	+	+	+	-	+	+	-	+
	32237	-	+	+	+	+	+	+	-	-
	32239	-	+	+	+	+	+	+	-	-
	32241	-	+	+	+	+	+	+	-	+
	32242	-	+	+	+	+	+	+	-	-
	32244	-	+	+	+	+	+	+	-	+
	32349	-	+	+	+	-	+	+	-	-
	32355	-	+	+	+	-	+	+	-	-
	32457	-	+	+	+	-	+	-	-	-
	B9	-	+	+	+	+	+	+	+ (starch)	-
	30M	-	+	+	+	+	+	-	-	+
	47M	-	+	+	+	+	+	-	-	-
	A046-5	-	+	+	+	+	+	+	-	-
VET	Km1008-06	-	+	+	+	+	+	+	+ (starch)	-
	OD474-06	-	+	+	+	-	+	+	-	-
	Km693-07	-	+	+	+	+	+	+	-	+
	Km323-09	-	+	+	+	+	+	+	-	-
	Km456-05	-	+	+	+	+	+	+	-	-
	Km1245-05	-	+	+	+	+	+	+		-
	Km765-04	-	+	+	+	+	+	+	-	-
	ACI_0638	-	+	+	+	-	+	+	-	-
	ACI_0639	-	+	+	+	+	+	+	-	+
	ACI_0644	-	+	+	+	+	+	+	-	+
	5966	-	+	+	+	+	+	+	+ (starch)	-
	132	-	+	+	+	+	+	+	+ (starch)	-
	4255	-	+	+	+	+	+	+	+ (starch)	-
	3204	-	+	+	+	+	+	+	+ (starch)	-
	P1697	-	+	+	+	+	+	+	+ (starch)	-
	1782	-	+	+	+	+	+	+	+ (starch)	-
	172	-	+	+	+	+	+	+	+ (starch)	-
	P1959	-	+	+	+	+	+	+	-	-
	4297	-	+	+	+	-	+	+	-	+
	196-1	-	+	+	+	-	+	+	-	+
	1231	-	+	+	+	+	+	+	-	-
	P869	-	+	+	+	-	+	+	-	-
	760	-	+	+	+	+	+	+	-	+
	1173	-	+	+	+	+	+	+	-	-
	1356	-	+	+	+	-	+	+	-	-
	3061	-	+	+	+	+	+	+	+ (starch)	-
	4199	-	+	+	+	+	+	+	-	-
HCL	A060	-	+	+	+	+	+	+	-	+
	A369	-	+	+	+	+	+	+	-	-
	A372	-	+	+	+	+	+	+	-	+
	A377	-	+	+	+	+	+	+	-	-
	A390	-	+	+	+	+	+	+	-	-
	A402	-	+	+	+	+	+	+	-	-
	A437	-	+	+	+	+	+	+	-	+
	A440	-	+	+	+	-	+	+	-	+
	A458	-	+	+	+	+	+	+	-	-
	A472	-	+	+	+	+	+	+	-	-

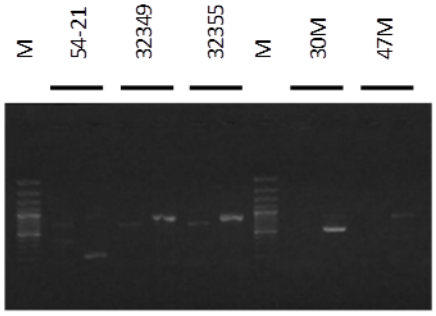
	A491	-	+	+	+	+	+	+	-	+
	50C	-	+	+	+	+	+	+	-	+
	139L	-	+	+	+	+	+	+	-	+
	175P	-	+	+	+	+	+	+	-	-
	141M	-	+	+	+	+	+	+	-	+
	82D	-	+	+	+	-	+	-	-	-
	3130	-	+	+	+	+	+	+	-	+
	3889	-	+	+	+	+	+	+	-	+
	4025	-	+	+	+	+	+	+	-	+
	3868	-	+	+	+	+	+	+	-	-
	3871	-	+	+	+	+	+	+	-	-
	3865	-	+	+	+	+	+	+	-	-
	3909	-	+	+	+	+	+	+	-	-
	ATCC 17978	-	+	+	+	+	+	+	-	-
	AYE	-	+	+	+	-	+	+	-	+
	ACICU	-	+	+	+	-	+	+	-	-
	RUH 5875	-	+	+	+	-	+	+	-	+

Table S3. Resistance to desiccation for NCL, VET and HCL *A. baumannii* isolates.

	Strain	**Log_{10} reduction of viability at time**			
		week 1	week 2	week 3	week 6
NCL	46-40	4	4	6	6
	109F5A	4	4	6	>7
	54-21	4	4	6	>7
	56-28	2	4	4	6
	114A14	4	4	6	6
	LUH01649	4	4	4	4
	LUH01650	4	4	4	6
	LUH08558	6	6	>7	>7
	31925	2	4	4	6
	31943	4	4	4	>7
	31950	4	4	6	6
	31953	4	4	6	6
	31954	4	4	6	6
	31957	2	4	6	6
	31961	4	4	4	>7
	32237	4	4	4	6
	32239	4	4	4	6
	32241	2	4	4	6
	32242	2	4	6	>7
	32244	2	4	4	4
	32349	2	4	4	4
	32355	2	4	4	6
	32457	4	6	6	6
	B9	4	4	4	4
	30M	4	6	>7	>7
	47M	2	4	4	6
	A046-5	2	4	4	4
VET	Km1008-06	2	4	4	4
	OD474-06	2	4	4	4
	Km693-07	2	4	4	6
	Km323-09	4	4	4	6
	Km456-05	2	4	4	6
	Km1245-05	4	4	4	4
	Km765-04	2	4	4	4
	ACI_0638	2	4	4	6
	ACI_0639	4	4	4	6
	ACI_0644	2	4	4	6
	5966	2	4	4	6
	132	2	4	4	6
	4255	4	4	4	6
	3204	4	4	4	6
	P1697	2	2	4	6
	1782	4	4	4	6
	172	2	4	4	4
	P1959	2	2	4	6
	4297	4	4	4	6
	196-1	4	4	4	>7
	1231	2	4	4	6
	P869	4	4	6	>7
	760	2	4	4	6
	1173	4	4	4	4
	1356	4	4	4	6
	3061	4	4	4	6
	4199	4	4	4	6
HCL	A060	4	4	6	6
	A369	4	6	6	6
	A372	4	4	4	6
	A377	4	4	4	6
	A390	2	4	4	6

	A402	4	4	4	6
	A437	4	6	6	>7
	A440	4	6	6	6
	A458	4	6	6	6
	A472	4	4	6	6
	A491	2	4	4	6
	50C	4	4	>7	>7
	139L	4	4	4	4
	175P	4	4	4	6
	141M	2	4	4	6
	82D	4	4	4	6
	3130	2	2	4	4
	3889	4	4	4	6
	4025	4	4	4	>7
	3868	2	4	6	6
	3871	4	4	4	4
	3865	2	2	4	6
	3909	2	4	4	6
	ATCC 17978	4	4	4	6
	AYE	4	4	4	4
	ACICU	4	4	4	6
	RUH 5875	4	6	6	>7

The log viability reduction at different times during the desiccation assay. The strains were grown in LB for 16 h and then adjusted in water to an OD_{600} of *ca*. 1. Aliquots of 10^7 10^5, 10^3 and 10 cells (in 10 µL water) were deposited on the flat bottom of microtiter plates, air dried for few minutes, and then placed in an airtight transparent plastic box. A constant 31% relative humidity (RH) was kept inside the plastic box by the presence of a saturated salt solution of $CaCl_2$ in an open becker. The time points considered for this experiment were: 7, 14, 21 and 42 days (respectively week 1, week 2, week 3 and week 6). At each time point fresh LB was added to each well, and the plate was incubated for up to 48 h at 37°C with mild agitation. Resistance to desiccation was assessed at different times through the determination of the highest dilution at which growth was rescued following LB addition.



Strain	Group 1 multiplex PCR			Group 2 multiplex PCR		
	csuE (702bp)	$bla_{OXA-51-like}$ (559bp)	*ompA* (355bp)	*csuE* (580bp)	*ompA* (343bp)	$bla_{OXA-51-like}$ (162bp)
54-21	+	-	+	-	-	+
32349	+	-	-	-	-	-
32355	+	-	-	-	-	-
30M	-	-	-	+	-	-
47M	-	-	-	-	-	-

Figure S1. Multiplex PCRs for identification of sequence groups (SGs). Five *A. baumannii* isolates from non-clinical sources (NCL) showed non-typical pattern profiles in multiplex PCRs targeting the *ompA*, *csuE* e $bla_{OXA-51-like}$ genes. PCR products from strains 54-21, 32349, 32355, 30M and 47M were loaded on 1% agarose gel. M is the 100 bp size ladder. For each isolates two lanes are shown corresponding to multiplex PCRs with primers for *csuE* (702 bp), $bla_{OXA-51-like}$ (559 bp), *ompA* (355 bp) on the left, and primers for *csuE* (580 bp), *ompA* (343 bp), $bla_{OXA-51-like}$ (162 bp) on the right.

Chapter 3

Iron and *Acinetobacter baumannii* biofilm formation

In the Chapter 2, pellicle formation and iron scavenging properties (siderophore production and hemolytic activity) are more in common in *A. baumannii* human clinical and veterinary isolates, respectively. Moreover, the ability to form biofilm is present and conserved in all *A. baumannii* isolates. Iron is essential for *A. baumannii* survival and pathogenicity, and its importance is also confirmed in the previous Chapter. The effect of iron availability on both planktonic and biofilm mode of *A. baumannii* growth deserves more in-depth investigation. In this Chapter, five well-characterized *A. baumannii* strains were used: AYE, representative for ICL-I, ACICU, representative for ICL-II; 50C, ICL-II pandrug resistant isolate; RUH5875, prototypic strain for ICL-III and ATCC 17978, reference strain.

Strains and optimal growth conditions for the generation *A. baumannii* biofilms were preliminarily established. Then, the role of iron in *A. baumannii* biofilm formation was also investigated. At last, the activity of a new therapeutic iron chelator was assessed in search for inhibitory drugs that could be repurposed as adjuvant antimicrobials in the treatment of biofilm-based *A. baumannii* infections.

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Article

Iron and *Acinetobacter baumannii* Biofilm Formation

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Abstract: *Acinetobacter baumannii* is an emerging nosocomial pathogen, responsible for infection outbreaks worldwide. The pathogenicity of this bacterium is mainly due to its multidrug-resistance and ability to form biofilm on abiotic surfaces, which facilitate long-term persistence in the hospital setting. Given the crucial role of iron in *A. baumannii* nutrition and pathogenicity, iron metabolism has been considered as a possible target for chelation-based antibacterial chemotherapy. In this study, we investigated the effect of iron restriction on *A. baumannii* growth and biofilm formation using different iron chelators and culture conditions. We report substantial inter-strain variability and growth medium-dependence for biofilm formation by *A. baumannii* isolates from veterinary and clinical sources. Neither planktonic nor biofilm growth of *A. baumannii* was affected by exogenous chelators. Biofilm formation was either stimulated by iron or not responsive to iron in the majority of isolates tested, indicating that iron starvation is not sensed as an overall biofilm-inducing stimulus by *A. baumannii*. The impressive iron withholding capacity of this bacterium should be taken into account for future development of chelation-based antimicrobial and anti-biofilm therapies.

Keywords: *Acinetobacter baumannii*; biofilm; chelator; deferasirox; deferiprone; deferoxamine; dipyridyl; iron; transferrin

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1. Introduction

Acinetobacter baumannii has emerged worldwide as a leading cause of hospital-acquired infections, especially among severely ill patients in intensive care units (ICUs) [1]. Although *A. baumannii* was initially regarded to as a low-grade pathogen, evidence has been accumulated suggesting that *A. baumannii* infections are associated with increased mortality in critically ill patients [2]. *A. baumannii* causes a broad range of nosocomial infections, including ventilator-associated pneumonia, urinary tract infections, wound infection, bacteremia, endocarditis, meningitis [3], and has recently been associated with very severe community-acquired infections, especially among individuals with predisposing factors in Southern Asia and other tropical regions [4]. *A. baumannii* can also be isolated from veterinary sources, and show common characteristics with strains described in human infection [5].

Tendency to the epidemic spread, resistance to antibiotics and persistence in the hospital setting are hallmarks of *A. baumannii* infection [3]. Successful strains of multidrug-resistant (MDR) *A. baumannii* are notorious for their ability to rapidly spread among hospitalized patients, overcome geographical borders, and become epidemic worldwide [6]. Epidemiologic and population genetics studies indicate that the majority of *A. baumannii* infections are caused by strains belonging to three international clonal lineages (ICLs) [1,3,6]. *A. baumannii* strains belonging to the most widespread ICLs are invariably characterized by an MDR phenotype, which is progressively evolving towards pandrug resistance, thereby challenging the current antimicrobial armamentarium [7,8]. This poses the urgent need for the development of novel treatment strategies to combat infections caused by MDR *A. baumannii* [9].

The capacity of MDR clinical isolates of *A. baumannii* to resist to desiccation and to form biofilms are regarded as crucial factors contributing to the clinical success and persistence of this species in healthcare facilities. *A. baumannii* can survive for up to months on the dry surface of inanimate objects [10,11], enabling transmission of infection for long times under both epidemic and endemic situations [12]. A number of reports have demonstrated that *A. baumannii* can form biofilms on several biotic and abiotic surfaces, providing the bacteria with protection against antibiotic/antiseptic treatment(s) and the host immune defenses *in vivo* (reviewed in [13,14]). Biofilm formation is crucial for several *A. baumannii* infections, since these are often associated with indwelling medical devices, e.g., vascular and urinary catheters, cerebrospinal fluid shunts, and endotracheal tubes [15]. While it is apparent that the capacity to form biofilms is a general phenotypic trait of *A. baumannii*, remarkable differences in the amount of biofilm formed by different strains have been reported, even if belonging to the same clonal lineage or epidemiological cluster [12,15–18]. A number of environmental factors can influence biofilm formation, including the presence of metal cations [16,19]. Among these, iron represents an essential nutrient for infecting bacteria, and a key determinant in host-pathogen interactions. This is because bacteria must counteract an iron-poor environment during infection, due to iron sequestration by iron carrier and storage proteins of the host and adaptive hypoferremia during infection [20]. *A. baumannii* has evolved an impressive capacity to acquire iron from the host, due to the production of multiple siderophores for Fe(III) transport, combined with uptake specificities for heme and Fe(II) [21,22].

Given the crucial role of iron in *A. baumannii*-host interactions [22–24], attention has recently been given to non-antibiotic approaches that target iron metabolism to achieve antibacterial activity,

including chelation therapy and use of iron mimetics (reviewed in [9]). Interestingly, it was noted that: (*i*) high concentrations of deferiprone (DFP, (Sigma Aldrich, St. Louis, MO, USA)), a compound used for chelation therapy in humans, inhibited to some extent logarithmic growth of *A. baumannii* ATCC 17978 in a chemically defined medium [25]; (*ii*) gallium, an iron-mimetic drug, suppressed the growth of MDR *A. baumannii* strains both *in vitro* and *in vivo*, acting through disruption of bacterial iron metabolism [25–27]; (*iii*) mutants impaired in production of the acinetobactin siderophore show reduced fitness *in vivo* [23]. On the other hand, it was also reported that biofilm formation on plastic by the type strain *A. baumannii* ATCC 19606^T was stimulated under conditions of iron scarcity imposed by the addition of the chelator 2,2′-dipyridyl (DIP) [19]. Therefore, the effect of iron availability on both planktonic and biofilm mode of *A. baumannii* growth deserves more in-depth investigation.

In this report, strains and optimal growth conditions for the generation *A. baumannii* biofilms were preliminarily established. Then, the role of iron in *A. baumannii* biofilm formation was investigated. Lastly, the activity of a new therapeutic iron chelator was assessed in search for inhibitory drugs that could be repurposed as adjuvant antimicrobials in the treatment of biofilm-based *A. baumannii* infections.

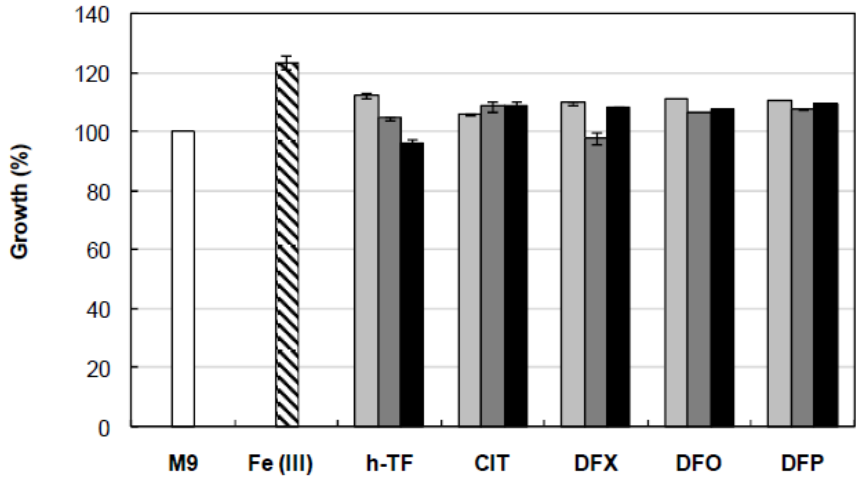
2. Results and Discussion

2.1. Definition of Culture Conditions for A. baumannii *Biofilm Formation*

Biofilm formation is a multifactorial phenotype [13,14], and in *A. baumannii* it can be modulated by iron availability, carbon sources, growth temperature, and different expression levels of adhesive and cell-aggregating factors [13,14,16,18,19,28]. Therefore, as a preliminary step to the investigation of the effect of iron on *A. baumannii* biofilm formation, we determined the growth response of the reference strain *A. baumannii* ATCC 17978 [25,29] to iron restriction imposed by different chelators in M9 minimal medium [30] containing 20 mM sodium succinate as the carbon source [26]. In line with previous observations [25], *A. baumannii* ATCC 17978 showed an impressive ability to multiply under conditions of iron deficiency, as those imposed by the addition of up to 128 μM human apo-transferrin (h-TF (Sigma Aldrich)), trisodium citrate (CIT (Sigma Aldrich)), desferrioxamine (DFO (Ciba Geigy, Origgio, Italy)), deferasirox (DFX (Novartis, Basel, Switzerland)) and DFP (Figure 1).

None of the tested chelators reduced *A. baumannii* ATCC 17978 growth yields at 48 h, even when 100 μM DIP (a chelator of the intracellular Fe(II) pool) was added to further reduce iron availability. As expected, growth in M9 was stimulated by *ca.* 25% in the presence of 100 μM $FeCl_3$. A similar resistance to exogenously supplied chelators in M9 was also observed for strains AYE [31] and ACICU [32], representatives for ICL-I and ICL-II, respectively (data not shown). These data can be explained by the presence in *A. baumannii* of very efficient iron uptake systems [11,21], capable of counteracting the iron withholding capacity of exogenously added chelators. The observation that DFX, DFO, and DFP do not stimulate bacterial growth in the presence of DIP (a chelator of the intracellular Fe(II) pool) suggests that these chelators are unlikely to serve as an iron source for *A. baumannii*.

Figure 1. Effect of different iron chelators on planktonic growth of *A. baumannii* ATCC 17978. Bacteria were grown for 48 h at 37 °C in 96-wells microtiter plates containing 100 µL M9 supplemented with the indicated iron chelator at different concentrations: 32 µM (light grey bars), 128 µM (dark grey bars) or 128 µM chelator + 100 µM DIP (black bars). Growth was measured as OD_{600} and expressed as percentage relative to the untreated control (*i.e.*, OD_{600} in M9). The average of the OD_{600} in control M9 was 0.318 ± 0.008 and represents 100% of growth (white bar). Relative growth in M9 supplemented with 100 µM $FeCl_3$ is reported (striped bar). Data represent the average of three independent experiments ± standard deviation. h-TF, tranferrin; CIT, citrate; DFX, deferasirox; DFO, desferrioxamine; DFP, deferiprone.



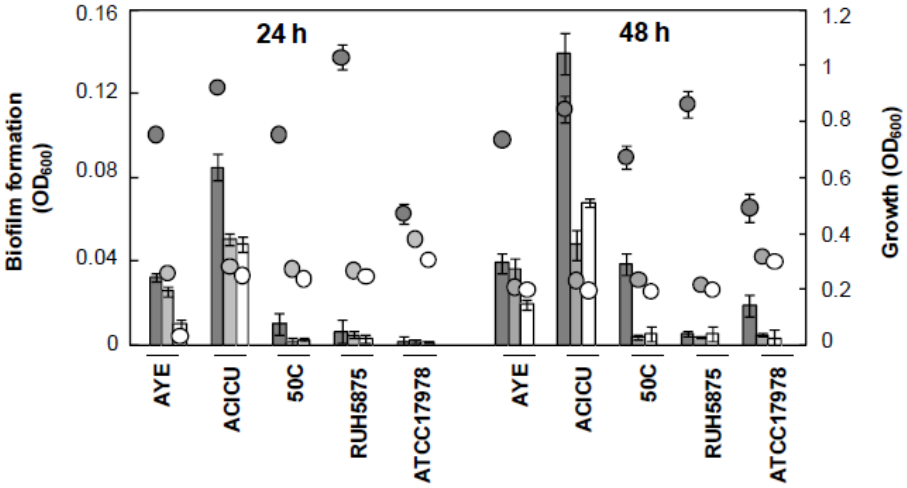
Next, the inter-strain variability and the growth medium-dependence of biofilm formation was investigated. Five well-characterized *A. baumannii* strains (AYE, representative for ICL-I [31]; ACICU, representative for ICL-II [32]; 50C, ICL-II pandrug resistant isolate [33]; RUH5875, prototypic strain for ICL-III [34]; ATCC 17978 [29]] were grown in three iron-poor media [M9, M9 supplemented with 100 µM DIP, and Chelex-100-treated Tryptic Soy Broth dialysate, TSBD [35]) in order to determine both growth and biofilm levels at 24 and 48 h. Quantitative estimation of the bacterial biomass in biofilms was assessed in 96-well polystyrene microtiter plates (BD Falcon, Milano, Italy), using the crystal-violet (CV) staining method [36]. There was a wide range of variation in growth and biofilm levels between *A. baumannii*, depending on strains and culture media, though for some strains moderate correlation was observed between growth yields and biofilm levels (Figure 2).

Remarkably, all strains produced more abundant biofilm in TSBD than in the other iron-poor media, and biofilm levels in strain ACICU were significantly higher ($p < 0.05$ in the student's *t*-test) than all the other strains tested (Figure 2). These findings corroborate the notion that biofilm levels in *A. baumannii* can vary even between closely related isolates (e.g., strains ACICU and 50C belonging to the same genetic cluster according to ref. [33]), and that different media have a profound impact on biofilm yields [15–18].

To rule out the possibility that differences in biofilm levels between TSBD and M9 or M9 plus DIP were due to different iron content of these media, an iron biosensor consisting of the Fur-controlled

basA promoter fused to the reporter *lacZ* gene [26] was used as a probe to determine the intracellular iron level in *A. baumannii* ATCC17978. Since the Fur repressor protein acts as an iron sensor, the activity of the Fur-controlled *basA* promoter provides an indirect estimate of the intracellular iron levels of bacteria grown in the different media. The β–galactosidase (LacZ) expression was higher in TSBD than in M9 or M9 plus DIP (Figure 3), and it was invariably repressed by iron, indicating that TSBD is sensed by *A. baumannii* as an iron-poor medium.

Figure 2. Growth and biofilm formation by selected *A. baumannii* strains in different iron-poor media. Bacterial cells were inoculated at OD_{600} of 0.01 in 100 µL of the different growth media, dispensed in a 96-wells microtiter plate, and grown at 37 °C without shaking for 24 and 48 h. Growth (circles) was measured spectrophotometrically (OD_{600}) and biofilm formation (bars) was evaluated using the CV staining assay [36]. Dark grey, TSBD; light grey M9; white M9 supplemented with 100 µM DIP. Data represent the average of three independent experiments ± standard deviation.



To visualize differences in biofilm structure among the five representative *A. baumannii* strains, biofilm formation on glass slides was monitored during seven days growth in TSBD by means of confocal microscopy, according to a previously described procedure [37] (Figure 4). High biofilm levels with formation of large cellular aggregates were observed for *A. baumannii* ACICU, and to a much lesser extent for the other strains (Figure 4A). Interestingly, *A. baumannii* biofilm cells were found to be embedded in a blue fluorescent material upon staining with calcofluor white (Figure 4B). In line with previous findings [18,29], this observation denotes the presence of exopolysaccharides in the matrix of *A. baumannii* biofilms, whose levels appear to be consistent with to the amount of biofilm formed in 96-well polystyrene microtiter plates (Figure 2).

Based on the above results, TSBD was considered as suitable iron-depleted medium that would allow robust biofilm formation and an easier evaluation of the effect of iron on this process. This is because the high biofilm levels achieved by *A. baumannii* in TSBD would facilitate the detection of

biofilm variations in response to iron levels. Moreover, the high peptide content and balanced formula of TSBD (35, see also [38]) make it more similar to a biological fluid than the M9 mineral salt medium.

Figure 3. Regulatory mechanism and activity of the *basA::lacZ* iron biosensor in the reference *A. baumannii* strain ATCC 17978. (A) Schematic of the regulatory mechanism the *basA::lacZ* iron-regulated transcriptional fusion carried by plasmid pMP220::P_{basA} [26]. Under iron proficient conditions (left), the Fur repressor protein binds the P_{basA} promoter and inhibits β–galactosidase (LacZ) expression; under iron deficient conditions Fur repression is relieved and the LacZ enzyme is expressed. (B) Activity of the *basA::lacZ* iron-regulated fusion in *A. baumannii* ATCC 17978 grown for 24 and 48 h in different media, as indicated, in the absence (white bars) or presence (black bars) of 100 μM $FeCl_3$. Data are the means (±standard deviations (SD)) of triplicate experiments.

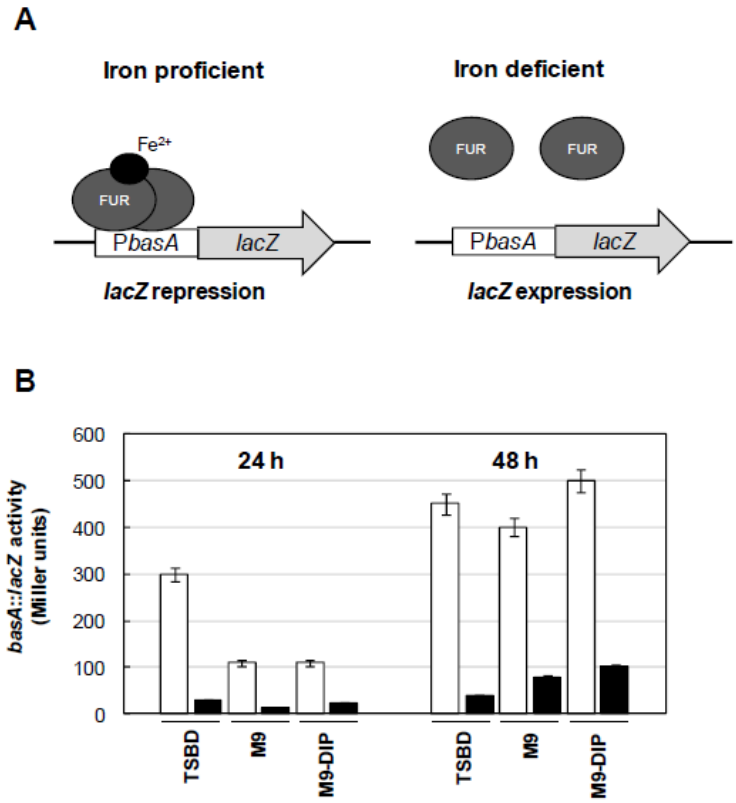
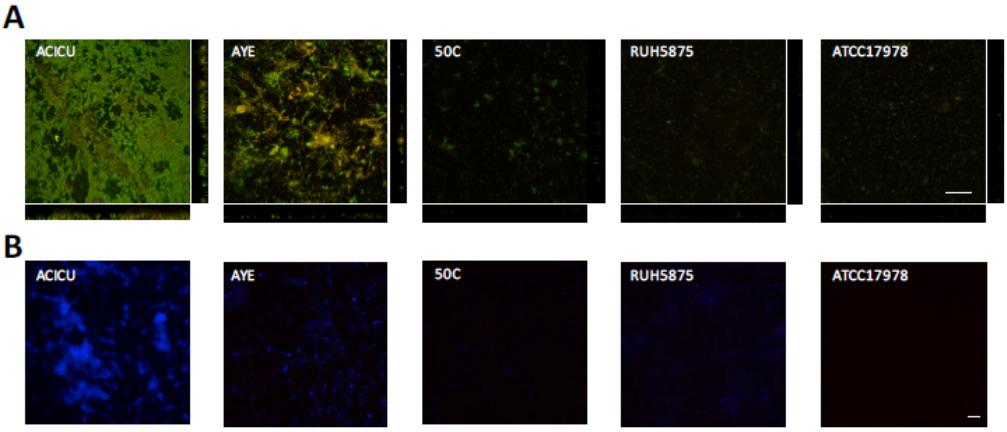


Figure 4. Seven-days biofilm of selected *A. baumannii* strains grown in TSBD. (**A**) Confocal microscope images (x-y plane and side view) of *A. baumannii* biofilms stained with acridine orange, a fluorescent dye which labels double-stranded nucleic acids (prevalently DNA) in green, and single-stranded nucleic acids (prevalently RNA) in red. (**B**) *A. baumannii* biofilms stained with the calcofluor white for exopolysaccharide labelling [19,28], and analyzed by fluorescence microscopy. Scale bar: 50 μm.



2.2. Effect of Iron Levels on Biofilm Formation by a Collection of Diverse A. baumannii Isolates

We investigated the effect of iron on biofilm formation in a representative collection of 54 *A. baumannii* strains (Table S1 in Supplementary Material), including 27 isolates from veterinary sources (67% MDR) and 27 isolates from clinical sources (96% MDR). Isolates were selected so to maximize diversity, as inferred by Random Amplified Polymorphic DNA fingerprints with primer DAF4 ([21,33]; data not shown), and represent the most widespread ICLs and some emerging lineages (Table S1 in Supplementary Material). High growth yields were observed for almost all isolates in TSBD (median OD_{600} = 0.716), which were significantly increased by the addition of 100 μM $FeCl_3$ (median OD_{600} = 1.031), consistent with iron being a nutritionally-limiting factor in TSBD (Figure 5A). Remarkably, biofilm formation was more abundant in $FeCl_3$-supplemented TSBD (median OD_{600} = 0.102) than in TSBD (median OD_{600} = 0.071) (Figure 5B). After having excluded from the analysis 4 biofilm non-producing isolates (namely, 4297, 196-1, 82D, RUH5875, see Table S1), the normalization of biofilm formation by growth yields resulted in minor differences between the two growth conditions (median values were 0.104 and 0.098 for the iron-limited and iron-rich condition, Figure 5C). This result is due to somehow opposite responses of *A. baumannii* isolates to iron starvation (*i.e.*, TSBD *vs.* $FeCl_3$-supplemented TSBD); in 21 isolates (42%) biofilm production was significantly enhanced by iron deficiency, in 12 (24%) it was significantly reduced, and in 17 (34%) iron had no effect on biofilm formation (significance in the Student's *t*-test was set at $p < 0.05$). Although stimulation of biofilm formation in response to iron-limited growth was observed for a minority (42%) of *A. baumannii* isolates, this may have relevant medical implications, since transition of these isolates from planktonic to biofilm-growing cells could be favored *in vivo*, where infecting bacteria are normally challenged with iron shortage. However, this behavior cannot be generalized,

since biofilm production was either unchanged or even inhibited by iron deficiency in the majority (66%) of the isolates.

2.3. Effect of Deferasirox on A. baumannii Biofilm Formation

We showed that planktonic *A. baumannii* has an impressive ability to grow in the presence of exogenously added therapeutic chelators DFP, DFO, and DFX (Figure 1), and previous data indicate only modest inhibition of *A. baumannii* growth at high DFP concentrations (*ca.* 200 μM, ref. [25]). To gain further insight into the effect of iron withholding on *A. baumannii* biofilm formation, we examined the effect of DFX, a newly developed orally active Fe(III) chelator, on our collection of 50 biofilm-producing isolates. DFX is a synthetic compound with high affinity and specificity for Fe(III) ($\log\beta_2 = 36.9$ according to ref. [39]), and is unlikely to serve as an iron carrier to *A. baumannii* based on growth assays (Figure 1). It was successfully used in combination therapy against murine staphylococcemia [40], and in treatment of invasive fungal infections [41].

Here, biofilm formation was tested in a DFX concentration range 4–128 μM, in order to match the DFX plasma levels achievable during treatment of iron overload in humans [42]. Notably, biofilm formation by most of the isolates was not significantly affected by DFX up to 128 μM, either in TSBD or in TSBD plus 100 μM $FeCl_3$ (Figure 6A).

Figure 5. Growth and biofilm formation in a representative collection of 54 *A. baumannii* strains from clinical and veterinary sources. (**A**) Growth of 54 *A. baumanni* strains for 48 h in 96-wells microtiter plates containing 100 μL TSBD supplemented (black circles) or not (white circles) with 100 μM $FeCl_3$, as indicated. (**B**) Absolute values of biofilm formation by the same isolates shown in panel A, evaluated by the CV staining assay (OD_{600}). Grey circles (B) represent the values for strains that in either or both conditions yielded negative biofilm values, and were excluded from calculations in panel C. (**C**) Relative values of biofilm formation (Biofilm formation (OD_{600})/Growth (OD_{600})) for a subset of 50 biofilm-producing isolates. The line bar represents the median value for each group. Values for each strain are the average of three independent experiments.

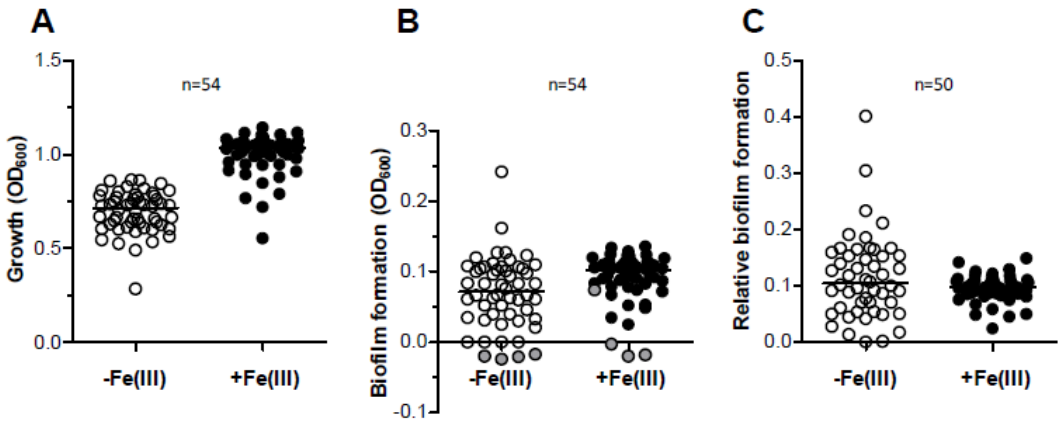
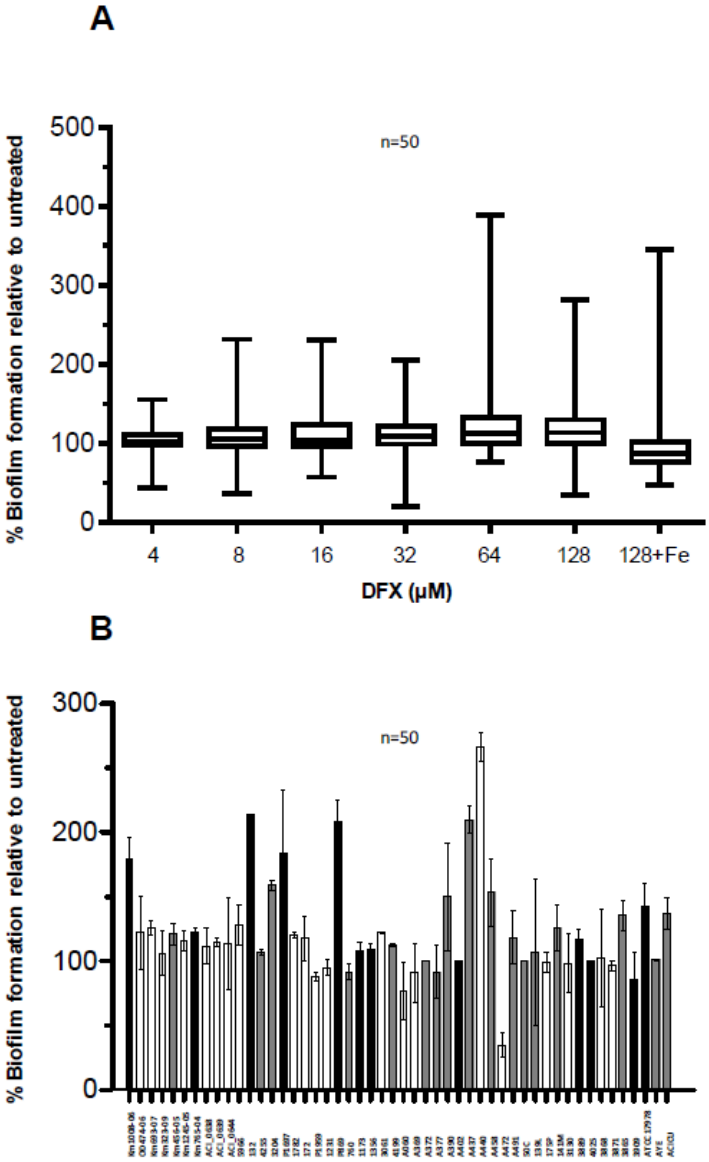


Figure 6. Effect of DFX on *A. baumannii* biofilm formation. (**A**) *A. baumannii* strains were grown statically for 48 h in microtiter plates containing 100 µL TSBD supplemented with DFX at indicated concentrations, or 128 µM DFX plus 100 µM $FeCl_3$. Biofilm formation (OD_{600} in the CV staining assay) was normalized by the growth yield (OD_{600} of the culture) and expressed as percentage relative to the DFX-untreated control (TSBD). Boxes represent medians, second and third interquartiles; whiskers represent range of 50 isolates tested. (**B**) Relative biofilm levels produced by individual isolates in presence of 128 µM DFX, expressed as % of the untreated control in TSBD. With reference to Figure 5C, the bar filling denotes: isolates in which biofilm production was significantly enhanced by iron deficiency (white, 21 isolates), or significantly reduced (black, 12 isolates), or in which iron had no effect on biofilm formation (grey, 17 isolates). In both panels data represent the average of three independent experiments.



No obvious correlation could be observed between the biofilm response to iron starvation in TSBD (see Figure 5 and associated text) and in the presence of 128 μM DFX (Figure 6B). The observation that a minority of isolates (*i.e.*, Km1008-06, 132, P1697, P869, A472, and ATCC17978) showed opposite responses to iron starvation induced by TSBD with and without iron (Figure 5), compared with TSBD and TSBD plus DFX (Figure 6B), suggests that either DFX exerts iron chelation-independent effects, or that the iron deficiency threshold that determines the biofilm response in *A. baumannii* can vary for these isolates. Apart from this, the ability to generate biofilms is scarcely influenced by the presence of DFX for most *A. baumannii* isolates, indicating that, at least *in vitro*, this therapeutic chelator is incapable of overcoming the iron withholding capacity of *A. baumannii* biofilms.

3. Experimental Section

3.1. Bacterial Strains and Growth Media

Relevant characteristics of the 54 *A. baumannii* strains used in this study are provided in Table S1 in the Supplementary Material. The collection includes representative strains for ICLs I, II, and III, namely AYE (ICL-I) [43], ACICU (ICL-II) [32], and RUH 5875 (ICL-III). Strain ATCC 17978 is a well-characterized clinical isolate dated 1950s and showing moderate antibiotic resistance [29]. Strain 50C is a pandrug resistant clinical isolate [33,44]. Other clinical and veterinary isolates were provided as part of the collections from various European laboratories [5,21,45–48]. Iron-poor culture media used in this study were the M9 minimal medium [30] supplemented with 20 mM sodium succinate as the carbon source, and TSBD, a Chelex 100-treated Trypticase Soy Broth dialysate [35]. When required, media were supplemented with either 100 μM DIP or 100 μM $FeCl_3$.

3.2. Chemicals

The chemicals used in this study were deferiprone (DFP, (Sigma Aldrich)), tri-sodium citrate [CIT, (Sigma Aldrich), desferrioxamine (DFO, (Ciba Geigy)); human apo-transferrin (h-TF, iron content ≤ 0.005%, (Sigma Aldrich)), and deferasirox (DFX, (Novartis)).

3.3. Growth Inhibitory Activity of Iron-Chelators

The activity of the iron chelators on bacterial growth was tested in 96-well microtiter plates (BD Falcon) containing increasing concentrations (4–128 μM) of iron chelators. Plates were inoculated at OD_{600} of 0.01 in a 100 μL final volume of M9 supplemented or not with 100 μM DIP at the highest iron-chelator concentration tested, or 100 μM $FeCl_3$, and incubated at 37 °C for 48 h with moderate shaking (100 r.p.m.). Spectrophotometric readings were performed in a Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer, Milano, Italy).

3.4. Biofilm Formation

Biofilm formation was measured according to the microtiter plate assay [36]. Briefly, bacterial cells were inoculated at OD_{600} of 0.01 in 100 μL of medium and grown at 37 °C for up to 48 h in 96-wells

microtiter plates without shaking. Planktonic cells were removed and the attached cells were gently washed three times with sterile PBS, air dried, and stained with 150 µL of 0.1% CV water solution for 20 min. The wells were gently washed four times with distilled water, and the surface-associated dye was eluted in 200 µL of 95% ethanol. The OD_{600} of the eluate was measured in a Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer).

3.5. Biofilm Inhibition

To investigate the effect of DFX on biofilm formation, fifty biofilm-producing *A. baumannii* strains were inoculated (OD_{600} of 0.01) into 96-well microtiter plates containing 100 µL TSBD supplemented with increasing DFX concentrations (4 to 128 µM) or 128 µM DFX plus 100 µM $FeCl_3$ The assay was performed as described above.

3.6. Microscopy Analysis

For microscopic visualization of *A. baumannii* biofilms, strains were grown in an 8-well chamber slide as previously described [37]. Briefly, bacteria were inoculated at OD_{600} of 0.01 in 200 µL of TSBD and incubated at 37 °C for 48 h to allow the adhesion of the bacterial cells on the glass surface. To maintain bacterial viability, the medium was changed every 24 h until the seventh day. To visualize biofilms structure, *A. baumannii* biofilms were stained with the acridine orange (0.1% water solution), a fluorescent dye, which labels double-stranded nucleic acids (prevalently DNA) in green, and single-stranded nucleic acids (prevalently RNA) in red, and examined using Leica TCS SP5 confocal microscope. For detection of matrix exopolysaccharides, samples were stained with calcofluor white (Fluka) and analyzed with an epifluorescence microscope. The Image J software [49] was used for image analysis.

3.7. β-Galactosidase Activity Assay

The *basA* promoter was cloned upstream of the *lacZ* reporter gene in plasmid pMP220::P*basA* (carrying the tetracycline-resistance (Tc^R) determinant) as previously described [26]. For reporter gene activity measurements, *A. baumannii* ATCC 17978 (Tc^S) was transformed with P*basA::lacZ* (Tc^R) and grown for 16 h at 37 °C in M9 medium supplemented with 10 µg/mL Tc. Cultures were then appropriately diluted in TSBD, M9 and M9 supplemented with 100 µM DIP, with or without 100 µM $FeCl_3$ to reach an initial cell concentration corresponding to OD_{600} ~ 0.01 and incubated at 37 °C with vigorous shaking. The β-galactosidase (LacZ) activity expressed by *A. baumannii* ATCC 17978 (pMP220::P*basA*) after 24 and 48 h growth was determined spectrophotometrically on toluene/SDS-permeabilized cells using *o*-nitrophenyl-β-D-galactopyranoside as the substrate, and expressed in Miller units [50].

$$\text{Miller units} = 1{,}000 \times [OD_{420} - (1.75 \times OD_{550})] / \text{Volume (ml)} \times \text{Time (min)} \times OD_{600}$$

4. Conclusions

The formation and maturation of *A. baumannii* biofilms depend on the complex interplay of many environmental and cell-associated factors [13,14]. In this study, attention has been focused on the role of iron, since this metal is essential for bacterial nutrition and virulence [22–24], and plays a central

role in host defense from bacterial infection [20]. In agreement with a previous study [25], we showed that planktonic *A. baumannii* cells can overcome iron restriction imposed by a variety of exogenous chelators, likely due to the presence in this species of multiple iron scavenging systems [21,22]. Then, we observed relevant differences in biofilm levels depending on *A. baumannii* strain and growth medium, and established suitable conditions for testing the effect of iron on biofilm formation. The two most relevant findings of these experiments were: (*i*) the strong influence of medium composition on biofilm yields; (*ii*) the high variability in biofilm levels produced by *A. baumannii* strains of clinical and veterinary origin, irrespective of their genetic relatedness or epidemic potential; (*iii*) the strain-dependent response of *A. baumannii* biofilms to iron scarcity. Since biofilm formation was either stimulated by iron or not responsive to this metal in the majority of strains tested, we conclude that iron starvation is not sensed as an overall biofilm-inducing stimulus by *A. baumannii*. Consistent with these findings, a recently developed clinical chelator, endowed with extremely high affinity for iron, showed no significant anti-biofilm activity in *A. baumannii*. Thus, while iron metabolism continues to represent a promising target for *A. baumannii* inhibition, the impressive iron withholding capacity of this bacterium should be taken into account for future development of chelation-based antimicrobial and anti-biofilm therapies.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2076-0817/3/3/704/s1.

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Author Contributions

Conceived and designed the experiments: Emanuela Frangipani, Paolo Visca. Supervised the laboratory work: Emanuela Frangipani, Paolo Visca. Performed the experimental work: Valentina Gentile, Carlo Bonchi, Federica Runci, Fabrizia Minandri. Wrote the manuscript: Paolo Visca.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

Table S1. Strains used in this study.

Strain (Alternative Designation)	Origin (Sample) [a]	Year of Isolation	Country	Antibiotic Susceptibility	Sequence Group	Reference
Km1008-06	VET (dog pus surgical wound)	2004–2009	Switzerland	MDR	1(ICL II)	5
OD474-06	VET (cat liver biopsy)	2004–2009	Switzerland		NV	5
Km693-07	VET (dog bronchial aspirate)	2004–2009	Switzerland	MDR	2(ICL I)	5
Km323-09	VET (dog eye swab)	2004–2009	Switzerland	MDR	1(ICL II)	5
Km456-05	VET (cat urine)	2004–2009	Switzerland		2(ICL I)	5
Km1245-05	VET (horse pus wound)	2004–2009	Switzerland		2(ICL I)	5
Km765-04	VET (dog pericardial effusion)	2004–2009	Switzerland		1(ICL II)	5
ACI_0638 (DVL5022)	VET (intravenous horse catheter)	2000–2008	Belgium	MDR	3(ICL III)	44
ACI_0639 (DVL4982)	VET (intravenous horse catheter)	2000–2008	Belgium	MDR	1(ICL II)	44
ACI_0644 (DVL5007)	VET (intravenous horse catheter)	2000–2008	Belgium		1(ICL II)	44
5966	VET (cat urine)	2000–2008	Germany		1(ICL II)	45
132	VET (dog pericardium)	2000–2008	Germany	MDR	2(ICL I)	45
4255	VET (dog abscess)	2000–2008	Germany	MDR	1(ICL II)	45
3204	VET (horse tendon)	2000–2008	Germany	MDR	1(ICL II)	45
P1697	VET (cat urine)	2000–2008	Germany		1(ICL II)	45
1782	VET (dog urine)	2000–2008	Germany	MDR	1(ICL II)	45
172	VET (dog urine)	2000–2008	Germany	MDR	1(ICL II)	45
P1959	VET (cat thorax)	2000–2008	Germany	MDR	1(ICL II)	45
4297	VET (dog wound)	2000–2008	Germany	MDR	3(ICL III)	45
196-1	VET (cat urine)	2000–2008	Germany	MDR	3(ICL III)	45
1231	VET (cat urine)	2000–2008	Germany	MDR	2(ICL I)	45
P869	VET (horse cervix)	2000–2008	Germany	MDR	3(ICL III)	45
760	VET (dog blood)	2000–2008	Germany	MDR	1(ICL II)	45
1173	VET (dog wound)	2000–2008	Germany	MDR	1(ICL II)	45
1356	VET (dog fistula)	2000–2008	Germany	MDR	3(ICL III)	45
3061	VET (cow udder)	2000–2008	Germany		14	45
4199	VET (dog vagina)	2000–2008	Germany		8	45
A60	HCL [a] (blood or respiratory secretions)	2001–2004	Argentina	MDR	1(ICL II)	21,47
A369	HCL [a] (blood or respiratory secretions)	2001–2004	Spain	MDR	1(ICL II)	21,47
A372	HCL [a] (blood or respiratory secretions)	2001–2004	Greece	MDR	2(ICL I)	21,47
A377	HCL [a] (blood or respiratory secretions)	2001–2004	Germany	MDR	3(ICL III)	21,47
A390	HCL [a] (blood or respiratory secretions)	2001–2004	Bulgaria	MDR	2(ICL I)	21,47
A402	HCL [a] (blood or respiratory secretions)	2001–2004	Taiwan	MDR	1(ICL II)	21,47
A437	HCL [a] (blood or respiratory secretions)	2001–2004	Croatia	MDR	2(ICL I)	21,47
A440	HCL [a] (blood or respiratory secretions)	2001–2004	Bulgaria	MDR	2(ICL I)	21,47
A458	HCL [a] (blood or respiratory secretions)	2001–2004	Estonia	MDR	2(ICL I)	21,47
A472	HCL [a] (blood or respiratory secretions)	2001–2004	Poland	MDR	2(ICL I)	21,47
A491	HCL [a] (blood or respiratory secretions)	2001–2004	India	MDR	1(ICL II)	21,47
50C	HCL (respiratory secretions)	2004	Italy	MDR	1(ICL II)	49

Table S1. *Cont.*

Strain (Alternative Designation)	Origin (Sample) [a]	Year of Isolation	Country	Antibiotic Susceptibility	Sequence Group	Reference
139L	HCL (respiratory secretions)	2007	Italy	MDR	1(ICL II)	49
175P	HCL (wound swab)	2008	Italy	MDR	1(ICL II)	49
141M	HCL (respiratory secretions)	2007	Italy	MDR	1(ICL II)	49
82D	HCL (wound swab)	2004	Italy	MDR	2(ICL I)	49
3130	HCL (blood)	2004	Lebanon	MDR	2(ICL I)	50
3889	HCL (bronchial aspirate)	2005	Greece	MDR	1(ICL II)	51
4025	HCL (bronchial aspirate)	2005	Lebanon	MDR	3(ICL III)	52
3868	HCL (bronchial aspirate)	2003	Turkey	MDR	12	51
3871	HCL (bronchial aspirate)	2003	Turkey	MDR	5	53
3865	HCL (blood)	2005	Turkey	MDR	12	53
3909	HCL (bronchial aspirate)	2007	Italy	MDR	8	54
ATCC 17978	HCL (respiratory secretions)	1951	France		NV	29
AYE	HCL (urine)	2001	France	MDR	2(ICL I)	31
ACICU	HCL (cerebrospinal fluid)	2005	Italy	MDR	1(ICL II)	32
RUH 5875	HCL (blood)	1997	Netherlands	MDR,	3 (ICL III)	34
ATCC 17978 (pMP220::P*basA*)	HCL (respiratory secretions)	1951	France		NV	26,29

Abbreviations: VET, veterinary; HCL, human clinical; MDR, strain resistant to three or more antimicrobial classes [55]; NV, new sequence group type variant; ICL, international clonal lineage. [a] human clinical isolates obtained from ARPAC project (A60–A491) derived from either respiratory secretion or blood, without specification from the sender laboratory.

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Chapter 4

Characterization of *Acinetobacter baumannii* iron-uptake mutants

A. baumannii is an emerging nosocomial pathogen responsible for infection outbreaks worldwide. The mechanisms which enable *A. baumannii* to infect and persist in the host are not yet fully understood. It is known that iron is an essential nutrient for this bacterium, thus iron uptake *in vivo* could contribute to its infectivity pathogenicity (see also Chapter 3). In the mammalian host, where there is paucity of iron, high-affinity iron uptake systems are likely to be essential for *A. baumannii* growth and infection. This reflects in the redundancy of iron uptake systems in this species; six gene clusters for iron acquisition have been identified in different *A. baumannii* strains, and four of these are carried by the type strain ATCC 19606^{T} (Antunes *et al.*, 2011a).

In this study, a markerless mutagenesis system (Stahl *et al.*, 2015) was used to generate single and multiple knock-out mutants of genes of the TonB and Feo systems, implicated in the uptake of ferric siderophores/heme or ferrous iron, or both. These iron uptake mutants were obtained in the ATCC 19606^{T} type strain, and compared to the wild type strain for growth capabilities under different conditions of iron availability (*e.g.* in the presence of an exogenous chelator and in human serum). Siderophore production by these mutants was also evaluated. Then, mutants were tested for their pathogenicity in the *Galleria mellonella* insect model of infection. These experiments allowed to clarify the essential functions of the TonB system, and provided more insights into the contribution of individual iron uptake systems to *A. baumannii* iron uptake capabilities both *in vitro* and *in vivo*.

Introduction

Acinetobacter baumannii is one of the most dreaded human opportunistic pathogens, being responsible for serious infections especially in immunocompromised and hospitalized patients (Bergogne-Berezin and Towner, 1996). *A. baumannii* pathogenicity is still poorly understood. Recently, it has been proposed that the ability to acquire iron from the environment could play a role in *A. baumannii* pathogenicity. (Zimbler *et al*., 2013). Iron is an essential nutrient for all living organisms, since it is required as a cofactor for several enzymes, as those implicated in vital pathways, e.g. electron transport and amino acid and DNA biosynthesis (Crosa *et al*., 2004; Wandersman *et al*., 2004). In aerobic environments iron is mainly present in the oxidized form Fe(III) which aggregates in insoluble oxy-hydroxyl polymers which are not readily usable by bacteria, while in anaerobic and/or reducing environments, the prevalent iron species is the reduced Fe(II) form. Once in the human host, *A. baumannii* is faced with the problem of iron acquisition. In human body fluid (e.g. serum, exudates), iron is sequestered by protein with high affinity for ferric iron Fe (III) such as trasferrin and lactoferrin, while in the cellular compartment iron is complexed in heme, iron sulfur cluster and ferritin (Ratledge and Dover, 2000). *A. baumannii* has developed several iron acquisition systems (Antunes *et al.*, 2011b) such as the production of low-molecular weight molecules with high affinity for Fe(III) able to scavenge this ion from different sources. Such systems are represented by the catechol-hydroxamate siderophore acinetobactin (Yamamoto *et al*., 1994) as well as systems implicated in the heme acquisition (Wandersman *et al*., 2012; Zimbler *et al.*, 2009). *A. baumannii* is also able to acquire ferrous iron by the Feo system (Antunes *et al.*, 2011b). This system has been studied extensively in *Escherichia coli* and other genera, and is nearly invariably composed of three proteins: FeoA, a small protein located in the cytosol; FeoB, a large protein with a cytosolic N-terminal G-protein domain and a C-terminal integral inner-membrane domain; FeoC, a small protein probably functioning as a transcriptional repressor (Lau *et al.*, 2015).

All systems involved in the acquisition of the ferric iron (either siderophore- or heme-bound) require the TonB energy transducing machinery, encoded by *tonB-exbB-exbD* genes, (Proschak *et al*., 2013; Noinaj *et al.*, 2010). This system, called TonB complex, transduces the proton motive force (PMF) into the energy required to internalize the ion carriers (siderophores, heme) into the periplasm. TonB is a periplasmic protein anchored into the inner membrane by its hydrophobic N-terminal domain and it is associated with both ExbB and ExbD proteins. A shuttle model has been proposed for the TonB-dependent energy

transduction process (Kadner, 1990). Uncharged TonB is converted to a charged TonB by the PMF thought the ExbB/D complex. Charged TonB shuttles to the outer membrane where it may recognize the TonB box on outer-membrane receptors for extracellular ligands. Ligand binding the receptor results in a conformational change in the receptor structure, which induces the interaction with the charged TonB and the release of the ligand into the periplasmic space concomitant with TonB deprotonation (Postle and Kadner, 2003). It has been described that in many Gram-negative bacteria the TonB-dependent receptors, which include receptors for ferric siderophores (Braun and Killmann, 1999), vitamin B12 (Kadner, 1990), heme and hemin (Wandersmann and Stojiljkovic, 2000), show conserved regions of homology called TonB box (Schramm, 1987). The TonB protein interacts with TonB box *via* its C-terminal domain, which is the best-characterized domain of TonB protein (Chu *et al*., 2007). Up to 21 putative TonB-dependent outer membrane receptor genes have been identified in the seven analysed *A. baumannii* genomes, presumably involved mainly in siderophore and heme uptake (Antunes *et al*., 2011b).

Although the TonB system has extensively been studied in several Gram negative bacteria, little is known about this system in *A. baumannii* (Chu *et al*., 2007; Seliger *et al*., 2001; Takase *et al*.; 2000). Recently, three putative *tonB* genes, namely *tonB1*, *tonB2* and *tonB3*, were identified in the chromosome of the *A. baumannii* type strain ATCC 19606^{T} (Zimbler *et al.*, 2013). The *tonB1* and *tonB3* genes are components of a typical *exbB-exbD-tonB* operon, while *tonB2* is a monocistronic element (Zimbler *et al.*, 2013). Using site-directed insertional mutagenesis in *tonB1* and *tonB2* genes it has been demonstrated that, although both the *tonB* genes somehow contribute to bacterial growth under iron limited condition, the *tonB2* plays a major role in the *A. baumannii* virulence (Zimbler *et al.*, 2013). Until now, no date are available on the role of *tonB3* in *A. baumannii* growth and virulence, albeit this gene has been demonstrated to be expressed at higher levels under iron deplete condition compared with *tonB1* and *tonB2*. The failure in the generation of the *tonB3* insertion mutant coupled with its iron-regulated expression suggests that probably the TonB3 protein plays a prominent role in the iron acquisition under iron starvation (Zimbler *et al*., 2013).

To gain more insight into the role of individual *tonB* alleles in the response of *A. baumannii* to iron starvation and in virulence, a markerless mutagenesis system was used to generate single, double and multiple mutants in the TonB and Feo systems of the type strain ATCC 19606^{T}. The generation of single and multiple mutants impaired in the acquisition of Fe(II) and/or Fe(III) allowed a better understanding of the contribution of each system to *A. baumannii* growth under conditions of iron scarcity and to virulence. Several cultural

conditions were tested mimicking different levels of iron availability. Mutants were also tested for their pathogenicity in the *Galleria mellonella* insect model of infection (Antunes *et al.*, 2011a). The results obtained highlight marked differences the contribution of each TonB system and of the Feo system to *A. baumannii* ATCC 19606^T iron acquisition both *in vitro* and in *vivo*.

Materials and methods

Strains and culture conditions

Strain and plasmid used in this study are listed in Table 1. *A. baumannii* ATCC 19606^T and *E. coli* DH5α were grown at 37°C in Luria Bertani medium (LB) or M9 minimal medium with 20 mM succinate as carbon source (Sambrook *et al*, 1989). Kanamycin and tetracycline were added when required to a final concentration of 20 μg/ml and 50 μg/ml, respectively.

Markerless mutagenesis

A markerless mutagenesis system (Stahl *et al.*, 2015) has been used to generate the *A. baumannii* iron uptake mutants. For each gene to be deleted, namely *feoB* (HMPREF0010_01984), *tonB1* (HMPREF0010_00773), *tonB2* (HMPREF0010_02677) and *tonB3* (HMPREF0010_01786), a DNA region of *ca.* 1,500 bp encompassing the start codon or the end codon of each gene of interest, was PCR amplified from the genome of *A. baumannii* ATCC 19606^T using the primer combinations listed in Table S1. Fragments containing upstream and downstream regions of each gene were cloned into the suicide vector pBIISK_*sacB/kanR* using BamHI, NotI, PstI and SacI restriction sites, resulting in the vectors designated pBIISK_*sacB/kanR*_"target gene"_updown (Table 1).

To generate electro-competent *A. baumannii* cells, an overnight culture of *A. baumannii* ATCC 19606^T was used to inoculate 400 ml of fresh LB medium and grown until it reached $OD_{600} \cong 0.5$. Cultures were cooled down in ice for 10 minutes and washed 4 times with sterile, ice-cold ion-free H_2O (obtained from a Milli-Q® Integral Water Purification System, Millipore), followed by one washing step in sterile, ice-cold 10% glycerol solution. Cell pellets were resuspended in 500 μl of the same solution. Then, 50 μl of the *A. baumannii* cell suspensions were used as recipient for electroporation of plasmid DNA (2–3 μg DNA). Electroporation was performed at 2.5 kV, 200 Ω and 25 μF in 2 mm pre-cooled electroporation cuvettes. After electroporation, 1 ml of pre-warmed SOC medium (2% tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20

mM glucose) was added. Cells were incubated for 1 hour at 37°C, and subsequent transformants were selected on LB-Agar containing 50 μg/ml kanamycin. Integration of the plasmid was monitored by PCR on single colony lysates using the primer combinations ctr_for + dw_rev and up_for + ctr_rev, for each target gene (Table S1). Correct integrants were subjected to counter-selection overnight at 37°C in 5 ml LB medium with 10% sucrose. Appropriate dilutions of the cultures were plated onto LB agar medium with 10% sucrose and incubated at 37°C for 18 h. Single colonies were tested for kanamycin sensitivity by replica plating onto LB agar with or without 50 μg/ml kanamycin. Kanamycin sensitive colonies were replicated, then lysed and subjected to PCR using the primers ctr_for + ctr_rev (Table S1, Figure S1). Double and triple mutants were obtained by insertion and segregation of the plasmids comprising the truncated genes of interest into single or double mutants. To successfully select for *tonB3* deletion mutants, 50 μM $FeSO_4$ was added to the agar plates and in liquid media in all integration and segregation steps.

Genetic complementation

The *A. baumannii* ATCC 19606^T *feoAB* and *tonB3* genes were PCR amplified from the genome of *A. baumannii* ATCC 19606^T using the long-range Phusion DNA polymerase (New England Biolabs) and primer pairs 25-26 and 27-28, respectively, which include EcoRI and XhoI restriction sites (Table S1). Amplicons were digested and ligated into the corresponding restriction sites of vector pME6031 (Heeb *et al*., 2000), to generate pME6031_*feoAB* and pME6031_*tonB3*, respectively (Table 1). These plasmids were introduced by electroporation in *A. baumannii* ATCC 19606^T Δ*feoB* and Δ*tonB3*, respectively, as described above. Transformants were selected on LB agar containing 50 μg/ml tetracycline. The presence of pME6031_*feoAB* and pME6031_*tonB3* in the complemented strains was confirmed by PCR and restriction analysis of plasmids extracted from *A. baumannii* cells grown in LB broth containing 50 μg/ml tetracycline.

Growth assays

The growth of the iron uptake mutants was monitored under different conditions of iron availability, and compared to that of the wild type strain. Bacteria were grown overnight at 37°C in LB, and then diluted to OD_{600} 0.005 in M9-succinate, in the presence of 100 μM 2, 2′-dipyridyl (DIP) and/or 100 μM $FeCl_3$. Growth was monitored for 72h at 37°C under aerated conditions (vigorous shaking at 120 rpm).

Growth in human serum

Growth of *A. baumannii* in heat-inactivated human serum (HS) was monitored in microtiter plates at 37°C, with mild-shaking (110 rpm). HS was obtained from 125 healthy donors, following illustration, approval and subscription of an informed consent. Complement was inactivated by incubation at 56 °C for 30 min, and the bulk of HS was sterilized by filtration as previously described (Antunes *et al*., 2012), then stored at +4 °C until used. Bulk HS chemistry was: total serum proteins 80 mg/ml; total iron 0.70 µg/ml; ferritin 0.243 µg/ml; Tf 2.63 mg/ml (64 µM total iron-binding sites); total iron binding capacity 4.27 mg/ml (20% Tf saturation, equivalent to 51.5 µM unsaturated iron binding sites). Bacteria were grown overnight at 37°C in LB and then diluted to OD_{600} 0.01 in 200 µl of HS supplemented or not with 100 µM Fe(III). Growth was monitored spectrophotometrically in a Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer), after 48h post inoculum.with mild-shaking

Siderophore production assay

The ability of *A. baumannii* ATCC 19606^T and isogenic mutants to produce siderophores was investigated using the Chrome Azurol S (CAS) assay on agar plates containing the CAS/HDTMA/iron complex (Schwyn and Neilands, 1987). Ten µl of a bacterial suspension containing *ca* 10^9 cells/ml were spotted on CAS agar plates and incubated for up to 48 h at 37°C. The halo around each strain provided a semi-quantitative estimation of the amount of released siderophores.

***Galleria mellonella* infection assay**

Galleria mellonella (Lepidoptera, Pyralidae) killing assay was performed as previously described by Peleg *et al*. (2009), with few modifications. Fifteen randomly chosen larvae (average weight 490 ± 90 mg) were used for each experimental group. Bacterial cells were cultured in LB for 14 h at 37°C with shaking, then washed twice with sterile saline and diluted to an OD_{600} of ca. 2.0, then diluted 10 times in saline so to obtain *ca*. 10^8 CFU/ml). One-ml BD Plastipak ™ insulin syringe with 0.3 mm needle, mounted on a Tridak stepper pipette, was used to inject 10 µl inocula (corresponding to 1×10^6 bacterial cells) into the hemocoel of each caterpillar, *via* the last left proleg. *G. mellonella* larvae were incubated at 37°C in petri dishes and their viability was monitored for 72 h. The number of viable bacteria in the inocula was determined by colony counts on LB agar. The chemical complementation of Δ*tonB3* mutant was performed adding Fe(III) before injection, in order to obtain a 10 µM

Fe (III) final concentration in each larva. Control groups included: larvae that were either not injected, or injected with sterile saline, or with sterile saline supplemented with 10 μM Fe(III), to rule out any toxic effect of Fe(III).

Table 1. Strains and plasmids used in this study.

Strains	Relevant characteristics	References
A. baumannii		
ATCC 19606^T	Clinical isolate; type strain	ATCC
Δ*feoB*	ATCC 19606^T containing a 1,681-bp deletion in the *feoB* gene	This study
Δ*tonB1*	ATCC 19606^T containing a 693-bp deletion in the *tonB1* gene	This study
Δ*tonB2*	ATCC 19606^T containing a 585-bp deletion in the *tonB2* gene	This study
Δ*tonB3*	ATCC 19606^T containing a 852-bp deletion in the *tonB3* gene	This study
Δ*tonB3*Δ*feoB*	Δ*tonB3* containing a 1,681-bp deletion in the *feoB* gene	This study
Δ*tonB1*Δ*tonB2*Δ*tonB3*	ATCC 19606^T deleted in all three *tonB* genes	This study
Δ*tonB1*Δ*tonB*Δ*tonB3*Δ*feoB*	ATCC 19606^T deleted in all three *tonB* genes and containing a 1,681-bp deletion in the *feoB* gene	This study
Δ*tonB3*_pME6031_*tonB3*	Δ*tonB3* harboring pME6031, containing *tonB3* gene	This study
Δ*feoB*_pME6031_*feoAB*	Δ*feoB* harboring pME6031, containing *feoAB* genes	This study
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 [ϕ80d*lacZ*Δ*M15*] F^- Nal^R	Sambrook *et al*., 1989

Plasmids		
pBIISK_*sacB/kanR*	Suicide vector for allelic replacement; *kanR*	Stahl *et al*., 2015
pME6031	Expression vector for complementation experiments; Tc^R	Heeb *et al*., 2000

Results

Mutation in *tonB3* impairs the ability of *A. baumannii* to grow under iron-limitated conditions

To gain insight into the role of TonB-dependent iron uptake in *A. baumannii* ATCC 19606^T, markless deletion mutants in the ferrous iron uptake system (*feoAB*) and in each of the known TonB energy transduction systems (*tonB1, tonB2* and *tonB3*), responsible for ferric iron acquisition, were generated. Growth of all mutants was monitored in M9-succinate in the presence or absence of DIP and/ or $FeCl_3$, and compared to that of the parental strain (Figures 1, 2). Growth of the Δ*tonB3* and Δ*tonB1*Δ*tonB2*Δ*tonB3* mutants was severely impaired in M9-succinate, displaying a 36 h lag phase (Figure 1A), and completely abolished in the presence of 100 μM DIP (Figure 1B) or when DIP was in combination with $FeCl_3$ (Figure 1C). An excess of Fe(III) was able to rescue the growth of all mutants, albeit a lag phase longer than that of the wild type or the complemented Δ*tonB3* mutant (12 h *vs* 4 h) was observed for the Δ*tonB3* deletion mutants (Figure 1D). The iron uptake deficient phenotype caused by the *tonB3* mutation was completely reversed when *tonB3* was provided *in trans* on a multicopy plasmid (strain Δ*tonB3*_pME6031_*tonB3*) (Figure 1). Interestingly, growth of mutants in the other TonB systems of *A. baumannii* ATCC 19606^T, namely Δ*tonB1* and Δ*tonB2* (Table 1), did not differ from that of the parental strain in all conditions tested (Figure S2). These results are in line with previous observations (Zimbler *et al*., 2013), and provide novel evidence of the prominent role of TonB3 in Fe(III) acquisition by *A. baumannii*.

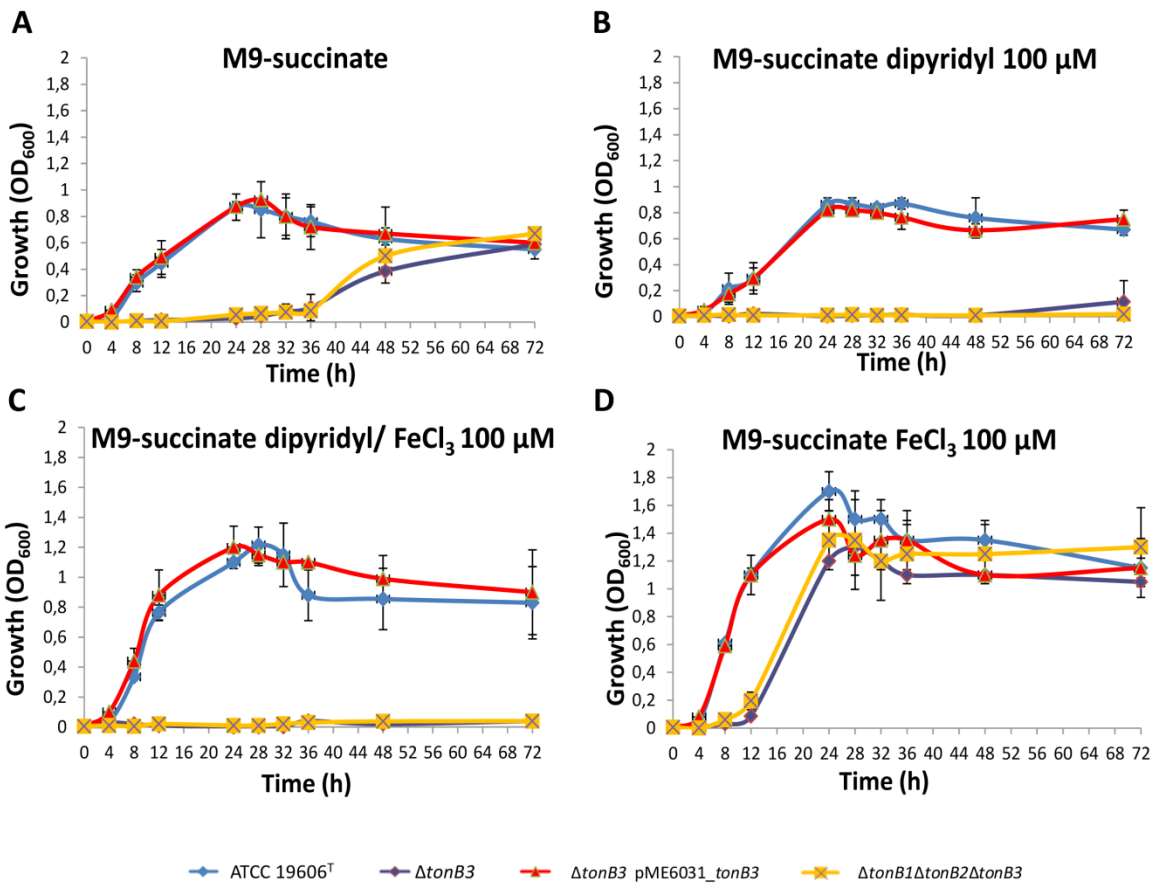

Figure 1. Effect of *tonB* mutations on *A. baumannii* growth under iron-limited conditions. The parental strain, ATCC 19606^T, isogenic mutants Δ*tonB3*, Δ*tonB1*Δ*tonB2*Δ*tonB3* and Δ*tonB3* complemented with pME6031, carrying the wild type *tonB*3 copy, was monitored in M9 minimal medium (A), supplemented with DIP 100 µM (B), or $FeCl_3$ (D), or both (C). Values are the means of three independent experiments ± standard deviation (SD).

Interestingly, inactivation of the Feo system did not affect the growth of *A. baumannii* under all conditions tested, even in the presence of the Fe(II) chelator DIP (Figure 2). Moreover, introduction of the Δ*feoB* mutation in the Δ*tonB3* and Δ*tonB1*Δ*tonB2*Δ*tonB3*, mutants did not cause any further growth defect; Δ*feoB*Δ*tonB3* and Δ*tonB1*Δ*tonB2*Δ*tonB3*Δ*feoB* mutants behaves similar to their *feoB*-positive parents, showing a delayed exponential phase in M9 succinate (Figure 2A) and no growth in the presence of DIP (Figure 2B) or DIP and $FeCl_3$ (Figure 2C). Moreover, growth of all mutants was rescued by an excess of Fe(III). No evident differences were detected between ATCC 19606^T, Δ*feoB* or Δ*feoB*_pME6031_*feoAB*, which carried a copy of *feoAB* genes on a multicopy plasmid (Figure 2).

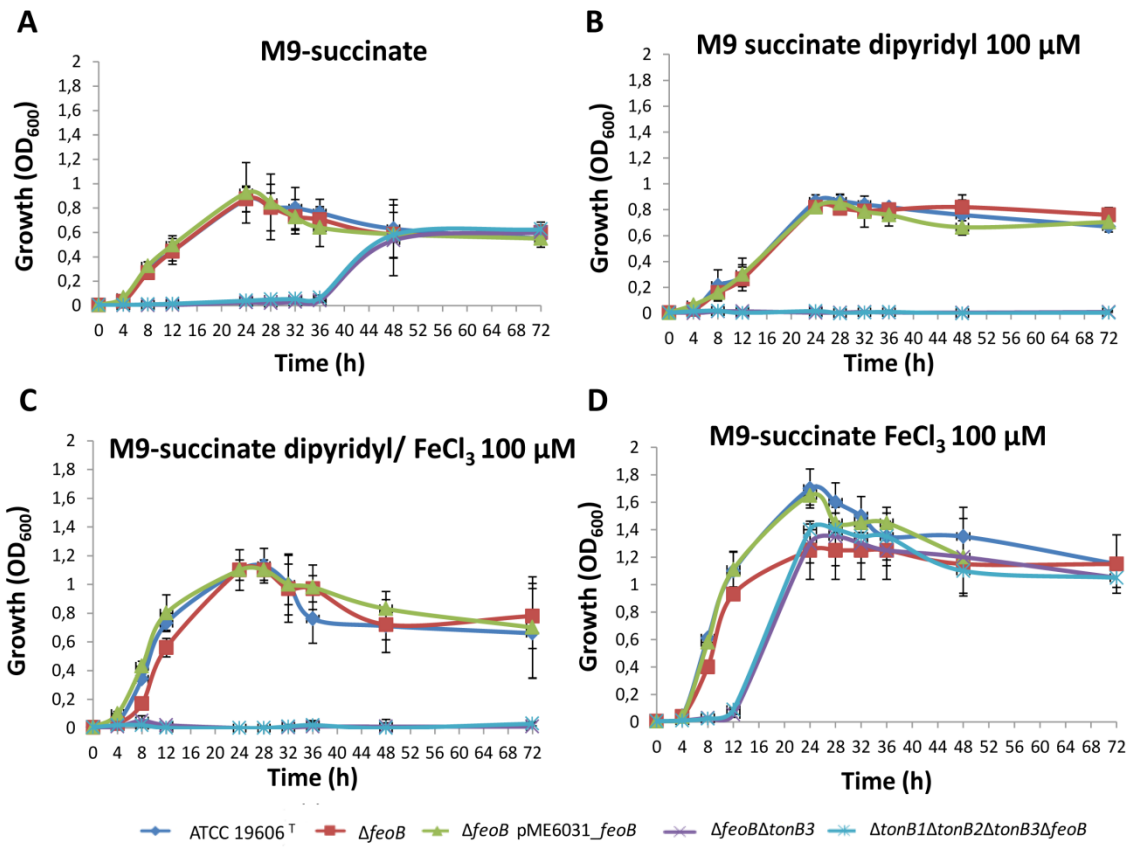

Figure 2. Effect of the *feoB* mutation on *A. baumannii* growth under iron-limitated conditions. The parental strain, ATCC 19606^T, and isogenic mutants Δ*feoB*, Δ*feoB*Δ*tonB3*, Δ*feoB*Δ*tonB1*Δ*tonB2*Δ*tonB3* and Δ*feoB* complemented with pME6031, carrying the wild type *feoB* copy, was monitored in M9 minimal medium (A), supplemented with DIP 100 µM (B), or $FeCl_3$ (D), or both (C). Values are the means of three independent experiments ± standard deviation (SD).

Ferrous iron uptake is required for optimal growth of *A. baumannii* in human serum

With the aim of evaluating the importance of iron uptake during *A. baumannii* infection in the human host, the wild type *A. baumannii* ATCC 19606^T and isogenic mutants defective in iron uptake were cultivated in HS in the presence or absence of an excess of Fe(III). Since *A. baumannii* growth yields in HS were undetectable after 24 h, growth of all strains was recorded after 48h post inoculation. As expected, Δ*tonB3* and Δ*tonB1*Δ*tonB2*Δ*tonB3*Δ*feoB* were unable to grow in HS, but, surprisingly, their growth was marginally restored by the addition of an excess of Fe(III) (Figure 3). Genetic complementation of the Δ*tonB3* with plasmid pME6031_*tonB3* rescued the growth to wild type levels. Different from what observed in M9 succinate medium, the *feoB* mutation caused a 4-fold decrease of growth in HS compared to the wild type (Figure 3), and could be restored upon addition of Fe(III).

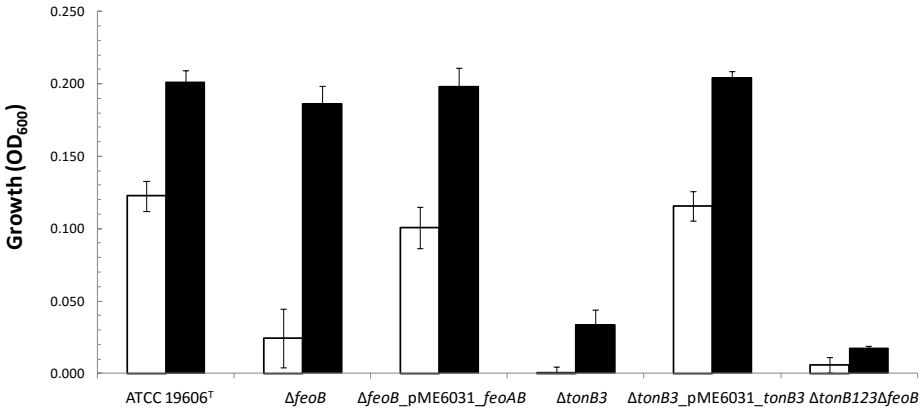

Figure 3. Growth of *A. baumannii* iron-uptake mutants in HS. *A. baumannii* strains were grown for 48 h in HS (white bars) and in HS supplemented with 100 μM $FeCl_3$ (black bars). Values are the means of three independent experiments ± standard deviation (SD).

Mutation in *tonB3* causes siderophore overproduction in *A. baumannii*

The inability of *tonB3* mutants to growth under conditions of iron deficiency suggests that the TonB3 is the functional energy-transducing device for Fe(III) uptake by *A. baumannii*. Thus, *tonB3* mutants appear severely iron-starved, and under such condition siderophore production is normally increased as physiological response to iron starvation. Thus, we wondered whether mutation in iron uptake systems could result in siderophore overproduction, as a consequence of the reduction of intracellular iron. To this aim, the ability of *A. baumannii* iron-uptake mutants to produce siderophores was qualitatively evaluated using the CAS assay on agar plates containing the CAS/HDTMA/iron complex (Figure 4; Schwyn and Neilands, 1987). Interestingly, we found that mutants carrying a *tonB3* deletion showed an increased production of siderophores compared to the parental strain (Figure 4). As expected, this phenotype was completely reversed when *tonB3* was provided *in trans* on a multicopy plasmid (strain Δ*tonB3*_pME6031_ *tonB3*) (Figure 4). Notably, neither mutation in *tonB1*, *tonB2* nor in *feoB* affected siderophore production, confirming that *tonB3* is the main energy transducing system for Fe(III) uptake in *A. baumannii*.

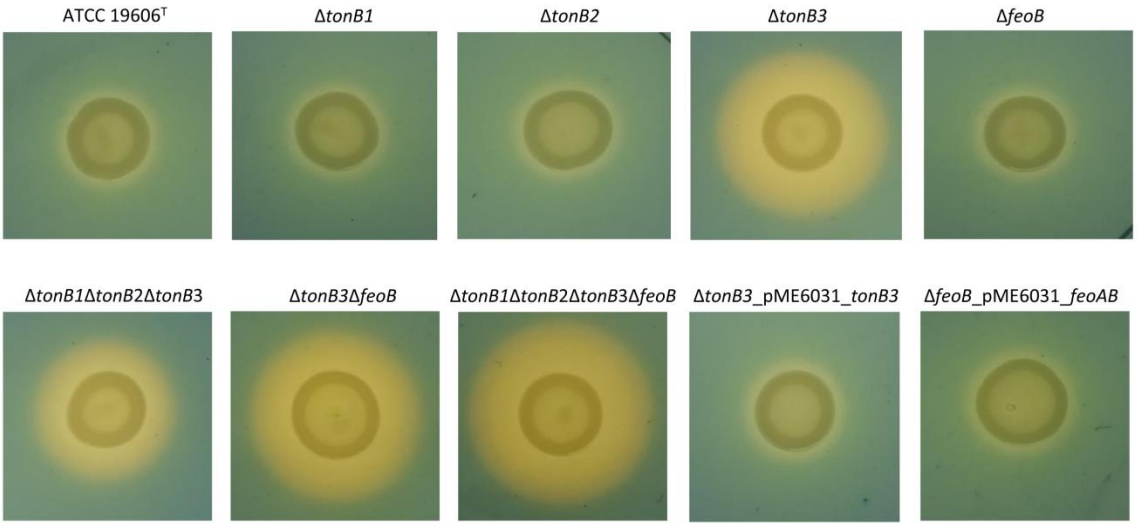

Figure 4. Siderophore production by different *A. baumannii* strains. The ability of iron-uptake mutants to produce siderophores was evaluated using the CAS assay on agar plates containing the CAS/HDTMA/iron complex. Siderophore production was visualised by the formation of an orange halo surrounding the colony after 48h of incubation. Images are representative of three independent experiments giving similar results.

Contribution of individual iron uptake systems to *A. baumannii* infectivity

The role of each iron uptake system in the virulence of *A. baumannii* ATCC 19606^T was examined using larvae of the greater wax moth *G. mellonella*, as an experimental animal infection model (Peleg *et al.*, 2009; Zimbler *et al.*, 2013). *G. mellonella* larvae were infected with 1×10^6 CFU of the wild type and of each mutant, and viability was daily monitored for 72 h (Figure 5). Sixty-six percent of caterpillars injected with the parental strain ATCC 19606^T were killed 3 days post infection (Figure 5), in line with previous results (Peleg *et al.*, 2009; Zimbler *et al.*, 2013). Interestingly, the *tonB3* deletion mutant killed only 6% of the injected caterpillars (Figure 5A), similarly to what observed for the Δ*tonB3*Δ*feoB* and the Δ*tonB1*Δ*tonB2*Δ*tonB3*Δ*feoB*, which killed 13% and 6% of larvae, respectively (Figure 5B). Surprisingly, the Δ*tonB1*Δ*tonB2*Δ*tonB3* was more virulent than all strains carrying the *tonB3* deletion, killing 40% of injected caterpillars (Figure 5A). The Δ*feoB* mutant behaved as the wild type strain, killing 60% of larvae (Figure 5B). Deletion in *tonB1* and *tonB2* did not alter *A. baumannii* virulence in the *G. mellonella* killing assay (Figure S3), as previously observed (Zimbler *et al.*, 2013). As negative controls, caterpillars were either not injected or injected with comparable volumes of saline alone and heat-killed ATCC 19606^T cells (Figure S3). While the not injected group as well as the group injected with saline showed the same low

mortality (6% killing), injection with heat-killed ATCC 19606^T cells killed 33% of larvae (Figure S3).

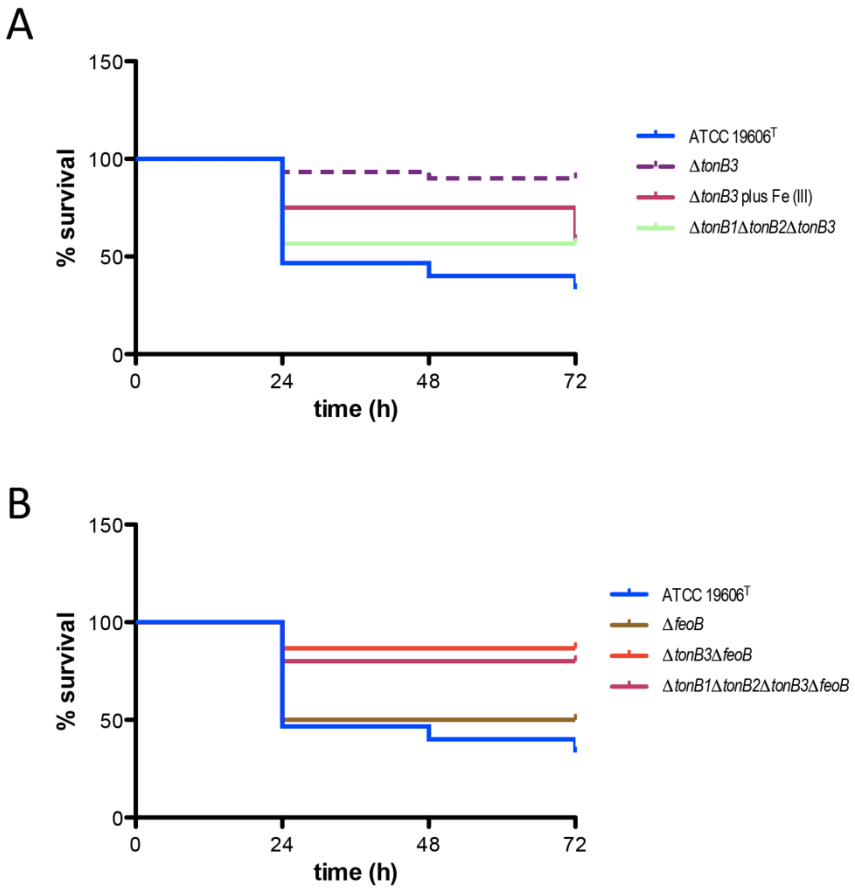

Figure 5. Role of iron uptake systems in *A. baumannii* virulence. *G. mellonella* caterpillars (n=30) were injected with 1×10^6 bacterial cells of the parental strain ATCC 19606^T, Δ*tonB3*, Δ*tonB3* + 10 μM $FeCl_3$, and Δ*tonB1*Δ*tonB2*Δ*tonB3* (A) or ATCC 19606^T, Δ*feoB*, Δ*tonB3*Δ*feoB* and Δ*tonB1*Δ*tonB2*Δ*tonB3*Δ*feoB* (B). Caterpillar death was determined daily for 3 days after incubation at 37°C in darkness.

We wonder whether the avirulent phenotype displayed by the Δ*tonB3* mutant could be chemically complemented by the addition of $FeCl_3$. To this aim, we preliminary assessed the minimal concentration of $FeCl_3$ required to rescue the growth of the Δ*tonB3* mutant in M9-succinate supplemented with increasing concentration of $FeCl_3$. The minimal $FeCl_3$ concentration required to restore the growth of the Δ*tonB3* mutant to wild-type levels was 25 μM (Figure 6). Then, we evaluated the toxicity of iron in the *G. mellonella* infection model. Injection of saline supplemented with 25 μM $FeCl_3$ resulted to be highly toxic (93% killing),

while injection of saline supplemented with 10 µM $FeCl_3$ killed 26% of larvae (Figure S3). Unfortunately, co-injection of Δ*tonB3* together with 10 µM $FeCl_3$ killed comparable percentage of larvae (Figure 5A), which cannot allow to chemically complement the avirulent phenotype of the Δ*tonB3* mutant, at this stage.

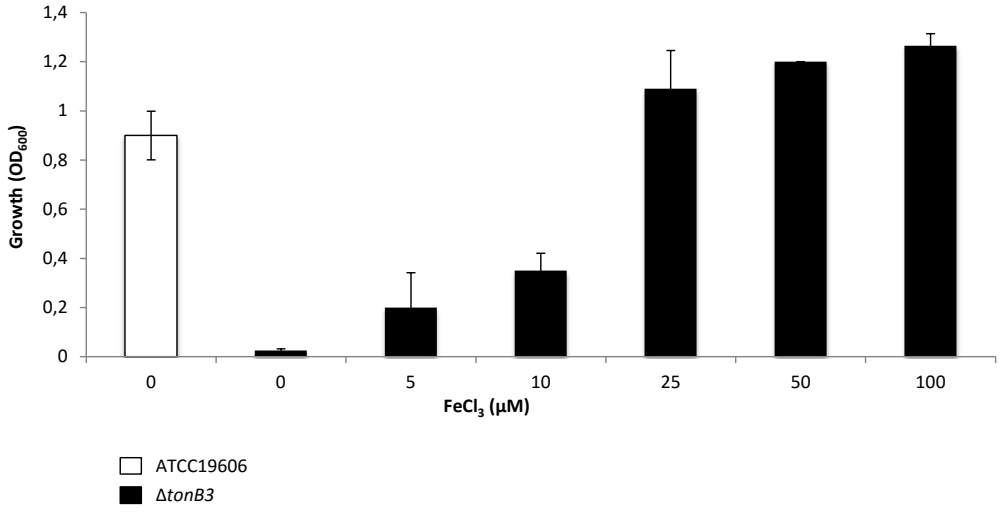

Figure 6. Chemically rescue of *tonB3* growth. The growth of the parental strain ATCC 19606^T(white bar) and Δ*tonB3* mutant (black bars) was measured in M9-succinate after 24h post inoculum. Addition of different $FeCl_3$ concentrations (0, 5, 10, 25, 50, 100 µM) rescued the growth of the Δ*tonB3* mutant in a dose-dependent manner.

Discussion

Colonisation and infection are dependent on the ability of pathogenic bacteria to acquire iron from their host (Ratledge and Dover, 2000). *A. baumannii* strains defective in siderophore production or lacking iron acquisition systems are less virulent compared to their parental strains, in different models of infections (Gaddy *et al.,* 2012; Zimbler *et al.,* 2013). However, the contribution of individual iron-uptake system to *A. baumannii* growth under conditions of iron scarcity as well as to virulence deserves more in-depth investigation. Although it has been shown that two of the three TonB energy transducing systems required for the internalisation of Fe(III), namely TonB1 and TonB2, played a role in supporting the growth of *A. baumannii* under iron-limiting conditions, and deletion of both systems significantly impaired *A. baumannii* virulence in the *G. mellonella* infection model, the contribution of the TonB3 system was only indirectly demonstrated, since all previous attempts to generate a *tonB3* mutant failed (Zimbler *et al.,* 2013). In this work, we successfully generated a complete set of *A. baumannii* iron uptake mutants (Table 1), including a *tonB3* deletion strain, using a new markerless mutagenesis system (Stahl *et al.,* 2015), and setting appropriate selection conditions. These were particularly important for the generation of the *tonB3* mutant, since this mutant would not grow unless fed with $FeCl_3$. Moreover, we have also generated a mutant in the Feo system, by deleting the *feoB* gene, whose homolog was previously been shown to be essential for Fe(II) uptake in *E. coli* (Kammler *et al*., 1993). Growth of all *tonB* mutants in the minimal medium M9 revealed that, differently to what was previously reported (Zimbler *et al.,* 2013), mutations in either *tonB1* or *tonB2* did not significantly impair *A. baumannii* ability to grow under iron limitation (Figure S2), while deletion of the *tonB3* gene caused a significant growth delay under the same growth conditions (Figure 1A). Moreover, growth of the Δ*tonB3* mutant was completely abolished under the severe iron limitation imposed by the Fe(II) chelator DIP (Figure 1B, C). Surprisingly, growth of the Δ*tonB3* mutant was not restored by the addition of an excess of $FeCl_3$ in the presence of DIP, suggesting that this mutant suffers from a more pronounced iron starvation, likely due to chelation of the intracellular Fe(II) pool by DIP concomitant with the lack of an efficient Fe (III) acquisition apparatus due to *tonB3* deletion. These data experimentally demonstrate, for the first time, the essential role of the TonB3 system in sustaining *A. baumannii* growth under iron limited conditions, arguing for a functional predominance of this ortholog over TonB1 and TonB2 systems. Although *E. coli* possesses a single copy of *tonB*, other bacteria, including *A. baumannii*, harbour more than one copy of

genes encoding for putative TonB proteins (Zimbler *et al*., 2013). The TonB protein is known for providing energy to many different high-affinity transport systems, which allow bacteria to acquire several nutrients, such as vitamin B12 (Heller and Kadner, 1985), ferric siderophores (Eick-Helmerich and Braun, 1989), haemin and heme (Jarosik *et al*., 1994). The specific receptors show the so-called TonB box, which is proposed to interact directly with TonB. Several TonB-dependent receptors (ranging from 8 to 21) have previously been identified in seven complete *A. baumannii* genomes (Antunes *et al*., 2011b). This suggests that the TonB complex could serve as a polyvalent energy coupler for functioning of multiple TonB-dependent receptors and that multiple TonB orthologs may accomplish different functions. In this scenario, albeit TonB1 and TonB2 seem to be dispensable for growth under the experimental condition used in this work, they could be involved in other TonB-dependent membrane-associated processes. This observation is supported by the evidence that, in *A. baumannii*, TonB2 has been demonstrated to be involved in cells adherence, other than in iron acquisition (Zimbler *et al*., 2013). Similar results were previously described in the opportunistic pathogen *P. aeruginosa*, which has three chromosomal copies of *tonB*, one of them being involved in motility and pilus assembly (TonB3), but not in iron transport (Huang *et al.*, 2004).

Different from Fe(III), Fe(II) can passively diffuse across the porins of the Gram-negative outer membrane, thus not requiring a TonB-energy transduction component to reach the periplasm. Once entered into the periplasm, Fe(II) can gain access to the cytoplasm via the Feo system, whose contribution to *A. baumannii* growth under iron limitation has never been investigated so far. Notably, loss of the Feo system did not affect the aerobic growth of *A. baumannii* under conditions of iron limitation, even in the presence of high concentrations of DIP (Figure 2B). This behaviour is probably due to the fact that, in the presence of oxygen, Fe(II) is rapidly oxidized to Fe(III), which can be internalized by the cells via TonB-dependent Fe(III) uptake systems. However, we found that Feo was crucial for *A. baumannii* growth in HS, suggesting a role of ferrous iron acquisition during systemic infection, which deserves further investigation. Indeed. It is still unclear why growth in HS of both the Δ*tonB3* and Δ*tonB1*Δ*tonB2*Δ*tonB3*Δ*feoB* mutants is not rescued by addition of Fe(III) levels exceeding the iron binding capacity of transferrin (51 µM vs. 100 µM exogenously added $FeCl_3$). Although no bactericidal activity was observed, it can be possible that the *tonB3* mutation renders the bacteria more susceptible to unspecific antibacterial components of HS (e.g., defensins).

The CAS assay revealed that all *tonB3* mutants were characterized by an increased siderophore production compared to the wild type strain, as opposed to the Δ*tonB1* and Δ*tonB2* mutants. These data suggest that, under the experimental conditions used in this work, the Δ*tonB3* mutant is intrinsically iron starved and responds to iron starvation by overproducing siderophores.

Results of experimental infections in the *G. mellonella* model revealed that mutants deleted in the *tonB3* gene were less virulent than the wild type, whereas mutations in either *tonB1* or *tonB2* did not affect *A. baumannii* lethality. Similarly, the *feoB* mutation had no effect on *A. baumannii* lethality.

The triple Δ*tonB1*Δ*tonB2*Δ*tonB3* deserves a special comment, since it showed an atypical behavior. This triple mutant unexpectedly produced less siderophores than the Δ*tonB3* and showed higher *G. mellonella* lethality than Δ*tonB3,* coherent with CAS assay results. While no obvious explanation can be provided for this behavior, it appears that deletion of both *tonB1* and *tonB2* in the *tonB3* background increases, rather than reduce, *A. baumannii* virulence. Further studies are needed to explain these apparently illogical observations.

In conclusion, our results support the prominent role of the TonB3 system for *A. baumannii* virulence and pathogenicity, and provide interesting insights to the contribution of iron uptake systems to *A. baumannii* growth under iron-restricted conditions and pathogenicity. Given the importance of the TonB3 system for *A. baumannii* growth both in HS and *in vivo*, TonB3 could represent a promising target for new antimicrobial drugs.

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Table S1. Primers used in this study.

N	Primer	Sequence 5`-3`	Application
1	*feoB*_up_for_BamHI	ATTAGGATCCTTGTGCTACATGCGTATAGATTTG	Forward primer used to amplify the upstream region of *feoB* to be cloned in pBIISK_*sacB/kanR*
2	*feoB*_up_rev_NotI	ATTAGCGGCCGCGGTCGCATTAAGACTGTATGTACC	Reverse primer used to amplify the upstream region of *feoB* to be cloned in pBIISK_*sacB/kanR*
3	*feoB*_dw_for_NotI	ATTAGCGGCCGCCATGTTGCAGCTATGACTACATATC	Forward primer used to amplify the downstream region of *feoB* to be cloned in pBIISK_*sacB/kanR*
4	*feoB*_dw_rev_SacI	ATTAGAGCTCCACCAACATTGGTACCTTTAGAGTC	Reverse primer used to amplify the downstream region of *feoB* to be cloned in pBIISK_*sacB/kanR*
5	*tonB1*_up_for_BamHI	CGGCGGATCCCTGAAGATGCCAATGTATGGAATG	Forward primer used to amplify the upstream region of *tonB1* to be cloned in pBIISK_*sacB/kanR*
6	*tonB1*_up_rev_ NotI	ATTAGCGGCCGCATGACTGCAGCAATACCAATTAGG	Reverse primer used to amplify the upstream region of *tonB1* to be cloned in pBIISK_*sacB/kanR*
7	*tonB1*_dw_for_NotI	ATTAGCGGCCGCGTGCCTCAGCAAGACTGGTATG	Forward primer used to amplify the downstream region of *tonB1*to be cloned in pBIISK_*sacB/kanR*
8	*tonB1*_dw_rev_ SacI	CGGCGAGCTCCACCTCTAGCATTACATCGTCATC	Reverse primer used to amplify the downstream region of *tonB1* to be cloned in pBIISK_*sacB/kanR*
9	*tonB2*_up_for_NotI	ATTAGCGGCCGCCGTGCTAATAGTCTTATTGCCAGG	Forward primer used to amplify the upstream region of *tonB2* to be cloned in pBIISK_*sacB/kanR*
10	*tonB2*_up_rev_BamHI	ATTAGGATCCTCGATTGTTCTGACGGCGCAGGC	Reverse primer used to amplify the upstream region of *tonB2* to be cloned in pBIISK_*sacB/kanR*
11	*tonB2*_dw_for_BamHI	CGGCGGATCCCAGTTCTAGATGAAGCAGCAAAGG	Forward primer used to amplify the downstream region of *tonB2* to be cloned in pBIISK_*sacB/kanR*
12	*tonB2*_dw_rev_PstI	CGGCCTGCAGCTGACCTGTTACACCTCTTGATTC	Reverse primer used to amplify the downstream region of *tonB2* to be cloned in pBIISK_*sacB/kanR*
13	*tonB3*_up_for_BamHI	ATTAGGATCCGAGCGCTAGAATACTCGGCTTATC	Forward primer used to amplify the upstream region of *tonB3* to be cloned in pBIISK_*sacB/kanR*
14	*tonB3*_up_rev_NotI	ATTAGCGGCCGCATGTGGCTGACTGCCCATAAGACC	Reverse primer used to amplify the upstream region of *tonB3* to be cloned in pBIISK_*sacB/kanR*
15	*tonB3*_dw_for_NotI	ATTAGCGGCCGCATTAAAGCAGAGCAACCGTTTGAC	Forward primer used to amplify the downstream region of *tonB3* to be cloned in pBIISK_*sacB/kanR*
16	*tonB3*_dw_rev_SacI	ATTAGAGCTCGCCAAGGTAATGATATTGGTACTC	Reverse primer used to amplify the downstream region of *tonB3* to be cloned in pBIISK_*sacB/kanR*
17	*feoB*_ctr_for	GTGCTACATGCGTATAGATTTG	Verification of the *feoB* mutation
18	*feoB*_ctr_rev	GACAGGCGCATCTTCACCAATC	Verification of the *feoB* mutation
19	*tonB1*_ctr_for	GTGCATCGGATGCTATCGTAAGTG	Verification of the *tonB1* mutation
20	*tonB1*_ctr_rev	CATAATAATGAGTACCAGCATGAC	Verification of the *tonB1* mutation
21	*tonB2*_ctr_for	CTCATCAAGCACACCTGCCTGCCAAGC	Verification of the *tonB2* mutation
22	*tonB2*_ctr_rev	GAGCCGAAGCGTTATTGCAACGAC	Verification of the *tonB2* mutation
23	*tonB3*_ctr_for	GAAGCGCTCATTATTAACCTCTGG	Verification of the *tonB3* mutation
24	*tonB3*_ctr_rev	CTTTGCAACGCATGATCCAACGAC	Verification of the *tonB3* mutation

25	*feoAB*_for_EcoRI	CCGGAATTCAGGGCTTGGAATACGCGGT	Forward primer used to amplify the *feoAB* genes including their promoter region, to be cloned in pME6031
26	*feoAB*_rev_XhoI	CCGCTCGAGATTGAAGCATATACAACCTCCT	Reverse primer used to amplify the *feoAB* genes including their promoter region, to be cloned in pME6031
27	*tonB3*_for_EcoRI	CCGGAATTCTAAGACTGTTGTTTTCTAACAAC	Forward primer used to amplify the *tonB3* gene including its promoter region, to be cloned in pME6031
28	*tonB3*_rev_XhoI	CCGCTCGAGCTCCTGATTAAGGTTAGCCG	Reverse primer used to amplify the *tonB3* gene including its promoter region, to be cloned in pME6031

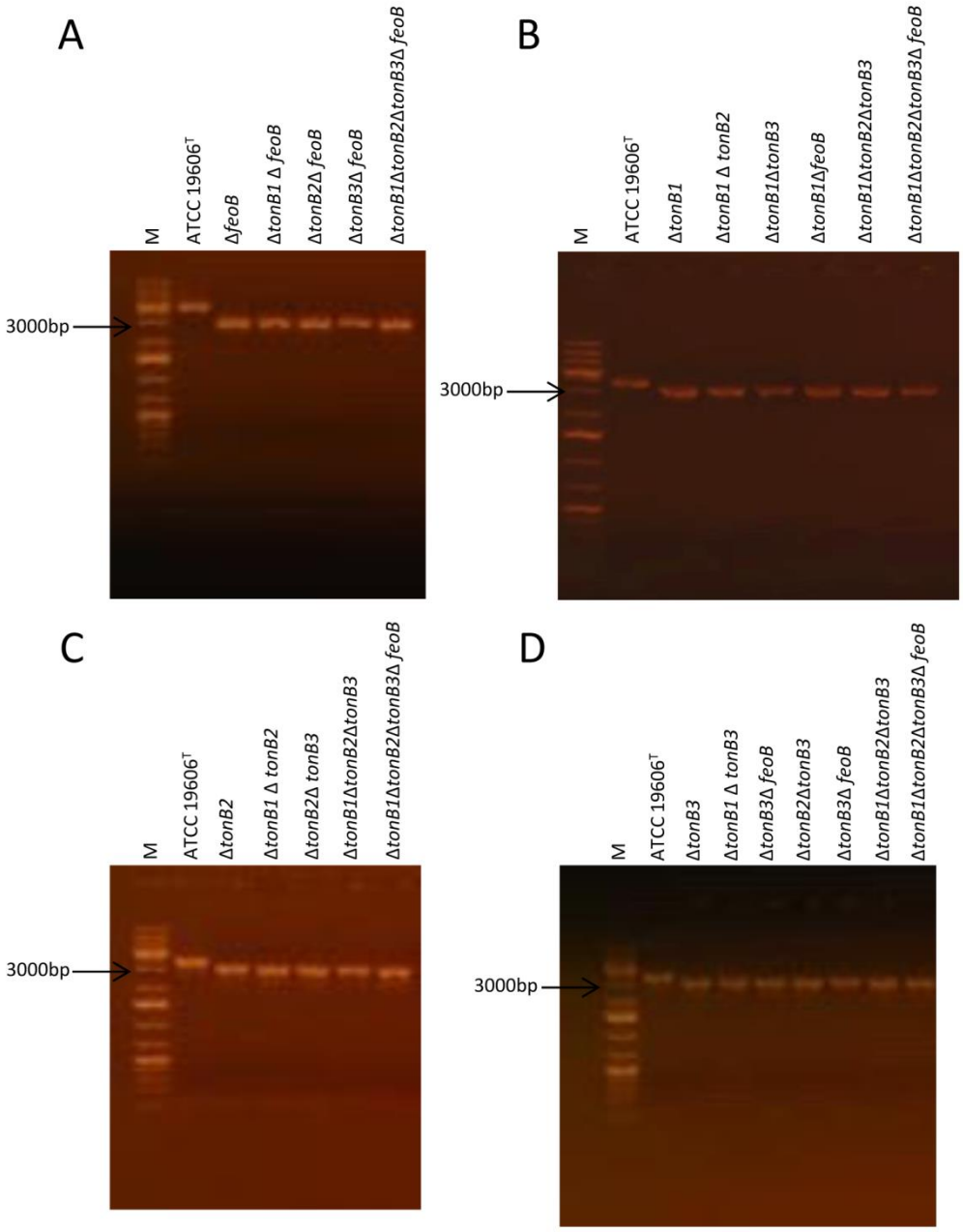

Figure S1. PCR verification of *A. baumannii* iron uptake mutants. Successful deletions of target genes were verified by PCR in the indicated strains, using different primers combinations: *feoB*_ctr_for and *feoB*_ctr_rev (A), *tonB1*_ctr_for and *tonB1*_ctr_rev (B), *tonB2*_ctr_for and *tonB2*_ctr_rev (C) and *tonB3*_ctr_for and *tonB3*_ctr_rev (D).

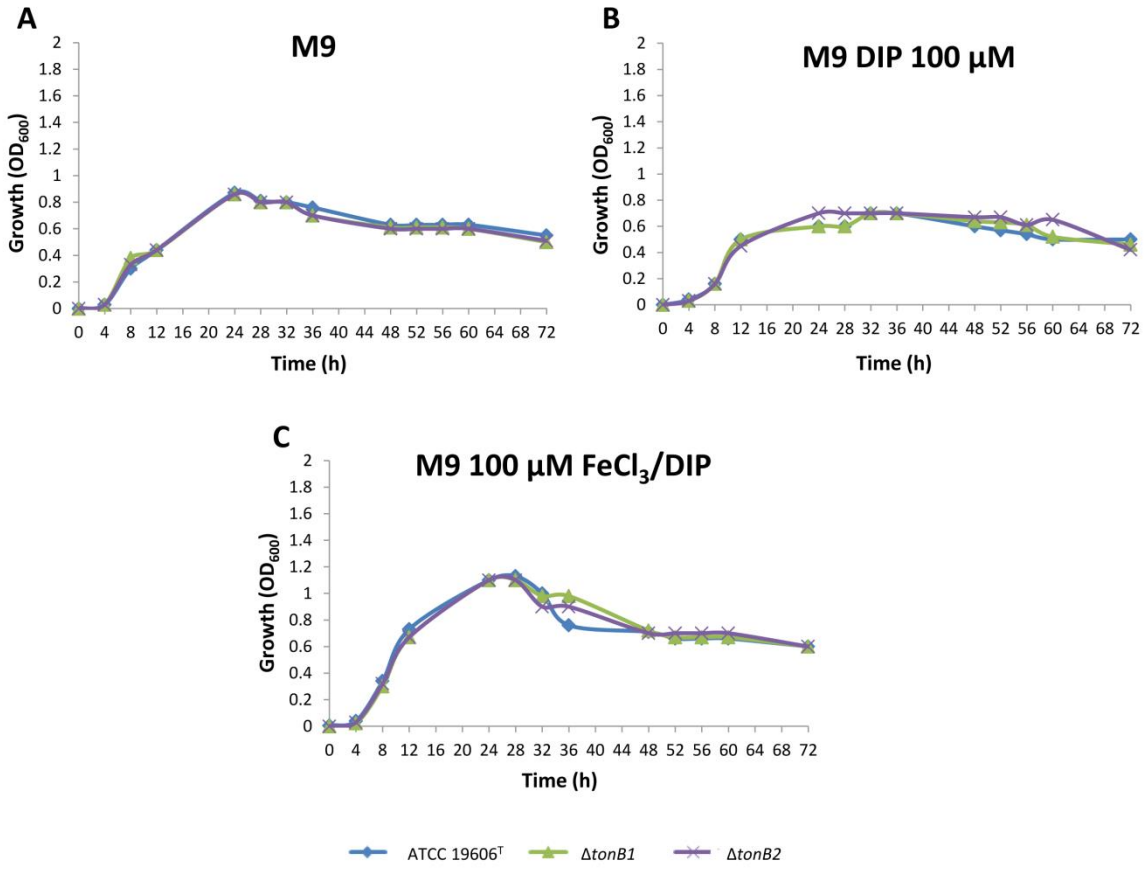

Figure S2. Effect of the *tonB1* and *tonB2* mutations on *A. baumannii* growth under iron limitation. Growth of the parental strain, ATCC 19606^T, and isogenic mutants Δ*tonB1* and Δ*tonB2* was monitored in M9 minimal medium (A), supplemented with DIP 100 µM (B), or 100 µM $FeCl_3$ and DIP (C). Values are the means of three independent experiments ± standard deviation (SD).

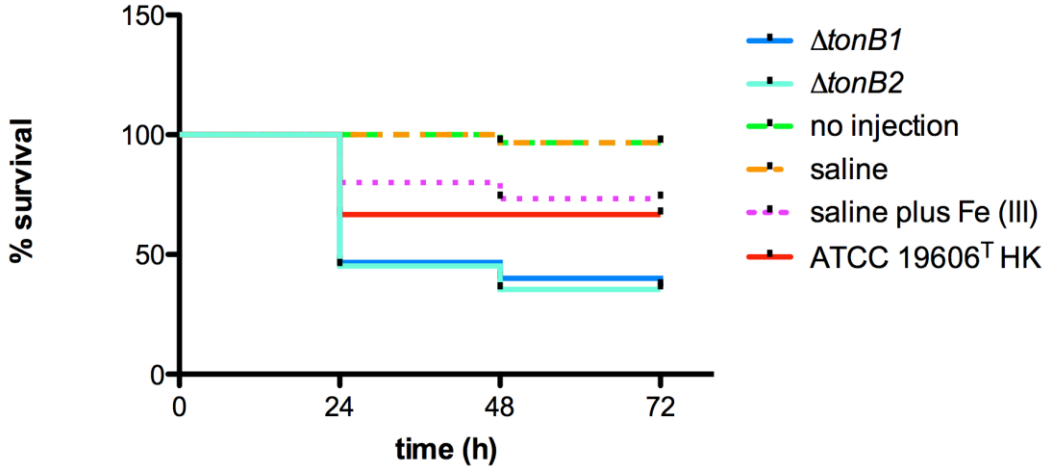

Figure S3. Role of *tonB1* and *tonB2* in *A. baumannii* virulence. *G. mellonella* caterpillars (n=30) were either not injected (no injection) or injected with 1×10^6 bacterial cells of Δ*tonB1*, Δ*tonB2*, heat-killed ATCC 19606^T (ATCC 19606^T HK) or saline ± 10 µM $FeCl_3$. Caterpillar death was determined daily for 3 days after incubation at 37°C in darkness.

Chapter 5

Concluding remarks

Concluding remarks

A. baumannii has emerged as one of the most worrying clinical entities among nosocomial pathogens. During the last 30 years this species has acquired, with surprising rapidity, an impressive array of antibiotic resistance determinants and has spread among hospitals and health care institutions worldwide. Infections caused by *A. baumannii* include ventilator-associated pneumonia, bloodstream infections, secondary meningitis, wound, skin, soft-tissue and urinary tract infections. Species of the genus *Acinetobacter* colonise a wide range of habitats, including soil, surface water, vegetables, animals and humans (Baumann, 1968). However, infection reservoirs of *A. baumannii* outside of the hospital are still unknown.

In this final chapter, main findings of this PhD thesis are summarized and major achievements briefly discussed. The first part of the thesis was aimed at addressing the question whether the colonization of different ecological niches has led to the differentiation of multiple intra-specific populations, or whether *A. baumannii* is a phenotypically plastic species, with an intrinsic capacity to adapt to a variety of environments. For this purpose, the genotypic and phenotypic diversity of a wide collection of *A. baumannii* strains isolated from multiple non-human sources (veterinary, soil, water, food-associated) was compared with that of representative clinical isolates. To understand the species diversity, a large collection of *A. baumannii* strains from environmental, veterinary and clinical sources were collected and characterized using genotypic and phenotypic methods. At the genotypic level, greater diversity was observed for isolates originating from non-pathological samples, compared with veterinary and clinical isolates which clustered in the three main epidemic lineages (ICLs I, II and III). Phenotypic diversity, *i.e.* variability in expression of a given phenotypic trait, was observed within each group of isolates. Comparison between the three groups, however, evidenced a few interesting phenotypic differences for some traits related to *in vivo* fitness and virulence, and these differences would provide some clues to understanding *A. baumannii* pathogenicity in animals. For instance, environmental isolates showed a narrower growth temperature range and reduced ability to multiply in serum and form surface pellicle, suggesting limited growth capabilities *in vivo*. It was also interesting to observe a different pattern of resistance to some toxic metals between groups; the group of environmental isolates was more sensitive to mercury and arsenic, two metals formerly used as ingredients of anti-infective drugs, but more resistant to nickel ad cadmium, two typical environmental pollutants. Most importantly, analysis of antibiotic resistance in the three groups delineated different patterns; one represented by the overall susceptible group of environmental isolates,

and the other including resistant isolates from animal and human sources. Altogether, these observations suggest that the *A. baumannii* population living in the extra-hospital environment is genetically distinct from epidemic ICLs and invariably susceptible to antibiotic since not previously exposed to these drugs. Conversely, strains infecting animals and humans belong to the epidemic ICLs and are endowed with much higher resistance to antimicrobials. This investigation provided a clearer understanding of the diversity of *A. baumannii* species and of the mechanisms that could have allowed it to become a successful nosocomial pathogen. Moreover, it appears that *A. baumannii* pathogenicity is multifactorial (not linked to a single character) and combinatorial (different combination of characters can account for it) and that antibiotic resistance rather than virulence has played a crucial role for the success of *A. baumannii* as a pathogen of both humans and animals.

Considering the crucial role of iron in *A. baumannii*-host interactions (Zimbler *et al*., 2009; Mortensen *et al*., 2013), in the second part of this thesis iron metabolism has been considered as a possible target for chelation-based antibacterial chemotherapy. The effect of iron restriction on *A. baumannii* growth and biofilm formation was assessed. Strains and optimal growth conditions for the generation of *A. baumannii* biofilms were preliminarily established. Substantial inter-strain variability and growth medium-dependence for biofilm formation by *A. baumannii* isolates were observed. Neither planktonic nor biofilm growth of *A. baumannii* were affected by exogenous chelators. Biofilm formation was either stimulated by iron or not responsive to iron in the majority of isolates tested, indicating that iron starvation is not sensed as an overall biofilm-inducing stimulus by *A. baumannii*. This study highlighted an impressive iron withholding capability of *A. baumannii*, which could pose serious limitations to chelation-based antimicrobial and anti-biofilm therapies.

The third part of the thesis was aimed at searches for new druggable targets in *A. baumannii*. Iron is an essential nutrient for bacteria, and plays a role in differential gene expression. Both *in silico* and *in vitro* data indicate that *A. baumannii* isolates have evolved multiple iron-uptake systems (Dorsey *et al*., 2004; Zimbler *et al*., 2009; Antunes *et al*., 2011; Eijkelkamp *et al*., 2011; Proschak *et al*., 2013). The TonB energy transducing machinery, encoded by *tonB-exbB-exbD* genes, is needed for translocation of ferric siderophores and heme through the outer membrane receptor of Gram negative bacteria (Proschak, 2013; Noinaj *et al*., 2010). Ferrous iron uptake is mediated by the Feo system. To gain more insight into the iron uptake-capability of *A. baumannii* 19606^{T}, different knock-out mutants both in “ferrous iron uptake” (in the *feoB* gene) and in “the ferric iron/heme uptake” (in the three *tonB* alleles) were generated. The iron uptake mutants were compared with the wild type strain for growth

capability under different conditions of iron availability (e.g. in human serum, or in the presence of exogenous chelators). To assess the uptake specificity of the mutated systems, growth promotion assays were also performed under conditions of extreme iron deficiency, and in the presence of exogenously added iron sources. Finally, mutants were tested for their lethality in the insect *G. mellonella*, which offers a simple animal model to assess *A. baumannii* pathogenicity. The most remarkable finding was that the *tonB3* gene is the principal allele (among the three *tonB* orthologs) for transport of the ferric ion into the cell. Notably, the *tonB3* mutation drastically reduced the ability of *A. baumannii* to multiply in iron poor environments, as those encountered by the bacterium during human or animal infection. The reduced fitness of the *tonB3* mutant was confirmed also *in vivo*, by the dramatic loss of lethality of the *tonB3* mutant in the *G. mellonella* model of infection. Ferrous iron uptake appeared to facilitate growth in human serum, but the *feoB* mutation had no effect on lethality in *G. mellonella*. It will be interesting to test the virulence of the different iron uptake mutants in more sophisticate animal models of infection, and to this purpose a new collaboration has been issued with Dr Wangxue Chen (National Research Council, Canada) for mouse pathogenicity studies.

In conclusion, the above results clarify the contribution of ferrous and ferric iron uptake systems to the survival and pathogenicity of *A. baumannii*, and point to the TonB3 system as a potential druggable target. Interfering with bacterial nutrition is now considered a viable strategy for antibacterial drug discovery, thus the identification in *A. baumannii* of an essential protein for iron transport opens a new perspective to the development of new target-based antimicrobials.

Chapter 5 References

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List of publications

- **Gentile, V., Frangipani, E., Bonchi, C., Minandri, F., Runci, F., Visca, P.** 2014. Iron and *Acinetobacter baumannii* Biofilm Formation. Pathogens. **3**(3):704-19. doi: 10.3390/pathogens3030704.

Manuscript in preparation

- **Gentile, V., Antunes, L. C., Visca, P.** Genotypic and phenotypic diversity among *Acinetobacter baumannii* isolates from human, veterinary and environmental sources.

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