UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

DOCTORAL IN PUBLIC HEALTH AND PREVENTIVE MEDICINE

XXIX DOCTORAL PROGRAM

Genomic epidemiology of multi-drug resistant *Acinetobacter baumannii*: characterization of antibiotic resistant determinants and virulence features of successful resistant clonal lineages

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Academic Year 2016-2017

Abstract

Acinetobacter baumannii is a globally important nosocomial pathogen associated with clinical infections that are difficult to treat due to broad antimicrobial resistance. *A. baumannii* epidemics are caused by a limited number of strains worldwide, belonging to the initially named European clones, but now regarded to as International clonal lineages (IC) I, II and III and a few additional lineages recently emerged as epidemic clones in some regions, such as the sequence types (ST)10, ST15, ST25, ST78 and ST79 according to Pasteur's multilocus sequencing typing (MLST) scheme.

The first chapter of the thesis analyzes the genomic epidemiology of 19 *A. baumannii* strains belonging to the emerging ST25 lineage. Seven genomes from the sequence type (ST)25 lineage were sequenced and compared to 12 ST25 genomes deposited in public databases. A recombination analysis identified multiple genomic regions that are homoplasious in the ST25 phylogeny, indicating active or historical recombination. Genes associated with antimicrobial resistance were differentially distributed between ST25 genomes, which matched our laboratory-based antimicrobial susceptibility typing. Differences were also observed in biofilm formation between ST25 isolates, which were demonstrated to produce significantly more extensive biofilm than an isolate from the ST1 clonal lineage. These results demonstrate that within *A. baumannii* , even a fairly recently derived monophyletic lineage can still exhibit significant genotypic and phenotypic diversity. These results have implications for associating outbreaks with sequence typing as well as understanding mechanisms behind the global propagation of successful *A. baumannii* lineages.

According to their antimicrobial susceptibility profile, *A. baumannii* strains can be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) if they are resistant to three or more, all but one or two, and all classes of potentially effective antimicrobial agents, respectively. MDR and XDR in *A. baumannii* is now an emerging issue worldwide. Strains responsible for epidemics are resistant to carbapenems and show intermediate resistance to tigecycline, but usually retain susceptibility to colistin. The second chapter of the thesis describes the molecular epidemiology of 194-single patient carbapenem resistant *A. baumannii* (CRAB) isolates collected

randomly during 2015 from 11 tertiary hospitals located throughout Greece. CRAB isolates were MDR or XDR, the most active antibiotics being trimethoprim/sulfamethoxazole (SXT) (34.6% of isolates susceptible), minocycline (71.6%), colistin (72.7%) and tigecycline (MIC50/90 values, 1/2mg/L). The *bla*OXA-23-like gene was identified in 188 isolates (96.9%), *bla*OXA-23-like together with *bla*OXA-58-like in 3 isolates (1.5%), *bla*OXA-58-like in 2 isolates (1.0%) and *bla*OXA-40-like in 1 isolate (0.5%). IS*Aba1* was found upstream of the *bla*OXA-23-like gene in all isolates. International clone (IC) 2 comprised 157 isolates (80.9%), IC1 comprised 36 isolates (18.6%) and ST78 comprised 1 isolate (0.5%). All IC2 and IC1 isolates tested by MLST were ST2 and ST1, respectively. Seven PFGE types were detected. IC2 isolates were resistant to more antibiotics than IC1, except for SXT. This nationwide study showed that CRAB isolates in Greek hospitals currently produce almost uniformly the OXA-23 carbapenemase and belong mainly to IC2 and, to a lesser extent, IC1. Of particular concern, colistin susceptibility is recently severely reduced.

The spread of extensively drug-resistant (XDR) gram-negative bacteria has boosted colistin use, with a resultant selection of colistin-resistant, often pandrug-resistant strains. Whether acquisition of further resistance mechanisms translates into a reduced virulence is the subject of active research. The third chapter of the thesis analyzes phenotypic and genotypic characteristics, molecular mechanisms of colistin resistance, and in vitro and in vivo fitness of sequential colistin-sensitive and colistin-resistant *A. baumannii* strains isolated from an immunocompromised patient who developed infection due to colistin-resistant *A. baumannii* while on long-term colistin therapy. Both colistin-sensitive and colistin-resistant strains were XDR and showed identical ST78 genotype. At variance with prior reports on colistin-resistant strains of *A. baumannii*, resistance to colistin due to P233S mutation in PmrB sensor kinase did not associate with any measurable reduction in strain fitness, growth characteristics, and virulence.

In addition to extensively antimicrobial resistance, *A. baumannii* strains responsible for epidemics show elevated resistance to desiccation, high biofilm-forming capacity on abiotic surfaces and adherence to host epithelial cells, virulence-related features which might have favored the spread and persistence in the hospital environment. As in other microrganisms, the formation of biofilm in *A. baumannii* is a redundantly organized, multifactorial process involving multiple cellular components. A giant protein called BAP (biofilm-associated protein) plays a role in biofilm formation and adhesion to host cells in *A.*

baumannii. Most of the protein is made by arrays of 80–110 aa modules featuring immunoglobulin-like (Ig-like) motifs. The fourth chapter of the thesis analyzes the organization of BAP coding sequences in wholly sequenced strains as in a large set of whole genome shotguns (WGS) of both *A. baumannii* and other *Acinetobacter* species. In silico analyses showed that BAP was highly polymorphic, distinguishable in three main types for changes both in the repetitive and the COOH region. Two additional proteins, BLP1 and BLP2 (for BAP-like proteins), structurally related to BAP because similarly containing Ig-like domains were identified among *Acinetobacter* species. BLP1 is missing in the majority of type-3 BAP strains. BLP2 was largely conserved, but was frequently missing in BAP-negative cells. The knock-out of either BLP1 or BLP2 genes of the *A. baumannii* ST1 AYE strain severely affected biofilm formation, as measured by comparing biofilm biomass and thickness, and adherence to epithelial cells. The above data suggest that multiple proteins sharing Ig-like repeats contribute to biofilm formation in *Acinetobacter* species. The uneven distribution of the different BAP types, BLP1, and BLP2 is highly indicative that alternative protein complexes involved in biofilm formation are assembled in different *A. baumannii* strains.

Riassunto

Acinetobacter baumannii è un importante patogeno nosocomiale diffuso a livello mondiale, associato ad infezioni cliniche che sono difficili da trattare a causa dall'ampia resistenza antimicrobica. Le epidemie di *A. baumannii* sono causate sia da un numero ristretto di ceppi diffusi in tutto il mondo, appartenenti ai cloni inizialmente denominati Europei, ma ora considerati cloni Internazionali (IC) I, II ed III sia da pochi cloni epidemici recentemente emersi in alcune regioni, come i sequence types (ST)10, ST15, ST25, ST78 and ST79 secondo lo schema di tipizzazione mediante multilocus sequencing typing (MLST) dell'Istituto Pasteur.

Il primo capitolo di questa tesi analizza l'epidemiologia genomica di 19 ceppi di *A. baumannii* appartenenti al complesso clonale emergente ST25. Sette genomi con genotipo (ST)25 sono stati sequenziati e confrontati a 12 genomi con genotipo ST25 depositati in pubbliche banche dati. Un'analisi di ricombinazione ha identificato regioni genomiche multiple le quali sono omeoplastiche nella filogenesi del complesso clonale ST25, indicando ricombinazioni attive o passate. Geni associati con la resistenza antimicrobica sono differentemente distribuiti tra i genomi di ST25, risultato che è in accordo con l'analisi di suscettibilità agli agenti antimicrobici effettuata in laboratorio. Tra gli isolati ST25 sono state osservate anche differenze nella formazione di biofilm; questi isolati mostrano produrre significativamente più biofilm rispetto a un isolato appartenente al complesso clonale ST1. Questi risultati dimostrano che all'interno di *A. baumannii*, anche un derivato monofiletico abbastanza recente può ancora mostrare una significatività genotipica e fenotipica. I nostri dati dimostrano inoltre che la tipizzazione dei ceppi a livello genomico è importante per identificare i meccanismi responsabili della propagazione globale delle linee clonali di *A. baumannii*.

I ceppi di *A. baumannii*, secondo il loro profilo di suscettibilità antimicrobica, possono essere classificati come multi-resistenti (MDR), estensivamente resistenti (XDR) e totalmente resistenti (PDR) se sono rispettivamente resistenti a tre o più, a tutti tranne uno o due, e a tutte le classi di agenti antimicrobici potenzialmente efficaci. Attualmente, la diffusione di ceppi di *A. baumannii* MDR e XDR è un problema emergente in tutto il mondo. I ceppi epidemici sono resistenti ai carbapenemi e mostrano una resistenza intermedia alla

tigeciclina, ma di solito risultano suscettibili alla colistina. Il secondo capitolo di questa tesi descrive l'epidemiologia molecolare di 194 ceppi di *A. baumannii* resistenti ai carbapenemi (CRAB) isolati casualmente da singoli pazienti nel corso del 2015 da 11 ospedali terziari situati in tutta la Grecia. CRAB isolati erano MDR o XDR, gli antibiotici farmacologicamente più attivi risultavano essere il trimetoprim/sulfametossazolo (SXT) (34,6% degli isolati suscettibili), la minociclina (71,6%), la colistina (72,7%) e la tigeciclina (valori di MIC50/90, 1/2mg/L). Il gene blaOXA-23 è stato identificato in 188 isolati (96,9%), blaOXA-23-like insieme a blaOXA-58-like in 3 isolati (1,5%), blaOXA-58-like in 2 isolati (1,0%) e blaOXA-40-like in 1 isolato (0,5%). In tutti gli isolati ISAba1 è stato trovato a monte del gene blaOXA-23-like. Il clone internazionale (IC) 2 comprende 157 isolati (80,9%), IC1 comprende 36 isolati (18,6%) e ST78 comprende 1 isolato (0,5%). Tutti gli isolati appartenenti al clone Internazionale (IC)2 e IC1 sono stati genotipizzati mediante MLST e corrispondevano rispettivamente al genotipo ST2 e ST1. Sono stati individuati sette profili di PFGE. Gli isolati IC2 erano resistenti a più antibiotici rispetto a IC1, ad eccezione di SXT. Questo studio nazionale ha mostrato che negli ospedali Greci gli isolati CRAB producono quasi uniformemente la carbapenemasi OXA-23 e appartengono principalmente a IC2 e, in misura minore, a IC1. Di particolare preoccupazione risulta essere la ridotta suscettibilità all'antibiotico colistina.

La diffusione di batteri gram-negativi estensivamente resistenti ai farmaci (XDR) ha determinato un aumento dell'impiego della colistina, con una conseguente selezione di ceppi colistina-resistenti, spesso ceppi totalmente resistenti. Se l'acquisizione di ulteriori meccanismi di resistenza si traduce in una ridotta virulenza è oggetto di ricerca attiva. Il terzo capitolo della tesi analizza le caratteristiche fenotipiche e genotipiche, i meccanismi molecolari della resistenza alla colistina e la fitness in vitro e in vivo di ceppi *A. baumannii* colistina-suscettibili e colistana-resistenti isolati da un paziente immunocompromesso che ha sviluppato l'infezione dovuta ad un ceppo di *A. baumannii* colistina-resistente, mentre era sottoposto per un lungo arco di tempo a una terapia con la colistina. Sia ceppi colistina-sensibili che colistina-resistenti erano XDR e hanno mostrato possedere un identico genotipo ST78. A differenza di precedenti relazioni su ceppi di *A. baumannii* colistina-resistenti, la resistenza alla colistina dovuta alla mutazione P233S nel sensore chinasi PmrB non è associata con nessuna riduzione misurabile della fitness, delle caratteristiche di crescita e della virulenza.

Oltre all' estesa resistenza antimicrobica, i ceppi di *A. baumannii* responsabili di epidemie mostrano un'elevata resistenza all'essiccazione, un'elevata capacità di formare biofilm sulle superfici abiotiche e adesione alle cellule epiteliali dell'ospite, caratteristiche correlate alla virulenza che potrebbero aver favorito la diffusione e la persistenza nell'ambiente ospedaliero. Come in altri microrganismi anche in *A. baumannii*, la formazione di biofilm è un processo multifattoriale che coinvolge diverse componenti. Una grande proteina chiamata BAP (biofilm-associated protein) svolge un ruolo nella formazione di biofilm e nell'adesione alle cellule dell'ospite in *A. baumannii*. La maggior parte delle proteine BAP è formata da un insieme di moduli di 80-110 amminoacidi caratterizzati da domini immunoglobulino-simili (Ig-like). Il quarto capitolo della tesi analizza l'organizzazione delle sequenze codificanti BAP in ceppi completamenti sequenziati in una ampia collezione di sequenze genomiche di *A. baumannii* e di altre specie di *Acinetobacter*. Nelle analisi "in silico" è emerso che BAP era altamente polimorfica, distinguibile in tre tipi principali per modifiche sia nelle regioni ripetute che nella regione COOH terminale. Due proteine addizionali, BLP1 e BLP2 (per BAP-like proteina), strutturalmente correlate a BAP, perché contengono domini immunoglobulino-simili, sono state identificate tra le specie di *Acinetobacter*. La BLP1 è mancante nella maggior parte dei ceppi che contengono la BAP di tipo-3. BLP2 è in gran parte conservata, ma è spesso assente nei ceppi in cui non è presente la BAP. L'eliminazione nel ceppo *A. baumannii* AYE con genotipo ST1 di entrambi i geni BLP1 o BLP2 ha gravemente influenzato la formazione di biofilm, misurata confrontando la biomassa e lo spessore del biofilm e l'aderenza alle cellule epiteliali. I dati suggeriscono che molteplici proteine che condividono ripetizioni Ig-like contribuiscono alla formazione di biofilm nelle specie di *Acinetobacter*. La distribuzione non omogenea dei differenti tipi di BAP, BLP1 e BLP2 è altamente indicativa che i complessi proteici alternativi coinvolti nella formazione del biofilm sono assemblati in diversi ceppi di *A. baumannii*.

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Chapter 1

Introduction

1.1 *Acinetobacter* genus

The Dutch microbiologist Beijerinck first isolated the organism in 1911 from soil using minimal media enriched with calcium acetate [1]. Originally described as *Micrococcus calco-aceticus*, the genus *Acinetobacter* (coming from the Greek "akinetos," meaning non-motile) was proposed by Brisou and Prevot in 1954 to separate the motile organisms within the genus Achromobacte [2]. The genus *Acinetobacter* is now defined as including Gram-negative coccobacilli, with a G+C content of 39–47%, that are strictly aerobic, non-motile, catalase positive, and oxidase negative [3]. Using DNA-DNA hybridization technique, the *Acinetobacter* genus consists of more than 33 species with valid names and at least ten genomic species (http://www.bacterio.net/*acinetobacter*.html, last accessed June 2014) [4]. Because phenotypic Identification of *Acinetobacter* isolates to the species level using phenotypic methods is difficult, several genotypic methods have been developed for genomic species identification, which include amplified 16S rRNA gene restriction analysis, high-resolution fingerprint analysis by amplified fragment length polymorphism, sequence analysis of the 16S–23S rRNA gene spacer region, *rpoB* sequencing and *gyrB* multiplex PCR [3,6-10]. Strains belonging to these species are genetically and phenotypically very similar to an environmental species, *Acinetobacter calcoaceticus*, and thus are often grouped together into the so-called *A. calcoaceticus–Acinetobacter baumannii* complex [4]. The most clinically successful species of the genus are *A. baumannii* (genospecies 2), *A. pittii* (genospecies 3) and *A. nosocomialis* (genospecies 13TU) [5]. The species that is most frequently recognized as a pathogen is *A. baumannii*, which causes a variety of healthcare-associated infections, comprising hospital-acquired and ventilator-associated pneumonia, bacteremia, urinary tract infection and surgical-site infection, especially in intensive care unit patients [5].

1.2 *Acinetobacter baumannii*

A. baumannii is a glucose-non-fermentative, Gram-negative coccobacillus, it is ubiquitous in nature and has been recovered from soil, water, animals, and humans. *A. baumannii* have emerged in recent years as a major cause of nosocomial infections associated with high morbidity and mortality. The capability to survive in dry conditions and resistance to disinfectants and antimicrobial agents contribute to the selection of *A. baumannii* in the hospital setting [5,11,12]. Although *A. baumannii* has been classically recognized as a hospital-acquired pathogen, an increased prevalence of multidrug-resistant *A. baumannii* isolates has been observed among older adults in community hospitals and nursing homes in the USA [13]. *A. baumannii* infections are often associated with epidemic spread, and outbreak strains are frequently multidrug resistant (MDR). A most concerning development is the increasing occurrence of strains resistant to carbapenems or even to last resource antimicrobial agents including colistin or the new antibiotic tigecycline [14].

1.3 Molecular epidemiological of *Acinetobacter baumannii*

A. baumannii is a globally distributed nosocomial pathogen. Infections caused by *A. baumannii* are increasing worldwide, due to the rapid expansion of a selected number of genetically distinct lineages [15,16]. Three of these lineages, known as international clones I to III, represent globally distributed and ubiquitous clades [17]. Other successful lineages, which spread in single institutions and/or worldwide, have been identified in the population structure of *A. baumannii* using different genotyping methods, such as such as the sequence types (ST)10, ST15, ST25, ST78 and ST79 according to Pasteur's multilocus sequencing typing (MLST) scheme. The population structure of *A. baumannii* has been studied using two MLST nomenclatures, which complicates advances of the epidemiological and population biology knowledge of this important pathogen [17,18,19]. The Oxford's MLST database assigned *A. baumannii* strains to 1584 different STs; similarly, the Pasteur's MLST scheme assigned *A. baumannii* strains to 1076 different STs (https://pubmlst.org/abaumannii/, last accessed July 2017). eBURST analysis [20] can group the Oxford's STs and the Pasteur's STs into at least 21 and 20 clonal complexes (CCs),

respectively, of which ten are corresponding and delineates ten distinct clonal lineages that spread in single institutions and/or worldwide (Table 2). In fact, international clone I and more recently international clone II prevailed worldwide; international clone III, CC10, CC15, CC32, ST25 and ST52 of Pasteur's MLST scheme were isolated in different European countries; CC79 of Pasteur's MLST scheme was isolated in Brazil and Spain; and ST78 of Pasteur's MLST scheme was isolated in several Italian hospitals. Additional typing techniques, such as the tri-locus sequencing-based typing and DiversiLab PCR-based typing schemes, are useful tools also to identify *A. baumannii* clonal lineages [16].

Table 2. *A. baumannii* clonal lineages

AFLP	Pasteur's MLST	Oxford's MLST	*bla*$_{\text{OXA51-like}}$ SBT	3LST	DiversiLab™
I	CC1 (23)	CC109 (26)	*bla*$_{\text{OXA-69}}$	SG2	WW1
II	CC2 (55)	CC92 (69)	*bla*$_{\text{OXA-66}}$	SG1	WW2
III	CC3 (5)	CC106 (5)	*bla*$_{\text{OXA-71}}$	SG3	WW3
	CC25 (11)	CC110 (19)	*bla*$_{\text{OXA-64}}$	SG4	WW7
Cluster A	CC15 (11)	CC103 (10)	*bla*$_{\text{OXA-51}}$	SG5	WW4
	CC78 (2)	CC944 (5)	*bla*$_{\text{OXA-90}}$	SG6	WW6
Cluster B	CC10 (6)	CC447 (12)	*bla*$_{\text{OXA-128}}$		WW8
Cluster 6	CC32 (5)	CC472 (3)	*bla*$_{\text{OXA-100}}$		
Cluster C	CC52 (3)	CC931(3)	*bla*$_{\text{OXA-98}}$		
	CC79 (10)	CC113 (15)	*bla*$_{\text{OXA-65}}$		

Modified by Zarrilli et al, Int J Antimicrob Agents, 41,11-19, 2013

1.4 Antimicrobial resistance in *Acinetobacter baumannii*

Resistance to antimicrobial agents is the main advantage of *A. baumannii* in the nosocomial environment. *A. baumannii* possesses several mechanisms of resistance to all existing antibiotic classes as well as a striking capacity to acquire new determinants of resistance. Resistance mechanisms involve antimicrobial-degrading enzymes, efflux pumps, target modification and porin deficiency. The acquisition of antimicrobial resistance can be supported by mobile genetic elements (plasmids, transposons and integrons) [5].

According to the actual antimicrobial susceptibility profile, *A. baumannii* strains can be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) if they are resistant to three or more, all but one or two, and all classes of potentially effective antimicrobial agents, respectively [21]. Molecular mechanisms of antimicrobial resistance in MDR, XDR and PDR *A. baumannii* are shown in Table 3 [22].

MDR and XDR in *A. baumannii* is now an emerging issue worldwide. Strains responsible for epidemics are resistant to carbapenems and show intermediate resistance to tigecycline, but usually retain susceptibility to colistin [16,23,24]. Colistin often constitutes the only active treatment alternative [25]. Colistin is rapidly bactericidal for Gram-negative bacteria, affecting the lipid A moiety of lipopolysaccharide (LPS) and thus disorganizing the outer membrane [26]. During the last few years, clinical isolates of *A. baumannii* expressing resistance to colistin have emerged, and outbreaks have been reported. The emergence of colistin resistance has been correlated with the selective pressure exerted by prolonged exposure to this drug [27,28].

Table 3. Mechanismis of antimicrobial resistance in MDR, XDR and PDR *A.baumannii*

Antimicrobial category	Antimicrobial agent	Major resistance mechanism
Penicillins + β-lactamase inhibitors	Ampicillin–sulbactam	blaTEM-1 overexpression
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin–tazobactam Ticarcillin–clavulanic acid	ADC class C β-lactamase overexpression
Extended-spectrum cephalosporins	Cefotaxime Ceftriaxone Ceftazidime Cefepime	ADC class C β-lactamase VEB-1, VEB-2, PER-1, PER-2, TEM-92, TEM-116, SHV-12, CTX-M-2, CTX-M-3 OXA-51-like class D β-lactamase
Antipseudomonal carbapenems	Imipenem Meropenem Doripenem	Class B carbapenemase (IMP, VIM, SIM, NDM) Class A carbapenemase (KPC, GES) Class D carbapenemase (OXA-23, OXA-24/40, OXA-58, OXA-143 clusters) Changes in outer membrane proteins (OprD-like OMPs and CarO)
Antipseudomonal fluoroquinolones	Ciprofloxacin Levofloxacin	Mutations in gyrase subunit topoisomerase IV subunit
Aminoglycosides	Gentamicin Tobramycin Amikacin Netilmicin	Aminoglycoside-modifying enzymes 16S rRNA methyltransferase
Folate pathway inhibitors	Trimethoprim–sulfamethoxazole	Dihydropteroate synthase Dihydrofolate reductase
Tetracyclines	Tetracycline Doxycycline Minocycline Tigecycline	Efflux pumps overexpression
Polymyxins	Colistin Polymyxin B	Mutations in *pmrAB* two component regulator and in genes of lipid A biosynthesis
Rifampicin		Mutations in *rpoB* Rifampicin ADP-ribosylating transferase Arr-2 Efflux pumps overexpression

Durante et al. Future Microbiol. 9(6), 773–789, 2014

1.5 Virulence determinants

Despite extensive research into the virulence potential of this emerging pathogen, few studies are available on the virulence traits and pathogenic potential of *A. baumannii*. *A. baumannii* strains responsible for epidemics show elevated resistance to desiccation, high biofilm-forming capacity on abiotic surfaces and adherence to host epithelial cells, virulence-related features which might have favored the spread and persistence in the hospital environment [29]. It has been reported that *A. baumannii* can form biofilms on several abiotic surfaces, including polystyrene, polypropylene, polytetrafluoroethylene and glass. Cells forming biofilms are embedded within a polymeric conglomerate of proteins and polysaccharides. Biofilms are resistant to host immune defenses, detergents and antibiotics, and antibiotic resistance of microrganisms in these habitats can be increased up to a thousand-fold [30].

Biofilm formation in *A. baumannii* has been shown to be positively correlated with the expression of chaperone–usher type I pili assembly system, the outer membrane protein OmpA, the extracellular polysaccharide poly-b-(1,6)-N-acetyl glucosamine (PNAG), a homolog of the staphylococcal biofilm-associated protein (BAP). Also, *A. baumannii* is able to form a tight biofilm structure at air-liquid interface, which is generally referred as pellicle and is associated with the presence of poly-Nacetylglucosamine (PNAG) polysaccharide and csuA/B usher protein of pili assembly system. Moreover, genes involved in motility, iron acquisition, quorum sensing and those encoding efflux system components such as RND efflux pump AdeT are over-expressed during biofilm growth of *A. baumannii*. Virulence determinants involved in biofilm growth, including OmpA, BAP, regulate adherence/invasion of *A. baumannii* to host epithelial cells, thus explaining the correlation between biofilm formation and adherence to host epithelial cells found in epidemic *A. baumannii* isolates [31-39].

The ability of *A. baumannii* strains to survive for a long time on dry surfaces is likely to contribute to their persistence in hospitals [39]. In this respect, it has been reported that RecA protein is involved in general stress response and resistance to heat shock and desiccation in *A. baumannii* [40].

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Chapter 2

Phylogenetic and genetic diversity within ST25 *Acinetobacter baumannii;* the propagation of a clonal lineage with non-clonal behavior

OPEN

Phylogenetic and genomic diversity in isolates from the globally distributed *Acinetobacter baumannii* ST25 lineage

received: 12 May 2015
Accepted: 22 September 2015
Published: 14 October 2015

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Acinetobacter baumannii **is a globally distributed nosocomial pathogen that has gained interest due to its resistance to most currently used antimicrobials. Whole genome sequencing (WGS) and phylogenetics has begun to reveal the global genetic diversity of this pathogen. The evolution of *A. baumannii* has largely been defined by recombination, punctuated by the emergence and proliferation of defined clonal lineages. In this study we sequenced seven genomes from the sequence type (ST)25 lineage and compared them to 12 ST25 genomes deposited in public databases. A recombination analysis identified multiple genomic regions that are homoplasious in the ST25 phylogeny, indicating active or historical recombination. Genes associated with antimicrobial resistance were differentially distributed between ST25 genomes, which matched our laboratory-based antimicrobial susceptibility typing. Differences were also observed in biofilm formation between ST25 isolates, which were demonstrated to produce significantly more extensive biofilm than an isolate from the ST1 clonal lineage. These results demonstrate that within *A. baumannii*, even a fairly recently derived monophyletic lineage can still exhibit significant genotypic and phenotypic diversity. These results have implications for associating outbreaks with sequence typing as well as understanding mechanisms behind the global propagation of successful *A. baumannii* lineages.**

Acinetobacter baumannii is an emergent nosocomial pathogen of increasing interest due to its widespread resistance to antimicrobials[1]. *A. baumannii* is truly a global pathogen, with isolates collected from hospitals around the world[2,3], including injured soldiers from Iraq[4] and Afghanistan[5]. The concern is the emergence of multidrug-resistant (MDR)[6] and extremely drug-resistant (XDR)[7] isolates that are resistant to most currently used therapeutics. Genes that confer resistance in *A. baumannii* have been documented, including class D beta-lactamases[8], such as $bla_{OXA\text{-}51\text{-}like}$, which appears to be highly conserved across *A. baumannii*[9]. The insertion element IS*Aba*1 is required for carbapenem resistance in $bla_{OXA\text{-}51\text{-}like}$ positive isolates[10].

The genome of *A. baumannii* is highly plastic[11], with much of the evolution characterized by recombination[12] and horizontal gene transfer[13]. The core genome phylogeny of *A. baumannii* demonstrates highly divergent genomes, with the emergence of a few highly successful clonal lineages[12,14]. While the evolution

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Isolate	Year/Country	Isolate Source	Pasteur ST[a]	Oxford ST[b]	Reference
RUH 1486	1985/Netherlands	Umbilicus	25	229	Diancourt *et al.*, 2010
NM3	2008/UAE	Sputum	25	229	Sonnevend *et al.* 2013
LUH 14601	1996/Singapore	Respiratory Tract	25	not typed	Unpublished
LUH 6220	2000/Netherlands	Sputum	25	not assigned	Unpublished
PV38/LUH 13606	2007/Italy	Upper Respiratory tract	25	not typed	Carretto *et al.* 2010
LUH 7841	2002/Netherlands	venuous catheter tip	402	229	Unpublished
4390	2003/Greece	Bronchial	25	not assigned	Gogou *et al.* 2011
66492	2012/Argentina	Blood	25	110	Stietz *et al.* 2013
AO-471	2005/Thailand	Wound	25	not typed	Karah *et al.* 2011
741019	2011/Argentina	Pleural fluid	25	not assigned	Stietz *et al.* 2013
66295	2012/Argentina	Blood	25	110	Stietz *et al.* 2013
NM133	2008/UAE	Bronchial aspirate	25	110	Sonnevend *et al.* 2013
3890	2003/Greece	Bronchial aspirate	25	not assigned	Di Popolo *et al.* 2011
3865	2005/Turkey	Blood	25	440	Di Popolo *et al.* 2011
AO-21841	2006/Sweden	Intra-abdominal isolate	25	not typed	Karah *et al.* 2011
65904	2009/Argentina	Inwelling catheter	25	not typed	Stietz *et al.* 2013
161/07	2007/Germany	Respiratory Tract	25	440	Bonnin *et al.* 2012
SLO	2008/Slovenia	Respiratory Tract	25	not typed	Bonnin *et al.* 2012
4190	2009/Italy	Blood	25	not assigned	Zarrilli *et al.* 2011

Table 1. Metadata associated with ST25 isolates analyzed in this study. [a]ST assigned using Pasteur'sMLST scheme (Diancourt *et al.* 2010) [b]ST assigned using Oxford's MLST scheme (Bartual *et al.* 2005)

of these lineages is anticipated to be clonal, no in depth evolutionary studies have been performed to look at the fine scale evolution, recombination, and gene composition of these clades.

Infections caused by *A. baumannii* are increasing worldwide, possibly due to the rapid expansion of a selected number of genetically distinct lineages[12,14]. Three of these lineages, known as international clones I to III, represent globally distributed and ubiquitous clades[15]. Other successful lineages, which spread in single institutions and/or worldwide, have been identified in the population structure of *A. baumannii* using different genotyping methods, including sequence type ST25[14]. *A. baumannii* strains assigned to ST25 were responsible for epidemics in different European countries[16–20] and the United Arab Emirates[21] and were isolated as endemic or sporadic isolates in South America[22] and Asia[18], respectively. ST25 genomes are of increasing interest due to increasing antimicrobial resistance[14] found within novel genomic resistance elements[23].

The aim of the current study was to analyze the genomic epidemiology of 19 *A. baumannii* strains belonging to the ST25 lineage according to Pasteur's MLST scheme[15]. Understanding the composition and evolution of one successful global lineage may help in understanding the genetic basis for the emergence and proliferation of global clones of *A. baumannii.*

Methods

Isolates. The collection of ST25 isolates analyzed in this study includes 19 strains: three sporadic strains from Leiden's collection isolated during 1985, 2000 and 2002; 13 strains representative of epidemics or endemic circulation in different countries; three additional sporadic isolates selected because of their antimicrobial susceptibility profile and mechanisms of antimicrobial resistance[15–22] (Table 1). Seven of these isolates were chosen for sequencing.

Pulsed-field gel electrophoresis (PFGE) typing and dendrogram analysis. *ApaI* DNA macrorestriction and PFGE of *A. baumannii* isolates were performed as previously reported[24]. PFGE profiles were compared using the GelCompar II v. 4.6 software package (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was based on the un-weighted pair-group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyze the similarities of the banding patterns with a tolerance of 1%. Interpretation of chromosomal DNA restriction patterns was based on the criteria of Tenover *et al.*[25] and also on a similarity of >85% at dendrogram analysis, to indicate strain relatedness.

MLST typing. Multi-locus sequence typing (MLST) analysis was performed using the Institut Pasteur's MLST scheme as previously described[15]. Allele sequence and MLST profile definitions were assigned using the sequence and profile definitions available at http://pubmlst.org/abaumannii/. The MLST results were confirmed from the whole genome sequence analysis using a publically available script: https:// github.com/Victorian-Bioinformatics-Consortium/mlst.

DNA extraction, sequencing, assembly. DNA was extracted with the GenElute DNA extraction kit (Sigma-Aldrich, Milan, Italy). Sequence libraries were generated from extracted DNA as reported previously[9]. Genomes were sequenced to high depth on the IlluminaMiSeq platform. Resulting reads were adapter trimmed with Trimmomatic[26], error corrected with Hammer[27], and assembled with SPAdes v3.1[28]. The read coverage across each contig was evaluated, and contigs of an anomalous coverage, due to read crossover in multiplexed runs, were manually removed. The assembly stats for each genome are shown in Supplementary Table S1. All assemblies and raw reads were deposited in public databases (accession numbers in Supplementary Table S1). Annotation was performed with the NCBI PGAP pipeline.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Vitek 2 system (bioMérieux, Marcy l' Étoile, France). Imipenem, meropenem and colistin minimum inhibitory concentrations (MICs) were determined by agar dilution and Etest (bioMérieux) and interpreted using the EUCAST[29] and CLSI 2012[30] interpretative criteria.

***in silico* antimicrobial susceptibility profiling.** To identify previously characterized genes associated with antimicrobial resistance in our dataset, raw reads were mapped to the ResFinder database[31] with the SRST2 pipeline[32]; raw reads were used to determine the percentage of the reference gene covered, but also could identify variants compared to the reference database. SRST2 produces a table of all positive hits identified in each genome.

Biofilm formation. Biofilm formation was determined as previously described[55]. Three independent experiments, each one performed in triplicate, were conducted for each strain. Biofilms were grown in the presence and absence of 0.5 mg/L imipenem.

Cell adhesion assays. Adherence of *A. baumannii* strains to A549 cells (human type 2 pneumocytes) was determined as described previously[55], with minor modifications. In brief, ~10^5 A549 cells were infected with ~10^7 bacterial CFU and incubated for 60 min at 37 °C in 5% CO2 (v/v) atmosphere. Non-adherent bacterial cells were removed by washing with PBS. Infected cells were lysed by the addition of 1 ml distilled water and serial 10-fold dilutions were plated on LB agar to determine the number of CFU of adherent bacteria. To determine adherent and invading bacteria, A549 cells were infected with *A. baumannii* strains as described above. The monolayers were then treated with 1 ml of fresh culture medium containing 5 mg/L of colistin sulfate (Sigma-Aldrich, Milan, Italy) for 30 min, the shortest time point that resulted in the killing of all extracellular bacteria added to the monolayers. Afterwards, the cells were washed with PBS, harvested with trypsin, and lysed with sterile distilled water. Dilutions from harvested samples were inoculated on LB agar plates and bacterial colony counts were estimated after overnight incubation at 37 °C. Each experiment was performed in triplicate.

Statistical analysis. Data were analyzed using a Statistical Package for the Social Sciences Version 13.1 (SPSS Inc., Chicago, IL, USA). Differences between mean values were tested for significance by performing either unpaired, two-tailed Student's t-tests or one-way ANOVA analysis followed by Tukey's multiple-comparison test, when appropriate. A P value < 0.05 was considered to be statistically signif- icant. Correlations were evaluated by regression analysis using the Pearson's correlation coefficient (r).

Single nucleotide polymorphism (SNP) identification and phylogenetics. For ST25 comparisons, all SNPs were identified by mapping raw reads against *A. baumannii* AB307-0294 (NC_011595)[33] with BWA-MEM[34] and calling SNPs with the UnifiedGenotyper method in GATK[35]. For external genome assemblies, whole genome alignments were generated with nucmer[36] and variants were identified by direct mapping of each query to the reference. These methods were wrapped by the Northern Arizona SNP Pipeline (NASP) (http://tgennorth.github.io/NASP/)[37]. A phylogeny was inferred from the resulting concatenated SNP alignment with a maximum likelihood algorithm in RaxML v8[38]. The Retention Index (RI) value[39], which demonstrates how consistent the nucleotide character states are with the phylogeny, was calculated with Phangorn[40].

For the global *A. baumannii* phylogeny, a set of 572 reference genomes (Supplementary Table S2) were downloaded from Patric[41]. Genome assemblies were aligned against AB307-0294 with NASP. A maximum likelihood phylogeny was inferred on this alignment with RaxML. Genomes were pruned from the phylogeny to only reflect the major sequence types. Clades were collapsed in ARB[42].

LS-BSR analysis. To look for differential gene conservation, the Large-Scale Blast Score Ratio (LS-BSR) pipeline[43] was employed. In this method, all coding regions (CDSs) predicted by Prodigal[44]

are clustered with USEARCH[45] at an ID of 90%. Each resulting centroid, which is the most representative sequence of each cluster, is then aligned against itself with BLAT[46] to obtain the reference bit score. Each centroid is then separately aligned against each genome assembly with BLAT to obtain the query bit score. Dividing the query bit by the reference bit score returns the BLAST Score Ratio (BSR)[47]. Unique genomic regions were identified by comparing all CDSs between groups and considering a region to be unique if it had a BSR value > 0.8 in target genomes and a BSR value < 0.4 in all non-target genomes.

Recombination analysis. The FASTA output of NASP was converted to NEXUS using Readseq[48]. The Retention Index for each base was then calculated with Paup v4a140[49]; the specific Paup commands are publicly available (https://gist.github.com/jasonsahl/a66afa55371d7d916a0e). The SNP density (SD), or number of parsimony-informative (PI) SNPs across a genomic interval, was calculated across 1-Kb, non-overlapping windows, compared to the reference genome of *A. baumannii* AB307-0294; PI SNPs are those that that contain at least two types of nucleotides and occur in a minimum of two genomes. The number of homoplasious SNPs, based on a per-base RI value < 0.5, was also calculated across the same window. The Homoplasy Density (HD) value was calculated by dividing the number of homoplasious SNPs (those SNPs that are inconsistent with the tree topology) by the total number of PI SNPs; a script to perform these functions has been published previously[50]. Visualization was performed by Circos[51]. Core genome regions, or those regions conserved across all genomes tested, were identified from the NASP output, where a call was made in all genomes.

Plasmid analysis. CDSs predicted by Prodigal for 40 plasmids identified in *A. baumannii* (Supplementary Table S3) were mapped across ST25 genomes with LS-BSR and BLAT. Following man- ual curation of screened CDSs, the conservation of genes in associated plasmids was visualized with the interactive tree of life[52]. Only a subset of CDSs was selected to demonstrate the variability in plasmid content across isolates.

Gene screen. The distribution of several genes associated with virulence or antimicrobial resistance was determined across all ST25 genomes. This included AbaR1, which has previously been shown to be missing from *A. baumannii* 4190[53]. This region (Coordinates 3702770-3602770 in *A. baumannii* AYE) was parsed out of *A. baumannii* AYE and coding regions were predicted with Prodigal. Other resistance islands, including AbaR4 (JN107991) and the G7 plasmid that contains AbaR3 (KF669606) were also screened. All CDSs were then compared against all ST25 genomes with LS-BSR and BLAT. An additional set of genes previously associated with virulence in *A. baumannii* (Supplementary Table S4) was also screened against ST25 genomes with LS-BSR.

Results

Isolates analyzed. In addition to ST25 genomes deposited in public databases, we selected a set of isolates to expand the diversity of this global lineage. The following seven isolates in our collection were selected for WGS analysis in addition to WGS data of strain 4190 already available in GenBank[54]: strains RUH1486 and NM3 are susceptible and MDR epidemic isolates, respectively; carbapenem-resistant strains 4390 and 741019 carry different carbapenemase genes (*bla*$_{\text{OXA-58}}$ versus *bla*$_{\text{OXA-23}}$) but both belong to the major PFGE type E (Fig. 1), isolated during epidemics in different countries (Table 1); strain 161/07 contains a distinct carbapenemase (NDM-1); strain LUH6220 has a MDR phenotype but is susceptible to carbapenems; strain LUH7841 is susceptible to most antimicrobials (Supplementary Table S5).

PFGE Analysis. PFGE analysis identified ten unrelated PFGE types (A-J), with six PFGE subtypes within these types (Fig. 1) (C1, E1-E4, I1). Interestingly, PFGE type A included the susceptible first isolate of our collection (RUH1486) and the NM3 MDR strain isolated during an epidemic in United Arab Emirates; six strains from Greece, Argentina, Sweden or Abu Dhabi Emirates were assigned to PFGE type E or PFGE subtypes E1-E4, while two XDR isolates from Argentina and Sweden showed identical PFGE type H (Table 1 and Fig. 1).

MLST. MLST with the Pasteur system[15] was performed on 19 ST25 genomes, although only 7 were subjected to whole genome sequencing (WGS). Of all ST25 isolates analyzed, including a set of reference genomes from GenBank, eighteen were assigned to ST25, while one to ST402 (LUH7841), which is a single-locus variant of ST25. *In silico* MLST confirmed sequence type assignments for all genomes where WGS data exists (Supplementary Table S1).

Antimicrobial susceptibility testing. The antimicrobial susceptibility profiles of 19 ST25 *A. baumannii* strains included in the study are shown in Supplementary Table S5. Two strains were classified as susceptible, six and eleven as MDR and XDR, respectively, according to Magiorakos *et al.*, 2012[29]. Five out of six MDR strains and all 11 XDR strains showed resistance to carbapenems and contained class D or class B carbapenemases.

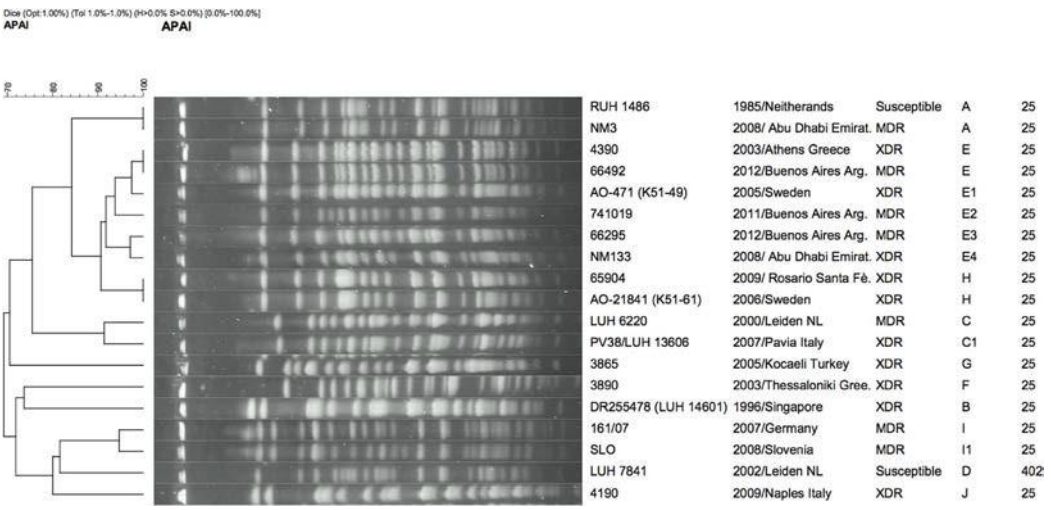

Figure 1. Genotypic analysis of PFGE profiles of *A. baumannii* strains included in the study. Percentage of similarity at dendogram analysis and position and tolerance values of the DICE correlation coefficient used in clustering are shown. Sizes in kilobases (kb) of lambda DNA molecular mass markers are indicated above the PFGE profiles. Strain number, year/country of isolation, PFGE types and subtypes and Multi-locus Sequence types are shown on the right of each profile.

Genome	Resistance phenotype	Aminoglycosides	Beta-lactams	Sulfonamides	Tetracyclines
4390	XDR	X07753 (aphA6)	$bla_{\text{OXA-64}}$	N/A	N/A
161/07	MDR	JN119852 (*aadB*), DQ336355 (OrfA), X00753 (aphA6), M96392 (*tnpA*)	$bla_{\text{NDM-1}}$, $bla_{\text{OXA-64}}$	GQ421466 (*glmM*)	N/A
741019	MDR	X57709 (aphA2), M96392 (*tnpA*)	$bla_{\text{OXA-64}}$, $bla_{\text{OXA-23}}$	GQ421466 (*glmM*)	AP000342 (*tetA*)
LUH7841	Susc.	N/A	$bla_{\text{OXA-64}}^{*}$	N/A	N/A
LUH6220	MDR	X57709 (aphA2), DQ336355 (OrfA), X07753 (aphA6)	$bla_{\text{OXA-64}}$, $bla_{\text{OXA-58}}$	N/A	N/A
NM3	MDR	X57709 (aphA2), M96392 (*tnpA*)	$bla_{\text{OXA-64}}$, $bla_{\text{OXA-23}}$	GQ421466 (*glmM*)	AP000342 (*tetA*)
RUH1486	Susc.	N/A	$bla_{\text{OXA-64}}^{*}$	N/A	N/A

Table 2. *in silico* analysis of genes associated with antimicrobial resistance. N/A = no detection *IS*Aba*1 negative

***In silico* antimicrobial resistance profiling.** As a complement to the laboratory-determined antimicrobial susceptibility profiles, *in silico* profiles were generated for each sequenced genome, using the ResFinder database[31] in conjunction with the SRST2 pipeline[32]. The results demonstrate that resistance mechanisms were identified in the genomes tested for only a few classes of antimicrobials (Table 2), demonstrating limitations in predicting the resistance phenotype from the genotype. Carbapenemase genes were found in the genomes of 161/07 ($bla_{\text{NDM-1}}$, $bla_{\text{OXA-64}}$), 741019 and NM3 ($bla_{\text{OXA-64}}$, $bla_{\text{OXA-23}}$), and 4390 and LUH6220 ($bla_{\text{OXA-64}}$). The $bla_{\text{OXA-64}}$ gene (AY750907), which is also known as $bla_{\text{OXA-51-like}}$, is conserved in all ST25 genomes tested (Table 2). However, the IS*Aba*1 insertion sequence is missing in carbapenem-susceptible isolates that are $bla_{\text{OXA-64}}$ positive, while present in resistant isolates, which confirms published results that this sequence is required for carbapenem resistance[10].

Biofilm formation and pneumocyte adherence. Our previous results demonstrated that the ability to form biofilm and adherence to cultured pneumocytes was significantly higher for *A. baumannnii* strains assigned to ST25 and ST2 compared to other STs[55]. The biofilm growth on abiotic surfaces and adherence/invasion to cultured A549 pneumocytes were assessed for the 19 strains included in this study. As demonstrated in Fig. 2 panel A, the 19 strains assigned to ST25 and ST402 and strain ACICU assigned to ST2 generally demonstrated significantly ($p < 0.05$) greater biofilm growth than strain AYE assigned to ST1, although variability in biofilm growth was observed among ST25 strains. Exposure to sub-inhibitory concentrations of imipenem significantly ($p < 0.05$) stimulated biofilm growth in strains

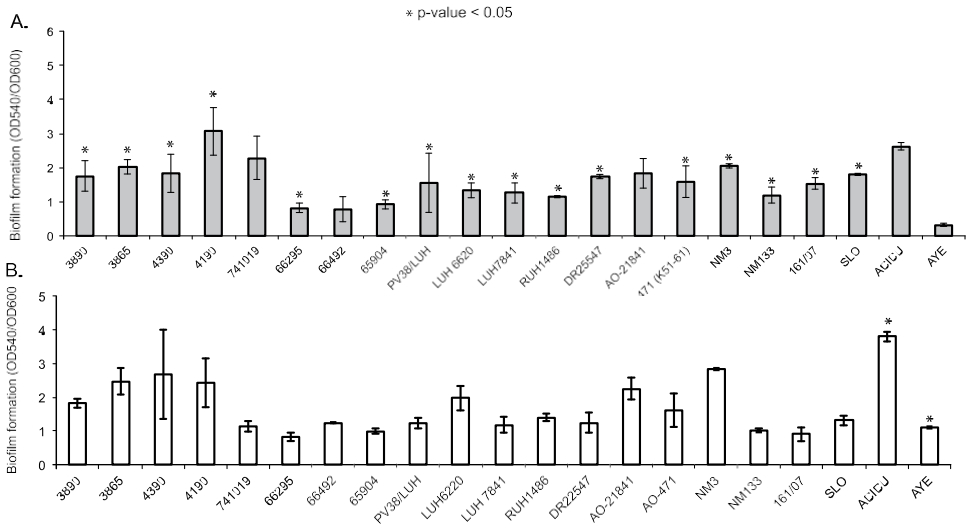

Figure 2. Biofilm variation between ST25 genomes, a ST1 genome (AYE), and a ST2 genome (ACICU). Error bars represent the standard deviation between biological replicates. Differences in biofilm production were calculated with a two-tailed t-test. Isolates were grown in the absence (**A**) or presence (**B**) of imipenem (0.5 mg/L).

AYE and ACICU assigned to ST1 and ST2, respectively, but not in the strains assigned to ST25 or ST402 (Fig. 2, panel B). We next investigated the ability of *A. baumannii* strains to adhere to A549 human alve- olar epithelial cells. All *A. baumannii* strains assigned to ST25 and ST2 (ACICU) strain showed a signif- icantly higher adherence to A549 human bronchial cells compared with ST1 strain AYE (Supplementary Fig. S1) ($p < 0.01$). On the other hand, ST25 *A. baumannii* strains were not able to invade A549 cells human alveolar cells. Also, a similar number of bacteria adhered to A549 cells when the monolayers were incubated with *A. baumannii* strains for 60 min at 4 °C, i.e. under conditions that do not allow for tissue invasion.

Sequencing and comparative genomics. WGS of seven strains was performed and compared to whole genome sequences of 12 *A. baumannii* strains assigned to ST25 available in GenBank (Supplementary Table S1) and to 572 non-ST25 *A. baumannii* reference genomes (Supplementary Table S2). The core genome phylogeny based on 1.15Mb of conserved sequence demonstrated the position of the ST25 lineage (Fig. 3) in relation to other globally-relevant lineages. The retention index (RI) of the concatenated SNP alignment was 0.85, demonstrating significant homoplasy likely due to recombination and introducing uncertainty in the phylogenetic placement, especially with regards to deeply branching nodes and long branches[56]. A phylogeny of just the ST25 genomes (Fig. 4) also demonstrated homoplasy (RI = 0.84), which demonstrates that although ST25 is a lineage with closely related genomes, the evolution of this group has also been partially driven by recombination; the core genome size of ST25 genomes in relation to AB307-0294 was 3Mb. As anticipated, the core genome phylogeny demonstrated much different relationships than were obtained by the PFGE cluster dendrogram (Figs 1 and 4). For example, genomes RUH1486 and NM3 both share the same PFGE type (Fig. 1), but are significantly different based on the core genome phylogeny (Fig. 4).

Recombination in the ST25 lineage. To demonstrate both the extent and location of recombination in the ST25 lineage in relation to the genome of AB307-0294, a homoplasy density analysis was performed[50]. Considering all of the *A. baumannii* genomes (n = 597), the homoplasy appears to be distributed equally across the reference chromosome, with no isolated regions of recombination (Supplementary Fig. S2). When only considering the ST25 genomes, clear regions have likely been recombined between isolates (Fig. 5, panel A). However, fragments in the core genome, using AB307-0294 as the reference, still generally give a strong phylogenetic signal (Fig. 5, panel B). The annotation of selected regions associated with recombination is shown in Supplementary Table S6. A comprehensive list of HD values across all regions in the reference chromosome is also available (https://gist.github.com/5e1cab0b85c-73de7c6d6.git).

Plasmid composition. The horizontal gene transfer of plasmids was analyzed in ST25 genomes, using the composition of 40 previously characterized *A. baumannii* plasmids (Supplementary Table S3). Coding regions were predicted for all the plasmids using Prodigal and they were compared against 19 ST25 genomes using LS-BSR. The results demonstrate that the plasmid content is highly variable across sequenced genomes (Fig. 6), although this method fails to discriminate between genes present on

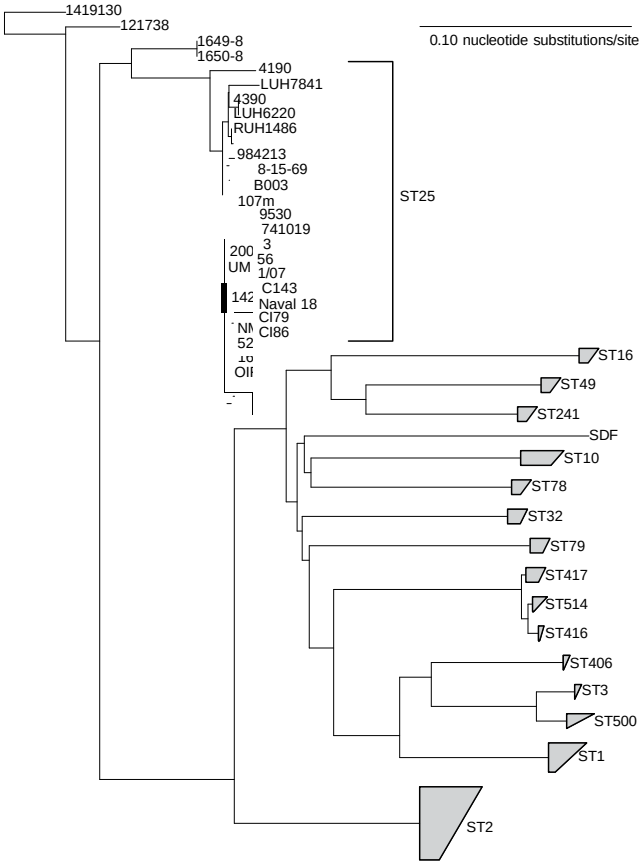

Figure 3. A core genome single nucleotide polymorphism (SNP) phylogeny of 597 *A. baumannii* genomes. The phylogeny was inferred with RAxML[38] from a concatenation of ~104,000 SNPs compared to the reference genome of AB307-0294. Sequence types were identified from genome assemblies. Genomes without close relatives in established sequence types were manually pruned from the tree and groups were collapsed with ARB[42]. The phylogeny was rooted by first including an outgroup from *A. nosocomialis*, then re-running the analysis with only *A. baumannii* and rooting on the most basal genome from the original analysis.

plasmids or the chromosome. The susceptible genome RUH1486 appeared to not contain any annotated plasmid.

Unique genomic regions. The complete genetic content for 597 *A. baumannii* genomes was compared using LS-BSR. By using default values in LS-BSR, a single coding region was found to be present in ST25 genomes and absent from all others; this region corresponds to a large (~14600 nucleotides) hemagglutinin repeat protein (WP_002016208.1). While portions of this gene are conserved in other *A. baumannii* genomes, the complete gene structure is unique to ST25 genomes and could potentially serve as a diagnostic tool for the surveillance of this global lineage.

Distribution of virulence associated genes. Virulence associated genes (Supplementary Table S4) were screened against ST25 genomes with LS-BSR and BLAT. The results demonstrate variability in composition across four genes. Perhaps the most striking difference is in *ompF*, which is highly conserved in only three genomes, each on the same branch of the phylogeny (Supplementary Fig. S3).

Distribution of resistance islands. Coding regions from three previously characterized resistance islands were screened against ST25 genomes with LS-BSR. The results demonstrate that AbaR1 and AbaR4 were sparsely distributed across ST25 genomes (Supplementary Fig. S4). However, the AbaR3 resistance island was highly conserved across two lineages in the ST25 phylogeny, likely demonstrating independent acquisition.

Discussion. *A. baumannii* is a globally distributed nosocomial pathogen associated with clinical infections that are difficult to treat due to widespread antimicrobial resistance. WGS has begun to demonstrate the phylogenetic diversity of this pathogen, which seems largely driven by homologous recombination[12].

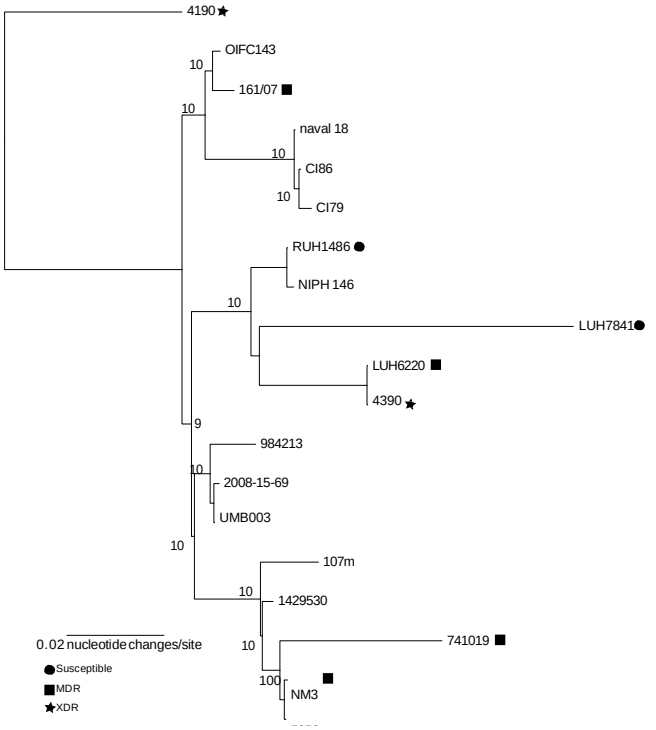

Figure 4. A core genome single nucleotide polymorphism (SNP) phylogeny of ST25 genomes. The phylogeny was inferred with RAxML from a concatenation of ~24,000 SNPs compared to the reference genome of AB307-0294[33] with 100 bootstrap replicates. Each genome was annotated with its antimicrobial susceptibility information, where available. The tree was rooted according to the most basal genome isolated from the global phylogeny (Fig. 3).

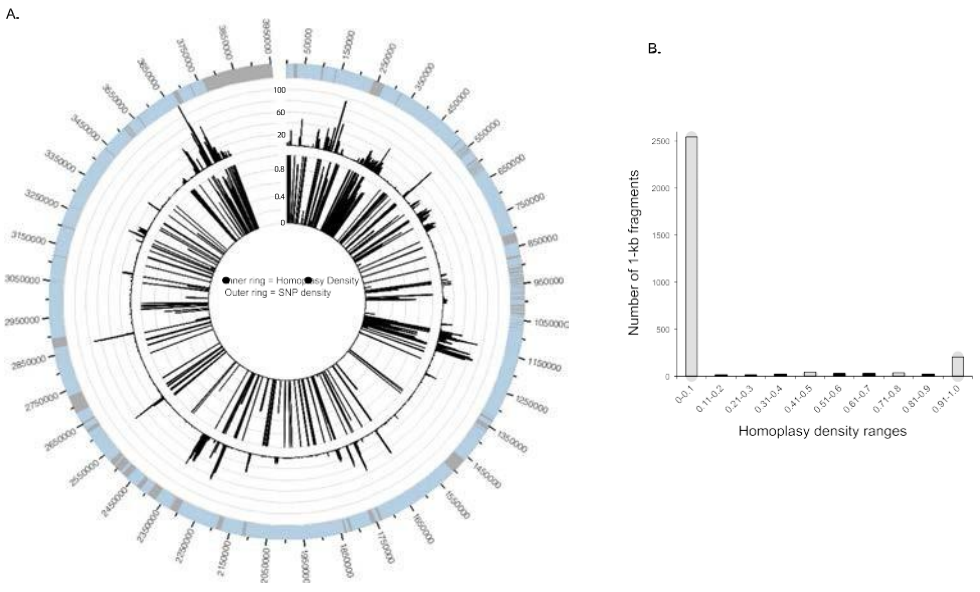

Figure 5. Homoplasy density (HD) ratio analysis of ST25 genomes. (Panel A): Parsimony informative (PI) single nucleotide polymorphisms (SNPs) were identified across 1-Kb, non-overlapping windows (SNP density, or SD), compared to the reference genome of AB307-0294. Homoplasious SNPs were identified by a Retention index value < 0.05. The HD was calculated by dividing the number of homoplasious SNPs by the total number of PI SNPs. The SD and HD values were visualized with Circos[51]. Core genome regions were identified where there was a call in all query genomes compared to the reference genome. (Panel B): The distribution of 1-kb, non-overlapping regions, based on the HD values.

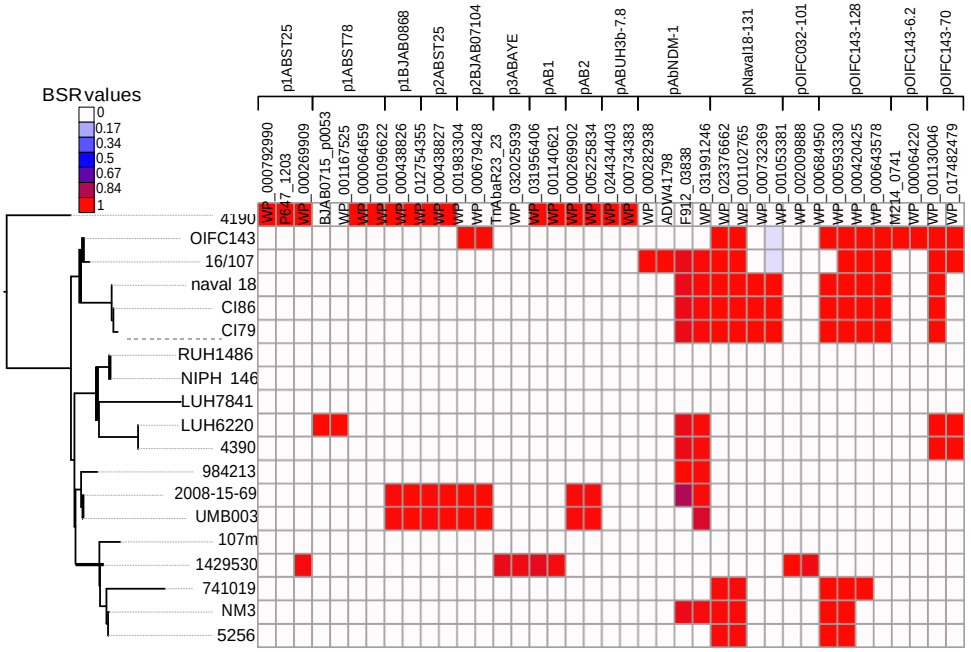

Figure 6. The ST25 phylogeny associated with a heatmap of coding regions predicted from plasmids identified in *A. baumannii* genomes. The heatmap was generated from LS-BSR[43] output and was visualized with the interactive tree of life[52]. GenBank accession numbers are listed for each queried coding region.

And although phylogenetic diversity has been documented, many genomes sequenced to date fall into clearly defined clonal lineages, such as ST1 and ST2, which have been identified worldwide[14]. Genetic diversity within each of these sequence types has largely focused on the diversity of antimicrobial resist- ance islands[57–60] or individual loci[61]. The focus of this study was to perform a comprehensive genomics analysis of the ST25 lineage, which was isolated in different countries and was responsible for epidemics worldwide[16–22], to better understand the genotypic and phenotypic properties behind the worldwide distribution and evolution of a successful lineage of *A. baumannii*.

Phenotypic diversity was observed within ST25, including differences in biofilm formation, antimicrobial susceptibility, and pneumocyte adherence. In terms of biofilm formation, ST25 isolates produce a significantly higher amount of biofilm than a single ST1 representative in the absence of antimicrobials (Fig. 2, panel A). Although the relationship between biofilm formation and virulence has not been solidified in *A. baumannii*[62], biofilms have been associated with resistance to antimicrobials[63], pathogenensis[64] as well as resistance to host factors[65]. The presence of imipenem did not affect biofilm production in ST25 isolates, suggesting that these mechanisms are constitutively expressed.

Antimicrobial susceptibility testing demonstrated varied susceptibility profiles within ST25 isolates (Supplementary Table S5). *In silico* profiles using WGS data against the ResFinder database could predict laboratory resistance for only a subset of antimicrobials (Table 2). This demonstrates that although antimicrobial resistance databases are useful for predicting resistance for some classes of antimicrobials, additional experimentation is required to fully understand the genetic basis for antimicrobial resistance in *A. baumannii*. In terms of adherence, ST25 genomes were demonstrated to adhere better than a representative from the ST1 clonal lineage. Although this may be due to the presence of a unique haemagglutinin identified in ST25 and absent from all other *A. baumannii* genomes, additional experimentation is required.

The genetic diversity of ST25 was demonstrated in multiple ways, including the visualization of a core genome single nucleotide polymorphism (SNP) phylogeny (Figs 3 and 4); a similar topology was observed compared to a recent CRISPR-subtyping analysis[66]. A homoplasy density approach demonstrated that much of the SNP density that defines the phylogenetic structure of ST25 is due to homoplasy (Fig. 5, panel A), most likely resulting from homologous recombination. The extent of homoplasy observed in the global phylogeny suggests that *A. baumannii* does not evolve in a tree like manner and different methods may better represent the evolution in this highly recombinant pathogen.

In addition to SNP analyses, comparative genomics demonstrated a much different gene content between ST25 genomes, primarily between mobile genetic elements. In the case of plasmids, the gene content was significantly different (Fig. 6), which was anticipated due to the movement of mobile genetic elements and has been demonstrated previously in *Acinetobacter*[67]. However, a screen of genes associated with virulence also demonstrated differences between closely related genomes (Supplementary Fig. S3).

The variable distribution of genes across the ST25 dataset, including those associated with antimicrobial resistance islands (Supplementary Fig. S4), may help explain the variable phenotypes. Overall, these results demonstrate the problem with assuming that isolates have similar gene content or phenotypes based solely on MLST or PFGE type analyses. In terms of either assigning isolates to outbreaks or understanding the evolution of clonal lineages, WGS offers the resolution to untangle the relationships between seemingly related isolates.

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Acknowledgements

The authors would like to thank the *Acinetobacter baumannii* ST25 clonal lineage study group, including Mariana Catalano and Maria Soledad Ramirez, University of Buenos Aires, Argentina; Tibor Pal, United Arab Emirates University, Al Ain, United Arab Emirates; Laurent Poirel, HFR - Hôpital Cantonal, Fribourg, Switzerland; Maria Triassi, University of Naples "Federico II", Italy; Athanassios Tsakris, University of Athens, Greece; Bernt Eric Uhlin, Umeå University, Sweden; and Haluk Vahaboglu, Medeniyet University, Istanbul, Turkey. This work was supported in part by grant from University of Naples "Federico II" (Fondo d'Ateneo per la Ricerca to RZ).

Author Contributions

Conceived and designed the experiments: J.W.S., S.P., L.D. and R.Z. Performed data analyses: J.W.S. Performed laboratory analyses: M.D.F., R.E.C., S.P. and N.K. Wrote the manuscript: J.W.S. and R.Z. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sahl, J. W. *et al.* Phylogenetic and genomic diversity in isolates from the globally distributed *Acinetobacter baumannii* ST25 lineage. *Sci. Rep.* **5**, 15188; doi: 10.1038/srep15188 (2015).

Chapter 3

Predominance of international clone 2 OXA-23-producing-*Acinetobacter baumannii* clinical isolates in Greece, 2015: Results of a nationwide study

International Journal of Antimicrobial Agents 49 (2017) 749–753

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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Predominance of international clone 2 OXA-23-producing-*Acinetobacter baumannii* clinical isolates in Greece, 2015: results of a nationwide study ★

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ARTICLE INFO

Article history:
Received 22 September 2016
Accepted 28 January 2017

Keywords:
Clonal complex
Molecular epidemiology
Multilocus sequence typing
Single-locus $bla_{OXA-51-like}$ sequence-based typing
Oxacillinase

ABSTRACT

In a previous nationwide study in Greece, OXA-58 was the sole carbapenemase present among carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolated between 2000 and 2009. In this study, the antibiotic resistances, carbapenemase gene content and clonal relatedness of 194 single-patient CRAB clinical isolates collected randomly during 2015 from 11 tertiary hospitals located throughout Greece were investigated. Antimicrobial susceptibility was determined using commercial and dilution methods. PCR assays for carbapenemase genes were performed. Clonality was tested by a scheme based on two multiplex PCRs and single-locus $bla_{OXA-51-like}$ sequence-based typing. Furthermore, Pasteur's multilocus sequence typing (MLST) scheme and pulsed-field gel electrophoresis (PFGE) were applied to 31 selected representative isolates. The most active antibiotics were trimethoprim/sulfamethoxazole (SXT) (34.6% of isolates susceptible), minocycline (71.6%), colistin (72.7%) and tigecycline ($MIC_{50/90}$ values, 1/2 mg/L). The $bla_{OXA-23-like}$ gene was identified in 188 isolates (96.9%), $bla_{OXA-23-like}$ together with $bla_{OXA-58-like}$ in 3 isolates (1.5%), $bla_{OXA-58-like}$ in 2 isolates (1.0%) and $bla_{OXA-40-like}$ in 1 isolate (0.5%). IS*Aba1* was found upstream of the $bla_{OXA-23-like}$ gene in all isolates. International clone (IC) 2 comprised 157 isolates (80.9%), IC1 comprised 36 isolates (18.6%) and ST78 comprised 1 isolate (0.5%). All IC2 and IC1 isolates tested by MLST were ST2 and ST1, respectively. Seven PFGE types were detected. IC2 isolates were resistant to more antibiotics than IC1, except for SXT. This nationwide study showed that CRAB isolates in Greek hospitals currently produce almost uniformly the OXA-23 carbapenemase and belong mainly to IC2 and, to a lesser extent, IC1. Of particular concern, colistin susceptibility is recently severely reduced.

1. Introduction

Acinetobacter baumannii isolates are increasingly causing severe infections among debilitated patients, particularly in intensive care units (ICUs) [1]. Carbapenem resistance is currently widespread among *A. baumannii*, with rates reaching or exceeding 90% in Southern and Eastern European countries [2]. Carbapenem-resistant *A. baumannii* (CRAB) are commonly resistant to most available antimicrobials except colistin, tigecycline and minocycline [3,4]. Tracking the evolution and clonal composition of CRAB isolates is important to support the implementation of control strategies. Molecular epidemiological studies usually reveal clonality of CRAB, with

★ Part of this work was presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, The Netherlands, 9–12 April 2016.

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http://dx.doi.org/10.1016/j.ijantimicag.2017.01.028
0924-8579/

outbreak strains usually belonging to international clones (IC) 1 and 2 and a few additional clonal lineages [5].

In Greek hospitals, during the last few years the vast majority of *A. baumannii* isolates are CRAB [2], which currently represent the most frequent infection pathogen in the ICU (http://www.mednet.gr/whonet/). Regarding their clonal nature and carbapenemase gene content, we showed previously in a Greek nationwide study conducted from 2000 to 2009 that CRAB were harbouring only the OXA-58 carbapenemase gene; IC1 was the most common lineage until 2004, with IC2 prevailing during 2005–2009 [6].

Studies in several European countries have shown that OXA-23 tends to gradually replace OXA-58 among CRAB [7,8]. Also, a preliminary study from a single Greek hospital in 2011 reported that OXA-23 was more common than OXA-58 [9]. Given the increased frequency and importance of CRAB, the present follow-up nationwide study was conducted to analyse the resistance phenotypes, carbapenemase gene content, genetic relatedness and evolution of clonal lineages among CRAB recovered from Greek patients during 2015.

2. Materials and methods

2.1. Bacterial strains

During 2015, as many as 2500 *A. baumannii* isolates were identified in 11 tertiary hospitals located in eight cities in Northern (Thessaloniki, Serres, Alexandroupolis), Western (Ioannina), Central (Athens, Piraeus, Larissa) and Southern (Heraklion) Greece. The rate of CRAB in these hospitals during 2015 was 95%, with virtually all clinically important *A. baumannii* being CRAB. The present study included 194 randomly selected non-repetitive CRAB isolates recovered during 2015 from clinical samples in these hospitals. All isolates were confirmed to be *A. baumannii* by PCR/sequencing for the intrinsic $bla_{\text{OXA-51-like}}$ gene [10].

2.2. Antimicrobial susceptibility

The susceptibilities of the isolates to ampicillin/sulbactam (SAM), ceftazidime, cefepime, ciprofloxacin, levofloxacin, gentamicin, amikacin, tobramycin, tetracycline and trimethoprim/sulfamethoxazole (SXT) were determined using a VITEK®2 system (bioMérieux, Marcy-l'Étoile, France). Minimum inhibitory concentrations (MICs) of imipenem, meropenem, minocycline and tigecycline were determined by agar dilution, whereas the broth microdilution method was used for colistin as recommended recently by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/guidance_documents/) and the Clinical and Laboratory Standards Institute (CLSI) [11]. CLSI interpretative criteria [11] were applied for the categorisation of isolates; for tigecycline, MIC_{50} and MIC_{90} values were calculated due to the lack of CLSI/EUCAST interpretative criteria for *Acinetobacter* spp. Multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) isolates were classified as recommended previously [12].

2.3. PCR for carbapenemase genes

Genes encoding common class D carbapenemases ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-143-like}}$) and class B metallo-β-lactamases (bla_{IMP}, bla_{VIM}, bla_{NDM}) [13–15] were detected by PCR, followed by sequencing of selected amplicons. The presence of IS*Aba1* upstream of $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-51-like}}$ was tested by PCR as described previously [16].

2.4. Typing by multiplex PCR, $bla_{\text{OXA-51-like}}$ sequence-based typing (SBT), multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE)

Two multiplex PCRs that selectively amplify group 1 or group 2 alleles of the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ were used as a screening method for rapid assignment of isolates to sequence groups (Gs) 1–3 and 6 as reported previously [17,18].

The single-locus $bla_{\text{OXA-51-like}}$ SBT method was also applied to all isolates to assign them into ICs [19]. A total of 31 isolates, at least 1 from each collaborating hospital, selected to represent all sequence groups and combinations of amplicons determined by the multiplex PCRs and also the ICs derived by SBT, were subjected to the Pasteur's MLST scheme (http://pubmlst.org/abaumannii/). *Apa*I DNA macrorestriction and PFGE of the 31 isolates were performed as previously described [20]. The PFGE profiles obtained were converted to TIFF files and were subjected to cluster analysis using GelCompar II v.4.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was based on the unweighted pair-group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyse the similarities of the banding patterns with a tolerance of 1.3%. Interpretation of chromosomal DNA restriction patterns was based on the criteria of Tenover et al. [21] and also on a similarity of >85% at dendrogram analysis in order to indicate strain relatedness.

3. Results

3.1. Patient data, susceptibility testing and carbapenemase genes

Of the patients that yielded CRAB, 70.1% were male and 29.9% female; most of them were hospitalised in ICUs (56.2%), 20.6% in medical wards, 17% in surgical wards, 2.1% in long-term care facilities and 4.1% were outpatients. Isolates were obtained from blood (45.9%), the respiratory tract (21.6%), skin and soft tissue (11.3%), urine (9.3%) and various other clinical samples (11.9%).

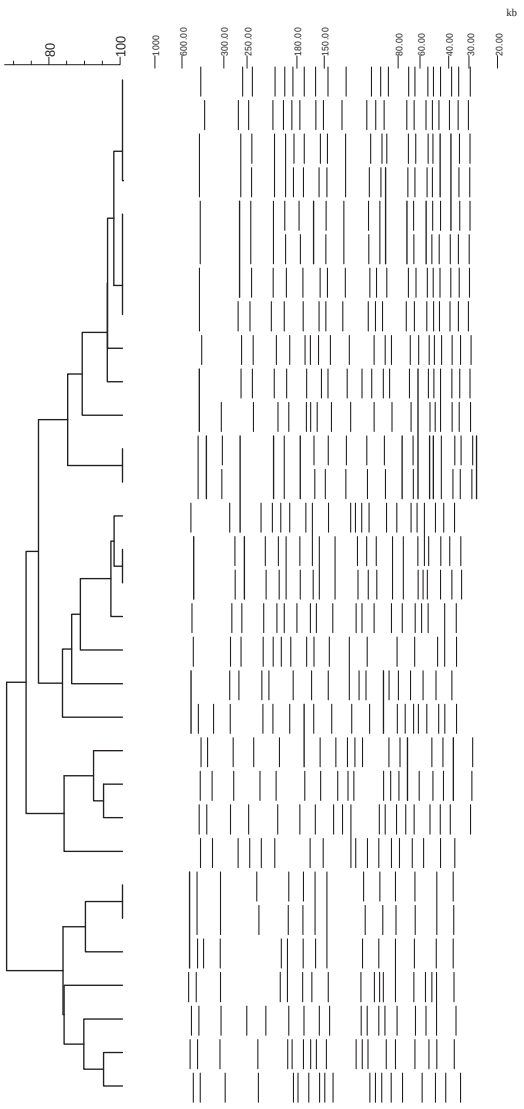
The 194 CRAB isolates showed imipenem and meropenem MICs ≥ 32 μg/mL and were non-susceptible to all fluoroquinolones and β-lactams tested except SAM (5.2% of isolates susceptible). Susceptibility rates to other antibiotics were as follows: gentamicin, 9.4%; amikacin, 15.5%; tobramycin, 22.9%; tetracycline, 10.3%; SXT, 34.6%; minocycline, 71.6%; and colistin, 72.7%. For tigecycline, $MIC_{50/90}$ values were 1/2 mg/L. Of the study isolates, 3.1% were MDR, 78.4% were XDR and 18.6% were PDR.

The $bla_{\text{OXA-23-like}}$ gene was identified in 188 isolates (96.9%), $bla_{\text{OXA-23-like}}$ together with $bla_{\text{OXA-58-like}}$ in 3 isolates (1.5%), $bla_{\text{OXA-58-like}}$ in 2 isolates (1.0%) and $bla_{\text{OXA-40-like}}$ in 1 isolate (0.5%). IS*Aba1* was detected upstream of the $bla_{\text{OXA-23-like}}$ gene in all 191 $bla_{\text{OXA-23-like}}$-positive isolates (98.5%) and upstream of $bla_{\text{OXA-51-like}}$ in 44 isolates (22.7%).

3.2. Molecular epidemiology

The multiplex PCR-based typing scheme assigned the majority of isolates (151; 77.8%) to G1 (IC2) and 14 isolates (7.2%) to G2 (IC1), whilst 1 isolate (0.5%) was assigned to G6. The remaining 28 isolates (14.4%) demonstrated three different banding patterns, as follows: amplification of G1 *ompA*/$bla_{\text{OXA-66}}$, 6 isolates; amplification of G1 *csuE*/G2 *ompA*/$bla_{\text{OXA-69}}$, 21 isolates; and amplification of G2 *ompA*/$bla_{\text{OXA-69}}$, 1 isolate.

Using SBT, 157 isolates (80.9%) belonged to IC2 harbouring $bla_{\text{OXA-66}}$, 36 isolates (18.6%) to IC1 ($bla_{\text{OXA-69}}$) and 1 isolate (0.5%) to G6 ($bla_{\text{OXA-90}}$). MLST confirmed these group assignments: all IC2 isolates tested were ST2, all IC1 isolates were ST1 and the isolate with $bla_{\text{OXA-90}}$ was ST78. Seven distinct PFGE types were identified: types



Isolate nr.	Date of isolation	Hospital/City	Source	PFGE Type	SequenceGroup	Pasteur MLST	SBT	Carbapenemase
146	02.01.2015	General Hospital/Serres	Urine	A	G1	ST2	IC2	OXA-23
37	23.10.2015	AHEPAUniv. Hospital/Thessaloniki	Trauma	A	G1	ST2	IC2	OXA-23
68	05.01.2015	Tzaneio Hospital/Piraeus	Blood	A	G1	ST2	IC2	OXA-23
72	06.03.2015	Tzaneio Hospital/Piraeus	VC	A	G1	ST2	IC2	OXA-23
31	23.07.2015	AHEPAUniv. Hospital/Thessaloniki	Tissue	A1	G1	ST2	IC2	OXA-23
40	09.05.2015	Univ. Hospital/Alexandroupolis	PeritonealFluid	A1	G1	ST2	IC2	OXA-23
51	28.09.2015	Univ. Hospital/Ioannina	Sputum	A1	G1	ST2	IC2	OXA-23
55	07.09.2015	Univ. Hospital/Ioannina	Urine	A1	G1	ST2	IC2	OXA-23
147	08.01.2015	General Hospital/Serres	Urine	A2	G1	ST2	IC2	OXA-23
69	28.01.2015	Tzaneio Hospital/Piraeus	Rectal	A3	G1*ompA*/*bla*oxa66	ST2	IC2	OXA-23
153	30.07.2015	General Hospital/Serres	Trauma	A4	G1*ompA*/*bla*oxa66	ST2	IC2	OXA-23
1	16.09.2015	Evaggelismos Hospital/Athens	Blood	B	G1	ST2	IC2	OXA-23
2	06.09.2015	Evaggelismos Hospital/Athens	Blood	B	G1	ST2	IC2	OXA-23
89	12.01.2015	Univ. Hospital/Heraklion	Blood	C	G1	ST2	IC2	OXA-23
189	16.07.2015	Eugenidio Hospital/Athens	Sputum	C1	G1	ST2	IC2	OXA-23
197	13.11.2015	Iaso General Hospital/Larissa	Urine	C1	G1	ST2	IC2	OXA-23
160	13.01.2015	G. Gennimatas Hospital/Athens	Blood	C2	G1*csuE*/G2*ompA*/*bla*oxa69	ST1	IC1	OXA-23
159	20.01.2015	G. Gennimatas Hospital/Athens	Blood	C3	G1	ST2	IC2	OXA-23
120	12.10.2015	KAT Hospital/Athens	Pleural Fluid	C4	G1	ST2	IC2	OXA-23
134	02.04.2015	General Hospital/Serres	Urine	D	G1	ST2	IC2	OXA-23
20	16.01.2015	AHEPAUniv. Hospital/Thessaloniki	CVC	E	G1*csuE*/G2*ompA*/*bla*oxa69	ST1	IC1	OXA-23
21	16.01.2015	AHEPAUniv. Hospital/Thessaloniki	Trauma	E1	G2	ST1	IC1	OXA-58
45	04.08.2015	Univ. Hospital/Alexandroupolis	Blood	E2	G1*csuE*/G2*ompA*/*bla*oxa69	ST1	IC1	OXA-23
124	09.11.2015	KAT Hospital/Athens	Blood	F	G6	ST78	CC78	OXA-40
131	31.01.2015	General Hospital/Serres	Bronchial	G	G2	ST1	IC1	OXA-23
38	12.03.2015	Univ. Hospital/Alexandroupolis	Blood	G	G2	ST1	IC1	OXA-23
130	28.01.2015	General Hospital/Serres	Blood	G1	G2	ST1	IC1	OXA-23
133	30.03.2015	General Hospital/Serres	Blood	G2	G2	ST1	IC1	OXA-23
136	12.06.2015	General Hospital/Serres	Bronchial	G3	G1*csuE*/G2*ompA*/*bla*oxa69	ST1	IC1	OXA-23
116	03.07.2015	KAT Hospital/Athens	Blood	G4	G2	ST1	IC1	OXA-23
166	16.02.2015	G. Gennimatas Hospital/Athens	Blood	G5	G2*ompA*/*bla*oxa69	ST1	IC1	OXA-23

Fig. 1. Characteristics and clonal composition of 31 representative *Acinetobacter baumannii* isolates tested using multiplex PCR-based typing, single-locus $bla_{\text{OXA-51-like}}$ sequence-based typing (SBT), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Percentage similarity at dendrogram analysis and sizes in kilobases (kb) of λ DNA molecular mass markers are indicated above the dendrogram and PFGE profiles, respectively. Isolate number, date of isolation, hospital/city, source, PFGE type/subtype, sequence group (G) assigned by multiplex PCR, MLST, international clone (IC) or clonal complex (CC) by SBT, and carbapenemase genes are shown on the right of each profile. VC, venous catheter; CVC, central venous catheter.

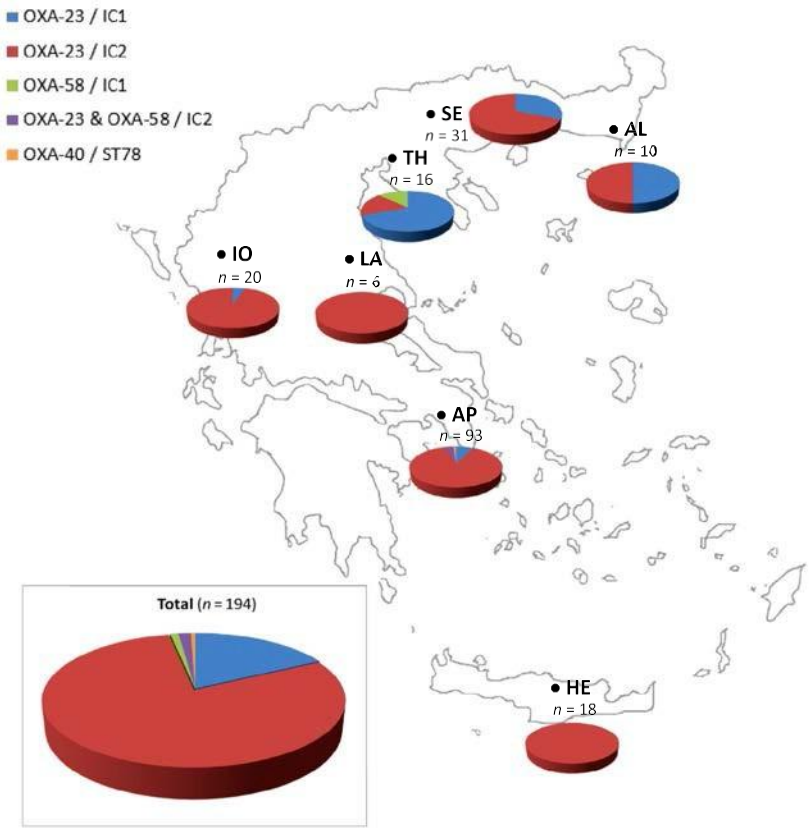


Fig. 2. Geographical distribution of the identified oxacillinase genes and international clones (ICs) of the carbapenem-resistant *Acinetobacter baumannii* (CRAB) study isolates. Black dots indicate the location of hospitals in which the isolates were recovered (AL, Alexandroupolis; SE, Serres; TH, Thessaloniki; IO, Ioannina; LA, Larissa; AP, Athens/Piraeus; HE, Heraklion). Coloured pie charts indicate the prevalence of different oxacillinases and ICs (in each region and in total). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A (with four subtypes), B, C (five subtypes) and D were found in ST2 isolates; and types E (three subtypes) and G (six subtypes) were found in ST1 isolates. The typing results of the 31 representative study isolates tested by all methods are shown in Fig. 1.

The IC2 isolates were detected in all study hospitals and comprised the vast majority of isolates (128/137; 93.4%) recovered from Western, Central and Southern Greece. The IC1 isolates were identified in six hospitals; three of them were located in Northern Greece, where IC1 was quite common, including 49.1% of the 57 CRAB tested.

Of the IC2 isolates, 154 had $bla_{\text{OXA-23-like}}$ and 3 had $bla_{\text{OXA-23-like}}$ plus $bla_{\text{OXA-58-like}}$. As far as IC1 isolates, 34 had $bla_{\text{OXA-23-like}}$ and 2 had $bla_{\text{OXA-58-like}}$. The ST78 isolate harboured $bla_{\text{OXA-40-like}}$. The geographical distribution of the identified oxacillinase genes and ICs of the study isolates are shown in Fig. 2.

Isolates belonging to IC1 were generally less resistant to antibiotics compared with IC2. Important differences in the susceptibility rates between IC1 versus IC2 isolates were observed for minocycline (100%vs.65.0%), tetracycline(53.3%vs.1.3%)and tobramycin(65.2% vs. 16.4%). An opposite trend was observed for SXT, against which 37.6% of IC2 isolates were susceptible compared with 13.9% for IC1 isolates.

4. Discussion

Acinetobacter baumannii exhibits particularly high carbapenem resistance rates in several regions worldwide [22]. Of note, during 2015 essentially no clinically important carbapenem-susceptible *A. baumannii* isolate has been recovered in the study Greek hospitals. The almost universal resistance to carbapenems and SAM precludes the use of β-lactams and considerably limits effective treatment options. Carbapenem resistance in *A. baumannii* is regularly due to the production of oxacillinases, the most frequent ones being OXA-23, OXA-40 and OXA-58 [5]. OXA-23 enzymes are recently increasing and tend to predominate among CRAB from distant regions worldwide [23], gradually replacing OXA-58 [7,8,24]. A similar observation was made in the present nationwide study, with OXA-23 being by far the commonest enzyme detected, displacing OXA-58, which was the sole carbapenemase present until 2009 among CRAB isolates throughout Greece [6]. It thus appears that the international shift towards OXA-23 predominance also applies to Greece.

During the last few years, isolates belonging to IC2 have expanded particularly and currently include the majority of CRAB isolates worldwide [5,25]. In the current study, very few lineages were shown to circulate in Greece, with IC2 being the commonest lineage detected. A similar trend was also observed already in our previous nationwide study, which showed that after 2005 IC2 was more prevalent in Greece than IC1 [6].

Among the few antimicrobials that remained potent, alarmingly high resistance rates (27.3%) were observed for colistin. We should note that until recently colistin was considered almost consistently active against CRAB and is most commonly included in combination therapies of infections caused by CRAB [26]. Minocycline exhibited a considerable degree of activity (71.6%), as was also found in a recent worldwide surveillance report showing minocycline susceptibility rates of 66.2% and 62.9% against MDR and XDR *A. baumannii*, respectively [3]. Tigecycline was also shown to retain efficient activity against the Greek CRAB isolates, with $MIC_{50/90}$ values

Appendix

The following members of the Greek Study Group on *Acinetobacter* Antimicrobial Resistance contributed significantly to the study design and strain collection: L. Skoura (AHEPA University Hospital, Thessaloniki, Greece); K. Themeli-Digalaki (Tzaneio Hospital, Piraeus, Greece); E. Perivolioti (Evangelismos Hospital, Athens, Greece); S. Tsiplakou (KAT Hospital, Athens, Greece); V. Karavassilis (IASO Hospital, Larissa, Greece); M. Panopoulou (University Hospital of Alexandroupolis, Alexandroupolis, Greece); M. Orfanidou ('G. Gennimatas' General Hospital, Athens, Greece); C. Hadjichristodoulou (Department of Hygiene and Epidemiology, Medical School, University of Thessaly, Larissa, Greece); S. Levidiotou (Department of Microbiology, Medical School, University of Ioannina, Ioannina, Greece); and A. Gikas (Department of Internal Medicine, Infectious Diseases Unit, University Hospital of Heraklion, Crete, Greece).

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Chapter 4

Emergence of colistin resistance uncoupled from virulence attenuation after prolonged colistin administration in a patient with extensively drug-resistant *Acinetobacter baumannii*

Diagnostic Microbiology and Infectious Disease 82 (2015) 222-226

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Emergence of colistin resistance without loss of fitness and virulence after prolonged colistin administration in a patient with extensively drug-resistant *Acinetobacter baumannii*

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article info

Article history:
Received 26 November 2014
Received in revised form 16 March 2015
Accepted 18 March 2015
Available online 25 March 2015

Keywords:
Acinetobacter baumannii
Colistin therapy
Colistin resistance
pmrB mutation
In vitro fitness
In vivo virulence

abstract

The spread of extensively drug-resistant (XDR) gram-negative bacteria has boosted colistin use, with a resultant selection of colistin-resistant, often pandrug-resistant strains. Whether acquisition of further resistance mechanisms translates into a reduced virulence is the subject of active research. In this report, we describe clinical features of an immunocompromised patient who developed infection due to colistin-resistant *Acinetobacter baumannii* while on long-term colistin therapy. We analyzed phenotypic and genotypic characteristics, molecular mechanisms of colistin resistance, and in vitro and in vivo fitness of sequential colistin-sensitive and colistin-resistant strains isolated from the patient. Both colistin-sensitive and colistin-resistant strains were XDR and showed identical ST78 genotype. At variance with prior reports on colistin-resistant strains of *A. baumannii*, resistance to colistin due to P233S mutation in PmrB sensor kinase did not associate with any measurable reduction in strain fitness, growth characteristics, and virulence.

1. Introduction

Extensively drug-resistant (XDR) *A. baumannii* is increasingly responsible for healthcare-associated infections (Zarrilli et al., 2013). These infections are difficult to treat, and a colistin-based regimen may represent the only available therapeutic option (Durante-Mangoni et al., 2014). The emergence of colistin resistance in XDR *A. baumannii* has been reported as a consequence of prolonged exposure to this drug (Lesho et al., 2013; Pournaras et al., 2014; Snitkin et al., 2013).

Two major mechanisms have been implicated in the development of resistance to colistin in *A. baumannii*. Mutations in the PmrA/B 2-component regulator, involved in the addition of phosphoethanolamine to hepta acylated lipid A, can lead to lipopolysaccharide (LPS) modifications (Adams et al., 2009; Beceiro et al., 2011; Lesho et al., 2013; Lopez-Rojas et al., 2011; Pournaras et al., 2014; Snitkin et al., 2013). Changes within either *lpxA*, *lpxC*, or *lpxD* lipid A biosynthesis pathway genes may in contrast result in the complete loss of the lipid A component of lipopolysaccharide (Moffatt et al., 2010). Mounting evidence indicates that colistin resistance in *A. baumannii* is associated with genomic instability, high fitness costs, and virulence attenuation (Beceiro et al., 2014; Lopez-Rojas et al., 2011; Pournaras et al., 2014; Snitkin et al., 2013). Aims of the present study were to analyze the mechanisms of colistin resistance, the in vitro fitness, and the virulence of consecutive colistin-susceptible (colistin-S) and colistin-resistant (colistin-R) *A. baumannii* strains isolated during colistin treatment from a single patient.

2. Materials and methods

2.1. Study design

This was a clinical-microbiological case study aimed at investigating the relationship between loss of susceptibility to colistin and virulence phenotypes in strains of *A. baumannii*. Colistin-R strains were grown from a single patient previously screened under informed consent for participation in the CoRAb trial (Durante-Mangoni et al., 2013) and not randomized due to lack of fulfillment of inclusion criteria. In this trial, inclusion criteria were age ≥18 years and microbiological evidence of a life-threatening pneumonia or bloodstream or intra-abdominal infection due to XDR *A. baumannii* susceptible to colistin, while exclusion criteria were previous treatment with or hypersensitivity to colistin. The index patient was initially deemed colonized not infected by *A. baumannii*. The study protocol had been approved by the Ethics Committee of the Monaldi Hospital. Patient's chart was evaluated, and relevant clinical data were collected after strain isolation. These included detailed antibiotic treatment regimens, prospective microbiology data, as well as clinical course.

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http://dx.doi.org/10.1016/j.diagmicrobio.2015.03.013
0732-8893/

Table 1
Epidemiological, phenotypic, and genotypic features of *A. baumannii* strains.

Strain	Year/country	Specimen	bla_{OXA} genes	PFGE type	MLST	MIC (μg/mL) of antibiotic				Mechanism of colistin resistance	Colistin (dose/route) at time of isolate growth	Reference
					ST	IPM	MEM	TIG	COL			
Ab4450[a]	07/06/2010/NA/IT	BA	bla_{OXA-58}, bla_{OXA-64}	A	78	32	16	4	0.5	None	None	Unpublished
Ab4451[a]	26/08/2010/NA/IT	BA	bla_{OXA-58}, bla_{OXA-64}	A1	78	8	8	2	128	*pmrB* mutation P233S	2 MU tid IV	Unpublished
Ab4452[a]	26/08/2010/NA/IT	BA	bla_{OXA-58}, bla_{OXA-64}	A1	78	64	8	2	128	*pmrB* mutation P233S	2 MU tid IV	Unpublished
Ab4453[a]	28/10/2010/NA/IT	BA	bla_{OXA-58}, bla_{OXA-64}	A2	78	32	8	2	0.5	None	None	Unpublished
3909	05/11/2007/IT	BA	bla_{OXA-58}, bla_{OXA-64}	A	78	32	8	2	0.5	None	None	(Giannouli et al., 2010; Zarrilli et al., 2011)
ATCC19606[T]	Before 1949/AT/USA	UC	bla_{OXA-98}	B	52	2	2	1	0.5	None	Not applicable	(Janssen et al., 1997)

Abbreviations: NA = Napoli; IT = Italy; AT = Atlanta; BA = bronchial aspirate; UC = urine culture; ST = sequence type; IPM = imipenem; MEM = meropenem; TIG = tigecycline; COL = colistin; MU = million units.

[a] *A. baumannii* strains isolated from the same patient.

2.2. Bacterial strain identification and genotyping

Bacterial strains included in the study are listed in Table 1. Strain identification of *A. baumannii* isolates was performed, as previously described (Giannouli et al., 2010). Genotyping was performed by *ApaI* DNA macrorestriction, pulsed-field gel electrophoresis (PFGE), and dendogram analysis and multilocus sequencing typing (MLST) analysis, as previously described (Giannouli et al., 2010). Interpretation of genetic relatedness of PFGE profiles was performed using the criteria of Tenover et al. (1995).

2.3. Antimicrobial susceptibility testing

Susceptibility testing of all antimicrobials was performed using the VITEK 2 system and the AST-GN089 card (bioMérieuxMarcy l'Étoile, France). Colistin susceptibility was evaluated by broth microdilution in Mueller-Hinton broth II (MHBII) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2010) using untreated polystyrene microplates. Colistin susceptibility of the 4 *A. baumannii* strains included in the study was confirmed by broth macrodilution in MHBII using borosilicate tubes, which was recently shown to exhibit excellent performance for colistin MIC testing of multidrug-resistant *A. baumannii* isolates (Pournaras et al., 2014). Tigecycline MIC results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (susceptible, ≤1 μg/mL; and resistant, ≥4 μg/mL) (EUCAST Steering Committee, 2006). In the absence of internationally recognized breakpoints, *A. baumannii* resistance to rifampicin was defined as an MIC N16 μg/mL according to the recommendations of the French Society of Microbiology (Bonnet et al., 2013). Multidrug-resistant and XDR phenotypes were defined according to Magiorakos et al. (2012).

2.4. PCR and sequencing

PCRs for the *bla*OXA-51-like, *bla*OXA-58-like, *bla*OXA-23-like, *bla*OXA-24-like, *bla*VIM, *bla*IMP, *bla*SIM, *pmrA*, *pmrB*, *pmrC*, *lpxA*, *lpxC*, and *lpxD* genes were performed as described previously (Giannouli et al., 2010; Pournaras et al., 2014). Nucleotide sequences obtained were compared with the deduced amino acid sequence available in GenBankusingBasicLocalAlignmentSearchToolsoftware.

2.5. Bacterial viability

A logarithmic-phase culture of each strain was adjusted to ca. 10^6 CFU/mL in MHBII. Five-milliliter cultures in glass tubes were incubated in a shaking water bath at 37 °C, and viable bacteria were counted after 0, 4, 8, 16, 24 and 48 h. At each time, after vortexing, a 0.5-mL sample was removed from the cultures, and serial 10-fold dilutions were made in MHBII. Bacterial viability was measured by pour-plating 25-μL aliquots of the appropriate dilution in MHBII onto 5% sheep blood agar plates (Oxoid SpA, Garbagnate Milanese, Italy) in duplicate and incubating for 24 h at 37 °C. All experiments were repeated 3 times. Differences in bacterial viability were calculated by the generalized linear model for multivariate comparisons. Differences were considered statistically significant at P b 0.05.

2.6. Galleria mellonella killing assay

The virulence of *A. baumannii* in vivo was assessed using the *G. mellonella* insect model of infection, as previously described (Giannouli et al., 2013). Survival curves were plotted using the Kaplan-Meier method, and differences in survival were calculated using the log-rank test for multiple comparisons. Differences were considered statistically significant at P b 0.05. Lethal dose 50% (LD50) values were calculated using GraphPad Prism v.5.04 (GraphPad Software, La Jolla, CA, USA), as previously described (Giannouli et al., 2013). All experiments were performed at least 3 times, and the results were shown as means ± SEM. Differences between mean values were tested for significance by performing 1-way analysis of variance followed by Tukey's multiple-comparison test. A P value b0.05 was considered to be statistically significant.

3. Results

3.1. Selection of colistin-R A. baumannii during colistin therapy

A 66-year-old woman was admitted to the Monaldi Hospital because of ischemic heart disease. On May 21, 2010, the patient underwent coronary artery bypass grafting complicated by severe biventricular failure. After extracorporeal cell membrane oxygenation device placement, as no functional recovery occurred, the patient was waitlisted for heart transplant. All surveillance cultures from blood, tracheal aspirate, surgical sites, and rectum were negative for sentinel microorganisms. On May 27, 2010, the patient received an AB0 compatible graft and was started on a standard immunosuppressive regimen including antithymocyte globulins, polyclonal immune globulins, high-dose metilpredisolone followed by cyclosporin A and micophenolate mofetil. Both surgeries were performed under 48 h prophylaxis with amoxicillin-clavulanic acid, 2.2 g thrice in a day (tid). Despite an uneventful transplantation procedure, the subsequent course was complicated by kidney failure requiring renal replacement therapy and prolonged intubation followed by tracheostomy. Antifungal prophylaxis with fluconazole was given for 6 days. Because of a pulmonary infiltrate on chest x-ray, meropenem 1 g tid intravenously (IV) was started. On June 7, a bronchial aspirate showed the presence

of 10^6 CFU/mL of XDR *A. baumannii*, i.e., nonsusceptible to all antimicrobial classes but susceptible to tigecycline and colistin (Magiorakos et al., 2012). Antimicrobial susceptibility studies showed MICs for meropenem and colistin of 16 and 0.5 μg/mL, respectively. Nebulized colistin methanesulphonate was started at the dose of 1 MU tid on June 9 for 7 days, and a tracheostomy was performed. Follow-up airway cultures repeatedly obtained from June 14 on showed the persistence of XDR-*A. baumannii* with the same antibiotype. This condition was deemed as airways colonization, and no further colistin was given. Patient clinical conditions only moderately improved, allowing withdrawal of all sedatives, but renal function remained significantly compromised with an estimated glomerular filtration rate below 30 mL/min. Multiple other microbial pathogens were isolated, including *Staphylococcus epidermidis* and *Enterococcus faecium* from blood, and MRSA repeatedly from bronchial aspirates, requiring treatment with vancomycin and, subsequently, linezolid. One month after transplant, KPC-producing *Klebsiella pneumoniae* was isolated from blood, lung, and urine cultures. Intravenous colistin, 2 MU q8h, was started and continued for 45 days. During this period and under this regimen, cultures from removed central venous catheter tips and airways remained persistently positive for XDR *A. baumannii* with MICs for colistin and tigecycline equal to 0.5 and 2 μg/mL.

On August 26, colistin-R *A. baumannii* strains were grown for the first time from 2 subsequent bronchial aspirates. These strains showed colistin MICs of 128 μg/mL and were also resistant to all beta-lactams, imipenem and meropenem carbapenems, aminoglycosides, and trimethoprim/sulfamethoxazole, but not tigecycline. Colistin was withdrawn, and no other antimicrobial active against gram-negative bacilli was given. Ten days later, another XDR-Acb strain was isolated in a bronchial aspirate, showing again a colistin MIC of 0.5 μg/mL. No further colistin was given, and all subsequent XDR-Acb strains showed an MIC ranging from 0.5 to 1 μg/mL. Tigecycline was started instead at the dose of 100 mg bid i.v. after a 200 mg loading dose. XDR-*A. baumannii* persisted also during this course, showing unchanged colistin MIC equal to 0.5 μg/mL. In the following days, patient conditions progressively deteriorated with development of multiorgan failure. Since September 2010, other infections supervened such as *Stenotrophomonas maltophilia* pneumonia and candidemia. Multiple colonizing microorganisms were also grown, including extended-spectrum beta-lactamase-producing *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and *S. epidermidis*, and *Enterococcus faecalis*. The patient eventually died on October 31, 2010.

3.2. Phenotypic and genotypic characterization of *A. baumannii* strains

All 4 *A. baumannii* strains isolated from the patient available were included in the analysis. Their phenotypic and genotypic characteristics are reported in Table 1. *A. baumannii* strain Ab4450 was isolated on June 7, before initiation of colistin treatment, and showed susceptibility to colistin (MIC 0.5 μg/mL). *A. baumannii* strains Ab4451 and Ab4452 were isolated on August 26, during colistin therapy, from 2 different samples of bronchial aspirate, and showed resistance to colistin (MIC 128 μg/mL). *A. baumannii* strain Ab4453 was isolated on October 28, 8 weeks after discontinuation of colistin therapy, and showed susceptibility to colistin (MIC 0.5 μg/mL). All 4 *A. baumannii* strains were XDR, i.e., nonsusceptible to at least 1 agent in all antimicrobial classes including carbapenems (imipenem MICs ranging from 8 to 64 μg/mL; meropenem MICs ranging from 8 to 16 μg/mL), intermediate/susceptible to tigecycline (MICs ranging from 2 to 4 μg/mL), and susceptible to rifampin (MICs ranging from 2 to 4 μg/mL). Molecular typing by PFGE assigned the 4 *A. baumannii* strains isolated from the patient to the same PFGE type, which we named A. PFGE type A could be further classified into subtypes A1 and A2, which differed in the migration of 3 bands from PFGE type A, respectively. The colistin-S strain Ab4450 showed identical PFGE profile (type A) to that of the epidemic *A. baumannii* strain 3909 isolated in the Monaldi Hospital in 2007 (Giannouli et al., 2010). The 2 colistin-R strains Ab4451 and Ab4452 showed identical PFGE profile A1, whereas the colistin-S strain Ab4453 isolated after colistin withdrawal was assigned to PFGE type A2 (Fig. 1 and Table 1). In particular, the 2 colistin-R strains assigned to PFGE type A1 showed a new larger fragment of 400 kb, and the absence of 2 smaller fragments of 190 and 230 kb compared to the colistin-S initial strain Ab4450 isolated from the patient assigned to PFGE type A, thus suggesting that a single genetic event might have caused the loss of a restriction site. *A. baumannii* colistin-S strain isolated after colistin therapy withdrawal showed a PFGE profile distinct from both types A and A1 and was assigned to PFGE type A2 (Fig. 1 and Table 1). All 4 *A. baumannii* strains Ab4450, Ab4451, Ab4452, and Ab4453 were assigned through Institute Pasteur's MLST typing scheme to ST78 epidemic clonal lineage (Giannouli et al., 2010). PCR and sequence analysis identified expression of a *bla*OXA-58 gene, which confers resistance to carbapenems, and a naturally occurring *bla*OXA-64 gene in all *A. baumannii* strains analyzed. Compared with colistin-S strains Ab4450, Ab4453, and 3909 (Zarrilli et al., 2011), colistin-R strains Ab4451 and Ab4452 harbored the amino acid substitution P233S in the PmrB sensor kinase of PmrAB 2-component system, which has been described as an activating mutation in colistin-R *A. baumannii* strains (Pournaras et al., 2014; Snitkin et al., 2013). No mutations were detected in genes *pmrA* and *pmrC*, coding for the response regulator and the lipid A phosphoethanolamine transferase of the PmrCAB operon, respectively. Likewise, there were no changes in lipid A biosynthesis pathway genes *lpxA*, *lpxC*, and *lpxD* in any of the analyzed *A. baumannii* strains (Table 1).

We also investigated the in vitro and in vivo fitness of the 2 colistin-R *A. baumannii* strains and compared it with that of colistin-S strains, including ATCC19606 reference strain (Table 1). The growth curves of colistin-R strains Ab4451 and Ab4452 showed no significant differences compared with colistin-S strains Ab4450, Ab4453, and ATCC19606 ($P = 0.548$ for multivariate comparisons by the generalized linear model) (Fig. 2). Consistently, there were no statistical significant differences of LD50 values in a *G. mellonella* killing assay among *A. baumannii* colistin-R strains Ab4451 and Ab4452 and colistin-S strains Ab4450, Ab4453, and ATCC19606 at any time postinfection ($P = 0.486$, $P = 0.0.483$, and $P = 0.0692$ at 24, 48, and 72 h, respectively, for multiple comparison test) (Table 2).

4. Discussion

In the last decade, colistin has become the last resource antibiotic for infections due to gram-negative rods resistant to carbapenems as well as multiple other classes of drugs. The spread of these pathogens across hospitals has translated into an exponential increase in the use of colistin, especially in intensive care units. Despite an inherently low barrier toward resistance, growing colistin utilization is posing the bases for the emergence of nonsusceptible strains.

This has occurred infrequently to date and has been coupled to a sharp reduction in fitness and virulence of *A. baumannii* strains (Beceiro et al., 2014; Lopez-Rojas et al., 2011; Pournaras et al., 2014; Snitkin et al., 2013). However, different mechanisms of colistin resistance might have a different impact on fitness and virulence. A more pronounced biological cost was observed in isolates lacking LPS (Beceiro et al., 2014) compared to those with phosphoethanolamine addition to LPS mediated through mutations in *pmrB* (Lopez-Rojas et al., 2011; Pournaras et al., 2014; Snitkin et al., 2013).

In this report, we describe the genotypic and phenotypic characteristics of 2 colistin-S and 2 colistin-R *A. baumannii* strains isolated from a single patient who developed in vivo resistance to colistin after prolonged exposure to the drug. We hypothesize that the persistence of XDR *A. baumannii* in the airways, coupled with a short course of inhaled colistin and a longer administration of intravenous colistin with little epithelial lining fluid penetration, favored sub-MIC concentrations of this drug at the infection site, leading to selection of colistin-R strains.

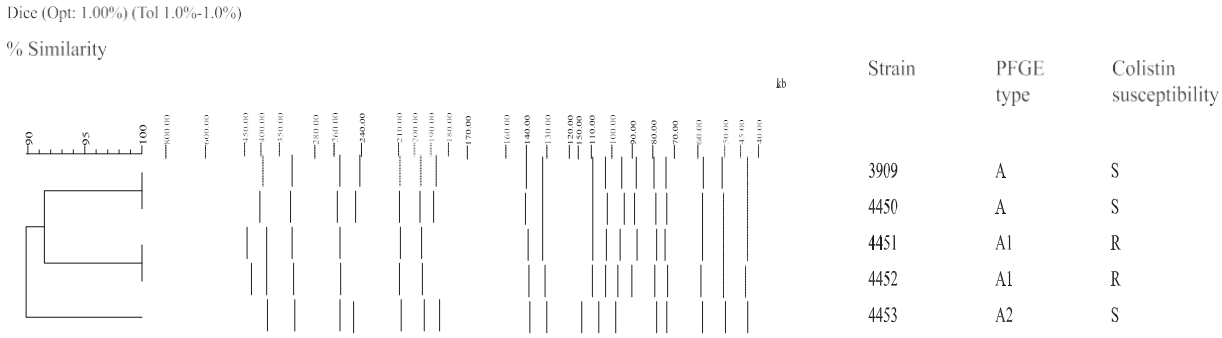

Fig. 1. Genotypic analysis of digitized *Apa*I PFGE profiles of *A. baumannii* strains included in the study. Strain numbers, PFGE profiles, and colistin susceptibility are shown. Percentage of similarity at dendogram analysis and position and tolerance values of the DICE correlation coefficient used in clustering are also shown.

In accordance with previous data (Snitkin et al., 2013), a strain susceptible to colistin outcompeted resistant strains upon colistin withdrawal. The reversion of colistin resistance in *A. baumannii* may occur through clonal replacement by an ancestral susceptible strain or by a compensatory inactivating mutation (Snitkin et al., 2013). Our data indicate that both susceptible and resistant *A. baumannii* strains belonged to the same major PFGE profile A but showed distinct PFGE subtypes A, A1, and A2. The above data suggest also that colistin-R strains of PFGE type A1 originated from the colistin-S strain of PFGE type A initially isolated from the patient because of at least 1 genetic mutation occurring in vivo during colistin therapy. A subsequent genetic mutation might have been responsible for the evolution, under further selection pressures, of the PFGE type A2 colistin-S strain, which outcompeted resistant isolates upon colistin withdrawal. All 4 *A. baumannii* strains belonged to ST78 epidemic clonal lineage, which emerged recently in Italy (Giannouli et al., 2010; Zarrilli et al., 2013). In prior reports, colistin-R *A. baumannii* strains isolated during patient treatment belonged to either international clonal lineage II (Pournaras et al., 2014; Snitkin et al., 2013) or international clonal lineage I (Snitkin et al., 2013) or to nonepidemic ST94 genotype (Lesho et al., 2013), suggesting that colistin resistance may develop in different genetic backgrounds. In accordance with previous data (Pournaras et al., 2014; Snitkin et al., 2013), the 2 resistant *A. baumannii* strains showed an identical point mutation in *pmrB* that generated the single amino acid substitution P233S in the PmrB sensor kinase of PmrAB 2-component system. No other nucleotide changes were detected in *pmr*CAB locus or *lpxA*, *lpxC*, and lpxD lipid A biosynthesis pathway genes. The absence of other nucleotide changes in *pmr*CAB locus excludes the possibility that reversion of the resistance phenotype was contributed by a compensatory inactivating mutation, as reported previously in *A. baumannii* (Snitkin et al., 2013). At variance with prior reports in *A. baumannii* colistin-R strains (Lopez-Rojas et al., 2011; Pournaras et al., 2014; Snitkin et al., 2013), *pmrB* mutation detected by us did not induce any measurable change in strain growth rate as well as no attenuation of lethality in a *G. mellonella* killing assay, suggesting that virulence attenuation paralleling resistance development might be dependent on additional unidentified genetic factors that may differ among *A. baumannii* strains and/or genotypes. Conversely, the data shown herein are in partial agreement with a recent study showing that colistin resistance in *A. baumannii* ATCC19606 due to mutation in *pmrB* has less impact on fitness and virulence than colistin resistance in *A. baumannii* ATCC19606 *lpxA*, *lpxC*, and lpxD isogenic mutants, showing complete loss of lipopolysaccharide (Beceiro et al., 2014). In agreement with our data, colistin resistance due to PmrB sensor kinase mutation was not associated with significant fitness costs in KPC-producing *Klebsiella pneumoniae* strains isolated during colistin treatment (Cannatelli et al., 2014).

There are some possible clinical drivers of the dynamics of colistin susceptibility in this case. The patient was a severely immunocompromised organ transplant recipient, who underwent 2 subsequent major surgeries with serial cardiac and renal dysfunction. Colistin had been administered initially nebulized via aerosol, subsequently intravenously, and with a possibly inadequate posology to reach inhibitory concentrations within the airways. These factors have likely contributed to emergence of colistin resistance in lung isolates. Following withdrawal of this drug, the P233S substitution-carrying strains disappeared. Based on the above data, we hypothesize that the selective pressure exerted by colistin was required for colistin resistance to emerge; on the other hand, despite conserved fitness and virulence, the *pmrB* mutant still had a disadvantage underlying its disappearance following colistin withdrawal.

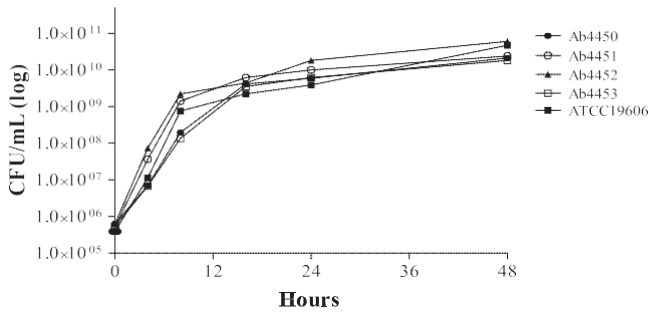

Fig. 2. Growth curves of the study and control isolates. y axis, CFU/mL from broth cultures: x axis, time of growth (hours).

5. Conclusions

We described the emergence of a colistin-R strain in an immunocompromised patient treated with a short course of inhaled colistin and a longer administration of relatively low doses of intravenous colistin. The mutation underlying colistin resistance involved the PmrB sensor kinase of PmrAB 2-component system and did not translate into an in vitro measurable reduction of growth capacity or virulence. However, withdrawal of colistin was followed by disappearance of the

Table 2
Lethal dose 50% of *A. baumannii* strains in *G. mellonella*.

Strains	LD_{50}		
	24 h	48 h	72 h
4450	9.4 (±0.4) × 106	8.6 (±0.4) × 10^6	8.0 (±0.4) × 10^6
4451	7.0 (±0.6) × 10^6	1.5 (±0.2) × 10^6	1.1 (±0.3) × 10^6
4452	1.0 (±0.2) × 10^7	5.5 (±0.8) × 10^6	1.2 (±0.4) × 10^6
4453	6.5 (±0.6) × 10^6	3.5 (±0.2) × 10^6	2.4 (±0.3) × 10^6
ATCC19606^T	8.0 (±0.7) × 10^6	5.6 (±0.3) × 10^6	4.0 (±0.2) × 10^6

The LD_{50} values are expressed in CFUs. Values represent the mean (±SEM) of 3 independent experiments.

resistant strain. We provide further evidence that selection of colistin resistance in XDR *A. baumannii* may be dependent on different molecular mechanisms and is not invariably associated with virulence attenuation.

Acknowledgment

Funding: This work was supported in part by grants from Agenzia Italiana del Farmaco, Italy (AIFA2007 contract no. FARM7X9F8K), and the EU 7th Framework Program (AIDA grant Health-F3-2011-278348).

Competing interests: None declared.

Ethical approval: Approval was obtained by the Ethics Committee of the Monaldi Hospital, decision n. 559 of the 20 October 2008.

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Chapter 5

Biofilm-associated proteins: news from *Acinetobacter*

De Gregorio et al. BMC Genomics (2015) 16:933
DOI 10.1186/s12864-015-2136-6

BMC Genomics

RESEARCH ARTICLE Open Access

Biofilm-associated proteins: news from *Acinetobacter*

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Abstract

Background: A giant protein called BAP (biofilm-associated protein) plays a role in biofilm formation and adhesion to host cells in *A. baumannii*. Most of the protein is made by arrays of 80–110 aa modules featuring immunoglobulin-like (Ig-like) motifs.

Results: The survey of 541 *A. baumannii* sequenced strains belonging to 108 STs (sequence types) revealed that BAP is highly polymorphic, distinguishable in three main types for changes both in the repetitive and the COOH region. Analyzing the different STs, we found that 29 % feature type-1, 40 % type-2 BAP, 11 % type-3 BAP, 20 % lack BAP. The type-3 variant is restricted to *A. baumannii*, type-1 and type-2 BAP have been identified also in other species of the *Acinetobacter calcoaceticus-baumannii* (ACB) complex. *A. calcoaceticus* and *A. pittii* also encode BAP-like proteins in which Ig-like repeats are replaced by long tracts of alternating serine and aspartic acid residues. We have identified in species of the ACB complex two additional proteins, BLP1 and BLP2 (BAP-like proteins 1 and 2) which feature Ig-like repeats, share with BAP a sequence motif at the NH2 terminus, and are similarly expressed in stationary growth phase. The knock-out of either BLP1 or BLP2 genes of the *A. baumannii* ST1 AYE strain severely affected biofilm formation, as measured by comparing biofilm biomass and thickness, and adherence to epithelial cells. BLP1 is missing in the majority of type-3 BAP strains. BLP2 is largely conserved, but is frequently missing in BAP-negative cells.

Conclusions: Multiple proteins sharing Ig-like repeats seem to be involved in biofilm formation. The uneven distribution of the different BAP types, BLP1, and BLP2 is highly indicative that alternative protein complexes involved in biofilm formation are assembled in different *A. baumannii* strains.

Background

Acinetobacter baumannii is a Gram-negative pathogen associated with multidrug resistance and hospital outbreaks of infection, particularly in the intensive care unit [1]. *A. baumannii* accounts for almost 80 % of all reported *Acinetobacter* infections, including ventilator-associated pneumonia, bacteremia, meningitis, peritonitis, urinary tract infections, and wound infections [1]. The rapid emergence of multidrug-resistant *A. baumannii* strains has resulted in limited treatment options, with most strains being resistant to clinically useful antibiotics [2]. *A. baumannii* cells readily form biofilms in vitro [3–5], and the ability of nosocomial strains to form biofilms on medical devices as in host tissues represents an important factor of microbial virulence. Cells forming biofilms are embedded within a polymeric conglomerate of proteins and polysaccharides. Biofilms are resistant to host immune defenses, detergents and antibiotics, and antibiotic resistance of microrganisms in these habitats can be increased up to a thousand-fold [6].

As in other microrganisms [7, 8] also in *A. baumannii* the formation of biofilm is a redundantly organized, multifactorial process involving multiple cellular components. The initial cell attachment to a surface is plausibly mediated by pili-like structures encoded by the *csu* locus [9], which are widespread among clinical isolates. However, the strong biofilm producer ATCC10696 strain lacks *csu*-encoded pili, as the ATCC17978 strain which expresses a different type of pili [10, 11]. Biofilms eventually grow by production of poly-beta-(1–6)-N-acetylglucosamine

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controlled by the *pga* locus [12]. The extracellular matrix provides adhesion between bacterial cells, enabling the formation of a multilayered structure. Several surface proteins are also involved in the process, and appear to differently contribute to the attachment of cells tobiotic or abiotic surfaces. The major outer membrane protein OmpA is essential for the attachment of *A. baumannii* to human alveolar epithelial cells, but plays a role also in the development of biofilms on plastic [13]. In contrast, both cell adhesiveness and biofilm formation are high in *A. baumannii* isolates expressing the PER-1 extended-spectrum beta-lactamase [14].

Inactivation of a protein called BAP (for biofilm-associated protein) in the ST (sequence type) 1 AB307-0294 strain resulted both in decreased biofilm growth on glass [15] and decreased adherence to human bronchial cells [16]. BAP is expressed at the cell surface, and biofilm formation by BAP-positive strains is inhibited by affinity-purified BAP antibodies [17]. BAPs are large multidomain proteins playing a role in the process of biofilm formation both in Gram-negative and Gram-positive bacteria [18, 19]. These proteins exhibit poor sequence similarity, but share structural similarities, as they are internally repetitious and feature multiple (3 to 50 copies) immunoglobulin-like domains. These domains have a peculiar three-dimensional structure known as Ig fold, composed of 70–100 amino acid (aa) residues in seven anti-parallel beta-strands organized in two beta-sheets packed against each other in a sandwich structure [18]. *In S. epidermidis,* the Embp (extracellular matrix-binding protein) protein, involved both in cell adherence and biofilm formation [20], is enriched in modules different from Ig-fold repeats called FIVAR (Found In Various Architectures, 59 copies) and GA (G-related albumin-binding, 38 copies).

In different species, BAP genes are accessory genome components. The *E. faecalis* BAP gene is inserted in a 153 Kb pathogenicity island, the *S. aureus* BAP gene in a composite transposon comprising an ABC transporter operon and a transposase, in turn inserted in the 27 kb mobile pathogenicity island SaPIbov2 [21]. The gene was identified in 5 % of the *S. aureus* bovine mastitis, but in none of the human *S. aureus* isolates studied [22]. *S. epidermidis* isolates from animal mastitis forming biofilms possess a gene highly homologous to the *S. aureus* BAP gene, but not other SaPIbov2 sequences. Similarly, the Bhp (Bap homologue protein) protein is present only in some human *S. epidermidis* biofilm producers, as the RP62A strain [23].

In contrast, the BAP gene is largely conserved in the *A. baumannii* population, having been identified in isolates belonging to different STs [15]. Differently from other species, in *A. baumannii* the BAP gene is unlinked to genes involved in protein secretion. The BAP detected in *A. baumannii* ST92 isolates is approximately four times smaller than the AB307-0294 protein [17]. Size heterogeneity plausibly denotes changes in the number of protein repeats due to *recA-independent* slipped-strand mispairing during DNA replication, as reported for *S. aureus* BAP variants [24].

In this work, we have studied the organization of BAP coding sequences in wholly sequenced strains as in a large set of whole genome shotguns (WGS) of both *A. baumannii* (Additional file 1) and other *Acinetobacter* species. In silico analyses had been carried out also for two surface proteins, that we have called BLP1 and BLP2 (for BAP-like proteins) structurally related to BAP because similarly containing Ig-like domains. We showed that *A. baumannii* BAPs come in different formats, for changes in the number and type of repeats, and organization of the COOH region. Data mining suggest functional hierarchy linking BAP to BLPs, a hypothesis strengthened by the observation that gene disruption of either BLP gene impaired biofilm formation and adhesiveness to epithelial cells.

Results

Heterogeneity of BAP proteins among *A. baumannii* strains

The BAP protein identified in the *A. baumannii* strain AB307-0294 contains 8621 aa, and features seven distinct repeat units ranging in size from 70 to 104 aa. Repeats A-D exhibit poor similarity to each other, but all share Big_3_4 (Bacterial Ig-like domain, group 3) motifs, fitting the consensus TDnAGN, found in many bacterial surface proteins (PFAM accession n. PF13754). B, C and D repeats are over-represented, and account for 2/3 of the AB307-0294 BAP. Repeats E, F and G lack Big_3_4 motifs, and are reiterated in tandem in the COOH region. A third G-like repeat is located downstream at 170 aa distance. A-G repeat sequences are reported in Additional file 2.

In BAP proteins found in wholly-sequenced *A. baumannii* genomes (Fig. 1), the NH2 region is conserved, the COOH region varies in length because either 2 or 4 copies of the EFG module are present (Additional file 2). Small and large COOH regions, which exhibit 44 % sequence identity, mark BAPs encoded by strains belonging to the abundant sequence types ST1 and ST2, which correspond to international clone I and II, respectively [25]. Accordingly, the corresponding proteins have been classified as type-1 and type-2 BAPs. Length and composition of the central repetitive region is also variable. According to GenBank annotations, intact BAPs are encoded by the AYE (8200 aa) and BJAB0715 (3059 aa) strains only, while the 8621 aa AB307-0294 protein described by Loehfelm and coworkers [15] is split in ORFs 776 and 777. The discrepancy may either signal that a mutation occurred in the AB307-0294 BAP gene prior

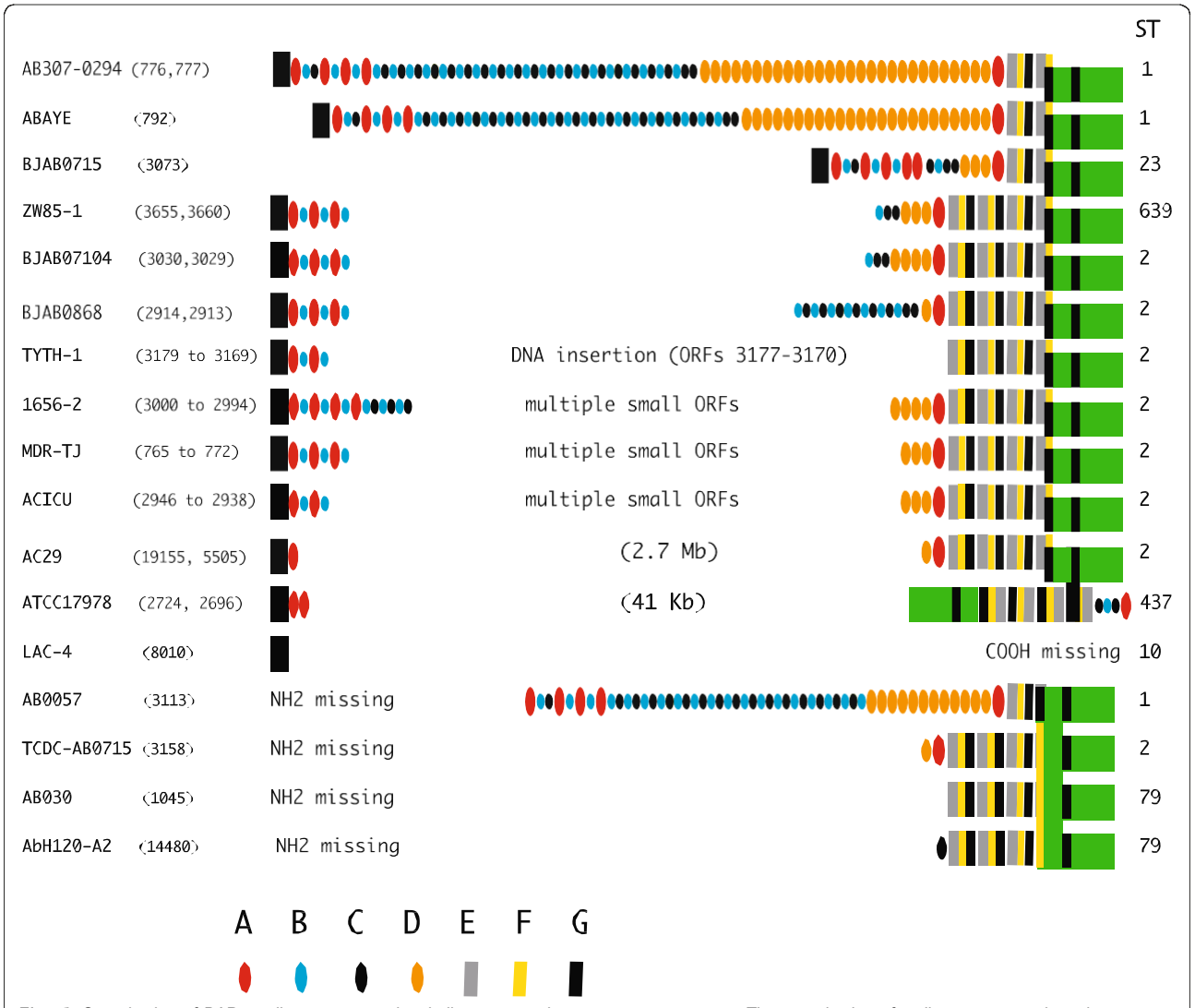

Fig. 1 Organization of BAPs coding sequences in wholly sequenced *A. baumannii* genomes. The organization of coding sequences homologous to BAP [15] in wholly sequenced *A. baumannii* genomes deposited at the KEGG (Kyoto Encyclopedia of Genes and Genomes) site is depicted. Boxes denote NH2 and COOH regions, ovals modules of the repetitive region. Modules within small ORFs spanning the repetitive region in different clones have not been reported for clarity. Numbers in parenthesis denote ORFs, numbers to the right of each protein the strain ST

to whole-sequencing, or denote sequencing errors. In this context, it may be worth noting that the *Salmonella thyphimurium* LT2 strain encodes a 386 kDa BAP, but the corresponding gene is annotated in GenBank as a pseudogene because of a frameshift mutation [26]. In other *A. baumannii* genomes, BAP homologous sequences are split in two or more ORFs (six in strains 1656-2, MDR-TJand ACICU). It is unlikely to hypothesize in all instances that the fragmentation reflects sequencing errors, sequencing data having been obtained by different investigators. In several ST2 strains (ZW85-1, BJAB0714, BJAB0868, MDR-TJ, MDR-ZJ06), BAP prematurely terminates at residue 737. The corresponding COOH region, preceded by a variable number of repeats, is encoded by downstream flanking ORFs. This finding and the size identity of small BAP ORFs in MDR-TJ and ACICU strains, both support the notion that allelic BAP-negative variants, spread by horizontal gene transfer, may be common in the bacterial population. In the ATCC17978 and A12 strains, the BAP gene was disrupted by a 7 Kb DNA insertion in TYTH-1, and by chromosomal rearrangements moving away, or flipping the NH2 and COOH regions. In six strains, either the NH2 or the COOH region is missing, in AC29 the two regions are at 2.7 Mb distance (Fig. 1).

BAP in *A. baumannii* draft genomes

Data in Fig. 1 suggest that BAP may be an accessory, dispensable gene expressed by subsets of the *A. baumannii* population only. To learn more, BAP homologs

were searched by tBLASTn in a large set of draft genomes of *A. baumannii*. MLST analyses were performed at the A. *baumannii* MLST website (http://pubmlst.org/abaumannii/) using the "Pasteur" MLST scheme [25]. Only strains which could be assigned to an ST were selected for further studies. The degree of identity of rpoB gene sequences helped to reassign several strains annotated as *A. baumannii* to other species. On the whole, the *A. baumannii* strains analyzed in this work (both complete and draft genomes) are 541 and belong to 108 different STs (Additional file 1). For sake of simplicity, hereon draft genomes and/or the corresponding strains will be referred to by the four-letter code in the GenBank accession number (see Additional file 1).

Intact BAP coding sequences have been identified only in the AYOI (6207 aa) and JEVZ (6219 aa) strains. In a few high quality draft genomes (AFDB, AFDK, AYOH, JFEL, JFXM), BAP coding sequences resided in one contig, but were interrupted by stop codons, and split into two or multiple ORFs, as in many wholly sequenced genomes shown in Fig. 1. In the remaining WGS, sequences encoding NH2 and COOH BAP regions, flanked by segments of the central repetitive region of variable lengths, were at the termini of two contigs. The stop codon at BAP residue 738 found in several wholly-sequenced genomes is a signature conserved in 42 % (118/282 genomes) of the ST2 strains analyzed. Given the prevalence of ST2 strains, interrupted BAP genes may be frequent in the *A. baumannii* population.

The NH2 and COOH regions were conserved in most of the clones analyzed. Changes in the region encompassing the terminal 397 aa mark BAP-2b and BAP-2c variants which exhibited 46 and 41 % identity to BAP-2 in the diversity region, respectively. BAP-2b have been found in all the 12 ST499 strains analyzed and two ST2 strains (AFTC and AMSX), BAP-2c in the single ST35 APRA and the ST504 JEXU strains.

Changes in the composition of the BAP repetitive region were also observed. A novel Ig-like Z repeat unrelated to A-D repeats was identified in 69 strains belonging to 26 different STs. As sketched in Fig. 2, the overall module organization in Z-positive and Z-negative regions differs. Type-3 BAPs have the same NH2 region of type-1 and type-2 BAPs, but differ both in the repetitive and the COOH regions. The repetitive region features D, Zb (a Z variant restricted to type-3 BAPs), W and Y modules (Fig. 2,; the sequence of the modules are in Additional file 2). The COOH region (1169 aa) of type-3 BAP exhibits only 40 % identity to the corresponding region of type-1 or type-2 BAPs. EFG modules are absent, but a tract of approximately 60 aa, exhibiting 50 % identity to G repeats, could be identified. The 26 clones carrying type-3 BAPs belong to 14 different STs, and about half of them belong to ST25. Type-3 BAPs

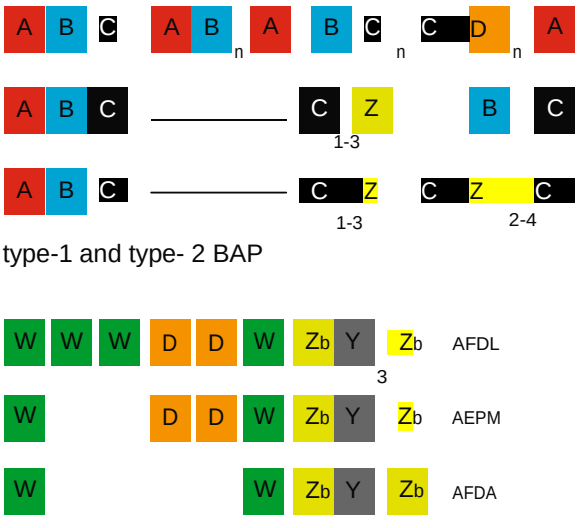

Fig. 2 Repeat modules in type-1, type-2 and type-3 BAPs. Bars denote undefined repeat regions in Z-plus type-1 and type-2 BAPs. AFDL, AEPM and AFDA clones potentially express a complete type-3 BAP

are relatively limited in size, the largest complete protein found in the AFDL strain measuring 2700 aa (Additional file 2). Here too, however, it must be emphasized that the complexity of the repetitive region might have been missed in sequencing assembly.

On the whole, strains encoding type-1, type-2 and type-3 BAP belong to 29, 40 and 11 % of the STs analyzed. The remaining STs lack BAP, or carry truncated forms of the protein.

BAP-like proteins encoded by the *A. baumannii* SDF strain

The *A. baumannii* SDF strain, isolated from a human body lice [27] encodes two unusual BAP-like proteins, ABSDF785 (2321 aa) and ABSDF2314 (2402 aa), which exhibit significant homology to BAPs at the NH2 terminus only. In both, Ig-like repeats are replaced by long stretches of alternating serine (S) and aspartic acid (D) residues (501 and 701 SD repeats in ABSDF785 and ABSDF2314, respectively).

BAPs in other *Acinetobacter* species

BAP proteins occur in the genomes of several non-*baumannii Acinetobacter* strains (Additional file 3). The list includes the *A. baylyi* ADP1, all the strains classified as *A. calcoaceticus* (taxid 471), *A. pittii* (taxid 48296), and *A. radioresistens* (taxid 40216), as several *A. haemolyticus* (taxid 29430) and *A. nosocomialis* (taxid 106654) strains, only one (APPX) of the six strains classified as *A. junii* (taxid 40215) BAP proteins are missing in *A. johnsonii* and *A. lwoffii*.

Acinetobacter BAPs have been classified as alpha or beta for the presence of either Ig-like or SD repeats as repetitive modules, respectively, and assigned to 8 types on the basis of their COOH regions (Fig. 3). In addition to *A. baumannii*, type-1 and type-2 BAPs have been found in *A. calcoaceticus*, *A. pittii* and *A. nosocomialis*, while type-3 BAPs are confined to *A. baumannii*. *A. baylyi* and *A. radioresistens* BAP-like proteins markedly differ from *A. baumannii* BAPs because the repetitive region features either highly divergent copies of a single Ig-like module (type-4), or novel Ig-like modules interrupted by foreign aa stretches (type-5) (Additional file 4).

Type-6 and type-7 beta-BAPs differ for the presence/absence of B modules in the repeat region. Curiously, *A. pittii* and *A. calcoaceticus* strains producing beta-BAPs potentially encode also alpha type-1 BAPs. *A. haemolyticus* type-8 BAPs are hybrid alpha/beta proteins which resemble type-1 BAPs in the COOH region, but feature long tracts of alternating aa in the repetitive region. The complete proteins encoded by the *A. haemolyticus* ATCC19194 and TG19602 strains feature 79 SD pairs and 148 AD pairs, respectively.

The NH2 region is largely conserved in all BAPs, but three main variants, denoted by the color code in Fig. 3, could be recognized (Additional file 5). Curiously, the *A. baumannii* and *A. haemolyticus* NH2 regions both feature a 38 aa insertion mostly made by tandem DA repeats. Significant similarities of the COOH regions of the various BAP types are limited to the terminal 100–150 aa. It may be worth noting that only G modules are conserved in many BAPs (Additional file 5).

The analysis of wholly sequenced genomes showed that alpha- and beta-BAP genes map at different chromosomal sites. The alpha gene region is conserved in *A. baumannii*, *A. calcoaceticus* PHE-A2 and *A. baylyi ADP1* genomes. In the *A. baumannnii* SDF strain, the region is also conserved, but is occupied by the beta-BAP ABSDF785 gene. The beta gene region is located on genomic islands, flanked by *lap*EBC genes coding for components of a type-I secretion system, both in *A. calcoaceticus* PHEA-2 and *A. baumannii* SDF strains (Additional file 6). SD-rich adhesion protein genes flanked by *lap* genes have been found in different Gram-negative microrganisms, such as *Klebsiella pneumoniae* MGH 78578 (ORF KPN_00994), and *Enterobacter aerogenes* EA1509E (ORF ST548_p6177).

Additional proteins with Ig-fold motifs encoded by *A. baumannii*

In *A. baumannii*, two additional proteins were found to contain Ig-like repeats. The proteins, variously named in

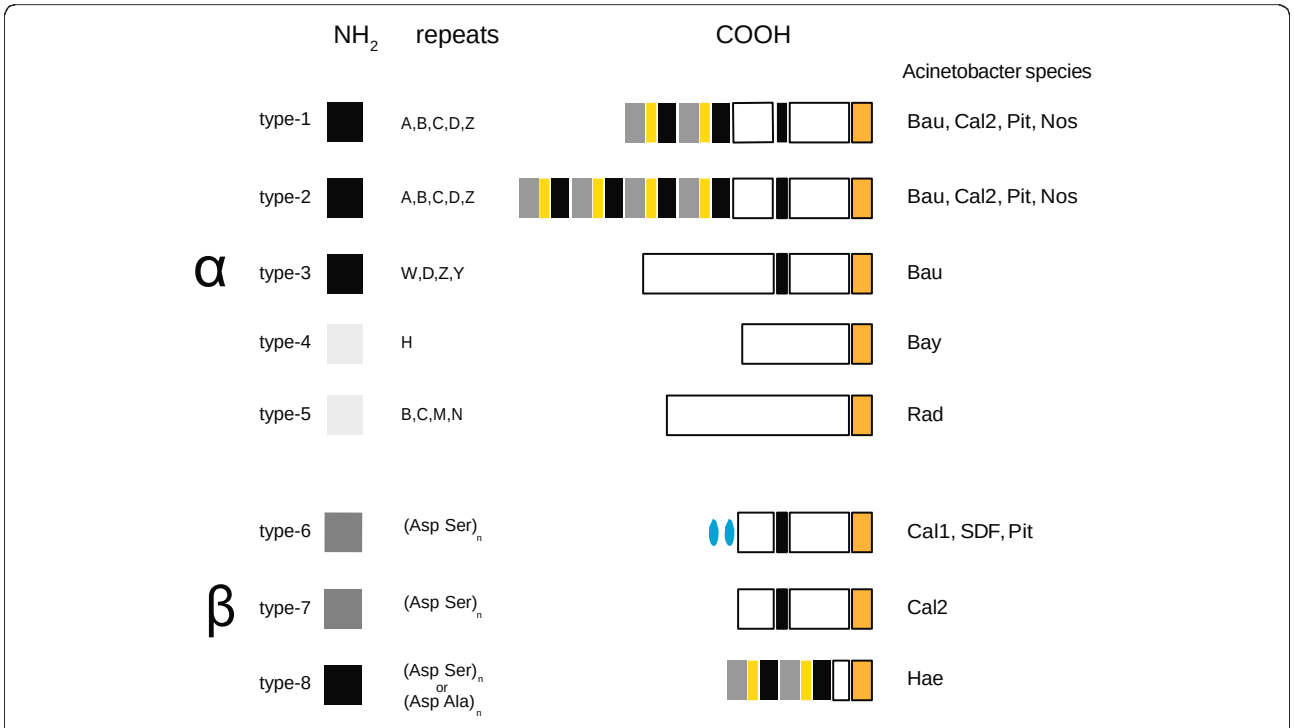

Fig. 3 Alpha and beta BAPs. Changes in the organization of the 8 BAP types found in the Acinetobacter genus are highlighted. Bau, *A. baumannnii*; SDF, *A. baumannnii* SDF; Hae, *A. haemolyticus*; Pit, *A. pittii*; Nos, *A. nosocomialis*; Bay, *A. baylyi*, Rad, *A. radioresistens*, Cal, *A. calcoaceticus*. Cal1 and Cal2 refer to the wholly sequenced *A. calcoaceticus* PHE-A2 [41] strain and the draft genomes deposited at the NCBI, respectively. Sequences conserved at the end of all BAP types are in orange. For sake of simplicity difference in the COOH regions among BAPs are not highlighted. E, G, F and B modules are depicted as in Fig. 1

different annotations, have been named, for sake of simplicity, BLP1 and BLP2. Both BLPs and BAP share a sequence motif at the NH2 terminus. BLP1 and BAP feature COOH-terminus aa sequences similar to those found in RTX toxins, proteins secreted through a type I secretion system ([28]; see Fig. 4). In the AYE strain, BLP1 and BLP2 correspond to ORFs 821 and 1037, respectively, and BAP to ORF 792. BAP and BLP1 genes are thus very close (approximately 30 kb away), and their relative position is conserved in all strains examined.

BLP1 and BLP2 exhibit the same tripartite structure of BAP (Fig. 5). In BLP1, the repetitive region is made up by four different repeat types (P, Q, R, S), all but Q featuring Big_3_4 motifs (Additional file 7). BLP1 vary in length from 3044 to 3356 aa. BLP1 has a small number of repeats, and complete proteins have been identified in about 60 % of the examined WGS. Aminoacids changes let to distinguish type-1 and type-2 BLP1 variants, exhibiting 86 % identity in a 552 aa domain of the COOH region. The nomenclature reflects the occurrence of type-1 and type-2 BLP1 in clones of the predominant ST1 and ST2 types. Type-2 variants featuring a more extensive remodeling of the variable domain are encoded by a few strains, each belonging to a different ST, and have been named H and K variants (see Additional file 7).

BLP2 features a simple repetitive region made by only four T repeats, and a relatively small (207 aa) COOH region. The protein is conserved in most of the strains examined, and four main sequence variants (A-D) could be recognized. The A-type has been found in >70 %(77/108) of the STs examined, including the predominant ST1 and ST2. The minor B, C and D variants, which exhibit decreasing similarity to the A-type protein, have been found in 11, 13 and just 2 STs,respectively. In both C- and D-type proteins, the Ig-like domain of one T repeat is mutated (Additional file 7).

In contrast to BLP2, BLP1 had been identified only in 80 % of the clones analyzed. BLP1-negative strains fall into two distinct groups. In the JFXH, APWV and ATGJ strains, the BLP1 coding region had been completely or partly deleted. In the majority of BLP1-negative strains, however, the BLP1 coding region is invariably replaced by a 99 bp DNA tract (Additional file 8).

BLPs in other *Acinetobacter* species

BLP1 and BLP2 genes were searched in non-*baumannii* *Acinetobacter* genomes (Additional file 3). *A. calcoaceticus* and *A. pittii* strains encode both BLPs, *A. haemolyticus* and *A. nosocomialis* strains BLP2 only. In all the BLP1-negative species, sequences homologous to the 99 bp DNA tract found in the *A. baumannii* BLP1-negative clones could be identified (Additional file 8). The presence/absence of BLP1 in the *A. baumannii* population can thus be rationalized by hypothesizing that the BLP1 gene region had been replaced by the corresponding "empty site" upon recombination with non-*baumannii* cells. Exchange of the BLP1 locus among *Acinetobacter* cells is frequent, as indicated by the spread in *A. baumannnii* of the H and K BLP1 variants found in *A. calcoaceticus* and *A. pittii* strains.

A. johnsonii, *A. lwoffii* and *A. junii* strains lack both BLP1 and BLP2. In 5/6 *A. junii* genomes analyzed, BLP2-like genes are inactivated by a frameshift mutation, separating NH2 and COOH regions into two ORFs, and the BAP gene is missing. Only the *A. junii* APPW strain encodes an intact BLP2, and is worth noting that only this strain potentially encodes also a BAP protein.

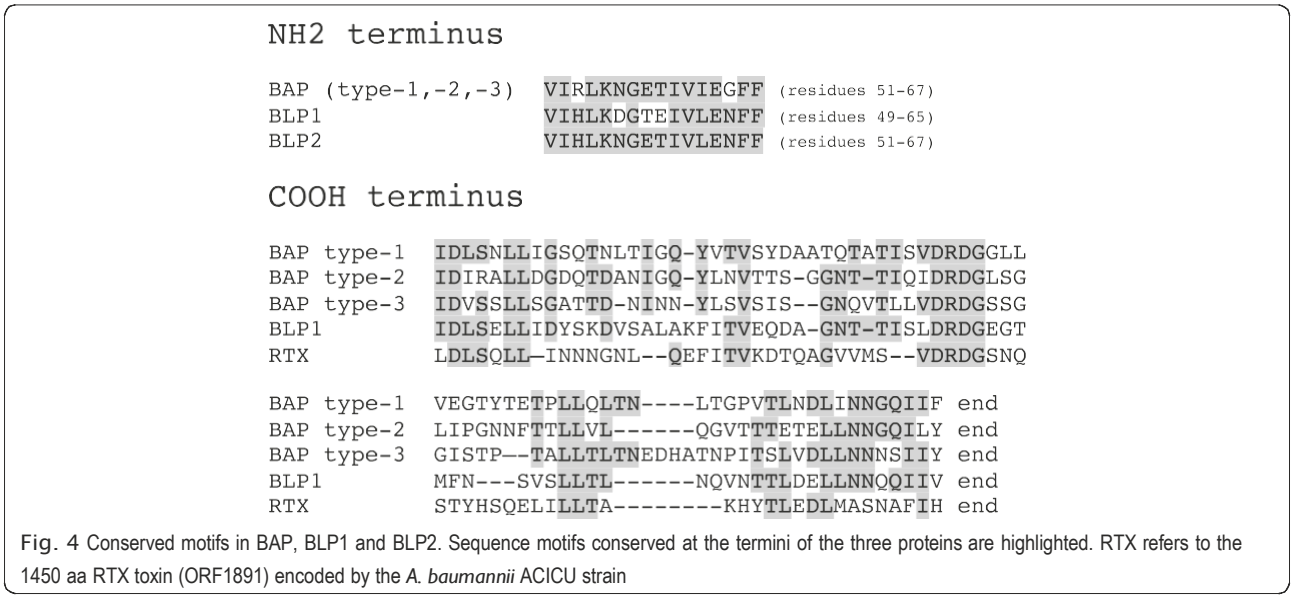

Fig. 4 Conserved motifs in BAP, BLP1 and BLP2. Sequence motifs conserved at the termini of the three proteins are highlighted. RTX refers to the 1450 aa RTX toxin (ORF1891) encoded by the *A. baumannii* ACICU strain

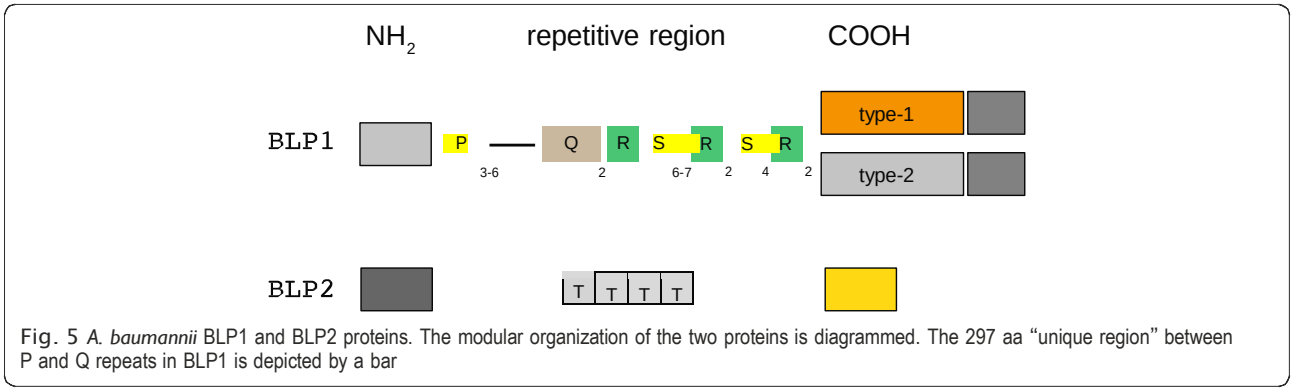

Fig. 5 *A. baumannii* BLP1 and BLP2 proteins. The modular organization of the two proteins is diagrammed. The 297 aa "unique region" between P and Q repeats in BLP1 is depicted by a bar

Sequence type distribution of BAP, BLP1 and BLP2

The distribution of BAP, BLP1 and BLP2 among analyzed STs is summarized in Additional file 9. A close look at data support the notion that the three proteins may be functionally related. BLP1 seems ancillary to BAP, since it is missing in almost all the STs (14/16) which lack BAP. Among BAP-positive strains, the BLP1 gene is unevenly distributed. BLP1 was found in most (42/47) STs expressing a type-2 BAP, 60 % of STs expressing a type-1 BAP, only in one of the 12 STs expressing a type-3 BAP. Moreover, type-2 BAP and type-2 BLP1 are more frequently associated than type-1 BAP and type-1 BLP1. Thus, the coexistence of some BLP1 and BAP variants may be preferred, while specific combinations may plausibly be disadvantageous, and this may explain the exchange of filled/empty BLP1 sites observed in the population. BLP2 is conserved in most of the clones examined. Several BAP-negative STs lack BLP2, others feature BLP2 variants which may be functional impaired because carrying mutations in one Ig-like domain (Additional file 9), suggesting that BAP and BLP2 may be interdependent. The hypothesis that BAP and BLP2 may functionally interact was reinforced by the analysis of *A. junii* genomes. BAP and BLP2 are absent in all *A. junii* strains examined but the APPW strain, in which both proteins are present.

Functional link of BAP, BLP1 and BLP2 was further provided by the results of quantitative RT-PCR analyses in which the levels of the corresponding transcripts were monitored. For all, RNA levels were undetectable in log-phase, high in late stationary phase (data not shown).

Biofilm formation in different *A. baumannii* strains

We thought of interest to evaluate differences in the type and extent of biofilm formation among *A. baumannii* strains belonging to different STs, and presenting a different combination of BAP and BLP1 proteins by CLSM (Confocal Laser Scanning Microscopy) analyses. The ACICU (type-2 BAP, BLP1-plus) and the 4190 (type-3 BAP, BLP1-negative) strains, assigned to international clone II and ST25 epidemic clonal lineage, respectively, were shown to be relatively strong biofilm producers [4]. Both strains carry *pga*, *csu* and *ompA* genes shown to be involved in biofilm production. Biofilms formed by the two strains have comparable heights, but different architectures (Fig. 6, panels a and b). The ACICU biofilm exhibited a total substratum coverage. By contrast, the 4190 biofilm was made up by large, evenly distributed microcolonies, separated by water channels. The ATCC17978 strain also carries *pga*, *csu* and *ompA* genes, but lacks both BLP1 and BAP1. Yet, this strain formed a dense biofilm, exhibiting an uniformely surface coverage. However, microcolonies were undetectable (Fig. 6, panel c). The heterogeneity of the biofilms formed by the analyzed strains is not surprising. The plethora of genes shown or hypothesized [11] to be involved in biofilm formation in *A. baumannii* makes difficult evaluating the role that different BAP types, and the presence/absence of BLP1, may play in the process. We thought therefore to address the problem by mutating, as done for the BAP gene in the B307-0294 strain [15], the BLP1 and BLP2 genes of the AYE strain. Either gene was inactivated by allelic replacement [29]. The test strain encodes a sequence-proved intact BAP, comparable in length and composition to the B307-0294 protein (see Fig. 1). BAP and BLP1 expressed by the AYE and B307-0294 strains, which both belong to sequence type 1, are functionally comparable, because unaffected by size changes which may influence their exposure on cell surface, as proved for repetitive *S. aureus* SDR proteins of different lengths [30]. The biofilms formed on glass by AYE and its BLP1 deletion derivative AYE-Δblp1 markedly differed. AYE formed a typical biofilm structure (Fig. 7a), in which distinct microcolonies could be distinguished. In contrast, AYE-Δblp1 formed just a thin monolayer. The width of the biofilm formed by AYE averaged 22 microns. The biofilm formed by the mutant (average 4 microns) was barely measurable (Fig. 7b). Inactivation of BLP2 also affected biofilm formation (Fig. 7c). The AYE-Δblp2 mutant produced a biofilm only twice thinner than AYE. However, in contrast to the AYE-Δblp1 biofilm, the coverage was

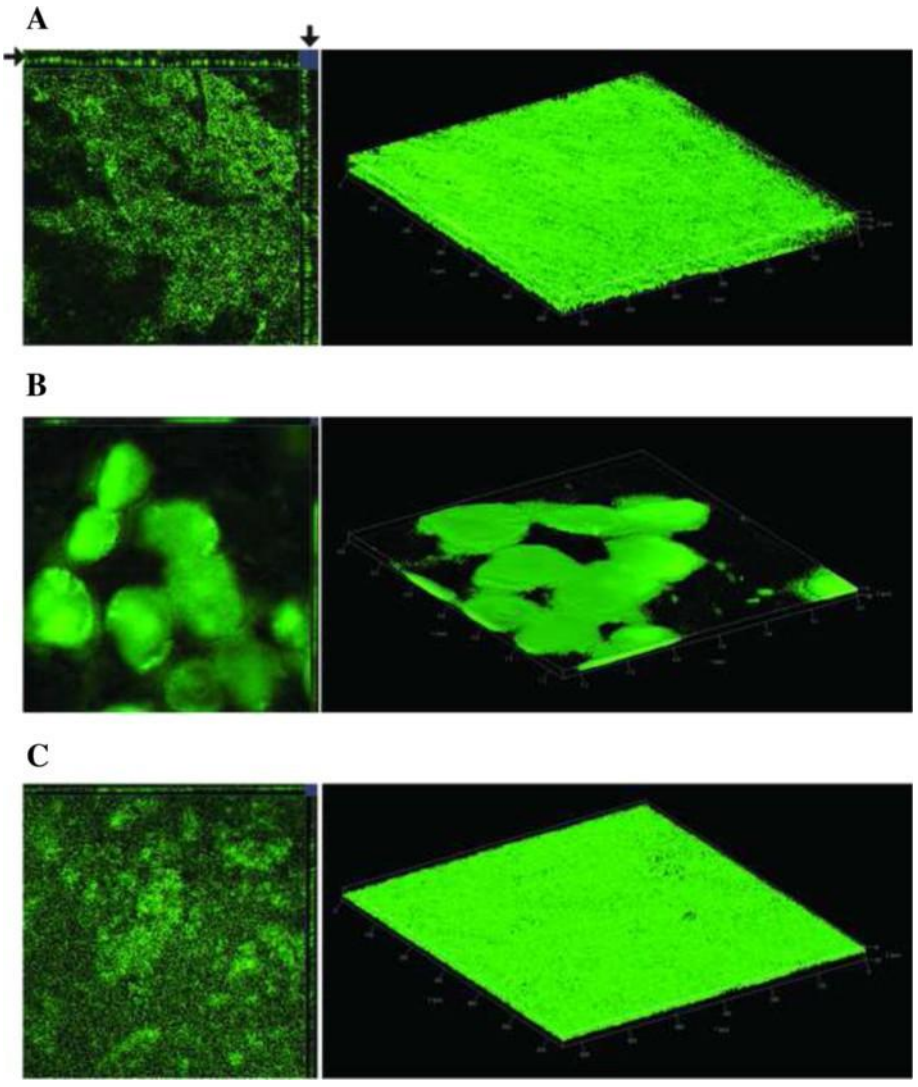

Fig. 6 CLSM analysis of biofilms formed by the *A. baumannii* strains ACICU (a), 4190 (b), and ATCC17978 (c). In each panel, to the left is shown the orthogonal view of Z-stacks, to the right the three-dimensional spatial distribution of the biofilm. Arrows denote biofilm heights. Bright and dark areas show cell clusters and voids in the biofilm

reduced, and microcolonies could not be detected. Quantitative estimates obtained using the IMARIS v7.0 software (Bitplane, Switzerland) allowed measuring the total biomass of biofilms as micrometers^3 [31]. Quantitative estimates of the biofilms formed by AYE, AYE-Δblp1 and AYE-Δblp2 are shown in Fig. 8. A significant correlation was found between biofilm biomass and biofilm thickness of AYE and AYE-Δblp1 and AYE-Δblp2 mutant strains ($r = 0.9974$, $p = 0.0455$).

Adherence of *A. baumannii* wild type AYE and AYE-Δblp1 and AYE-Δblp2 mutant strains to A549 human bronchial cells

We next investigated the ability of AYE, AYE-Δblp1 and AYE-Δblp2 strains to adhere to A549 human alveolar epithelial cells. As shown in Fig. 9, AYE showed a statistically significant higher adherence to A549 human bronchial cells compared with AYE-Δblp1 and AYE-Δblp2 mutant strains, which showed a reduction in adherence by 2.9- and 1.7 fold, respectively. Moreover, significant correlations were found between adhesiveness to epithelial cells and biofilm biomass ($r = 0.9999$, $p = 0.0104$) and bacterial adhesiveness to epithelial cells and biofilm thickness ($r = 0.9985$, $p = 0.0351$) for AYE, as for AYE-Δblp1 and AYE-Δblp2 mutants. On the other hand, AYE and mutant strains were not able to invade A549 cells human alveolar cells (data not shown). Also, a similar number of bacteria adhered to A549 cells when the monolayers were incubated with *A. baumannii* strains for 60 min at 4 °C, i.e. under

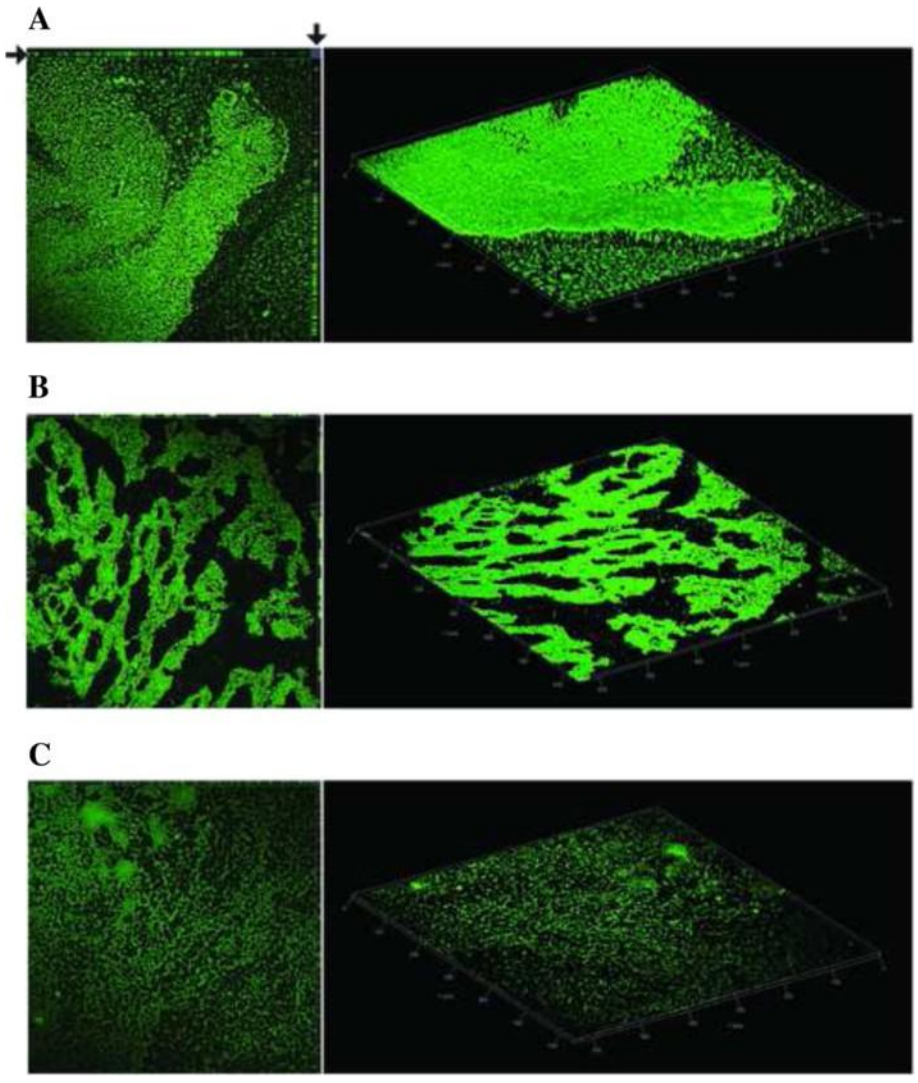

Fig. 7 Biofilms CLSM analysis. Biofilms formed by the AYE (a), the AYE-ΔBLP1 (b) and AYE-ΔBLP2 (c) strains. Orthogonal view of Z-stacks, three-dimensional spatial distribution of the biofilm and arrows are as in Fig. 6

conditions that do not allow for tissue invasion (data not shown).

Variable assortment of other surface proteins

We checked next whether the distribution of other surface proteins may similarly vary among *A. baumannii* strains. OmpA, the abundant 356 aa protein involved in biofilm formation [13] is highly conserved (>95 % identity) in most isolates. In 16 clones, however, a protein variant exhibiting only 70 % identity at the NH2 side (residues 35–183 aa) was detected (Additional file 10). Interestingly, all these clones but one potentially expressed a type-3 BAP. SURP-1 (SUrface repetitive protein 1) is a 904 aa protein with a central region made by 84–93 aa repeats (Additional file 10) unrelated to either BAP or BLP1. SURP-1 has been found only in 65 % of the ST. The protein is missing in all STs featuring type-3 BAP, except ST 215, 50 % of BAP-negative STs, most of the BAP-plus/BLP1-negative STs. The distribution of OmpA and SURP-1 among the various STs is summarized in Additional file 9.

Discussion

Most bacterial species form biofilms, and the identification of the genetic determinants controlling this complex process became an active field of research in recent years, given the implications that biofilm formation has in medical care. As in other pathogens, a large protein expressed at the *A. baumannii* cell surface, known as biofilm-associated protein or BAP, has a role in biofilm formation as in host cell adherence [15, 17]. Comparative genome analyses carried out over hundreds of

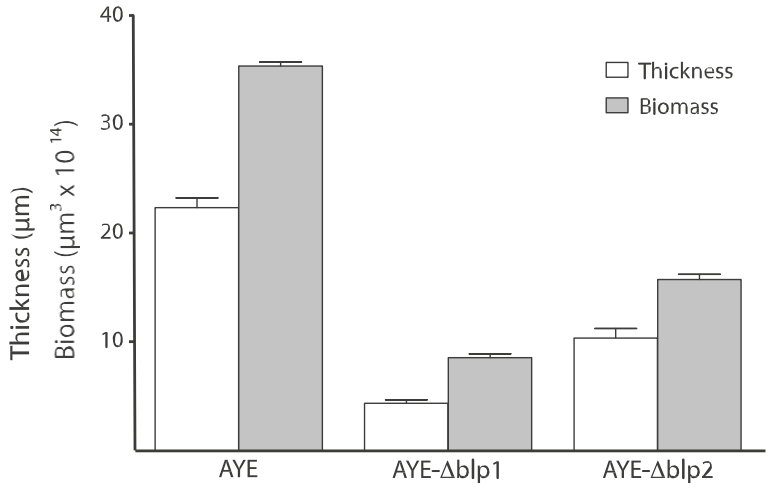

Fig. 8 Quantitative analysis of biofilm formation. The thickness (*white bars*) and the biomass (*grey bars*) of biofilms formed by the *A. baumannii* AYE, AYE-ΔBLP1 and AYE-ΔBLP2 strains are shown

sequenced strains revealed that the *A. baumannii* BAP is highly polymorphic. The central repetitive region has a variegated structure, and type and number of repeat modules both vary among isolates. This region connects a relatively small NH2 domain, conserved in all BAPs which is plausibly exposed on the cell surface and involved in binding, to large COOH regions in turn hosting a few, additional repeats. BAP COOH regions also vary, and three main types of proteins could be distinguished. Type 1 and type 2 proteins are expressed by strains of the predominant clonal complexes 1 and 2 strains, respectively, and have been identified also in *A. calcoaceticus*, *A. pittii* and *A. nosocomialis*, which form with *A. baumannii* the monophyletic ACB complex [32, 33]. Type 3 BAPs feature short repetitive regions, which include novel repeat modules, and a COOH region exhibiting only 40 % similarity to the corresponding regions of either type-1 or type-2 BAPs. This protein variant seems restricted to *A. baumannii*. However, taking into the account that the variant had been identified only in 13/108 STs, and a relatively small number of non-*baumannii Acinetobacter* strains had been sequenced, it cannot be ruled out that type 3 BAP may be expressed also by other species of the ACB complex.

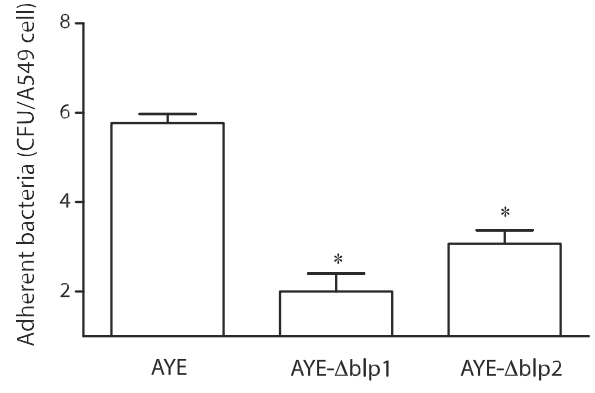

Fig. 9 Bacterial adherence of *A. baumannii* AYE, AYE-ΔBLP1 and AYE-ΔBLP2 strains to A549 bronchial epithelial cells. Cell surface-associated bacteria after 60 min incubation at 37 °C. Asterisks denote statistically significant (p <0.001) differences in the degree of cell adhesion of AYE and derivative clones

The genus *Acinetobacter* hosts a variety of BAP proteins, all featuring large central repetitive regions made either by 80–100 aa long Ig-like repeats (alpha-type BAP), or homopolymeric stretches of alternating serine and aspartic acid, or serine and alanine residue containing up to 500 dipeptides (beta-type BAP). Repetitive regions in both alpha- and beta-type BAPs span the cell-wall allowing exposure of the N-terminal region to the environment [15, 30, 34]. The length of the repeat region has been shown to influence exposure of the *S. aureus* SDR proteins on cell surface [30], and the same may plausibly hold true for alpha-BAPs. In alpha-like BAPs found in other species, the repeat region originated by the expansion of a single module ([35, 36]). The rule is subverted in *A. baumannii* BAPs, which feature multiple repeats varying in length, composition and relative arrangement. A variegated repetitive region may be beneficial for such a large protein, which is a gain of specific isolates in many species, because encoded by mobile DNA, but is in contrast a conserved gene product in *A. baumannii*. Modules diversification should decrease the chance that the protein may vary in size because of strand slippage during DNA replication. Moreover, a low sequence identity between modules strongly inhibits misfolding and aggregation of repetitive proteins, and

consecutive homologous domains in large multi-domain proteins almost exclusively show sequence identities of less than 40 % [37].

As shown in this report, differences in size and extent of module variation among *A. baumannnii* BAPs both remain largely unknown. The number of isolates in which the BAP gene is functional, or had been inactivated by mutations within the repetitive region, as reported for different genomes (see Fig. 1), remains also to be established, and the issue may be settled for individual strains only by expression analyses. Mapping repetitive DNA regions is a major bias in whole sequencing projects. Reconstructing the organization of the baroque BAP repetitive region is a tremendous, pitfall-rich task, and most of the sequence data on *A. baumannnii* BAP could be wrong, and need to be amended. Translation of the BAP gene RNA is interrupted at residue 737 of the protein in a high number of ST2 clones, but a –1 frameshifting could let translation extending into downstream BAP coding sequences. While the occurrence of both short and long BAP isoforms in these clones may be experimentally tested, data denote variation in the state of the BAP gene among strains of the same ST. In many isolates, the BAP is truncated, or absent. Altogether, the fraction of BAP-negative isolates belonging to different STs is elevated (Additional file 9). Thus, the BAP gene may be catalogued as a variable dispensable gene.

In *A. baumannii*, two additional proteins, BLP1 and BLP2, include a central repetitive region made by Ig-like repeats. BAP, BLP1 and BLP2 share a large sequence motif at the NH2 terminus, are co-expressed at the end of the stationary growth phase, and may work in conjunction. BLP1 is a large (3044 up to 3356 aa) protein which features a composite repetitive region, and can be typed, as BAP, into main sequence variants according to aa changes in the COOH region. BLP1 has been found in *A. baumannii*, *A. calcoaceticus* and *A. pittii*. In several *A. baumannii* isolates, the BLP1 coding region is missing, and is invariably replaced by the same piece of DNA which is highly homologous to DNA tracts found, at the same relative chromosome position, in *A. nosocomialis* and other *Acinetobacter* BLP1-negative species. The coexistence of empty and filled BLP1 sites in the population implies recombination of *A. baumannii* with non-*baumannii Acinetobacter* cells. The genetic exchange plausibly occurred once, and the empty site status had been eventually maintained, probably because the absence of BLP1 turned out to be advantageous in specific genetic milieu. The hypothesis stems from the uneven distribution of BLP1, conserved in most isolates expressing type-2 BAP, but only in half of those expressing type-1 BAP, missing in isolates expressing type-3 BAP. BLP1 is also missing in most BAP-negative isolates.

Altogether, data suggest that BAP and BLP1 may be interdependent. The proteins may interact and/or share a partner on the cell surface, and the combination of BLP1 with different BAP types may be beneficial or harmful. BLP2 is a smaller (728 aa) protein with a repetitive region made by only 4 Ig-like repeats. The protein, found in all the species of the ACB complex, is highly conserved in *A. baumannii*. BLP2 may also be a component of surface protein complexes involved in biofilm formation. The conclusion stems from the observation that BAP-negative strains belonging to different STs lack BLP2, or feature BLP2 with mutated Ig-like domains. Moreover, BAP and BLP2 are both absent or present in all *A. junii* strains examined.

A link between BAP and BLPs is suggested by the observation that gene disruption of either BLP gene impaired biofilm formation, as proved by SCLM analyses (Figs. 7 and 8). Similarly to BAP, the BLP1- and BLP2-negative derivatives AYE-Δblp1 and AYE-Δblp2 also showed a reduced adherence to human epithelial cells (Fig. 9). Other surface proteins, known or hypothesized to be involved in biofilm formation, may interact and/or be assembled with BAP and BLPs on the cell surface. OmpA has a role in biofilm formation [13] and is highly conserved in the *A. baumannii* population. Interestingly, the protein varies significantly in sequence composition in most clones expressing type-3 BAP. SURP-1, a repetitive protein structurally related to BAP and BLPs, is missing in most type-3 BAP, as in many BLP1-negative clones. The uneven distribution of the various BAP types, OmpA variants, BLP1 and SURP-1 proteins among isolates is indicative of alternative combinations of surface proteins in the *A. baumannii* population. Consequently, the architecture of the biofilm formed by different isolates may greatly vary.

Conclusions

The construction of clones expressing BAP and BLPs isoforms in all possible combinations is important to clarify the role played by each protein in biofilm formation of *A. baumannii*, establish whether the proteins interact, assess underneath hierarchies. In parallel, mutational analyses may shed light on the functional organization of the repetitive regions of BAPs and BLPs, and establish how the combination of specific modules may influence secretion and surface exposure of each protein.

The assay of type-3 BAP derivatives may be crucial to assess whether the unicity of this protein, which seems mutually exclusive relative to other surface components, correlates with the particular combination of Ig-like modules in the repetitive region and/or their paucity, or resides in the COOH region.

Methods

In silico data

Tblastn searches of BAP and BAP-like proteins in *A. baumannii* and other *Acinetobacter* species WGS were carried at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Modules in BAP repetitive regions were marked according to the AB307-0294 BAP protein nomenclature ([15]; accession number EU117203).

Tandem repeats in BAP, BAP-like and other repetitive proteins were identified by using the XSTREAM (variable Sequence Tandem Repeats Extraction and Architecture Modeling, [38]) and the RADAR (Rapid Automatic Detection and Alignment of Repeats, [39]) algorithms. Searches are restricted to Whole Genome Shotgun (WGS) genomes deposited at GenBank before March 2014.

Construction of AYE-Δblp1 and AYE-Δblp2 mutants

The genes encoding the BLP1 and BLP2 proteins of the *A. baumannii* AYE strain [27] were mutagenized by allelic replacement, according to the published procedure [29]. Flanking fragments located immediately upstream and downstream from the AYE *A. baumannii* BLP1 and BLP2 genes were amplified by PCR and cloned into the suicide vector pMo130-TelR, which contains the tellurite-resistance marker and the sacBR genes conferring sucrose sensitivity [29]. Upstream and downstream BLP1 and BLP2 fragments were amplified using the pairs of BLP1 and BLP2 up and dw primers shown below:

BLP1up-fw: tatgcggccgcaacagcctgaagtgattgttgtg (*NotI* site underlined)
BLP1up-rv: gctggatccttaatatcaatttttgcgattaccttc (*BamHI* site underlined)
BLP1dw-fw: tatggatcctagacgaattactaaacaaccagca (*BamHI* site underlined)
BLP1dw-rv: tatgcatgcatcagctggtttagcaatagaacg (*SphI* site underlined)
BLP2up-fw: tatgcggccgcaaaggtgacattcaggcatc (*NotI* site underlined)
BLP2up-rv: gctggatccatcttgcagaacgtccaaac (*BamHI* site underlined)
BLP2dw-fw: tatggatcctccagttgtttagggtatttgag (*BamHI* site underlined)
BLP2dw-rv: tatgcatgctattggtaaacttgaactcaatgc (*SphI* site underlined)

PCR products were digested with *NotI-BamH*I (upstream fragments) and *BamHI–SphI* (downstream fragments), and cloned into *NotI-SphI* restricted pMo130-TelR DNA, to create pMo130-TelR-ΔBPL1 and pMo130-TelR-ΔBPL2, respectively.

Each pMo130-TelR derivative was introduced into *E. coli* S17-1 by transformation, and subsequently mobilized to the *A. baumannii* AYE strain via conjugation as described [29]. pMo130-TelR derivatives integrated into the AYE genome by homologous recombination (first crossovers) were selected on LB agar containing 30 μg/ml tellurite and 50 μg/ml ampicillin. Transconjugants were cultured in LB broth containing 10 % sucrose to select double recombinants, and serial dilutions were spread onto LB plates containing 10 % sucrose. Sucrose-resistant colonies were screened for tellurite sensitivity to monitor excision of the suicide vector. The inactivation of BLP1 and BLP2 genes in tellurite sensitive colonies was confirmed by PCR amplification, using the primers that annealed to DNA immediately flanking the deleted BLP1 and BLP2 gene regions shown below:

BLP1mut-fw: tgtagtagatccgaaggtaatcg
BLP1mut-rv: ccagataggtacagaagatgaagc
BLP2mut-fw: tttggacgttctgcaagatg
BLP2mut-rv: aattggcgcaatcctctatg

Control PCR assays were performed to rule out that merodiploids containing both the intact and the deleted gene had been selected, using the "wild-type" primers listed below:

BLP1wt-fw: cgaaggtaatcgcaaaaattg
BLP1wt-rv: atgtaatggacgaatgttgctc
BLP2wt-fw: ccagatgttccacaagctca
BLP2wt-rv: ccgcttcactggttaatggt

Biofilm assay

Experiments were carried out in TSB (Tryptic soy broth; [31]). Overnight bacterial cultures were diluted with TSB to concentration of 10^8 CFU/ml. Approximately 2×10^5 bacteria was added to cell culture plates containing glass coverslips and incubated in static conditions at 37 °C for 48 h. The coverslips were then washed 3 times with PBS to remove non-adherent bacteria. Biofilms were stained with LIVE/DEAD® BacLight™ Bacterial Viability kit for microscopy (Molecular Probes) as described prior [40]. The images were captured using LSM 710 inverted confocal laser-scanning microscope (Zeiss). Quantitation of mean biomass was determined using the IMARIS v7.0 software package [31]. Each experiment was performed in triplicate.

Cell adhesion assays

Adherence of *A. baumannii* strains to A549 cells (human type 2 pneumocytes) was determined as described previously [4], with minor modifications. In brief, ~ 10^5 A549 cells were infected with ~ 10^7 bacterial CFU and incubated for 60 min at 37 °C in 5 % CO2 (v/v) atmosphere. Non-

adherent bacterial cells were removed by washing with PBS. Infected cells were lysed by the addition of 1 ml distilled water and serial 10-fold dilutions were plated on LB agar to determine the number of CFU of adherent bacteria. To determine adherent and invading bacteria, A549 cells were infected with *A. baumannii* strains as described above. The monolayers were then treated with 1 ml of fresh culture medium containing 5 mg/L of colistin sulfate (Sigma-Aldrich, Milan, Italy) for further 30 min, the shortest time point that resulted in the killing of all extracellular bacteria added to the monolayers. Afterwards, the cells were washed with PBS, harvested with trypsin, and lysed with sterile distilled water. Dilutions from harvested samples were inoculated on LB agar plates and bacterial colony counts were estimated after overnight incubation at 37 °C. Each experiment was performed in triplicate.

Statistical analysis

Data were analysed using Statistical Package for the Social Sciences Version 13.0 (SPSS Inc., Chicago, IL, USA). Differences between mean values were tested for significance by performing either unpaired, two-tailed Student's t-tests or one-way ANOVA analysis followed by Tukey's multiple-comparison test, when appropriate. A P value <0.05 was considered to be statistically significant. Correlations were evaluated by regression analysis using the Pearson's correlation coefficient (r).

Additional files

Additional file 1: *A. baumannii* strains analyzed. (XLS 84 kb)

Additional file 2 A) ABCD BAP modules in AB307-0294 BAP. B) EGF region in type-1 and type-2 BAPs. C) Organization of type-3 BAP D) Z and Zb modules alignment. (DOC 65 kb)

Additional file 3: BAP, BLP1 and BLP2 in non-*baumannii Acinetobacter* strains. (XLSX 37 kb)

Additional file 4: Organization of type-4 (A) and type-5 BAPs (B). (DOC 42 kb)

Additional file 5: A) NH2 and COOH regions in *A. baumannii* BAP genes. B) solo G modules in BAPs. (DOC 52 kb)

Additional file 6: Alpha and beta-BAP genes regions in *A. baumannii* SDF, *A. calcoaceticus* PHEA-2, *A. baumannii* AYE and *A. baylyi* ADP1 strains. (PDF 39 kb)

Additional file 7: A) Organization of BLP1 B) Organization of BLP2 C) BLP2 types. (DOCX 156 kb)

Additional file 8: BLP1 empty chromosomal site A) DNA replacing BLP1 coding sequences in BLP1-negative strains. B) Alignment of BLP1 gene empty sites in *A. baumannii and* non-*baumannii* species. (DOCX 125 kb)

Additional file 9: Distribution of surface proteins in strains expressing different BAP types. (XLS 49 kb)

Additional file 10: OmpA sequence variants. Organization of SURP-1. (DOCX 117 kb)

Abbreviations

aa: Aminoacids; ACB: Acinetobacter calcoaceticus-Acinetobacter baumannii; AD: Alanine and aspartic acid; BAP: Biofilm associated protein; Big_3_4: Bacterial Ig-like domain, group 3; BLP: BAP-like protein; bp: Base pair; CFU: Colony forming unit; CLSM: Confocal laser scanning microscopy; dw: Downstream; Embp: Extracellular matrix-binding protein; FIVAR: Found in various architectures; GA: G-related albumin-binding; Ig-like: Immunoglobulin-like; kb: Kilobase; KEGG: Kyoto encyclopedia of genes and genomes; LB: Luria broth; Mb: Megabase; mg: Milligram; MLST: Multilocus sequence typing; mut: Mutant; OmpA: Outer membrane protein A; ORF: Open reading frame; PBS: Phosphate saline buffer; PCR: Polymerase chain reaction; PFAM: Protein families; RADAR: Rapid automatic detection and alignment of repeats; RTX: Repeat in toxin; SD: Serine and aspartic acid; SDR: SD repeats; ST: Sequence type; SURP-1: Surface repetitive protein 1; tBLASTn: Basic local alignment sequence tool using a protein as query against nucleotide 6-frame translation; TSB: Tryptic soy broth; up: Upstream; WGS: Whole genome shotguns; wt: Wild type; XSTREAM: Variable sequence tandem repeats extraction and architecture modeling.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EDG, MM and MDF obtained the BPL1 and BLP2 mutants, MM and ER analyzed biofilm formation of wt and mutant *A. baumannii* strains, MDF and RZ monitored clones STs and performed the adhesion assays of *A. baumannii* strains to A459 cells, PPDN performed in silico analyses, PPDN, EDG and RZ wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported in part by PON Ricerca e competitività 2007–2013 of the Agency of the Italian Ministry of University and Scientific Research (PON01_02589).

Received: 7 July 2015 Accepted: 23 October 2015
Published online: 14 November 2015

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Chapter 6

Concluding remarks

Concluding remarks

A. baumannii is a globally important nosocomial pathogen responsible for widespread and persistent outbreaks among hospitalized patients. *A. baumannii* had a clonal population structure dominated by three international clonal lineages I–III and a few additional epidemic lineages, including emerging genotype ST25. *A. baumannii* epidemic clonal lineages were selected because of extensive antibiotic resistence, in particular the majority of epidemic *A. baumanni* isolates (>90%) are carbapenem-resistant and express OXA-23 carbapenem-hydrolyzing class D (CHDL) oxacillinase. This extremely rapid development of antimicrobial resistance is due to the widespread use of antimicrobials in the hospital environment and to the ability of *A. baumannii* to respond rapidly to challenges issued by antimicrobials. The widespread administration of polymyxin antibiotics such as colistin in the hospital setting has exerted selective pressure for the development of resistance in *A. baumannii* clinical isolates. Colistin-resistant (colistin-R) *A. baumannii* strains showed activating mutations in the PmrB sensor kinase of PmrAB 2-component system. In addition to In addition to extensive antimicrobial resistance, *A. baumannii* strains responsible for epidemics show elevated resistance to desiccation, high biofilm-forming capacity on abiotic surfaces and adherence to host epithelial cells, virulence-related features which might have favored the spread and persistence in the hospital environment. Biofilm-forming capacity on abiotic surfaces and adherence to host epithelial cells is dependent on the expression of biofilm associated proteins BAB, BLP1 and BLP2.

List of pubblications

List of papers included in the thesis

Durante-Mangoni E, Del Franco M, Andini R, Bernardo M, Giannouli M, Zarrilli R. Emergence of colistin resistance without loss of fitness and virulence after prolonged colistin administration in a patient with extensively drug-resistant Acinetobacter baumannii. *Diagnostic Microbiology and Infectious Disease*. 2015; 82: 222-6.

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Additional papers not included in the thesis

Del Franco M, Paone L, Novati R, Giacomazzi CG, Bagattini M, Galotto C, Montanera PG, Triassi M, Zarrilli R. Molecular epidemiology of carbapenem resistant Enterobacteriaceae in Valle d'Aosta region, Italy, shows the emergence of KPC-2 producing Klebsiella pneumoniae clonal complex 101 (ST101 and ST1789). *BMC Microbiology*. 2015; 15: 260.

Acknowledgments

I would like to express my special appreciation and thanks to my tutor, Prof. Raffaele Zarrilli for guidance, encouraging, support and constructive criticism to my PhD work. My sincere thanks also goes to my colleagues and friends of the molecular biology lab, at the Department of Public Health of University Federico II of Napoli, Eliana Pia Esposito and Laura Paone who made my experience unique, better and fun. I want to thank Dr. Anna Di Popolo, Dr. Miriam Bagattini and Dr. Francesca Pennino their precious help and fruitful discussions over these three years. I would also like to thank all my beautiful friends for their support and encouragement over these years, especially Imma. I wish to express my deep gratitude to my special family for their continuous and unconditional support, starting from the beginning of my studies to the end of my PhD. A special thank to my husband Luca for his precious and lovely support, as for his inestimable encouragement to me and my work. Finally, a special thought for my son, who despite his age, he spurs me to give the best, surpassing my limitations in every experience that life puts me in front of.