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Patterns of Biofilm Structure and Formation Kinetics among *Acinetobacter baumannii* Clinical Isolates with Different Antibiotic Resistance Profiles

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Abstract

Acinetobacter baumannii is a ubiquitous organism that has been involved in a wide range of nosocomial infections. Its ability to produce biofilms, among other characteristics, allows it to persist in hospitals for prolonged periods. In this study, in order to check a possible relationship between the resistance to different antibiotics and the ability to form biofilms on inert surfaces, the rate of biofilm formation as well as siderophore production and detection of *OmpA* and *CsuE* by PCR were investigated for 12 *A. baumannii* clinical isolates. Biofilms were cultured at 37ºC on steel coupons immersed in BHI broth and attached viable cells were counted after 5, 24 and 48h. Confocal Laser Scanning Microscopy (CLSM) images were obtained for some of the strains that were noted to produce a brown pigment. Biofilm volume and substratum coverage were estimated with image analysis software. Our data, though preliminary, show that the quicker biofilm formers were strains susceptible to aminoglycosides, whereas the biofilms providing thicker and more uniform surface coverage were produced by carbapenem resistant strains, producing a brown pigment with a plausible siderophore role. Further investigation on a wider set of isolates could help better understand the relationship between biofilm formation and various clinical findings.

Key words: *Acinetobacter baumannii*, antibiotic resistance, biofilms, Spain

Introduction

Acinetobacter spp. are Gram-negative bacteria that have diverse roles that range from bioremediation agents and oil extraction aids to antibiotic-resistant nosocomial pathogens. They possess an outstanding chemical ability to degrade xenobiotic compounds, from alkanes to herbicides and even pharmaceuticals[1,2]. *A. baumannii* is known to easily acquire resistance to various antimicrobials, thanks to its wide array of natural resistance mechanisms, such as the production of efflux pumps, down-regulation of porins, production of degrading enzymes and/or modifying their target site of antibiotics[3,4]. This has converted this ubiquitous microorganism into one of the most concerning and dangerous nosocomial pathogens, contributing to ventilator associated pneumonia, bloodstream, burn, wound and catheter related infections. Very frequent antibiotic resistances are found in clinical isolates and mortality rates as high as 20-60% have been registered for associated infection cases[5-7].

The ability of clinical isolates of *A. baumannii* to produce biofilms has often been reported[8-10] as well as their involvement in a wide range of genetic expression changes. Regulation of the formation mechanisms, including quorum sensing ones, are progressively being characterized[11-13]. Biofilm formation is considered to provide bacteria with protection against many hazards, ranging from antimicrobial agents to macrophage attacks as well as stress

conditions such as desiccation. They assist in the development of antibiotic recalcitrance by different mechanisms[7,14-16]. They differ according to the antibiotic, nature and history of the bacteria forming the biofilm and various environmental factors. Biofilms not only provide diffusion limitations due to the charged macromolecular mesh of the matrix, but also act as a protective barrier for embedded cells. Sub-lethal exposure to antimicrobial agents is prone to induce resistance responses such as the production of degradation enzymes. In addition, dissemination of resistance plasmids is favored by the high rate of gene exchange operating at high cell densities. Moreover, slowly dividing cells at deep biofilm layers are scarcely susceptible targets for agents that hinder cell division. Besides, the high cell densities in biofilms give rise, under antimicrobial exposure, to very small subpopulations of persistor cells that survive antimicrobial exposure and will eventually repopulate the site.

In short, antibiotic resistance and biofilm formation depend both on genetic diversity and genetic expression. Understanding the connections among these phenomena has, as Badmasti *et al.*[17] recently pointed out, a threading potential to understand *A. baumannii* persistence in the hospital environment and its colonization of medical equipment. In the present study, 12 clinical isolates from a Spanish hospital have been compared in terms of their kinetics of biofilm formation and the structures formed on abiotic surfaces. Association between these features, antibiotic resistance profiles, presence of *CsuE* and *OmpA* genes, siderophore and pigment production have also been explored. Association between clinical findings and the ability to attach and form biofilms on abiotic surfaces is important from an infection control perspective where special care could be taken for strains with certain clinical characteristics so as to avoid the colonization of abiotic surfaces and persistence in the hospital of these isolates for prolonged periods of time.

Results and Discussion

Details of the materials and methods used in this study are available as supporting information.

Antibiotic susceptibility and characteristics of A. baumannii clinical strains

The twelve isolates here studied are clinical bloodstream isolates and were deliberately chosen for their heterogeneity in antibiotic susceptibility and clinical history. Their origin, according to the patient's unit assignment and/or treatment, is shown in Table 1. Five were isolated from the general Intensive Care Unit (ICU) and four from the Burn Unit. Strain 30 was obtained from Hematology/Oncology and strain 59 came from Internal Medicine. Strain 20 also came from Internal Medicine, but from a patient not previously admitted to the ICU. Two thirds of the isolates were sampled from patients previously exposed to mechanical ventilation and catheterization, both of which are regarded as risk factors for biofilm formation and subsequent *A. baumannii* infection[6]. 75% of the source patients, except those infected by isolates 9, 20, and 38, had undergone antibiotic treatment in the 15 days prior to strain isolation.

Table 1. Clinical data of the patients harboring the isolates. Risk factors that may contribute to biofilm formation ("MV= Mechanical Ventilation; CVC= Central Venous Catheterization, and UC= Urethral Catheterization), previous antibiotic treatment (15 days prior to isolation), primary infection prior to bacteremia, underlying disease and clinical outcome of the patient.

Strain	Risk Factors	Previous Antimicrobial Treatment	Disease	Primary infection	Patient Outcome
3	MV CVC UC	Carbapenems	2nd degree burns on 70% of body surface	Unknown	Recovered
9	None	None	Rapidly progressive glomerulonephritis	Urinary Tract Infection	Recovered
12	MV CVC UC	Carbapenems Colistin Linezolid	Flame burn on 26% of the body surface	Respiratory Infection	Died
15	MV CVC UC	Carbapenems Colistin Linezolid	Amputation of left leg	Respiratory Infection and Soft Tissue Infection	Recovered
20	None	None	Multifactorial chronic anemia Septic shock	Soft Tissue Infection	Recovered
26	MV CVC	Carbapenems Linezolid	Bilateral eosinophilic Pneumonia	Respiratory Infection	Recovered
30	None	Carbapenems Vancomycin	Severe combined immunodeficiency Bone marrow transplant	Unknown	Recovered
35	MV CVC UC	Carbapenem Colistin Linezolid	Flame burn on 70% of the body surface	Respiratory Infection	Died
38	MV CVC UC	None	Intracranial hematoma	Respiratory Infection	Recovered
45	MV CVC UC	Carbapenems Colistin Tigecycline	Non-Hodgkin lymphoma Allogeneic transplant	Respiratory Infection	Died
52	MV CVC UC	Carbapenems Colistin Vancomycin	Acute lymphoblastic leukemia Bone marrow transplant	Respiratory Infection	Died
59	None	Piperacillin/ Tazobactam	Crohn's Disease	Unknown	Recovered

According to AST criteria (Table 2) the only entirely antibiotic-susceptible strains were strains 38 and 59. The rest were resistant to β-lactams and quinolones. This was not surprising since *A. baumannii* is known to have a wide array of intrinsic resistance mechanisms (causing resistance to first and second generation cephalosporins and most third generation cephalosporins) and an outstanding ability to acquire resistance from the environment[5].

Table 2. Resistance of the *A. baumannii* strains to antimicrobials agents. "MIC" is the Minimum Inhibitory Concentration acquired in μg mL^{-1}. "R" stands for Resistant, "I" stands for Intermediate resistance, and "S" stands for Susceptible, as interpreted according to the CLSI guidelines. "TIC" stands for Ticarcillin, "PIP" for Piperacillin, "A/S" for Ampicillin/Sulbactam, "P/T" for Piperacillin/Tazobactam, "CTZ" for Ceftazidime, "CFP" for Cefepime, "IMI" for Imipenem, "MER" for Meropenem, "COL" for Colistin, "G" for Gentamycin, "TO" for Tobramycin, "AK" for Amikacin", "MIN" for Minocyclin, "CIP" for Ciprofloxacin, "LEV" for Levofloxacin, and "T/S" for Trimethoprim/Sulfamethoxazole".

Strain		Antimicrobial Agents														
		β-Lactams								Polymyxin	Aminoglycosides			Tetracyclin	Fluoroquinolones		Folic Acid Synthesis Inhibitors
		TIC	PIP	A/S	P/T	CTZ	CFP	IMI	MER	COL	G	TO	AK	MIN	CIP	LEV	T/S
3	MIC	>64	>64	16/8	>64/4	>32	16	>8	>8	≤0.5	≤1	≤1	≤2	2	>2	>4	>4/76
	S-I-R	R	R	I	R	R	I	R	R	S	S	S	S	S	R	R	R
9	MIC	>64	>64	16/8	>64/4	>32	32	>8	>8	≤0.5	>8	>8	>32	8	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	R	I	R	R	R
12	MIC	64	>64	4/2	>64/4	16	32	>8	>8	16	>8	>8	8	≤1	>2	>4	>4/76
	S-I-R	I	R	S	R	I	R	R	R	R	R	R	S	S	R	R	R
15	MIC	>64	>64	16/8	>64/4	>32	>32	>8	>8	≤0.5	>8	>8	16	≤1	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	S	S	R	R	R
20	MIC	>64	>64	16/8	>64/4	>32	>32	>8	>8	≤0.5	>8	>8	>32	8	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	R	I	R	R	R
26	MIC	32	>64	4/2	>64/4	>32	16	≤1	1	≤0.5	≤1	≤1	≤2	2	>2	>4	>4/76
	S-I-R	I	R	S	R	R	I	S	S	S	S	S	S	S	R	R	R
30	MIC	>64	>64	4	64/4	16	16	2	2	≤0.5	>8	8	4	≤1	>2	>4	>4/76
	S-I-R	R	R	S	I	I	I	S	S	S	R	I	S	S	R	R	R
35	MIC	>64	>64	>16/8	>64/4	>16	8	>8	8	≤0.5	>8	>8	≤2	≤1	>2	>4	>4/76
	S-I-R	R	R	R	R	R	I	R	I	S	R	R	S	S	R	R	R
38	MIC	≤8	≤8	≤2/1	≤4/2	4	2	≤1	≤0.25	≤0.5	≤1	>8	≤2	≤1	≤0.25	≤0.12	≤2/38
	S-I-R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
45	MIC	>64	>64	>16	>64/4	>32	8	>8	8	8	>8	8	≤2	2	>2	>4	>4/76
	S-I-R	R	R	R	R	R	I	R	I	R	R	I	S	S	R	R	R
52	MIC	>64	>64	>16/8	>64/4	>32	>32	>8	>8	≤0.5	4	≤1	≤2	≤1	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	S	S	S	S	R	R	R
59	MIC	≤8	≤8	≤2/1	≤4/2	2	2	≤1	1	≤0.5	≤1	≤1	≤2	≤1	≤0.25	≤0.12	≤2/38

	S-I-R															
	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Our data show that eight of the twelve strains were resistant to carbapenems. Protection mechanisms against these agents in *A. baumannii* strains is a global concern, since they tend to be the most commonly used ones to deal with recalcitrant strains. Carbapenem resistance has been also associated with increased mortality rates among infected individuals[18]. Strains 12 and 45 were also resistant to colistin. Use of this antibiotic has re-emerged in the clinical setting as an alternative to deal with carbapenem resistant *A. baumannii* isolates[19]. Resistance to both carbapenems and colistin leaves the clinician with very few options for therapy and may result in treatment failure. Moreover, previous patient treatment with carbapenems and colistin has been defined as a risk factor for ventilator associated pneumonia by Inchai *et al.*[20].

All strains tested here were still relatively susceptible to aminoglycosides and tetracyclins. The exceptions were strains 9 and 20 that were resistant to aminoglycosides and had an intermediate resistance to tetracyclins. This conclusion however was mainly based on susceptibility to amikacin, as strains 9, 12, 15, 30, 35 and 45 were resistant to gentamycin and tobramycin and strains 30 and 45 were moderately resistant to tobramycin. According to the definitions proposed by Magiorakos *et al.*[21], strains 9, 12, 15, 20, 35 and 45 would be classified as Extensively Drug Resistant (XDR) and strains 3, 26, 30 and 52 as Multi Drug Resistant (MDR); strains 38 and 59 were susceptible. As is shown further on, all tested strains, including XDR and MDR isolates, were found to produce biofilms, a significant aspect from a public health point of view, as it allows them to persist for long periods of time in hospital settings and give rise to repeated outbreaks.

Biofilm formation patterns of A. baumannii clinical strains

The isolates were tested for biofilm formation ability on abiotic surfaces, which is linked to the attachment densities of the cells on steel coupons. As shown in Figure 1, all of the strains were able to attach to stainless steel coupons at 37ºC, though their development kinetics differed. A quick formation pattern is presumably due to an earlier adherence to the substratum, acting as a bottleneck step for attached cell division and overall inert surface colonization. The speed of attachment and subsequent biofilm formation on abiotic surfaces reflect the bacterium's ability to quickly adhere unto surfaces in the hospital setting and start protecting itself through the formation of biofilms. This, in turn, leads to a longer persistence in the hospital and a possible source of repeated infections and outbreaks.

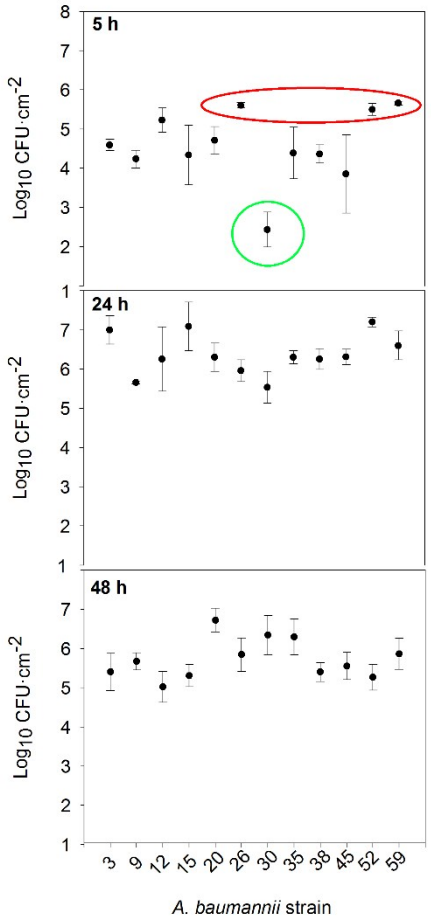

Figure 1. Attached cell density of each *A. baumannii* strain, after 5 h (A), 24h (B) and 48h (C) incubation. Dots represent the average of three independent experiments (n=6) where two coupons were sampled for each time point in each experiment, while the bars represent the standard deviation.

According to the density of attached cells per surface unit attained after 5 hours incubation (Fig. 1a), the strains could be classified into three different groups. Group 1 (in red) includes strains 26, 52 and 59: quick biofilm formers, reaching more than 5 Log CFU cm^{-2}. Strains 26 and 52 came from patients with a respiratory infection, who had stayed at the ICU and undergone mechanical ventilation and catheterization. Strain 59 was isolated from a Crohn's disease patient. These individuals tend to have an altered intestinal microbiota, allowing/selecting for strongly adherent microbial strains[22]. Group 1 strains were susceptible to all aminoglycosides; only strain 52 was resistant to carbapenems. Group 2 (in green) represented the slow biofilm forming mode; consisted only of strain 30, which attained just around 2 Log CFU cm^{-2} after 5h. This rather special strain was isolated from an oncology immunodeficient patient not exposed to catheterization; it was sensitive to carbapenems, resistant to gentamycin and moderately resistant to tobramycin. The rest of the strains, attaining intermediate attached cell densities after 5h, constituted Group 3. Except for strain 38, they were all resistant to carbapenems; strains 12 and 45 were besides resistant to colistin. Strains 9, 12, 15, 20, 35, and 45 were resistant to at least one aminoglycoside. All XDR isolates belonged to this group 3, with intermediate attachment densities.

The distribution of antibiotic susceptibility profiles and biofilm forming patterns suggests some association between good susceptibility to aminoglycosides and rapid biofilm formation, as in the case of our group 1. Hoffman *et al.*[23] reported that sub-inhibitory concentrations of aminoglycoside antibiotics induced biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli*. This induction was found to be inhibited by GTP. These authors postulated that

biofilm formation could be a specific defensive reaction to the presence of antibiotics, whose molecular basis are linked to alterations in the level of c-di-GMP. Rodriguez-Baño *et al.*[8] observed that previous aminoglycoside use was associated with *A. baumannii* biofilm-forming isolates, whereas treatment in an intensive care unit, ciprofloxacin resistance and isolation from a respiratory sample were associated with non-biofilm forming isolates. He *et al.*[24] have described biofilm formation being induced by levofloxacin and a correlation between biofilm induction and an upregulation of the transcription of the gene encoding the adeG efflux pump; they suggest a link between low dose antimicrobial therapy and a high risk of infection caused by biofilm forming *A. baumannii* strains. The fact that XDR strains showed intermediate rates of biofilm formation (our group 3) may correspond to an energy allocation tradeoff between taking advantage of the biofilms' physical protection and expressing antibiotic resistance mechanisms.

After 24h incubation (Fig. 1b) most of the strains had similar counts of viable biofilm forming cells and beyond 48h (Fig. 1c) dispersal caused a general decrease in cell numbers. For only two out of the twelve tested strains (strains 20 and 30), attached cell densities continued to increase between 24 to 48h, either as a result of a slow biofilm development pattern, or a less active dispersal mechanism.

Biofilm formation on abiotic surfaces has been previously tested in clinical *A. baumannii* strains[9,25] and rather variable scores after 24h were observed. De Breij *et al.*[26] also reported variable biofilm formation abilities in a collection of *A. baumannii* strains. Rao *et al.*[27] and Gurung *et al.*[28] found a positive relationship between antibiotic resistance and biofilm formation ability. Orsinger-Jacobsen *et al.*[29] described biofilm formation on steel for 13 strains of this organism; they also observed wide variation in cell densities and rather scarce matrix formation, as seen by Scanning Electron Microscopy (SEM). The variations these authors observed could be due to quantitative differences in the expression of specific biofilm phenotype genes or in Quorum Sensing (QS) mechanisms involved in their regulation, as it may also be in our case.

In our study, the presence of two biofilm formation-related genes was checked in the isolates. One was Outer Membrane Protein A (*OmpA*), considered to be a virulence factor in this species and required for the production of robust biofilms on abiotic surfaces and for attachment to epithelial cells[11]. All tested strains in this study were positive for the gene encoding OmpA.

The other one was the *CsuE* gene, coding for a protein included in an assembly system of the pili, usually involved in biofilm formation and cellular attachment[30]. All tested strains except strains 12 and 59 had the *CsuE* gene. The absence of the *CsuE* gene in strain 59, coming from the Crohn's disease patient, may imply an alternative, though rather effective mechanism, to either build the pili, or do without them; we should note that this strain belonged to the fast-biofilm forming group. Gaddy *et al.*[11] reported that even though the CsuE protein seems essential for the production of pili and the formation of biofilms, some *A. baumannii* strains without this gene were still able to form biofilms on abiotic surfaces when cultured on certain chemically controlled media. De Breij *et al.*[31] also showed that certain *A. baumannii* strains not producing pili through the pathway involving the CsuE protein, were still able to attach to human epithelial cells.

Pigment production and biofilm structure of A. baumannii clinical strains

Strains 3, 26, 45 and 52 were noted to produce a brown pigment when cultured in BHI broth while the rest presented a neutral hue. Pigment production was thus not related to the speed of biofilm formation. Colors displayed by biofilms of the pigmented strain 52, belonging to group 1, the quick biofilm forming group, and the non-pigmented strain 38 (in group 3, with intermediate biofilm formation speed) are shown in Figure 2. To check whether pigment production had any relationship with biofilm features, Confocal Laser Scanning Microscopy (CLSM) images of 24h biofilms of two pigment producing strains (52 and 45) and two non-pigmented strains (30 and 38) were analyzed, to estimate substratum surface coverage and biofilm thickness and volume (Table 3).

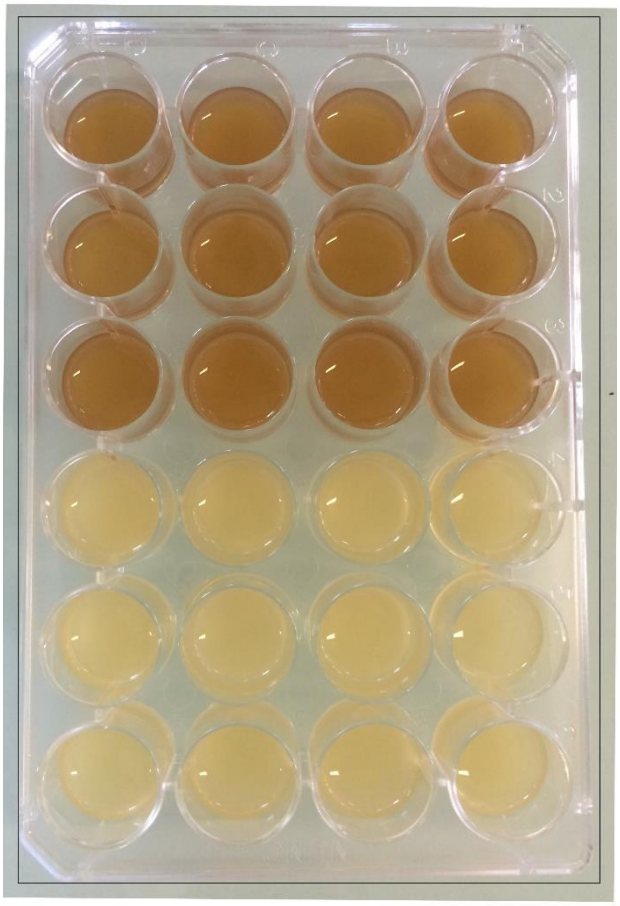

Figure 2. Pigment production during incubation of strain 52 (above) and 38 (below).

Table 3. Structural parameters of *Acinetobacter baumannii* biofilms (n=2).

***A. baumannii* strain**	**Biofilm thickness (µm)**	**Biovolume (μm^3)**	**Biovolume distribution (%)**	
			Cells	**Matrix**
38	6	$1.6*10^3$	90	10
30	2	$3.3*10^3$	51	49
45	9	$112*10^3$	83	17
52	9	$12.3*10^3$	73	27

Pigmented strains developed thicker biofilms than non-pigmented ones and managed to cover almost the whole surface of the available substratum, showing, at least apparently, better colonization of steel coupons (Fig. 3). The biofilms of strain 45, an XDR organism and intermediate rate biofilm former, attained the highest biovolume value after 24h. How much of the extensive antibiotic resistance exhibited by this strain could be due to the protection provided by its dense biofilms, is hard to say at this point. The biofilm could have been forming while resistance was being assayed, and besides, possible previous exposure of each strain to low antibiotic concentrations was not recorded. Strain 52, a MDR organism and quick biofilm former (Fig. 1) also developed relatively thick biofilms (Table 3). Both pigmented strains 45 and 52,

which gave rise to relatively homogeneous biofilms with wide surface coverage (Fig. 3), happened to be resistant to imipenem and meropenem (Table 2).

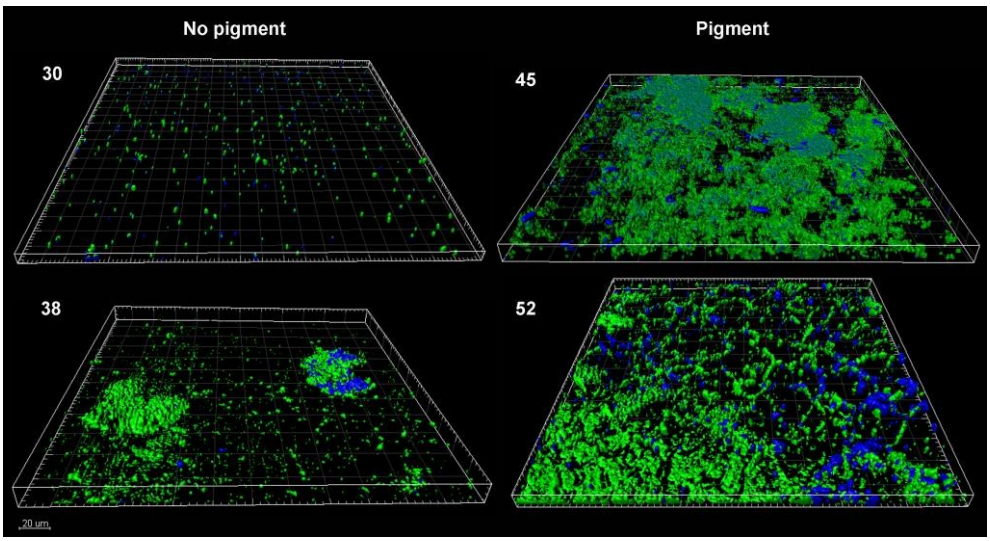

Figure 3. CLSM images (zenital view) of 24h-biofilms formed by *A. baumannii* strains pigment (+): 45 and 52 and pigment (-): 30 and 38. Cells were in green and EPS in blue.

On the other hand, the non-pigmented strains, which were sensitive to those β-lactam antibiotics, gave rise to small volume biofilms which did not homogeneously cover the substratum surface. Strain 38, a medium rate biofilm former, fully sensitive to all the antibiotics tested here, produced a heterogeneous biofilm mostly consisting of scattered colonies unable to cover the whole surface (Fig. 3). This sort of pattern usually develops from few and separate adhered cells, which later divide at a relatively fast rate, giving rise to cell stacks. Strain 30, the MDR slow biofilm former (single member of Group 2) produced a light and thin homogeneous coating (Fig. 3) with a higher proportion of matrix to cells than the others (Table 3).

Vilacoba *et al.*[32] reported an outbreak caused by an XDR indigo-pigmented *A. baumannii* strain isolated at an acute care hospital unit in Argentina. In that case, there was a link between pigment formation and virulence and/or antibiotic resistance. The specific nature or mechanism of action of the brown pigment observed in this study is not yet established, but one could think it might be a siderophore. There is a well described need for iron in biofilm development in general[33], and in this species in particular[34]. Iron acquisition systems of *A. baumannii* have been reviewed by Mortensen *et al.*[35] and McConnell *et al.*[6]. When all of our isolates were screened for unspecific siderophore production, all turned up to be positive, except for strain 30, the strain which showed very feeble biofilm production. Thus, the features of the pigment producing strains are compatible with a siderophore role for the brown pigment, but further characterization work is still needed.

Conclusions

Though the number of studied isolates does not allow for more than preliminary conclusions, the tested *A. baumannii* strains showed rates of biofilm formation that could be grouped into fast, moderate, and slow biofilm forming groups on steel coupons after 5 hours of growth. The fast biofilm forming group seems to be associated with good susceptibility to aminoglycosides. Under CLSM, the pigmented strains that produced more homogenous and voluminous biofilms were resistant to carbapenems, suggesting an interplay between the density of the formed biofilms and resistance to this class of antibiotics. This also highlights the importance of biofilms in MDR strains that could lead to their persistence in the hospital for prolonged periods of time. No association between a brown pigment noted for certain strains and siderophore production was found. It was however observed that pigmented strains produce a

more voluminous and homogeneous biofilm. All the tested strains were positive for *OmpA*. One fast biofilm forming strain, in addition to one with a moderate rate of biofilm formation, lacked *CsuE*. Further investigation into the mechanisms of biofilm formation for these strains could be of interest. Finally, investigation of a broader set of clinical isolates may shed a clearer light on the interplay between the various clinical and laboratory findings and biofilm formation patterns.

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Supporting Information

Materials and Methods

1. Bacterial strains

Acinetobacter baumannii strains were isolated at the "Hospital Universitario La Paz", Madrid (Spain) from bloodstream infections in patients with different conditions and primary sites of infection between 2009 and 2013. *Pseudomonas fluorescens* B52 that was originally isolated from cold bulk raw milk[1] was used as a control strain.

The Bactec™ (Becton Dickinson, Franklin Lakes, NJ, US) or the BacT/Alert (bioMérieux, Marcy l'Etoile, France) automated systems were used in order to process blood cultures. The isolates were identified using the Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH) as previously described[2]. Briefly, blood cultures that were flagged as positive for bacterial growth were centrifuged at 140 × g for 5 minutes. The supernatant was then centrifuged at 16,000 × g for 10 minutes in order to harvest the bacterial cells. The pellet was washed with 1 mL deionized water and a solution containing 300 µL water and 900 µL absolute ethanol was added. The mixture was centrifuged at 29,000 × g for 2 minutes and the supernatant was discarded. 20 µL of 70% (v/v) formic acid was added to the pellet and the solution was mixed vigorously. Then, 20 µL of acetonitrile was added to the resultant mixture and the solution was mixed again and centrifuged at 29,000 × g for 1 minute. 1 µL of the supernatant was transferred unto a steel target plate (Bruker Daltonik GmbH, Germany) and gently mixed with 1 µL of α-cyano-4-hydroxy-cinnamic acid matrix solution in an organic solvent containing 50% acetonitrile and 2.5% trifluoroacetic acid. The plates were allowed to air dry and mass spectra were obtained using a Microflex LT Mass Spectrometer (Burker Daltonik, GmbH). The spectra were compared to reference libraries provided by the manufacturer (Reference library 3.0.10) using the MALDI-BIOTYPER 2.0 software (Bruker Daltonik, GmbH).

The strains were stored at -20°C in Tryptone Soy Broth (TSB, Oxoid) supplemented with 15% glycerol until used. Pre-inocula were obtained after overnight incubation in Brain Heart Infusion broth (BHI, Oxoid) at 37ºC. Cells were harvested by centrifugation at 4000 × g for 10 min and washed twice in sterile BHI; their OD_{600} was adjusted to obtain 10^3 CFU mL^{-1} of each strain after inoculation.

2. Biofilm experimental system

Biofilms were cultured in BHI broth at 37ºC on disposable 24-well microtiter plates (Thermo Fisher Scientific) holding 10x10mm 304 stainless steel (SS) coupons as substratum surfaces. Before use, coupons were gently swabbed with a postsurgical toothbrush and soap solution, rinsed with distilled water, placed in a glass Petri dish and autoclaved. In each well, one sterile coupon was immersed into 1 mL of the corresponding bacterial suspension. In order to prevent evaporation, the whole system was wrapped in aluminum foil during incubation and a tray filled with water was placed under the microplate. In this system, only the upper side of the coupon was considered for the quantification of attached biofilm forming cells whereas the lower side was marked so that it would remain downwards all along the assay.

3. Cell Recovery and Counting

For cell recovery and counting, the surface of the steel coupon was scraped repeatedly in several directions in order to remove as much of the attached cells as possible at 5, 12, and 24 hours after incubation. Those cells were transferred into a tube containing 1.5 mL peptone water

and vigorously stirred using a vortex to break up cell aggregates; later, they were serially diluted in peptone water and plated on Tryptone Soy Agar (TSA, Oxoid). Counting of viable cells was performed after 24h incubation of the TSA plates at 37ºC. Two coupons for each time point were taken per strain and the entire experiment was repeated independently three times.

4. Siderophore determination in CAS solution

4.1 Bacterial growth in liquid media

In order to detect siderophore production, the 12 *A. baumannii* clinical isolates and *P. fluorescens* strain B52 (used as positive control) were cultured in an iron free mineral medium (PMS_7-Ca) that contains (per liter): N,N-bis-(2-hydroxymetyl)-2-aminoethanesulfonic acid (BES) (10.7 g), sodium pyruvate (11.00 g), dibasic potassium phosphate (0.86 g), ammonium chloride (0.65 g), and magnesium sulphate (0.20 g); the solution was adjusted to pH 7.0 and after autoclaving, supplemented with 0.111 g L^{-1} of filter-sterilized calcium chloride. Cells were harvested by centrifugation at 4000×g for 10 min, washed twice with the same medium and diluted with it in order to reach an initial concentration of 10^3 CFU mL^{-1} after inoculation. The concentrations were adjusted using a spectrophotometer. Cultures were carried out for 24h, at 37ºC for *A. baumannii* strains, and at 21ºC for *P. fluorescens* B52.

4.2. Siderophore detection assay

For siderophore detection, the Chrome Azurol S (CAS) assay initially described by Schwyn and Neilands[3] was used. In order to obtain CAS solution as described by Louden *et al.*[4], three solutions were prepared. 0.06 g of CAS (Sigma Aldrich) were dissolved in 50 mL ultra-pure water (solution 1); 0.0027 g of $FeCl_3$-$6H_2O$ were dissolved into 10 mM HCl (solution 2); 0.073 g of hexadecyl trimethyl ammonium bromide (HDTMA) were dissolved in 40 mL ultra-pure water (solution 3). Solution 1 was mixed with 9 ml of solution 2 and then mixed with solution 3. The final mixture had an intense blue color. This liquid was stored in a plastic container and protected from light until used. For siderophore detection, 1 mL of cell-free supernatant from the tested strain culture was mixed with 1 mL of the CAS solution. A negative control was prepared with the same volume of PMS_7-Ca medium instead of the culture's supernatant. *P. fluorescens* B52's supernatant was used as a positive control for siderophore production. A blue to green change of color was indicative of the presence of siderophores in the supernatant.

5. Antibiotic Susceptibility Testing

The Minimum Inhibitory Concentrations (MICs) were determined through the broth microdilution method using the automated Vitek2 system, (bioMérieux, Marcy l'Etoile, France) with AST-N-245 cards, according to contemporary Clinical and Laboratory Standards Institute (CLSI) standards. The antimicrobial agents included in the AST-N-245 cards, with the concentration ranges tested (expressed in parenthesis in μg mL^{-1}), were: ticarcillin (4-128), piperacillin (4-128), ampicillin/sulbactam (2/2-32/16), piperacillin/tazobactam (4/4-128/4), ceftazidime (1-64), cefepime (1-64), imipenem (0.25-16), meropenem (0.25-16), colistin (0.5-16), gentamicin (1-16), tobramycin (1-16), amikacin (2-64), minocycline (1-16), ciprofloxacin (0.25-4), levofloxacin (0.12-8), and trimethoprim/sulfamethoxazole (1/19-16/304). The concentration ranges standardized by the CLSI (document M100-S23) were used for the determination of whether a strain is resistant, susceptible, or has an intermediate resistance to the tested antibiotics[5]. The results were reported as "R" if the strain had an MIC value higher than the cutoff value for resistance, "I" if it the MIC was between the cutoff values of resistance and susceptibility, and "S" if the MIC value was below the cutoff value for susceptibility for each antibiotic (Table 2).

6. Polymerase Chain Reaction

DNA was extracted from the tested strains using a kit and according to manufacturer's instructions (Qiagen, Netherlands). Polymerase Chain Reaction (PCR) for the tested strains was performed in order to detect the presence of the *OmpA* and *CsuE* genes. The master mix contained 1X PCR Buffer with 1.5 mM $MgCl_2$, 12.5 pmol of each primer, 200 µM of each dNTP, and 1 U of Taq polymerase. The primers used for the *OmpA* gene were: F-5'-CAATTGTTATCTCTGGAG-3' and R-5'-ACCTTGAGTAGACAAACGA-3'. The primers for the *CsuE* gene were F-5'-ATGCATGTTCTCTGGACTGATGTTGAC-3' and R-5'-CGACTTGTACCGTGACCG TATCTTGATAAG-3'. PCR conditions were 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 50°C and 65°C for *OmpA* and *CsuE* respectively for 45 seconds, 72°C for all for 1 minute and a final extension step at 72°C for 5 minutes[6]. PCR products were then run on 1.5% agarose gels and visualized using a gel documentation system (BioRad, Germany).

7. Confocal Laser Scanning Microscopy (CLSM)

For CLSM observations, biofilms developed on SS coupons were rinsed with sterile 0.9% NaCl and then stained with Syto 13 (S7575, Life Technologies), which generally labels all the bacteria present, and Calcofluor White (18909, Fluka), a non-specific fluorochrome that binds to cellulose, chitin, and other polysaccharides commonly present in the biofilm matrix. Hence, the green color observed in CLSM corresponds to bacterial cells, whereas blue corresponds to Extracellular Polymeric Substances (EPS). CLSM images of various regions of the coupons (0.12 x 0.12 mm) were obtained using a Fluoview® FV 1200 Laser Scanning Confocal Microscope (Olympus) with an oil immersion 60X objective lens. Three-dimensional projections (Maximun Intensity Projection, MIP) were reconstructed from z-stacks using IMARIS® 7.7 software (Bitplane AG, Zurich, Switzerland). To calculate biovolume figures using the MeasurementPro module of the above mentioned software, the whole image was segmented into channels that was analyzed to obtain the total volume occupied by cells (i.e. green) and EPS (i.e. blue).

8. Statistical Analysis

Three independent experiments for biofilm attachment on steel coupons were performed and two coupons sampled every time (in total, n=6). Results were analyzed using one-way ANOVA with STATGRAPHICS PLUS 5.0 software (Statistical Graphics Corporation, Rockville, Md., USA). To check if there were differences among strains in terms of biofilm formation ability, a multiple range test was performed. Mean comparisons were carried out to determine significant differences at a 95.0% confidence level ($p < 0.05$).

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Table of Contents Entry:

This study evaluates the rates of biofilm formation in light of different characteristics among twelve *A. baumannii* clinical isolates.