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 ($p < 0.05$).

References

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