Colistin-Resistant *Acinetobacter baumannii* Clinical Strains with Deficient Biofilm Formation

Konstantina Dafopoulou,a,b Basil Britto Xavier,b An Hotterbeekxb, Lore Janssens,b Christine Lammens,b Emmanuelle Dě,c Herman Goossens,b Athanasios Tsakis,a Surbhi Malhotra-Kumar,b Spyros Pournarasb

Department of Microbiology, National and Kapodistrian University of Athens, Athens, Greece; Laboratory of Medical Microbiology, Vaccine and Infectious Disease Institute, University of Antwerp, Antwerp, Belgium; CNRS UMR 6270, Polymers, Biopolymers and Surfaces Laboratory, University of Rouen, Rouen, France

In two pairs of clinical colistin-susceptible/colistin-resistant (Cst'/Cst) *Acinetobacter baumannii* strains, the Cst' strains showed significantly decreased biofilm formation in static and dynamic assays (*P* < 0.001) and lower relative fitness (*P* < 0.05) compared with those of the Cst' counterparts. The whole-genome sequencing comparison of strain pairs identified a mutation converting a stop codon to lysine (241K) in LpsB (involved in lipopolysaccharide [LPS] synthesis) in one Cst' strain and a frameshift mutation in CarO and the loss of a 47,969-bp element containing multiple genes associated with biofilm production in the other.

Colistin-resistant (Cst') *Acinetobacter baumannii* clinical isolates are increasingly recovered worldwide (1), which is causing major clinical concerns. Thus far, two primary colistin resistance mechanisms have been described in *A. baumannii*, (i) modification of the lipid A moiety of lipopolysaccharide (LPS) mediated by mutations and/or overexpression in the two-component regulatory system pmrAB and (ii) loss of LPS caused by either mutations or insertional inactivation of lipid A biosynthesis genes (2).

The development of colistin resistance in clinical and laboratory-derived Cst' *A. baumannii* due to changes in the PmrAB system has been correlated with impaired fitness and virulence (3, 4) and lower infectivity (5, 6). Also, reduced biofilm formation has been observed in laboratory-generated Cst' *A. baumannii* (7, 8).

We previously reported the characteristics of two pairs of colistin-susceptible (Cst'')/Cst' *A. baumannii* clinical strains (Ab248/Ab249 and Ab299/Ab347; colistin MICs of 0.5/128 and 0.5/32 μg/ml, respectively) sequentially obtained from two patients after prolonging colistin exposure (6). Briefly, compared to Cst' strains, Cst' strains harbored a single pmrB mutation (P233S for Ab249 and P170L for Ab347) and had significantly slower growth. Strains within each pair had identical pulsed-field gel electrophoresis (PFGE) profiles, and all were assigned to the international clone 2. Important differences in the antibiotic resistance phenotypes other than colistin were not observed (6). One Cst' strain underexpressed the CsuA/B and CsuC proteins, which are involved in biofilm formation (6). In the present follow-up study, we compared these strain pairs in terms of fitness, biofilm-forming ability, and whole-genome sequencing (WGS) focusing specifically on genes involved in virulence and biofilm formation.

In *in vitro* competition assays were performed in triplicate at 5 h and 20 h (4), and relative fitness was calculated as previously described (9). To study adhesion (6 h) and biofilm formation (24 h) under static conditions, the crystal violet method was used (10) with some modifications. Briefly, inoculum was prepared by adjusting exponential cultures grown in Luria-Bertani (LB) broth to a 0.5 McFarland standard, and this was followed by a 1:10 dilution in LB broth. Then, 200-μl aliquots (approximately 2 × 10^6 CFU) were loaded into a 96-well polystyrene microplate (8 replicates/strain) and were incubated for 6 h or 24 h at 37°C. The plates were washed 3 times with sterile phosphate-buffered saline (PBS), fixed with methanol, and stained with 0.2% crystal violet. The biofilm was quantified by eluting the dye in 33% acetic acid, and then the optical density at 620 nm (OD_{620}) was measured using a Multiskan FC photometer (Thermo Fisher Scientific, Bremen, Germany). Assays were performed in triplicate and at three independent time points. *A. baumannii* ATCC 19606 was utilized as a positive control and uninoculated wells as negative controls.

Initial adhesion or early biofilm formation under dynamic conditions was also determined using the BioFlux system (Fluxion Biosciences Inc., South San Francisco, CA) (10) with some modifications. Briefly, inocula containing approximately 10^6 CFU of each strain were loaded in a BioFlux plate (5 replicates/strain) and were allowed to attach for 30 min. The plate was incubated for 6 h at 37°C with a flow speed of 0.5 dyne/cm², and the biomass was stained by Live/Dead BacLight stain (Invitrogen, Life Technologies) and visualized by fluorescence microscopy (Observer Z1; Carl Zeiss Inc., Oberkochen, Germany). Three independent experiments were performed. Biofilms were quantified using the ZEN 2012 (Zeiss) and ImageJ (http://imagej.nih.gov/ij/) software, and fluorescence levels were recorded as integrated density (Int Den) on the total area of the channel.

In *in vitro* competition experiments within the two pairs showed a significant fitness burden in the Cst'’ strains (Fig. 1). The Cst' strain Ab249 showed an average fitness reduction of 17.0% at 5 h (relative fitness, 0.91; standard deviation [SD], 0.03; *P* = 0.001) and of 47.9% at 20 h (relative fitness, 0.70; SD, 0.06; *P* = 0.001) compared to the Cst’ competitor Ab248. Similarly, the Cst’ strain Ab347 exhibited an average fitness decrease of 20.7% at 5 h (relative fitness, 0.91; standard deviation [SD], 0.03; *P* = 0.001).
the ACICU strain that is available under the GenBank accession number CP000863. The obtained pseudochromosomes were independently assembled using SPAdes v3.1.0 (11), and scaffolds were aligned against the reference genome of the ACICU strain that is available under the GenBank accession number CP000863. The obtained pseudochromosomes were compared using Mauve v2.3.1 for genome rearrangements. Next, the two Cst’ strains were compared to their Cst counterparts as reference templates, and reference mapping and single nucleotide polymorphism (SNP) extractions were performed using the CLC Genomics Workbench v7.5.1 (CLC bio, Denmark) with default parameters. All changes identified by WGS were confirmed by PCR and Sanger sequencing.

Intrapair WGS comparisons identified the previously detected single amino acid change in the PmrB protein in the two Cst’ strains (6). Also, a mutation converting a stop codon to lysine (*241K) in LpsB, a highly conserved glycosyltransferase that is involved in the biosynthesis of the LPS core (12) and is potentially important for A. baumannii virulence and colistin resistance (13), was observed in the Cst’ strain Ab249. Interestingly, in the second Cst’ strain Ab347, we observed the loss of a 47,969-bp genomic region containing, among others, the genes mrkC, mrkD, modA, modB, modC, modD, and ppk, which have been previously associated with biofilm production in Enterobacteriaceae and in Pseudomonas strains (14–16). Of note is the removal of the genes mrkC (pilin) and mrkD (assembly chaperone), which are part of the chaperone-usher (CU) system assembling pili. It was previously shown that the disruption of such pili CU systems, like Csu or Fim, induce a severe decrease in biofilm formation in A. baumannii (17, 18). The loss of these genes and of the genomic element was confirmed by Sanger sequencing and by whole-genome mapping (data not shown) (19). Also, in the strain Ab347, a frameshift mutation (A19fs) was observed in the outer membrane protein CarO, which was previously implicated in biofilm formation (20). In concordance, proteomic data generated for strain Ab347 also showed significant (−28.34-fold change; P = 5.00 × 10⁻¹⁵) underexpression of CarO (6). The above genetic modifications identified in Ab347 may partly explain the almost complete absence of biofilm production observed in this strain.

Our study demonstrated that acquisition of colistin resistance by these two clinical A. baumannii strains was associated with a significant loss of biofilm forming capacity. To the best of our knowledge, there are no studies comparing the impact of colistin resistance on biofilm formation among clinical Cst’/Cst A. baumannii strains, and the only available surveys investigated laboratory-generated Cst’ mutants, which also produced less biofilm than their Cst counterparts (7, 8). Also, in vitro competition experiments showed that Cst’ A. baumannii strains demonstrated considerably lower relative fitness than their Cst’ ancestors. Ge-
A genome-wide analysis identified, in the two pairs, unique changes. As the changes observed in the Cst' strains were primarily localized in genes affecting the surface properties, changes in initial adhesion and therefore in biofilm formation capabilities may be expected. In addition, the decreased fitness of these strains measured in competitive growth experiments also raises the possibility of a reduced growth rate of the Cst' strains being the cause of the reduced biomass of these strains in the biofilm experiments. From a clinical perspective, reduced biofilm formation as well as fitness may affect the infectivity and actually facilitate treatment of infections caused by Cst' A. baumannii.

Overall, the findings of the current study support previous findings (6) regarding the reduced clinical invasiveness of Cst' strains. However, further research with a larger set of isolates is needed to fully elucidate the relationship between colistin resistance and biofilm formation and the underlying mechanisms responsible for these developments in clinical strains.

(As of this work was presented at the 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark, 25 to 28 April 2015, and at the 10th International Symposium on the Biology of Acinetobacter, Athens, Greece, 3 to 5 June 2015.)

ACKNOWLEDGMENTS

K.D. was supported by a grant from the Federation of European Microbiological Societies (FEMS Research Fellowship). B.B.X. is supported by University of Antwerp Research Funds (BOF-DOCPRO 2012-27450). We declare no conflict of interest.

FUNDING INFORMATION

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors and was supported by internal funding.

REFERENCES


