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

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In two pairs of clinical colistin-susceptible/colistin-resistant (Cst^s/Cst^r) *Acinetobacter baumannii* strains, the Cst^r 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^s 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^r 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^r) *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^r *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^r *A. baumannii* (7, 8).

We previously reported the characteristics of two pairs of colistin-susceptible (Cst^s)/Cst^r *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 prolonged colistin exposure (6). Briefly, compared to Cst^s strains, Cst^r 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^r 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 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), fixated with methanol, and stained with 0.2% crystal violet. The biomass 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. baumanni* 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/cm^2, and the biomass was stained by Live/Dead *Bac*Light stain (Invitrogen, Life Technologies) and was 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 vitro competition experiments within the two pairs showed a significant fitness burden in the Cst^r strains (Fig. 1). The Cst^r 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^s competitor Ab248. Similarly, the Cst^r strain Ab347 exhibited an average fitness decrease of 20.7% at 5 h (rela-

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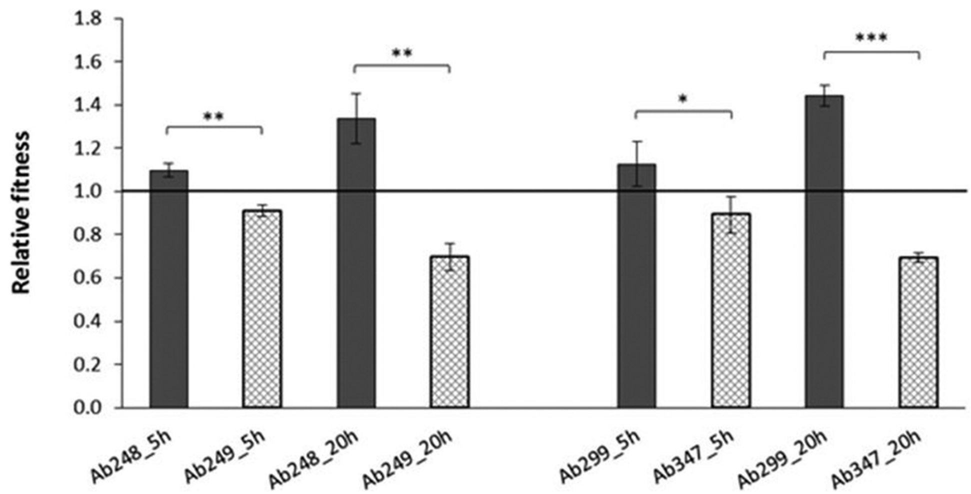

FIG 1 Relative fitness of Cst[s]/Cst[r] *A. baumannii* strains. Values represent the means ± SD from three independent experiments performed at 5 and 20 h. Fitness values below 1 denote fitness cost, while those above 1 denote a fitness benefit. Asterisks indicate statistically significant differences between strains (*, $P < 0.05$; **, $P = 0.001$; ***, $P < 0.001$).

tive fitness, 0.89; SD, 0.08; $P = 0.038$) and of 51.9% at 20 h (relative fitness, 0.69; SD, 0.02; $P < 0.001$) relative to the Cst[s] strain Ab299.

The two Cst[r] *A. baumannii* strains showed significantly lower biofilm production in the 24-h static assay than Cst[s] strains. In particular, the Cst[r] strain Ab249 produced 55.1% less biomass than its Cst[s] counterpart Ab248 (Ab249, $OD_{620} = 0.060$ and SD = 0.023; Ab248, $OD_{620} = 0.134$ and SD = 0.05; $P < 0.001$) (Fig. 2B). The Cst[r] strain Ab347 showed a 96.4% reduction relative to Cst[s] strain Ab299 (Ab347, OD_{620} = 0.002 and SD = 0.005; Ab299, $OD_{620} = 0.068$ and SD = 0.035; $P < 0.001$) (Fig. 2B). Our findings for these clinical strains support previous studies, which showed that laboratory-generated Cst[r] *A. baumannii* strains were weaker biofilm producers than their Cst[s] counterparts (7, 8). We also utilized a 6-h static assay protocol to test for differences in the initial adhesion capacity of the strain pairs; Ab248 did show higher biomass than that of Ab249 ($P < 0.001$), while the Ab299/Ab347 pair showed a visible growth delay compared to the growth of the control strain (ATCC 19606); the two strains essentially did not form biofilms (Fig. 2A). In the more sensitive 6-h dynamic assay, the Cst[r] strain Ab249 had 43.6% lower surface coverage than its Cst[s] counterpart Ab248 (Ab249, IntDen = 6.11E + 07 and SD = 1.48E + 07; Ab248, IntDen = 1.08E + 08 and SD = 7.51E + 06; $P < 0.001$). The Cst[r] strain Ab347 demonstrated 89.2% less surface coverage ($P < 0.001$) than Ab299 (Ab347, IntDen = 7.36E + 06 and SD = 5.35E + 06; Ab299, IntDen = 6.78E + 07 and SD = 1.50E + 07; $P < 0.001$) (Fig. 2C and D).

WGS was performed using 2 × 150 b paired-end sequencing (Nextera XT sample preparation kit and MiSeq; Illumina). Sequences 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[r] strains were compared to their Cst[s] 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 each of the two Cst[r] 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[r] strain Ab249. Interestingly, in the second Cst[r] 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 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; $P = 5.00 \times 10^{-15}$) 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[s]/Cst[r] *A. baumannii* strains, and the only available surveys investigated laboratory-generated Cst[r] mutants, which also produced less biofilm than their Cst[s] counterparts (7, 8). Also, *in vitro* competition experiments showed that Cst[r] *A. baumannii* strains demonstrated considerably lower relative fitness than their Cst[s] ancestors. Ge-

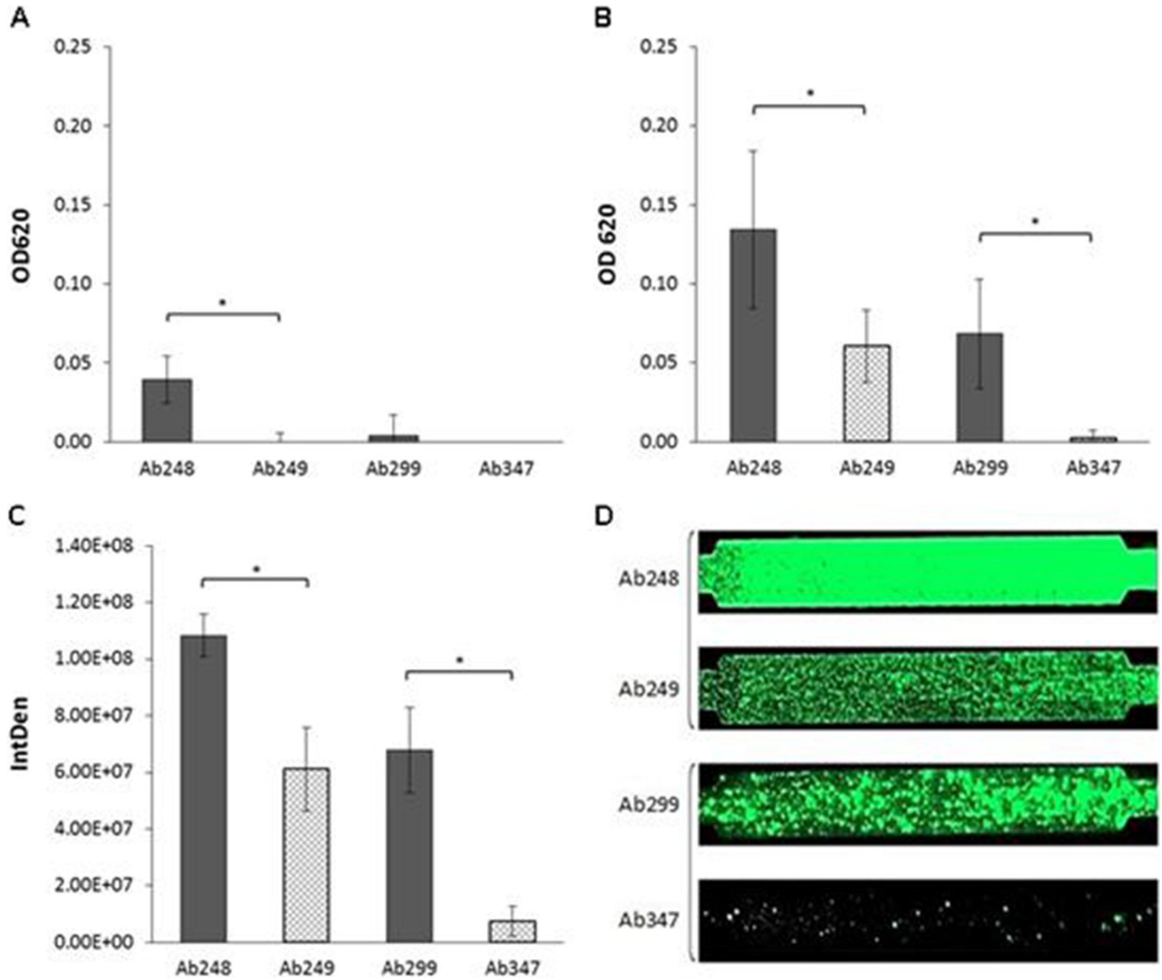

FIG 2 Adhesion and biofilm formation by Cst^s/Cst^r *A. baumannii* strains at 6 h (A) and 24 h (B) in a static model. Results represent the mean values ± SD from three independent experiments (OD, optical density). Asterisks indicate statistically significant differences between strains (*, $P < 0.001$). (C) Biofilm formation in a dynamic model; results represent the mean values ± SD from three independent experiments (IntDen, integrated density). Asterisks indicate statistically significant differences between strains (*, $P < 0.001$). (D) Fluorescence microscopy images of BioFlux channels.

nome-wide analysis identified, in the two pairs, unique changes. As the changes observed in the Cst^r 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^r strains being the cause of the reduced biomass of these stains 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^r *A. baumannii*.

Overall, the findings of the current study support previous findings (6) regarding the reduced clinical invasiveness of Cst^r 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.

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