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Growth Retardation, Reduced Invasiveness, and Impaired Colistin-Mediated Cell Death Associated with Colistin Resistance Development in *Acinetobacter baumannii*

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Two colistin-susceptible/colistin-resistant (Col^s/Col^r) pairs of *Acinetobacter baumannii* strains assigned to international clone 2, which is prevalent worldwide, were sequentially recovered from two patients after prolonged colistin administration. Compared with the respective Col^s isolates (Ab248 and Ab299, both having a colistin MIC of 0.5 μg/ml), both Col^r isolates (Ab249 and Ab347, with colistin MICs of 128 and 32 μg/ml, respectively) significantly overexpressed *pmrCAB* genes, had single-amino-acid shifts in the PmrB protein, and exhibited significantly slower growth. The Col^r isolate Ab347, tested by proteomic analysis in comparison with its Col^s counterpart Ab299, underexpressed the proteins CsuA/B and C from the *csu* operon (which is necessary for biofilm formation). This isolate also underexpressed aconitase B and different enzymes involved in the oxidative stress response (KatE catalase, superoxide dismutase, and alkyl hydroperoxide reductase), suggesting a reduced response to reactive oxygen species (ROS) and, consequently, impaired colistin-mediated cell death through hydroxyl radical production. Col^s isolates that were indistinguishable by macrorestriction analysis from Ab299 caused six sequential bloodstream infections, and isolates indistinguishable from Ab248 caused severe soft tissue infection, while Col^r isolates indistinguishable from Ab347 and Ab249 were mainly colonizers. In particular, a Col^s isolate identical to Ab299 was still invading the bloodstream 90 days after the colonization of this patient by Col^r isolates. These observations indicate considerably lower invasiveness of *A. baumannii* clinical isolates following the development of colistin resistance.

Acinetobacter baumannii has been an important nosocomial pathogen for the past 30 years, being frequently implicated in ventilator-associated pneumonia and bloodstream, urinary tract, and soft tissue infections (1). The propensity to develop antibiotic resistance makes *A. baumannii* a difficult-to-treat pathogen (2).

As severe infections caused by multidrug-resistant *A. baumannii* clinical isolates are increasing worldwide, colistin often constitutes the only active treatment alternative (3). Colistin is rapidly bactericidal for Gram-negative bacteria, affecting the lipid A moiety of lipopolysaccharide (LPS) and thus disorganizing the outer membrane (4). During the last few years, clinical isolates of *A. baumannii* expressing resistance to colistin have emerged, and outbreaks have been reported (5). The exact mechanism of resistance to colistin still needs to be elucidated, although two unlinked hypotheses have been expressed, involving (i) mutations and overexpression of PmrCAB proteins, leading to LPS modifications (phosphoethanolamine addition on lipid A), or (ii) complete loss of LPS production through inactivation of a lipid A biosynthesis gene (5).

The emergence of colistin resistance has been correlated with the selective pressure exerted by prolonged exposure to this drug (6, 7). There are also preliminary observations in laboratory-derived strains that colistin-resistant *A. baumannii* may exhibit impaired virulence and *in vivo* fitness (7). To investigate this hypothesis and also the mechanisms that confer colistin resistance in *A. baumannii*, we characterized two colistin-susceptible/colistin-resistant (Col^s/Col^r) pairs of *A. baumannii* clinical isolates, with each pair to be recovered from the same patient and to include isolates with identical macrorestriction patterns. We report here that compared with the respective Col^s isolates, the Col^r isolates may exhibit significant growth retardation, impaired virulence, and also considerably lower clinical invasiveness.

MATERIALS AND METHODS

Study isolates and susceptibility testing. The study included two Col^s/Col^r pairs of *A. baumannii* isolates that were recovered consecutively from two intensive-care unit (ICU) patients, as well as the Col^s *A. baumannii* strain ATCC 19606 as a control. The isolates were provisionally identified as belonging to the *A. baumannii* complex by API 20NE (bioMérieux, Marcy l'Etoile, France) and were all identified as *A. baumannii* by positive PCR/sequencing that revealed the carriage of a $bla_{\text{OXA-51-like}}$ variant gene. Susceptibilities to β-lactams, co-trimoxazole, rifampin, aminoglycosides, and quinolones were determined by Etest (bioMérieux). Colistin MICs were initially determined by Etest and subsequently determined by broth macrodilution (8) using glass tubes (tube dilution [TDS]), which was recently shown to exhibit excellent performance for colistin MIC testing of multidrug-resistant *A. baumannii* isolates (9).

Typing assays. The genetic relationship of all Col^s/Col^r isolates that were consecutively recovered from the study patients during the study period was tested by pulsed-field gel electrophoresis (PFGE) of ApaI-digested genomic DNA (10); the banding patterns were compared visually using previously proposed criteria (11). The first Col^s/Col^r isolations of

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TABLE 1 Characteristics of the *A. baumannii* study isolates

Isolate	Date of isolation (mo/day/yr)	Specimen	Colistin TDS MIC (μg/ml)/susceptibility status[a]	bla_{OXA} genes	PFGE type	ST	*pmrB* genotype
Ab248	8/3/2008	Pus	0.5/S	bla_{OXA-66}, bla_{OXA-58}	Ia	2	Wild type
Ab249	8/26/2008	Pus	128/R	bla_{OXA-66}, bla_{OXA-58}	Ia	2	P233S
Ab299	10/17/2008	Bronchial secretion	0.5/S	bla_{OXA-66}, bla_{OXA-58}	Ib	2	Wild type
Ab347	1/30/2009	Bronchial secretion	32/R	bla_{OXA-66}, bla_{OXA-58}	Ib	2	P170L

[a] S, susceptible; R, resistant.

each pair were further tested with the multilocus sequence typing (MLST) scheme developed by the Institute Pasteur (12) and were assigned an MLST (ST) type.

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 (3, 13–16). The nucleotide sequences of both strands of PCR products were determined at Macrogen Inc., Seoul, South Korea, and sequence analysis was carried out with DNAStar software (version 5.07; Lasergene, Madison, WI).

qRT-PCR. The expression of the *pmrA*, *pmrB*, and *pmrC* genes was tested by quantitative real-time reverse transcription-PCR (qRT-PCR) with the two pairs of clinical isolates in comparison with the *A. baumannii* control strain ATCC 19606, as described previously (3, 17). In particular, bacteria were grown to the mid-logarithmic phase (as shown in the growth analysis), and total cellular RNA was extracted with an RNeasy minikit (Qiagen, West Sussex, United Kingdom). RNA abundance was quantified spectrophotometrically at 260 nm, and contaminating DNA was removed by DNase I treatment (Promega, Madison, WI, USA). The quantitative RT-PCR was performed with the SuperScript III Platinum SYBR green one-step qRT-PCR kit (Invitrogen Corporation, Carlsbad, CA, USA) with 12 ng of total RNA and previously used primers (3). The 16S rRNA gene was used as an internal control for quantification of relative gene expression (3). Control reactions with untranscribed RNA were also included to detect DNA contamination. The expression of genes was described as the mean values from three independent experiments.

Growth curves. Growth rates were determined for the two pairs of clinical isolates and the control strain ATCC 19606. Growth curves were performed in triplicate by diluting equal numbers of CFU of each isolate (approximately 5×10^5 CFU/ml) in Muller-Hinton broth, followed by incubation at 37°C under constant shaking. CFU were enumerated after serially 10-fold diluting broth cultures and plating in antibiotic-free medium at each time point from 12 h to 48 h. Growth of isolates at each time point was statistically compared by using the paired *t* test and Minitab software (version 13.31; Minitab Inc., State College, PA); a *P* value of <0.05 indicated statistical significance.

Proteomic analysis. Proteins of bacterial envelopes from the clinical isolates Ab299 and Ab347 were extracted as described previously (18) from cultures with final optical densities at 600 nm (OD_{600}) of 1.5 and 1.7, respectively. Protein concentration, trypsin digestion, and mass spectrometry analyses were performed as described previously (19, 20), except for the injection volume, which was 1 μl. For protein quantification, we used Progenesis LC-MS software (Nonlinear Dynamics) with the same parameters as used previously (19, 20). The merged peak list was searched against the *A. baumannii* ATCC 17978 database (www.genoscope.cns.fr) using a local version of Mascot (version 2.2; Matrix Science, United Kingdom). Protein fold change was taken into account when it was above 2. At least 3 peptides were used for protein identification and quantification.

RESULTS

First Col^s/Col^r *A. baumannii* pair. A 61-year-old-patient was hospitalized during October 2008 due to severe neurological disease and respiratory distress. The patient was transferred to the intensive-care unit (ICU), underwent tracheostomy and mechanical ventilation, and was given treatment with ampicillin-sulbactam. Blood, urine, and bronchial cultures showed no growth. On hospitalization day 17, an *A. baumannii* isolate (Ab299) that was resistant to many antibiotic classes and susceptible to colistin (MIC, 0.5 μg/ml) was first recovered from bronchial secretions. During the next 100 days, another 14 *A. baumannii* isolates with the same susceptibility profile were intermittently grown from bronchial secretions and five isolates from blood. During this period, the patient suffered from bloodstream infection episodes not only due to this *A. baumannii* strain but also due to *Burkholderia cepacia* and received multiple antibiotic regimens (including ampicillin-sulbactam, gentamicin, tigecycline, ciprofloxacin, and meropenem for more than 10 days each) and also colistin for a total of 28 days. On day 122, a Col^r *A. baumannii* isolate (Ab347; MIC, 32 μg/ml) was first recovered from bronchial secretions with no signs of infection, and subsequently, another 12 Col^r isolates phenotypically similar to Ab347 and indistinguishable from Ab347 by PFGE were grown from bronchial secretions up to day 206. It should be noted that a Col^s isolate indistinguishable from Ab299 caused another bloodstream infection episode on day 212, while no Col^r isolate was recovered from blood during the whole hospitalization. The patient was discharged on day 224 in a good condition.

Second Col^s/Col^r *A. baumannii* pair. A 77-year-old-patient with previous coronary artery bypass surgery was admitted to the hospital in July 2008 due to sternotomy abscess and mediastinitis. The patient was transferred to the ICU; surgical debridement of the sternotomy was conducted, and empirical treatment with imipenem, teicoplanin, and metronidazole was started. On hospitalization day 14, a carbapenem-resistant, Col^s *A. baumannii* isolate (Ab248; MIC, 0.5 μg/ml) was recovered from cultures of drained pus, and treatment with colistin plus tigecycline was administered. On day 37, when the patient's condition was considerably improved, drainage cultures yielded the Col^r *A. baumannii* isolate Ab249 (MIC, 128 μg/ml), and subsequently another three phenotypically similar Col^r isolates were recovered, but no antibiotic treatment was given due to the absence of clinical signs of infection. The patient discharged on day 65.

Characteristics of the isolates. The characteristics of the four *A. baumannii* study isolates are listed in Table 1. Both pairs of isolates were carbapenem resistant (imipenem and meropenem MICs of >32 μg/ml) and also resistant to most available antibiotics except tigecycline and ampicillin-sulbactam. Ab248, Ab249, and Ab299 were intermediate susceptible, but Ab347 was resistant, to gentamicin and tobramycin, while all four isolates had low rifampin MICs (3 to 6 μg/ml). Colistin MICs determined by TDS were 0.5 μg/ml for the Col^s isolates Ab248 and Ab299, while being 128 μg/ml and 32 μg/ml for the Col^r isolates Ab249 and Ab347, respectively. It should be noted that both Col^r isolates exhibited Etest MICs of 4 μg/ml when tested using the same inoculum with TDS; similar discrepancies between Etest and TDS and other di-

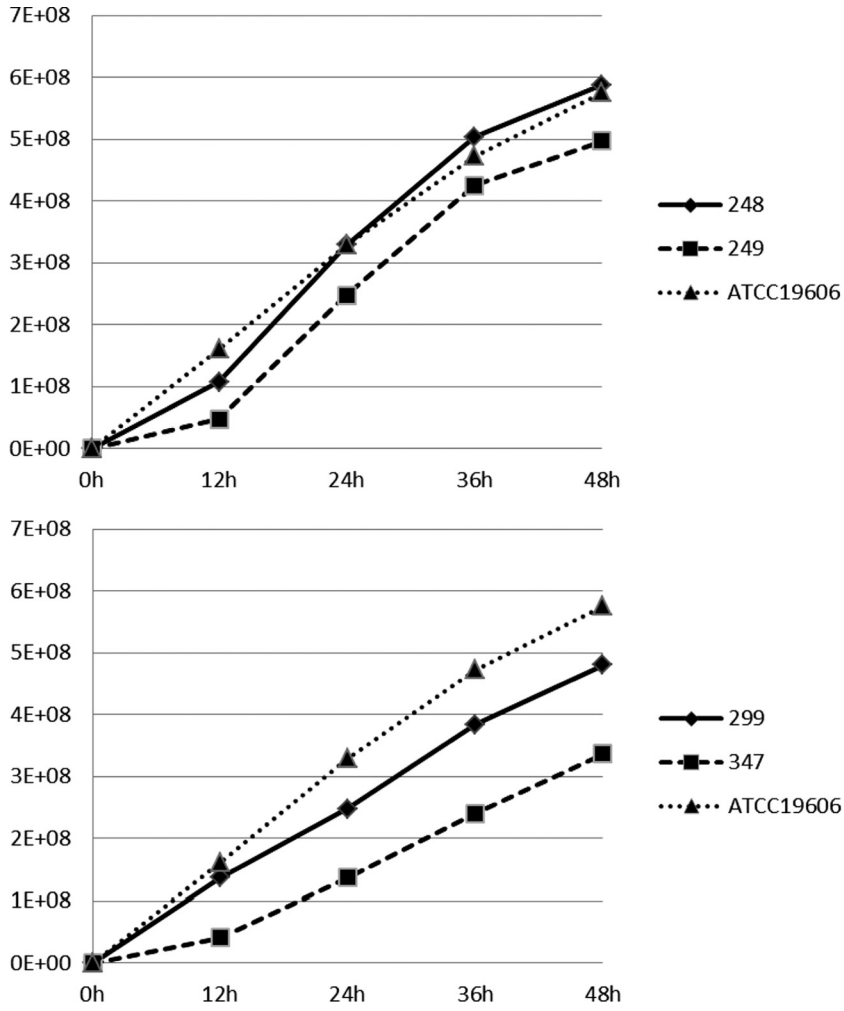

FIG 1 Growth curves of the study and control isolates. *y* axis, CFU/ml from broth cultures; *x* axis, time of growth (hours).

lution methods for the determination of colistin MICs in multiresistant *A. baumannii* isolates were also shown recently (9).

Typing assays. All Col^s/Col^r clinical isolates within each pair had identical PFGE profiles, with the PFGE profile of each pair differing by 2 bands from that of the other pair. All four isolates (Ab299/Ab347 and Ab248/Ab249) tested by MLST belonged to ST2 (international clone 2), which is currently predominant in most regions worldwide (21, 22).

PCR and sequencing. PCR for β-lactamase genes was positive for the $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58-like}}$ genes and negative for the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and class B carbapenemase genes. By nucleotide sequencing, all Col^s/Col^r study isolates were shown to carry the oxacillinase alleles $bla_{\text{OXA-66}}$ and $bla_{\text{OXA-58}}$ and did not have any mutations in genes *pmrA*, *pmrC*, *lpxA*, *lpxC*, and *lpxD*. Compared with the Col^s isolate Ab299, the Col^r isolate Ab347 harbored one nucleotide shift in *pmrB*, leading to the amino acid replacement P170L in the PmrB protein, which is involved in colistin resistance via LPS modifications (23). Similarly, the Col^r isolate Ab249 harbored the amino acid replacement P233S in the PmrB protein compared with the Col^s isolate Ab248.

qRT-PCR. The Col^r isolates Ab249 and Ab347 had increased expression of genes *pmrA* (3.6- and 10.9-fold, respectively) and *pmrB* (5.7- and 23.7-fold, respectively) compared with that of their Col^s counterparts Ab248 and Ab299, respectively, whereas they exhibited smaller increases in *pmrC* expression (1.6- and 2.4-fold, respectively). The relative gene expression represents the mean from three independent experiments. The differences in *pmrCAB* gene expression were significant in both pairs of isolates ($P < 0.05$ for all genes).

Growth analysis. The growth curves of the two pairs of Col^s/Col^r clinical isolates and the ATCC 19606 control are shown in Fig. 1. The growth of the Col^r isolates was considerably slower than that of the respective Col^s isolates, with the difference being significant ($P < 0.05$) at all time points for the pair Ab299/Ab347 and at 24 to 48 h for the pair Ab248/Ab249. It should be noted that the colonies grown from the growth analysis tubes of the Col^r isolate Ab347 were considerably smaller than those of the Col^s isolate Ab299.

Proteomic analysis. Interestingly, the proteomic comparison between the envelopes of Col^s/Col^r isolates Ab299/Ab347 showed a significant underexpression of the CsuA/B and CsuC proteins in Ab347 (Table 2). These proteins are part of the chaperone-usher pilus assembly system that produces pili necessary for biofilm formation. This underexpression supported the impaired biofilm formation that has already been observed by Fernandez-Reyes et

TABLE 2 Proteins related to antibiotic resistance and virulence differentially expressed in Col^r strain Ab347

Accession no.	No. of peptides[a]	Score[b]	ANOVA[c] (*P*)	Fold change[d]	Description
ABYAL1639|katE	39	2,919	1.78×10^{-13}	−35.39	Catalase hydroperoxidase II
ABYAL3026	4	207	5.00×10^{-15}	−28.34	Putative porin protein associated with imipenem resistance, CarO
ABYAL2670	6	549	3.27×10^{-13}	−27.38	Putative protein CsuA/B
ABYAL2667	3	130	1.24×10^{-6}	−14.64	Putative protein CsuC
ABYAL2566|acnB	9	410	1.64×10^{-9}	−3.13	Fragment of aconitate hydratase 2 (part 3)
ABYAL2567|acnB	7	332	1.43×10^{-7}	−2.46	Fragment of aconitate hydratase 2 (part 2)
ABYAL3715|sodC	7	376	1.41×10^{-4}	−2.15	Superoxide dismutase precursor (Cu-Zn)
ABYAL1416|ahpC	14	881	1.81×10^{-8}	−2.13	Alkyl hydroperoxide reductase (detoxification of hydroperoxides)
ABYAL2568|acnB	7	291	3.17×10^{-6}	−2.12	Fragment of aconitate hydratase 2 (part 1)
ABYAL1790|bla_{OXA-66}	3	104	2.17×10^{-7}	−2.03	Carbapenem-hydrolyzing oxacillinase OXA-66
ABYAL0009	9	578	1.08×10^{-9}	+3.15	Putative RND-type efflux pump involved in aminoglycoside resistance (AdeT)

[a] Number of peptides for identification and quantification.
[b] Confidence score for identification by Mascot software.
[c] ANOVA, analysis of variance.
[d] Negative values indicate underexpression in Col^r strain Ab347.

al. (24) for Col^r strains. The Col^r isolate also underexpressed different enzymes involved in the oxidative stress response, i.e., the KatE catalase, the superoxide dismutase, and the alkyl hydroperoxide reductase, suggesting a reduced capacity or necessity to respond to reactive oxygen species (ROS). Furthermore, of note, the aconitate hydratase (aconitase B) enzyme of the tricarboxylic acid (TCA) cycle is underexpressed in the Col^r isolate, suggesting that the production of NADH may be reduced and that the rapid cell death caused by colistin through hydroxyl radical production may be impaired (25). As already observed by Fernandez-Reyes et al. (24) in an *A. baumannii* reference strain where colistin resistance was induced, the carbapenem resistance-associated protein CarO was also underexpressed. Finally, among proteins associated with antibiotic resistance, the carbapenem-hydrolyzing oxacillinase OXA-66 was significantly underexpressed and the putative RND-type efflux pump AdeT, which is involved in aminoglycoside resistance, was significantly overexpressed in the Col^r isolate. It should be noted that Ab299 and other indistinguishable Col^s isolates were resistant to amikacin and netilmicin and intermediate to gentamicin and tobramycin (MICs of 6 and 8 μg/ml, respectively), while Ab347 and indistinguishable Col^r isolates exhibited considerably elevated MICs to gentamicin and tobramycin (32 and 64 μg/ml, respectively).

DISCUSSION

The growing worldwide issue of antibiotic resistance among *A. baumannii* isolates often necessitates the use of colistin for the treatment of severe infections, particularly in ICUs (3). However, the widespread administration of colistin in hospital settings has exerted selective pressure for the development of resistant *A. baumannii* clinical isolates, which are increasingly reported from many regions (5, 26).

The exact colistin resistance mechanism has not yet been elucidated, although two unrelated resistance mechanisms affecting lipid A, the target molecule of colistin, have been described in *A. baumannii* isolates from different locations. The first hypothesis suggested the complete loss of LPS through inactivation of lipid A biosynthesis gene *lpxA*, *lpxC*, or *lpxD* (16). The second proposed mechanism showed mutations in the genes *pmrA* and *pmrB* leading to phosphoethanolamine addition to hepta-acylated lipid A to be linked to colistin resistance in *A. baumannii*, along with increased expression of the PmrCAB system (3, 23, 27). In the present study, the underlying mechanisms of colistin resistance development were mutations in PmrB protein along with *pmrCAB* upregulation, while the *lpxA*, *lpxC*, and *lpxD* genes were not affected. It should be noted that in most of the previous clinical or laboratory reports of colistin resistance mechanisms in *A. baumannii*, resistance development was linked to exposure to colistin (3, 5, 6), similarly to our clinical cases.

It has been recently demonstrated that the mechanism of bacterial killing by colistin could be via the production of hydroxyl radicals, leading to rapid cell death (25). This mechanism remained active in *A. baumannii* multidrug-resistant strains, but hydroxyl radical production might be abolished when colistin resistance appeared (25). In this study, the proteomic comparison of envelopes from isolates Ab299/Ab347 showed an underexpression of the aconitase B protein, a component of the TCA cycle, in the Col^r isolate Ab347. Therefore, in accordance with the model and studies described by Kohanski et al. (28), this underexpression should reduce the production of NADH, decrease superoxide generation (and then decrease ferrous iron availability for the Fenton reaction), and finally increase the bacterial resistance to colistin. In accordance with a reduced cell death following colistin exposure, the large underexpression in the Col^r isolate Ab347 of enzymes such as catalase, alkyl hydroperoxide reductase, and superoxide dismutase may be linked to a reduced necessity to counteract ROS production.

Col^r *A. baumannii* laboratory strains or, very recently, clinical isolates have been associated with reduced virulence relative to that of their Col^s counterparts, as reflected by reduced experimental fitness and mortality in animals (7, 29). In the current study, the two Col^r isolates, compared with their respective Col^s clinical isolates, exhibited significantly slower growth, indicating a reduced fitness. Further, the Ab347 Col^r isolate underexpressed the Csu system, which is necessary for biofilm formation, the outer membrane protein CarO (30), and antioxidant proteins that could protect it from ROS toxic effects generated by macrophages (31), overall possibly indicating a lower virulence of the Col^r isolates and supporting the previous observations (7, 29). Furthermore, it had been reported that a Col^r isolate was associated with a prolonged clinical carriage without signs of infection, in contrast to the case for its Col^s counterpart causing bloodstream infections

(6). In our case patients, of particular importance, the Col^r isolates probably had a reduced ability to cause invasive infection, as the patients remained carriers for prolonged time periods. Furthermore, their Col^s counterparts repeatedly caused severe clinical infections, including multiple bloodstream infections, while a Col^s isolate from case patient 1 was still evading the bloodstream long after the emergence of Col^r isolates. These observations could suggest that the changes contributing to colistin resistance development confer a considerable fitness cost and affect the capacity to produce clinical infections.

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