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

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Running title: Lower infectivity of colistin-resistant *A. baumannii*

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ABSTRACT

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

Keywords: pili; antioxidant enzymes; Csu; Pmr
INTRODUCTION

Acinetobacter baumannii has been an important nosocomial pathogen for the past 30 years, frequently implicated in ventilator-associated pneumonia, 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), 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 (ColS/ColR) pairs of A. baumannii clinical isolates, with each pair to be recovered from the same patient and to include isolates with identical macrorestriction pattern. We report herein that compared with the respective ColS isolates, the ColR isolates may exhibit significant growth retardation, impaired virulence and also considerably lower clinical invasiveness.

METHODS
Study isolates and susceptibility testing. The study included two ColS/ColR pairs of *A. baumannii* isolates consecutively that were recovered from two ICU patients, as well as the ColS *A. baumannii* strain ATCC19606 as control. The isolates were provisionally identified as belonging to *A. baumannii* complex by API 20NE (bioMerieux, Marcy l’ Etoile, France) and were all identified as *A. baumannii* by positive PCR/sequencing that revealed the carriage of a *bla*<sub>OXA-51-like</sub> variant gene. Susceptibility status to β-lactams, cotrimoxazole, rifampin, aminoglycosides and quinolones was performed by Etest (bioMerieux). Colistin MICs were initially performed 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. Genetic relationship of all ColS/ColR isolates that were consecutively recovered from the study patients during the study period was tested by PFGE of *Apa*I-digested genomic DNA [10]; the banding patterns were compared visually using previously proposed criteria (11). The first ColS/ColR isolations of each pair were further tested by the multi locus sequence typing (MLST) scheme developed by the Institute Pasteur (12) and were assigned an MLST (ST) type.

PCR and sequencing. PCR reactions for *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-58-like</sub>, *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-24-like</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>SIM</sub>, pmrA, pmrB, pmrC, lpxA, lpxC and lpxD genes were performed as described previously (3, 13-16). Nucleotide sequence of both strands of PCR products was determined at Macrogen Inc, Seoul, South Korea, and sequence analysis was carried out with DNAStar software (version 5.07; Lasergene, Madison, WI).

Quantitative real-time PCR. The expression of the pmrA, pmrB, and pmrC genes was tested by quantitative real-time RT–PCR (QRT–PCR) in the two pairs of clinical isolates in comparison with the *A. baumannii* control strain ATCC19606, 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 RNase minikit (Qiagen, West Sussex, United Kingdom). RNA abundance was quantified spectrophotometrically at 260 nm and contaminating DNA was removed with DNaseI 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 of untranscribed RNA were also included to detect DNA contamination. The expression of genes was described as the mean of three independent experiments.

Growth curves. Growth rates were determined for the two pairs of clinical isolates and the control strain ATCC19606. Growth curves were performed in triplicate by diluting equal numbers of colony-forming units (CFUs) of each isolate (approx. 5x10^5 CFUs/mL) in Muller-Hinton broth, followed by incubation at 37°C under constant shaking. CFUs 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; www.minitab.com); P <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 OD_{600} of 1.5 and 1.7 respectively. Protein concentration and 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 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 above 2. At least 3 peptides were used for protein identification and quantification.
RESULTS

First ColS/ColR 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) resistant to many antibiotic classes and susceptible to colistin (MIC 0.5 µg/ml) was first recovered from bronchial secretions. In 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 ColR A. baumannii isolate (Ab347, MIC 32 µg/ml) was first recovered from bronchial secretions with no signs of infection and, subsequently, another 12 ColR isolates phenotypically similar and indistinguishable to Ab347 by PFGE were grown from bronchial secretions up to day 206. It should be noted that a ColS isolate indistinguishable to Ab299 caused another bloodstream infection episode on day 212, while no ColR isolate was recovered from blood during the whole hospitalization. The patient was discharged on day 224 in a good condition.

Second ColS/ColR A. baumannii pair. A 77-year-old-patient with previous coronary artery bypass surgery was admitted to the hospital at 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, ColS A. baumannii isolate (Ab 248, MIC 0.5 µg/ml) was recovered from cultures of drained pus and colistin plus tigecycline treatment was administered. On day 37,
when the patient’s condition was considerably improved, drainage cultures yielded the ColR A. baumannii isolate Ab 249 (MIC 128 µg/ml) and, subsequently, another three phenotypically similar ColR isolates, 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 > 32 µg/ml) and also resistant to most available antibiotics except tigecycline and ampicillin/sulbactam. Ab248, Ab249 and Ab299 were intermediate susceptible, but Ab347 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 ColS isolates Ab248 and Ab299, while being 128 µg/ml and 32 µg/ml for the ColR isolates Ab249 and Ab347, respectively. It should be noted that both ColR isolates exhibited Etest MICs of 4 µg/ml when tested using the same inoculum with TDS; similar discrepancies of Etest with TDS and other dilution methods for the determination of colistin MICs in multiresistant A. baumannii isolates were also shown recently (9).

Typing assays. All ColS/ColR clinical isolates within each pair had identical PFGE profiles, with the PFGE profile of each pair to differ by 2-bands from that of the other pair. All four isolates (Ab299/Ab347; 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 blaOXA-51-like and blaOXA-58-like and negative for blaOXA-23-like, blaOXA-24-like and class B carbapenemase genes. By nucleotide sequencing, all ColS/ColR study isolates were shown to carry the oxacillinase alleles blaOXA-66 and blaOXA-58 and did not have any mutations in genes pmrA, pmrC, lpxA, lpxC and lpxD. Compared with the ColS isolate Ab299, the ColR isolate Ab347 harboured one nucleotide shift in pmrB
leading to the aminoacid replacement P170L in PmrB protein that is involved in colistin resistance via LPS modifications (23). Similarly, the ColR isolate Ab249 harbourd the aminoacid replacement P233S in PmrB protein, compared with the ColS isolate Ab248.

qRT-PCR. The ColR 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 ColS 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* genes expression were significant in both pairs of isolates (P < 0.05 for all genes).

Growth analysis. The growth curves of the two pairs of ColS/ColR clinical isolates and the ATCC19606 control are shown in Figure 1. The growth of the ColR isolates was considerably slower than that of the respective ColS isolates, with the difference to be significant (P < 0.05) at all time points for the pair Ab299/Ab347 and at 24-48 h for the pair Ab248/249. It should be noted that the colonies grown from the growth analysis tubes of the ColR isolate Ab347 were considerably smaller than those of the ColS isolate Ab299.

Proteomic analysis. Interestingly, the proteomic comparison between the envelopes of ColS/ColR 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 pili 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 al.* (24) for ColR strains. The ColR isolate underexpressed also 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 204
underexpressed in the ColR isolate, suggesting that the production of NADH may be reduced and 205
that the rapid cell death caused by colistin through hydroxyl radical production may be impaired 206
(25). As already observed by Fernandez-Reyes et al. (24) in an A. baumannii reference strain where 207
colistin resistance was induced, the carbapenem resistance associated protein, CarO, was also 208
underexpressed. Finally, among proteins associated with antibiotic resistance, the carbapenem-
hydrolyzing oxacillinase OXA-66 was significantly underexpressed and the putative RND type 209
efflux pump AdeT, involved in aminoglycoside resistance, was significantly overexpressed in the 210
ColR isolate. It should be noted herein that Ab299 and other indistinguishable ColS isolates were 211
resistant to amikacin and netilmicin and intermediate to gentamicin and tobramycin (MICs 6 and 8 212
µg/ml, respectively), while Ab347 and indistinguishable ColR isolates exhibited considerably 213
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 218
necessitates the use of colistin for the treatment of severe infections, particularly in the ICUs (3). 219
However, the widespread administration of colistin in the hospital settings exerted selective 220
pressure for the development of resistant A. baumannii clinical isolates, which are increasingly 221
reported from many regions (5, 26).

The exact colistin resistance mechanism has not yet been elucidated, although two unrelated 223
resistance mechanisms affecting lipid A, the target molecule of colistin, have been described in A. 224
baumannii isolates from different locations. The first hypothesis suggested the complete loss of 225
LPS through inactivation of a lipid A biosynthesis genes lpxA, lpxC or lpxD (16). The second 226
proposed mechanism showed mutations in the genes pmrA and pmrB leading to 227
phosphoethanolamine addition to hepta-acylated lipid A to be linked to colistin resistance in A. 228
baumannii, along with increased expression of the PmrCAB system (3, 23, 27). In the present
report, the underlying mechanisms of colistin resistance development were mutations in PmrB protein along with pmrCAB upregulation, while 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 with exposure to colistin (3, 5, 6), similarly with our clinical cases.

It has been recently demonstrated that the mechanism of bacterial killing by colistin could happen 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 isolates Ab299/Ab347 envelopes showed an underexpression of the aconitase B protein, a component of the TCA cycle, in the ColR 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 (then decrease ferrous iron availability for 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 ColR isolate Ab347 of enzymes like catalase, alkyl hydroperoxide reductase and superoxide dismutase may be linked to a reduced necessity to counteract ROS production.

ColR A. baumannii laboratory strains or, very recently, clinical isolates have been associated with reduced virulence relatively with their ColS counterparts, as reflected by reduced experimental fitness and mortality in animals (7, 29). In the current study, the two ColR compared with their respective ColS clinical isolates, exhibited significantly slower growth, indicating a reduced fitness. Further, the Ab347 ColR isolate underexpressed the Csu system, necessary for biofilm formation, the outer membrane protein CarO (30) and antioxidant proteins that could protect it from ROS toxic effect generated by macrophages (31), overall possibly indicating a lower virulence of the ColR isolates and supporting the previous observations (7, 29). Furthermore, it had been reported that a ColR isolate was associated with a prolonged clinical carriage without signs of infection, in
In our case patients, of particular importance, the ColR isolates had probably reduced ability to cause invasive infection, as they remained carriers for prolonged time periods. Furthermore, their ColS counterparts repeatedly caused severe clinical infections including multiple bloodstream infections, while a ColS isolate of case patient 1 was still evading the bloodstream long time after the emergence of ColR isolates. These observations could suggest that the changes contributing to colistin resistance development confer a considerable fitness cost and affect their capacity to produce clinical infections.

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REFERENCES


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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Date of isolation (m/d/y)</th>
<th>Specimen</th>
<th>Colistin TDS MIC (µg/mL) / Susceptibility status</th>
<th>bla<em>OXA</em> genes</th>
<th>PFGE type</th>
<th>ST</th>
<th>pmrB genotype</th>
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<td>0.5/S</td>
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<td>Ia</td>
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<td>Wild type</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>OXA<em>58</em></td>
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<tr>
<td>Ab249</td>
<td>8/26/2008</td>
<td>Pus</td>
<td>128/R</td>
<td><em>bla</em>OXA<em>66</em></td>
<td>Ia</td>
<td>2</td>
<td>P23S</td>
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<td></td>
<td></td>
<td></td>
<td><em>bla</em>OXA<em>58</em></td>
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<td>0.5/S</td>
<td><em>bla</em>OXA<em>66</em></td>
<td>lb</td>
<td>2</td>
<td>Wild type</td>
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<tr>
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TABLE 2 Proteins related to antibiotic resistance and virulence differentially expressed in the ColR strain 347

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<th>Score (^b)</th>
<th>Anova (p)</th>
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<td>ABYAL2670</td>
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<td>putative RND type efflux pump involved in aminoglycoside resistance (AdeT)</td>
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\(^a\) number of peptides for identification and quantification

\(^b\) confidence score for identification by Mascot software

\(^c\) fold change, negative value for underexpression in ColR 347 strain
FIG 1 Growth curves of the study and control isolates. Y-axis, CFUs/ml yielded from broth cultures; x-axis, time of growth (hours)