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Short Communication

Impaired growth under iron-limiting conditions associated with the acquisition of colistin resistance in *Acinetobacter baumannii*

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ABSTRACT

Acquisition of colistin resistance in *Acinetobacter baumannii* has been associated with reduced bacterial fitness and virulence, although the mechanisms underlying this fitness loss have not been well characterised. In this study, the role played by environmental iron levels on the growth and survival of colistin-resistant strains of *A. baumannii* was assessed. Growth assays with the colistin-susceptible ATCC 19606 strain and its colistin-resistant derivative RC64 [colistin minimum inhibitory concentration (MIC) of 64 mg/L] demonstrated that the strains grew similarly in rich laboratory medium (Mueller–Hinton broth), whereas RC64 demonstrated impaired growth compared with ATCC 19606 in human serum (>100-fold at 24 h). Compared with RC64, ATCC 19606 grew in the presence of higher concentrations of the iron-specific chelator 2,2′-bipyridine and grew more readily under iron-limiting conditions in solid and liquid media. In addition, iron supplementation of human serum increased the growth of RC64 compared with unsupplemented human serum to a greater extent than ATCC 19606. The ability of 11 colistin-resistant clinical isolates with mutations in the *pmrB* gene to grow in iron-replete and iron-limiting conditions was assessed, demonstrating that eight of the strains showed reduced growth under iron limitation. Individual mutations in the *pmrB* gene did not directly correlate with a decreased capacity for growth under iron limitation, suggesting that mutations in *pmrB* may not directly produce this phenotype. Together these results indicate that acquisition of colistin resistance in *A. baumannii* can be associated with a decreased ability to grow in low-iron environments.

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1. Introduction

Colistin resistance is emerging as an increasingly important problem in infections caused by *Acinetobacter baumannii* [1]. Mutations in the PmrAB two-component system can produce colistin resistance in *A. baumannii* by increasing the addition of phosphoethanolamine molecules to the lipid A component of lipopolysaccharide (LPS) through increased expression of the phosphoethanolamine transferase PmrC [2–4]. Increasing evidence suggests that the acquisition of colistin resistance is associated with reduced bacterial fitness and virulence. A laboratory-derived colistin-resistant mutant (RC64) of the *A. baumannii* ATCC 19606 strain with two point mutations in *pmrB* showed reduced in vivo fitness and decreased virulence in a mouse model of peritoneal sepsis [5]. Two subsequent studies have independently reported that colistin-resistant clinical isolates with mutations in the PmrAB system, both of which were isolated from patients during colistin treatment, have decreased virulence in animal models compared with their parental strains [6,7]. Although these studies establish that acquisition of colistin resistance is associated with decreased bacterial fitness, the underlying mechanisms that produce this effect have not been identified. The aim of the present study was to characterise the role played by environmental iron levels on the growth and survival of colistin-resistant strains of *A. baumannii* using a colistin-resistant derivative of the ATCC 19606 strain that was previously shown to have reduced in vivo fitness and virulence [5] as well as 11 colistin-resistant clinical isolates with mutations in the *pmrB* gene [8].

2. Materials and methods

2.1. Bacterial strains

A. baumannii ATCC 19606 is a colistin-susceptible [colistin minimum inhibitory concentration (MIC) of 0.5 mg/L] reference strain

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[5]. RC64 is a colistin-resistant derivative of ATCC 19606 (colistin MIC of 64 mg/L) obtained by selection during growth in colistin [9]. RC64 contains two point mutations in the *pmrB* gene producing R134C and A227V amino acid substitutions [5]. Eleven colistin-resistant clinical isolates obtained from Seville, Spain, in 2002 were used [8]. Sequencing of the *pmrA* and *pmrB* genes was performed as described previously [5]. LPS biosynthesis was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by carbohydrate-specific silver staining using a previously described protocol [10]. The clonal lineages of the clinical isolates were determined by multilocus sequence typing (MLST) using the Institut Pasteur scheme [11].

2.2. Growth in laboratory medium and human serum

Growth of ATCC 19606 and RC64 in Mueller–Hinton broth (MHB) (Sigma-Aldrich, St Louis, MO) and human serum (type AB; Sigma-Aldrich) was assessed by inoculating 20 mL of static cultures to a concentration of 5×10^5 CFU/mL followed by incubation at 37 °C for 24 h. At 0, 2, 4, 8 and 24 h, 100 μL aliquots were taken and serial $\log_{10}$ dilutions were plated on solid medium for bacterial quantification.

2.3. Effect of iron depletion on growth

MICs of the iron-specific chelator 2,2′-bipyridine (Bip) (Sigma-Aldrich) were determined in a microdilution assay. Strains were cultured in MHB to a concentration of 10^8 CFU/mL and then 100 μL of the bacterial suspension was added to the wells of a 96-well plate containing increasing concentrations of Bip (final concentrations 0.125–64 mg/L). Bacterial growth was assessed visually after 24 h at 37 °C. For plate dilution assays, ATCC 19606 and RC64 grown on solid medium were re-suspended in physiological saline and were adjusted to an optical density at 600 nm (OD_{600}) of 0.6. Serial 10-fold dilutions of the bacterial suspensions were made in physiological saline and 3 μL drops of each dilution were plated on Mueller–Hinton agar (MHA) (Sigma-Aldrich) or on MHA containing 32 mg/L Bip.

2.4. Growth in iron-supplemented human serum

A microdilution assay was used to characterise the effect of iron supplementation on growth in human serum. Single colonies were grown during 4 h in 20 mL of MHB. Bacteria were washed twice with physiological saline and were re-suspended to a 0.5 McFarland standard. Then, 100 μL of the cell suspension and 100 μL of human serum supplemented with concentrations from 0.125–64 mg/L $FeCl_2$ were combined in 96-well plates and growth was assessed visually after incubation at 37 °C for 24 h.

The effect of iron supplementation on bacterial growth was assessed by culturing ATCC 19606 and RC64 in 20 mL of human serum supplemented with either 2 mg/L or 32 mg/L $FeCl_2$ (five independent cultures for each condition). After 24 h, the concentration of bacteria in the cultures was determined by plating on solid medium. Results were normalised to bacterial concentrations obtained after 24 h of growth in unsupplemented human serum.

2.5. Growth of clinical isolates in iron-limiting conditions

Plate dilution assays in which 10-fold dilutions of overnight cultures of the clinical isolates were plated on MHA or on MHA containing 32 mg/L Bip were performed. Serial 10-fold dilutions of the bacterial suspensions were made in physiological saline and 3 μL drops of each dilution were plated. The highest dilution at which bacterial growth occurred was determined for each growth condition after incubation at 37 °C for 24 h. For growth curve analysis, overnight cultures were diluted 1:1000 in the indicated media. Briefly, 100 μL of the diluted bacterial suspensions were placed into a 96-well plate and the OD_{595} was determined every hour using a Tecan i-control 1.6.19.2 plate reader (Tecan Austria GmbH, Salzburg, Austria) during incubation at 37 °C. Doubling times for each strain both in iron-replete and iron-limiting conditions were calculated. The experiment was performed in triplicate.

2.6. Statistical analysis

Mean fold changes in bacterial concentrations and doubling times were compared using Student's *t*-test.

3. Results

3.1. Bacterial growth in laboratory medium and human serum

RC64 has previously been shown to have lower in vivo fitness and virulence than the ATCC 19606 parental strain [5]. To begin to characterise the mechanisms underlying this reduced fitness, bacterial growth was assessed in nutrient-rich laboratory medium and human serum. Both strains showed similar growth in MHB (Fig. 1A). However, RC64 showed reduced growth compared with ATCC 19606 when grown in human serum, with >100-fold fewer viable bacteria at 24 h compared with ATCC 19606 (Fig. 1A). Together these findings raised the possibility that the reduced growth observed with RC64 was due to a decreased ability to grow under the nutrient-limited conditions encountered in human serum. On the basis that human serum is known to contain low concentrations of free iron [12], we aimed to characterise how the environmental iron concentration affected the growth of ATCC 19606 and RC64.

3.2. Bacterial growth under iron-limiting conditions

To assess the effect of iron limitation on bacterial growth, the concentrations of Bip required for suppressing bacterial growth were determined for ATCC 19606 and RC64. A concentration of 64 mg/L Bip was required to inhibit the growth of ATCC 19606, whereas for RC64 a concentration of 32 mg/L was required. This aspect was assessed further by culturing 10-fold dilutions of ATCC 19606 and RC64 on MHA or on MHA containing 32 mg/L Bip. Both strains grew similarly on MHA (Fig. 1B). In the presence of Bip, growth of ATCC 19606 was seen at the same dilution as in the absence of Bip, although with somewhat reduced colony size, whereas in the case of RC64 growth was only observed up to the 10^{-2} dilution in the presence of Bip compared with the 10^{-5} dilution in the absence of Bip. These results indicate that iron limitation affects the growth/survival of RC64 to a greater extent than ATCC 19606.

3.3. Effect of iron supplementation on growth

We next determined whether iron supplementation could increase the growth of RC64 in human serum. ATCC 19606 showed growth in serum without iron supplementation, whereas RC64 required supplementation with ≥0.5 mg/L $FeCl_2$ in order to achieve visible bacterial growth at 24 h. The ability of iron to facilitate the growth of ATCC 19606 and RC64 was further assessed by supplementing serum with either 2 mg/L or 32 mg/L $FeCl_2$ and determining the fold-increase in bacterial growth compared with growth in serum without iron supplementation. As shown in Fig. 2, supplementation with 2 mg/L $FeCl_2$ resulted in increased growth of both ATCC 19606 and RC64, although the differences in fold change compared with unsupplemented serum between the strains was not significant ($P = 0.067$, Student's *t*-test). When serum was supplemented with 32 mg/L $FeCl_2$, ATCC 19606 demonstrated a 33-fold increase in growth compared with growth in unsupplemented

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