Pyomelanin production: a rare phenotype in *Acinetobacter baumannii*

*Acinetobacter baumannii* has been deemed one the major nosocomial pathogens of our time. Ability to generate biofilm, production of capsule, presence of lipopolysaccharide and outer-membrane proteins, secretion of hydrolytic enzymes, and ability to accumulate a great variety of resistance mechanisms have been highlighted as virulence factors of *A. baumannii* (Roca *et al.*, 2012). Melanin is a substance dark in colour and has been linked with virulence and pathogenicity of microbes able to produce this pigment (Nosanchuk & Casadevall, 2003). Melanin potentially reduces the susceptibility of the microbe to host defence mechanisms and environmental insults. Depending on the pathway of synthesis, melanin may be given a different designation; the term pyomelanin was proposed for the brown pigment produced from tyrosine or phenylalanine through the accumulation of homogentisic acid (Yabuuchi & Ohyama, 1972).

From 20 to 22 July 2010, production of a brown diffusible pigment was observed in five isolates identified as *A. baumannii* obtained from three adult patients, two from the intensive care unit and one from a general clinic ward of a 592-bed, tertiary care, public hospital in Rio de Janeiro city, Brazil. Specimen sources were tracheal secretion (two), venous catheter tip (two) and blood (one). As this pigmentation is highly atypical, isolates were sent to our research laboratory. The aim of the present study was to confirm the identification of these isolates and to characterize the pigment.

Study isolates were originally identified with the NC50 panel of MicroScan Walk-Away (Siemens), software LabPro version 3.01, and stored as suspensions in a 10% (w/v) skimmed milk solution containing 10% (v/v) glycerol at −20 °C. Identification was subsequently performed by biochemical tests, and species were classified by rpoB gene sequencing (La Scola *et al.*, 2006). PCR amplicons were purified with the QIAquick kit (Qiagen) and sequenced at the Federal University of Rio de Janeiro. Sequences were compared with a set of reference strains (La Scola *et al.*, 2006; Gundi *et al.*, 2009) with CLUSTAL W and deposited in GenBank. The presence of a *blaOXA-51*-like gene was investigated by PCR (Turton *et al.*, 2006). Isolates were typed by random-amplified polymorphic DNA (RAPD)-PCR (Grundmann *et al.*, 1997).

Susceptibility to amikacin, ampicillin–sulbactam, ceftazidime, cefepime, ciprofloxacin, imipenem, gentamicin, meropenem, piperacillin–tazobactam, tobramycin and trimethoprim–sulfamethoxazole was determined by disc diffusion following Clinical and Laboratory Standards Institute (CLSI, 2009) guidelines. MICs of tigecycline were determined by Etest (bioMérieux) and interpreted as suggested by the US Food and Drug Administration (FDA) for Enterobacteriaceae.

A substance has been considered a melanin if it is dark in colour, insoluble in aqueous and organic fluids, resistant to acid and susceptible to bleaching by oxidizing agents (Nosanchuk & Casadevall, 2003). Thus, a pigment pellet was obtained by acidification of culture supernatant to pH 2.0 and boiling in the presence of ethanol, as described (Sajjan *et al.*, 2010), and subjected to solubility tests.

To investigate whether the pigment was linked to tyrosine, the following experiments were conducted with three *A. baumannii* strains: pigment-producing 456MDp as study isolate; non-pigment-producing clinical isolate 522A, and ATCC 19606 strain, as negative controls. As tyrosine is a precursor of different kinds of melanin, the effect of this amino acid on pigment production was tested by growing isolates in three minimal defined media: Y-medium (Yabuuchi & Ohyama, 1972), TL and T-medium (Arai *et al.*, 1980), with the sole carbon sources tyrosine, glutamate and glutamate plus tyrosine, respectively. Results were read after 48 h of incubation.

The potential effect of sulcotrione [2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanediione; Sigma], an inhibitor of tyrosine metabolism via homogentisic acid (Turick *et al.*, 2008), was evaluated to characterize the metabolic pathway involved in pigment production. Accordingly, isolates were grown in T-broth with sulcotrione at five different concentrations (0, 2.5, 10, 15, 20 μM), and the absorbance curves were read at A_{400} after 48 h incubation. All cultures were grown in ambient air at 37 °C.

In the present study, the production by *A. baumannii* of a brown pyomelanin-like diffusible pigment visible on Mueller–Hinton agar is reported. Production of such a pigment has not, to our knowledge, been previously recorded in the clinical laboratory of the large hospital where the isolates were obtained, nor in our research laboratory after studying about 400 clinical isolates confirmed by genetic tests as *Acinetobacter* sp., from three different cities in Brazil (data not shown). Indeed, pigment production is not mentioned in a microbiology reference manual (Vanechoutte *et al.*, 2011). However, in a study including 291 *Acinetobacter* isolates with no species identification, brown pigment production was observed in about 40 isolates from aquatic environments (Pagel & Seyfried, 1976). The brown pigment described in the present study is not equivalent to another phenotype described in *Acinetobacter* sp. – a brown discoloration of media supplemented with blood and an aldose sugar (Siau *et al.*, 1998); neither this brown discoloration nor the production of any pigment was observed in media without blood and sugar supplementation by these authors.

All five isolates were identified as *A. baumannii* (99.9%) by MicroScan Walk-Away, and phenotypically characterized as oxidase-negative, catalase-positive, glucose
oxidizing, non-motile, Gram-negative coccobacilli. The rpoB gene sequences of isolates had 99–100 % similarities with sequences of A. baumannii deposited in the GenBank database (nos JF914986–JF914990); all carried the blaOXA-51-like gene and shared an indistinguishable RAPD fingerprint (data not shown).

The five isolates were resistant to all antimicrobial drugs tested, except ampicillin–sulbactam and tigecycline (MIC 0.38–0.75 µg ml⁻¹). After 18 h incubation, brown pigmented colonies were observed for the five isolates on Mueller–Hinton agar plates. Representative isolate 456MDp is shown in Fig. 1(a).

The dark pellet obtained from A. baumannii 456MDp was insoluble in distilled water, 1 M HCl, ethanol, acetone, chloroform, phenol and benzene; it was soluble in 1 M NaOH and phenol, and blanched by H₂O₂ 30 %. These results indicate that the pigment may be classified as a melanin (Nosanchuk & Casadevall, 2003).

After incubation on the set of minimal defined media, A. baumannii 456MDp showed the following profile: poor growth with no pigment on Y-medium (Fig. 1d), normal growth with no pigment on TL-medium (Fig. 1g), and normal growth with a brown diffusible pigment on T-medium (Fig. 1j). The control A. baumannii 522A strain showed heavy growth with no pigmentation on Y-, TL- and T-media (Fig. 1e, h, k, respectively). The control A. baumannii ATCC 19606 strain showed a similar growth profile, except for slight pigmentation on T-medium (Fig. 1l). The growth profile of 456MDp in Y-, TL- and T-medium is typical for strains with impairment of the homogentisate 1,2-deoxigenase metabolic pathway, as described for melaninogenic Vibrio cholerae (Ruzafa et al., 1995), Burkholderia cenocepacia (Keith et al., 2007) and Pseudomonas aeruginosa (Rodríguez-Rojas et al., 2009). In this case, the enzyme defect leads to accumulation of the tyrosine metabolism intermediate homogentisic acid, which is exported and spontaneously oxidized to melanin products in the culture medium (Ruzafa et al., 1995). The profile of A. baumannii 522A strain was that expected for successful degradation of tyrosine to fumaric and acetoacetic acids. Pigment production of A. baumannii ATCC 19606 in T-medium might have resulted from the excess of tyrosine in the medium, leading to accumulation of homogentisic acid.

By growing A. baumannii 456MDp in T-medium broth containing sulcotrione, a concentration-dependent reduction in the final pigmentation was observed (Fig. 2). As sulcotrione is an inhibitor of tyrosine metabolism one step before the formation of the melanin precursor, the decreased

**Fig. 1.** Growth characteristics of Acinetobacter baumannii isolates on Mueller–Hinton agar (a–c), and minimal amino acid defined Y- (d–f), TL- (g–i) and T- (j–l) media. Isolate 456MDp (a, d, g, j) shows normal growth on all media except Y (d), and produces pigment on Mueller–Hinton agar (a) and T-medium (j) only. Isolate 522A (b, e, h, k) shows normal growth with no pigment on all media. Strain ATCC 19606 (c, f, i, l) shows normal growth on all media and produces only slight amounts of pigment on T-medium (l).

**Fig. 2.** Acinetobacter baumannii 456MDp pigment production in T-medium broth after 48 h of incubation (A₄₀₀) in the presence of growing concentrations of sulcotrione. Data represent the mean ± SD from two independent experiments.
pigmentation observed in the presence of this substance suggests that the homogenetic acid metabolic pathway is involved in the production of the pigment, a pyomelanin, by this strain. Disc diffusion susceptibility test of the 456MDp isolate in the presence of T-medium containing 2.5 μM sulcotrione, a concentration that inhibited pigment production substantially (Fig. 2), had no impact on antimicrobial inhibition haloes. This observation suggested pigment production has no impact on antimicrobial resistance.

In the present study, the production of a brown diffusible pigment by five clonal clinical A. baumannii isolates is reported. Strains were identified by biochemical and molecular methods and characterized as highly antibiotic resistant. The pigment was biochemically characterized as a melanin, a product related to tyrosine utilization most likely via the homogenetic acid metabolic pathway. Further studies are required to establish the impact of pigment production on the pathogenesis of A. baumannii. To our knowledge, this is the first published report of such a phenotype in clinical strains of A. baumannii.

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The GenBank/EMBL/DDBJ accession numbers for the rpoB gene sequences of five A. baumannii pigment-producing isolates are JF911496 to JF9114990.


