Burkholderia dolosa phenotypic variation during the decline in lung function of a cystic fibrosis patient during 5.5 years of chronic colonization

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Although rarely isolated from cystic fibrosis (CF) patients, Burkholderia dolosa is associated with accelerated lung function decline. During 18 years of epidemiological surveillance in the major Portuguese CF centre in Lisbon, only one patient was infected with B. dolosa. Pulmonary deterioration, associated with the evolution of forced expiratory volume in 1 s, occurred during 5.5 years of colonization with this B. dolosa clone (with the new sequence type ST-668). Transient co-colonization with Burkholderia cenocepacia and other bacterial and fungal pathogens occurred, but B. dolosa prevailed until the patient’s death. The systematic assessment of relevant phenotypes for the sequential clonal isolates examined in this retrospective study (14 of B. dolosa and four of B. cenocepacia) showed that they were variants, although in general no isolation time-dependent pattern of alteration was identified. However, the first B. dolosa isolate retrieved was more susceptible to gentamicin, imipenem and tobramycin, and exhibited a higher swarming motility compared with most of the isolates obtained during the later stages of disease progression and antimicrobial therapy.

INTRODUCTION

Airway infection with opportunistic microbial pathogens remains the leading cause of morbidity and mortality among cystic fibrosis (CF) patients due to irreversible decline in lung function (Döring et al., 2011; LiPuma, 2010). Most of the respiratory infections are caused by Pseudomonas aeruginosa, but infections with Burkholderia cepacia complex (Bcc) bacteria are associated with a worse prognosis and decreased life expectancy (LiPuma, 2010). Although most of the 18 described Bcc species have been recovered from CF patients, Burkholderia cenocepacia and Burkholderia multivorans account for >80% of all Bcc infections worldwide (Drevinek & Mahenthiralingam, 2010; LiPuma, 2010; Peeters et al., 2013). The importance of both species as the predominant Bcc pathogens in CF lung colonization is well documented for continental Europe and the UK, even though the prevalence of each species varies with the country and the CF centre under consideration (Drevinek & Mahenthiralingam, 2010; Govan et al., 2007). A remarkable exception was registered in the major Portuguese CF center at Hospital de Santa Maria (HSM), in Lisbon, where ~30% of the Bcc infections were caused by B. cepacia (Coutinho et al., 2011b; Cunha et al., 2007). The exceptionally high representation of this species was associated with a contamination of non-sterile saline solutions for nasal application with two B. cepacia strains (Cunha et al., 2007). Since this contamination was detected the number of B. cepacia isolates obtained at HSM has gradually declined, but still remain high due to persistent colonization of living patients (Coutinho et al., 2011b). The evolution over two decades of an epidemiological survey of Bcc infections in this CF population was also described recently by our group (Coutinho et al., 2011b).

As B. cenocepacia is generally considered the most serious Bcc pathogen in CF, most of the research concerning Bcc pathogenicity in CF has been focused on this species (Drevinek & Mahenthiralingam, 2010; Mahenthiralingam...
et al., 2005). Moreover, as a result of the predominance of B. cenocepacia and B. multivorans, microbiological and clinical information on infections caused by other species within the Bcc is scanty. However, epidemiological studies have shown that other Bcc species are also associated with poor prognosis, patient-to-patient transmission, chronic or transient infections and cepacia syndrome (Biddick et al., 2003; Cunha et al., 2003, 2007; Drevinek & Mahenthiralingam, 2010; Kalish et al., 2006; LiPuma, 2010). Although isolated rarely from CF patients, B. dolosa is associated with accelerated loss of lung function and decreased survival in CF (Kalish et al., 2006). More than 100 B. dolosa isolates, involved in a small outbreak among CF patients receiving care in a Boston hospital, were recovered and examined recently by whole-genome sequencing and comparative genomic analysis (Biddick et al., 2003; Kalish et al., 2006; Lieberman et al., 2011). This extensive and pioneer study identified 17 genes as targets of selection, and presumably involved in B. dolosa pathogenesis and adaptive evolution in CF lungs (Lieberman et al., 2011). The occurrence of clonal expansion and emergence of phenotypic variants from a clonal B. cenocepacia population during long-term colonization was also suggested to occur based on the systematic phenotyping of 11 sequential isolates retrieved from a CF patient during 3.5 years of colonization until death with cepacia syndrome (Coutinho et al., 2011a). Genome-wide expression analyses of three of these isolates implied the occurrence of genetic adaptation to changing selection pressures, presumably resulting from challenges in the immune system, antimicrobial therapy and oxygen limitation (Döring et al., 2011; Lieberman et al., 2011; Madeira et al., 2011, 2013; Mira et al., 2011).

Over 18 years of epidemiological surveillance of respiratory infections involving Bcc at HSM, the first and only case of infection by B. dolosa was detected in 2005. This case study is described in this paper The novel chemical structure and biological activity of the lipoooligosaccharide endotoxin produced by this B. dolosa clone was characterized recently and its strong proinflammatory activity demonstrated (Lorenzo et al., 2013). Although this paper describes a longitudinal study of a single CF patient, it provides important evidence of the potential clinical impact of B. dolosa.

**METHODS**

**Bacterial isolates and culture conditions.** The 18 Bcc isolates examined in this study (Table 1) were obtained from the respiratory secretions of the same chronically colonized CF patient under surveillance at HSM CF centre. Isolates were obtained from August 2005 to January 2011, as part of the hospital routine. Sputum samples are obtained from CF patients every 2–3 months, during periodic consultations to monitor their clinical status or more often for patients showing clinical deterioration. These were grown in selective B. cepacia Selectatab medium (Mast Diagnostics) for 2 days at 35 °C, followed by 3 days of incubation at room temperature. Bacteria were cultured in Lysogeny Broth (Lennox) agar (LB agar; Conda/Pronadisa) plates or in Pseudomonas isolation agar (PIA; Difco) plates.

**Species identification and genotyping.** Total genomic DNA was extracted from bacterial cultures using the Puregene DNA isolation kit (cell and tissue kit) (Gentra Systems) and the concentration estimated using a ND-1000 spectrophotometer (NanoDrop Technologies).

Multilocus sequence typing (MLST) analysis was performed using the amplification primers described previously for housekeeping genes (Baldwin et al., 2005). The reaction mixture and the amplification reaction were performed as described by Coutinho et al. (2011a). Amplification was carried out using a GeneAmp PCR System 2700 (Applied Biosystems) and amplification was confirmed following separation of the PCR products by 0.8 % (w/v) horizontal agarose gel electrophoresis. The bands were excised, purified using a gel extraction kit (JETquick spin column technique; Genomed) and sequenced. DNA sequences were compared to known sequences from the Bcc MLST database (http://pubmlst.org/bcc) using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and the allelic profile (string of seven integers) was used to define the sequence type (ST).

**Antimicrobial susceptibility tests.** Susceptibility to several antimicrobials belonging to different classes was compared using the broth microdilution method. Liquid cultures grown in LB broth at 37 °C with orbital agitation until mid-exponential phase were harvested by centrifugation at 10 000 g and 4 °C for 10 min. These cell cultures were suspended in Mueller–Hinton broth (Sigma-Aldrich) and diluted to a standardized culture OD640 0.2 ± 0.02. Of these cell suspensions, 190 µl was used to inoculate the wells of a 96-well polystyrene microtitre plate (Greiner Bio-One) containing 10 µl antibiotic solution to be tested. All antimicrobials were in powder form and were obtained from Sigma-Aldrich. Culture OD640 in the wells was measured in a VERSAmax tunable microplate reader (Molecular Devices) after 24 h of incubation at 37 °C without shaking. The microplate reader was connected to a computer running SoftMax Pro 6.0 Microplate Data Acquisition and Analysis Software (Molecular Devices). Positive (without antibiotic) and negative (not inoculated) controls were included.

**Colony morphology.** Colony morphology in LB agar plates was observed as described previously (Bernier et al., 2008). Isolates were grown in LB broth at 37 °C for 24 h with shaking at 250 r.p.m. and, after serial dilution into fresh LB broth, 50 µl was plated onto LB agar plates and incubated at 37 °C for 48 h, following an additional period of 24 h at room temperature to more clearly distinguish the different colony morphologies. A Panasonic digital camera using a Stemi 2000-C stereomicroscope at ×16 magnification was used to capture images of the colonies.

**Motility.** For the swarming assay, dry swarm agar plates containing 20 ml 0.8 % (w/v) nutrient broth (Difco), 0.5 % (w/v) glucose and 0.5 % (w/v) agar (Oxoid) were point-inoculated using a sterile toothpick with bacterial cultures from isolated colonies grown on PIA for 24 h at 37 °C. These swarm agar plates were incubated at 37 °C for 24 h and the diameter of the zone of growth around the point of inoculation was measured.

For the swimming assay, dry swim agar plates containing 20 ml 1 % (w/v) tryptone (Difco), 0.5 % (w/v) NaCl and 0.3 % (w/v) agar were point-inoculated and incubated as described for the swarming assay. The circular turbid zone formed by the bacterial cells migrating away from the point of inoculation was measured.

**Biofilm formation assay.** The biofilm formation assay was based on the methodology described previously (O’Toole & Kolter, 1998). Liquid cultures grown in LB broth at 37 °C and 250 r.p.m. until mid-exponential phase were diluted to a standardized culture OD640 0.05 and then 200 µl of this cell suspension was used to inoculate the wells of a 96-well polystyrene microtitre plate (Greiner Bio-One). Wells
containing sterile growth medium were used as negative controls. These microtitre plates were incubated at 37 °C for 24 h without shaking. Growth was assessed by measuring the absorbance of cultures in wells at 640 nm with a VERSAmax tunable microplate reader (Molecular Devices). Cellular biomass attached to the microtitre dish in wells at 640 nm with a VERSAmax tunable microplate reader shaking. Growth was assessed by measuring the absorbance of cultures containing sterile growth medium were used as negative controls. These microtitre plates were incubated at 37 °C for 24 h without shaking. Growth was assessed by measuring the absorbance of cultures in wells at 640 nm with a VERSAmax tunable microplate reader (Molecular Devices). Cellular biomass attached to the microtitre dish was quantified by crystal violet staining (Cunha et al., 2004).

Statistical analysis. The statistical significance of the differences registered in the values obtained for the phenotypes assessed in this work was determined using ANOVA and the Tukey post-test provided in Prism GraphPad software. P<0.05 was considered statistically significant.

Patient’s clinical course. The patient’s clinical course before the detection of Bcc infection and throughout the period of chronic infection was assessed based on values of forced expiratory volume in 1 s (FEV1) during regular lung function tests performed at the hospital to monitor the patient’s respiratory capacity.

Ethics. Studies involving clinical Bcc isolates obtained as part of the hospital routine were approved by the hospital ethics committee and the patient's anonymity was preserved. Consent was obtained from the patient for the use of these isolates in research.

RESULTS

Identification and molecular typing of sequential Bcc isolates

Eighteen sequential isolates of the Bcc were recovered from a CF patient who was under surveillance at HSM from 2005 until death in 2011 following severe lung deterioration. During these 65 months of surveillance, sputum cultures were found to grow not only Bcc bacteria but also, transiently, Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus parainfluenzae, Aspergillus fumigatus and Candida albicans. Before the isolation of the first Bcc bacterium, the single FEV1 value recorded was 70 % and this decreased to 31 % over the next 9 months, reaching a minimum of 15 % after 4.5 years of the onset of infection (Fig. 1). Due to severe deterioration of the pulmonary function and the patient’s clinical condition, no further FEV1 values were available for the last 9 months of the patient’s life (Fig. 1).

Fourteen of the isolates examined were identified as B. dolosa and produced a new ST, designated ST-668 and deposited in the Bcc MLST database on 12 July 2011 (Table 1). Five of the seven loci of ST-668 were associated to the same MLST profile, but exhibited differences in relevant nucleotide. All the B. dolosa isolates examined shared the same MLST profile, but exhibited differences in relevant phenotypes, being clonal variants. Four B. cenocepacia isolates related to a 3-month infection were assigned to ST-43 (Table 1).

<table>
<thead>
<tr>
<th>No.</th>
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<th>Isolation date</th>
<th>Species</th>
<th>Allelic profile</th>
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*Pairs of isolates IST4484VI/IST4484IIIB (isolation date 30 April 2010), IST4481VI/IST4481IIIB (isolation date 19 May 2010) and IST4601S/ IST4601R (isolation date 12 January 2011) were obtained in the same isolation procedure.
†Belonging to recA lineage III-B.
Antimicrobial susceptibility variation of Bcc isolates

In general, the MIC of the antimicrobials tested on the B. dolosa isolates retrieved from the patient over 5.5 years of chronic infection was above the MIC values exhibited by the B. cenocepacia isolates obtained during a 3-month period (Fig. 2). This difference was marked for imipenem and meropenem, but not detectable for ceftazidime. For all the antimicrobials tested, the MIC values for this B. cenocepacia clone were significantly below those reported previously for another B. cenocepacia clone that had chronically colonized another CF patient for 3.5 years (Coutinho et al., 2011a).

An increased resistance towards the aminoglycosides gentamicin and tobramycin, and the carbapenem imipenem, was registered when the majority of the isolates obtained during the later stages of disease progression and antimicrobial therapy were compared to the first B. dolosa isolate obtained from the patient (Fig. 2). However, no significant variation in resistance was observed for the carbapenem meropenem, the cephalosporin ceftazidime or the fluoroquinolone ciprofloxacin. No detectable alteration was registered in the antimicrobial resistance of the B. cenocepacia isolates obtained during the short colonization period of 3 months.

Variation of colony morphotype, cell motility and biofilm formation by the Bcc isolates

The colony morphology of the first B. dolosa isolate obtained from the patient (IST4208) and the morphotypes exhibited by most of the B. dolosa isolates were considered smooth (Fig. 3a). Isolate IST4601R, which gave rise to rough colonies, was the only exception to the smooth morphotype. This isolate was recovered immediately before the patient’s death, during the same isolation procedure as IST4601S. The colony morphology of the B. cenocepacia isolates also varied from smooth (IST4484IIIb, IST4481IIIb and IST4499) to semi-rough (IST4503) (Fig. 3a). B. cenocepacia IST4499 colonies were smooth under standardized growth conditions, but after an additional period of incubation of 24 h at room temperature, part of the colonies showed a semi-rough morphotype (IST4499b), whilst others (e.g. IST4499a) maintained the smooth morphotype.

The swarming motility of the B. dolosa isolates obtained during the later stages of disease progression, namely IST4601S and IST4601R, showed a significant reduction when compared with the first B. dolosa isolate obtained from the patient (Fig. 3b). No consistent variation pattern was found for the swimming motility values (Fig. 3c). No consistent pattern of swarming motility variation could be found for B. cenocepacia isolates obtained during the 3-month colonization period (Fig. 3b).

All the serial B. dolosa and B. cenocepacia isolates were capable of producing biofilm, and the cellular biomass in the biofilms produced by the isolates varied during the colonization period, although no clear pattern could be identified (Fig. 3d). Remarkably, the cellular biomass in biofilms formed by isolate IST4601S was 50% higher than the cellular biomass in the biofilm produced by IST4601R, obtained in the same isolation procedure.

DISCUSSION

This retrospective study focused on the Bcc isolates obtained from a 25-year-old CF patient found to be infected with the same B. dolosa for 5.5 years until death following continuous lung function deterioration, as indicated by FEV1 values.
The B. dolosa clone under investigation was the sole case of B. dolosa infection over 18 years of epidemiological surveillance in the major Portuguese CF centre in Lisbon. A transient (3 months) co-colonization with B. cenocepacia was detected 9 months before death, when the patient’s clinical condition was already highly deteriorated (FEV1, 15 %), and lung transplantation was considered but denied.

Remarkably, although the two B. dolosa isolates recovered on the same date during the last isolation procedure, immediately before the patient’s death, do share the same sequence type, they exhibit different phenotypes. This result is consistent with the idea that there is a genetic and phenotypic heterogeneous colonizing clonal population in the CF lung (Lieberman et al., 2011). The suggested heterogeneity of this B. dolosa clone in the patient’s airways is presumably the result of an adaptive evolution occurring in the mutagenic environment of the CF lung under selective environmental pressures, in particular those exerted by the immune defences, antibiotic therapy, nutrient availability and oxygen limitation (Döring et al., 2011; Harrison, 2007).

Given that the B. dolosa isolates examined in this study were selected at random from a heterogeneous colonizing population, this circumstance might be the reason for the MICs and swarming motility values that apparently failed to follow the proposed pattern for the sequential B. dolosa isolates obtained at the later stages of infection compared with the first isolate obtained from the patient. However,

**Fig. 2.** MIC values determined using the broth microdilution method for the activities of the indicated antimicrobial agents of different classes against B. dolosa (black bars) or B. cenocepacia (grey bars) sequential clonal isolates retrieved from the same CF patient during long-term infection. MIC values are the results of at least three independent experiments; bars, sd. *P<0.05 when each B. dolosa isolate was compared to IST4208.
an increase in the level of resistance between the first and most of the latter B. dolosa isolates to gentamicin, tobramycin and imipenem was registered, as described previously for other patients and isolates (Leitão et al., 2008). Although there was no indication of regular administration of gentamicin and tobramycin to the patient, the antibiotic therapy administered included the aminoglycoside amikacin, of the same antimicrobial class as gentamicin and tobramycin. However, no variation of resistance was found for meropenem and ceftazidime, both included in the patient’s therapy during the course of infection. According to the hospital records, intravenous therapy administered to the patient included the intercalated use of these two antibiotics – a practice that might have contributed to counteracting the development of resistance to meropenem and ceftazidime.

With regard to B. cenocepacia isolates, no significant alteration in the level of antimicrobial susceptibility could be identified between the first and subsequent isolates obtained, consistent with the short-term colonization period during which these bacteria were exposed to antibiotic therapy in the patient’s lungs. Moreover, the MIC values for most of the antimicrobials tested for the B. cenocepacia isolates were below those estimated for B. dolosa isolates or for another B. cenocepacia clone obtained during long-term infection of another CF patient (Coutinho et al., 2011a). This observation is consistent with the idea that the B. cenocepacia infection described here was recent and of environmental origin, which might have contributed to the fact that, in contrast to the generalized idea that B. cenocepacia bacteria replace other

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**Fig. 3.** (a) Colony morphology (I, smooth; II, rough; III, semi-rough), (b) swarming motility, (c) swimming motility and (d) cellular biomass in the biofilms formed by the sequential clonal isolates of B. dolosa (black bars) and B. cenocepacia (grey bars) retrieved from the same persistently colonized CF patient. Values are the results of at least three independent experiments; bars, sd. *P*<0.05 when each B. dolosa isolate was compared to IST4208.
Bcc species (B. multivorans, Burkholderia vietnamiensis, B. cepacia or B. dolosa) present in the lungs (Bernhardt et al., 2003), such replacement was not observed in this case study.

The cellular biomass in the biofilms produced by B. dolosa and B. cenocepacia isolates obtained along the colonization period also varied, but no clear variation pattern was found, as reported previously (Coutinho et al., 2011a; Cunha et al., 2003). Biofilm formation by Bcc has been linked with the high antibiotic resistance exhibited by these bacteria that when grown in biofilms exhibit a higher antibiotic resistance compared with planktonically grown bacteria (Carahe et al., 2007). However, no correlation could be established between the antibiotic resistance levels assessed in vitro and the cellular biomass produced by the corresponding isolates, reinforcing the idea that other mechanisms are likely to underlie the variation of antibiotic resistance levels.

A significant decrease in B. dolosa swarming motility from the first to the later isolates was registered. The remarkable exception of isolate IST4370 may be related to the higher biomass concentration that could be attained for this isolate at the stationary phase compared with all the other B. dolosa isolates, suggesting a more efficient growth in the testing medium. The reduction of swarming motility during the course of colonization was already described for other clonal isolates of B. cepacia retrieved during long-term colonization (Coutinho et al., 2011a), reinforcing the idea that this trait may be among the strategies used by Bcc bacteria to adapt to the CF lung during the progress of infection and deterioration of lung function. Differences registered at the level of swarming motility were attributed previously to the altered production of surfactants, which reduce surface friction and enable the smooth migration of groups of cells on semi-solid surfaces (Verstraeten et al., 2008).

This extensive and systematic study correlates the decline of FEV₁ values, the clinical parameter used to describe clinical deterioration in the patient, during long-term infection by a B. dolosa clone together with transient infections with other bacterial and fungal pathogens, including B. cepacia during the last stages of the disease. The inexorable decline in FEV₁ values from 18 months before isolation of B. dolosa until the patient’s death, 84 months later, also raises the question whether B. dolosa infection was a cause or a result of this decline. The phenotypic characterization of the Bcc isolates examined here reinforces the concept that clonal expansion, presumably reflecting the adaptive evolution of these bacteria, occurs in the lungs of CF patients during long-term colonization and the progress of the disease, in particular, involving an increased level of antimicrobial resistance and decrease of swarming motility. This systematic longitudinal study provides new insights into and important evidence of the potential clinical impact of B. dolosa species, rarely associated with CF.

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