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Hypermutation in *Burkholderia cepacia* complex is mediated by DNA mismatch repair inactivation and is highly prevalent in cystic fibrosis chronic respiratory infection

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ABSTRACT

The *Burkholderia cepacia* complex (Bcc) represents an important group of pathogens involved in long-term lung infection in cystic fibrosis (CF) patients. A positive selection of hypermutators, linked to antimicrobial resistance development, has been previously reported for *Pseudomonas aeruginosa* in this chronic infection setting. Hypermutability, however, has not yet been systematically evaluated in Bcc species. A total of 125 well characterized Bcc isolates recovered from 48 CF patients, 10 non-CF patients and 15 environmental samples were analyzed. In order to determine the prevalence of mutators their spontaneous mutation rates to rifampicin resistance were determined. In addition, the genetic basis of the mutator phenotypes was investigated by sequencing the *mutS* and *mutL* genes, the main components of the mismatch repair system (MRS). The overall prevalence of hypermutators in the collection analyzed was 13.6%, with highest occurrence (40.7%) among the chronically infected CF patients, belonging mainly to *B. cenocepacia, B. multivorans, B. cepacia*, and *B. contaminans* –the most frequently recovered Bcc species from CF patients worldwide. Thirteen (76.5%) of the hypermutators were defective in *mutS* and/or *mutL*. Finally, searching for a possible association between antimicrobial resistance and hypermutability, the resistance-profiles to 17 antimicrobial agents was evaluated. High antimicrobial resistance rates were documented for all the Bcc species recovered from CF patients, but, except for ciprofloxacin, a significant association with hypermutation was not detected. In conclusion, in the present study we demonstrate for the first time that, MRS-deficient Bcc species mutators are highly prevalent and positively selected in CF chronic lung infections. Hypermutation therefore, might be playing a key role in increasing bacterial adaptability to the CF-airway environment, facilitating the persistence of chronic lung infections.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a heterogeneous group of Gram-negative bacteria that comprises at least 18 closely related species (Mahenthiralingam et al., 2008; Peeters et al., 2013; Vanlaere et al., 2009, 2008). Bcc bacteria posses an extraordinary metabolic versatility, allowing an adaptation to a wide range of environments, such as water and soil, and also the infection and colonization of plants (Mahenthiralingam et al., 2008; Vanlaere et al., 2009). In addition, Bcc species have emerged as highly problematic human pathogens causing severe respiratory infections in cystic fibrosis (CF) patients and immunocompromised individuals (Baldwin et al., 2007; Drevinek and Mahenthiralingam, 2010).

Bcc species are intrinsically resistant to most clinically available antimicrobials, including aminoglycosides, quinolones, polymyxins, and β-lactams (Correia et al., 2008; Leitão et al., 2008; Mahenthiralingam et al., 2005; Zhou et al., 2007). In many instances these bacteria show multi- or panresistance so as to make their

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Table 1
Characteristics of the collection of 125 isolates of the *Burkholderia cepacia* complex used in this work.

Patient[a]	Strain	Species	Source	Sample	Chron. infec.	Time of chron. infec. (years)[b]	Year Isol.	Origin[c]	RFLP-*recA*[d]	BOX-PCR[e]	Mutation rate[f]
P1*	CBC001	*B. cepacia*	CF	sputum	+	2.8	2009	HST	E	–	6.01E−09
	CBC002	*B. cepacia*	CF	sputum	+	3	2009	HST	E	–	4.83E−09
	CBC003	*B. cepacia*	CF	sputum	+	3.1	2009	HST	E	–	**6.10E−08**
	CBC004	*B. cepacia*	CF	sputum	+	3.3	2009	HST	E	–	3.62E−08
	CBC005	*B. cepacia*	CF	sputum	+	4.1	2010	HST	E	–	6.69E−09
	CBC006	*B. cepacia*	CF	sputum	+	4.2	2010	HST	E	–	1.75E−08
	CBC007	*B. cepacia*	CF	sputum	+	4.5	2010	HST	E	–	5.98E−09
	CBC008	*B. cepacia*	CF	sputum	+	5	2011	HST	E	–	6.30E−09
	CBC009	*B. cepacia*	CF	sputum	+	5	2011	HST	E	–	**6.54E−07**
P2*	CBC010	*B. cepacia*	CF	sputum	+	1.8	2010	HNLP	E	–	5.04E−09
P3	CBC011	*B. cepacia*	CF	sputum			2010	HST	E	–	4.75E−09
P4	CBC012	*B. cepacia*	CF	sputum			2009	HCBA	E	–	1.26E−08
P5*	CBC013	*B. multivorans*	CF	sputum			2008	HNLP	F	–	2.74E−09
	CBC014	*B. multivorans*	CF	sputum	+	1.3	2009	HNLP	F	–	1.20E−08
P6	CBC015	*B. multivorans*	CF	sputum			2010	HNLP	F	–	5.25E−09
P7	CBC016	*B. multivorans*	CF	sputum			2012	HST	F	–	6.34E−09
P8*	CBC017	*B. multivorans*	CF	sputum			2010	HNLP	F	–	1.27E−08
	CBC018	*B. multivorans*	CF	sputum	+	0.4	2010	HNLP	F	–	**7.91E−07**
	CBC019	*B. multivorans*	CF	sputum	+	1.3	2011	HNLP	F	–	5.89E−09
	CBC020	*B. multivorans*	CF	sputum	+	1.5	2011	HNLP	F	–	1.21E−09
	CBC021	*B. multivorans*	CF	sputum	+	2.1	2012	HNLP	F	–	5.93E−09
	CBC022	*B. multivorans*	CF	sputum	+	2.3	2012	HNLP	F	–	2.25E−09
P9	CBC023	*B cenocepacia*	CF	sputum			2009	HNLP	J'	–	7.98E−09
	CBC024	*B. cenocepacia*	CF	sputum			2012	HNLP	G	–	3.77E−08
P10*	CBC025	*B. cenocepacia*	CF	sputum	+	3	2009	HST	G	–	**1.09E−07**
P11	CBC026	*B. cenocepacia*	CF	sputum			2012	HNLP	H	–	3.83E−09
P12*	CBC027	*B. cenocepacia*	CF	sputum	+	2.8	2009	HNLP	G	–	1.04E−08
	CBC028	*B. cenocepacia*	CF	sputum	+	4.2	2011	HNLP	G	–	2.86E−08
	CBC029	*B. cenocepacia*	CF	sputum	+	4.7	2011	HNLP	G	–	**5.43E−07**
	CBC030	*B. cenocepacia*	CF	sputum	+	5.3	2012	HNLP	G	–	**1.01E−06**
P13*	CBC031	*B. cenocepacia*	CF	sputum	+	1	2010	HNLP	G	–	2.90E−09
	CBC032	*B. cenocepacia*	CF	sputum	+	1	2010	HNLP	G	–	**6.88E−08**
	CBC033	*B. cenocepacia*	CF	sputum	+	1.6	2011	HNLP	G	–	3.30E−09
	CBC034	*B. cenocepacia*	CF	sputum	+	1.6	2011	HNLP	G	–	2.48E−08
P14*	CBC035	*B. cenocepacia*	CF	sputum	+	1.2	2009	HNLP	G	–	5.56E−09
P15	CBC036	*B. cenocepacia*	CF	sputum			2009	HNLP	G	–	4.70E−09
P16	CBC037	*B vietnamiensis*	CF	sputum			2008	HNLP	A	–	4.67E−09
P17*	CBC038	*B vietnamiensis*	CF	sputum	+	3.1	2012	HNLP	A	–	1.76E−09
P18*	CBC039	*B. seminalis*	CF	sputum			2003	HST	H	–	1.27E−09
	CBC040	*B. seminalis*	CF	sputum	+	4.6	2009	HST	H	–	1.30E−09
	CBC041	*B. seminalis*	CF	sputum	+	6.2	2009	HST	H	–	3.32E−09
	CBC042	*B. seminalis*	CF	sputum	+	6.3	2007	HST	H	–	4.66E−09
	CBC043	*B. seminalis*	CF	sputum	+	8.6	2011	HST	H	–	5.18E−09
P19*	CBC044	*B. contaminans*	CF	sputum			2004	HNLP	K	B8	4.84E−08
	CBC045	*B. contaminans*	CF	sputum	+	5.4	2009	HNLP	K	B8	9.68E−09
P20*	CBC046	*B. contaminans*	CF	sputum			2004	HST	K	B7	2.39E−09
	CBC047	*B. contaminans*	CF	sputum	+	1.2	2005	HST	K	B7	6.37E−09
P21*	CBC048	*B. contaminans*	CF	sputum	+	0.5	2010	HST	K	B3	3.20E−09
	CBC049	*B. contaminans*	CF	sputum	+	0.8	2010	HST	K	B3	1.90E−08
	CBC050	*B. contaminans*	CF	sputum	+	0.8	2010	HST	K	B4	3.13E−09
	CBC051	*B. contaminans*	CF	sputum	+	1.2	2010	HST	K	B1	3.46E−09
	CBC052	*B. contaminans*	CF	sputum	+	1.4	2011	HST	K	B1	4.80E−09
	CBC053	*B. contaminans*	CF	sputum	+	1.5	2011	HST	K	B1	8.13E−09
	CBC054	*B. contaminans*	CF	sputum	+	1.8	2011	HST	K	B1	**1.08E−06**
	CBC055	*B. contaminans*	CF	sputum	+	1.9	2011	HST	K	B1	1.79E−09

Table 1 (*Continued*)

Patient[a]	Strain	Species	Source	Sample	Chron. infec.	Time of chron. infec. (years)[b]	Year Isol.	Origin[c]	RFLP-*recA*[d]	BOX-PCR[e]	Mutation rate[f]
P22	CBC056	*B. contaminans*	CF	sputum			2012	HNLP	K	B2	6.90E−09
P23	CBC057	*B. contaminans*	CF	sputum			2009	HNLP	K	B8	3.55E−09
P24*	CBC058	*B. contaminans*	CF	sputum			2003	HST	K	B9	1.47E−09
	CBC059	*B. contaminans*	CF	sputum	+	0.9	2004	HST	K	B8	5.16E−09
	CBC060	*B. contaminans*	CF	sputum	+	0.9	2004	HST	K	B8	8.16E−09
P25*	CBC061	*B. contaminans*	CF	sputum			2005	HST	K	B8	4.95E−09
	CBC062	*B. contaminans*	CF	sputum	+	4.7	2009	HST	K	B1	**9.61E−08**
	CBC063	*B. contaminans*	CF	sputum	+	4.8	2009	HST	K	B1	4.81E−09
	CBC064	*B. contaminans*	CF	sputum	+	6.7	2011	HST	K	B1	5.49E−09
P26*	CBC065	*B. contaminans*	CF	sputum			2005	HST	K	B8	2.60E−09
	CBC066	*B. contaminans*	CF	sputum	+	2.1	2006	HST	K	B8	8.44E−09
P27*	CBC067	*B. contaminans*	CF	sputum			2004	HST	K	B1	4.56E−09
	CBC068	*B. contaminans*	CF	sputum	+	0.9	2005	HST	K	B1	1.67E−09
P28*	CBC069	*B. contaminans*	CF	sputum	+	2.6	2010	HNLP	K	B1	**5.96E−07**
	CBC070	*B. contaminans*	CF	sputum	+	3.3	2011	HNLP	K	B1	7.22E−09
	CBC071	*B. contaminans*	CF	sputum	+	3.4	2011	HNLP	K	B1	1.61E−09
P29*	CBC072	*B. contaminans*	CF	sputum	+	3.5	2011	HNLP	K	B1	2.55E−09
	CBC073	*B. contaminans*	CF	sputum	+	4.1	2012	HNLP	K	B1	**1.81E−06**
	CBC074	*B. contaminans*	CF	sputum	+	4.1	2012	HNLP	K	B1	**5.23E−05**
P30	CBC075	*B. contaminans*	CF	sputum			2010	HST	K	B1	3.99E−09
P31*	CBC076	*B. contaminans*	CF	sputum			2008	HNLP	K	B8	2.99E−09
	CBC077	*B. contaminans*	CF	sputum	+	1.2	2008	HNLP	K	B8	5.11E−08
	CBC078	*B. contaminans*	CF	sputum	+	2.2	2010	HNLP	K	B1	2.88E−09
P32	CBC079	*B. contaminans*	CF	sputum			2004	HNLP	K	B6	3.95E−09
P33	CBC080	*B. contaminans*	CF	sputum			2012	HNLP	K	B2	1.50E−08
P34*	CBC081	*B. contaminans*	CF	sputum	+	3.8	2009	HNLP	K	B5	6.66E−09
P35*	CBC082	*B. contaminans*	CF	sputum	+	1	2010	HNLP	K	B1	1.04E−08
P36	CBC083	*B. contaminans*	CF	sputum			2004	HST	K	B1	3.08E−09
P37	CBC084	*B. contaminans*	CF	sputum			2004	HNLP	K	B8	1.11E−08
P38	CBC085	*B. contaminans*	CF	sputum			2010	HST	K	B8	1.20E−09
P39	CBC086	*B. contaminans*	CF	sputum			2005	HNLP	K	B6	3.11E−09
P40*	CBC087	*B. contaminans*	CF	sputum	+	0.9	2004	HNLP	K	B6	1.87E−08
P41*	CBC088	*B. contaminans*	CF	sputum	+	0.7	2011	HNLP	K	B1	**1.07E−06**
	CBC089	*B. contaminans*	CF	sputum	+	1.0	2011	HNLP	K	B1	1.41E−08
P42*	CBC090	*B. contaminans*	CF	sputum			2004	HNLP	K	B6	3.30E−09
	CBC091	*B. contaminans*	CF	sputum	+	7.8	2011	HNLP	K	B1	3.54E−08
P43	CBC092	*B. contaminans*	CF	sputum			2010	HNLP	K	B1	1.04E−08
P44*	CBC093	*B. contaminans*	CF	sputum			2004	HNLP	K	B8	1.12E−08
	CBC094	*B. contaminans*	CF	sputum	+	5.3	2009	HNLP	K	B8	9.85E−09
	CBC095	*B. contaminans*	CF	sputum	+	6.7	2010	HNLP	K	B8	1.87E−09
	CBC096	*B. contaminans*	CF	sputum	+	6.8	2011	HNLP	K	B1	2.29E−09
P45	CBC097	*B. contaminans*	CF	sputum			2004	HNLP	K	B8	1.03E−08
P46	CBC098	*B. contaminans*	CF	sputum			2009	HNLP	J	B13	5.05E−09
P47*	CBC099	*B. contaminans*	CF	sputum	+	4.2	2009	HNLP	K	B8	**1.07E−06**
P48	CBC100	*B. contaminans*	CF	sputum			2009	HNLP	K	B14	7.73E−09
P49	CBC101	*B. multivorans*	No-CF	blood			2005	HNLP	F	–	5.46E−09
P50	CBC102	*B. cenocepacia*	No-CF	blood			2005	HNLP	G	–	7.83E−09
P51	CBC103	*B. cenocepacia*	No-CF	blood			2005	HCBA	H	–	**3.11E−07**
P52	CBC104	*B. contaminans*	No-CF	blood			2012	HNLP	K	B1	1.18E−08
P53	CBC105	*B. contaminans*	No-CF	blood			2011	HNLP	K	B9	7.16E−10
P54	CBC106	*B. contaminans*	No-CF	blood			2005	HCBA	K	B8	7.70E−09
P55	CBC107	*B. contaminans*	No-CF	blood			2005	HCBA	K	B5	3.28E−09
P56	CBC108	*B. contaminans*	No-CF	blood			2005	HCBA	AT	B5	**9.30E−06**
P57	CBC109	*B. contaminans*	No-CF	blood			2005	HCBA	K	B5	5.95E−09
P58	CBC110	*B. contaminans*	No-CF	blood			2005	HCBA	K	B8	9.99E−09

M1	CBC111	*B. cepacia*	Env	soil	2001	UNC	–	–	1.39E−08
M2	CBC112	*B.vietnamiensis*	Env	catheter	2009	HST	A	–	6.63E−09
M4	CBC113	*B. contaminans*	Env	filt. air	2004	HNLP	K	B5	6.10E−09
M5	CBC114	*B. contaminans*	Env	water	2004	HNLP	AT	B5	1.54E−08
M6	CBC115	*B. contaminans*	Env	water	2009	HNLP	AT	B4	5.07E−09
M7	CBC116	*B. contaminans*	Env	gel	2009	HNLP	AT	B12	2.73E−08
M8	CBC117	*B. contaminans*	Env	water	2008	HST	K	B5	1.27E−08
M9	CBC118	*B. contaminans*	Env	water	2007	HST	AT	B11	9.58E−09
M10	CBC119	*B. contaminans*	Env	ult. gel	2007	HST	AT	B11	3.01E−09
M11	CBC120	*B. contaminans*	Env	water	2011	HST	K	B11	2.71E−09
M12	CBC121	*B. contaminans*	Env	catheter	2005	HST	K	B1	9.65E−09
M13	CBC122	*B. contaminans*	Env	instrument	2009	HST	AT	B12	1.08E−08
M14	CBC123	*B. lata*	Env	water	2009	HST	K	–	**2.18E−06**
M15	CBC124	*B. lata*	Env	water	2009	HST	K	–	6.15E−10
M16	CBC125	*B. lata*	Env	water	2009	HST	K	–	1.01E−08

Mutation rate values indicated by boldface letters correspond to hypermutator isolates.

[a] Patients indicated with an asterisk (*) were considered as chronically infected.

[b] Time determined in years since each CF patient was defined as chronically infected by Bcc.

[c] HST, HNLP, HCBA and UNC correspond to bacterial isolates provided by Hospital de Niños de la Santísima Trinidad de Córdoba, Córdoba Province; Hospital de Niños de La Plata Sor María Ludovica, La Plata, Buenos Aires Province, Hospital de Clínicas, Buenos Aires city, and Universidad Nacional de Córdoba (Dr. A. Smania), respectively.

[d] All isolates were genotyped by *recA*-restriction-fragment-length polymorphism (PCR-*recA* RFLP) through digestion by the *HaeIII* enzyme.

[e] The BOX-PCR DNA fingerprinting technique was performed according to Martina et al. (2013).

[f] Mutation rate was measured as the occurrence of spontaneous resistance to 300 μg/ml rifampicin (Oliver et al., 2000). Isolates were considered hypermutators when their mutation rates were $>5 \times 10^{-8}$.

eradication extremely difficult. Thus, although for some patients a respiratory tract infection may occur that is only transient, the acquisition of Bcc most typically results in a chronic infection with acute exacerbations and a gradual decline in lung function. Among the Bcc species *B. multivorans, B. cenocepacia*, and *B. cepacia* have been reported to be the most frequently recovered worldwide, with some of their constituent strains being highly transmissible and pathogenic (Drevinek and Mahenthiralingam, 2010; Lipuma, 2010). In Argentina for many years Bcc species have infected a relatively small fraction of CF patients (less than 2.3%). Nevertheless, in early 2004 an outbreak involving many CF care centers and hospitals occurred and the incidence of Bcc reached values between 19 and 36% depending on the hospital (Bosch et al., 2008; Jordá-Vargas et al., 2008; Martina et al., 2013; Miñán et al., 2009). Since that outbreak occurred, despite the implementation of strict infection control protocols, Bcc species have been recovered from both CF and non-CF patients, industrial products, and environmental samples. In an epidemiological study of this Bcc population we have recently reported a remarkable high representation of *B. contaminans* isolates –almost 60% in CF patients– followed by *B. cenocepacia*, *B. cepacia*, *B. multivorans* (Martina et al., 2013).

During chronic infection, the CF airways represent an ecosystem in which the opportunistic bacterial pathogens evolve in response to stressing selection pressures –such as immune-defence challenges, antimicrobial therapy, and oxygen limitation– thus producing multiple phenotypic variants that emerge from an initial clonal lineage (Harrison, 2007; Oliver et al., 2000). In *Pseudomonas aeruginosa* the occurrence of diversification processes –including conversion to the mucoid phenotype, inactivation of quorum-sensing functions, resistance to antibiotics, alterations in lipopolysaccharides, loss of the Type-III secretion system, and an increase in the mutation rate leading to a hypermutator phenotype– have been extensively studied (Ciofu et al., 2005; Feliziani et al., 2010; Hoffman et al., 2009; Marvig et al., 2013; Oliver et al., 2000). Very recent studies suggest the existence of an important genetic variation and specific selection signatures in *B. dolosa* isolates recovered from CF patients (Lieberman et al., 2014). Therefore, an understanding of how these bacteria adapt to the CF-airway environment and resist both host defences and antimicrobial therapies would be most relevant and of a high priority.

Heritable hypermutator bacteria are those that have increased spontaneous-mutation rates that could result from defects in one of the several DNA-repair or error-avoidance systems (Oliver and Mena, 2010). In natural bacterial populations, an inactivation of any of the genes involved in the mismatch repair system (MRS) (i.e., *mutS, mutL, mutH*, or *uvrD)* has been found to be the most frequent mechanism leading to the emergence of mutator phenotypes (LeClerc et al., 1996). In this regard, the CF lung chronically infected by *P. aeruginosa* was the first natural environment to reveal a high prevalence of hypermutators, in contrast to what had been observed in acute infections (LeClerc et al., 1996; Oliver and Mena, 2010; Oliver et al., 2000). Soon thereafter, a marked increase in the retrieval of hypermutable strains from long-term infections of CF patients was reported for *Staphylococcus aureus* (Prunier et al., 2003), *Haemophilus influenzae* (Román et al., 2004), *Stenotrophomonas maltophilia* (Turrientes et al., 2010), and *Streptococcus pneumoniae* (Del Campo et al., 2005). These findings therefore, suggest that hypermutation might be playing a key role in increasing bacterial adaptability to a continuously changing and challenging environment, thus facilitating the persistence of chronic lung infections.

To our knowledge, hypermutator prevalence has not been reported in Bcc species so far. Therefore, the objective of this work was to evaluate the prevalence and mechanisms of hypermutation in populations of Bcc isolates obtained from different sources, including CF patients, non-CF patients and environmental

samples; the potential linkage between hypermutator phenotypes and chronic persistence or antimicrobial resistance was also explored.

Materials and methods

Bacterial isolates

A total of 125 Bcc isolates collected from 2004 to 2010 were used in this study (Table 1). The collection consisted in 100 clinical isolates belonging to 48 CF patients, with 10 retrieved from non-CF patients (Non-CF), plus 15 nonclinical isolates recovered from hospital settings and environmental samples (ENV). The clinical isolates were obtained from three different hospitals or CF reference centers: Hospital de Niños de La Plata "Sor María Ludovica" (HNLP), located in La Plata, Buenos Aires province; Hospital de Niños de la Santísima Trinidad (HST), in Córdoba province; and Hospital de Clínicas Buenos Aires (HCBA), located in Buenos Aires city (Table 1). Twenty-seven out of the 48 CF patients –indicated with an asterisk in Table 1– were chronically infected having had at least 3 positive Bcc cultures documented within a 6-month period (Nørskov-lauritsen et al., 2010). These patients were infected by Bcc species for time periods ranging from 6 months to almost 10 years. Among the cohort of isolates belonging to each chronically infected patient, 1 to 9 isolates was examined (Table 1).

All isolates were genotyped by PCR-*recA* sequencing and, -*gyrB* sequencing when the identification remained ambiguous. The isolates were maintained as both lyophilized and frozen stocks at −70 °C in Trypticase-soy broth with 10% (v/v) glycerol until further analysis.

Determination of mutation rates

The procedure used for the estimation of spontaneous mutation rates was adapted from previously established protocols (Harrison, 2007; Mena et al., 2008). Stated in brief, *ca.* 10^3–10^4 cells from an overnight culture in LB medium obtained from a single colony were subcultured into 3 ml of LB and incubated for 12 h at 37 °C. Subsequently, 100 μl from serial dilutions were seeded onto LB agar plates, and 100 μl onto LB agar plates supplemented with 300 μg/ml rifampicin. Colonies were counted after 24 h of incubation on LB plates and 36 h on LB-rifampicin plates. The results from at least five independent cultures were considered. Mutation rates were then estimated through a fluctuation assay by means of the FT program (Gould et al., 2007; Sniegowski et al., 2000). The *P. aeruginosa* PAO1 reference strain and its isogenic PAO1 *mutS* mutant (Mena et al., 2008) were used as nonmutator and hypermutator controls, respectively. Strains were considered hypermutators when their mutation rates were $>5 \times 10^{-8}$ mutations per cell, which is approximately 1 Log higher than the median mutation rate values obtained for all Bcc tested isolates (6.4×10^{-9}) and very similar to the break point that would be established (5.6×10^{-8}) according to previous recommendations based in values 20-fold higher than those of *P. aeruginosa* reference strain (Oliver et al., 2000).

PCR amplification, sequencing, and analysis of mutS and mutL genes

Genomic DNA was extracted through the use of a Qiagen DNA-isolation kit (QIAGEN). Table 2 indicates the primers used to amplify and sequence the *mutS* and *mutL* genes. The temperature protocol for the PCR amplifications was the following: 94 °C for 3 min, 28 amplification cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1–3 min, depending on the size of the DNA fragment to be amplified. The amplicons were analyzed by electrophoresis on an agarose-ethidium-bromide gel and further cleaned with the Gel Purification kit (QIAGEN); both strands were then directly sequenced by using their respective PCR primers (Macrogene Corp., USA). To score for mutations within the genes, the sequencing results were compared with the corresponding gene sequences by means of the BLAST program of the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/blast/).

Table 2
Primers used to amplify the *Burkholderia cepacia* complex MRS genes.

Primer	Sequence (5′ → 3′)[a]
mutS-F1	ATGACCACGCTGTCGCC
mutS-R1	GGTCTGGCCGCGCGTGAC
mutS-F2	GCCAGATCGCCGACGTC
mutS-F2.1	TTACGCGTGCCCGTCGG
mutS-R2	CGCAGTTCGTACAGCAGGT
mutL-F1	ATGTCCGATATCACCGAAACGG
mutL-F1.2	CGTGCTGCACGGCGACCG
mutL-R1	TCAGCGTGAGCTGATACCA

[a] Primers were designed according to the homologous regions recognized by the alignment of *mutS* and *mutL* sequences from different *Burkholderia cepacia* complex species reported in the Genbank (http://www.genbank.com) as follows: *B. cenocepacia* J2315 (AM747720.1); *B. cenocepacia* MC0-3 (CP000958.1); *B. cenocepacia* AU 1054 (CP000380.1); *B. lata* (CP000151.1); *B. cepacia* GG4 (CP003774.1); *B. ambifaria* MC40-6 (CP001025.1); *B. ambifaria* AMMD (CP000440.1); *B. vietnamiensis* G4 (CP000614.1); and *B. multivorans* ATCC 17616 (AP009385.1).

Sequence similarities to already sequenced Bcc MRS genes were determined through the use of the BLAST algorithm at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments were performed by means of the software ClustalW at MEGA 4.0 (http://www.megasoftware.net/mega4/mega.html). For the assembly of partial sequences of the *mutS* and *mutL* genes, the *Vector* NTI *Advance* 10.1.1 was used.

The effects of missense mutations on protein functioning were predicted by the SIFT software with default parameters (Ng and Henikoff, 2003). Mutations were considered as "not tolerated" at a $p < 0.05$.

All sequence data can be retrieved from the NCBI Sequence Read Archive (SRA) under the accession numbers indicated in Table 4.

Antimicrobial-susceptibility testing

The minimal inhibitory concentration (MIC) profile for the 125 Bcc isolates was determined through the use of the Vitek-automated-sensitivity-card (AST-N082) system (BioMérieux, France) for 17 antimicrobial agents according to the manufacturer's instructions. Isolates were classified as susceptible (S), intermediate (I), or resistant (R) according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) guidelines for *B. cepacia* (CLSI, 2011). For the antimicrobials which the CLSI does not include recommendations for Bcc, the breakpoints recommended for *P. aeruginosa* or *E. coli* were considered (CLSI, 2011). *B. cepacia* ATCC 25416, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 were used as controls.

Statistical analysis

Quantitative variables were compared by means of the Mann–Whitney *U*-test or the Student *t* test as appropriate. Categorical variables were compared by the χ^2 test. These statistics tests were performed through the use of the SPSS Statistics 11.0 package. *p* Values of less than 0.05 were considered statistically significant.

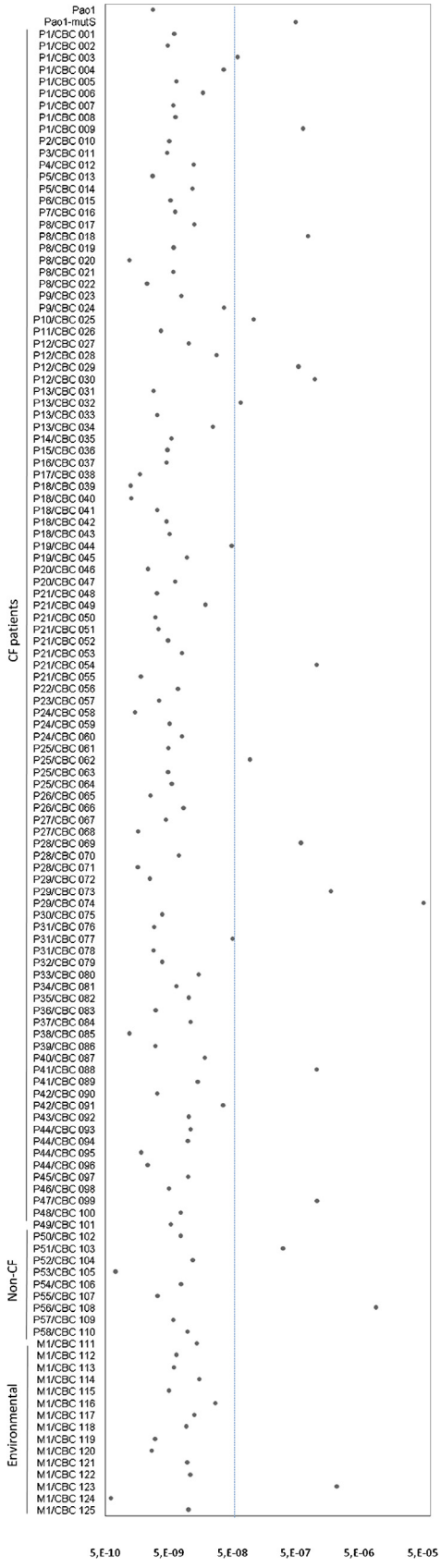

Fig. 1. Rifampicin mutation rates for the 125 *Burkholderia cepacia* complex isolates. Isolates were grouped according to their source: CF patients (100 isolates), non-CF (10 isolates), and environmental (15 isolates). The mutation frequency was measured as the occurrence of spontaneous resistance to 300 μg/ml rifampicin (Oliver et al., 2000). The *Pseudomonas aeruginosa* standard laboratory strain PAO1 and its isogenic PAO1mutS mutant were included as controls. The dashed line indicates the breakpoint to classify the isolates as hypermutators, established at $>5 \times 10^{-8}$ mutations per cell.

Table 3
Distribution of hypermutator isolates among the *Burkholderia cepacia* complex species.

Species	Total isolates ($n = 125$)	Mutator isolates (%) ($n = 17$)
B. cepacia	13	2 (15.4)
B. multivorans	11	1 (9.1)
B. cenocepacia	16	5 (31.3)
B. vietnamiensis	3	0
B. seminalis	5	0
B. contaminans	74	8 (10.8)
B. lata	3	1 (33.3)

Results

A high prevalence of hypermutators among the Bcc strains isolated from chronic respiratory infections as revealed through a determination of the mutation rates

We determined the mutation rates for the collection of 125 Bcc isolates. Seventeen isolates displayed hypermutator phenotypes (Fig. 1), where 14 belonged to 11 of the 48 CF patients (23%), giving a median mutation frequency of 7.23×10^{-7} (from 6.10×10^{-7} to 5.23×10^{-5}; Table 1). That all these patients were chronically infected by Bcc is notable. Indeed, hypermutator strains could be detected in up to 40.7% of the chronically infected CF patients, while none of the CF patients in which chronic infection was not documented showed hypermutator isolates. Moreover, the overall proportion of hypermutators among isolates from chronically infected CF patients (14 of 78) was also significantly higher ($p = 0.02$) than that of CF patients in which chronic infection was not documented (0 of 22). In addition, 2 hypermutators belonged to the group of non-CF patients and 1 to an environmental sample, giving mutation rates of 3.1×10^{-7}, 9.3×10^{-6}, and 2.2×10^{-6}, respectively, for those three sample groups. That the 2 non-CF patients from whom these hypermutators were retrieved were not chronically infected patients is also highly relevant.

Distribution of hypermutator strains among the Bcc species

Table 3 shows the distribution of hypermutator phenotypes among the different Bcc species. Almost 50% of the hypermutators encountered were *B. contaminans* isolates, with those comprising 8 recovered from 7 different CF patients, 6 of which hypermutators notably possessed the same genotype (BOX-PCR subtype B1, Table 1). Of the positive species *B. cenocepacia* exhibited an especially high representation of hypermutator phenotypes (5 isolates out of 16 recovered from 4 different CF patients; cf. Table 3). Nevertheless, *B. cepacia* was represented by only 2 hypermutator strains belonging to the same patient. The only environmental hypermutator isolate found was *B. lata*. Neither the *B. vietnamiensis* nor the *B. seminalis* strains manifested hypermutator phenotypes, though we must consider that small populations of both species were included in this study.

MRS inactivation: the main cause underlying hypermutability in Bcc strains

The *mutS* and *mutL* sequences in the Bcc hypermutator strains were analyzed to determine whether a mutation in these genes might have been responsible for the hypermutator phenotypes within this population. Our results revealed that 13 out of the

Table 4
Mutations in the *mutS* and *mutL* genes of the *Burkholderia cepacia* complex hypermutator strains.

Species	Patient	Strains	Mutation rate	*mutS* Accession number[a]	Mutation in *mutS*	*mutL* Accession number[b]	Mutation in *mutL*
B. cepacia							
	P1	CBC 003	6.10E−08	KJ540232	NF		ND
		CBC 009	6.54E−07	KJ540233	NF	KJ540249	C → G at 49 (A16G) G → T at 1102 (A368S) C → A at 1209 (T419P)
B. multivorans							
	P8	CBC 018	7.91E−07	KJ540234	G → A at 1405 (A469T) A → C at 2245 (I749L)		NA NA
B. cenocepacia							
	P10	CBC 025	1.09E−07	KJ540235	+1 C at 1473 (Stop at 632)		NA
	P12	CBC 029	5.43E−07	KJ540236	NF	KJ540250	C → T at 973 (Stop at 265)
		CBC 030	1.01E−06	KJ540237	+1 G at 1093 (Stop at 632)		NA
	P13	CBC 032	6.88E−08	KJ540238	+1 G at 1093 (Stop at 632)		NA
	P51	CBC 103	3.11E−07	KJ540246	−1 G at 1527 (Stop at 519)		NA
B. contaminans							
	P21	CBC 054	1.08E−06	KJ540239	NF	KJ540251	NF
	P25	CBC 062	9.61E−08	KJ540240	NF	KJ540252	NF
	P28	CBC 069	5.96E−07	KJ540241	NF	KJ540253	G → A at 1519 (E507 K)
	P29	CBC 073	1.81E−06	KJ540242	−1 A at 1561 (Stop at 632)	KJ540254	NF
		CBC 074	5.23E−05	KJ540243	G → C at 1888 (G630R)		NA
	P41	CBC 088	1.07E−06	KJ540244	NF	KJ540255	C → G at 810 (N270 K)
	P47	CBC 099	1.07E−06	KJ540245	NF		G → C at 1314
	P56	CBC 108	9.30E−06	KJ540247	C → T at 466 C → T at 1035 C → T at 1152 −3 ACG at 1328	KJ540256	G → A at 1185 (S395P) T → C at 1195 (S399P) G → A at 1396 (A469 T)
B. lata							
	M14	CBC 123	2.18E−06	KJ540248	G → T at 1078 (Stop at 360)		NA

NF, no mutation found; ND, not determined; NA, not PCR amplified; −, deletion; + insertion; amino-acid substitutions are shown between parentheses.
[a,b] GenBank accession numbers.

17 hypermutator isolates showed mutations in these main components of the MRS system (Table 4). In two additional isolates (CBC003 and CBC099) the *mutL* gene could not be efficiently amplified by PCR, suggesting that this strain could also be *mutL* deficient –though genetic complementation was not performed. As indicated in Table 4, 8 isolates were defective in *mutS*, 4 in *mutL*, and one (CBC 108 isolate) in both genes. These results suggested that *mutS* seemed to be the main target for the acquisition of a stable hypermutable state within the Bcc population. While mainly frameshift mutations were responsible for *mutS* inactivation –including deletions and insertions– that type of mutation was not observed for *mutL*. In *B. cenocepacia*, all the mutations led to premature (i.e., upstream) stop codons through frameshift and nonsense mutations (Table 4). In addition, the substitutions accounting for the inactivation of the MRS genes through missense and nonsense mutations were mostly found to be transversions (62.5%).

Antibiotic resistance: not strictly associated with hypermutability in Bcc species

For the entire collection of 125 Bcc isolates, we determined the minimal inhibitory concentration of 17 antimicrobial agents and the corresponding susceptibilities (Table S1 in the supplemental material). Taken as a whole, these results clearly confirmed the antibiotic-multiresistance trait that characterizes the Bcc species (Coenye and Vandamme, 2003; Mahenthiralingam et al., 2005; Zhou et al., 2007). Furthermore, the clinical isolates exhibited a resistance that was higher and to a greater number of antimicrobials than did the environmental isolates. Although different numbers of strains were investigated for each Bcc species, diverse patterns of antibiotic resistance were detected at the species level, as well as among isolates of the same species (see Tables S1 and S2 in the supplemental material).

The association between antimicrobial resistance and hypermutability has been well documented for other pathogens. In fact, particularly in *P. aeruginosa*, hypermutator strains recovered from CF patients were found to be resistant to a higher number of antibiotics than nonmutator strains (Oliver et al., 2000), and they also displayed an increased rate of acquisition of resistance to new antibiotics (Ferroni et al., 2009). In line with this, and considering that during long-term antibiotic treatment *B. cenocepacia* also develop higher-level resistance and resistance to different classes of antimicrobials (Mira et al., 2011), we wanted to investigate if, despite the overall high levels of antibiotic resistance observed in Bcc, hypermutator isolates from CF patients showed higher and to a greater number of antimicrobials resistance than nonmutator isolates. We therefore analyzed the percent antibiotic resistance to 17 different antimicrobial agents among the 14 hypermutator and the 86 non-hypermutator isolates recovered from the CF patients (Fig. 2). Although a tendency toward increased resistance among the hypermutator strains was observed for certain antibiotics, the differences reached statistical significance only for the fluoroquinolone ciprofloxacin. Moreover, no significant differences were observed in the number of antibiotics to which the strains were resistant. In fact, the hypermutators were resistant to an average of 10.6 ± 4.4 antibiotics versus the 10.3 ± 3.7 for the non-hypermutators. Therefore, no strict link between antibiotic resistance and MRS inactivation could be established in our collection of Bcc strains from CF patients.

Discussion

The bacteria of the Bcc are particularly threatening for CF patients. Although CF patients may be culture positive for Bcc without symptoms or long-term effect, in general long term culture positivity leads to chronic infection with progressive decline in

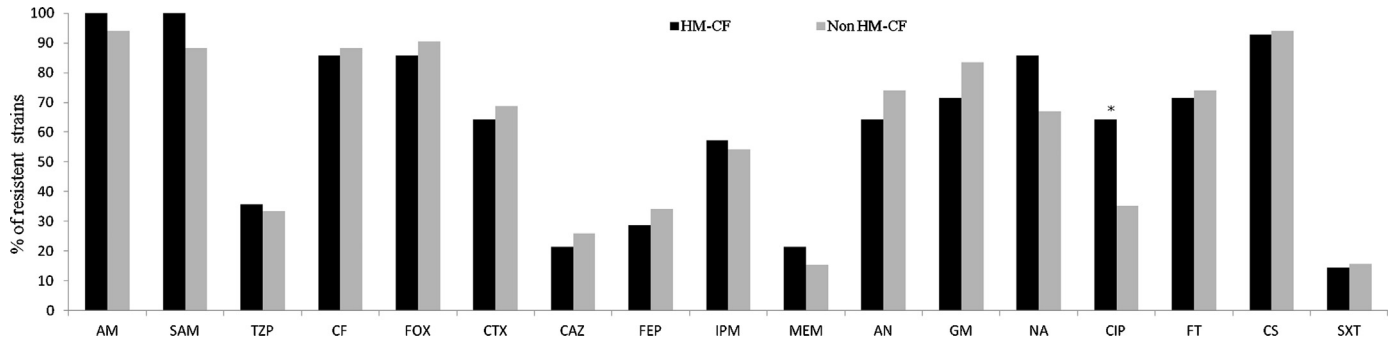

Fig. 2. Comparison between the antibiotic resistance rates of the *Burkholderia cepacia* complex hypermutator and nonhypermutator isolates. Hypemutator CF isolates (HM-CF) $n = 14$, black bars; nonhypermutator CF isolates (Non-HM-CF) $n = 86$, gray bars. Statistically significant differences are indicated with an asterisk. AM, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CF, cefalotin; FOX, cefoxitin, CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MEM, meropenem; AN, amikacin; GM, gentamicin; NA, nalidixic acid, CIP, ciprofloxacin; FT, nitrofurantoin, CS, colistin; SXT, trimethoprim sulfamethoxazole.

pulmonary function (Mahenthiralingam et al., 2002). Moreover, because of the intrinsic antimicrobial resistance of many strains, the eradication of Bcc chronic infections is a continuous challenge to patient management. For those reasons, a better understanding of how these bacteria adapt to the CF-airway environment and resist antibiotics along with the host defence mechanisms is critical. In the example of *P. aeruginosa*, an initial clonal lineage evolves during the course of a chronic infection leading to the emergence of multiple phenotypic variants as the result of genetic adaptation (Cramer et al., 2011; Mowat et al., 2011; Workentine et al., 2013). Previous reports have suggested that hypermutation may act as a catalyser of this process (Mena et al., 2008). Furthermore, although several studies in the field of infectious diseases caused by *P. aeruginosa* and other bacteria have provided evidences for a relevant role of mutators in the evolution of natural bacterial populations (Ciofu et al., 2005; Ferroni et al., 2009; LeClerc et al., 1996; Mena et al., 2008; Oliver et al., 2000; Román et al., 2004), the information on the genetic evolution of the Bcc during chronic respiratory infections is still limited.

In the present study, we assessed the prevalence of hypermutators within a wide and diverse Bcc population. By determining the mutation rates of 125 Bcc strains, we found an overall high prevalence (13.6% of the isolates) of hypermutators (Fig. 1). To our knowledge, hypermutability had been previously evaluated, but not observed, in a panel of *B. cenocepacia* ($n = 6$) and *B. multivorans* ($n = 2$) isolates. Nevertheless, those negative results might likely have been distorted because of the small number of strains analyzed (Pope et al., 2010). However, a very recent study detected one hypermutator strain among 5 CF patients infected with *B. dolosa* (Lieberman et al., 2014). In fact, although in the present work only one isolate was recovered as hypermutator among the 15 environmental samples, and 2 from early colonization and/or acute infections among the 10 non-CF isolates; we detected a high prevalence of hypermutator isolates among the chronically infected CF patients, with up to 40.7% of those chronically infected patients carrying such hypermutators. These figures are comparable only to those of chronic respiratory infections by *P. aeruginosa*, the natural setting with the highest reported prevalence of hypermutators (Harrison, 2007; Macia et al., 2005; Mena et al., 2008; Oliver et al., 2000). Nevertheless, the presence of Bcc hypermutators in non-CF patients or in the environmental populations analyzed here, notably enough, seems to be indicating that –as had been previously reported for *P. aeruginosa* (Kenna et al., 2007)– a long-term antibiotic therapy and/or an inflammatory host response along with the general stressing conditions of the CF lung might not be the only conditions influencing hypermutability in Bcc.

Another observation that is relevant to mention is that, 7 out 11 CF patients harboring hypermutator strains showed intermittent presence of hypermutator Bcc. It has been previously shown in *P. aeruginosa* obtained from CF chronic patients a coexistence of hypermutator and nonmutator isolates within the same sputum sample (Mowat et al., 2011; Oliver et al., 2000). Hence, one possible explanation for the apparently intermittent occurrence of hypermutators in our collection of Bcc isolates is the simultaneous presence of both, wild-type and hypermutator phenotypes, in CF Bcc populations. The sampling design in the present study –one Bcc isolate per sputum sample– does not permit to explore further this possibility.

In order to explain the molecular basis underlying hypermutability, we characterized the mutations that occurred in the *mutS* and/or *mutL* genes, the main components of the MRS. A large proportion of the hypermutator strains (76.5%) were the result of a MRS deficiency, and the prevalence of mutations in *mutS* indicated that gene as being the main site of hypermutability in Bcc (Table 4). This observation reinforces the notion that MRS inactivation is a frequent driver of stable hypermutability within clinical settings, as had been previously described for other species such as *P. aeruginosa* and *Staphylococcus aureus* (Oliver and Mena, 2010; Prunier et al., 2003). Nevertheless, since a minor proportion of hypermutable strains did not reveal any change in *mutS* or *mutL*, mutations in other genes might also be responsible for the occurrence of hypermutable phenotypes in Bcc isolates.

Previous studies had shown that the elevated mutation capacity of hypermutator strains accelerated genetic adaptation, thus enhancing the accumulation of new mutations (Mena et al., 2008). In particular, hypermutability could constitute a source of mutations in genes conferring antibiotic resistance since alterations in such genes (*e.g.*, *ampC, emrB, ftsI, fusA, gyrA/B, mexB/Y, pmrB, pprA, oprD*, and *rpoB/C*) have been found to confer resistance against a wide range of antibiotics (Oliver et al., 2002; Strateva and Yordanov, 2009). Accordingly, the increased rates of antibiotic resistance documented in CF patients have been shown to be related to the high frequency of hypermutable *P. aeruginosa*, *S. aureus*, and *H. influenzae* isolated from those patients' airways (Feliziani et al., 2010; Harrison, 2007; Hoboth et al., 2009; Román et al., 2004). Our present results from a systematic comparison of the antimicrobial susceptibilities of serial isolates of different Bcc species belonging to diverse sources (CF, non-CF, and nonclinical isolates) indicated the highest rates of antibiotic resistance in that collection of Bcc isolates to be found in the strains harbored by the CF patients (Tables S1 and S2 in the supplemental material). Nevertheless, we observed a significant association between increased antibiotic resistance and hypermutators for only fluoroquinolone ciprofloxacin (Fig. 2). Whether or not that specific association was influenced by the well established mutagenic effect of the fluoroquinolones (Blázquez et al., 2012), however, remains to be determined. In conclusion,

since no significant differences were observed between hypermutator and non-mutator CF isolates with respect to the distribution of the multidrug-resistant phenotype –except for ciprofloxacin– the high prevalence of Bcc hypermutators observed could instead be explained by their co-selection with other mutations involved in the bacterial pathoadaptability to the CF lung environment. Thus, further investigations on the nature of those selected genetic traits will be needed to reveal key aspects of the pathogenesis, the evolution of virulence, transmissibility and persistence of Bcc in CF patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.08.011.

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