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Burkholderia cenocepacia strains isolated from cystic fibrosis patients are apparently more invasive and more virulent than rhizosphere strains

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Summary

Given the widespread presence of *Burkholderia cenocepacia* in the rhizosphere it is important to determine whether rhizosphere strains are pathogenic for cystic fibrosis patients or not. Eighteen *B. cenocepacia* strains of rhizosphere and clinical origin were typed by multi-locus sequence typing (MLST) analysis and compared for their ability to invade pulmonary epithelial cells and their virulence in a mouse model of airway infection. Although there was great variability, clinical strains were the most invasive *in vitro.* Almost all the rhizosphere and two clinical strains were defined as non-invasive, six clinical strains as invasive, and two strains of both clinical and environmental origin as indeterminate. Exposure of murine airways to clinical strains caused higher acute mortality than that seen after challenge with rhizosphere strains. Furthermore, both clinical and environmental strains were able to persist in the lungs of infected mice, with no significant differences in bacterial loads and localization 14 days after challenge. DNA dot blot analyses of AHL synthase, porin and amidase genes, which play a role in *B. cenocepacia* virulence, showed that they were present in *B. cenocepacia* strains irrespective of their origin. Overall, our results suggest that rhizosphere strains do not differ from clinical strains in some pathogenic traits.

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Introduction

Burkholderia cenocepacia bacteria are widely present in a variety of habitats such as agricultural soils, urban settings, freshwater habitats and the rhizosphere of several crop plants (for a review see Chiarini *et al*., 2006). In particular, *B. cenocepacia* can be recovered in high numbers from the rhizosphere of maize (*Zea mays*), where it represents one of the predominant species of the *Burkholderia cepacia* complex (BCC) (Coenye and Vandamme, 2003; Vandamme *et al*., 2003). *Burkholderia cenocepacia* is also an important opportunistic human pathogen responsible for devastating lung infections in cystic fibrosis (CF) patients (Mahenthiralingam and Vandamme, 2005; Reik *et al*., 2005), leading to unpredictable outcomes, ranging from asymptomatic carriage to a fulminant and fatal pneumonia, the so-called 'cepacia syndrome' (Govan and Deretic, 1996; Mahenthiralingam *et al*., 2002). The wide environmental spread of *B. cenocepacia* has raised concern about the existence of natural reservoirs of bacterial strains that may exhibit pathogenic traits when acquired by CF patients. Indeed, infection control measures, including patient segregation, have reduced but not eliminated new infections, and CF patients may occasionally become infected by isolates showing novel fingerprint types. In addition, genotypically identical *B. cenocepacia* strains have been isolated from both CF patients and environmental sources, suggesting that the acquisition of pathogenic strains may occur directly from the natural environment (LiPuma *et al*., 2002; Speert *et al*., 2002; Payne *et al*., 2005; Baldwin *et al*., 2007; Mahenthiralingam *et al*., 2008).

Due to a high content of nutrients, the rhizosphere may be a natural reservoir for opportunistic human pathogenic bacteria (Berg *et al*., 2005). Various bacterial species, including *B. cenocepacia*, contain root-associated strains that may enter bivalent interactions with plant and human hosts (Coenye and Vandamme, 2003; Chiarini *et al*., 2006). It has been speculated that *B. cenocepacia* can

colonize both human lung epithelial and plant root cells through similar mechanisms responsible for recognition and adherence to host cells (Cao *et al.*, 2001). To date, studies on the infectivity and pathogenicity of *B. cenocepacia* have been performed on clinical strains only, providing growing evidence that these strains are endowed with a wide range of virulence determinants (Scordilis *et al.*, 1987; Speert, 2001; Schwab *et al.*, 2002; Mahenthiralingam *et al.*, 2005), which have been related to their ability to survive and persist in host cells (Valvano *et al.*, 2005). Although the pathogenic potential of rhizosphere *B. cenocepacia* isolates has been poorly addressed, it has been found that some phenotypic traits (i.e. biofilm formation, antibiotic susceptibility, exopolysaccharide production) and genetic markers associated with virulence and transmissibility are also spread among environmental isolates of *B. cenocepacia* (Bevivino *et al.*, 2002; Chiarini *et al.*, 2002; 2004; Baldwin *et al.*, 2004). Nevertheless, the virulence of environmental strains has not been investigated yet.

In the present work, we explored the virulence of some environmental *B. cenocepacia* strains, most of them isolated from the maize rhizosphere, and clinical *B. cenocepacia* strains isolated from patients with CF, all typed by multi-locus sequence typing (MLST) analysis, by using *in vitro* and *in vivo* models of airway infection. Furthermore, the presence/absence of cci genes relevant in virulence and persistence was investigated (Baldwin *et al.*, 2004). The purposes of this study were (i) to determine whether rhizosphere and clinical *B. cenocepacia* strains differ in their capacity to invade respiratory epithelial cells (A549) and to cause lung pathogenesis in a murine infection model, and (ii) to investigate the association between invasiveness in the *in vitro* and *in vivo* models and the AHL synthase, porin and amidase genes, previously shown to be involved in both virulence and persistence.

Results

Molecular typing

Eight environmental and 10 clinical *B. cenocepacia* strains were analysed by a MLST scheme, recently validated for both BCC strain and species discrimination (Baldwin *et al.*, 2005). Among the *B. cenocepacia* strains used in this work, nine strains had already been typed by MLST in previous studies (Table 1), while the other nine were typed in this work. The latter were successfully sequenced at all

Table 1. *Burkholderia cenocepacia* strains used in this work.

Isolate name	Origin	Reference or source	RFLP type	ST	Allelic profile						
					atpD	*gltB*	*gyrB*	*recA*	*lepA*	*phaC*	*trpB*
B. cenocepacia recA lineage IIIA											
LMG16656^T (J2315)	CF-e (UK)	Hutchison *et al.* (1998)	G	28	16	11	10	14	11	6	79
FC43[a]	CF (Italy)	Manno *et al.* (2004)	G	234	15	11	182	14	11	6	147
FC50[a]	CF-e (Italy)	Manno *et al.* (2004)	G	452[b]	16	11	10	238	11	6	79
Mex1[a]	Maize rhizosphere (Mexico)	Kindly provided by J. Caballero-Mellado	G	423[b]	15	11	358	15	271	6	147
POPR8	Radish (Mexico)	Baldwin *et al.* (2005)	G	32	16	11	10	14	11	6	79
B. cenocepacia recA lineage IIIB											
PHDC (AU1482)[a]	CF-e (USA)	LiPuma *et al.* (2002)	J′	448[b]	23	16	57	15	93	8	14
LMG16654 (J415)	CF (UK)	Glass and Govan (1986)	J′	34	17	107	119	15	93	6	13
FC24[a]	CF (Italy)	Manno *et al.* (2004)	J′	453[b]	16	136	57	15	93	8	14
FC67[a]	CF-e (Italy)	Manno *et al.* (2004)	I	451[b]	55	39	369	39	24	30	38
FC87[a]	CF (Italy)	Manno *et al.* (2004)	I	447[b]	67	252	227	15	198	6	185
MDII-151p	Maize rhizosphere (Italy)	Dalmastri *et al.* (2007)	I	158	23	135	86	122	93	8	14
MDII-129r	Maize rhizosphere (Italy)	Dalmastri *et al.* (2007)	I	159	16	136	57	15	128	8	14
MDII-143p	Maize rhizosphere (Italy)	Dalmastri *et al.* (2007)	I	139	16	128	141	49	117	105	124
MCII-168	Maize rhizosphere (Italy)	Dalmastri *et al.* (2007)	J′	138	107	127	139	49	116	41	122
BC-1	Maize rhizosphere (USA)	Baldwin *et al.* (2005)	H	37	17	65	57	15	69	8	14
B. cenocepacia recA lineage IIIC											
LMG19230	Wheat-root endophyte (Australia)	Balandreau *et al.* (2001)	H2	44	65	49	41	47	33	36	44
B. cenocepacia recA lineage IIID											
FC7[a]	CF-e (Italy)	Manno *et al.* (2004)	U	46	55	39	32	39	24	30	38
FC49[a]	CF-e (Italy)	Manno *et al.* (2004)	U	46	55	39	32	39	24	30	38

a. Strains typed by multi-locus sequence typing (MLST) in the present study.
b. New STs obtained in the present study. The other STs are present in the *Burkholderia cepacia* complex MLST database (http://pubmlst.org/bcc/).
ST, sequence type; CF, infection of a CF patient; CF-e, strain that has spread epidemically among patients with CF; RFLP type, *recA* Restriction Fragment Length Polymorphism (RFLP) with *HaeIII*; superscript T, type strain for *B. cenocepacia* species.

the loci, resulting in a total of eight sequence types (STs), of which six were novel when compared with existing STs in the MLST database, and were designed with novel arbitrary numbers (Table 1). Indeed, only two already known STs were recovered: ST-234, which was assigned to the *B. cenocepacia* IIIA strain FC43 and was also present in six CF Canadian strains belonging to this *recA* lineage, as reported in the database (http://pubmlst.org/bcc/), and ST-46, which was assigned to the two *B. cenocepacia* IIID isolates FC7 and FC49, and had been already observed in one CF Italian isolate belonging to this *recA* lineage (Baldwin *et al*., 2005). There were no shared STs between clinical and environmental strains examined in this study; however, the environmental strains POPR8 and BC-1 shared their STs (ST-32 and ST-37 respectively) with some clones isolated from CF patients' sputum (Baldwin *et al*., 2005; 2007).

Internalization of rhizosphere and clinical B. cenocepacia *strains into human respiratory epithelial cells*

To investigate whether there were differences between rhizosphere and clinical *B. cenocepacia* strains in their ability to invade human respiratory epithelial cells, all environmental and clinical strains (Table 1) were used to infect human alveolar epithelial A549 cells. There was great variability in the invasion levels among *B. cenocepacia* strains, with the mean percentage of invasion ranging from 0.0013% (MDIIr129) to 0.025% (LMG19230) for environmental strains and from 0.0053% (FC87) to 10.35% (FC43) for clinical strains (Table 2). Overall, clinical *B. cenocepacia* strains appeared to be more invasive than rhizosphere strains (Mann–Whitney test, $P < 0.001$). Next, by using a statistical model we compared the degree of invasiveness of clinical and rhizosphere strains with positive and negative controls. We used the clinical invasive *B. cenocepacia* strain LMG16656^T as positive control (Cieri *et al*., 2002) and the non-invasive *Escherichia coli* strain DH5α as negative control. Almost all the rhizosphere *B. cenocepacia* strains were defined as non-invasive, as well as two clinical strains; in addition, six clinical strains were defined as invasive. No strain was found to be statistically more invasive than the positive control, although strain FC43 was 10-fold more invasive. One clinical strain and one environmental strain were categorized as indeterminate, as they were more invasive than the negative control but less invasive than the positive control. When we compared the degree of invasiveness of five rhizosphere and five clinical strains belonging to the *recA* lineage IIIB, which is the only situation that allowed a proper comparison between environmental and clinical isolates of a *recA* III subgroup, the rhizosphere IIIB strains invaded epithelial cells with significantly lower percent invasion than the clinical ones (Mann–Whitney test, $P < 0.001$).

Table 2. Invasiveness of clinical (CF) and environmental (ENV) *B. cenocepacia* strains in the *in vitro* model.

Strain	Origin	% Invasion[a]
Invasive strains[b]		
FC43	CF	10.3559 (5.9230)
FC7	CF	1.2176 (1.5090)
FC49	CF	0.7176 (0.7176)
LMG16654	CF	0.1969 (0.0932)
PHDC	CF	0.1799 (0.2515)
FC67	CF	0.0541 (0.0252)
Non-invasive strains[c]		
MCII-168	ENV	0.0134 (0.0019)
MDII-143p	ENV	0.0121 (0.0026)
FC50	CF	0.0111 (0.0059)
POPR8	ENV	0.0062 (0.0047)
FC87	CF	0.0053 (0.0049)
BC-1	ENV	0.0040 (0.0013)
MDII-151p	ENV	0.0033 (0.0014)
Mex1	ENV	0.0032 (0.0021)
MDII-r129	ENV	0.0013 (0.0007)
Indeterminate strains[d]		
LMG19230	ENV	0.0251 (0.0125)
FC24	CF	0.0139 (0.0107)
Control strains[e]		
LMG16656^T	CF	1.0120 (0.6846)
E. coli DH5α	Laboratory	0.0006 (0.0003)

a. Mean (standard deviation) based on the results of three A549 cell invasion assays.
b. Significantly more invasive than the negative control ($P < 0.001$) and not significantly different from the positive control ($P > 0.05$).
c. Not significantly different from the negative control ($P > 0.05$) and significantly less invasive than the positive control ($P < 0.001$).
d. Significantly more invasive than the negative control ($P < 0.001$) and significantly less invasive than the positive control ($P < 0.001$).
e. *B. cenocepacia* strain LMG16656^T and *E. coli* DH5α were used as positive and negative controls respectively; superscript T, type strain for *B. cenocepacia* species.

Screening of rhizosphere and clinical B. cenocepacia *strains in a murine model of airway infection*

First, the clinical *B. cenocepacia* reference strain LMG16656^T was embedded in agar beads and challenged intratracheally in C57Bl/6 mice. A total dose of 5×10^7 colony-forming units (cfu) per mouse was found to cause chronic lung infection during 1 month after challenge (Fig. S1). All of the mice challenged with strain LMG16656^T developed chronic lung infection with a mean of 3×10^4 cfu per lung at day 7. Thereafter, no significant differences in the cfu per lung ($P > 0.05$, *t*-test) and in the percentage of infected mice ($P > 0.05$, Fisher's test) were detected during 1 month after challenge (Fig. S1). Spleens and livers of infected mice were free of bacteria at all time points and no mortality was detected, indicating that the strain LMG16656^T had adapted to establish chronic infection and to persist in the murine lung but did not induce acute mortality.

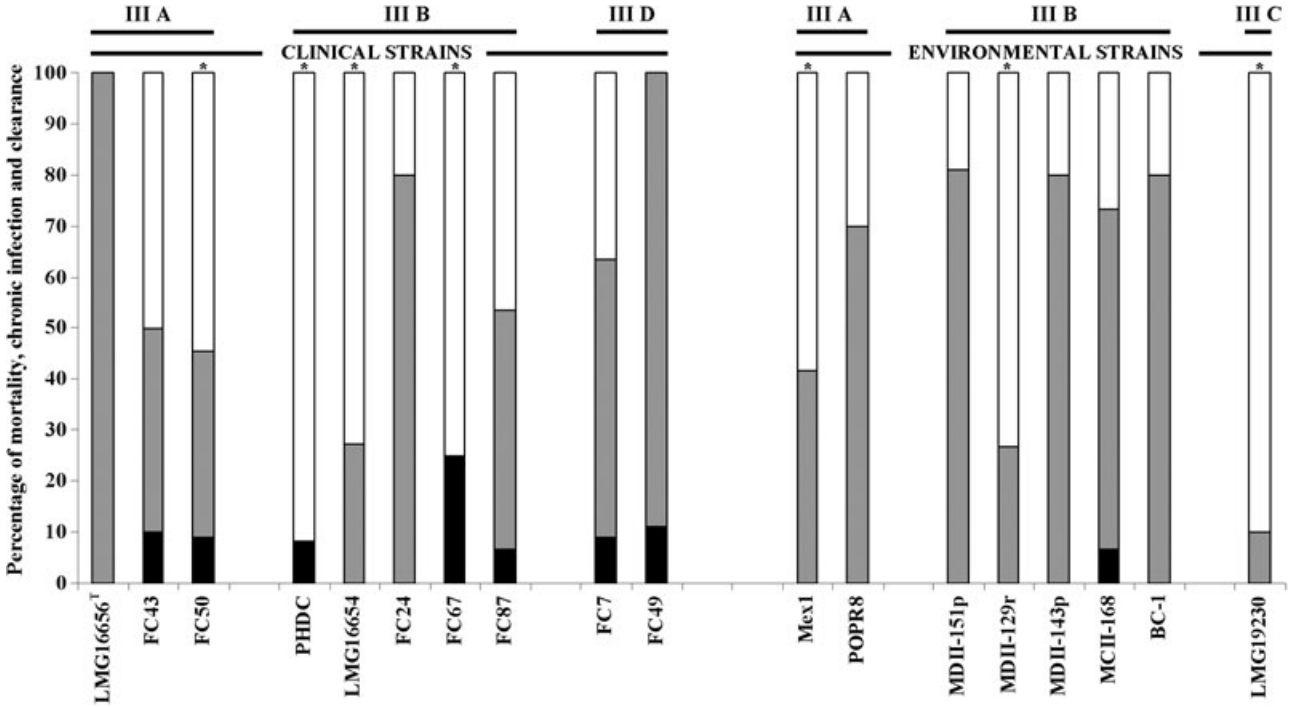

Fig. 1. Virulence of clinical and environmental *B. cenocepacia* strains embedded in agar beads and introduced intratracheally in C57Bl/6 mice. The *x*-axis reports *B. cenocepacia* strains, and the *y*-axis reports the percentage of infected (grey), dead (black) and not infected (white) mice for each strain. C57Bl/6 mice were infected with 5×10^7 cfu per lung of *B. cenocepacia* strains and infection, mortality or clearance were determined at 14 days after challenge. Strains indicated by an asterisk (*) were significantly less virulent than LMG16656^T.

Next, all *B. cenocepacia* strains were evaluated for their capacity to induce acute mortality and chronic persistence 14 days after challenge. By analysing all *in vivo* data (mortality, chronic infection and clearance), statistical analysis revealed that all *B. cenocepacia* strains, irrespective of their origin, showed pathogenic properties (Table S1 and Fig. 1). When we considered acute mortality and chronic infection separately, *B. cenocepacia* strains of environmental and clinical origin showed statistical differences both in term of mortality (logistic regression model: environmental strains versus clinical strains $P < 0.05$) and in term of chronic infection (environmental strains versus clinical strains, $P < 0.05$). Exposure of murine airways to *B. cenocepacia* clinical strains caused higher acute mortality than challenge with environmental isolates (clinical strains versus environmental strains 7.6% versus 0.8%). However, *B. cenocepacia* environmental strains were able to persist in murine airways more efficiently than those of clinical origin (clinical strains versus environmental strains 45.7% versus 61.7%) pointing to the ability of all *B. cenocepacia* strains to persist in murine airways. Among the mice surviving to the infection there were no significant differences in the bacterial loads recovered from the lung indicating that chronic infection is always established with a defined dose, independently of the strain and its origin (clinical strains versus environmental strains, $P > 0.05$).

Comparison within the *recA* lineage IIIB revealed no statistically significant difference in mortality between *B. cenocepacia* IIIB strains of clinical and rhizosphere origin ($P > 0.05$) and a higher persistence of environmental strains than clinical ones in the murine airways ($P < 0.05$), with no statistically significant difference in number of recovered bacteria from the lung of infected mice ($P > 0.05$). Finally, when comparing the degree of virulence of single *B. cenocepacia* strains with that of strain LMG16656^T, five strains of environmental origin (POPR8, MDII-151p, MDII-143p, MCII-168 and BC-1) and five strains of clinical origin (FC24, FC43, FC87, FC7, FC49 and LMG16654) were found to be as virulent as the strain LMG16656^T. A moderate association between invasiveness in the *in vitro* model and *in vivo* virulence was found (Cramer's $V = 0.257$).

Localization of B. cenocepacia *in the murine lung*

Lung histology of infected mice and localization by indirect immunofluorescence of clinical strain LMG16656^T and rhizosphere strain Mex1, belonging to the *recA* lineage IIIA, were investigated. The results showed that the agar beads were deposited in the bronchial lumen and contained *B. cenocepacia* LMG16656^T (Fig. 2A–C) and Mex1 microcolonies 1 day after challenge (data not shown). At this time point, an intense recruitment of inflammatory cells

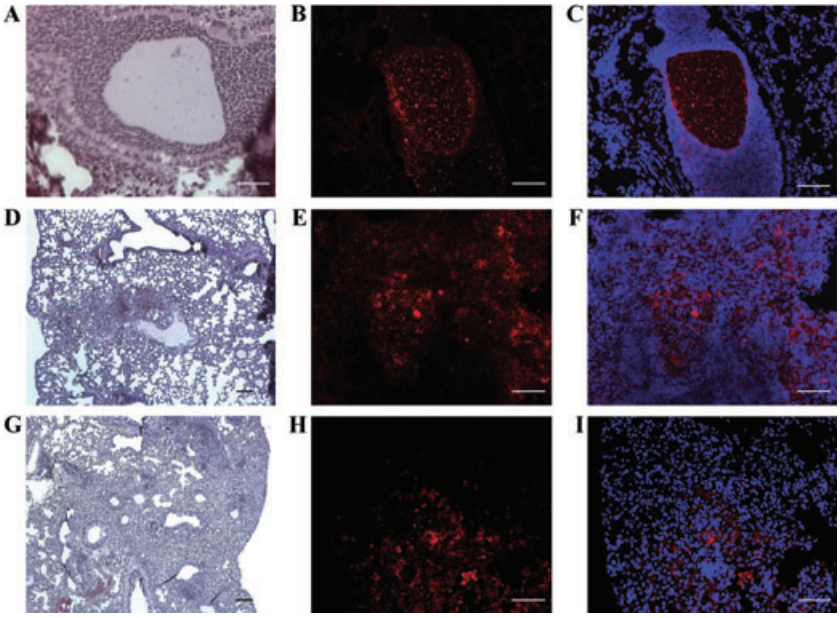

Fig. 2. Localization of *B. cenocepacia* within agar beads (A–C) and lung tissues of infected mice (D–I). C57Bl/6 mice were infected with *B. cenocepacia* embedded in agar beads, and lungs were investigated histologically by indirect immunofluorescence.
A. Agar beads containing bacterial microcolonies of LMG16656^T, deposited in the bronchial lumen, 1 day after challenge.
B. Bacterial microcolonies detected by indirect immunofluorescence in an agar bead 1 day after intratracheal injection.
C. 4′,6′-Diamidino-2-phenylindole dihydrochloride (DAPI) staining merged with the immunostaining of the same tissue section as that in (B).
D and G. Inflammatory cell infiltration in the thickened alveolar septa of murine lung infected with strain LMG16656^T(D) and Mex1 (G) 14 days after challenge.
E and H. Localization of LMG16656^T bacterial microcolonies (E) and Mex1 (H) by indirect immunofluorescence.
F and I. Merge between immunofluorescence and DAPI staining of the same tissue section as that in (E) and (H).
Bars in (A)–(I), 50 μm.

was observed that surrounded the agar beads in the bronchial lumen. Fourteen days after bacterial challenge, microscopic examination of haematoxylin and eosin (H&E)-stained lung tissue sections from mice challenged with LMG16656^T and Mex1 strains revealed an extensive inflammatory cell infiltrate in the thickened alveolar septa (Fig. 2D and G). LMG16656^T and Mex1 cells were visualized by indirect immunofluorescence as microcolonies in the alveoli (Fig. 2E, F, H and I).

Presence of B. cenocepacia *genomic island (cci) genes*

B. cenocepacia strains were analysed by Southern hybridization for the presence of genes included in the cci genomic island: AHL synthase gene (*ccil*), porin gene (*opcl*), amidase gene (*amil*), arsenic resistance gene (BCAM0236), IS transposase (BCAM0248), stress protein (BCAM0278) and the hypothetical gene (BCAM0280). The results (Table 3) revealed that these genes are differently distributed among the strains analysed with the exception of LMG16656^T and FC43 strains that harbour the entire set of genes (Table 3). No differences in the presence of *cci* genes between clinical and rhizosphere strains were observed ($P > 0.05$). We then analysed our data focusing on the AHL synthase and porin genes, both involved in virulence, and on the amidase gene that has been shown to play a role in persistence (Baldwin *et al*., 2004). In our study, these genes were not closely linked to virulent strains; in fact, strains not possessing these genes were able to invade epithelial cells and to be virulent in mice (strains FC7 and FC49). When we examined the correlation between the presence of these genes and the degree of virulence of bacterial strains *in vitro*, we found a strong correlation for the presence of the porin gene (Cramer's $V = 0.584$), whereas the correlation was moderate and weak for AHL synthase and amidase genes respectively (Cramer's $V = 0.24$ and 0.134). Correlation analysis performed with *in vivo* data revealed that the presence of the amidase gene is not correlated to the degree of virulence in the murine model (Cramer's $V = 0.04$), and a moderate correlation was found for the presence of AHL synthase and porin genes (Cramer's $V = 0.316$ and 0.255 respectively). In addition, we found that the genes mentioned above (AHL synthase, porin and amidase) were present among *B. cenocepacia* strains irrespective of their origin (Cramer's $V = 0.079$, 0.040 and 0.255 respectively).

Discussion

This study compares rhizosphere and clinical *B. cenocepacia* strains for their potential pathogenicity by using *in vitro* and *in vivo* models of airways infection. To the best of

Table 3. Distribution of *B. cenocepacia* genomic island (cci) genes mapped by DNA probings of *B. cenocepacia* DNA dot blots.

Isolate name	*recA* lineage	Arsenic resistance gene (BCAM0236)	AHL synthase (*ccil*)	IS transposase (BCAM0248)	BCESM (*esmR*)	Amidase (*amil*)	Porin (*opcl*)	Stress protein (BCAM0278)	Hypothetical gene (BCAM0280)
Clinical									
LMG16656^T	IIIA	+	+	+	+	+	+	+	+
FC43	IIIA	+	+	+	+	+	+	+	+
FC50	IIIA	+	+	–	+	+	+	+	+
PHDC	IIIB	+	+	+	–	–	+	–	+
LMG16654	IIIB	+	–	+	–	–	–	–	–
FC24	IIIB	+	+	–	+	+	+	–	+
FC67	IIIB	–	–	+	–	–	+	–	–
FC87	IIIB	+	+	–	+	+	+	–	–
FC7	IIID	–	+	–	–	–	+	–	–
FC49	IIID	+	–	–	–	–	+	+	+
Environmental									
Mex1	IIIA	+	+	+	+	+	+	+	–
POPR8	IIIA	–	–	+	+	+	–	+	–
MDII-151p	IIIB	–	+	–	+	+	+	–	+
MDII-129r	IIIB	+	+	–	+	+	+	–	+
MDII-143p	IIIB	–	–	–	–	–	+	–	+
MCII-168	IIIB	+	+	+	+	+	+	–	–
BC-1	IIIB	+	+	–	+	+	+	–	–
LMG19230	IIIC	–	–	–	–	–	+	–	–

Superscript T, type strain for *B. cenocepacia* species.

our knowledge, this is the first evaluation of the pathogenic characteristics of rhizosphere strains of *B. cenocepacia.* We asked whether environmental *B. cenocepacia* strains could behave as pathogens showing virulence properties similar to those of clinical and virulent strains.

Lung epithelia represent the first barrier against bacterial infection; consequently, interaction of bacteria with epithelial cell surfaces represents a crucial step in the colonization and subsequent establishment of the infection process (Mohr *et al*., 2001). Previous studies suggested that the level of epithelial cell invasion by an environmental isolate of *Burkholderia multivorans* did not differ significantly from that of strains of clinical origin, whereas environmental *Burkholderia cepacia* and *Burkholderia vietnamiensis* isolates appeared to be non-invasive (Martin and Mohr, 2000; Cieri *et al*., 2002). Until now, no environmental *B. cenocepacia* strain had been tested in the *in vitro* invasion assays. In the present work, we tested rhizosphere *B. cenocepacia* isolates in comparison with clinical strains in an *in vitro* invasion assay, by using the pulmonary epithelial cell line A549, which has been used to quantify BCC invasion of a range of strains in an antibiotic protection assay (Burns *et al*., 1996; Mahenthiralingam *et al*., 2005). Overall, we found that *B. cenocepacia* strains were internalized by epithelial cells at a low level, unlike other intracellular pathogens such as *Legionella pneumophila*, *Salmonella typhimurium* and *Brucella abortus* (Small *et al*., 1987). In addition, as *B. cenocepacia* has intrinsically low invasiveness (Burns *et al*., 1996), variability can affect the ability to reveal differences among strains. Thus, we used a systematic statistical model to analyse our data, which accounts for the variability and relatively low-level invasiveness shown by *B. cenocepacia.* This kind of statistical analysis, which has the advantage of permitting comparisons of data from a large number of strains, has some limitations, such as the selection of control strains which are used to define whether a strain is invasive or not. To overcome this limit, we accurately chose and verified the control strains; thus we used as positive control the LMG16656^T strain, which had been previously reported to be invasive (Cieri *et al*., 2002), and as negative control the *E. coli* DH5α strain. The results obtained revealed that six clinical strains were invasive and all but one rhizosphere strain were non-invasive. However, individual rhizosphere strains showed a similar or even higher percentage of invasion than some clinical strains, with a maximum invasion frequency of 0.02%.

As chronic infection is a characteristic of colonization of individuals with BCC, to further investigate the pathogenic potential of environmental strains, we used the agar bead mouse model of chronic infection (Mahenthiralingam *et al*., 2005; Valvano *et al*., 2005). Although it bypasses the normal entry and colonization route of BCC, this system permits to monitor for 7–21 days infection occurring in the lungs by BCC or other CF-related pathogens such as *Pseudomonas aeruginosa* (van Heeckeren and Schluchter, 2002; Mahenthiralingam *et al*., 2005; Montanari *et al*., 2007). We have previously shown that the agar beads provide microaerobic/anaerobic conditions for bacterial growth (Bragonzi *et al*., 2005), resembling those found in the lung of CF patients (Worlitzsch *et al*., 2002), and it has recently been used to test virulence of clinical strains isolated from CF patients (Montanari *et al*., 2007).

Our results revealed a highly efficient chronic infection with the clinical *B. cenocepacia* strain LMG16656^T lasting for at least 1 month in a high number of treated mice. The observation that *B. cenocepacia* LMG16656^T is capable of persisting in the host appears to be consistent with the clinical findings that *B. cenocepacia* infections in CF patients are chronic and difficult to eradicate (Govan and Deretic, 1996).

Next, all *B. cenocepacia* strains were evaluated for their capacity to induce mortality and chronic infection 14 days after challenge. By analysing mortality and chronic infection data together, statistical analysis revealed that rhizosphere strains were found to be as virulent as the clinical ones. Owing to the nature of the agar bead infection model, overall mortality of mice infected with *B. cenocepacia* strains was low; however, we observed that mortality was significantly greater in mice infected with clinical strains than in those treated with environmental ones, in agreement with the *in vitro* data. This result is of particular concern as *B. cenocepacia* can invade human lung epithelia and cause bacteraemia in CF patients (Duff *et al*., 2006). When we examined the ability to establish chronic infection at 14 days post-infection, rhizosphere *B. cenocepacia* strains were able to persist in the murine lung longer than clinical strains. Histological analysis of mouse lung sections showed that although chronic infection is initiated in the bronchi where agar beads were deposited soon after challenge, long-term chronic infection is maintained in the alveolar septa where bacterial cells, released by agar beads, were localized. An extensive inflammatory cell infiltrate in the thickened alveolar septa of mice following infection with strain LMG16656^T was found. Although similar findings have been reported for *B. cenocepacia* strain J2315 by Tomich and colleagues (2003) and for *B. cepacia* in other animal models (Sokol *et al*., 1999; Chiu *et al*., 2001; Sajjan *et al*., 2001a), long-term chronic infection has not previously been observed in lung tissues of infected mice. Interestingly, indirect immunofluorescence staining of infected lungs from mice infected with the rhizosphere *B. cenocepacia* strain Mex1 revealed an extensive inflammatory cell infiltrate in the thickened alveolar septa, suggesting that *B. cenocepacia* strains of clinical and rhizospheric origin have a similar capacity to maintain a chronic respiratory infection that may be due to production of similar virulent factors in strains of different origin. A moderate association between invasiveness in the *in vitro* model and *in vivo* virulence was found. This suggests that in addition to a set of virulence factors required for pathogenicity in all hosts, there are also factors that are specific for particular hosts.

During chronic colonization bacterial pathogens become able to adapt to the 'local environmental' conditions of CF airways (Smith *et al*., 2006; Menard *et al*., 2007), and evolve specific virulence mechanisms to evade host defences contributing to establish a chronic and destructive lung infection (Nguyen and Singh, 2006; Tümmler, 2006). Virulence factors characterized in *B. cenocepacia* and known to play a role in the pathogenic process include factors inducing necrosis in lung tissue, such as porins and *N*-acyl homoserine lactones, and factors that promote survival and persistence during *in vivo* growth, for example, amidase, a protein involved in amino acid metabolism (Baldwin *et al*., 2004). Southern hybridization analysis of the AHL synthase, porin and amidase genes on the *B. cenocepacia* cci, demonstrated that they do not provide evidence of distinctive characteristics that could be used to differentiate clinical and rhizosphere strains, as well as virulent and avirulent *B. cenocepacia* strains; in fact, they were absent in strains that were found to be invasive in epithelial cells and virulent in mice (FC7 and FC49). Our findings corroborate previous data on the presence of these genes in *B. cenocepacia* environmental strains and their absence in significant numbers of *B. cenocepacia* strains which were all associated with CF infection (Baldwin *et al*., 2004), suggesting that although they play a role in virulence and persistence of *B. cenocepacia* in the lung, they are not essential for pathogenicity, as already pointed out by Van Baarlen and colleagues (2007).

To date, no clear pathogenic potential to humans has been established for plant-associated *B. cenocepacia*. There is no clear evidence that strains from the rhizosphere directly colonize the human body, but the appearance of unique clones in individual patients suggests that they may be independently acquired from the environment (Parke and Gurian-Sherman, 2001). Indeed, LiPuma and colleagues (2002) reported that the *B. cenocepacia* type strain HI2424, isolated from an onion field, was indistinguishable by several typing methods [pulsed-field gel electrophoresis (PFGE) genomic fingerprinting and repetitive extragenic palindromic (rep)-PCR] from isolates of the epidemic CF lineage PHDC, recovered from a high number of CF patients in the mid-Atlantic region of the USA. In addition, a recent analysis of a diverse collection of BCC isolates performed by MLST identified identical *B. cenocepacia* sequence types (STs) from environmental and clinical sources, one of which (ST-32) was found to be a predominant global epidemic strain (Baldwin *et al*., 2007). These findings suggest that acquisition of pathogenic strains can occur directly from the natural environment. Our study reinforces the idea that potential reservoirs outside of clinics may play a role in the acquisition of infections. Interestingly, we found that strains POPR8 (isolated from a radish in Mexico) and BC-1 (isolated from the maize rhizosphere in the USA), which were identified as ST-32 and ST-37, respectively, and for which some clones were also

isolated from the sputum of CF patients (Baldwin *et al.*, 2005; 2007), were able to persist in the lungs of infected mice, with 70% and 80% of infection rate 14 days after challenge, respectively, suggesting the pathogenic potential of environmental strains.

In conclusion, the results of the present study, in which both *in vitro* and *in vivo* models were used, clearly demonstrate that rhizosphere *B. cenocepacia* strains do not differ from strains isolated from CF patients in some pathogenic traits. Overall, there is no correlation between the origin of isolates and their pathogenicity, and different strains regardless of their origin may display different patterns of pathogenicity. The presence of numerous insertion sequences and of at least one pathogenicity island may enable the exchange and recombination of virulence factors, favouring the adaptation of these microbes to multiple environments.

Experimental procedures

Bacterial strains

A total of 18 *B. cenocepacia* strains of environmental (eight) and clinical (10) origin were used in this study (Table 1), representative of the different *recA* phylogenetic subgroups. We have not included environmental isolates for *B. cenocepacia* IIID because this lineage has not been recovered from natural habitats; similarly, as no isolates of *B. cenocepacia* IIIC have been recovered from CF patients yet, we only included the environmental strain LMG19230 (Mahenthiralingam *et al.*, 2008). The 10 clinical strains included organisms obtained from the CF Centre of Gaslini Hospital, Genoa, Italy (Manno *et al.*, 2004), and from the following collections: the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Gent, Belgium, and the US *Burkholderia cepacia* Research Laboratory and Repository, Ann Arbor, Michigan, USA. The eight environmental strains included organisms isolated from the maize rhizosphere in Italy and Mexico, and organisms obtained from the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Gent, Belgium, and from the Cardiff University Collection, Cardiff, Wales, UK. All strains were cryo-preserved at –80°C in 30% (v/v) glycerol. Prior to assays, bacteria were streaked from frozen stock preparations onto Nutrient Agar (NA, Difco) plates and incubated at 30°C for 24–48 h.

Multi-locus sequence typing

Bacterial DNA was extracted by following the procedure described by Giovannetti and colleagues (1990). The nucleotide sequences of seven independent genetic loci (*atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC* and *trpB*) were determined using nested primers as described previously (Baldwin *et al.*, 2005). Sequencing reactions were prepared by using Applied Biosystem Big Dye® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analysed with a ABI PRISM 310 Genetic Analyser Perkin-Elmer, at the ENEA Genome Research Facility DNA Sequencing Laboratory (Genelab, ENEA C.R. Casaccia, Italy). The forward and reverse sequences of each locus for each isolate were aligned, trimmed to the desired allele length using SeqMan II (DNA Star software), and compared with existing alleles in the BCC MLST database at http://pubmlst.org/bcc/ (Jolley *et al.*, 2004). The respective alleles numbers were then assigned for each locus. The allelic profiles were compared using the MLST database in order to assign the proper ST to each strain allelic profile, indicative of each genetically distinct strain (Baldwin *et al.*, 2005). New sequences as well as new allelic profiles, when found, were submitted to the website curator A. Baldwin to assign their arbitrary numbers and respective STs and add the new data to the BCC MLST database.

Detection of B. cenocepacia *genomic island (cci) genes*

Non-radioactive Southern hybridization was performed by dot blot using 1 μg and 0.5 μg of genomic DNA samples from each *B. cenocepacia* strain which were heat-denatured at 100°C for 10 min and aliquoted into 96-well plates (BIO-DOT™ APPARATUS, Bio-Rad) using positively charged nylon membranes (Roche Diagnostic). Each filter contained two genomic DNA samples from *P. aeruginosa* PAO1 as a negative control, and from LMG16656^T, as positive control as it was positive by PCR screening to all the *cci* genes. The spotted DNAs were fixed on the membrane by cross-linking with UV light 1800 μJ (Stratalinker, Stratagene). Hybridization, washing and detection with CSPD were performed in accordance with the manufacturer's protocols (Roche Diagnostic). The membranes were exposed to autoradiography film (Lumi-Film for chemiluminescent detection, Roche Molecular Biochemicals) at room temperature for up to 18 h. Control hybridizations to the genomic DNA present in each spot were performed using a 16S rRNA gene probe. The autoradiograms were photographed and analysed by Kodak-ID software for quantitative analysis.

PCR and probe labelling

cci genes probes were prepared by PCR in the presence of the non-radioactive label digoxigenin (DIG)-UTP as described by the manufacturer (Roche Diagnostic) and using *Taq* DNA polymerase (Fermentas) in 25 μl of reaction mixture containing 20 ng of template DNA and 10 pmol of each primer. Primers and reaction conditions were as reported by Baldwin and colleagues (2004). The control 16S rDNA probe was generated pooling the PCR products amplified with the universal eubacterial primers P0 and P6 (5′-GAGAGTTTGATCCTGGCTCAG-3′; 5′-GAGAGTTTGATCCTGGCTCAG-3′; Di Cello *et al.*, 1997) from DNA of each *B. cenocepacia* strain.

Cell line and invasion assay

The human alveolar epithelial carcinoma cell line A549 from American Type Culture Collection CCL-185 was grown in Ham's F12 culture medium (Gibco, USA), supplemented with 1% Penicillin-Streptomycin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA) in a humidified 5% CO_2 incubator

at 37°C. Bacteria invasion assay was performed by using ceftazidime-amikacin protection assay with minor modifications (Martin and Mohr, 2000). First, we assessed that all strains used in this study were susceptible to the antibiotic concentrations used in the invasion assay. We found that a combination of ceftazidime (1 mg ml^{-1}) and amikacin (1 mg ml^{-1}) incubated with each *B. cenocepacia* strain for 2 h resulted in more than 99.99% killing (less than 5 and 10 cfu were recovered from environmental and clinical strains, respectively, with an initial inoculum of $1–2 \times 10^7$ cfu). *B. cenocepacia* strains were grown in Nutrient Broth (NB, Difco) medium until mid-exponential phase (OD_{600} of 0.5–0.6). Cells were harvested at 4°C, washed twice with phosphate-buffered saline (PBS; pH 7.0) and diluted in tissue culture serum-free medium. The cell suspension was used to infect cell monolayers (approximately 80% confluence) in 24-well tissue culture plates at a multiplicity of infection (MOI) of 100:1 (2.5×10^7 bacteria per 2.5×10^5 cultured cells). The infected monolayers were centrifuged (800 *g* per 5 min) and incubated at 37°C in 5% CO_2 for 2 h to allow bacterial entry. The monolayers were then washed three times with PBS, and tissue culture containing a combination of ceftazidime and amikacin was added. After 2 h of incubation, the cell monolayers were washed and lysed with H_2O_{dd}. The intracellular bacteria were quantified by plating serial dilutions of the lysates on NA (Difco) plates. All quantitative invasion assays were performed in triplicate, on three different occasions. Cell viability after bacteria infection was determined by trypan-blue counting. The non-invasive strain *E. coli* DH5α was routinely used as a negative control, and LMG16656^T strain, which has been previously reported to be invasive (Cieri *et al.*, 2002), was used as a positive control. These control strains were selected previously based on an approximately 3-log difference in invasiveness in the *in vitro* model.

Agar bead preparation and mouse model

Agar beads (100–200 μm in diameter) containing *B. cenocepacia* strains were prepared according to the method of Cash and colleagues (1979), with minor modifications. Bacteria were cultured overnight in NB to the stationary phase. The cells were harvested by centrifugation and re-suspended in 1 ml of PBS (pH 7.4). Bacteria were added to 9 ml of 1.5% NA (w/v) in PBS, which was kept at 50°C for bead preparation. The number of *B. cenocepacia* cfu in the beads was determined by plating serial dilutions of the homogenized bacteria-bead suspension on NA plates. The inoculum was prepared by diluting the bead suspension with PBS to 5×10^9 cfu ml^{-1}. C57Bl/6 male mice (Charles-River; weight, 22–24 g) were infected as described elsewhere (Starke *et al.*, 1987). Briefly, mice were anaesthetized by an intraperitoneal injection of a solution of 2.5% Avertin (2,2,2-tribromoethanol and tert-amyl alcohol) (Sigma-Aldrich, Italy) in 0.9% NaCl and administered in a volume of 0.015 ml g^{-1} body weight. Mice were then placed in dorsal recumbency and the trachea was directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22 g cannula (Becton Dickinson, Italy) attached to a 1 ml syringe. A 100 μl inoculum of an agar-bead suspension was implanted via the cannula into the lung, with both lobes inoculated. After 1, 7, 14 and 28 days, mice were killed by an overdose of CO_2, and the lungs, livers and spleens were removed aseptically, and homogenized in PBS. For quantitative bacteriology, 100 μl of appropriately serial diluted lung homogenate samples were plated on NA, and incubated at 37°C. The experiments were performed twice by using groups of mice ranging from 8 to 15 individuals for each *B. cenocepacia* strain (Table S1). The animal research protocol was reviewed and approved by the IACUC, HS-Raffaele Animal Care and Use Committee.

Histology and immunolocalization of B. cenocepacia *in murine lung sections*

Murine lungs were removed *en bloc*, fixed in 4% paraformaldehyde/PBS for 24 h at 4°C, and processed for paraffin embedding according to the procedure reported in Bragonzi and colleagues (2005). Five-micrometre longitudinal sections were taken with a microtome at regular intervals from the proximal, medial and distal lung regions. Mounted sections were stained with H&E and evaluated for qualitative pathological changes. De-paraffinized sections of murine lung tissue were analysed by indirect immunofluorescence using a polyclonal rabbit antibody to *B. cepacia* (R418) kindly provided by U. Sajjan (Sajjan *et al.*, 2001b). Non-specific binding sites were blocked with 10% swine serum (Dako) and the slides were incubated with R418 (dilution 1:50). The secondary antibody was Texas red-labelled goat anti-rabbit IgG (Molecular Probes). The slides were examined using Axioplan fluorescence microscope (Zeiss) and images were taken by KS 300 imaging system (Kontron).

Statistical analysis methods

Analysis of in vitro data. In order to summarize cell invasion assay results in terms of percentage of invasion for each *B. cenocepacia* strain, we computed descriptive statistics using the invasive strain LMG16656^T as a positive control (Martin and Mohr, 2000; Cieri *et al.*, 2002) and *E. coli* DH5α as a negative control, following the analysis of covariance models described in Cieri and colleagues (2002). This approach has been made non-parametric by using the rank transformation described in Conover and Iman (1982). The ranked number of bacteria recovered for each strain was adjusted for the ranked number of bacteria inoculated and day effects. Pairwise comparisons between each strain and the positive control strain and between each strain and the negative control strain were made, by using a two-sample *t*-test for the adjusted means. The significance level of the tests was adjusted for multiple comparisons by using the Bonferroni correction method. Invasive, non-invasive and indeterminate strains were classified according to Cieri and colleagues (2002) as follows: invasive strain, significantly more invasive than the negative control and not significantly different from the positive control; non-invasive strain, not significantly different from the negative control but significantly less invasive than the positive control; indeterminate strain, significantly more invasive than the negative control and significantly less invasive than the positive control. The statistical significance of data obtained from different groups of strains (clinical versus environmental strains; clinical IIIB

versus environmental IIIB strains) was determined by calculating the *P*-values with the Mann–Whitney test.

Analysis of in vivo data. First, we estimated the effect of each strain with respect to the number of cfu for infected mice, if there was more than one infected mouse for a given strain. We used the non-parametric Kruskal–Wallis test as follows: cfu = $\alpha + \beta 1$ strain, conditionally on infection. We then defined a new categorical response variable 'status': whether a mouse cleared the infection, was infected or dead. To analyse clearance, mortality and chronic infection data together, we fitted a polytomic logistic ANOVA model: logit (pj) = $\alpha j + \beta 1$ strain, where pj is the probability for a mouse of being in status j (not infected, infected or dead). Significance was assessed obtaining confidence intervals for the log-odds ratios through the profile likelihood. Second, we investigated mortality and persistence separately with the use of binary logistic ANOVA models logit (p) = $\alpha + \beta 1$ strain, where p was, respectively, the probability of a mouse to be dead (versus alive, even if infected) or to be infected (versus clear). The effect of strain origin (clinical or environmental) was investigated similarly.

Analysis of Southern hybridization data. The relationships between the presence or absence of cci genes, *recA* lineage status and source of bacterial strains were analysed by means of chi-square tests applied to the opportune contingency tables. The significance levels were estimated by means of Monte Carlo simulations to cope with the lack of asymptotic properties of chi-square statistics. We finally standardized the chi-square statistics, thus obtaining Cramer's *V* statistics, to evaluate the strength of the relationship found. The magnitudes of association were interpreted using Rea and Parker's (1992) conventions for describing the magnitude of association in contingency tables: no relationship, the Cramer's *V* index ranging from 0.00 to 0.10; weak relationship, the Cramer's *V* index ranging from 0.10 to 0.20; moderate relationship, the Cramer's *V* index ranging from 0.20 to 0.40; strong relationship, the Cramer's *V* index greater than 0.40.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Subpopulation of *B. cenocepacia* strain LMG16656^T selected in the lungs of agar bead-infected C57Bl/6 mice. C57Bl/6 mice were infected with 5×10^7 colony-forming units (cfu) per lung of *B. cenocepacia* strain LMG16656^T, and mortality was determined. Various groups of mice were killed at indicated time points, for the determination of the number of cfu per lung and the percentage of infected mice.
A. The growth curve of strain LMG16656^T in murine lungs is shown. Dots represent individual measurements of cfu per lung, and horizontal lines represent median values.
B. Mortality, percentage of LMG16656^T-infected mice and number of cfu per lung (median) are also shown. Values were selected from two different experiments.
n, number of pooled mice analysed.

Table S1. Mortality and persistence of clinical and environmental *B. cenocepacia* strains in the murine airways.

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