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Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae

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Members of the taxonomically diverse *Burkholderia cepacia* complex have become a major health risk for patients with cystic fibrosis (CF). Although patient-to-patient transmission of *B. cepacia* strains has been well-documented, very little is known about possible vehicles of transmission and reservoirs for these micro-organisms. In this work, it is shown that strains of the *B. cepacia* complex can survive within different isolates of the genus *Acanthamoeba*. Trophozoites containing bacteria developed profuse cytoplasmic vacuolization. Vacuolization was not detected in trophozoites infected with live *Escherichia coli* or heat-killed *B. cepacia*, or by incubation of trophozoites with filter-sterilized culture supernatants, indicating that metabolically active intracellular bacteria are required for the formation of vacuoles. Experiments with two different *B. cepacia* strains and two different *Acanthamoeba* isolates revealed that bacteria display a low level of intracellular replication approximately 72–96 h following infection. In contrast, extracellular bacteria multiplied efficiently on by-products released by amoebae. The findings suggest that amoebae may be a reservoir for *B. cepacia* and possibly a vehicle for transmission of this opportunistic pathogen among CF patients.

Keywords: *Burkholderia cepacia*, *Acanthamoeba*, cystic fibrosis, intracellular survival

INTRODUCTION

Burkholderia cepacia is a soil micro-organism first described by Burkholder (1950) as the agent causing bacterial soft rot in onions. *B. cepacia* strains display an extraordinary metabolic versatility, being capable of degrading a wide variety of organic compounds, as well as surviving in nutritionally limited environments (Beckman & Lessie, 1979). Such properties have attracted considerable interest from agricultural researchers attempting development of *B. cepacia* strains for use as biological control agents to combat soilborne plant pathogens, and to decontaminate soils containing toxic pesticides and herbicides (Govan & Vandamme, 1998). However, in recent years *B. cepacia* has increasingly been found associated with life-threatening lung infections in humans, which may occur in patients requiring mechanical ventilation, as well as those with cystic fibrosis (CF) and chronic granulomatous disease (Govan *et al.*, 1996; Govan & Deretic, 1996; Isles *et al.*, 1984; Tablan *et al.*, 1985). Therapy of *B. cepacia* infections is problematic since these micro-organisms are intrinsically resistant to a wide range of antimicrobial agents (Prince, 1986). Recent taxonomic studies have shown that *B. cepacia* isolates, cultured from clinical or environmental sites, belong to at least five different genomovars, collectively referred to as the *B. cepacia* complex (Vandamme *et al.*, 1997). The available taxonomic evidence indicates that there is no clear distinction between environmental and clinical species within the complex.

A major concern with *B. cepacia* in CF is that some strains can spread from patient-to-patient, both within and outside the hospital. Molecular typing of isolates from patients and contacts demonstrated that transmission is strain-specific (LiPuma *et al.*, 1990). Clinical isolates can survive for long periods in respiratory droplets on environmental surfaces typically found in CF clinics (Drabick *et al.*, 1996). Very little is known about vehicles for transmission of *B. cepacia*. Recently,

Abbreviation: CF, cystic fibrosis.

Michel & Hauröder (1997) reported the presence of intracellular *Ralstonia* (*Burkholderia*) *pickettii* in a strain of *Acanthamoeba* isolated from the tiles of a physiotherapy room. Free-living amoebae are widely distributed in the environment, and humans may frequently come into contact with these organisms, particularly with their resistant cysts (Mergeryan, 1991). *Acanthamoeba* and other types of amoebae can colonize humans and may be isolated from respiratory sources (De Jonckheere, 1991; Kilvington & White, 1994; Michel & Hauröder-Philippczyk, 1994). Intracellular survival of bacteria in amoebae offers additional protection against disinfectants (Kilvington & Price, 1990) and may contribute to their enhanced environmental survival (Boulanger & Edelstein, 1995). Microbial pathogens with well-established intracellular survival strategies in mammalian cells such as *Legionella pneumophila*, *Mycobacterium avium*, *Chlamydia pneumoniae* and *Listeria monocytogenes* have been shown to survive within amoebae (Birtles *et al.*, 1997; Cirillo *et al.*, 1997; Essig *et al.*, 1997; Ly & Muller, 1990; Rowbotham, 1980).

Since the genera *Ralstonia* and *Burkholderia* are taxonomically related, we hypothesized that *B. cepacia* would also be capable of intracellular survival in free-living amoebae. In the current study, we present evidence that clinical and environmental isolates belonging to different genomovars of the *B. cepacia* complex can survive within several different strains of the genus *Acanthamoeba*, and can also grow saprophytically on by-products released by the amoebae. We propose that free-living amoebae may be a reservoir for the acquisition, and perhaps transmission, of *B. cepacia*.

METHODS

Bacterial strains. Clinical isolates of *B. cepacia* were obtained from the Vancouver collection, the London Health Sciences Centre, and from an outbreak at the Nephrology Unit, CEMIC, Buenos Aires, Argentina. Many of these isolates were genetically differentiated into the various genomovars by P. Vandamme, Laboratory of Microbiology, University of Gent, Belgium, and others by E. Mahenthiralingam, Department of Pediatrics, University of British Columbia, Vancouver, British Columbia, Canada. Environmental strains were obtained from the American Type Culture Collection. *Escherichia coli* DH5α, *Enterobacter cloacae* and *Pseudomonas aeruginosa* PAO1 were from our laboratory stocks. All strains were grown on Luria agar plates. For some experiments bacteria were grown in amoeba-conditioned medium. This medium was prepared by growing amoebae for 4 d at 25 °C in Acanthamoeba buffer (see below). Amoebae were removed by centrifugation and the medium was filter-sterilized using a 0·22 μm filter membrane (Millipore).

Amoebae. *Acanthamoeba castellanii* ATCC 30234 and *Acanthamoeba polyphaga* strain JAC/S2 (ATCC 50372) were obtained from the American Type Culture Collection, Manassas, VA, USA. *Acanthamoeba* spp. Vic-1 and HLA were clinical isolates from cases of human keratitis. *Acanthamoeba* sp. Wi III 8/2 was isolated from the wet area of a hospital physiotherapy unit while *A. castellanii* strain C3 came from a water reservoir used for drinking water (Michel & Hauröder, 1997). Amoebae were maintained axenically in peptone/yeast extract/glucose (PYG) medium (Page, 1976) at 25 °C as monolayers in 25 cm^2 flasks (Falcon 3018; Becton Dickinson). Cells were resuspended by tapping the flask, and cell counts were determined with a haemocytometer.

Infection of amoebae with bacteria. Amoebae and bacteria were cocultivated on non-nutrient agar (NNA) plates (Page, 1976). Axenically grown amoebae were washed with Acanthamoeba buffer (Page, 1976) and 200–300 cells were spotted in the centre of an NNA plate that had been seeded with 5×10^4 bacteria previously grown in Luria broth (LB) and washed twice with Acanthamoeba buffer. Plates were incubated at 25 °C and observed daily by phase-contrast microscopy and differential interference microscopy for up to 15 d.

Determination of intracellular multiplication of bacteria. Amoebae were grown to confluence in a 25 cm^2 dish (Falcon 3018), washed three times with Acanthamoeba buffer, and counted with a haemocytometer. Aliquots (1 ml) containing approximately 6×10^4 amoebae were placed in each well of a 24-well tissue culture dish (Falcon 3047) and allowed to settle for 2 h. Bacteria from an overnight culture in Luria broth were washed and resuspended in Acanthamoeba buffer to an approximate density of $1{\cdot}2 \times 10^8$ c.f.u. ml^{-1} and 100 μl of this suspension was added to each well containing amoebae to give an approximate m.o.i. of 200. Dishes were centrifuged for 3 min at 1000 r.p.m. and incubated at 25 °C. Bacterial invasion was permitted to continue for 4 h, after which time the wells were washed three times with Acanthamoeba buffer and 500 μg ceftazidime ml^{-1} (Tazidime; Eli Lilly) to kill residual extracellular bacteria. One hundred microlitres of 10^7 heat-killed *E. coli* was added to each well as a food source to avoid stress by starvation of amoebae. Wells were processed at 24, 48, 72, 96, 120 and 144 h. Processing at each time point was as follows. Buffer was carefully aspirated and the number of amoebae was counted using an inverted microscope. One millilitre of fresh Acanthamoeba buffer was added and a 100 μl aliquot was sampled to determine the number of extracellular bacteria. One hundred microlitres of 20% Triton X-100 (final concentration 0·4%) was added to lyse the amoebae. The extent of lysis was monitored for 10–15 min under the inverted microscope until approximately 90% of the trophozoites were lysed. A 100 μl aliquot of the lysate was taken for bacterial counts to determine the number of total bacteria (intracellular + extracellular bacteria). Wells to be processed at a later time were washed with Acanthamoeba buffer and ceftazidime, and heat-killed *E. coli* was added as indicated above. Bacterial counts were carried out as described by Jett *et al.* (1997). The number of intracellular bacteria was calculated by subtracting the number of extracellular bacteria from the number of total bacteria. All experiments were carried out in duplicate.

Electron microscopy. Co-cultures with amoebae and bacteria at different times were washed from agar plates and fixed in 2·5% glutaraldehyde/4% paraformaldehyde buffered in cacodylate. Fixed samples were processed as previously described (Michel & Hauröder, 1997) and examined in a Zeiss EM910 electron microscope.

RESULTS

B. cepacia CEP021 survives within cytoplasmic vacuoles in *Acanthamoeba* spp. Vic-1 and HLA

Initial cocultivation experiments were conducted with *Acanthamoeba* spp. Vic-1 and HLA and the *B. cepacia* complex genomovar VI strain CEP021. Cocultivations

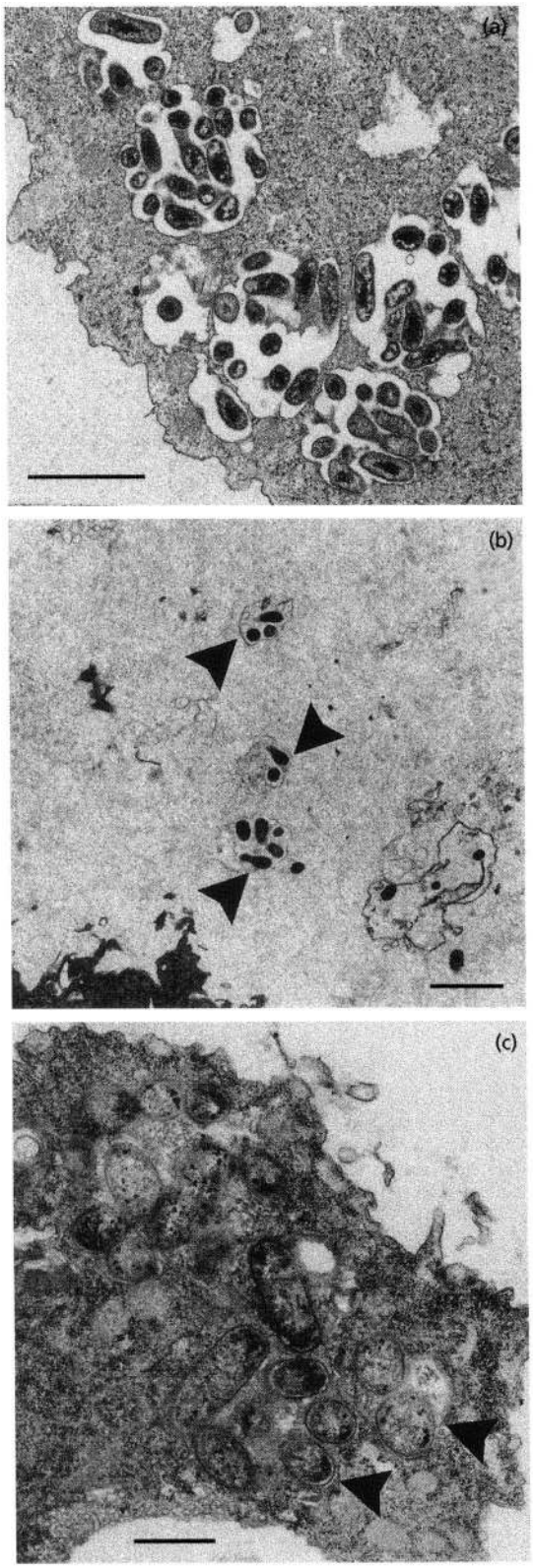

Fig. 1. Transmission electron microscopy of *Acanthamoeba* sp. HLA cells cocultivated with *B. cepacia* CEP021 and *Ent. cloacae*. (a) Trophozoites infected with *B. cepacia* after 5 d incubation showing spacious vacuoles with intact bacteria (bar, 2 μm). (b) Arrowheads indicate small membrane-bound vesicles containing bacteria drifting off a disintegrating amoeba (bar, 2 μm). (c) Amoeba cells cocultivated with *Ent. cloacae* showing tight-fitting vacuoles with bacteria (solid arrowheads) (bar, 1 μm).

were carried out by seeding NNA plates containing axenically grown trophozoites with 5×10^4 bacteria per plate and changes in the morphology of trophozoites were followed by phase-contrast and differential interference contrast microscopy. At approximately 48 h after infection, trophozoites on the agar surface began to show several vacuoles, which increased in size and number over 5 d. Some trophozoites were completely filled with vacuoles. Motile bacteria were seen within giant vacuoles even after 15 d (data not shown). The trophozoites appeared to be viable since they were able to move on the agar plate, albeit with reduced motility, as the trophozoites became heavily vacuolated and round. In addition, the contractile vacuole remained free of bacteria, and was opening and closing normally, indicating that its function was preserved. In control experiments using live *E. coli* DH5α or heat-killed *B. cepacia* CEP021, trophozoites did not show any abnormal vacuolization. A large number of cysts appeared on plates cocultivated with *E. coli* DH5α after 3–4 d incubation. Combined data of four independent experiments revealed that after 4 d, $92 \pm 3\%$ of the amoeba cells cocultivated with live *E. coli* DH5α had formed cysts, while only $7 \pm 4\%$ of cysts were found at a similar time point when trophozoites were infected with live CEP021 cells. Progressive encystation was found as a normal occurrence in amoebae fed with heat-killed *B. cepacia*, as well as with dead or live *E. coli* after several days of incubation. The absence of profuse encystation in plates infected with CEP021 suggested that *B. cepacia* infection interfered with the differentiation process from trophozoites into cysts. We also concluded from these experiments that extensive vacuolization was caused only by living bacteria. An experiment using filter-sterilized bacterial culture supernatants from strain CEP021 did not show any apparent vacuolization of trophozoites, suggesting that the presence of microorganisms is required for vacuolization.

The localization of intracellular CEP021 organisms in vacuoles was confirmed by transmission electron microscopy. Fig. 1(a) shows intracellular bacteria in spacious vacuoles, surrounded by a membrane structure. Some of the bacteria showed indications of partial septa, suggesting intravacuolar replication. No bacteria were identified free in the cytoplasm, suggesting that they always remain within the phagocytic vacuole. No apparent changes in the fine structure of the mitochondria of the infected trophozoites were observed as has been documented in the case of amoebae infected by *R. pickettii* (Michel & Hauröder, 1997). However, some amoeba cells appeared to be disintegrating, showing lamellated structures, some of which resemble myelin-like coils (data not shown). Intracellular bacteria in these cells remained intact within vacuoles or were observed drifting off disintegrating amoebae in small

Table 1. Properties of strains of the *B. cepacia* complex examined in this study

Strain	Genomovar*	Source†	Infection in *Acanthamoeba*‡		
			Vic-1	*A. castellanii* 30234	*A. polyphaga* JAC/S2
B. cepacia					
ATCC 25416	I	Onion	+	(+)	+
CEP163	I	Soil	−	ND	+
CEP509§	I	CF	++	+	+
FC124§	I	CGD	−	(+)	−
MC76	I	Onion	++	+	+
MC81	I	Onion	+	ND	+
MC135	I	Soil	−	+	+
MC353	I	Onion	++	ND	ND
C1484	III	CF	+	(+)	−
C3865	III	CF	+	++	+
C4455	III	CF	−	ND	−
C5424§	III	CF	−	+	−
C6061	III	CF	−	ND	+
CEP024	III	CF	−	ND	−
CEP54	III	CF	−	ND	−
CEP511§	III	CF	−	ND	ND
CP 706-J	III	CF	−	ND	ND
F28369-82	III	CF	+	ND	−
F38192-89	III	CF	(+)	ND	ND
L10	III	CF	−	ND	+
PC 527-I	III	CF	−	ND	ND
PC 701-J	III	CF	−	ND	ND
CEP559	IV	CF	−	ND	−
FC362	IV	Soil	ND	ND	−
FC473	IV	CF	ND	ND	+
CEP021§	VI	CF	++	−	+
Ce1258	Unknown	Blood	+	ND	+
CEP455	Unknown	CF	−	ND	−
CEP493	Unknown	CF	−	ND	ND
CFLG	Unknown	CF	+	ND	+
L6	Unknown	CF	++	++	++
MC366	Unknown	Soil	+	ND	ND
B. multivorans					
C0514§	II	CF	−	ND	(+)
C3430§	II	CF	−	+	−
C4297§	II	CF	−	ND	−
C5274	II	CF	(+)	ND	−
C5393	II	CF	−	ND	−
C5568	II	CF	−	−	−
C7072	II	CF	−	+	+
CEP484	II	CF	−	ND	−
B. gladioli					
CEP89	N/A	CF	ND	ND	+
CEP22	N/A	CF	−	ND	+
CEP30	N/A	CGD	−	+	+
CEP32	N/A	Soil	ND	ND	−
CEP654	N/A	CF	−	+	−

Table 1 *(cont.)*

Strain	Genomovar*	Source†	Infection in *Acanthamoeba*‡		
			Vic-1	*A. castellanii* 30234	*A. polyphaga* JAC/S2
B. vietnamiensis					
C2822	N/A	CF	+	ND	+
CEP40	N/A	CF	++	+	+++
FC369	N/A	Soil	+	+	+
FC441	N/A	CGD	+	ND	+
FC466	N/A	CF	ND	ND	+
P. aeruginosa					
PAO1	N/A	Keratitis	+++	ND	ND

* Genomovars I, II, III and IV were determined by P. Vandamme as previously described (Vandamme *et al.*, 1997). Genomovar VI is a newly determined genomovar of the *B. cepacia* complex (P. Vandamme, personal communication). Unknown, genomovar assignment not done; N/A, not applicable.

† CF, cystic fibrosis; CGD, chronic granulomatous disease.

‡ Infection was determined by the appearance of vacuoles containing bacteria in cocultivation assays performed on NNA plates incubated at 25 °C. (+), Small vacuoles that disappeared after 72 h (abortive infection?); +, two or three vacuoles with motile bacteria appearing after 72 h; ++, five or more vacuoles with motile bacteria appearing after 72 h; +++, five or more vacuoles with motile bacteria appearing after 24 h. ND, Not done.

§ Same results as with *Acanthamoeba* sp. Vic-1 were obtained using *Acanthamoeba* sp. HLA.

vacuoles of approximately 5–6 μm in size harbouring two to five intact bacteria (Fig. 1b). In contrast, examination of strain HLA trophozoites that had been fixed and embedded 72 h after addition of *Ent. cloacae* as a food source revealed that the ingested bacteria were enclosed by tightly fitting membranes of food vacuoles (Fig. 1c). Some of these bacterial cells appeared to be in a state of degradation, probably caused by lysosomal enzymes.

Infection of amoebae is host-dependent and varies with the temperature of incubation

To determine whether *B. cepacia* CEP021 could infect other amoeba isolates we conducted infections with *A. castellanii* 30234, *A. castellanii* C3, *Acanthamoeba* sp. Wi III 8/2 and *A. polyphaga* JAC/S2. Vacuolization of trophozoites was observed in the case of *A. polyphaga* JAC/S2 (Table 1 and data not shown). However, neither *A. castellanii* 30234 nor *Acanthamoeba* sp. Wi III 8/2 and *A. castellanii* C3, which could be infected with *R. pickettii* (Michel & Hauröder, 1997), were infected by *B. cepacia* CEP021. These results suggest that not all amoeba strains can support intracellular survival of *B. cepacia*.

Since temperature may affect the outcome of infection of amoebae in cocultivation assays we also examined the relationship between infection and growth temperature. Cocultivations were carried out at room temperature (approx. 20 °C), 25 °C, 30 °C and 37 °C. Uninfected trophozoites rapidly developed into cysts within 24–48 h upon incubation at 37 °C. The number of trophozoites infected with CEP021 rapidly declined and a substantial amount of debris was observed on the plates, suggesting that under these conditions infection with *B. cepacia* results in the rapid lysis of the amoebae. At 20, 25 and 30 °C infections proceeded normally and uninfected amoebae in control plates looked healthy.

B. cepacia complex strains can grow saprophytically in the presence of amoebae

We carried out quantitative studies to determine whether isolates of *B. cepacia* can replicate intracellularly. For these experiments we utilized two strains of *Acanthamoeba* (*A. polyphaga* and *Acanthamoeba* sp. Vic-1) and two isolates of the *B. cepacia* complex (the genomovar I strain CEP509 and *B. cepacia* strain CEP021). We utilized ceftazidime (Burns *et al.*, 1996) to kill extracellular bacteria since these strains are highly resistant to gentamicin. The experiments with the ceftazidime killing assay showed comparable results (Fig. 2) revealing a 15–20-fold increase in the number of intracellular bacteria between 96 and 120 h over the values at earlier time points. The number of extracellular bacteria remained low at all times. We also determined the mean number of bacteria per trophozoite, which demonstrated similar kinetics, ranging from 10 to 35 bacteria per trophozoite at 120 h in contrast to one to five bacteria per trophozoite at earlier time points (data not shown). Parallel control experiments using live *E.*

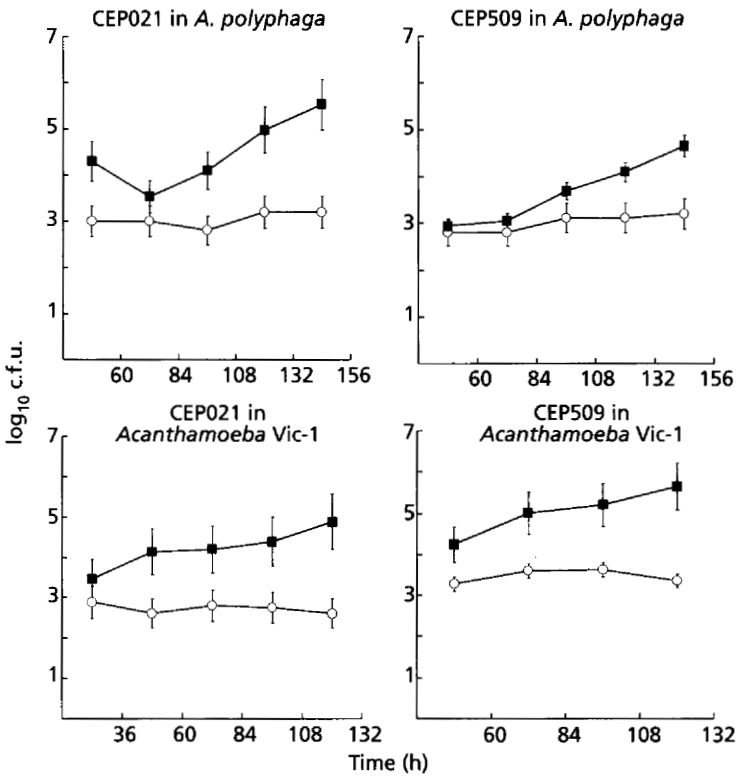

Fig. 2. Kinetics of intracellular growth of *B. cepacia* CEP021 (genomovar VI) and *B. cepacia* CEP509 (genomovar I) in *A. polyphaga* and *Acanthamoeba* sp. Vic-1. The data points are the mean of two independent experiments. ■, Intracellular bacteria; ○, extracellular bacteria.

coli DH5α and *Ent. cloacae* revealed a mean of about 100 intracellular bacteria at 24 h, and failed to detect any viable intracellular bacteria at 48, 72 and 96 h. The data are consistent with the fact that these micro-organisms serve as a food source for the amoebae.

In preliminary experiments, we determined that ceftazidime at 500 μg ml^{-1} killed 99·9 % of micro-organisms in Acanthamoeba buffer with an initial bacterial inoculum between 10^4 and 10^5 c.f.u. ml^{-1}. However, previous experience with invasion assays as used in this study has revealed that antibiotics often fail to kill all extracellular bacteria in the presence of the eukaryotic cells (Elsinghorst, 1994). Therefore, we also examined the efficacy of antibiotic killing of extracellular bacteria in the presence of amoebae. In these experiments, ceftazidime was added as before, but bacteria were cultivated from medium supernatant without any washes. The results (Fig. 3) show that ceftazidime efficiently killed at least 99·8 % of the bacterial inoculum. The number of recovered bacteria following antibiotic treatment was less than 100 c.f.u. ml^{-1} at 24, 72, 96 and 120 h. We therefore concluded that the antibiotic treatment was effective in killing extracellular bacteria. It is also possible that some extracellular bacteria remained attached to the amoeba cell surface and therefore escaped killing by ceftazidime. This could easily be excluded by microscopic observation since strain CEP021 forms filamentous cells in Acanthamoeba buffer that are about five times the cell length of normal rods. Although many filamentous bacteria attached to amoebae were visible during the first 24 h following infection, none were seen beyond such time after inspecting 10 fields with a magnification of ×400 (data not shown). We conclude, therefore, that the possibility of extracellular bacteria escaping killing by attaching to the amoebae is unlikely.

Additional experiments were conducted to determine the growth rate of bacteria in fresh Acanthamoeba buffer and in amoebae-conditioned Acanthamoeba buffer (see Methods). Fig. 3 shows that bacteria do not grow well in regular Acanthamoeba buffer but were capable of rapid growth in amoebae-conditioned medium. This suggests that by-products released to the medium by amoebae may contribute to the saprophytic growth of the *B. cepacia* CEP021. A comparison between the saprophytic growth rate and intracellular growth clearly shows that the former is the main mechanism of growth (Fig. 3). Therefore, we conclude that although *B. cepacia* can survive and probably even replicate at low levels intracellularly, replication is primarily extracellular and aided by the presence of by-products produced by amoebae.

Infection experiments using various amoebae and clinical and environmental isolates of *B. cepacia*

We investigated whether other isolates of the *B. cepacia* complex can infect amoebae using *A. polyphaga* JAC/S2, *A. castellanii* 30234 and *Acanthamoeba* sp. Vic-1 as hosts. We screened several taxonomically well-characterized isolates belonging to known genomovars, as well as other isolates identified in the laboratory as *B. cepacia* but not genetically characterized as yet. Isolates from soil and onions were also used in this screen. Data in

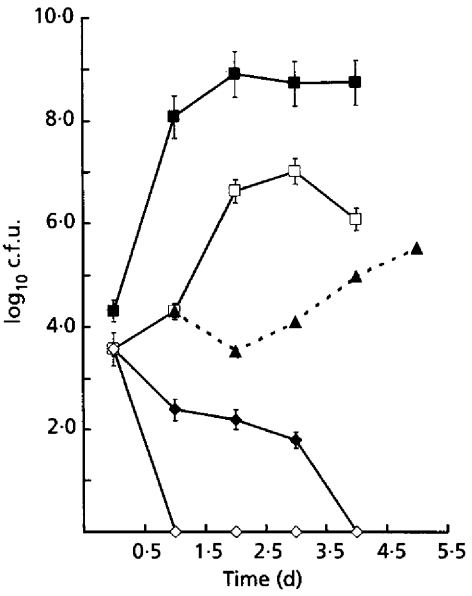

Fig. 3. Growth rate of *B. cepacia* CEP021 (genomovar VI) under different conditions. ■, Amoebae-conditioned medium, no amoebae, no antibiotic; □, Acanthamoeba buffer with *A. polyphaga*, no antibiotic; ◆, Acanthamoeba buffer, no amoebae, no antibiotic; ◇, Acanthamoeba buffer with *A. polyphaga* + 500 μg ceftazidime ml^{-1}. For comparison, data from Fig. 2 corresponding to intracellular growth rate are also included (▲- - -▲), except that error bars were omitted for clarity.

Table 1 show that strains causing vacuolization in amoebae were found in all genomovar groups of *B. cepacia* and also within *Burkholderia multivorans*, *Burkholderia gladioli* and *Burkholderia vietnamiensis*. Most of the invasive bacteria caused extensive vacuolization of trophozoites 48–72 h following infection as previously observed with CEP021, and the vacuoles were also filled with motile bacteria. We also found some strains that took 72 h or more to induce the formation of visible vacuoles in the amoebae. In the case of strains ATCC 25416 in *A. polyphaga* and *A. castellanii*, *B. multivorans* C5274 and *B. cepacia* F38192-89 in Vic-1, and *B. cepacia* FC124 in *A. castellanii*, the cytoplasm of trophozoites was filled with small vacuoles during the first 3 d following infection. These vacuoles then disappeared, suggesting an abortive infection. Strain FC124 was unusual in that it appeared to be toxic for *Acanthamoeba* sp. Wi III 8/2, although it was unable to infect the other amoebae examined. *B. multivorans* C3430, *B. gladioli* CEP654 and *B. cepacia* C5424 were only moderately infective in *A. castellanii* 30234. We also showed that intracellular survival was not restricted to *B. cepacia*, since *P. aeruginosa* strain PAO1 would cause vacuolization of *Acanthamoeba* sp. Vic-1 (Table 1), as has been shown previously with another strain of *P. aeruginosa* isolated from tap water (Michel & Hauröder-Philippczyk, 1992). From all these results, we concluded that the property of intracellular survival within amoebae is not restricted to a single genomovar of the *B. cepacia* complex. Furthermore, our data suggest that it is useful to conduct vacuolization assays for *B. cepacia* isolates using a panel of various strains of amoebae to maximize the detection of isolates capable of intracellular survival.

DISCUSSION

We demonstrate in this study that many clinical and environmental isolates of *B. cepacia* belonging to all genomovars associated with CF are capable of intracellular survival in *Acanthamoeba* cells. Recently, Burns *et al.* (1996) reported that a clinical isolate of *B. cepacia* could invade and survive within a respiratory epithelial cell line. Also, *B. cepacia*-like micro-organisms are endosymbiotically associated with arbuscular mycorrhizal fungi, and reside within cytoplasmic vesicles (Perotto & Bonfante, 1997). Our results add more support to the idea that isolates of the *B. cepacia* complex may be able to become intracellular at least at some stage during the infectious process. Preliminary data in our laboratory also show that *B. cepacia* CEP021 is capable of surviving within murine macrophages (L. Saini & M. A. Valvano, unpublished). Intracellular survival in professional phagocytes may protect invading micro-organisms from antibiotics and host defences while promoting, at the same time, an enhanced inflammatory response that may ultimately cause lung tissue damage in CF patients.

We also propose that *Acanthamoeba* and perhaps other amoebae can serve as a reservoir for *B. cepacia* and may serve as vehicles for transmission of *B. cepacia* from patient to patient. The fact that infections of amoebae were more pronounced at 30 °C is interesting, since in humans amoebae can be found colonizing the anterior nasal mucosa (De Jonckheere, 1991; Kilvington & White, 1994) where the temperature is typically 30–32 °C (Willatt, 1993). Recently, it has been demonstrated that *Acanthamoeba* strains release membrane-bound vesicles with bacterial content (Berk *et al.*, 1998). These vacuoles are 2–5 μm in diameter on average, which means that they can be transported directly to the lower respiratory tract by the airflow. Bacteria in these vacuoles are presumably also protected from environmental conditions in the airways. Similar small vacuoles containing *B. cepacia* CEP021 were noticed in our study, suggesting that infected amoebae colonizing the upper respiratory tract could potentially disintegrate and release intracellular bacteria within vacuoles, facilitating the colonization and establishment of *B. cepacia* in the patients' airways. Respirable vesicles containing bacteria may also be breathed out, allowing person-to-person transmission and colonization of the environment. To test these ideas we are currently undertaking a clinical study to determine colonization by amoebae in patients with CF, as well as experiments with an animal model. It is possible that amoebae may also be a reservoir for strains of *P. aeruginosa*. In a previous work, the isolation of amoebae carrying *P. aeruginosa* from a hospital environment has been described (Michel *et al.*, 1995), and we have shown in this study that *P.*

aeruginosa PAO1 also causes the formation of extensive vacuolization in *Acanthamoeba* sp. Vic-1.

Several lines of evidence from our study suggest that intracellular *B. cepacia* is metabolically active: (i) bacteria in the vacuoles remain motile; (ii) some intracellular bacteria examined by electron microscopy show evidence of septum formation; (iii) the vacuoles of amoebae cocultivated with live *B. cepacia* are characteristically spacious, in contrast with the tight-fitting vacuoles of amoebae cocultivated with either heat-killed *B. cepacia* or live *E. coli*. Our results also suggest that intracellular *B. cepacia* displays a low level of intracellular replication, as indicated by increasing numbers of intracellular bacteria recovered at the latter stages of infection, in contrast to the small number of extracellular bacteria (between 100 and 1000 c.f.u. ml^{-1}) remaining in the presence of the antibiotic ceftazidime. The overall increase in intracellular bacteria was, however, at best no more than two logarithmic units higher than the amount of intracellular bacteria at the beginning of the experiment. This is in marked contrast to the rapid multiplication of bacteria in amoeba-conditioned media. Recently, Steinert *et al.* (1998) showed conclusively that *M. avium* can grow saprophytically on products secreted by the amoebae, although their experiments did not rule out intracellular multiplication. Since intracellular *M. avium* was also able to survive within amoebae it is conceivable that these micro-organisms may receive nutrients and grow within the protozoan. The fact that in our study, *B. cepacia* CEP021 grows much better in amoebae-conditioned medium suggests that these micro-organisms can grow saprophytically, and perhaps the primary mechanism of growth in nature is extracellular in the presence of products secreted by amoebae. Intracellular bacteria may benefit in terms of survival from the protective shield conferred by amoebae, and growth mediated by amoebae by-products. It is not clear how the amoeba kills bacteria but presumably killing is mediated by non-oxidative mechanisms. *B. cepacia* is known to resist non-oxidative killing by neutrophils (Speert *et al.*, 1994), and this property could also be important for survival within amoebae. Further studies, currently under way in our laboratories, are required to characterize the molecular basis of *B. cepacia* survival within amoebae. Presumably, these properties may also be important in the pathogenesis of *B. cepacia* infections in CF patients.

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REFERENCES

Beckman, W. & Lessie, T. G. (1979). Response of *Pseudomonas cepacia* to β-lactam antibiotics: utilization of penicillin G as the carbon source. *J Bacteriol* **140**, 1126–1128.

Berk, S. G., Ting, R. S., Turner, G. W. & Ashburn, R. J. (1998). Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. *Appl Environ Microbiol* **64**, 279–286.

Birtles, R. J., Rowbotham, T. J., Storey, C., Marrie, T. J. & Raoult, D. (1997). *Chlamydia*-like obligate parasite of free-living amoebae. *Lancet* **349**, 925–926.

Boulanger, C. A. & Edelstein, P. H. (1995). Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. *Appl Environ Microbiol* **61**, 1805–1809.

Burkholder, W. H. (1950). Sour skin, a bacterial rot of onion bulbs. *Phytopathology* **40**, 115–117.

Burns, J. L., Jonas, M., Chi, E. Y., Clark, D. K., Berger, A. & Griffith, A. (1996). Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. *Infect Immun* **64**, 4054–4059.

Cirillo, J. D., Falkow, S., Tompkins, L. S. & Bermudez, L. E. (1997). Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect Immun* **65**, 3759–3767.

De Jonckheere, J. F. (1991). Ecology of *Acanthamoeba*. *Rev Infect Dis* **13** (Suppl. 5), S385–S387.

Drabick, J. A., Gracely, E. J., Heidecker, G. J. & LiPuma, J. J. (1996). Survival of *Burkholderia cepacia* on environmental surfaces. *J Hosp Infect* **32**, 267–276.

Elsinghorst, E. A. (1994). Measurement of invasion by gentamicin resistance. *Methods Enzymol* **236**, 405–420.

Essig, A., Heinemann, M., Simnacher, U. & Marre, R. (1997). Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Appl Environ Microbiol* **63**, 1396–1399.

Govan, J. R. W. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.

Govan, J. R. W. & Vandamme, P. (1998). Agricultural and medical microbiology: a time for bridging gaps. *Microbiology* **144**, 2373–2375.

Govan, J. R., Hughes, J. E. & Vandamme, P. (1996). *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J Med Microbiol* **45**, 395–407.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P. & Levison, H. (1984). *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr* **104**, 206–210.

Jett, B. D., Hatter, K. L., Huycke, M. M. & Gilmore, M. S. (1997). Simplified agar plate method for quantifying viable bacteria. *Biotechniques* **23**, 648–650.

Kilvington, S. & Price, J. (1990). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *J Appl Bacteriol* **68**, 519–525.

Kilvington, S. & White, D. G. (1994). *Acanthamoeba*: biology, ecology and human disease. *Rev Med Microbiol* **5**, 12–20.

LiPuma, J. J., Dasen, S. E., Nielson, D. W., Stern, R. C. & Stull, T. L. (1990). Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. *Lancet* **336**, 1094–1096.

Ly, T. M. & Muller, H. E. (1990). Ingested *Listeria monocytogenes* survive and multiply in protozoa. *J Med Microbiol* **33**, 51–54.

Mergeryan, H. (1991). The prevalence of *Acanthamoeba* in the human environment. *Rev Infect Dis* **13** (Suppl. 5), S390–S391.

Michel, R. & Hauröder-Philippczyk, B. (1992). Cocultivation of *Acanthamoeba castellanii* and *Pseudomonas aeruginosa* leads to infection of the amoebae. *Publ Jpn-Ger-Cent Berlin Ser B* **5**, 174–178.

Michel, R. & Hauröder-Philippczyk, B. (1994). *Acanthamoeba* from human mucosa infected with an obligate intracellular parasite. *Eur J Protistol* **30**, 104–110.

Michel, R. & Hauröder, B. (1997). Isolation of an *Acanthamoeba* strain with intracellular *Burkholderia pickettii* infection. *Zentbl Bakteriol* **285**, 541–557.

Michel, R., Burghardt, H. & Bergmann, H. (1995). Natürliche intrazelluläre Infektion bei Acanthamoeben mit *Pseudomonas aeruginosa* nach ihrer Isolierung aus einer mikrobiologisch beanstandeten Trinkwasser-Hausinstallation eines Krankenhauses. *Zentbl Hyg* **196**, 532–544.

Page, F. C. (1976). *An Illustrated Key to Freshwater and Soil Amoebae*. Ambleside, Cumbria: The Ferry House.

Perotto, S. & Bonfante, P. (1997). Bacterial associations with mycorrhizal fungi: close and distant friends in the rhizosphere. *Trends Microbiol* **5**, 496–501.

Prince, A. (1986). Antibiotic resistance of *Pseudomonas* species. *J Pediatr* **108**, 830–834.

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J Clin Pathol* **33**, 1179–1183.

Speert, D. P., Bond, M., Woodman, R. C. & Curnutte, J. T. (1994). Infection with *Pseudomonas cepacia* in chronic granulomatosis disease: role of non-oxidative killing by neutrophils in host defense. *J Infect Dis* **170**, 1524–1531.

Steinert, M., Birkness, K., White, E., Fields, B. & Quinn, F. (1998). *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Appl Environ Microbiol* **64**, 2256–2261.

Tablan, O. C., Chroba, T. L., Schidlow, D. V. & 7 other authors (1985). *Pseudomonas cepacia* colonization in patients with cystic fibrosis: risk factors and clinical outcome. *J Pediatr* **107**, 382–387.

Vandamme, P., Holmes, B., Vancanneyt, M. & 8 other authors (1997). Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**, 1188–1200.

Willatt, D. J. (1993). Continuous infrared thermometry of the nasal mucosa. *Rhinology* **31**, 63–67.

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