Burkholderia multivorans survival and trafficking within macrophages

Crystal L. Schmerk¹² and Miguel A. Valvano¹²

¹Microbiology and Immunology, University of Western Ontario, London, Ontario, N6A 5C1, Canada
²Center for Human Immunology, University of Western Ontario, London, Ontario, N6A 5C1, Canada

INTRODUCTION

Cystic fibrosis (CF) patients are at great risk of opportunistic lung infection, particularly by members of the Burkholderia cepacia complex (Bcc). This group of bacteria can cause damage to the lung tissue of infected patients and are difficult to eradicate due to their high levels of antibiotic resistance. Although the highly virulent Burkholderia cenocepacia has been the focus of virulence research for the past decade, Burkholderia multivorans is emerging as the most prevalent Bcc species infecting CF patients in North America. Despite several studies detailing the intramacrophage trafficking and survival of B. cenocepacia, no such data exist for B. multivorans. The results of this study demonstrated that the clinical CF isolates C5568 and C0514 and an environmental B. multivorans isolate, ATCC 17616, were able to replicate and survive within murine macrophages in a manner similar to that of B. cenocepacia strain K56-2. These strains were also able to survive but were unable to replicate within human THP-1 macrophages. Differences in macrophage uptake were observed among all three B. multivorans strains; these variances were attributed to major differences in O-antigen production. Unlike B. cenocepacia-containing vacuoles, which delay phagosomal maturation in murine macrophages by 6 h, all B. multivorans-containing vacuoles co-localized with lysosome-associated membrane protein-1, a late endosome/lysosomal marker, and the lysosomal marker dextran within 2 h of uptake. Together, these results indicated that, whilst both Bcc species were able to survive and replicate within macrophages, they utilized different intramacrophage survival strategies. To observe differences in virulence, the strains were compared using the Galleria mellonella (wax worm) model. When compared with the B. multivorans strains tested, B. cenocepacia K56-2 was highly virulent in this model and killed all worms within 24 h when injected at 10⁷ c.f.u. B. multivorans clinical isolates C5568 and C0514 were significantly more virulent than the soil isolate ATCC 17616, which was avirulent even when worms were injected with 10⁷ c.f.u. These results suggest strain differences in the virulence of B. multivorans isolates.

Abbreviations: Bcc, Burkholderia cepacia complex; BcCV, Burkholderia cenocepacia-containing vacuole; BmCV, Burkholderia multivorans-containing vacuole; CF, cystic fibrosis; IL-1β, interleukin-1β; LAMP-1, lysosome-associated membrane protein-1; p.i., post-infection; PMA, phorbol 12-myristate 13-acetate.
they are resistant to antimicrobial peptides and many clinically useful antibiotics, making infections caused by them very difficult to treat (Aaron et al., 2000; Nzula et al., 2002; Hamad et al., 2010).

Burkholderia cenocepacia and Burkholderia multivorans are the Bcc species most commonly isolated from CF patients, with B. cenocepacia being widely considered the most prevalent species. Due to the high virulence and patient-to-patient spread associated with B. cenocepacia infection, the majority of research groups have focused on the study of this bacterium. However, B. multivorans infection rates have been increasing steadily compared with those of B. cenocepacia (Govan et al., 2007), with B. multivorans becoming the most prevalent Bcc strain infecting CF patients in North America (Bumford et al., 2010; Zloznik et al., 2011). With few studies focusing on B. multivorans, there is still much to learn concerning the infective process of this bacterium, particularly with respect to its interaction with immune cells. To date, studies have demonstrated that B. multivorans can infect human lung epithelial cells (Duff et al., 2006; Moura et al., 2008), monocytes (Zelazny et al., 2009) and dendritic cells (MacDonald & Speert, 2008), and have elucidated some differences in B. cenocepacia and B. multivorans infections. During the infection of dendritic cells, only B. cenocepacia was able to impair cell function and induce necrosis, even though B. multivorans elicited a similar release of cytokines (MacDonald & Speert, 2008). However, invasion of human epithelial cells resulted in similar levels of cytokine release and induction of apoptosis by B. multivorans and B. cenocepacia strains (Moura et al., 2008). To date, there has been no study evaluating the infection of phagocytic cells by B. multivorans at the intracellular level.

In this study, we investigated the differences between environmental and CF isolates of B. multivorans with respect to intramacrophage trafficking, replication and survival. The behaviour of these strains was compared with the previously characterized intramacrophage trafficking of B. cenocepacia strain K56-2. In contrast to B. cenocepacia, which causes a 6-h delay in phagosomal maturation, all B. multivorans-containing vacuoles (BmCVs) were found to be associated with lysosomal markers within 2 h of infection. Despite these major differences in intramacrophage trafficking, all B. multivorans and B. cenocepacia strains were able to survive in RAW264.7 and THP-1 macrophages. To determine differences in virulence, the strains were also compared using the Galleria mellonella (wax worm) model of infection. B. multivorans clinical isolates C5568 and C0514 exhibited increased virulence in the wax worm model when compared with the environmental isolate ATCC 17616. These results suggest that strain differences exist among B. multivorans isolates that determine differential survival within a eukaryotic host.

**METHODS**

**Bacterial strains, cell lines and growth conditions.** Bacteria were grown on Luria–Bertani (LB) agar plates or in LB broth with shaking at 37 °C. Escherichia coli cultures were supplemented, as required, with the following final concentrations of antibiotics: 50 µg trimethoprim ml⁻¹, 40 µg kanamycin ml⁻¹ and 30 µg tetracycline ml⁻¹. When needed, B. multivorans cultures were supplemented with 100 µg trimethoprim ml⁻¹ and 150 µg tetracycline ml⁻¹. B. cenocepacia and B. multivorans carrying the plasmid pIN62 (Vergunst et al., 2010) were grown in the presence of 100 µg chloramphenicol ml⁻¹. The murine macrophage cell line RAW264.7 (ATCC TIB-71) was maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 with 10% FBS. Cell lines were maintained at 37 °C in a 95% humidified atmosphere with 5% CO₂.

**General molecular techniques.** DNA manipulations and cloning were performed as described previously by Sambrook et al. (1989). PCR amplification of DNA was performed using Tag or HotStar HiFidelity DNA polymerases (Qiagen). Antarctic phosphatase (New England Biolabs) and T4 DNA ligase (Roche Applied Science) were used as recommended by the manufacturer. E. coli GT115 cells were transformed via standard electroporation methods (Sambrook et al., 1989). DNA sequencing was completed at the sequencing facility in York University (Toronto, Canada). Mobilization of plasmids into B. cenocepacia and B. multivorans was performed by triparental mating using the helper plasmid pRK2013 (Figurski & Helinsky, 1979; Craig et al., 1989). The sequenced genome of B. multivorans strain ATCC 17616 was analysed using the BLAST and Artemis programs.

**Construction of gentamicin-sensitive B. multivorans strains.** The construction of unmarked, non-polar mutant strains was accomplished using the method described by Flannagan et al. (2008). The deletion mutagenesis plasmids were created by amplifying 550 bp DNA fragments flanking genes homologous to those deleted in B. cenocepacia to create the gentamicin-sensitive strain MH1K (Hamad et al., 2010). The sequenced genome from B. multivorans strain ATCC 17616 was used as the basis for all primer design. The upstream fragment was amplified using primers 4733 (5’-ATCGATAATTT-CGCGGCGACGACGGCGCCGTTTCTGTC-3’; Spl site underlined) and 4734 (5’-GTCATGTTACGGCCCTTTCCGAGCGG-GCGGGGCCCA-C3’; KpnI site underlined). The downstream fragment was amplified using primers 3856 (5’-GATCGGTAC GCCCGACGCGGAGCGGACG-3’; KpnI site underlined) and 4732 (5’-GTCGACATCTAGATCCTGTCGACGACCC-CAATCG-3’; XbaI site underlined). The flanking regions upstream of the gene Bmml_1614 (see Results) were digested with SspI and KpnI, and the flanking regions downstream of Bmml_1616 were digested with KpnI and XbaI (Roche Applied Science). Both digested, amplified fragments were ligated into pGPI-Scl to create the pDelGmATCC17616, pDelGmC5568 and pDelGmC0514 deletion plasmids. The mutagenic plasmids were mobilized into B. multivorans ATCC 17616, C5568 or C0514 by triparental mating and co-integrants were selected using 100 µg trimethoprim ml⁻¹. Selection against the E. coli donor and helper strains after the triparental mating was accomplished using 100 µg ampicillin ml⁻¹ in combination with 25 µg polymyxin B ml⁻¹. pDAl-Sce-I-SacB, used in the final stage of mutagenesis to induce the second recombination event leading to an unmarked gene deletion, was mobilized into B. multivorans co-integrants. Exconjugants selected with 150 µg tetracycline ml⁻¹ were patched onto plates containing 50 µg gentamicin ml⁻¹ to test for gentamicin sensitivity (indicative of appropriate gene deletions). Deletion mutants were cured of the levansucrase-encoding (SacB) plasmid by growing the B. multivorans strains in LB broth overnight and then plating on LB agar supplemented with 5% (w/v) sucrose. The resulting tetracycline-sensitive colonies were further screened by PCR to confirm gene deletions.

**Intracellular survival in macrophages.** Bacterial intracellular survival was assayed as described previously, with slight modifications (Hamad et al., 2010). RAW264.7 macrophages and THP-1 monocytes...
were seeded in 12-well plates at a density of $3 \times 10^5$ and $8 \times 10^5$ cells per well, respectively, and incubated overnight. To induce differentiation into macrophages, the THP-1 growth medium was supplemented with 50 μg phenol 12-myristate 13-acetate (PMA) (Sigma) ml$^{-1}$ for 24 h. The THP-1 macrophages were then incubated in PMA-free medium for 24 h prior to infection. Bacterial strains were grown overnight in LB broth at 37 °C with shaking. The bacterial cultures were washed three times with DMEM plus FBS and were used to infect RAW264.7 or THP-1 macrophages at an m.o.i. of 50. Plates were centrifuged for 2 min at 300 g to synchronize the infection and incubated for 1 h at 37 °C under 5% CO$_2$. Infected macrophages were washed with PBS three times to remove extracellular bacteria. DMEM plus FBS containing 100 μg gentamicin ml$^{-1}$ was added to kill any remaining extracellular bacteria. After 1 h, the macrophages were washed twice in PBS, and fresh medium containing 10 μg gentamicin ml$^{-1}$ was added for the remainder of the experiment. To enumerate intracellular bacteria, infected macrophages were lysed with 0.1% sodium deoxycholate (w/v) at 0, 24 and 48 h post-infection (p.i.). Lysates were serially diluted in PBS and plated on LB agar.

**LPS analysis.** LPS was extracted as described previously (Marolda et al., 1990) and resolved on 16% polyacrylamide gels using a tricine-SDS gel electrophoresis system (Schägger & von Jagow, 1987; Lesse et al., 1990). LPS was visualized by silver staining (Marolda et al., 1990).

**Measurement of phagocytic index.** The number of infected macrophages and the number of bacteria per macrophage were determined using the method of Saldias et al. (2009). RAW264.7 macrophages seeded on glass coverslips were infected at an m.o.i. of 50. Plates were centrifuged for 2 min at 300 g to synchronize the infection and incubated for 2 h at 37 °C under 5% CO$_2$. Infected macrophages were then washed with PBS five times to remove extracellular bacteria. Intracellular bacteria were differentiated by visual observation using differential interference contrast microscopy at ×100 magnification. Each experiment was repeated three times.

**Phagosomal maturation assays.** RAW264.7 macrophage infections were performed as described above for the intracellular survival assay, the only difference was that glass coverslips were placed in each well before seeding with macrophages. When needed, 0.1 μM LysoTracker Green DND-26 (Invitrogen) was added for 30 s prior to visualization. Lysosome labelling was performed by incubating macrophages with 30 μg fluorescein-labelled dextran ml$^{-1}$ overnight. External fluorescein–dextran was removed by serial washes with PBS and chased for 1 h in DMEM plus FBS before infection. For immunostaining, infected cells were fixed at room temperature for 20 min using 4% (v/v) paraformaldehyde and then permeabilized using 0.1% (v/v) Triton X-100 at room temperature for 20 min. Coverslips were blocked in a solution of 3% (w/v) BSA, 2% (v/v) FBS and 0.1% (v/v) Triton X-100 for 1 h at room temperature. The coverslips were then incubated with rabbit anti-lysosome-associated membrane protein-1 (LAMP-1) (clone 1D4B; BD Pharmingen) at a 1:200 dilution at 4 °C overnight. Alexa Fluor 488-labelled chicken anti-rat secondary antibody (Invitrogen) was added at a 1:5000 dilution for 45 min prior to visualization at ×100 magnification. Fluorescence and phase-contrast images were acquired using a QImaging RETIGA-SRV camera on an AxioScope 2 (Carl Zeiss) microscope. In each experiment, 21 fields of view were examined.

**G. mellonella infection.** Killing assays were performed as described by Seed & Dennis (2008) with some changes. Briefly, sixth-instar larvae of G. mellonella were obtained from Recorp and stored in wood shavings in the dark at 13 °C prior to infection. Larvae were given 10 μl bacterial injections into the haemocoel through the hindmost right proleg. Injections contained either $6 \times 10^7$ or $6 \times 10^5$ cfu diluted in 10 mM MgSO$_4$ with 1.0 mg ampicillin ml$^{-1}$. Larval survival was monitored at 24 h intervals over a period of 72 h and was judged based on visual appearance and lack of movement in response to stimuli. Control larvae were injected with 10 μl 10 mM MgSO$_4$ with 1.0 mg ampicillin ml$^{-1}$. Ten larvae were used for each condition and the experiment was repeated on three independent occasions.

**Statistics.** All experiments were performed at least in triplicate. Unless specified otherwise, all data are presented as means ± SEM of the indicated number of experiments. For comparisons of means, one-way analysis of variance was used. A level of $P<0.05$ was considered significant. The Mantel–Cox test was used to compare the survival curves in the G. mellonella virulence model.

**RESULTS AND DISCUSSION**

**B. multivorans strains replicate and survive within murine macrophages.**

The B. multivorans strains chosen for study were ATCC 17616, C5568 and C0514. Strain ATCC 17616 (Leslie & Gaffney, 1986) is derived from a soil sample and was used as our environmental isolate, whilst strains C5568 and C0514 were isolated from CF patients and are stored in the Canadian B. cepacia complex Research and Referral Repository. C5568 has been shown to persist in the lungs of infected BALB/c mice (Chu et al., 2004) and to replicate in human dendritic cells (Chu et al., 2004). C5568 also displayed low levels of pathogenicity in a Caenorhabditis elegans infection model, whereas C0514 was non-pathogenic (Cardona et al., 2005).

Like other Bcc species, B. multivorans is highly resistant to gentamicin and other aminoglycosides. Thus, to determine the intramacrophage growth phenotype of B. multivorans using a gentamicin protection assay, we first had to delete the cluster of genes required for bacterial resistance to gentamicin. In a study by Hamad et al. (2010), the deletion of a cluster of genes encoding an AmrAB–OprA-like efflux pump resulted in the creation of B. cenocepacia MH1K, a gentamicin-sensitive derivative of B. cenocepacia K56-2. Performing a BLASTP analysis of B. multivorans strain ATCC 17616, we found three genes (annotated Bmul_1614, Bmul_1615 and Bmul_1616) encoding proteins that shared 86, 92 and 85% amino acid sequence identity to B. cenocepacia J2315 OprA, AmrB and AmrA proteins, respectively. Bmul_1614-Bmul_1616 was deleted in all three B. multivorans strains and the resulting gentamicin-sensitive strains, ATCC 17616 Gm$, C5568 Gm$ and C0514 Gm$, were used to infect RAW264.7 murine macrophages and THP-1 human macrophages.

Previous studies have shown that B. cenocepacia MH1K is able to replicate within RAW264.7 macrophages (Hamad et al., 2010; Schmerk et al., 2011), so this strain was included as a reference strain and allowed direct comparison of the replication and survival of B. multivorans strains with B. cenocepacia. RAW264.7 macrophages were infected with all three strains of B. multivorans as well as B. cenocepacia MH1K at an m.o.i. of 50. Bacterial replication and survival were monitored over a period of 48 h (Fig. 1a). All strains tested were able to replicate within the murine macrophages. The majority of intramacrophage
induced to differentiate into macrophages, after which they were infected at an m.o.i. of 50. The THP-1 cells engulfed all four *Burkholderia* strains at a greater rate than the RAW264.7 macrophages (over 4 log_{10} units more, Fig. 1b). However, unlike in the RAW264.7 cells, none of the strains was able to replicate in THP-1 cells and bacterial burdens diminished slightly after 48 h of infection. Interestingly, the THP-1 macrophages appeared to clear MH1K more effectively than the *B. multivorans* strains. Although this difference was not statistically significant, it is known that *B. cenocepacia* activates the pyrin inflammasome in THP-1 cells, resulting in the processing and release of interleukin-1β (IL-1β) (Gavrilin et al., 2012). When used to infect the lungs of BALB/c mice, *B. cenocepacia* was effectively cleared, whereas *B. multivorans* persisted for days, establishing a pulmonary infection. The effective clearance of *B. cenocepacia* was attributed, in part, to a much greater IL-1β response when compared with that induced by *B. multivorans* (Chu et al., 2004). This induction of IL-1β in THP-1 cells may account for the increased clearance of MH1K in comparison with the *B. multivorans* strains.

**B. multivorans** strains are internalized differentially by macrophages

During the macrophage infection experiments described above, it was noted that the different strains of *B. multivorans* appeared to be engulfed by macrophages at varying levels (Fig. 1). To investigate this more closely, the rates of internalization were determined for all Bcc strains in RAW264.7 macrophages. The phagocytic index was calculated by infecting macrophages with *B. cenocepacia* K56-2 or *B. multivorans* ATCC 17616, C5568 or C0514 at an m.o.i. of 50 for 2 h. The results showed that C0514 and K56-2 were internalized at the lowest rates, with only 1.2 and 1% of macrophages being infected, respectively. The phagocytic index of C0514 and K56-2 did not differ significantly. *B. multivorans* ATCC 17616 was internalized at the highest frequency, with 5.2% of macrophages being infected (Fig. 2a; ATCC 17616 versus K56-2, P<0.001). *B. multivorans* C5568 followed with 3.7% (Fig. 2a; C5568 versus K56-2, P<0.05), a difference that did not differ significantly from that of ATCC 17616. These results were consistent with the pattern observed during the macrophage infections seen in Fig. 1. Similarly, the mean numbers of intracellular bacteria per infected macrophage were higher for ATCC 17616 and C5568 compared with those for K56-2 and C0514; however, only the difference between K56-2 and C5568 was statistically significant (P<0.05, Fig. 2b). These results indicated that the strains ATCC 17616 and C5568 were internalized at a higher rate than C0514 and K56-2.

**Correlation between internalization and O-antigen production**

A study performed by Saldías et al. (2009) demonstrated that differences in the internalization of *B. cenocepacia*...
strains J2315 and K56-2 were attributed to the absence or presence of O-antigen polysaccharide, a component of the LPS molecule found on the surface of Gram-negative bacteria. The internalization of strains lacking O-antigen was greatly increased when compared with that of O-antigen-producing strains. To determine whether differences in the internalization of B. multivorans strains could be attributed to differences in O-antigen production, the LPS profiles of these strains were compared. B. cenocepacia strains K56-2 and J2315 were included as representative O-antigen-producing and -non-producing strains, respectively.

Upon comparison of the B. multivorans LPS profiles, it could be observed that, in contrast with clinical isolates C5568 and C0514, the environmental isolate ATCC 17616 did not produce any O-antigen (Fig. 2c). Thus, similar to J2315, the lack of O-antigen production by ATCC 17616 is probably responsible for the high level of macrophage internalization. Although C5568 did produce O-antigen, there was great variation in the LPS profile, with shorter O-antigen chains being produced. The variation in O-antigen chain length is likely to account for its high rate of internalization when compared with C0514, which produces O-antigen chains of much greater length than those seen in C5568 and K56-2. C0514 produced an LPS profile comparable to that seen in B. cenocepacia K56-2, and these strains displayed similar rates of macrophage internalization. Together, these results suggested that the differences in the phagocytic uptake of B. multivorans strains could be attributed to differences in O-antigen production.

LPS plays a vital role in the pathogenesis of many Gram-negative bacteria (Raetz & Whitfield, 2002). In particular, the O-antigen has been shown to contribute to the intracellular survival and/or replication of several pathogenic bacteria, including Burkholderia pseudomallei (Arjcharoen et al., 2007), Francisella tularensis (Maier et al., 2007) and Brucella species. (Fernandez-Prada et al., 2003; Porte et al., 2003; Rajashekara et al., 2008). However, much like B. cenocepacia (Saldias et al., 2009), it appeared that O-antigen production did not alter the intramacrophage survival or replication of B. multivorans, as all three strains behaved similarly in both RAW264.7 and THP-1 macrophages.

**BmCVs do not exhibit any delay in maturation**

The process of phagosome maturation leads to the clearance of invading bacteria from host macrophages. Many bacterial pathogens have developed sophisticated methods to avoid this host defence mechanism (Scott et al., 2003). To date, only Coxiella burnetii has been found to survive and replicate within the acidic environment of fully mature phagolysosomes (Howe et al., 2010). Recent studies of B. cenocepacia have indicated that this opportunistic pathogen may also replicate within a mature phagolysosome. However, this Bcc species delays the maturation of the bacteria-containing vacuole by 6 h (Lamothe et al., 2007; Lamothe & Valvano, 2008), possibly giving the bacterium time to make changes that would allow it to adapt to and survive within the phagolysosome (Tolman & Valvano, 2012). To determine whether B. multivorans causes a similar delay in phagosome maturation, RAW264.7 macrophages were infected with ATCC 17616 Gm$, C5568 Gm$ and C0514 Gm$. B. cenocepacia MH1K was included as a control strain as it is known to cause a delay in phagosomal maturation in this cell line (Hamad et al., 2010). Macrophage infections were observed at 2, 6 and 24 h p.i. using various markers to assess the state of BmCV maturation.
LysoTracker is an acidotropic dye that preferentially accumulates in the most acidic cellular compartments, chiefly lysosomes (Via et al., 1998; Al-Younes et al., 1999; Zheng & Jones, 2003). Hence, we used LysoTracker Green to qualitatively evaluate the acidity of BmCVs. At 2 h p.i., all B. multivorans strains resided in vacuoles that co-localized with LysoTracker, whilst vacuoles containing B. cenocepacia did not (Fig. 3a). Quantification of these results showed that, at 2 h p.i., only 25 ± 1.8% of B. cenocepacia-containing vacuoles (BcCVs) were positive for LysoTracker, whilst 92 ± 1.4, 89 ± 1.9 and 90 ± 0.6% of BmCVs containing ATCC 17616 Gm³, C5568 Gm³ and C0514 Gm³, respectively, were positive for LysoTracker (Fig. 3b). The association of BcCVs with LysoTracker Green continued to increase slowly over time with co-localization of 44 ± 1.0% after 6 h and 94 ± 1.2% after 24 h of infection. The association of BmCVs with LysoTracker Green remained constant throughout the infection (Fig. 3b). These results demonstrated that vacuoles containing B. multivorans appeared to acidify normally, unlike vacuoles containing B. cenocepacia, which exhibited a pronounced delay in acidification.

To determine whether vacuoles containing B. multivorans fused with late endosomes soon after infection, we observed the accumulation of LAMP-1 in BmCVs by immunostaining. At 2 h p.i., 91 ± 0.9, 92 ± 1.5 and 84 ± 1.8% of the vacuoles containing ATCC 17616 Gm³, C5568 Gm³ and C0514 Gm³, respectively, accumulated LAMP-1 (Fig. 4a, b). In contrast, only 15 ± 1.3% of BcCVs accumulated LAMP-1 after 2 h of infection, with levels increasing to 53 ± 2.0% after 6 h (Fig. 4a, b). The accumulation of LAMP-1 by BmCVs after 6 h of infection was similar to that seen after 2 h (Fig. 4b). Accumulation of LAMP-1 at 24 h p.i. was not observed due to the cross-reactivity of unknown ATCC 17616 surface molecules with the primary and secondary antibodies. These data indicated that BmCVs, unlike BcCVs, exhibit typical interactions with late endosomes.

LAMP-1 is a protein that is found on both late endosomes and lysosomes (Rabinowitz et al., 1992); as such, the previous experiments could not be used to determine whether BmCVs had fused with lysosomes. Fluorescein-labelled dextran is internalized by fluid-phase endocytosis and this hydrophilic polysaccharide specifically labels lysosomes (Eissenberg et al., 1988; Racoosin & Swanson, 1993; Hewlett et al., 1994; Strasser et al., 1999; Hmama et al., 2004). Prior to infection, RAW264.7 macrophages were incubated with 30 μg fluorescein-labelled dextran ml⁻¹ overnight. The following morning, the cells were subjected to a 1 h chase in dextran-free medium before infection. After 2 h of infection, 92 ± 2.0, 84 ± 5.0 and 91 ± 1.2% of the vacuoles containing ATCC 17616 Gm³, C5568 Gm³ and C0514 Gm³, respectively, were found to be associated with fluorescein-labelled dextran (Fig. 5a, b). As expected, only 19 ± 2.1% of BcCVs co-localized with dextran after 2 h of infection (Fig. 5a, b). The number of BcCVs associated with dextran increased gradually, with levels increasing to 45 ± 6.6% after 6 h and 89 ± 3.5% after 24 h (Fig. 5b). The association of BmCVs with fluorescein-labelled dextran remained steady throughout the infection (Fig. 5b). Once again, these results indicated that BmCVs exhibited normal trafficking within macrophages, fusing with lysosomes <2 h after infection.

Work by Lamothe et al. (2007) demonstrated that BcCVs exhibit delayed acidification and lysosomal fusion. Inactivation of the late endosomal marker Rab7 by an as

---

**Fig. 3.** B. multivorans strains reside in an acidic phagosome throughout infection. (a) RAW264.7 macrophages were infected with *Burkholderia* strains expressing the DsRed fluorescent protein. Acidic vacuoles were labelled with LysoTracker Green DND-26 at 2 h p.i. and the merged images are shown in the far right panels. Bar, 5 μm. (b) Quantification of BmCV and BcCV co-localization with LysoTracker Green DND-26 at 2, 6 and 24 h p.i. (a) MH1K; (b) ATCC 17616 Gm³; ▲, C5568 Gm³; ▼, C0514 Gm³. Data are means ± SEM.
yet undetermined mechanism contributes to this delay in lysosomal fusion (Huynh et al., 2010). It is generally thought that this delay in BcCV maturation allows B. cenocepacia adequate time to activate genes that are required for survival in the severe phagolysosomal environment. However, it would appear that this delay in phagosomal maturation is not required for the intramacrophage survival of Bcc species, as all of the B. multivorans strains tested in this study resided in vacuoles that exhibited normal progression through the endocytic pathway.

Nevertheless, with only three markers of phagosomal progression being evaluated in this study, it is possible that other aspects of phagosomal maturation are altered by B. multivorans. Despite early fusion of BmCVs with lysosomes, B. multivorans strains were all able to survive and replicate within RAW264.7 macrophages, indicating

**Fig. 4.** Vacuoles containing B. multivorans do not exhibit a delay in the accumulation of LAMP-1. (a) RAW264.7 macrophages were infected with Burkholderia strains expressing the DSRed fluorescent protein. After 2 h of infection, the cells were fixed and immunolabelled using rat anti-LAMP-1 and a secondary antibody conjugated to Alexa Fluor 488. Bar, 5 μm. (b) Quantification of BmCV and BcCV co-localization with LAMP-1 at 2 and 6 h p.i. ●, MH1K; ■, ATCC 17616 Gm3; ▲, C5568 Gm3; ▼, C0514 Gm3. Data are means ± SEM.

**Fig. 5.** BmCVs fuse with lysosomes early in infection. (a) RAW264.7 macrophages were pre-loaded with the lysosomal marker fluorescein–dextran and infected with Burkholderia strains expressing the DSRed fluorescent protein. The far right panels show co-localization of the Burkholderia species with dextran at 2 h p.i. Bar, 5 μm. (b) Quantification of BmCV and BcCV co-localization with dextran at 2, 6 and 24 h p.i. ●, MH1K; ■, ATCC 17616 Gm3; ▲, C5568 Gm3; ▼, C0514 Gm3. Data are means ± SEM.
that *B. multivorans* may be replicating within mature phagolysosomes.

**B. multivorans ATCC 17616 induces large-vacuole formation during macrophage infection**

An interesting observation was made concerning RAW264.7 macrophage infection with the environmental *B. multivorans* strain ATCC 17616 Gm<sup>+</sup>. After 24 h of infection, this strain induced the formation of numerous large vacuoles, a phenomenon not observed during infection with either of the CF isolates or *B. cenocepacia* MH1K (Fig. 6) or during infection of THP-1 human macrophages (data not shown). The vacuolization was similar to that observed during epithelial cell infection with *Vibrio cholerae* (Figueroa-Arredondo et al., 2001) and *Helicobacter pylori* (Papini et al., 1994) and fibroblast infection with *Aeromonas hydrophila* (Abrami et al., 1998). In these instances, the vacuolization is due to the production of specific exoenzymes, VacA and HlyA, produced by *H. pylori* and *V. cholerae*, respectively, and aerolysin, produced by *A. hydrophila*. Vacuole formation caused by the cytotoxin VacA of *H. pylori* results from interference with the trafficking of molecules, such as lysosomal hydrolases, and an impairment of lysosomal function. It has also been hypothesized that this vacuolization may contribute to virulence by wasting cellular resources and energy due to the production of excess internal membranes and subsequent disruption of cellular homeostasis (Satin et al., 1997). As such, it may be possible that this vacuolar phenotype is indicative of some type of interference with the function of the ATCC 17616-containing phagosome.

Bcc species produce a variety of secreted enzymes, including proteases, lipases, phospholipase C and haemolysin (Carvalho et al., 2007). The production of secreted enzymes by *B. multivorans* strains was tested using a variety of characteristic growth media (Fehlner-Gardiner et al., 2002). However, using these simple phenotypic assessment methods, we were unable to detect any differences that might account for the vacuolization occurring during ATCC 17616 Gm<sup>+</sup> infection (data not shown). Further research is required to discover any putative effector(s) responsible for this vacuolar phenotype, as well as to discern its significance in *B. multivorans* infection.

**Clinical B. multivorans strains are more virulent in the G. mellonella infection model**

*G. mellonella* larvae have been used as an infection model for the study of many bacterial pathogens, including *P. aeruginosa* (Jander et al., 2000; Hendrickson et al., 2001; Miyata et al., 2003), *F. tularensis* (Aperis et al., 2007) and several *Burkholderia* species (Schell et al., 2008; Seed & Dennis, 2008, 2009; Wand et al., 2011). Because the innate immune system of these insects shares a high degree of homology with the innate immune systems of mammals, this infection model can provide an accurate indication of the mammalian response to a pathogen (Hoffmann, 1995; Kavanagh & Reeves, 2004). All three *B. multivorans* strains utilized in this study, as well as *B. cenocepacia* MH1K, were injected into *G. mellonella* larvae, whose survival were monitored every 24 h for 72 h. There were no significant differences observed between the virulence of the wild-type and gentamicin-sensitive derivative strains of *B. cenocepacia* and *B. multivorans* (data not shown). *B. cenocepacia* K56-2 is highly virulent in this model (Seed & Dennis, 2008); as such, it was not surprising that MH1K was the most virulent strain tested, with only 10±5.5% of larvae surviving after injection with 10<sup>3</sup> c.f.u. (Fig. 7a). The *B. multivorans* strains exhibited minimal virulence at this inoculum level, with survival rates being >90% for all three strains. The differences in larval survival among *B. multivorans* ATCC 17616 Gm<sup>+</sup>, C5568 Gm<sup>+</sup> and C0514 Gm<sup>+</sup> were not statistically significant. The virulence of all *B. multivorans* strains differed significantly from MH1K (*P*<0.0001, Fig. 7a). Variances in the virulence of *B. multivorans* strains were evident when larvae were injected with 10<sup>7</sup> c.f.u. At this inoculum level, survival rates of 97±3.3, 20±7.4 and 0% were observed for larvae injected with ATCC 17616 Gm<sup>+</sup>, C5568 Gm<sup>+</sup> and C0514 Gm<sup>+</sup>, respectively (Fig. 7b). *B. cenocepacia* MH1K killed all larvae within 24 h of injection. The difference in survival rates of all strains was statistically significant at this inoculum level.

---

**Fig. 6.** The environmental *B. multivorans* isolate ATCC 17616 induces the formation of large vacuoles during macrophage infection. Shown are phase-contrast images of RAW264.7 macrophages infected for 24 h with *B. cenocepacia* MH1K or *B. multivorans* ATCC 17616 Gm<sup>+</sup>, C5568 Gm<sup>+</sup> or C0514 Gm<sup>+</sup>. Two independent viewing fields for each infection are shown. Bar, 10 μm.
virulence of B. multivorans strains. In conclusion, although the B. multivorans and B. cenocepacia strains exhibited similar survival and replication within macrophages, the characteristics of their phagosomal maturation were vastly different. Whilst B. cenocepacia utilized unknown virulence factors to delay maturation of the BcCV, B. multivorans did not, allowing the BmCV to fuse with lysosomes within 2 h of bacterial uptake. This variance in intramacrophage survival strategies may, in part, help to explain the marked differences in virulence among various Bcc strains, which has been observed in a variety of animal models (Chu et al., 2002, 2004; Cardona et al., 2005; Seed & Dennis, 2008), as well as in CF patients (Mahenthiralingam et al., 2001; Jones et al., 2004; Manno et al., 2004). The increased virulence of B. multivorans strains, isolated from CF patients, in the G. mellonella infection model combined with the vacuolar intramacrophage growth phenotype of the soil isolate ATCC 17616 suggest that this Bcc species may make adaptations that prove advantageous in a mammalian host environment. However, it is also possible that these strain-specific differences occur naturally in the environment, conferring specific advantages immediately after contact with CF patients. Together, these data provide the first in-depth insight into the intramacrophage survival strategies of B. multivorans, whilst simultaneously allowing their direct comparison with those of the highly virulent B. cenocepacia K56-2.

ACKNOWLEDGEMENTS

We thank D. Aubert for a critical review of the manuscript. This work was supported by a grant from Cystic Fibrosis Canada to M. A. V. C. L. S. was supported by a Postdoctoral Fellowship from Cystic Fibrosis Canada. M. A. V. holds a Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.

REFERENCES


