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A *Burkholderia cenocepacia* gene encoding a non-functional tyrosine phosphatase is required for the delayed maturation of the bacteria-containing vacuoles in macrophages

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Abstract

*Burkholderia cenocepacia* infects patients with cystic fibrosis. We have previously shown that *B. cenocepacia* can survive in macrophages within membrane vacuoles (BcCVs) that preclude fusion with the lysosome. The bacterial factors involved in *B. cenocepacia* intracellular survival are not fully elucidated. We report here that deletion of BCAM0628, encoding a predicted low-molecular weight protein tyrosine phosphatase (LMW-PTP) that is restricted to *B. cenocepacia* strains of the transmissible ET-12 clone, accelerates the maturation of the BcCVs. Compared to parental strain and deletion mutants in other LMW-PTPs that are widely conserved in *Burkholderia* species, a greater proportion of BcCVs containing the ∆BCAM0628 mutant were targeted to the lysosome. Accelerated BcCV maturation was not due to reduced intracellular viability since ∆BCAM0628 survived and replicated in macrophages similarly to the parental strain. Therefore, BCAM0628 was referred to as *dpm* (delayed phagosome maturation). We provide evidence that the Dpm protein is secreted during growth *in vitro* and upon macrophage infection. Dpm secretion requires an N-terminal signal peptide. Heterologous expression of Dpm in *B. multivorans* confers to this bacterium a similar phagosomal maturation delay as found with *B. cenocepacia*. We demonstrate that Dpm is an inactive phosphatase, suggesting that its contribution to phagosomal maturation arrest must be unrelated to tyrosine phosphatase activity.
INTRODUCTION

The *Burkholderia cepacia complex* (Bcc) is a diverse group of at least 17 species widely distributed in the environment (Lipuma, 2005). Bcc species display extraordinary metabolic versatility and have the ability to colonize and adapt to various ecological niches (Coenye & Vandamme, 2003). Bcc strains are potentially beneficial bacteria to promote plant growth, pest control, and bioremediation (Parke & Gurian-Sherman, 2001). Unfortunately, Bcc are also opportunistic pathogens causing chronic infection in immunocompromised individuals such as those with chronic granulomatous disease and cystic fibrosis (Loutet & Valvano, 2010; Mahenthiralingam et al., 2001; Vandamme et al., 1997). *Burkholderia cenocepacia* and *Burkholderia multivorans* are the two Bcc species most commonly isolated worldwide from cystic fibrosis patients (Drevinek & Mahenthiralingam, 2010; Govan et al., 2007).

Chronic infection with *B. cenocepacia* can result in acute, fatal necrotizing pneumonia and septicemia, termed “cepacia syndrome” (Courtney et al., 2004; Mahenthiralingam et al., 2008). Due to their intrinsic resistance to most available antibiotics the treatment of *Burkholderia* infections becomes difficult (George et al., 2009; Waters & Ratjen, 2006). Macrophages are crucial in the early stage of host defense against infections; they ingest and destroy incoming pathogens upon phagocytosis and recruit inflammatory cells to the site of infection (Hume et al., 2002; Kinchen & Ravichandran, 2008). Previous work has demonstrated that *B. cenocepacia* survive within a membrane-bound vacuole (hereafter referred as BcCV) in amoebae, human respiratory epithelial cells (Marolda et al., 1999; Saldias & Valvano, 2009) and macrophages (Hamad et al., 2010; Lamothe et al., 2007). Intramacrophage survival of *B. cenocepacia* has been associated with an arrest of the phagosomal maturation, since the BcCV shows impaired acidification and delayed fusion with lysosomes during the initial 6-8 h post infection (Huynh et al., 2010; Lamothe et al., 2007; Lamothe & Valvano, 2008). The delayed fusion of the BcCV with lysosomes can be attributed, at least in part, to impaired assembly of the NADPH oxidase complex on the BcCV membrane (Keith et al., 2009) as consequence of Rac1 inactivation (Flannagan et al., 2012; Rosales-Reyes et al., 2012b). Inactivation of Rab7 (Huynh et al., 2010), a small GTPase important for the endosome maturation (Wang et al., 2011), is also critical for this delay. Furthermore, recent evidence indicates that the BcCV can lose membrane integrity resulting in bacteria coming into contact with cytosol components (Al-Khodor et al., 2013) and promoting macrophage cell death by pyroptosis (Rosales-Reyes et al., 2012a). Loss of BcCV permeability partly depends on the activity of a type VI secretory system, while effector proteins secreted by a Type II secretory pathway can alter the BcCV maturation (Rosales-Reyes et al., 2012a). These observations suggest a complex interplay of *B. cenocepacia* effectors contributed by at least two different secretory pathways in the manipulation of the vesicular trafficking in macrophages. However, the specific bacterial components required are largely unknown.

Protein tyrosine phosphorylation is a covalent modification that regulates numerous cellular functions in eukaryotic and bacterial cells (Cozzone et al., 2004). Bacterial tyrosine kinases catalyze the phosphorylation of the side hydroxyl groups of tyrosine residues in protein substrates leading to the formation of the corresponding phosphomonoesters (Shi et al., 1998), while bacterial protein tyrosine phosphatases (PTPs) catalyze the reverse reaction. Bacterial PTPs can be categorized based on structural basis into three subfamilies: (i) the low molecular weight protein-tyrosine phosphatases (LMW-PTPs), composed by small and acidic enzymes.
present also in eukaryotes; (ii) the classic type I Cys-based PTPs, also known as eukaryotic-like phosphatases (Böhmer et al., 2013); and (iii) the polymerase and histidinol phosphatase family of phosphoesterases, which is found mainly in Gram-positive bacteria (Morona et al., 2002). Bacterial PTPs operate in two major processes: production and translocation of exopolysaccharides (reviewed in (Cozzone et al., 2004)), and interference with host signal transduction upon infection (Cozzone, 2005; Whitmore & Lamont, 2012). Examples of PTPs affecting host signaling pathways include YopH from Yersinia, which is injected into human epithelial cells where it targets host focal adhesion proteins, such as p130Cas, paxillin, and focal adhesion kinase (FAK) (Black & Bliska, 1997; Persson et al., 1997). Similarly, SptP from Salmonella enterica serovar Typhimurium inhibits the activation of the MAPK pathway by dephosphorylating Raf (Lin et al., 2003), disrupts the intermediate filaments distribution after vimentin binding (Murli et al., 2001), and regulates the biogenesis of an intracellular niche through VCP dephosphorylation (Humphreys et al., 2009). Mycobacterium tuberculosis secretes two PTPs, both required for intracellular survival in macrophages. PtpA inhibits V-ATPase trafficking to the mycobacterial phagosome and blocks phagolysosome fusion (Bach et al., 2008; Wong et al., 2011), while PtpB has a possible role subverting the host immune response (Zhou et al., 2010).

Manipulation of host signal transduction pathways by intracellular B. cenocepacia strongly suggests that bacterial secreted virulence factors are operating to allow the subversion of the host cell (Abdulrahman et al., 2013; Rosales-Reyes et al., 2012a). On this basis, we evaluated four predicted LMW-PTPs: BCAM0208, BCAM0628, BceD and BCAL2200, for their contribution to intracellular survival and phagosome maturation arrest. We demonstrate that B. cenocepacia secretes BCAM0628 (herein designated as dpm (delayed phagosome maturation) during growth and upon macrophage infection. Deletion of dpm accelerates the maturation of the BeCVs. Translocation of Dpm requires an N-terminal signal peptide plus an unperturbed C-terminal region. We also demonstrate that Dpm is an inactive phosphatase, suggesting that the maturation arrest of phagocytic vacuoles is unrelated to tyrosine phosphatase activity.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used here are listed in Table 1. Bacteria were cultured in Luria broth (LB; Difco) at 37 °C with shaking. Escherichia coli cultures were supplemented, as required, with the following antibiotics (final concentrations): tetracycline (25 µg ml\(^{-1}\)), kanamycin (40 µg ml\(^{-1}\)), trimethoprim (50 µg ml\(^{-1}\)) and chloramphenicol (30 µg ml\(^{-1}\)). B. cenocepacia cultures were supplemented, as required, with trimethoprim (100 µg ml\(^{-1}\)), tetracycline (100 µg ml\(^{-1}\)), and chloramphenicol (120 µg ml\(^{-1}\)). To assess growth rates of parental and mutant strains of B. cenocepacia MH1K, overnight cultures were inoculated into fresh medium to give a starting optical density at 600 nm (OD\(_{600}\)) of 0.01. Growth rates were determined in 100-well microtiter plates using a Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems, Inc.). E. coli strains for cloning and production of recombinant proteins were DH5α and BL21(DE3), respectively. E. coli GT115 was used for cloning into the suicide vector pGPI-SceI.
General molecular techniques. DNA manipulations and cloning were performed as described previously (Sambrook & Russell, 2001). PCR amplification was performed using Taq or HotStar HiFidelity DNA polymerases (Qiagen). Antarctic phosphatase (New England Biolabs), restriction enzymes (New England Biolabs) and T4 DNA ligase (Roche Applied Science) were used as recommended by the manufacturers. DNA sequencing was completed at the sequencing facility in York University (Toronto, Canada) and in Eurofins MWG Operon (Alabama, USA). Plasmids were mobilized into B. cenocepacia and B. multivorans by triparental mating (Craig et al., 1989; Figurski & Helinski, 1979).

Mutagenesis of B. cenocepacia K56-2. The construction of unmarked, non-polar mutant strains was accomplished as described (Flannagan et al., 2008). The deletion mutagenesis plasmids were created by amplifying 400-550 bp DNA fragments flanking the corresponding target genes using chromosomal DNA from B. cenocepacia K56-2 as template and the indicated primer pairs (Table 2). The amplicons were double digested with the corresponding restriction enzymes (Table 2) and cloned into pGPISce-I resulting in mutagenic plasmids pDelM0208, pDelM0628, pDelM0857 and pDelL2200 (Table 1). Mutagenic plasmids were mobilized into B. cenocepacia MH1K strain by triparental mating and co-integrants were selected using 100 µg ml\(^{-1}\) trimethoprim. Selection against the E. coli donor and helper strains after the triparental mating was accomplished using 100 µg ml\(^{-1}\) ampicillin in combination with 25 µg ml\(^{-1}\) polymyxin B. pDAI-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. cenocepacia co-integrants. Exconjugants were selected with 100 µg ml\(^{-1}\) tetracycline, 100 µg ml\(^{-1}\) ampicillin, and 25 µg ml\(^{-1}\) polymyxin B. Identification of the appropriate gene deletions was accomplished by PCR. Deletion mutants were cured of the levansucrase (SacB)-encoding plasmid by growing the B. cenocepacia mutant strains in LB broth overnight and then plating on LB agar supplemented with 5% (w/v) sucrose.

Motility assays. For swimming assays, 2 µl of overnight culture, adjusted to an OD\(_{600}\) of 1.0, was inoculated within the agar of a swim plate (LB; 0.3% agar). For swarming assays, 2 µl of overnight culture, adjusted to an OD\(_{600}\) of 1.0, was spotted on top of the swarm plate agar (nutrient broth; 0.5% agar, 0.2% glucose). The plates were incubated at 37 °C for 24 h, after which the diameters of the swimming and swarming zones were measured.

Cloning of predicted phosphotyrosine phosphatase genes and site-directed mutagenesis. The open reading frames BCAM0208, bceD, BCAL2200 and the full length dpm or the dpm truncated version were PCR-amplified using chromosomal DNA from B. cenocepacia K56-2 as template with the corresponding primer pairs (Table 2). Amplicons were digested with the restriction enzymes Ndel-XbaI and cloned into a similarly digested pDA17 plasmid, giving rise to pM0208, pbceD, pL2200, pdpm and pA27 (Table 1). To produce histidine tagged recombinant proteins, Dpm and BCAL2200 the plasmids pdpm or pL2200 were digested with Ndel-HindIII and the resulting inserts were cloned into a similarly digested pET28a plasmid, giving rise to pHisdpm and pHisL2200, respectively. To generate pdpmHis, dpm was amplified from chromosomal DNA with the primer pair 6829-6830 (Table 2), the PCR product was digested with Ncol-XhoI and cloned into a similarly digested pET28a. The site directed mutagenesis of dpm was performed by PCR with Pfu DNA polymerase (Stratagene) using the primer pairs 6496-6517 (Table 2) and the plasmid pHisdpm or pdpmHis as template for the reaction. DpnI was
added to PCR reactions for overnight digestion of parental plasmid DNA at 37 °C. The resulting DNA was introduced into *E. coli* DH5α by transformation, and transformants were selected on LB-agar containing 40 µg ml⁻¹ kanamycin. Directed mutagenesis in the resulting plasmids, pHisdpm D36C and pdpmHis D36C (Table 1), respectively, was confirmed by sequencing.

**Protein analysis.** Overnight cultures were diluted 1:100 in 7 ml of LB. After 2 h of incubation at 37 °C, the cultures were centrifuged for 15 min at 16,100 ×g and the resulting pellets were resuspended in 1× SDS–PAGE sample buffer; the volume was normalized to the OD₆₀₀. Supernatants were sterilized through a 0.22-µm filter (Millipore), and proteins were precipitated during 4 h at 4 °C with 10% (vol/vol) of trichloroacetic acid (final concentration). The precipitates were isolated by centrifugation at 16,100 ×g for 30 min at 4 °C, and the pellet was air-dried and resuspended in 2× SDS–PAGE sample buffer containing 10% (vol/vol) of saturated Tris base. Protein concentration of the different cultures was normalized to the OD₆₀₀ value. After SDS–PAGE, the gel was transferred onto a nitrocellulose membrane. The membrane was then blocked overnight with western blocking reagent (Roche Diagnostics) in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% % Tween-20). The primary antibodies, anti-FLAG M2 monoclonal antibody (Sigma) or anti-αRNAPol (*E. coli*) (Neoclon), were diluted to 1:15,000 in TBST, anti-beta lactamase (TEM-1, *E. coli*) (Thermo Fisher) was diluted to 1:5,000 in TBST. Primary antibodies were applied for 1.5 h. Secondary antibody, goat anti-mouse Alexa Fluor 680 IgG antibodies (Invitrogen), was diluted to 1:15,000 and applied for 1 h. Western blots were developed using LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

**Intracellular survival in macrophages.** Bacterial intracellular survival in RAW264.7 murine macrophages was assayed as described previously (Schmerk & Valvano, 2013). Bacteria were added to macrophages at multiplicity of infection (MOI) of 50. Plates were centrifuged for 1 min at 300 × g and incubated for 2 h at 37 °C under 5% CO₂. Infected macrophages were washed with PBS three times to remove extracellular bacteria and fresh medium containing 100 µg ml⁻¹ gentamicin was added to kill any remaining extracellular bacteria. After 1 h, the macrophages were washed twice in PBS, and fresh medium containing 10 µg ml⁻¹ gentamicin was added for the remainder of the experiment. To enumerate intracellular bacteria infected macrophages were lysed with 0.1% sodium deoxycholate (w/v), and the lysates were serially diluted in PBS before plating on LB agar.

**Phagosomal maturation assays.** For lysosome labeling, macrophages were incubated overnight with 250 µg ml⁻¹ fluorescein isothiocyanate–dextran (Invitrogen). External fluorescein–dextran was removed by serial washes with PBS and chased for 1 h in fresh medium. Infections of RAW264.7 macrophages with *B. cenocepacia* strains carrying pIN62 (for bacteria labeling) or *B. multivorans* ATCC 17616 Gms carrying pDA17 or pdpm were performed as described above. After infection extracellular bacteria were removed by washes and fresh medium containing 50 µg ml⁻¹ gentamicin was added. At 2 h post infection cells were fixed at room temperature for 25 min using 4% (v/v) paraformaldehyde and visualized at × 100 magnification. The *B. multivorans* infected macrophages were similarly treated but the infection proceeded during 1 h, similarly to (Schmerk & Valvano, 2013). For immunostaining, fixed cells were permeabilized using 0.1% (v/v) Triton X-100 at room temperature for 30 min. Coverslips were blocked in a solution of 3% (w/v) BSA, 2% (v/v) FBS in PBS for 2 h at 4 °C. The coverslips were then incubated at 4 °C overnight with rat anti-lysosome-associated membrane protein-1 (LAMP-1) (clone 1D4B; BD...
Pharmingen) at a 1:200 dilution, for complementation assays due to plasmid incompatibility. Bacteria were labeled with rabbit antiserum against *B. cenocepacia* K56-2 (Rosales-Reyes et al., 2012b) at a 1:4 000 dilution. Alexa Fluor 488-labelled chicken anti-rat (Invitrogen) and Alexa Fluor 594-labelled goat anti-rabbit (Invitrogen), when necessary, were added at a 1:4 000 dilution for 90 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.

Intramacrophage Dpm secretion. RAW264.7 macrophages were infected with *B. cenocepacia* ∆dpm strain carrying the plasmid pdpm or pΔ27 (Table 1) as described above. At 1 h post-infection cells were fixed and treated as described for immunostaining. The coverslips were then incubated at 4 °C overnight with mouse anti-FLAG M2 monoclonal antibody (Sigma) at a 1:200 dilution and with rabbit antiserum against *B. cenocepacia* K56-2 at a 1:4 000 dilution. Then Alexa Fluor 488-labelled goat anti-mouse (Invitrogen) and Alexa Fluor 594-labelled goat anti-rabbit (Invitrogen) were added at a 1:4 000 dilution for 90 min prior to visualization at × 100 magnification.

Production and purification of recombinant proteins. Plasmids pHisdpm, pHisL2200 and pdpmHis (Table 1) were expressed in *E. coli* BL21(DE3). 200 ml LB cultures were grown at 30°C until they reached an OD_{600} 0.7. Then 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added and bacteria continued growing for 4 h. Induced bacteria were harvested by centrifugation and bacterial pellets were resuspended in binding buffer, BB (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl) containing protease inhibitor cocktail (Roche Diagnostics). Cell lysis was achieved using a cell disruptor (Constant Systems Ltd) at 20 kpsi. Lysates were centrifuged for 50 min at 24,000 ×g, 4 °C, to pellet undisrupted cells and inclusion bodies. Soluble fractions were applied to Ni^{2+} charged sepharose beads for 30 min at 4 °C. After extensive washing with BB containing 40 mM imidazole, proteins were eluted with BB containing 200 mM imidazole. Purified recombinant proteins were dialyzed overnight at 4 °C against 1.0 l of TND buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl and 1 mM DTT). Protein concentration was determined by using the Bio-Rad protein assay dye-binding reagent (Bio-Rad).

Phosphatase activity. Phosphatase activity was determined by monitoring, at 405 nm, the p-nitrophenol (PNP) formed from p-nitrophenol phosphate (PNPP) as previously described in (Cowley et al., 2002) with minor modifications. Mixtures reaction contained, in a final volume of 1 ml, 2.5 µg of purified and dialyzed protein in 7 mM HEPES pH 7.0, 5 mM MgCl₂ and 1 mM DTT. Enzymatic reactions were started adding 1 mM PNPP and incubated at 37 °C. After the determinate time intervals reactions were stopped adding 0.2 N NaOH (final concentration) and the absorbance was measured at 405 nm. The concentration of PNP formed was estimated using a molar extinction coefficient of 1.78 × 10⁴ M⁻¹ cm⁻¹. And phosphatase activity was calculated from the slope obtained in time course analyses.

Statistical analyses. All experiments were performed at least in triplicate. All data are presented as means ± SEM of the indicated number of experiments. Statistical analyses were done by Student’s t-test and one-way analysis of variance for comparisons of means using GraphPad Prism version 4.03.
RESULTS

Identification of *B. cenocepacia* putative LMW-PTPs

Analysis of the *B. cenocepacia* J2315 genome (J2315 is clonally related to strain K56-2) with Conserved Domain Database (Marchler-Bauer et al., 2011) tools revealed 4 open reading frames encoding proteins with putative LMWPc domains (pfam01451). These enzymes catalyze the removal of a phosphate group attached to a tyrosine residue, using a cysteinyl-phosphate enzyme intermediate (Wang et al., 2000). The predicted LMW-PTPs were BCAL2200, BCAM0208, BCAM0628 (Dpm) and BCAM0857 (BceD). The genes encoding these proteins are located in different clusters within the first (BCAL) and second (BCAM) chromosomes (Fig. 1). The cluster containing bceD was previously identified as part of the bce exopolysaccharide biosynthetic gene cluster (Moreira et al., 2003), which is widespread within all the sequenced *Burkholderia* strains, with the exception of *B. rhizoxinica* and *B. mallei* (Ferreira et al., 2010). BCAM0208 resides in another exopolysaccharide biosynthesis cluster next to the predicted tyrosine kinase BCAM0207 (Fig. 1b), in a similar arrangement as the described bceD/bceF pair of genes (Moreira et al., 2003). BCAM0208/BCAM0207 are also widespread in the *Burkholderia* genus. BCAL2200 is located within a region highly conserved in other *Burkholderia* species, which presumably is involved in regulating sulfur amino acid metabolism (Fig. 1b and data not shown). In contrast, dpm is located in a poorly conserved region of the genome, and homologues to dpm are only found in *B. cenocepacia* J2315, K56-2, and H111, as well as in *B. cepacia* GG4 and *B. lata* (Fig. 1b and data not shown). Analysis of the translated sequences of *B. cenocepacia* LMW-PTPs showed a common secondary structure prediction pattern (data not shown) and indicated that all except Dpm have the conserved amino acids of the tyrosine phosphatase motif, CX₅R(S/T) (Fig. 1a). Dpm has an aspartate substitution in the position of the catalytic cysteine (Fig. 1a) and a larger N-terminus with strong prediction to a signal peptide sequence (data not shown), suggesting it is a secreted protein.

Dpm is secreted to the growth medium

The genes encoding BCAM0208, Dpm, BceD and BCAL2200 were cloned into pDA17 under the expression of the constitutive *dhfr* promoter. Resulting plasmids, pM0208, pdpm, pbceD and pL2200 (Table 1) were mobilized into *B. cenocepacia* K56-2 and secretion assays were performed as described in Methods. The pDA17 vector enables to fuse a C-terminal FLAG tag, which facilitates detection of the expressed products by immunoblotting. Our results show that except for BceD, the other proteins had similar expression levels (Fig. 2, upper left panel), as compared to the β-lactamase TEM-1, which was used as a loading control (Fig. 2, lower left panel). However, only Dpm was found in the supernatant (Fig. 2, upper panels). The absence of TEM-1 in supernatants (Fig. 2, lower panels) rules out spontaneous cell lysis or outer membrane leakage, confirming that Dpm is a secreted protein. Also, we did not find cytoplasmic RNA polymerase α-subunit in supernatants (data not shown), which provided an additional control indicating the cell lysis, did not occur during these experiments.

Characterization of *B. cenocepacia* tyrosine phosphatase mutants

Single gene deletion mutants of BCAM0208, dpm, bceD and BCAL2200 were constructed to evaluate the contribution of individual LMW-PTPs to *B. cenocepacia* infectivity in
macrophages. The genetic background for the construction of the mutants was *B. cenocepacia* MH1K, an aminoglycoside sensitive K56-2 mutant strain useful for intracellular infections (Hamad *et al*., 2010). No differences in growth rate at 37 or 42 °C were found in the ∆*BCAM0208*, ∆*dpm*, ∆*bceD* and ∆*BCAL2200* mutants in comparison to parental MH1K (Fig. 3a). Also, the mutants did not differ in swimming and swarming motility (Fig. 3b), except for ∆*bceD*, which had a noticeable swarming defect (Fig. 3b).

**∆dpm cannot delay BeCV lysosomal fusion**

We have previously reported that the BeCV in infected macrophages has a maturation arrest demonstrated by failure to acidify and delayed fusion with lysosomes up to 6-8 h post infection (Huynh *et al*., 2010; Lamothe *et al*., 2007; Lamothe & Valvano, 2008). To determine whether vacuoles containing *B. cenocepacia* mutant strains display delayed phagosome maturation we preloaded macrophages with fluorescein isothiocyanate–dextran overnight prior to infection with parental and mutant bacteria. At 2 h post infection, BeCVs in ∆*dpm* infected macrophages showed significant differences in colocalization with the fluorescein-labelled lysosomes (Fig. 4a). Quantification indicated that 66 ± 2 % of BeCVs containing ∆*dpm* were dextran positive, while 40 ± 2 % of BeCVs containing the parental strain colocalized with dextran rich compartments (*p*<0.01; Fig. 4b). In contrast, BeCVs of macrophages infected with ∆*BCAM0208*, ∆*bceD* and ∆*BCAL2200* indicated 27 ± 6 %, 39 ± 4 % and 39 ± 2 % colocalization with dextran (Fig. 4b). These results suggest that loss of Dpm increases the traffic of BeCVs to the lysosome.

To further characterize the differential endocytic trafficking observed in macrophages infected with ∆*dpm* we compared the accumulation of the lysosome-associated membrane protein (LAMP-1) on the BeCVs by immunostaining (Fig. 5a). At 2 and 4 h postinfection, 47 ± 3 and 59 ± 1.5 % of BeCVs containing ∆*dpm* accumulated LAMP-1, respectively (Fig. 5b). In contrast, 30 ± 0.5 and 32 ± 7 % of BeCVs containing the parental strain colocalized with LAMP-1 (Fig. 5b), respectively, as it has been previously shown for *B. cenocepacia* strains (Schmerk & Valvano, 2013). LAMP-1 accumulation of ∆*dpm* BeCVs was restored to parental levels (25 ± 3 % and 45 ± 3 % after 2 and 4 h, respectively) by expressing *dpm* from the pdpm plasmid (Fig. 5a and b). Together, these results indicate that Dpm contributes to the delayed phagolysosomal fusion in macrophages infected with *B. cenocepacia*.

**Dpm is secreted intracellularly upon macrophage infection**

We tested whether DpmFLAG is secreted after bacterial internalization by macrophages using antiserum against *B. cenocepacia* in combination with monoclonal antibodies against the FLAG epitope. Macrophages were infected with ∆*dpm* carrying pdpm (encoding full length Dpm), or pΔ27 (encoding Dpm lacking the predicted leader peptide Table 1). Immunostaining of macrophages infected with ∆*dpm* (pdpm) revealed FLAG-specific immunofluorescence at the periphery of 62.74 ± 2.2 % BeCVs, which overlapped with the localization of *B. cenocepacia* (Fig. 6a and b), as expected by a secreted effector able to escape from the phagosome (Bach *et al*., 2008). In contrast, only 5.4 ± 0.2 % of FLAG-specific signal was observed colocalizing with BeCVs of macrophages infected with ∆*dpm* (pΔ27) (Fig. 6a and b), this value can be attributed to the detergent treatment during permeabilization for immunostaining or even to bacteria cell lysis. Immunoblot analysis of bacterial lysates revealed that *dpm* and ∆27*dpm* were equally expressed (Fig. 6c, left upper panel), confirming that both proteins are synthesized at similar levels. We also noticed that ∆27DpmFLAG, which has a predicted mass of 18 kDa, migrated
slightly above full-length \textit{Dpm}_{\text{FLAG}}, which has a predicted mass of 20.9 kDa (Fig. 6c, upper panel). We interpreted this result as an indication of the presence of uncleaved \textit{Δ27Dpm}_{\text{FLAG}} polypeptide and efficiently processed \textit{Dpm}_{\text{FLAG}}. This is supported by the demonstration that \textit{Dpm}_{\text{FLAG}} but not the truncated derivative was detected in the culture supernatants (Fig. 6c, upper panel). The lack of β-lactamase TEM-1 in the supernatants indicated that the bacteria had an intact outer membrane leakage (Fig 6c, lower panel). Together, our results indicate that Dpm requires an N-terminal (signal peptide) for secretion.

**Single LMW-PTPs are not required to \textit{B. cenocepacia} intramacrophage survival**

We also assessed whether the effect of \textit{Δdpm} in the acceleration of the BcCV maturation correlates with decreased intracellular bacterial survival. Gentamicin protection assays of macrophages infected with \textit{ΔBCAM0208}, \textit{Δdpm}, \textit{ΔbceD}, and \textit{ΔBCAL2200} revealed no differences in the recovery of intracellular bacteria at 1 h and 24 h post infection in comparison to the parental strain (Fig. 7). Similar results were also obtained using human THP-1 macrophages (data not shown), a more restrictive cell line to \textit{B. cenocepacia} infection (Schmerk & Valvano, 2013). Therefore, none of the LMW-PTPs encoding genes is required for \textit{B. cenocepacia} intramacrophage survival.

**Dpm delays maturation of \textit{B. multivorans}-containing vacuoles (BmCVs)**

Unlike \textit{B. cenocepacia}, \textit{B. multivorans} strains do not delay phagosomal maturation in murine macrophages (Schmerk & Valvano, 2013). No homolog to \textit{dpm} is present in the \textit{B. multivorans} ATCC 17616 genome (data not shown). To investigate whether Dpm could alter \textit{B. multivorans} trafficking pdpm and pDA17 (vector control) were mobilized into \textit{B. multivorans} ATCC 17616 Gm\textsuperscript{S} (Table 1) and these strains were used in infection experiments. Macrophages were preloaded with fluorescein isothiocyanate–dextran and subsequently infected with ATCC 17616 Gm\textsuperscript{S} (pDA17) or ATCC 17616 Gm\textsuperscript{S} (pdpm). At 2 h post infection, 45 ± 0.2% of BmCVs carrying ATCC 17616 Gm\textsuperscript{S} (pdpm) colocalized with fluorescent dextran. In contrast, colocalization with fluorescent dextran was observed in 74 ± 0.4% of ATCC 17616 Gm\textsuperscript{S} (pDA17) BmCVs (Fig. 8a and b). Furthermore, we confirmed that \textit{B. multivorans} supports the secretion of Dpm, as demonstrated by detection of this protein in the supernatant of \textit{B. multivorans} at similar levels as found with \textit{B. cenocepacia} (Fig. 8c). Together, the results of these experiments strongly support the notion that Dpm is a bacterially secreted protein affecting phagosomal maturation.

**Dpm lacks phosphatase activity**

Despite the absence of a critical Cys for catalysis in its predicted phosphatase motif we investigated if the Dpm has any phosphatase activity. We constructed inducible plasmids encoding N- or C-terminal histidine tagged Dpm (pHisdpm and pdpmHis, respectively, since the position of the histidine tag may alter phosphatase activity (Ueda & Wood, 2009). A plasmid expressing BCAL2200 carrying an N-terminal 6x-His tag (pHisL2200) was used as a control. Recombinant proteins were overproduced in \textit{E. coli} BL21(DE3) and purified by Ni\textsuperscript{2+}-affinity chromatography (Fig. 9a). A double banded pattern appeared using Dpm-His (carrying a C-terminal His tag), suggesting the purification of both processed and unprocessed forms of Dpm, which agreed with the predicted masses of 20.9 and 17.9 kDa, respectively (Fig. 9a). In contrast, the presence of the histidine tag plus 10 additional residues in the Dpm N-terminus blocks processing of the protein resulting in a polypeptide with an apparent mass of 24 kDa, as expected.
(Fig. 9a). All of the purified proteins were assayed for their ability to cleave the general phosphatase substrate p-nitrophenyl phosphate (PNPP). Recombinant BCAL2200 efficiently hydrolyzed the synthetic substrate (Fig. 9b) with an estimated specific activity of 1.44 ± 0.4 nmoles min⁻¹ µg⁻¹, a value that is in the range of that reported for other bacterial LMW-PTPs (Ferreira et al., 2007; Vincent et al., 1999). However, we could not detect PNPP hydrolysis by any of the Dpm derivatives under the same conditions used for BCAL2200 or by adding a 10-fold excess of substrate and/or protein into the reaction mix (Fig. 9b and data not shown). We attempted the reconstruction of the catalytic site by replacing the aspartate at position 36 with cysteine. The corresponding recombinant proteins His-DpmD36C and Dpm-HisD36C were purified to homogeneity (Fig. 9a) and assayed for PNPP hydrolysis, but they remained inactive (Fig. 9b). Together, these experiments indicate that Dpm lacks phosphatase activity, but this is not only due to the absence of a cysteine at the D36 position (Fig. 9b), suggesting additional mutations in this protein that alter its putative phosphatase activity.

**DISCUSSION**

Persistence of *B. cenocepacia* and other Bcc bacteria within the airways of cystic fibrosis patients has been associated with their ability to survive intracellularly in macrophages and epithelial cells (Valvano et al., 2014). We have previously shown abnormal maturation of the BcCVs in infected macrophages, but very little is known about the mechanism used by intracellular *B. cenocepacia* to arrest phagolysosome fusion. In this work, we show that deletion of *dpm* accelerates the maturation of the BcCVs in macrophages cells.

Dpm was initially identified as one member of a group of four predicted LMW-PTPs in *B. cenocepacia*. These putative proteins were investigated since bacterial protein tyrosine phosphatases commonly interfere with host signaling (Whitmore & Lamont, 2012) and could be candidates for effector proteins mediating the phagosomal maturation arrest in *B. cenocepacia*-infected macrophages. However, *dpm* was the only gene whose deletion resulted in loss of the phagosomal maturation defect in *B. cenocepacia*-infected macrophages. Several pieces of evidence support this conclusion: (i) the ∆*dpm* strain rapidly acquired the lysosomal marker LAMP-1, a phenotype that was abrogated by restoration of a functional *dpm* expressed from a recombinant plasmid; (ii) BcCVs containing ∆*dpm* bacteria colocalized more rapidly with dextran rich compartments; and (iii) heterologous expression of *dpm* in *B. multivorans*, which normally traffics rapidly to the lysosome, caused a delay in the traffic of bacteria-containing vacuoles to the lysosome compartment. Together, these observations indicate that Dpm directly contributes to the phagosome maturation arrest observed for intracellular *B. cenocepacia*.

The role of Dpm as an effector protein is also supported by its ability to be secreted to the culture supernatant and in macrophages. Indeed, Dpm was the only predicted tyrosine phosphatase that was secreted both *in vitro* and also during macrophage infection, as it could be detected at the periphery of the BcCVs. Deletion experiments revealed that Dpm secretion requires a signal peptide, suggesting that the protein crosses the inner membrane in a Sec-dependent manner. The specific mechanism involved in Dpm secretion across the outer membrane remains to be discovered. Nevertheless, once Dpm is transported outside the bacteria we can hypothesize that it escapes of the vacuole through the BcCV membrane damage induced via T6SS, in a similar
manner as has been shown for type II secreted proteins (Rosales-Reyes et al., 2012a). Despite the relevance of Dpm delaying phagosome maturation, bacterial intramacrophage survival was not affected in the Δdpm strain suggesting that other factors are required to enable B. cenocepacia survival in the lysosomes. The trafficking delay in B. cenocepacia is not observed with B. multivorans although both bacteria can survive in a lysosomal compartment (Schmerk & Valvano, 2013). This suggests that other factors likely common to both species play a role in intramacrophage survival. A similar scenario can be anticipated to other Bcc members; however additional work describing specific intracellular behavior of different Bcc members remains to be revealed.

The apparently restricted conservation of dpm to only a few Burkholderia species is puzzling. This gene in B. cenocepacia is located next to BCAM0627, encoding a putative endonuclease VII protein that is homologous to gp-49 from the T4 phage, which resolves four-way junctions in branch DNAs (Jensch & Kemper, 1986). BCAM0627 is predicted to be co-transcribed with BCAM0626, which encoded a helix-turn-helix protein. Both BCAM0626-BCAM0627 form a predicted toxin-antitoxin cassette, which is proposed to stabilize heterologous replicons (Dziewit et al., 2007). Therefore, it is possible that dpm and its neighboring genes may have been acquired by horizontal transmission, thus explaining their restricted conservation.

Remarkably, Dpm lacks phosphatase activity and this defect cannot be corrected by reconstructing the critical catalytic cysteine at the position corresponding to aspartate 36. However, a highly conserved second cysteine and a proline at positions 41 and 44, respectively, are also missing in the Dpm predicted phosphatase motif. These residues could also be important for proper enzymatic activity, since both cysteines of the phosphatase motif may form an intramolecular disulfide bridge that protects the nucleophilic cysteine from oxidation (Raugei et al., 2002). Alternatively, other mutations abolishing phosphatase activity appear to stabilize substrate binding (Bach et al., 2008; Bliska et al., 1992; Mukhopadhyay & Kennelly, 2011; Murli et al., 2001). Therefore, we speculate that Dpm could potentially recognize and remain attached to a tyrosine phosphorylated host cell substrate, partially blocking its function. Another possibility is that Dpm might recognize a non-tyrosine phosphorylated host substrate. For instance PtpA from M. tuberculosis prevents phagosomal acidification by binding to the H-subunit of the macrophage vacuolar-H+-ATPase, even though subunit H is not a tyrosine-phosphorylated protein (Wong et al., 2011). The region of PtpA required to bind H subunit was mapped at it C-terminal α-helix and is also independent of the presence of the PtpA catalytic cysteine (Wong et al., 2011). Unfortunately, attempts to isolate a putative macrophage host cell substrate for Dpm by pull-down assays, similar to those performed with M. tuberculosis were unsuccessful (data not shown), and further experiments are underway to elucidate its molecular mechanism. In summary, our results indicate that Dpm is a novel effector protein contributing to the ability of B. cenocepacia to subvert the phagocytic pathway in macrophages by a mechanism that is independent of its predicted phosphatase activity.

Acknowledgements
The authors thank to D. Aubert, R. Rosales-Reyes, C. Schmerk and O. El-Halfawy for technical advice. This research was supported by grants from Cystic Fibrosis Canada and the United
Kingdom Cystic Fibrosis Trust. A. A. was supported by a Post Doctoral fellowship from the Government of Canada and from the Consejo Nacional de Ciencia y Tecnología of Mexico.

References


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<thead>
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<th>Strain or plasmid</th>
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<th>Source/reference</th>
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<td>K56-2</td>
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<td>(Hamad et al., 2010)</td>
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**Plasmids**

- **pDAI-Scel-SacB**: ori<sub>pper</sub>, Tet<sup>R</sup>, Pdhfr, mob+, expressing I-Scel, SacB (Hamad et al., 2010)
- **pDA17**: Expression vector, ori<sub>pper</sub>, Tet<sup>R</sup>, mob<sup>+</sup>, Pdhfr, FLAG epitope (D. Aubert, unpublished)
- **pET28**: Expression vector, ori<sub>pper</sub>, Kan<sup>R</sup>, P<sub>77</sub> Novagen
- **pGPI-Scel**: ori<sub>pper</sub>, ΩTp<sup>R</sup>, mob<sup>+</sup>, I-Scel restriction site, Tp<sup>R</sup> (Flannagan et al., 2008)
- **pIN62**: ori<sub>pper</sub>, mob<sup>+</sup>, Cm<sup>R</sup>, DsRed (Vergunst et al., 2010)
- **pRK2013**: ori<sub>pper</sub>, RK2 derivative, Kan<sup>R</sup>, mob<sup>+</sup>, tra<sup>+</sup> (Figurski & Helinski, 1979)
- **pDelM0208**: pGPI-Scel with fragments flanking the BCAM0208 This study
- **pDelM0628**: pGPI-Scel with fragments flanking the dpm This study
- **pDelM0857**: pGPI-Scel with fragments flanking the BCAM0857 This study
- **pDelL2200**: pGPI-Scel with fragments flanking the BCAL2200 This study
- **pbceD**: pDA17 encoding bceD This study
- **Pdpm**: pDA17 encoding dpm This study
- **pL2200**: pDA17 encoding BCAL2200 This study
- **pM0208**: pDA17 encoding BCAM0208 This study
- **pΔ27**: pDA17 encoding dpm without their first 27 codons This study
- **pHisL2200**: pET28 encoding 6xHis-BCAL2200-FLAG tagged This study
- **pHisdpm**: pET28 encoding 6xHis-dpm-FLAG tagged This study
- **pHisdpm D36C**: pHisdpm, with the substitution D36C This study
- **pdpmHis**: pET28 encoding dpm-6xHis tagged This study
- **pdpmHis D36C**: pdpmHis, with the substitution D36C This study

<sup>a</sup>BCRRC, B. cepacia complex Research and Referral Repository for Canadian Cystic Fibrosis Clinics; Cm, chloramphenicol; Gm, gentamicin; Kan, kanamycin; Tet, tetracycline; Tp, trimethoprim.
### Table 2. Oligonucleotides primers

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<th>Purpose</th>
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*Restriction endonuclease sites incorporated in the oligonucleotide sequences are underlined.

*N/A indicates the absence of restriction site.
Figure Legends

Fig. 1. *B. cenocepacia* J2315 genome encodes 4 predicted LMW-PTPs. (a) Partial ClustalW sequence alignment of *Burkholderia cenocepacia* Dpm, BCAM0208, BceD, BCAL2200, *Mycobacterium tuberculosis* PtpA (MtB PtpA), *Saccharomyces cerevisiae* LTP1 (YEAST_LTP1), *Homo sapiens* LMWPc (HUMAN_LMWPc), and *Sus scrofa* LMWPc (SWINE_LMWPc). Bar indicates the tyrosine phosphatase motif, CX_R(S/T), and the asterisk denotes the position of the catalytic cysteine. (b) Schematic representation of the genetic environment around the predicted *B. cenocepacia* LMW-PTPs (black) and their associated tyrosine kinases (grey) as annotated in strain J2315. Predicted iron-sulfur metabolism related genes are marked by an asterisk. Arrows denote the directionality of gene transcription.

Fig. 2. Secretion of LMW-PTPs into the growth medium. pDA17 derivatives encoding the various predicted LMW-PTPs were expressed in *B. cenocepacia* K56-2. Immunodetection of whole-cell lysates and secreted proteins was performed using antibodies specific for the FLAG epitope (α-FLAG) and the beta lactamase of *E. coli* (α-TEM-1). Molecular masses of protein standards are indicated.

Fig. 3. Characterization of single-deletion mutant strains. (a) Growth curves in LB medium at 37 °C (straight lines) and 42 °C (dashed lines). (b) Bacterial motility assays in soft agar at 37 °C. Graph represents the mean ± SEM from three independent experiments. *** p<0.001 relative to MH1K (WT).

Fig. 4. Bacterial colocalization with dextran-rich vacuoles. Pulse-chase experiments were conducted with RAW264.7 macrophages. Lysosomes were pre-loaded with fluorescein-dextran and infected with strains expressing the DSRed fluorescent protein. Colocalization was assessed at 2 h post infection. (a) Immunofluorescence microscopy images of infected macrophages with MH1K (wild-type) and Δdpm strains. (b) Quantification of BcCV colocalizing with dextran at 2 h post infection. Graph represents the mean ± SEM from three independent experiments. ** p<0.01 relative to MH1K (WT).

Fig. 5. Colocalization of BcCVs with LAMP-1. RAW264.7 macrophages were infected with MH1K and Δdpm strains expressing the DSRed fluorescent protein or Δdpm expressing pdpm. (a) Colocalization at 2 h post infection. Cells were fixed and immunolabelled using rat anti-LAMP-1 and a secondary antibody conjugated to Alexa Fluor 488. For complementation assays *B. cenocepacia* did not express the DSRed encoding plasmid, as it was incompatible with the complementing plasmids. Therefore bacteria were detected in these cases by antiserum against K56-2, plus a secondary anti-rabbit antibody conjugated to Alexa Fluor 594. (b) Quantification of BcCVs colocalizing with LAMP-1 at 2 and 4 h post infection. Graph represents mean ± SEM from three independent experiments. ** p<0.01 relative to MH1K (WT).

Fig. 6. Secretion of Dpm during macrophage infection. (a) RAW264.7 macrophages were infected with Δdpm carrying the plasmid pdpm or pΔ27 and examined for immunolocalization of the Dpm protein together with *B. cenocepacia* at 1 h post infection. (b) Quantification of FLAG colocalizing with BcCV. Graph represents mean ± SEM from three independent experiments. *** p<0.001 relative to FLAG colocalization of Δdpm containing pdpm. (c) Secretion assays in *B. cenocepacia* expressing pdpm or pΔ27. Immunodetection of whole-cell-lysates and secreted
proteins was performed using antibodies specific for the FLAG epitope (α-FLAG) and the *E. coli* β-lactamase (α-TEM-1). Molecular masses of protein standards are indicated.

**Fig. 7.** Intramacrophage survival of *B. cenocepacia* mutants with deletion of LMW-PTPs encoding genes. Infected macrophages were lysed at 1 or 24 h post infection, and intracellular bacteria were enumerated by serial dilution and colony counts on LB agar plates. The results represent the averages and standard errors of three independent experiments. No significant differences in replication or survival were found using an unpaired *t* test.

**Fig. 8.** Dpm modifies *B. multivorans* phagosome traffic. (a) Pulse-chase experiments in RAW264.7 macrophages. Lysosomes were pre-loaded with fluorescein-dextran and infected with *B. multivorans* carrying pdpm or the vector pDA17. (b) Quantification of BmCV colocalization with dextran at 2 h post infection. Graph represents mean ± SEM from three independent experiments. *** p<0.001 relative to dextran colocalization of *B. multivorans* containing pDA17. (c) Immunodetection of Dpm in whole-cell-lysates (P) and supernatants (S) of *B. cenocepacia* and *B. multivorans* carrying pdpm. Detection was performed using antibodies specific for the FLAG epitope (α-FLAG) and the α subunit of the *E. coli* RNA polymerase (α-RNAPo) as cell lysis control. Molecular masses of protein standards are indicated at the left of each panel.

**Fig. 9.** Phosphatase activity assay. (a) Coomassie blue-stained SDS-PAGE of purified Dpm and BCAL2200 recombinant proteins. (b) Phosphatase activity of purified BCAL2200 and Dpm at 37 °C using 1mM PNPP as substrate.
(a)

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(b)

Fig. 1

819

Fig. 2
Fig. 3
Fig. 4

(a)

Phase contrast

wild type

Dextran

Merged

Phase contrast

Δdpm

Dextran

Merged

(b) Bacteria-containing vacuoles reaching dextran-labeled compartments

% of vacuoles containing bacteria

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**
Fig. 5

(a) Phase contrast images of wild type, Δdpm, Δdpm + pdpm, and their merged images. (b) Bar graph showing the percentage of LAMP-1 colocalization with WT, Δdpm, and Δdpm + pdpm. ** indicates statistical significance.
Fig. 6

Fig. 7
**Fig. 8**

- **B. multivorans + pDA17**
  - Phase contrast
  - Dextran
  - Merged

- **B. multivorans + pdpm**
  - Phase contrast
  - Dextran
  - Merged

**Fig. 9**

- (a) [Diagram showing protein expression levels for B. multivorans and B. cepacia using Western blots.]
  - **B. multivorans**
    - **Hist-Dpm**
    - **Hist-Dpm D36C**
    - **Dpm-His**
    - **Dpm-His D36C**

- (b) [Graph showing time course of optical density changes for His-BCAL200 and other protein samples.]