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A Type IV Secretion System Contributes to Intracellular Survival and Replication of *Burkholderia cenocepacia*▽

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***Burkholderia cenocepacia* is an important respiratory pathogen in persons with cystic fibrosis (CF). Recent studies indicate that *B. cenocepacia* survives within macrophages and airway epithelial cells in vitro by evading endosome-lysosome fusion. We investigated the role of a plasmid-encoded type IV secretion system in the intracellular survival, replication, and processing of *B. cenocepacia*. Both a wild-type strain (K56-2) and its type IV secretion system mutant (designated LC101) entered and replicated in CF airway epithelial cells and monocyte-derived macrophages. However, significantly more intracellular K56-2 than LC101 bacteria were found in both cell types at 24 h postinfection. Colocalization of bacteria with markers of the classical endocytic pathway indicated that although both K56-2 and LC101 reside transiently in early endosomes, a greater proportion of the mutant bacteria are targeted to lysosomal degradation. In contrast, wild-type bacteria escape from the classical endocytic pathway and traffic to the endoplasmic reticulum, where they replicate. Our results show that the intracellular processing of *B. cenocepacia* is similar in both professional and nonprofessional phagocytes and that a functional plasmid-encoded type IV secretion system contributes to the survival and replication of *B. cenocepacia* in eukaryotic cells.**

Burkholderia cenocepacia, a member of the *Burkholderia cepacia* complex, causes respiratory tract infection in persons with cystic fibrosis (CF). *B. cenocepacia* is a facultative intracellular bacterium that has been observed within airway epithelial cells and alveolar macrophages in vivo (5, 27). Previous work has demonstrated that the species enters and replicates within airway epithelial cells and monocyte-derived macrophages cultured in vitro (3, 20, 30, 32, 34). We recently demonstrated that *B. cenocepacia* interferes with the normal endocytic pathway, prevents acidification of bacterium-containing vacuoles, and traffics to and replicates in the endoplasmic reticulum (ER) of CF airway epithelial cells in vitro (30). Others have shown that *B. cenocepacia* persists with little replication within endosomes in murine macrophages and amoebae by delaying phagosome maturation and acidification (16, 17). The bacterial factors involved in these events have yet to be identified.

Several pathogenic bacterial species survive and replicate within host cells by modulating the processing of bacterium-containing vacuoles to evade their fusion with lysosomes (21, 23). This vacuole hijacking may involve bacterial effectors that interfere with host factors that are important in vacuole trafficking and membrane fusion. For example, the Spi/Ssa type III secretion system of *Salmonella enterica* exports SpiC into the host cell cytosol, where it affects host vacuolar trafficking machinery (35). The Inc proteins from *Chlamydia trachomatis* interfere with the processing of nascent chlamydial vacuoles (33). Effector proteins exported by the *Dot/Icm* type IV secretion system (T4SS) of *Legionella pneumophila* and the *VirB* T4SS of *Brucella* species are required to modulate the maturation of bacterium-containing phagosomes (8, 14, 25, 38). In *Bartonella henselae*, effector proteins exported by the *VirB/VirD4* T4SS similarly play roles in host cell invasion and intracellular replication (31).

As an opportunistic human pathogen, clear distinctions between virulent and avirulent *B. cenocepacia* strains are difficult; however, clinical-outcome data suggest the epidemic ET12 clonal lineage is, in general, more virulent than other *B. cenocepacia* strains (13, 18). Recently, two T4SSs belonging to the ET12 lineage (which includes strain K56-2) have been identified in *B. cenocepacia* by genomewide analysis (9). One of these is encoded on chromosome 2 and bears homology to the VirB/D4 T4SS of *Agrobacterium tumefaciens* (6). The other, termed the Ptw T4SS, is a chimeric system composed of VirB/D4 and F-specific subunits encoded on a resident 92-kb megaplasmid (6, 9). Insertional inactivation of *ptwD4*, which has homology to coupling proteins involved in effector translocation in other T4SSs, of the plasmid-encoded Ptw system abolished the plant water-soaking phenotype of *B. cenocepacia* strain K56-2 (9). In the study reported here, we investigated whether the *B. cenocepacia* Ptw T4SS is involved in intracellular bacterial replication and/or bacterium-containing vacuole processing. We constructed a *ptwD4* mutant and compared its capacity to invade and replicate in airway epithelial cells and macrophages to that of the wild-type parent.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. cenocepacia* K56-2 was isolated from a CF patient and is a representative of the epidemic ET12 lineage. For bacterial-invasion assays, strains were grown on Luria-Bertani (LB) agar (Fisher Scientific, Hampton, NH), with or without 300 μg ml^{-1} of tetracycline, for 24 h. A single colony was inoculated into 5 ml of LHC-8 medium (Invitrogen, Carlsbad, CA) for use with IB3 cells or RPMI 1640 medium (Invitrogen) for use with U937-derived macrophages and grown overnight at 37°C on an orbital shaker at 230 rpm. Bacteria were harvested by centrifugation, washed once with phos-

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phate-buffered saline (PBS), and suspended to the desired concentration in LHC-8 or RPMI 1640.

Construction of the *ptwD4* mutant. A 2.0-kb genomic fragment containing the *ptwD4* gene was amplified from *B. cenocepacia* K56-2 with primers PD4-1 (5′-TGACTCAGCGAAGGAA-3′) and PD4-2 (5′-ATCCGGTGGAAGCAA-3′) as previously described (9) and cloned into pCR2.1 (Invitrogen). To inactivate *ptwD4*, a 1,299-bp tetracycline resistance (Tc^r) cassette was amplified from pBR325 (2) using primers containing AatII sites and cloned into the AatII site of *ptwD4*. The disrupted *ptwD4* gene was excised from pCR2.1 by double digestion with SacI and XbaI and cloned into SacI/XbaI-linearized pEX18Tc, which is a suicide vector in *B. cenocepacia* (12). The suicide construct was introduced into K56-2 by triparental mating using pRK2013 as a helper (11). Mutants produced by allelic exchange were selected on LB agar containing 300 μg ml^{-1} tetracycline. Tc^r clones were cultured on medium containing 5% sucrose to select for double-crossover events. Mutagenesis of *ptwD4* was confirmed by PCR and Southern blot analyses. The K56-2 isogenic mutant containing the insertionally inactivated *ptwD4* gene was designated strain LC101.

RT-PCR. Reverse transcription (RT)-PCR was performed to evaluate the expression of genes downstream of *ptwD4*. Total RNA was isolated from mid-log-phase cultures of K56-2 or LC101 using the PureLink RNA Mini Kit (Invitrogen) and then treated with DNase I (Invitrogen) for 15 min. RT-PCR was performed using the SuperScript III One-Step RT-PCR System (Invitrogen) to target four open reading frames (ORFs) immediately downstream of *ptwD4*. The following primers were used: PC1 (5′-TCTGCAGCAGTTCTCGTACACGTT-3′) and PC2 (5′-TTGACGGAGTGGTGGCTAATCACA-3′), ORF1-1 (5′-AGTTCTCGGCGATCAATGAGCGAT-3′) and ORF1-2 (5′-GTGCAAACTCACGCGACACAAGAT-3′), ORF2-1 (5′-TTCCATGCATTCCTACTCGACGGT-3′) and ORF2-2 (5′-GTTTGAATCTCGTCCGCCTTGACA-3′), and ORF3-1 (5′-ATCAGTCGATGAAGCGCGTGAGAT-3′) and ORF3-2 (5′-ACATTGACCGGTAGTTCCGATGGAC-3′). Negative control reactions were performed by substituting *Taq* DNA polymerase for SuperScript III RT/*Taq* mix.

Complementation of the *ptwD4* mutant. A 3.1-kb genomic fragment containing the *ptwD4* gene was amplified from *B. cenocepacia* K56-2 with primers PD4-2B (5′-CGCAGACTGCGCGAATGC-3′) and PD4-3 (5′-CCGTACCTTACCGGGTGAAGG-3′) and cloned into pCR2.1. The *ptwD4* gene with a 1.1-kb flanking upstream region was excised from pCR2.1 by double digestion with XbaI and HindIII and cloned into XbaI/HindIII-linearized pBcRLaR-CL, which is derived from pBBR1 by the addition of the chloramphenicol cassette from EZ::Tn (EpiCentre) and the multiple cloning site from pCR2.1 (Invitrogen). The complement construct was introduced into LC101 by triparental mating as described above. Mutants containing the complementing plasmid were selected for on LB agar containing 300 μg ml^{-1} tetracycline and 200 μg ml^{-1} chloramphenicol and confirmed by miniprep isolation of plasmid DNA and PCR. Stability of the complement, designated strain LC102, was confirmed by serial passage on nonselective medium. LC102 was found to be stable at least through five serial passages on nonselective medium.

Growth curves. Overnight cultures of bacterial isolates were adjusted to an optical density at 600 nm of 0.05 in cell culture medium (RPMI 1640 or LHC-8) without serum or antibiotics. The optical density at 600 nm was measured every hour for 24 h.

Cell culture. IB3 cells are an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (kindly provided by P. Zeitlin, Johns Hopkins University). IB3 cells were grown in LHC-8 cell culture medium with glutamine supplemented with 5% fetal bovine serum, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. The human monocytic cell line U937 was obtained from the American Type Culture Collection (Manassas, VA). U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. Prior to use in infection experiments, U937 cells were stimulated with phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO) for 72 h in collagen-coated plates to promote their differentiation into macrophages. For bacterial-invasion and -replication experiments, IB3 or U937 cells (5×10^4 cells/well) were seeded in 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ). For immunofluorescence experiments, cells (10^5 cells/well) were cultured in collagen-coated four-well chamber slides (BD BioCoat, Bedford, MA). The cultures were grown at 37°C with 5% CO_2. Prior to infection, cells were incubated overnight in antibiotic- and serum-free medium.

Binding assays. Binding of bacteria to cells was determined as previously described (29). IB3 cells grown in six-well plates were lightly fixed with 0.5% paraformaldehyde for 30 min and washed with Dulbecco's PBS (D-PBS) containing 0.5% bovine serum albumin (BSA). Fluorescein isothiocyanate (FITC)-labeled K56-2 or LC101 (25) bacteria at a multiplicity of infection (MOI) of 100 were centrifuged ($250 \times g$ for 15 min) onto the monolayers, which were then incubated at 37°C for 2 h. The cells and adherent bacteria were detached from the plate by using Cell Dissociation Buffer (Invitrogen), washed with PBS-BSA, fixed in 1% paraformaldehyde, and analyzed with a Becton Dickinson FACSCalibur using CellQuest software.

Bacterial-invasion and intracellular-replication assays. Invasion and intracellular replication by *B. cenocepacia* were determined as previously described (30). Briefly, IB3 cells or macrophages were infected with K56-2 or LC101 at an MOI of 1 or 10 for 2 h. Extracellular bacteria were then killed by incubating the cells for 2 h in medium containing 500 μg ml^{-1} ceftazidime (Sigma) and 250 μg ml^{-1} gentamicin (Sigma). The cells were washed with D-PBS, and an aliquot of the final wash was plated to determine the number of viable extracellular bacteria remaining. In order to determine the efficacy of killing bacteria that might be adherent to cells or to the culture well, IB3 cells or macrophages grown in 24-well plates were fixed as in the binding assays. Cells were incubated with K56-2 or LC101 at an MOI of 10 for 2 h and then washed and treated with ceftazidime and gentamicin as in the invasion assays. Following antibiotic treatment, the cells were washed, collected in 0.1% Triton X-100, serially diluted, and plated to determine the number of viable bacteria. Greater than 99.9% of K56-2 and LC101 bacteria were killed by the antibiotic treatment. For invasion assays, cells were lysed in 0.1% Triton X-100, serially diluted, and plated to determine the number of intracellular bacteria. For intracellular-replication assays, cells were incubated for a further 20 h in medium containing 100 μg ml^{-1} gentamicin before being lysed for determination of the number of intracellular bacteria.

Cytotoxicity assay. The viability of cells after bacterial infection was measured using the Live/Dead Viability/Cytotoxicity Kit (Invitrogen) following the manufacturer's instructions. In brief, 8×10^3 IB3 cells or macrophages were seeded in 96-well tissue culture plates and infected with K56-2 or LC101 at an MOI of 1 for 2 h. Extracellular bacteria were killed as described above for bacterial-replication assays, and the cells were incubated for a further 20 h in medium containing 100 μg ml^{-1} gentamicin. The cells were washed three times in 300 μl D-PBS and then incubated with 1 μM ethidium homodimer 1 and 1 μM calcein AM for macrophages or 5 μM ethidium homodimer 1 and 2.5 μM calcein AM for IB3 cells. After 40 min, fluorescence was measured using a SpectraMax GeminiEM plate reader (Molecular Devices, Sunnyvale, CA).

Primary and secondary antibodies. Monoclonal antibody to LAMP1 was purchased from the Hybridoma Facility, University of Iowa, Iowa City. Monoclonal antibody to cathepsin D and polyclonal antibodies to EEA1, Rab7, and calnexin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibody (R418) to whole lysed *B. cenocepacia* has been described previously (27). Secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were purchased from Molecular Probes (Carlsbad, CA).

Immunofluorescence staining and microscopy. Immunofluorescence experiments were performed as previously described (30). IB3 cells or macrophages were infected with K56-2 or LC101 at an MOI of 20 and incubated for 30 min, 2 h, 4 h, or 24 h. The cells were washed three times in D-PBS to remove unbound bacteria and then fixed and permeabilized in cold methanol for 5 min at −20°C. The slides were blocked with 5% BSA in D-PBS for 1 h and then incubated with the appropriate primary antibody overnight at 4°C. Bound antibodies were detected by anti-mouse (LAMP1 and cathepsin D) or anti-goat (EEA1, Rab7, and calnexin) and anti-rabbit (R418) antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594. The cells were counterstained with DAPI (4′,6′-diamidino-2-phenylindole) for 5 min, mounted, and visualized using a Zeiss LSM 510 confocal laser microscope. The proportion of intracellular bacteria colocalizing with each marker of interest was determined by counting all intracellular bacteria in 10 to 12 microscopic fields.

Labeling cells with LysoTracker Red. Wild-type K56-2 and LC101 were labeled with FITC as previously described (28). IB3 cells were infected with bacteria at an MOI of 20 and incubated for 1 h or 5 h. The cells were washed to remove unbound bacteria and incubated with 100 nM LysoTracker Red (Invitrogen) for 1 h. Macrophages were infected with bacteria at a MOI of 20, incubated for 5 h, washed, and incubated with 100 nM LysoTracker Red. For 30-min infections, macrophages were preloaded with 100 nM LysoTracker Red for 30 min and then infected with bacteria at an MOI of 20 in medium containing 100 nM LysoTracker Red and incubated for another 30 min. The cells were washed to remove unbound bacteria, fixed with 3.7% formaldehyde (vol/vol) for 30 min, mounted, and visualized by fluorescence microscopy.

Statistical analysis. Statistical analyses were carried out using Sigma Stat (SPSS Inc., Chicago, IL). Data were expressed as geometric means and standard errors of the mean (SEM). Student's *t* test was used to compare two groups, and parametric one-way analysis of variance with the Tukey posttest was used to compare three or more groups. Differences were considered statistically significant if the *P* value was <0.05.

RESULTS

Mutagenesis of the *B. cenocepacia* Ptw T4SS. We inactivated the *ptwD4* gene of the plasmid-encoded Ptw T4SS of *B. cenocepacia* K56-2 to determine the potential role of the system in intracellular bacterial trafficking and replication. *ptwD4* encodes a protein with Walker A and B nucleotide binding/hydrolysis motifs (9). Its strong homology to the coupling proteins of other T4SSs (e.g., VirD4) suggests a role in effector protein translocation (1). PCR and Southern blot analyses of wild-type K56-2 and the novel *ptwD4* mutant (designated LC101) confirmed the insertion of a tetracycline resistance cassette in *ptwD4*. Mutation of *ptwD4* resulted in loss of the plant water-soaking phenotype as previously observed (9) but did not result in any observable growth defects of planktonic cultures of K56-2 or LC101 grown in cell culture medium (RPMI 1640 or LHC-8) without serum or antibiotics to reproduce the conditions used during infection of cell cultures (data not shown). Wild-type K56-2 and LC101 were used in parallel in all binding, invasion, replication, and colocalization experiments.

Invasion and intracellular replication of K56-2 and LC101. We examined whether mutation of *ptwD4* attenuates the capacity of *B. cenocepacia* to invade, survive, or replicate within CF airway epithelial (IB3) cells. As we observed previously, wild-type K56-2 invaded and replicated within IB3 cells (30). In invasion assays, IB3 cells were infected with bacteria at an MOI of 10 and incubated for 2 h. After removal of unbound bacteria, the cells were incubated with gentamicin and ceftazidime for a further 2 h to kill extracellular bacteria. The cells were then washed, lysed, and plated to determine the number of intracellular bacteria. Plating from the final wash fluid revealed negligible numbers of viable bacteria for both K56-2 and LC101 (data not shown). Cells infected with wild-type K56-2 had a geometric mean of 4.4×10^4 CFU per cell culture well, whereas cells infected with LC101 showed significantly fewer intracellular bacteria (geometric mean, 2.4×10^4 CFU per well) (Fig. 1A). We determined the binding of bacteria to cells by fluorescence-activated cell sorter analysis. Both K56-2 and LC101 bound to IB3 cells in very low numbers, with the wild-type bacteria showing 1.5-fold more binding than the mutant LC101 (data not shown).

In intracellular-replication assays, cells were infected with bacteria at an MOI of 1.0 and processed as in the invasion assay but then incubated for a further 20 h before being lysed and plated for bacterial counts. Cells incubated with K56-2 showed a geometric mean of 1.3×10^6 CFU per well. In contrast, cells infected with LC101 showed a geometric mean of 8.3×10^4 CFU per well (Fig. 1B). To confirm intracellular replication, cells infected with either the wild type or mutant were fixed/permeabilized and immunostained with antibody to *B. cenocepacia* and subjected to confocal microscopy. Microcolonies of both wild-type and mutant bacteria were clearly observed inside the cells, indicating intracellular replication (Fig. 2). However, while large clumps of wild-type bacteria were observed in the perinuclear area, groups of only three or four LC101 bacteria were observed, primarily in the perinuclear area and sometimes in the cytoplasm. These results indicate that LC101 is significantly attenuated in its capacity to bind, invade, and replicate in IB3 cells compared to the K56-2 parent.

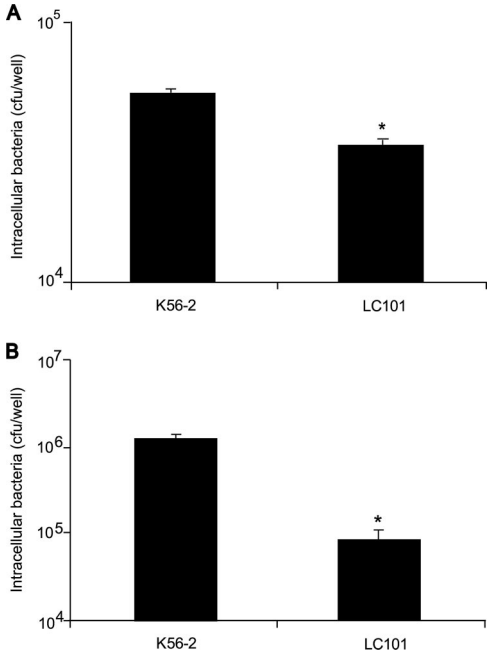

FIG. 1. Invasion and intracellular replication of K56-2 and LC101 in IB3 airway epithelial cells. IB3 cells were infected with K56-2 or LC101 at an MOI of 1 (replication) or 10 (invasion) and incubated for 2 h. Extracellular bacteria were killed by treating them with gentamicin and ceftazidime. The cells were either lysed with Triton X-100 (invasion experiments) or incubated for a further 20 h in the presence of gentamicin (replication experiments). The cells were washed, lysed with Triton X-100, and plated to determine the number of live bacteria. (A and B) Invasion and intracellular replication, respectively. The data represent geometric means (±SEM) calculated from three or four independent experiments, each performed in triplicate. (*, $P < 0.05$; Student's *t* test.)

B. cenocepacia was previously shown to enter and replicate in U937-derived macrophages (20). We therefore examined the effect of inactivating the Ptw T4SS on this process. U937 cells were stimulated with PMA to promote their differentiation into macrophages and infected with wild-type K56-2 or LC101 at an MOI of 1.0. At 4 h postinfection, four times fewer intracellular LC101 than wild-type K56-2 bacteria were found, although this difference was not statistically significant (Fig. 3A). For both K56-2 and LC101, negligible numbers of bacteria were recovered after washing (data not shown). At 24 h postinfection, significantly fewer LC101 bacteria than the K56-2 parent were found within macrophages (Fig. 3B). Confocal microscopy revealed large numbers of intracellular wild-type bacteria with no degraded bacterial particles within the cells. In contrast, only two or three LC101 bacteria were observed, with copious degraded bacterial particles within the cells (Fig. 4). These findings suggest that although the wild-type strain and the LC101 mutant are internalized by macrophages at comparable rates, the intracellular replication and survival of the wild-type strain are greater than those of the mutant.

We evaluated the viability of IB3 cells and macrophages after bacterial infection by using a Live/Dead assay. We found

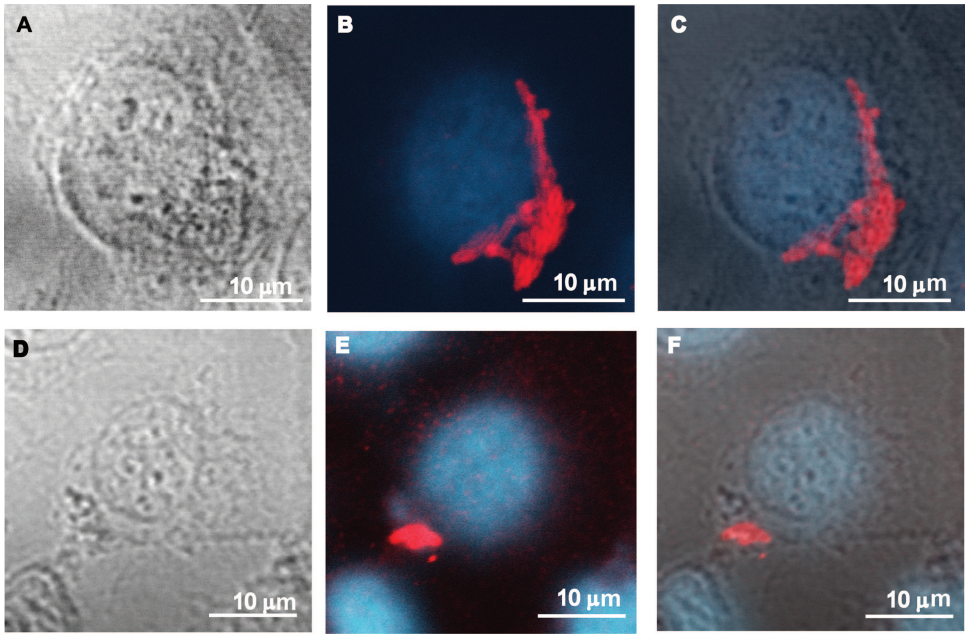

FIG. 2. Replication of K56-2 and LC101 inside IB3 cells. IB3 cells were infected with K56-2 (A to C) or LC101 (D to F) at an MOI of 100 and incubated for 2 h. Extracellular bacteria were killed by treating them with gentamicin and ceftazidime, and the cells were incubated for another 16 h in the presence of gentamicin. The cells were washed with PBS, fixed with cold methanol, and blocked, and bacteria were detected by using antibody to *B. cenocepacia*. The cells were imaged by confocal microscopy. (A and D) Differential interference contrast images of panels B and E, respectively. (C and F) Overlays of panels A and B, and D and F, respectively.

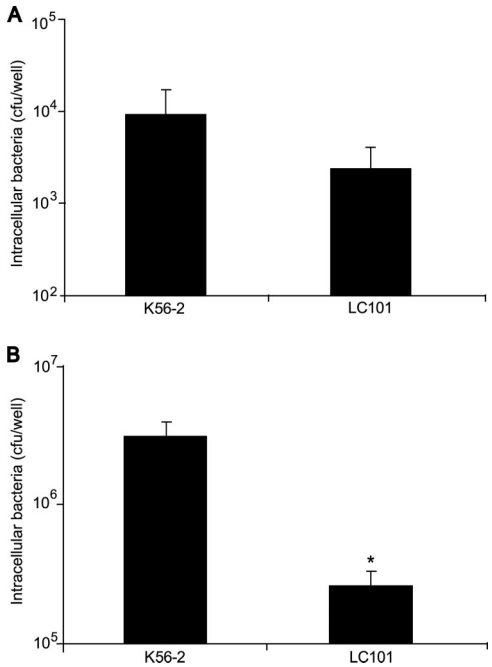

FIG. 3. Invasion and intracellular replication of K56-2 and LC101 in U937 cells. Monocyte-derived macrophages (U937) were infected with K56-2 or LC101 at an MOI of 1, and invasion and replication were determined as described in the legend to Fig. 1. (A and B) Invasion and intracellular replication, respectively. The data represent geometric means (±SEM) calculated from three or four independent experiments, each performed in triplicate. (*, $P < 0.05$; Student's *t* test.)

that 71.7% ± 4.5% of IB3 cells infected with K56-2 were viable, whereas 64.7% ± 4.0% of IB3 cells infected with LC101 were viable; this difference was not statistically significant. We further found that 57.6% ± 12.0% of macrophages infected with K56-2 were viable, whereas 47.4% ± 11.1% of macrophages infected with LC101 were viable; again, this was not a statistically significant difference.

We complemented LC101 with the full-length *ptwD4* gene, along with its upstream predicted promoter region, and were able to detect the wild-type gene by PCR for at least five serial passages on nonselective medium. We then examined the capacity of the complemented mutant to invade and replicate in both cell types. Although complementation with *ptwD4* partially restored the capacity of LC101 to invade and replicate in IB3 cells, we found no complementation of these phenotypes in macrophages (data not shown). RT-PCR analyses detected transcripts of all four ORFs immediately downstream of the targeted *ptwD4* gene in K56-2, as well as LC101, suggesting that the mutation is not polar. We also sought to investigate the possible roles of other genes in the plasmid-encoded T4SS using previously constructed mutants that were shown to abrogate water soaking (9). None of these other mutants, however, was stable under the current experimental conditions.

Intracellular processing of K56-2 and LC101. To investigate whether the differences we observed in intracellular survival between the wild-type K56-2 and LC101 involved differences in intracellular bacterial processing, we colocalized bacteria in infected IB3 cells and macrophages with markers of the endocytic pathway. We previously demonstrated that following infection of airway epithelial cells, wild-type K56-2 enters early endosomes but escapes from late endosomes to transiently

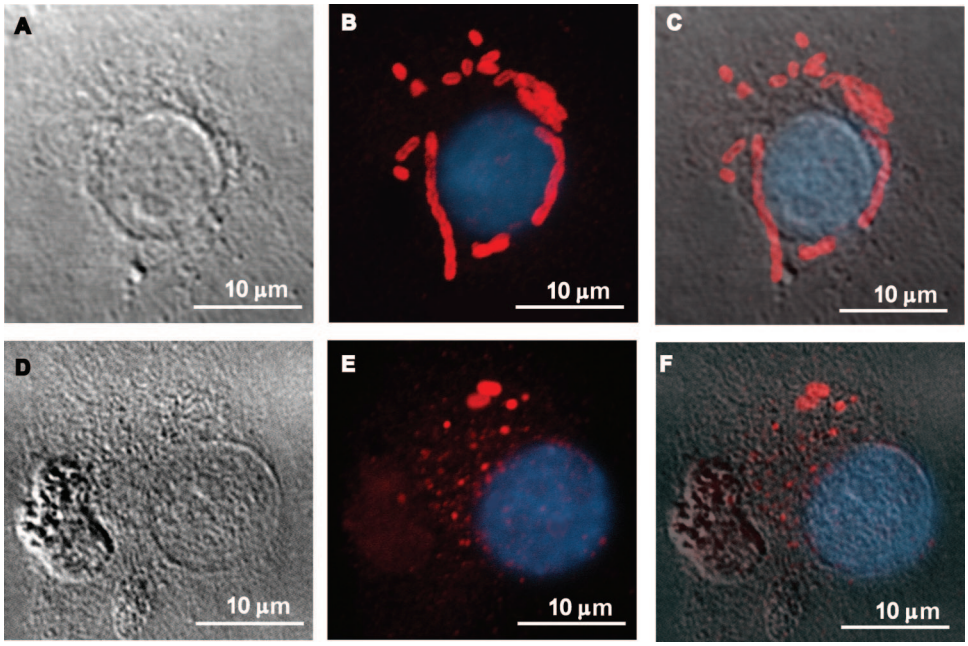

FIG. 4. Replication of K56-2 and LC101 in macrophages. PMA-treated U937 cells were infected with K56-2 (A to C) or LC101 (D to F) at an MOI of 100 and incubated for 2 h. Extracellular bacteria were killed by treating them with gentamicin and ceftazidime, and the cells were incubated for another 16 h in the presence of gentamicin. The cells were washed with PBS, fixed with cold methanol, and blocked, and bacteria were detected by using antibody to *B. cenocepacia*. The cells were imaged by confocal microscopy. (A and D) DIC images of panels B and E, respectively. (C and F) Overlays of panels A and B, and D and E, respectively.

reside in autophagosomes that apparently fail to fuse with mature lysosomes (30). The bacteria ultimately colocalize with ER-derived vesicles, where they appear to replicate. Heat-killed K56-2, in contrast, appeared to be targeted to the classical endocytic degradative pathway, entering late endosomes that fused with lysosomes. In the present study, K56-2 and LC101 were observed in early endosome marker EEA1-positive vacuoles in both IB3 cells and macrophages 30 min postinfection (data not shown). At 2 h postinfection, however, 9.5% of LC101 bacteria colocalized with the late endosome marker Rab7 while only 4.3% of wild-type K56-2 did so (Fig. 5), suggesting that unlike wild-type bacteria, LC101 is deficient in its ability to escape from late endosomes.

To investigate whether internalized wild-type K56-2 and LC101 enter autophagosomes, we assessed bacterial colocalization with the lysosomal/endosomal marker LAMP1 and with the ER marker calnexin, which are characteristic components of autophagosomes. The acid hydrolase cathepsin D was used as a marker for mature autophagolysosomes (15). Four hours after infection of IB3 cells, >80% of the intracellular wild type K56-2 bacteria were observed in calnexin-positive vacuoles. At that time point, ~20% of intracellular K56-2 bacteria colocalized with LAMP1, indicative of their presence in autophagosomes (24). Fewer than 10% of intracellular K56-2 bacteria colocalized with cathepsin D. In contrast, only ~50% of LC101 bacteria colocalized with calnexin and fewer than 5% colocalized with LAMP1. Approximately 30% of LC101 bacteria colocalized with vacuoles positive for cathepsin D. We also observed that intracellular LC101 bacteria were often disintegrated by 4 h postinfection; however, this was not observed in K56-2-infected cells (data not shown). At 24 h postinfection, 98% of intracellular K56-2 and 38% of LC101 bacteria were found in vacuoles positive for calnexin (Fig. 6A). However, whereas 28% of LC101 bacteria were in cathepsin D-positive vacuoles, only 9% of K56-2 bacteria colocalized with cathepsin D at that time point (Fig. 6B).

We next similarly infected and examined U937 monocyte-derived macrophages. At 4 h postinfection, we observed that greater than 90% of intracellular bacteria in both K56-2- and

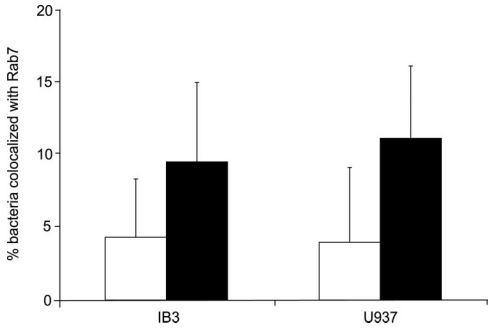

FIG. 5. Colocalization of K56-2 and LC101 with the late endosome marker Rab7 in infected IB3 cells and macrophages. IB3 cells and U937-derived macrophages were infected with K56-2 or LC101 at an MOI of 20 and incubated for 2 h. The infected cells were incubated with antibodies to Rab7 and *B. cenocepacia*, and bound antibody was visualized by confocal microscopy. The proportion of intracellular K56-2 (open bars) and LC101 (closed bars) colocalizing with Rab7 in each cell type is shown. The bars represent geometric means and SEM from counting intracellular bacteria in 10 microscopic fields from three independent experiments. A total of 692 bacteria in 40 fields were counted. The differences between K56-2 and LC101 are not statistically significant.

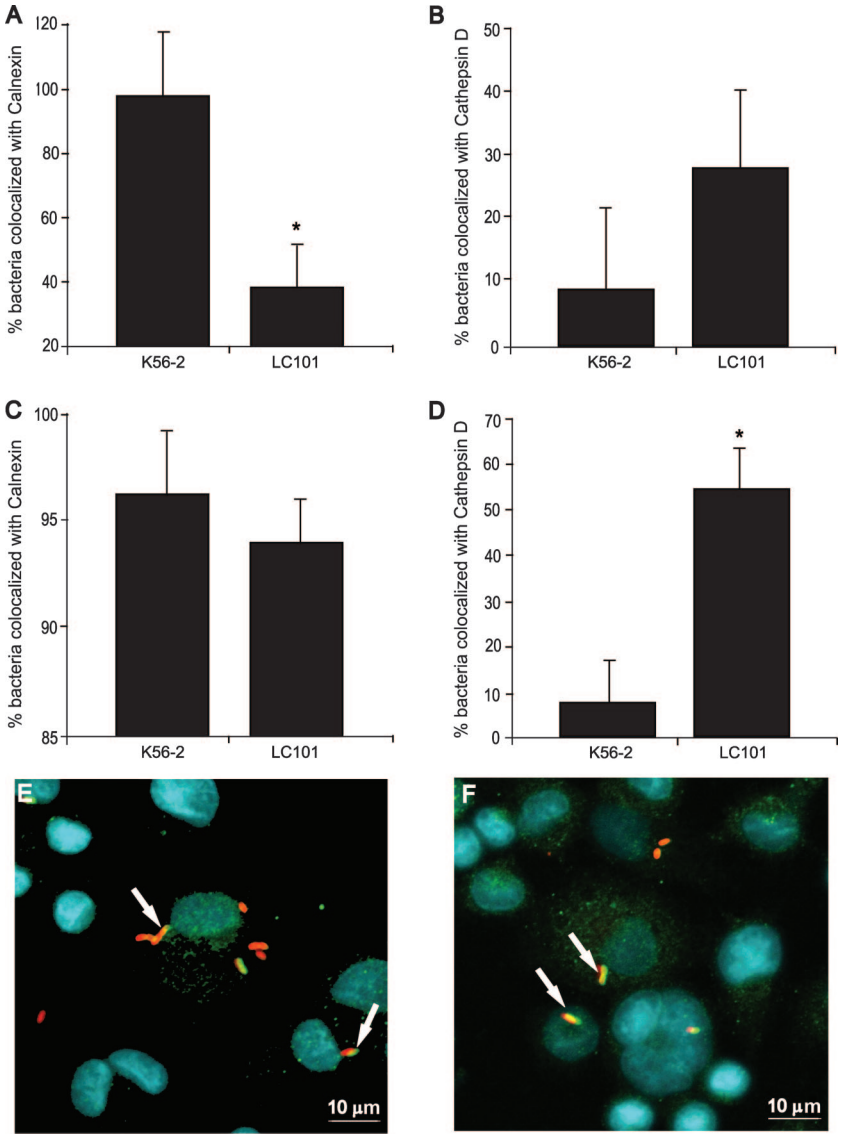

FIG. 6. Colocalization of K56-2 and LC101 with autophagosome markers in infected IB3 cells and macrophages. Cells were infected with K56-2 or LC101 at an MOI of 20 and incubated for 24 h as described in Materials and Methods. The infected cells were incubated with antibodies to *B. cenocepacia* and then, in separate experiments, with either calnexin or cathepsin D; bound antibody was visualized by confocal microscopy. The proportions of intracellular K56-2 and LC101 colocalized with calnexin (A and C) or cathepsin D (B and D) at 24 h in IB3 cells (A and B) and macrophages (C and D) are shown. The bars represent geometric means and SEM from counting intracellular bacteria in 10 to 12 microscopic fields from three independent experiments. A total of 1,716 bacteria in 42 fields were counted for calnexin and 2,362 bacteria in 40 fields for cathepsin D. (E and F) Colocalization of K56-2 (E) or LC101 (F) and calnexin (arrows) in macrophages 24 h postinfection showing bacteria in the perinuclear area. (*, $P < 0.05$; Student's t test; the absence of an asterisk indicates that the difference is not statistically significant.)

LC101-infected macrophages colocalized with calnexin (data not shown). A majority (>90%) of both K56-2 and LC101 bacteria were found to colocalize with calnexin even at 24 h postinfection (Fig. 6C). In contrast, while 55% of LC101-containing vacuoles were positive for cathepsin D, only 7.7% of K56-2 bacteria resided in cathepsin D-positive vacuoles (Fig. 6D). At that time point, we also observed bacteria in the perinuclear area colocalizing with the ER marker calnexin (Fig. 6E and F).

Acidification of *B. cenocepacia*-containing vacuoles. We showed previously that live *B. cenocepacia* bacteria appear to prevent acidification of bacterium-containing vacuoles in infected CF airway epithelial cells (30). To examine whether the Ptw T4SS contributes to this process, IB3 cells were infected with K56-2 or LC101 and incubated with LysoTracker Red, which labels acidified vacuoles. Fewer acidified vacuoles were observed in IB3 cells infected with K56-2 than in cells infected with LC101 (Fig. 7A and B). At 2 h postinfection, <4% of both K56-2 and LC101 bacteria were observed in acidified vacuoles (Fig. 7C). By 6 h postinfection, however, 36% of intracellular LC101 bacteria colocalized with acidified vacuoles, whereas 13% of K56-2 bacteria were in acidified vacuoles (Fig. 7C).

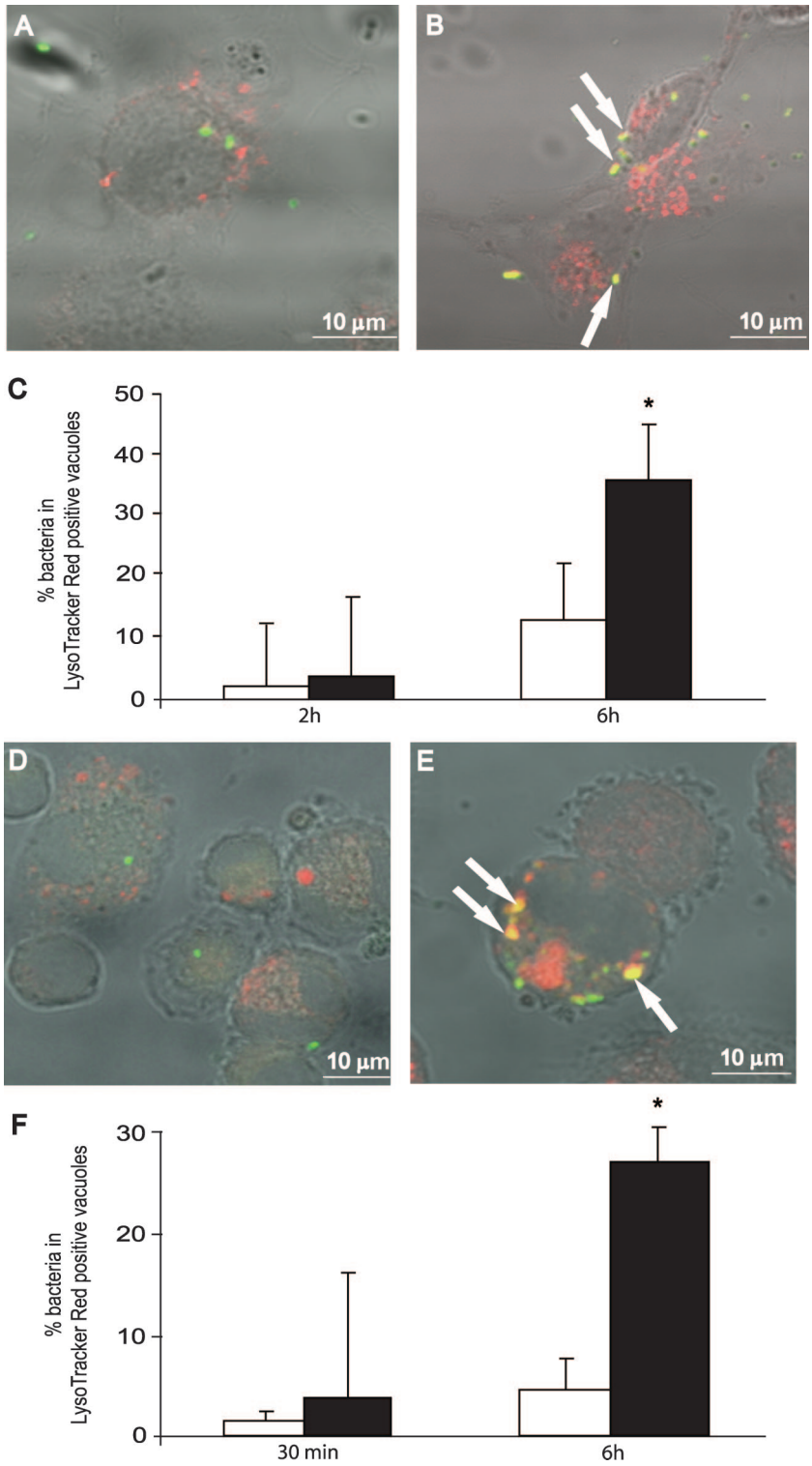

FIG. 7. Colocalization of K56-2 or LC101 and LysoTracker Red-positive vacuoles in IB3 cells and macrophages. IB3 cells (A and B) and macrophages (D and E) were infected with FITC-labeled K56-2 (A and D) or LC101 (B and E) at an MOI of 20 and processed as described in Materials and Methods. Green and red represent bacteria and LysoTracker Red-positive vacuoles, respectively. The arrows indicate bacteria colocalized with LysoTracker Red-positive vacuoles. The proportions of K56-2 (open bars) or LC101 (closed bars) colocalized with LysoTracker Red vacuoles in infected IB3 cells (C) or macrophages (F) are shown. The bars represent geometric means and SEM from counting intracellular bacteria in 10 microscopic fields from three independent experiments. A total of 1,179 bacteria in 80 fields were counted. (*, $P < 0.05$; Student's *t* test.)

We similarly examined infected U937-derived macrophages (Fig. 7D and E). At both 30 min and 6 h postinfection, a greater proportion of LC101 than of the parent K56-2 bacteria colocalized with acidified vacuoles (Fig. 7F).

DISCUSSION

B. cenocepacia, a species within the *B. cepacia* complex, is a bacterial pathogen of considerable concern for persons with CF. Infection of the respiratory tracts of CF patients by this species is most often chronic, refractory to antimicrobial therapy, and associated with significantly increased rates of morbidity and mortality (19). Unfortunately, relatively little is known about the pathogenic mechanisms of *B. cenocepacia* in CF.

The capacity of *B. cenocepacia* to survive and replicate within eukaryotic host cells may contribute to the organism's ability to persist and evade host defenses in vivo during chronic infection. Recently, we and others have shown that the intracellular survival of *B. cenocepacia* appears to involve modulation of the normal endocytic pathway of macrophages and CF airway epithelial cells (16, 30). Similar intracellular survival and replication of other pathogenic bacteria, including *L. pneumophila* and *Brucella*, *Chlamydia*, and *Salmonella* species, often involves bacterial effector proteins secreted by type III secretion systems or T4SSs (8, 14, 25, 33, 35, 38). The recent identification of T4SSs in *B. cenocepacia*, therefore, led us to investigate whether they similarly play a role in the species' intracellular survival.

To construct a mutant deficient in the plasmid-encoded T4SS, we focused on *ptwD4*, a gene that predicts a protein with strong homology to coupling proteins of other T4SSs. Inactivation of coupling proteins abrogates the secretion of T4SS effector proteins in other species (4). We found that although both wild-type K56-2 and LC101 were capable of entering and surviving in CF airway epithelial cells and monocyte-derived macrophages, fewer mutant bacteria were recovered from cells 4 h after infection. By 24 h postinfection, the numbers of T4SS mutant bacteria found within both epithelial cells and macrophages were significantly reduced compared to the wild-type strain. Although wild-type K56-2 showed 1.5 times more binding to cells than LC101, the overall level of bacterial binding was quite low. Furthermore, this observed difference in binding between K56-2 and LC101 seems quite unlikely to account for the greater than 10-fold difference between viable K56-2 and LC101 bacteria recovered from cells after 24 h. We also measured the viability of IB3 and U937 cells 24 h after infection to determine whether the observed differences in levels of intracellular bacteria (between K56-2- and LC101-infected cells) were due to differences in infected-cell viability. We did not find a significant difference in viability between cells infected with K56-2 and cells infected with LC101. In a previous study, Saini et al. (26) found that strains of *Burkholderia vietnamiensis* and *Burkholderia dolosa* survived without replication in murine macrophages for several days. Our results are consistent with those of Martin and Mohr (20), who found replication of *B. cenocepacia* J2315 (another representative of the ET12 lineage) in U937 monocyte-derived macrophages. Also similar to our observations, Martin and Mohr (20) found visible disruption of macrophage (U937) and epithelial cell monolayers 24 h after infection with *B. cenocepacia*, suggesting that the species is cytotoxic to cultured human cells. Our results suggest that the plasmid- encoded T4SS plays a role in the intracellular survival of *B. cenocepacia* in both professional and nonprofessional phagocytes.

We previously reported that internalized *B. cenocepacia* bacteria escape from the normal endocytic pathway and enter autophagosomes (based on colocalization with monodansyl cadaverine) that fail to mature into autophagolysosomes (30). In the current study, we compared the intracellular processing of the T4SS mutant and the parent strain to determine if the plasmid-encoded T4SS plays a role in modulating phagosome maturation, which could explain the decreased capacity of the mutant to persist intracellularly. Both wild-type K56-2 and LC101 appeared to enter the normal endocytic pathway, as both strains colocalized with the early endosome marker EEA1, irrespective of the host cell type. Shortly after infection of either epithelial cells or macrophages, however, more than twice as many mutant-containing vacuoles colocalized with late endosomal and lysosomal markers as did vacuoles containing wild-type bacteria, suggesting that a proportion of internalized LC101 mutant bacteria were quickly targeted to the classic endocytic pathway. At 24 h postinfection, approximately two or three times as many LC101 mutant bacteria were found in cathepsin D-positive vacuoles as the parent strain in both epithelial cells and macrophages, and LC101 bacteria were often observed as degraded particles. The relatively low level of colocalization of wild-type bacteria and cathepsin D, a marker of mature autophagolysosomes, suggests that the plasmid-encoded T4SS may allow *B. cenocepacia* to inhibit fusion of bacterium-containing autophagosomes with lysosomes.

We previously demonstrated that CF epithelial cells treated with heat-killed *B. cenocepacia* showed many more bacteria colocalizing with acidified vacuoles than were observed in cells infected with live *B. cenocepacia* bacteria (30). In the current study, we similarly found more than twice as many acidified bacterium-containing vacuoles in LC101 mutant-infected epithelial cells and macrophages as in cells infected with the wild-type strain. Collectively, these results suggest that more LC101 bacteria are killed via the endocytic pathway soon after entering host cells. Effector substrates delivered by the T4SS may be involved in diverting wild-type *B. cenocepacia* from this pathway, altering lysosome fusion with autophagosomes and preventing acidification of bacterium-containing vacuoles.

Our results are consistent with observations of other intracellular pathogens whose T4SSs are capable of altering membrane trafficking. Effectors secreted by the Dot/Icm T4SS of *L. pneumophila* alter host membrane trafficking and impede phagosome-lysosome fusion, establishing a vacuole permissive of bacterial replication (37). In *Brucella abortus*, a functional T4SS is necessary to escape the degradative endocytic pathway and to establish an ER-derived replication vacuole (7).

Inactivation of *ptwD4* did not completely abolish the capacity of the mutant to persist intracellularly or to evade phagosome-lysosome fusion. It is possible that the chromosomally encoded VirB/VirD T4SS system of *B. cenocepacia* may compensate for the deficiency of the plasmid-encoded T4SS. It is also possible that separate classes of effectors are responsible for evasion of lysosome fusion and for transport of bacterium-containing phagosomes to the ER (22). Other mechanisms distinct from the T4SS may act in addition to or instead of

secreted bacterial substrates to form the intracellular niche permissive for *B. cenocepacia* persistence. T4SS-independent mechanisms that inhibit phagosome-lysosome fusion have been identified in *L. pneumophila* (10). Further studies to look at these alternatives and to identify the T4SS effector proteins that are required for intracellular persistence of *B. cenocepacia* are in progress. Although several candidate proteins containing a positively charged C-terminal transport signal motif similar to that found *A. tumefaciens* effector proteins (36) have been identified, none has yet been shown to be involved in the Ptw T4SS.

In summary, the studies described in this report indicate that a functional plasmid-encoded T4SS contributes to the ability of *B. cenocepacia* to evade endocytic degradation and to survive and replicate in both airway epithelial cells and monocyte-derived macrophages. The contribution of the Ptw T4SS to this potential virulence phenotype provides ample incentive for further studies, such as identification of the effector protein(s) and construction of double (Ptw and VirB/VirD) mutants, to characterize the role of these T4SSs in *B. cenocepacia* virulence.

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REFERENCES

1. **Baron, C.** 2005. From bioremediation to biowarfare: on the impact and mechanism of type IV secretion systems. FEMS Microbiol. Lett. **253:**163–170.
2. **Blank, R. D., and D. B. Wilson.** 1982. Isolation and characterization of 2000-bp derivative of pBR322. Plasmid **7:**287–289.
3. **Burns, J. L., M. Jonas, E. Y. Chi, D. K. Clark, A. Berger, and A. Griffith.** 1996. Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. Infect. Immun. **64:**4054–4059.
4. **Cascales, E., and P. J. Christie.** 2003. The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. **1:**137–149.
5. **Chiu, C. H., A. Ostry, and D. P. Speert.** 2001. Invasion of murine respiratory epithelial cells in vivo by *Burkholderia cepacia*. J. Med. Microbiol. **50:**594–601.
6. **Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales.** 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu. Rev. Microbiol. **59:**451–485.
7. **Comerci, D. J., M. J. Martinez-Lorenzo, R. Sieira, J. P. Gorvel, and R. A. Ugalde.** 2001. Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. Cell Microbiol. **3:**159–168.
8. **Delrue, R. M., M. Martinez-Lorenzo, P. Lestrate, I. Danese, V. Bielarz, P. Mertens, X. De Bolle, A. Tibor, J. P. Gorvel, and J. J. Letesson.** 2001. Identification of *Brucella* spp. genes involved in intracellular trafficking. Cell Microbiol. **3:**487–497.
9. **Engledow, A. S., E. G. Medrano, E. Mahenthiralingam, J. J. LiPuma, and C. F. Gonzalez.** 2004. Involvement of a plasmid-encoded type IV secretion system in the plant tissue watersoaking phenotype of *Burkholderia cenocepacia*. J. Bacteriol. **186:**6015–6024.
10. **Fernandez-Moreira, E., J. H. Helbig, and M. S. Swanson.** 2006. Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes. Infect. Immun. **74:**3285–3295.
11. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
12. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
13. **Jones, A. M., M. E. Dodd, J. R. Govan, V. Barcus, C. J. Doherty, J. Morris, and A. K. Webb.** 2004. *Burkholderia cenocepacia* and *Burkholderia multivorans*: influence on survival in cystic fibrosis. Thorax **59:**948–951.
14. **Kirby, J. E., J. P. Vogel, H. L. Andrews, and R. R. Isberg.** 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27:**323–336.
15. **Kirkegaard, K., M. P. Taylor, and W. T. Jackson.** 2004. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. **2:**301–314.
16. **Lamothe, J., K. K. Huynh, S. Grinstein, and M. A. Valvano.** 2006. Intracellular survival of *Burkholderia cenocepacia* in macrophages is associated with a delay in the maturation of bacteria-containing vacuoles. Cell. Microbiol. **9:**40–53.
17. **Lamothe, J., S. Thyssen, and M. A. Valvano.** 2004. *Burkholderia cepacia* complex isolates survive intracellularly without replication within acidic vacuoles of *Acanthamoeba polyphaga*. Cell. Microbiol. **6:**1127–1138.
18. **Ledson, M. J., M. J. Gallagher, M. Jackson, C. A. Hart, and M. J. Walshaw.** 2002. Outcome of *Burkholderia cepacia* colonisation in an adult cystic fibrosis centre. Thorax **57:**142–145.
19. **LiPuma, J. J.** 1998. *Burkholderia cepacia* epidemiology and pathogenesis: Implications for infection control. Curr. Opin. Pulm. Med. **4:**337–341.
20. **Martin, D. W., and C. D. Mohr.** 2000. Invasion and intracellular survival of *Burkholderia cepacia*. Infect. Immun. **68:**24–29.
21. **Meresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, and J. P. Gorvel.** 1999. Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. Nat. Cell Biol. **1:**E183–E188.
22. **Nagai, H., and C. R. Roy.** 2003. Show me the substrates: modulation of host cell function by type IV secretion systems. Cell. Microbiol. **5:**373–383.
23. **Pizarro-Cerda, J., E. Moreno, M. Desjardins, and J. P. Gorvel.** 1997. When intracellular pathogens invade the frontiers of cell biology and immunology. Histol. Histopathol. **12:**1027–1038.
24. **Reggiori, F., and D. J. Klionsky.** 2005. Autophagosomes: biogenesis from scratch? Curr. Opin. Cell Biol. **17:**415–422.
25. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.
26. **Saini, L. S., S. B. Galsworthy, M. A. John, and M. A. Valvano.** 1999. Intracellular survival of *Burkholderia cepacia* complex isolates in the presence of macrophage cell activation. Microbiology **145:**3465–3475.
27. **Sajjan, U., M. Corey, A. Humar, E. Tullis, E. Cutz, C. Ackerley, and J. Forstner.** 2001. Immunolocalisation of *Burkholderia cepacia* in the lungs of cystic fibrosis patients. J. Med. Microbiol. **50:**535–546.
28. **Sajjan, U., Y. Wu, G. Kent, and J. Forstner.** 2000. Preferential adherence of cable-piliated *Burkholderia cepacia* to respiratory epithelia of CF knockout mice and human cystic fibrosis lung explants. J. Med. Microbiol. **49:**875–885.
29. **Sajjan, U. S., M. B. Hershenson, J. F. Forstner, and J. J. LiPuma.** 2008. *Burkholderia cenocepacia* ET12 strain activates TNFR1 signalling in cystic fibrosis airway epithelial cells. Cell. Microbiol. **10:**188–201.
30. **Sajjan, U. S., J. H. Yang, M. B. Hershenson, and J. J. LiPuma.** 2006. Intracellular trafficking and replication of *Burkholderia cenocepacia* in human cystic fibrosis airway epithelial cells. Cell. Microbiol. **8:**1456–1466.
31. **Schulein, R., P. Guye, T. A. Rhomberg, M. C. Schmid, G. Schroder, A. C. Vergunst, I. Carena, and C. Dehio.** 2005. A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. Proc. Natl. Acad. Sci. USA **102:**856–861.
32. **Schwab, U., M. Leigh, C. Ribeiro, J. Yankaskas, K. Burns, P. Gilligan, P. Sokol, and R. Boucher.** 2002. Patterns of epithelial cell invasion by different species of the *Burkholderia cepacia* complex in well-differentiated human airway epithelia. Infect. Immun. **70:**4547–4555.
33. **Scidmore-Carlson, M. A., E. I. Shaw, C. A. Dooley, E. R. Fischer, and T. Hackstadt.** 1999. Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. Mol. Microbiol. **33:**753–765.
34. **Tomich, M., C. A. Herfst, J. W. Golden, and C. D. Mohr.** 2002. Role of flagella in host cell invasion by *Burkholderia cepacia*. Infect. Immun. **70:**1799–1806.
35. **Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman.** 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. **18:**3924–3933.
36. **Vergunst, A. C., M. C. van Lier, A. den Dulk-Ras, T. A. Stuve, A. Ouwehand, and P. J. Hooykaas.** 2005. Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proc. Natl. Acad. Sci. USA **102:**832–837.
37. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
38. **Zuckman, D. M., J. B. Hung, and C. R. Roy.** 1999. Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. Mol. Microbiol. **32:**990–1001.

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