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

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

Construction of the *ptwD* mutant. A 2.0-kb genomic fragment containing the *ptwD* gene was amplified from *B. cenocepacia* K56-2 with primers PD4-1 (5'-TACGTACCGGAGAA-3') and PD4-2 (5'-AATCGTGTAAGGCAA-3') as previously described (9) and cloned into pCR2.1 (Invitrogen). To inactivate *ptwD*, a 1.299-bp tetacycline resistance (*Tc*) cassette was amplified from pBR325 (2) using primers containing AatII sites and cloned into the AatII site of *ptwD*. The disrupted *ptwD* gene was excised from pCR2.1 by double digestion with SacI and XbaI and cloned into SacI/XbaI-linearized pEX151CTc, 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 tetacycline. *Tc* clones were cultured on medium containing 5% sucrose to select for double-crossover events. Mutagenesis of *ptwD* was confirmed by PCR and Southern blot analyses. The *K56-2* isogenic mutant containing the insertional inactivated *ptwD* gene was designated strain LC101.

RT-PCR. Reverse transcription (RT)-PCR was performed to evaluate the expression of genes downstream of *ptwD*. 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 *ptwD*. The following primers were used: PCI (5'-TCTCGAGCGTCTTGCATACGCT-3') and PC2 (5'-TTGACGGAGTGGTGGCTAATCACA-3'). ORF1-1 (5'-AGTTCGCGGCACTCGCTGGA-3') and ORF1-2 (5'-GTGACGACTACGCCGACAACAAGT-3'). ORF2-1 (5'-TTCATGAGCTTCTACCTAAGGCCT-3') and ORF2-2 (5'-TGCATGGGATTCGGCTCGCAAAGT-3'). ORF3-1 (5'-TCTGCAGCAGTTCTCGTACACGTT-3') and ORF3-2 (5'-ACATTAGGGTGATTCCGGATGAC-3'). Negative control reactions were performed by substituting Taq DNA polymerase for SuperScript III RT/TAq mix.

Complementation of the *ptwD* mutant. A 3.1-kb genomic fragment containing the *ptwD* gene was amplified from *B. cenocepacia* K56-2 with primers PD4-2B (5'-CGAGACTCCGGCCAATGTC-3') and PD4-3 (5'-CCGTACCTTACCGGGGCC-3') and cloned into pCR2.1. The *ptwD* 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 pBRRLaRCL, which is derived from pBBR1 by the addition of the chloramphenicol cassette from EZ-Tn (EpiCentre) and the multiple cloning site from pCR2.1. 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 100 µg ml⁻¹ gentamicin. Bacteria were then lysed by lysozyme for 1 h 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 and lysed by lysozyme for determination of the number of intracellular bacteria.
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/hydrosis 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 x 10^4 CFU per cell culture well, whereas cells infected with LC101 showed significantly fewer intracellular bacteria (geometric mean, 2.4 x 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 x 10^6 CFU per well. In contrast, cells infected with LC101 showed a geometric mean of 8.3 x 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.

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...
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 \textit{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 \textit{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 \textit{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. 2. Replication of K56-2 and LC101 inside IB3 cells.](image)

![FIG. 3. Invasion and intracellular replication of K56-2 and LC101 in U937 cells.](image)
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
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 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 monocytic-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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