

## Involvement of a Plasmid-Encoded Type IV Secretion System in the Plant Tissue Watersoaking Phenotype of *Burkholderia cenocepacia*

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***Burkholderia cenocepacia* strain K56-2, a representative of the *Burkholderia cepacia* complex, is part of the epidemic and clinically problematic ET12 lineage. The strain produced plant tissue watersoaking (ptw) on onion tissue, which is a plant disease-associated trait. Using plasposon mutagenesis, mutants in the ptw phenotype were generated. The translated sequence of a disrupted gene (*ptwD4*) from a ptw-negative mutant showed homology to VirD4-like proteins. Analysis of the region proximal to the transfer gene homolog identified a gene cluster located on the 92-kb resident plasmid that showed homology to type IV secretion systems. The role of *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10* in the expression of ptw activity was determined by conducting site-directed mutagenesis. The ptw phenotype was not expressed by K56-2 derivatives with a disruption in *ptwD4*, *ptwB4*, or *ptwB10* but was observed in a derivative with a disruption in *ptwC*. Complementation of ptw-negative K56-2 derivatives in *trans* resulted in complete restoration of the ptw phenotype. In addition, analysis of culture supernatants revealed that the putative ptw effector(s) was a secreted, heat-stable protein(s) that caused plasmolysis of plant protoplasts. A second chromosomally encoded type IV secretion system with complete homology to the VirB-VirD system was identified in K56-2. Site-directed mutagenesis of key secretory genes in the VirB-VirD system did not affect expression of the ptw phenotype. Our findings indicate that in strain K56-2, the plasmid-encoded Ptw type IV secretion system is responsible for the secretion of a plant cytotoxic protein(s).**

The *Burkholderia cepacia* complex (Bcc) consists of nine genomovars recently elevated to species status (11, 65, 66, 68). Members of the Bcc include plant and animal pathogens as well as catabolically active soil saprophytes (64). Some Bcc members can cause life-threatening respiratory infections, particularly in persons with cystic fibrosis (CF) (43). Studies indicate that 85 to 90% of strains isolated from infected CF patients are *B. cenocepacia* or *B. multivorans*, with other Bcc species being infrequently isolated (41). Several epidemic clonal lineages of *B. cenocepacia* have been identified (12), including the ET12 lineage, which is responsible for infecting many CF patients in Canada and the United Kingdom (32, 39); the PHDC lineage, responsible for nearly all Bcc infections in the mid-Atlantic region of the United States (8); and the Midwest lineage, which is responsible for infecting numerous patients in CF centers in the midwestern region of the United States (42). Factors that account for the apparent enhanced capacity of epidemic clones for human infection are unknown.

Postulated clinically associated virulence determinants for *B. cenocepacia* include hemolysins (30, 67), siderophores (62), and cable pili (56). The hemolysin induces apoptosis and degranulation of phagocytes (30), and siderophore production, regulated by quorum sensing (40), plays a role in the primary

colonization process of animal lung and spleen tissues (62). Cable pili are important in adherence to the respiratory mucosal blanket and epithelial cells (57).

In *B. cepacia* type strain ATCC 25416, a plant pathogenic representative of the Bcc, a plasmid-encoded pectate hydrolase (Peh) is a virulence factor necessary for maceration of onion tissue (24). Derivatives of ATCC 25416 that have been cured of the Peh-encoding plasmid do not macerate onion tissue. However, the Peh-negative derivatives remain capable of causing a plant tissue watersoaking (ptw) phenotype that results from loss of cell membrane integrity and the accumulation of fluids in the intracellular spaces of plant tissue (22). In a survey of isolates from the Bcc experimental strain panel (47), we found that all of the *B. cepacia* and *B. cenocepacia* strains tested and one *B. vietnamiensis* strain produced the ptw phenotype, suggesting that members of these genomic species may produce a cytotoxic factor(s) that affects plant cell integrity (50).

In recent years, a body of research has substantiated the concept that bacterial pathogens share common secretion mechanisms for the delivery of virulence determinants (9, 54). There are numerous examples of the importance of type III (54) and type IV secretion systems (10) in the infection process for both plant and animal pathogens, with evidence that elements of the secretion systems show an ancestral relationship to bacterial transport machinery. It has been hypothesized that type III secretion systems are derived from flagellar assembly constituents modified to function as a transport mechanism for virulence factors (46). The type IV secretion system from *Agrobacterium tumefaciens* was the first to be identified and is

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics <sup>a</sup>	Reference or source
pK56-2	92-kb resident plasmid of <i>B. cenocepacia</i> strain K56-2 containing the <i>ptw</i> cluster	This study
pDrive	Cloning vector; Km <sup>r</sup> , Ap <sup>r</sup> , pUC origin, T7, SP6, <i>lacZα</i> <sup>+</sup>	Qiagen
pRK2013	Km <sup>r</sup> , Tra <sup>+</sup> , Mob <sup>+</sup> , ColE1 replicon	20
pBR325	Tc <sup>r</sup> , Ap <sup>r</sup> , Cm <sup>r</sup> , ColE1 replicon	4
pR388	IncW, Su <sup>r</sup> , Tp <sup>r</sup>	13
pTnMod-RTp <sup>r</sup>	Tn5, R6K <i>oriR</i> , Tp <sup>r</sup>	15
pScosBC1	Cosmid with K56-2 genomic library	62
pSBC-9F5	pScosBC1 with K56-2 genomic DNA containing <i>ptwD4</i> and <i>ptwC</i>	This study
pSBC-3H3	pScosBC1 with K56-2 genomic DNA containing <i>ptwB4</i> and <i>ptwB10</i>	This study
pJQ200SK	Gm <sup>r</sup> <i>sacB</i> Mob <sup>+</sup> , P15A replicon	55
pURF047	IncW, Amp <sup>r</sup> , Gm <sup>r</sup> , Mob <sup>+</sup> , <i>lacZα</i> <sup>+</sup> , Par <sup>+</sup> , derivative of pURF043	63
pASE101	pURF047 with 2.0-kb Tp <sup>r</sup> cassette inserted at <i>ScaI</i> site of Ap <sup>r</sup> cassette	This study
pASE102	pJQ200SK containing 10-kb BamHI fragment (pSBC-9F5) containing <i>ptwD4</i> and <i>ptwC</i> inserted in the MCS	This study
pASE103	pJQ200SK containing 11-kb BamHI fragment (pSBC-3H3) containing <i>ptwB4</i> and <i>ptwB10</i> inserted in the MCS	This study
pASE104	pASE101 containing 10-kb BamHI fragment (pSBC-9F5) containing <i>ptwD4</i> and <i>ptwC</i> inserted in the MCS	This study
pASE105	pASE101 containing 11-kb BamHI fragment (pSBC1-3H3) containing <i>ptwB4</i> and <i>ptwB10</i> inserted in the MCS	This study
pASE106	pASE102 with 1.9-kb Tc <sup>r</sup> cassette from pBR325 inserted in the <i>ScaI</i> site of <i>ptwD4</i>	This study
pASE107	pASE102 with 1.9-kb Tc <sup>r</sup> cassette from pBR325 inserted in the <i>EcoRV</i> site of <i>ptwC</i>	This study
pASE108	pASE103 with 1.9-kb Tc <sup>r</sup> cassette from pBR325 inserted in the <i>ApaI</i> site of <i>ptwB4</i>	This study
pASE109	pASE103 with 1.9-kb Tc <sup>r</sup> cassette from pBR325 inserted in the <i>SacI</i> site of <i>ptwB10</i>	This study
pASE110	pDrive containing 1.5-kb PCR product containing <i>virB6</i>	This study
pASE111	pDrive containing 1.9-kb PCR product containing <i>virB11</i>	This study
pASE112	pJQ200SK containing <i>virB6</i> from pASE110	This study
pASE113	pJQ200SK containing <i>virB11</i> from pASE111	This study
pASE114	pASE112 with 1.9-kb Tc <sup>r</sup> cassette from pBR325 inserted into the <i>ScaI</i> site of <i>virB6</i>	This study
pASE115	pASE113 with 1.9 kb Tc <sup>r</sup> cassette from pBR325 inserted into the <i>ScaI</i> site of <i>virB11</i>	This study

<sup>a</sup> Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Su, sulphonamide; Tc, tetracycline; Tp, trimethoprim; *ptw*, plant tissue watersoaking phenotype.

used to deliver oncogenic transfer DNA and effector proteins to plant cells during infection (10, 37). Presently, type IV secretion systems can be categorized as (i) conjugation systems that mediate DNA transfer to recipient cells, (ii) effector translocator systems that transfer molecules termed effectors to eukaryotic cells during infection, or (iii) DNA uptake or release systems mediating exchange of DNA with the milieu (18). The *Legionella pneumophila* Dot/Icm transporter is an example of a type IV secretion system that is capable of both conjugation and effector translocation (e.g., RalF) (52). *Helicobacter pylori* possesses two type IV secretion systems with differing roles. The Cag secretory apparatus functions in the translocation of CagA into host cells, and the Com system is used to uptake DNA to facilitate genetic variation and enhance cell survival (16, 18).

In this study we report on the presence of both a plasmid and a chromosomally encoded type IV secretion system in *B. cenocepacia* strain K56-2 and describe the involvement of the plasmid-encoded system in the export of a putative protein(s) responsible for the *ptw* phenotype.

#### MATERIALS AND METHODS

**Media and growth conditions.** Descriptions of plasmids and bacterial strains used in this study are listed in Tables 1 and 2, respectively. Luria Bertani (LB) medium was used for routine maintenance of cultures. Minimal medium Vogel Bonner (69) amended with 1.0% glucose (VBG) was used in mating experiments. Minimal medium M9 (59) containing 1.0% glucose was used for culturing bacteria for supernatant analysis. *B. cenocepacia* and *Escherichia coli* strains were grown at 37°C. Media were supplemented with appropriate concentrations of antibiotics as needed for selection. Antibiotics were added to media at the

following concentrations: 50 µg of tetracycline (Tc)/ml and 100 µg of trimethoprim (Tp)/ml for *B. cenocepacia* and 10 µg of Tc/ml, 10 µg of gentamicin (Gm)/ml, 40 µg of ampicillin/ml, and 100 µg of Tp/ml for *E. coli*.

**Plant tissue assay and growth study.** Plant tissue watersoaking activity was determined as previously described (24). Onion cultivar 1015Y was used for plant tissue assays. Onion bulb scales were inoculated, in triplicate, with 10 µl of an aqueous suspension of the isolate being tested as previously described (24). Aqueous bacterial suspensions were adjusted spectrophotometrically ( $A_{425} = 0.5$ ) to yield a final concentration of  $\sim 10^6$  CFU/scale. Sterile double-distilled deionized water was used as a negative control and strain K56-2 served as the positive control in all experiments. Scales were placed on a sheet of aluminum foil that had been surface sterilized with 70% ethanol in containers layered with paper towels that were moistened with double-distilled deionized water, sealed, and incubated at 37°C. Plant tissue watersoaking activity was assessed at 24, 48, and 72 h postinoculation by measuring the vertical and horizontal diameters of the zones for triplicate samples in three independent experiments. Data were reported as the average area of tissue watersoaking  $\pm$  standard deviations (SD) for the three independent experiments in triplicate.

To obtain a quantitative measure of growth in plant tissue for strains K56-2, AE307, and AE310, bulb scales were inoculated with an aqueous suspension of the strains as described above. At 0, 24, 48, and 72 h postinoculation watersoaked zones were measured and tissue was processed to determine the bacterial population. Scales were commuted and crushed in 0.0125 M phosphate buffer (pH 7.1) containing Triton X-100 (0.01%) by using a sterile mortar and pestle. A dilution series of each tissue slurry was plated to LB agar containing Gm (10 µg/ml) and Tc (10 µg/ml) and was incubated at 37°C for 48 h. Population data for each time interval was expressed as the average CFU/gram of tissue  $\pm$  SD for three independent experiments in triplicate. Tissue watersoaking data were reported as the average area of watersoaking  $\pm$  SD for three independent experiments in triplicate.

**Activity testing of culture supernatants.** Cultures of strains K56-2, AE307, and AE310 were grown at 37°C and harvested at late exponential phase ( $A_{425} = 0.9$ ) by centrifugation (16,000  $\times$  g, 15 min, at 5°C). Supernatants (1 liter) were filter sterilized using 0.22 µm filters (Pall) and were concentrated to 3 ml by using an Amicon ultrafiltration stirred cell (molecular weight cutoff, 10,000). Protein

concentration of supernatant concentrates was determined by the method of Koch and Putnam (36). Concentrated uninoculated medium (1 liter) served as the negative control.

Plant protoplasts were used as the plant tissue system to obtain a quantitative measurement of activities of concentrated culture supernatants. Initial testing demonstrated that onion and carrot protoplasts were similar in sensitivity, and carrots were chosen because the presence of chromoplasts facilitated a more accurate evaluation of plasmolytic activity. Protoplasts were obtained using the following procedure (33). Young carrots were placed in a 10% sodium hypochlorite solution for 5 min and then were rinsed four times with sterile double-distilled deionized water. The epidermis was resected with a sterile scalpel and discarded. Cortical and phloem tissues were obtained, diced into fragments ~2 mm in width, and placed in a sterile petri dish containing 20 ml of a filter-sterilized enzyme solution. The enzyme solution contained 10% mannitol (Fisher), 1.5% cellulase (CalBiochem), 0.25% macerace (CalBiochem), and 0.75% bovine serum albumin fraction V (Sigma) (33). Tissue was digested in the dark for 5 h at 28°C with gentle shaking (50 rpm). The carrot tissue was strained through nylon mesh and centrifuged ( $825 \times g$  for 5 min at 25°C). The supernatant was removed using a pipette, avoiding disturbance of the protoplasts. The protoplasts were washed with 20 ml of 10% mannitol by resuspending with gentle swirling and centrifuged. The supernatant was removed and the pellet was gently resuspended in 10 ml of 10% mannitol, and a 5-ml 20% sucrose cushion was carefully layered in the bottom of the tube. The sample was centrifuged and the protoplasts were collected from the interface and counted with a hemacytometer (Hausser Scientific). On average,  $1.8 \times 10^6$  protoplasts/ml were obtained.

The plasmolytic activity of supernatants was determined by using concentrates standardized for protein concentration and adjusted to 10% mannitol. The standardized concentrates, as well as dilutions (1:1, 1:5, and 1:10), were added to protoplasts. Assay mixtures and controls were incubated in 96-well microtiter plates (Corning) with an average concentration of 50 protoplasts per well. Assays, done in triplicate, were observed for changes in protoplast integrity at 2-h intervals for 8 h using a Zeiss inverted microscope. In addition, the effect of temperature on plasmolytic activity was tested by heating concentrates to 100°C for 10 min or at 80, 65, or 37°C for 1 h. Concentrates were also individually treated with 1-mg/ml final concentrations of proteinase K (Boehringer Mannheim) dissolved in 0.05 M Tris-HCl, pH 8; DNase I (3,200 U/mg; Sigma) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, and 50 µg of bovine serum albumin/ml; or RNase A (100 U/mg; Sigma) in 0.25 M Tris-HCl, pH 6.8. All reaction mixtures were incubated at temperatures optimal for each enzyme's activity and were then adjusted to 10% mannitol before being mixed with protoplasts. Each incubation duration was performed in duplicate in three independent experiments with an average of 50 protoplasts examined at 0, 2, 4, 6, and 8 h. Data were expressed as percent plasmolysis  $\pm$  SD for three independent experiments. Plasmolysis was analyzed as a function of time, dilution, treatment, and their interactions. Level of statistical significance of the different variables was determined with an analysis of variance. Statistical analysis was performed using SAS software and the general linear model procedure (SAS Institute Inc.).

**Mating experiments.** For triparental matings, donor, mobilizer, and recipient suspensions were made in LB broth from cultures grown on solid media under selective conditions, as appropriate, for 18 h. Bacterial suspensions were adjusted spectrophotometrically ( $A_{600} = 0.5$ ), mixed at an equal ratio (1:1:1), and transferred to a positively charged sterile membrane layered on a 100- by 15-mm LB agar petri dish. Following a 6-h incubation period, the cells from the mating and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation ( $12,096 \times g$  for 10 min at 5°C). The bacterial pellets were suspended in phosphate buffer and dilution plated to media with appropriate antibiotic selection. Single-colony isolates of individual transconjugants were obtained by streaking on selective media. Cultures were subjected to survey lysis followed by agarose gel electrophoresis (23) to confirm plasmid transfer.

**Plasposon mutagenesis.** The plasposon pTnMod-RTP' was employed to obtain initial ptw-negative mutants of strain K56-2 (15). Transconjugants were selected on VBG supplemented with Tp. After 48 h of incubation at 37°C, isolated colonies were transferred to homologous media to confirm selection. Trimethoprim-resistant transconjugants were evaluated for ptw activity by using the onion tissue assay. Transconjugants that no longer demonstrated the ptw phenotype were single-colony purified, retested, and retained for further analysis.

**DNA isolation and sequence analysis.** Genomic DNA was extracted from plasposon-generated ptw-negative K56-2 mutants using a DNeasy kit (QIAGEN). Three micrograms of DNA were digested with PstI and self ligated by using T4 DNA ligase (New England Biolabs). The resulting plasmids were transformed into *E. coli* CC118Apir by CaCl<sub>2</sub> transformation (59) and were selected on LB agar amended with Tp. Survey lysis and agarose gel electrophore-

sis were conducted to confirm the presence of plasmid DNA in the transformants. Plasmid DNA for sequencing was extracted using a HiSpeed Plasmid Midi kit (QIAGEN). Primers designed from the 5' and 3' termini of the plasposon insert were designated RTP1 and RTP2 (Table 3), respectively, and were used to sequence the cloned genomic DNA from *B. cenocepacia* strain K56-2. Sequencing was conducted by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory, Texas A&M University. MacVector 7.0 (Oxford Molecular, Ltd.) was used for DNA sequence analysis. DNA sequence for *B. cenocepacia* strain J2315 was produced by the Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge, and can be obtained from [http://www.sanger.ac.uk/Projects/B\\_cenocepacia/](http://www.sanger.ac.uk/Projects/B_cenocepacia/).

**Site-directed mutagenesis.** Using PCR analysis, *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10* were found to be located on two cosmids from a previously constructed K56-2 library (62). Primer sets PD4 (PD4-1 and PD4-2), PC (PC-1 and PC-2), PB4 (PB4-1 and PB4-2), and PB10 (PB10-1 and PB10-2) (Table 3) were designed based on sequence data from the *B. cenocepacia* Genome Project. Each PCR (50 µl) was performed using a Taq PCR Core kit (QIAGEN) and a GeneAmp 2700 (Applied Biosystems). Reactions were run for 25 cycles, and parameters were as follows: denaturation at 95°C for 30 s; annealing at 55, 52, 56.5, and 54°C for 1 min for primer sets PD4, PC, PB4, and PB10, respectively; and extension at 72°C for 75 s. Resulting products were analyzed by agarose gel electrophoresis. It was determined that cosmid pSBC-9F5 contained *ptwD4* and *ptwC* and that pSBC-3H3 harbored *ptwB4* and *ptwB10*, respectively. Genes *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10*, were disrupted using site-directed mutagenesis. A 10-kb BamHI fragment from pSBC-9F5 and an 11-kb BamHI fragment from pSBC-3H3 were individually inserted into the multiple cloning site (MCS) of pJQ200SK (55) to construct pASE102 and pASE103, respectively. Plasmids pASE102 and pASE103 were transformed into *E. coli* DH10B by electroporation (25 µF, 2.5 kV, 200 Ω) followed by selection on LB agar amended with Gm. Orientation of inserted DNA was determined by restriction enzyme digest analysis using SmaI. Only plasmids containing insertions that were in frame were used in the subsequent experiments. Using pASE102, plasmids pASE106 and pASE107 were constructed by inserting a Tc-resistance cassette, obtained as a 1.9-kb fragment from pBR325 (4), into the ScaI and EcoRV sites of *ptwD4* and *ptwC*, respectively. Plasmids pASE108 and pASE109 were constructed by inserting the Tc cassette into the ApaI and SacI sites of *ptwB4* and *ptwB10* of pASE103, respectively. Constructs pASE106, pASE107, pASE108, and pASE109 were electroporated into *E. coli* DH10B, and transformants were selected on LB agar amended with Tc and Gm. Insertion of the Tc resistance cassette was confirmed by restriction enzyme digest analysis using BamHI and PCR using primer sets PD4, PC, PB4, and PB10 to confirm a 1.9-kb increase in plasmid size and PCR product, respectively. Plasmids pASE106, pASE107, pASE108, and pASE109 were individually mobilized into K56-2 by utilizing pRK2013 in triparental matings to yield genetic exchange mutants. Tetracycline-resistant transconjugants were single-colony purified and retested for Tc<sup>r</sup>. PCR using primer sets PD4, PC, PB4, and PB10 and plasmid survey lysis were used to confirm allelic exchange, reflected by the increase in PCR product and loss of the suicide vector. Single-colony isolates were tested for the ptw phenotype.

Genes *virB6* and *virB11*, from the chromosomally located type IV secretion system, were disrupted by site-directed mutagenesis. Primer sets VB6 (VB6-1 and VB6-2) and VB11 (VB11-1 and VB11-2) (Table 3) were designed based on sequence data from the Sanger Centre. Each PCR was performed as described above, except that the cycle parameters were as follows: denaturation at 95°C for 30 s; annealing at 57.5 and 56°C for 1 min for primer sets VB6 and VB11, respectively; and extension at 72°C for 75 s. PCR products were individually ligated into vector pDrive (QIAGEN) and were transformed into *E. coli* DH10B by electroporation followed by selection on LB agar amended with Ap to yield pASE110 and pASE111, respectively. Genes *virB6* and *virB11* were excised from pASE110 and pASE111, respectively, by double digestion using restriction enzymes PstI and XhoI and were individually inserted into the MCS of pJQ200SK to construct pASE112 and pASE113, respectively. Plasmids pASE112 and pASE113 were transformed into *E. coli* DH10B by electroporation followed by selection on LB agar amended with Gm. Using pASE112 and pASE113, plasmids pASE114 and pASE115 were constructed by inserting a Tc resistance cassette, from pBR325, into the ScaI site of *virB6* or *virB11*, respectively. Constructs pASE114 and pASE115 were individually electroporated into *E. coli* DH10B and transformants were selected on LB agar amended with Tc and Gm. Insertion of the Tc resistance cassette was confirmed by restriction enzyme digest analysis using BamHI and PCR primer sets VB6 and VB11 to confirm a 1.9-kb increase in plasmid size and PCR product, respectively. Plasmids pASE114 and pASE115 were individually mobilized into K56-2 by triparental matings to yield genetic exchange mutants. Tetracycline-resistant transconjugants were single-colony purified and retested for Tc<sup>r</sup>. PCR using primer sets VB6 and VB11 and

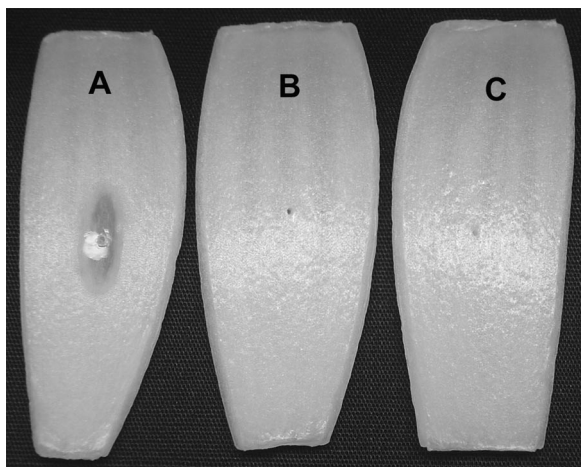


FIG. 1. Plant tissue watersoaking assay. (A) Pierced onion bulb scale inoculated with *B. cenocepacia* strain K56-2 showing partial tissue collapse and translucence characteristic of the *ptw* phenotype. (B) Pierced onion bulb scale inoculated with sterile water (*ptw* negative). (C) Pierced onion bulb scale inoculated with *ptw*-negative mutant AE307. All *ptw*-negative mutants expressed no *ptw* activity on onion tissue, and inoculated scales showed the same symptoms as that in panel C.

plasmid survey lysis were used to confirm allelic exchange, reflected by the increase in PCR product and loss of the suicide vector. Single-colony isolates were tested for the *ptw* phenotype.

**Complementation of disrupted genes.** Complementation of the disrupted *ptwD4*, *ptwB4*, or *ptwB10* was accomplished in *trans*. Plasmid pURF047 (63) was amended by inserting a Tc resistance cassette, obtained from R388 as a BamHI fragment, into the ScaI site in the *bla* gene to obtain pASE101. A 10-kb BamHI fragment from pSBC-9F5 that contained *ptwD4* and an 11-kb BamHI fragment from pSBC-3H3 that contained *ptwB4* and *ptwB11* were individually inserted into the MCS of pASE101, resulting in pASE104 and pASE105, respectively. Plasmids pASE104 and pASE105 were electrotransformed into *E. coli* DH10B, followed by selection on LB agar amended with Tc and Gm. Orientation of inserted DNA was determined by restriction enzyme digest analysis using *ScaI*.

Only plasmids containing in-frame insertions were used in the subsequent experiments. Plasmid pASE104 was mobilized into AE307, and pASE105 was mobilized into AE321 and AE322 to complement insertionally disrupted *ptw* genes. Tetracycline-resistant and Tc<sup>r</sup> transconjugants were single-colony purified and retested for antibiotic resistance. Plasmid survey lysis and PCR were used to confirm plasmid transfer, which was reflected by the presence of both the resistant and complement plasmid as well as inserted and uninserted PCR products.

## RESULTS

**Isolation and characterization of *ptw*-negative mutants.** A total of 5,000 transconjugants of strain K56-2 were obtained by using the plasposon mutagenesis system. Genomic DNA from 20 randomly selected transconjugants were digested with PstI, which does not cleave the TnMod-RTp' insert. The DNA fragments were separated by agarose gel electrophoresis, and Southern blots were probed with the Tc cassette to determine the randomness of insertion. It was determined that the plasposon system was suitable for the generation of random mutations in the genome of *B. cenocepacia* strain K56-2 (data not shown).

From the pool of 5,000 transconjugants, 56 mutants were verified as *ptw*-negative using the plant tissue assay (Fig. 1) and demonstrated no other apparent phenotypic differences compared to phenotype of the parental strain. The DNA flanking the plasposon insertion site of 10 *ptw*-negative mutants was cloned and sequenced, and a BLAST search was performed. The translated gene products disrupted in clones GM237 and GM242 (Table 2) showed homology to VirD4-like proteins, whereas GM241 and GM243 (Table 2) showed homology to a putative regulator and promoter, respectively. Thus, our initial inquiry was focused on determining if strain K56-2 possessed and/or utilized a similar transfer system. Characterization of the remaining *ptw*-negative mutants will be addressed in a separate report.

Using sequence data from the *B. cenocepacia* Genome Project, a 60-kb region proximal to the identified *virD4*-like

TABLE 2. Bacterial strains used in this study

Strain	Relevant characteristics <sup>a</sup>	Reference or source
<i>B. cenocepacia</i>		
K56-2	CF respiratory isolate, <i>ptw</i> <sup>+</sup>	62
GM237	K56-2 <i>ptwD4</i> ::RTp', <i>ptw</i> <sup>-</sup>	This study
GM238	K56-2::RTp', <i>ptw</i> <sup>+</sup>	This study
GM241	K56-2::RTp', <i>ptw</i> <sup>-</sup>	This study
GM242	K56-2 <i>ptwD4</i> ::RTp', <i>ptw</i> <sup>-</sup>	This study
GM243	K56-2::RTp', <i>ptw</i> <sup>-</sup>	This study
AE307	K56-2 <i>ptwD4</i> ::Tc, <i>ptw</i> <sup>-</sup>	This study
AE320	K56-2 <i>ptwC</i> ::Tc, <i>ptw</i> <sup>+</sup>	This study
AE321	K56-2 <i>ptwB4</i> ::Tc, <i>ptw</i> <sup>-</sup>	This study
AE322	K56-2 <i>ptwB10</i> ::Tc, <i>ptw</i> <sup>-</sup>	This study
AE310	AE307 (pASE104), <i>ptw</i> <sup>+</sup>	This study
AE323	AE321 (pASE105), <i>ptw</i> <sup>+</sup>	This study
AE324	AE322 (pASE105), <i>ptw</i> <sup>+</sup>	This study
AE360	K56-2 <i>virB6</i> ::Tc, <i>ptw</i> <sup>+</sup>	This study
AE361	K56-2 <i>virB11</i> ::Tc, <i>ptw</i> <sup>+</sup>	This study
<i>E. coli</i>		
DH10B	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>llacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ( <i>ara, leu</i> ) 7697 <i>galU</i> <i>galK1</i> <i>rpsL</i> <i>nupG</i>	Life Technologies, Inc. (Gaithersburg, Md.)
HB101	F <sup>-</sup> <i>recA13</i>	6
CC118λpir	Δ <i>lacX74</i> <i>galE</i> <i>galK</i> <i>thi-1</i> <i>rpsE</i> <i>rpoB</i> <i>recA1</i> , lysogenized with λ pir phage	27

<sup>a</sup> Tc, tetracycline; *ptw*, plant tissue watersoaking phenotype.



FIG. 2. A 45-kb segment from plasmid pK56-2 containing the *ptw* cluster. Designation of genes was based on homology to gene products of transfer- and translocation-related proteins and are depicted by solid arrows. Open reading frames having no homologs are illustrated by patterned arrows.

gene was analyzed. Strain J2315, the subject of the genome sequence project, is a member of the ET12 clonal lineage, as is strain K56-2 (47), and hence the arrangement of genes and their sequence was expected to be similar. A cluster of 11 genes potentially involved in the delivery of the *ptw* effector(s) was identified and designated the *ptw* cluster (Fig. 2). Southern analysis of K56-2 lysates (data not shown) determined that the *ptw* cluster was located on a 92-kb resident plasmid. Other isolates of the ET12 lineage (J2315, BC7, and C5424) were also shown to harbor the *ptw* cluster on their resident plasmids (data not shown). The resident plasmid, designated pK56-2, has a G-C ratio of 62.7%, and the *ptw* cluster has a G-C ratio of 61%, whereas the value for the whole genome is 66.9%.

#### DNA sequence and protein similarities of the *ptw* cluster.

The encoded products of the *ptw* cluster showed homology to various translocation and/or conjugation related proteins from other bacteria, including *Agrobacterium tumefaciens*, *Salmonella enterica* serovar Typhi, *Vibrio cholerae*, *Novosphingobium aromaticivorans*, and *E. coli*. Based on amino acid sequence similarity to TraD and other VirD4-like proteins, the predicted function of *ptwD4* could involve nucleoside triphosphatase activity (NTPase) and may therefore serve as an active motor for secretion (60, 61). PtwB4 and its homolog VirB4 of the VirB-VirD system may also exhibit NTPase activity (2). Based on homology to R388 TrwC, PtwC may function as a component of a relaxosome (44). PtwB7, PtwB8, PtwB9, and PtwB10 were predicted to form complexes in the periplasm and/or membrane to create the pore (74). PtwB11 was also predicted to have NTPase activity and is part of the TraG subfamily, which belongs to the large superfamily of type II/IV secretion NTPases (53). PtwN, as well as PtwU, had no VirB-VirD homologs; however, they possessed homology to TraN and TraU of the F plasmid, respectively. In the F plasmid system TraN is an adhesin and TraU is involved in mating pair stabilization (38).

Based on analysis of gene products with sequence similarity and the probable involvement in the expression of the *ptw* phenotype, *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10* were chosen for further analysis. Translation of the *ptwD4* sequence resulted in a protein that was 640 amino acids in length, with a predicted molecular size of 72 kDa and a pI of 6.6. Further analysis of the PtwD4 amino acid sequence, using reverse position specific BLAST (rpsBLAST) and the conserved domain database, revealed a predicted Walker A (P-loop) and B site for nucleotide binding (70) that is characteristic of type IV secretion ATP-binding proteins (74). The predicted P-loop nucleotide binding motif extends from 117 to 124 in the amino acid sequence, and the Walker B motif extends from 338 to 344. The translated sequence of *ptwB4* was 1,013 amino acids in length, with a predicted molecular size of 108 kDa and a pI of 6.0. Further analysis of the PtwB4 amino acid sequence for hydrophobicity

revealed four putative transmembrane domains, which is characteristic of type IV secretion NTPases (74). Translation of the *ptwB10* sequence resulted in a protein that was 278 amino acids in length, with a predicted molecular size of 30 kDa and a pI of 5.2. Using rpsBLAST and the conserved domain database, PtwB10 was shown to contain a well-conserved C-terminal hydrophobic region (74). Translation of the *ptwC* sequence resulted in a protein that was 940 amino acids in length, with a predicted molecular size of 104 kDa and a pI of 6.0. A Pustell protein matrix analysis comparing PtwC and TrwC from R388 illustrated regions of similarity between the sequences. At the N terminus (amino acids 1 to 200), PtwC and TrwC showed 42% identity and signature motifs (2 and 3) that are conserved in proteins with DNA-nicking activities (44). An analysis of the PtwC C-terminal sequence indicated that PtwC contained 7 motifs (I, Ib, II, III, IV, V, and IV) characteristic of helicases (28).

**Site-directed disruption of the *ptw* or *virB-virD4* clusters and its effect on the *ptw* phenotype.** Translated products of the *ptw* cluster showed homology to proteins from known type IV secretion systems. Based on their predicted function, the role of *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10* in the expression of the *ptw* phenotype was determined. Watersoaking mutants of K56-2 with a disrupted *ptwD4* were obtained in the initial screening of the plasposon generated mutants and by site-directed mutagenesis (Fig. 1 and Table 2). Site-directed mutagenesis of *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10* was accomplished by individual mobilization of pASE106, pASE107, pASE108, and pASE109, respectively, into K56-2. Tetracycline-resistant transconjugants showed a 1.9-kb increase in PCR product, which reflected the insertion of the Tc cassette (data not shown). Insertions in *ptwD4*, *ptwB4*, and *ptwB10* resulted in loss of the *ptw* phenotype, whereas disruption of *ptwC* did not. Representative insertionally inactivated *ptw*-negative mutants of *ptwD4*, *ptwB4*, and *ptwB10* were designated AE307, AE321, and AE322, respectively, whereas the inserted *ptwC* derivative was designated AE320.

Complementation of *ptw*-negative mutants was accomplished by mobilization of pASE104 into AE307 and pASE105 into AE321 or AE322 and selecting for Tc<sup>r</sup> and Tp<sup>r</sup> transconjugants. All transconjugants tested were *ptw* positive (Fig. 3). Plasmid pURF047 derivatives were used in complementation studies, because pURF047 is a derivative of pURF034 that contains the *par* locus and is maintained stably without selective pressure (>95% retention over 36 generations), which was a desirable parameter for plant tissue studies (14). A survey of transconjugant lysates confirmed plasmid transfer (data not shown). Representatives of *ptw*-positive complemented *ptwD4*, *ptwB4*, and *ptwB10* mutants were designated AE310, AE323, and AE324, respectively (Fig. 3).

Genomic analysis of the J2315 sequence from the Sanger Centre identified a second cluster on chromosome II, which showed homology to the VirB-VirD type IV secretion system (Fig. 4). Using PCR analysis, the presence of *virB1*, *virB4*, *virB6*, *virB11*, and *virD4* (Table 3), which span the entire *virB-virD* cluster, were confirmed in K56-2 (data not shown). In *A. tumefaciens*, *virB6* is thought to mediate assembly of the pilus and a functional secretion machine through its effects on *virB7* and *virB9* multimerization (31), and *virB11* is predicted to function as an NTPase, which provides the necessary energy

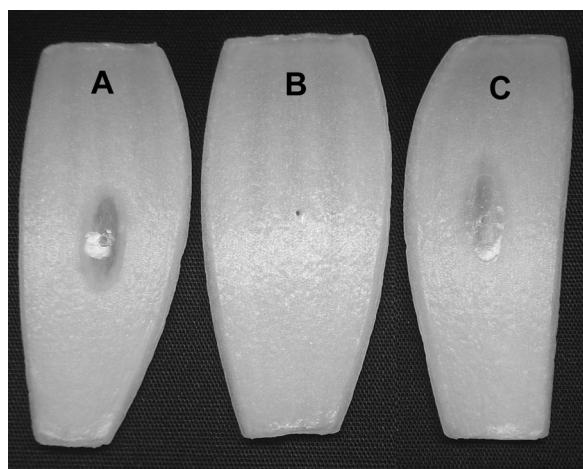


FIG. 3. Complementation of *ptw*-negative mutants. (A) Pierced onion bulb scale inoculated with strain K56-2 showing characteristic *ptw* activity. (B) Pierced onion bulb scale inoculated with *B. cenocepacia* *ptw*-negative mutant AE307 showing no *ptw* activity. (C) Pierced onion bulb scale inoculated with complemented AE307 (AE310) showing restored *ptw* activity.

for secretion (74). Based on their predicted function, the role of *virB6* and *virB11* in the expression of the *ptw* phenotype was assessed. Site-directed mutagenesis of *virB6* and *virB11* was accomplished by individual mobilization of pASE114 and pASE115 into K56-2, yielding AE360 and AE361, respectively. Tetracycline-resistant transconjugants showed a 1.9-kb increase in PCR product, which reflected the insertion of the Tc cassette (data not shown). Disruption of *virB6* or *virB11* did not result in loss of the *ptw* phenotype.

**Partial characterization of watersoaking effector(s) and population dynamics.** Plant tissue assays indicated that the parental strain K56-2 and the complemented mutant (AE310) caused watersoaking, whereas the *ptwD4* mutant (AE307) did not (Fig. 3). Growth and watersoaking were monitored over a 72-h period using the plant tissue assay to determine the contribution of *ptw* activity in survival and growth. Over the 72-h period both K56-2 and AE310 showed an approximate 100-fold increase in CFU/gram of tissue and an increase in the watersoaking area, whereas AE307 showed no watersoaking activity and an approximate 1,000-fold decrease in CFU/gram of tissue over the same period (Table 4).

Based on analysis of the *ptw* cluster and the putative role of a type IV secretion system in the export of an effector molecule(s), it was of interest to determine if culture supernatant concentrates showed effector activity. Over an 8-h duration,

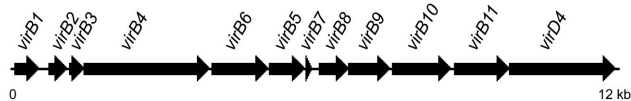


FIG. 4. A 12-kb segment containing a VirB-VirD-like type IV secretion system cluster located on chromosome II of *B. cenocepacia* strain J2315. Designation of genes was based on homology to gene products of transfer- and translocation-related proteins and are depicted as solid arrows. Genes *virB1*, *virB4*, *virB6*, *virB11*, and *virD4* have been identified in strain K56-2.

TABLE 3. Primers used in this study

Primer	Sequence
RTp1	5'-GGTACCGTCGACATGCATGG-3'
RTp2	5'-CAGTGCAAATTTATCCTGTG-3'
PD4-1	5'-TGA CT CAGCGAAGGAA-3'
PD4-2	5'-ATCCGGTGGAAAGCAA-3'
PC-1	5'-CACTCCGACATCGAAT-3'
PC-2	5'-AACTGTGTAGGACACT-3'
PB10-1	5'-ATGCTTGTTCGATGTTGCA-3'
PB10-2	5'-TTCCGACGACTTCAT-3'
PB4-1	5'-AACTCAAGATGAATCAT-3'
PB4-2	5'-AGAACGCATGGTGTGTA-3'
VB1-1	5'-GACAGATCATGCGTGT-3'
VB1-2	5'-AGGAGGACACAGAGT-3'
VB4-1	5'-ATCGCACTGTTGATCGTTG-3'
VB4-2	5'-TCACGTTACGTGTGCATAC-3'
VB6-1	5'-AAGGTGGTGTCTATGAA-3'
VB6-2	5'-ACAGTACGATATGCGTGAA-3'
VB11-1	5'-TTCAAGTAACGATTGCTGA-3'
VB11-2	5'-TGCAAATCGAGTTCGGTA-3'
VD4-1	5'-TGTTATGAGACAGATTGCA-3'
VD4-2	5'-AGATAAGGATGGTCAGGT-3'

carrot protoplasts exposed to M9 medium concentrate did not experience significant plasmolysis compared to that of the protoplast controls ( $P > 0.05$ ) (Table 5). However, significant ( $P \leq 0.05$ ) plasmolysis was observed for protoplasts exposed to the K56-2 or complemented mutant (AE310) concentrates as early as 2 h, and plasmolysis increased at a significant rate for dilutions tested (up to 1:5) over the 8-h time period compared to that of medium controls. Concentrated supernatant from the *ptwD4* mutant (AE307) caused 6.7% plasmolysis after 8 h, which was not statistically different ( $P > 0.05$ ) from plasmolysis resulting from treatment with medium controls. Dilution of the K56-2 and AE310 concentrates to a protein concentration of 0.02  $\mu\text{g/ml}$  reduced activity to levels which approximated those of the control and AE307. Temperatures of 37, 65, and 80°C for 1 h or 100°C for 10 min did not affect the plasmolysis activity of the K56-2 concentrate. Complete inactivation of plasmolysis activity was observed when the K56-2 concentrate was treated with proteinase K; however, incubation with DNase I or RNase A showed no effect on activity.

## DISCUSSION

Previously, the role of a polygalacturonase (PehA) as a virulence factor in *B. cepacia* strain ATCC 25416 was investigated (24). Derivatives cured of a resident plasmid that codes for the PehA no longer macerated onion tissue; however, the derivatives still produced a phenotype described as *ptw*. Of interest in the present study was the observation that all *B. cepacia* and *B. cenocepacia* isolates and a *B. vietnamiensis* strain from the Bcc experimental strain panel (47) produced the *ptw* phenotype in onion tissue (50). In this study K56-2, a *B. cenocepacia* strain that produced the plant disease-associated phenotype, was genetically analyzed and found to contain a plasmid-borne gene cluster, designated *ptw*, that encodes a type IV secretion system responsible for the translocation of the *ptw* effector protein(s).

The identified gene products in the *ptw* cluster had homology to proteins from known type IV secretion systems. Bacteria

TABLE 4. Growth and watersoaking activity of *B. cenocepacia* strain K56-2 and its derivatives in onion tissue

Time (h)	Strains		
	K56-2	AE307	AE310
0	$6.5 \times 10^5 \pm 2.5 (0 \pm 0)^a$	$4.2 \times 10^5 \pm 1.7 (0 \pm 0)$	$7.3 \times 10^5 \pm 2.0 (0 \pm 0)$
24	$6.1 \times 10^4 \pm 3.3 (90 \pm 20)$	$5.6 \times 10^4 \pm 1.0 (0 \pm 0)$	$4.4 \times 10^4 \pm 2.9 (120 \pm 11)$
48	$4.5 \times 10^6 \pm 1.7 (178 \pm 42)$	$6.7 \times 10^3 \pm 1.6 (0 \pm 0)$	$5.1 \times 10^6 \pm 3.5 (193 \pm 14)$
72	$4.4 \times 10^7 \pm 3.1 (304 \pm 18)$	$5.8 \times 10^2 \pm 3.1 (0 \pm 0)$	$8.6 \times 10^7 \pm 5.5 (347 \pm 33)$

<sup>a</sup> Average CFU/gram of tissue  $\pm$  SD for three independent experiments in triplicate. Numbers in parentheses are the average area of tissue watersoaking (in square millimeters)  $\pm$  SD for three independent experiments in triplicate.

have evolved type IV secretion systems to transfer DNA or protein macromolecules to a wide array of target cell types (2, 10, 52). Originally, the nomenclature referred to the VirB-VirD-encoded translocation system of *A. tumefaciens* and two closely related systems encoded by the transfer region of the IncN plasmid pKM101 and the *ptl* operon of *Bordetella pertussis* (10). In the past decade, the type IV family has expanded considerably in number with the relaxation of defining criteria. Currently, type IV secretion systems are defined as translocation systems ancestrally related to any conjugation system of gram-negative or -positive bacteria (10, 25). However, it is important to distinguish functional translocation machines from mobile elements characteristically found in bacterial genomes. Ding et al. (18) have suggested that mutagenesis of a putative type IV system should yield a phenotype at least consistent with a translocation defect.

Christie (10) has suggested subclassification of type IV secretion systems based on ancestral lineage. Thus, the VirB-VirD type has been designated IVA, and the CollB-P9 Tra and *L. pneumophila* Dot-Icm types have been designated IVB. This subclassification left open the possibility of further division of gram-negative and -positive secretion systems that differ from IVA and IVB. Therefore, an alternative grouping scheme has been suggested by Ding et al. (18) that separates systems based

on function and does not replace the one previously described but rather expands its usefulness. This classification method groups type IV secretion systems as (i) conjugation systems, (ii) effector translocator systems, or (iii) DNA uptake or release systems (18). By definition, the conjugation systems deliver DNA substrates by establishing direct physical contact with target cells. Examples include the well-studied *A. tumefaciens* T-DNA transfer system and the F, RP4, and R388 plasmid transfer systems. Although the conjugation systems are known mainly for their role in distributing DNA among bacterial populations, they can also translocate protein substrates independently of DNA (73). There is also a subset of these systems that can transfer DNA and protein substrates to a range of eukaryotic cell types, including plant, fungal, and human (3, 71, 72). Most of the members of the type IV effector translocator group inject their substrates directly into the eukaryotic cytosol. This type of translocation is now recognized as the dominant virulence mechanism of the phytopathogen *A. tumefaciens* and of several medically important pathogens, including *H. pylori*, *L. pneumophila*, *Brucella* spp., and *Bartonella* spp. (7). Also included in this subfamily is the Ptl system of *B. pertussis*, even though this system exports its protein substrate independently of host-cell contact. Presently, at least 10 type IVA and several type IVB systems can be grouped as effector

TABLE 5. Effect of supernatant concentrates from K56-2, *ptwD4* mutant (AE307), and complemented *ptwD4* mutant (AE310) on carrot protoplasts

Supernatant concentrate	Concentrate dilution <sup>a</sup>	% Plasmolysis <sup>b,c</sup> at time point (h):			
		2	4	6	8
M9 medium	0	$1.2 \pm 1.1x$	$2.7 \pm 1.6x$	$6.6 \pm 1.7x$	$8.2 \pm 3.4x$
	1:1	$1.5 \pm 1.7x$	$3.4 \pm 1.2x$	$6.4 \pm 2.0x$	$7.5 \pm 2.8x$
	1:5	$2.6 \pm 1.0x$	$3.1 \pm 1.6x$	$4.3 \pm 1.4x$	$6.9 \pm 2.7x$
	1:10	$1.9 \pm 1.5x$	$2.9 \pm 1.0x$	$5.6 \pm 2.3x$	$7.3 \pm 2.6x$
K56-2	0 <sup>d</sup>	$18 \pm 4.0y$	$37 \pm 4.1y$	$57 \pm 5.1y$	$75 \pm 5.8y$
	1:1	$18 \pm 2.1y$	$38 \pm 5.0y$	$56 \pm 6.1y$	$76 \pm 7.6y$
	1:5	$18 \pm 4.3y$	$36 \pm 5.0y$	$55 \pm 5.6y$	$73 \pm 6.4y$
	1:10	$1.6 \pm 1.4x$	$3.6 \pm 1.0x$	$5.9 \pm 0.6x$	$7.3 \pm 1.4x$
AE307	0 <sup>d</sup>	$1.8 \pm 1.2x$	$3.3 \pm 0.7x$	$4.9 \pm 1.7x$	$6.7 \pm 1.7x$
	1:1	$2.2 \pm 1.3x$	$3.8 \pm 2.1x$	$5.4 \pm 1.0x$	$7.4 \pm 1.9x$
	1:5	$2.3 \pm 1.4x$	$3.5 \pm 0.7x$	$5.8 \pm 1.5x$	$8.4 \pm 1.4x$
	1:10	$2.3 \pm 1.5x$	$4.5 \pm 1.1x$	$6.1 \pm 0.8x$	$7.7 \pm 0.6x$
AE310	0 <sup>d</sup>	$19 \pm 1.0y$	$37 \pm 3.0y$	$52 \pm 4.0y$	$78 \pm 7.7y$
	1:1	$19 \pm 1.4y$	$36 \pm 4.9y$	$52 \pm 3.7y$	$70 \pm 6.6y$
	1:5	$18 \pm 2.0y$	$39 \pm 4.5y$	$56 \pm 4.2y$	$77 \pm 7.0y$
	1:10	$1.5 \pm 1.0x$	$1.8 \pm 1.0x$	$6.0 \pm 1.6x$	$7.2 \pm 1.0x$
None	0	$2.0 \pm 1.6x$	$3.1 \pm 7.7x$	$6.7 \pm 1.6x$	$11 \pm 2.2x$

<sup>a</sup> All dilutions were adjusted to 10% mannitol.

<sup>b</sup> Data are mean values  $\pm$  SD of two replicates for three independent experiments. At 0 h all plasmolysis values were 0x.

<sup>c</sup> Values within the columns scored by the same letter were not statistically different at  $P = 0.05$ .

<sup>d</sup> Undiluted concentrate adjusted to 0.2  $\mu$ g of protein/ml in 10% mannitol.

translocators and are thought to be essential for infection (18). The DNA uptake and release group, which presently contains three members, translocates DNA substrates across the cell envelope to or from the extracellular milieu (18). *Neisseria gonorrhoeae* uses a system encoded by the gonococcal genetic island to export DNA (17, 26). Recent studies have established that this system is very closely related to the F plasmid conjugation system of *E. coli*, even though it is not performing the same function (17). The two other members of this subfamily, *Campylobacter jejuni* and *H. pylori*, translocate DNA in the opposite direction, promoting genetic variation and cell survival (1, 29).

A notable feature of the type IV secretion systems is their extreme versatility. These systems can recognize a wide array of DNA and protein substrates, translocate substrates by both cell-contact-dependent and -independent mechanisms, and deliver substrates to an exceptionally wide range of prokaryotic and eukaryotic taxa (18). The *ptw* cluster contains homologs to components of multiple type IV secretion systems (Fig. 2). With respect to function, the plasmid-encoded type IV secretion system we have identified in *B. cenocepacia* strain K56-2 was involved in export of an effector(s) that was responsible for the *ptw* phenotype. The facts that the Ptw system was involved in the translocation of a protein(s), that it did not contain all of the necessary genetic components to support conjugation, and that no *oriT* homolog was identified indicate that functionally it is a member of the effector translocator subfamily. The difference in the G-C ratio between pK56-2 harboring the *ptw* cluster (62.7%) and the G-C ratio for the entire *B. cenocepacia* genome (66.9%) suggests acquisition by horizontal transfer (34).

The presence of a second type IV secretion system in *B. cenocepacia* is not unprecedented, because many bacteria, such as *H. pylori* and *A. tumefaciens*, have been found to harbor multiple secretion systems (18, 35). The type IV secretion system located on chromosome II showed most similarity to that of the *A. tumefaciens* VirB-VirD translocation system with respect to arrangement and gene product similarity (Fig. 4). The difference in the G-C ratio of the chromosomally located type IV secretion system (63%) and the G-C ratio of the entire genome (66.9%) suggests horizontal acquisition (34), as with the Ptw translocation system. Evidence suggests that the Ptw system is responsible for export of the protein(s) involved in expression of the *ptw* phenotype; however, the substrate(s) translocated by the VirB-D4 system is unknown.

Identification of a cluster of genes that encoded proteins with similarities to components of a type IV secretion system allowed for strategic generation of K56-2 mutants to support the hypothesis that such a system was involved in expression of the *ptw* phenotype. The genes selected for functional characterization were *ptwD4*, *ptwC*, *ptwB4*, and *ptwB10*. VirD4-like proteins (PtwD4 homologs) are cytoplasmic membrane NTP-binding proteins that are essential for coupling the relaxosome to the macromolecular transport system (51, 61). It is thought to interact with the *oriT*-bound relaxosome, which is made up of TrwC and TrwA, to facilitate DNA transfer (19, 45). In the Ptw system, however, there is no TrwA homolog. The *ptwD4* product appears to have functions similar to those of Hp0524 of *H. pylori*. The *hp0524* product is critical for the transfer of CagA from the bacterium to epithelial cells (21). Further anal-

ysis revealed that the PtwD4 amino acid sequence contained P-loop and Walker B sites, which are characteristic of type IV secretion ATP-binding proteins. The *ptwC* product had homology to TrwC, which is both a relaxase that cleaves at the *nic* site within the *oriT* in a strand- and sequence-specific manner, and a helicase, which is essential for transfer (48, 49). Analysis of PtwC revealed that the N terminus possessed signature motifs (2 and 3) conserved in proteins with DNA-nicking activities (44), and the C terminus possessed signature motifs (I, Ib, II, III, IV, V, and VI) conserved in DNA helicases (28); however, there is no identified *oriT* to facilitate the cleavage and thus linearization required for DNA transfer. VirB10, the PtwB10 homolog, is a protein located in the periplasmic space that is believed to form the core of the transfer machinery and is possibly part of the pore that spans the inner and outer membranes (74). PtwB10 was shown to contain the C-terminal hydrophobic region that is characteristic of VirB10-like proteins. PtwB4 showed similarity to VirB4 of *A. tumefaciens*, which is a putative nucleoside triphosphatase that may also serve as an active motor for secretion (74). The PtwB4 amino acid sequence was shown to possess four transmembrane regions that are characteristic of VirB4-like NTPases (74). The *ptw* phenotype was not expressed by K56-2 derivatives with a disruption in *ptwD4*, *ptwB4*, or *ptwB10* but was expressed by a derivative with a disruption in *ptwC*. These results correlated with the predicted function of the gene products, because successful secretion of the *ptw* effector protein(s) would likely involve the following: a protein that potentially couples the moiety to the secretion system and supplies the necessary energy for export (PtwD4); a protein that acts in the assembly of the pore necessary for the passage of the substrate and provides energy necessary for the system to function (PtwB4); and a protein involved in structural formation of a pore (PtwB10), but not one involved in the generation of a relaxosome (PtwC).

By combining mutational analysis with cytotoxic assays employing both plant tissue and protoplasts, we have identified a type IV secretion system that appears to export an effector molecule(s) that is proteinaceous in nature and is responsible for the activity. This activity plays an important role in the ability of *B. cenocepacia* strain K56-2 to cause watersoaking and to grow in plant tissue (Fig. 2, Table 4). It appears that in the plant tissue assay the role of the effector(s) is to provide bacterial cells with needed nutrients by causing leakage of the plant cell cytosol. Presently, the role of the putative virulence effector protein(s) in contributing to growth and infection in a pulmonary environment is unknown; however, the *ptw* phenotype appears to be common among isolates belonging to *B. cenocepacia* that infect CF patients.

Protein DspE of *Erwinia amylovora* and WtsE of *Pantoea stewartii* subsp. *stewartii* are involved in elicitation of the watersoaking phenotype in their respective hosts. The *dspE* product is a 198-kDa protein that is required for the production of fireblight disease symptoms that include watersoaking of apple and pear tissue (5). A disruption in *wtsE* renders *P. stewartii* subsp. *stewartii* incapable of eliciting an observable watersoaking phenotype and causing a systemic corn leaf infection (22). In contrast to the *ptw* effector molecule(s), a type III secretion system is presumed to export DspE and WtsE from the phytopathogen to the plant hosts.

There are two types of substrates typically transported by



both type III and type IV secretion systems (52, 58). The first group alters host processes by mimicking the function of a eukaryotic protein. Substrate proteins in this category, however, do not have significant sequence similarities with the eukaryotic factor they mimic, which makes identification difficult. The second class are genes that were "stolen" from eukaryotic host cells and then reshaped. The RalF protein of the *L. pneumophila* Dot/Icm system falls into the latter group (52). Substrates in this category are relatively easy to identify with genomic analysis. Secreted substrates from either group can vary in size from ~22 kDa for *A. tumefaciens* VirF to ~145 kDa for *H. pylori* CagA. They also vary in composition, from monomers to multisubunit structures that may include protein and/or DNA (18). Thus, there are no universally conserved primary sequence motifs or physical characteristics that are readily discernible for secreted effectors.

In conclusion, our study has identified a plasmid-encoded type IV secretion system in *B. cenocepacia* strain K56-2 that was functionally an effector translocator. The chromosomally located type IV secretion system showed modular similarity to the *A. tumefaciens* VirB-VirD translocation system; however, it was not responsible for the ptw phenotype in *B. cenocepacia* strain K56-2.

There are multiple examples for the role of type IV secretion systems in translocation of both plasmid DNA and proteins associated with plant and human pathogenesis (9, 10). The role of this putative virulence effector(s) in contributing to disease is being determined in studies using a murine model of pulmonary infection. Studies to identify the structural gene(s) for the effector protein(s) and regulatory subunits are in progress.

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