ROLE OF TYPE IV SECRETION SYSTEMS IN TRAFFICKING OF VIRULENCE DETERMINANTS OF *Burkholderia cenocepacia*

A Dissertation

by

AMANDA SUZANNE ENGLEDOW

Submitted to the Office of Graduate Studies
of Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Carlos F. Gonzalez
Committee Members,	Dennis C. Gross
	Daniel J. Ebbole
	James E. Samuel
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ABSTRACT

Role of Type IV Secretion Systems in Trafficking of Virulence Determinants of *Burkholderia cenocepacia*. (August 2006)

Amanda Suzanne Engledow, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Carlos F. Gonzalez

Type IV secretion systems have been identified in several human pathogens including *Bordetella pertussis, Helicobacter pylori,* and *Legionella pneumophila.* These systems are responsible for the translocation of virulence proteins and/or DNA, thereby playing an important role in the pathogenesis of infection and plasticity of genomes. *Burkholderia cenocepacia* is an important opportunistic human pathogen, particularly in persons with cystic fibrosis (CF). Respiratory tract infection by *B. cenocepacia* in CF patients is often associated with a decline in respiratory function, and can result in acute systemic infection. *Burkholderia cenocepacia* strain K56-2 is part of the epidemic and clinically problematic ET12 lineage. Two type IV secretion systems have been identified in this strain; one system is plasmid encoded (designated the Ptw type IV secretion system) whereas the other is chromosomally encoded (designated the VirB/D type IV secretion system) and shows homology to the *Agrobacterium tumefaciens* VirB/D4 type IV secretion system. It was determined that the plasmid encoded Ptw system is a chimeric type IV secretion system composed of VirB/D4-like elements and F-specific subunits. More recently, it was found that this system translocates a protein effector (PtwE1) that is cytotoxic to plant cells. It was also determined that the positively

charged C-terminal region of PtwE1 is important for translocation via the Ptw type IV secretion system. Strains of the epidemic *B. cenocepacia* PHDC lineage contain only a chromosomal VirB/D4-like type IV secretion system (designated BcVirB/D); and a putative effector protein associated with this system has been identified that has C-terminal transport signal and sequences different from the effectors of the Ptw type IV secretion system. It has also been shown that a competing plasmid substrate and a plasmid fertility inhibition factor act to render *B. cenocepacia* of the PHDC lineage incapable of expressing a plant phenotype. Thus, three type IV secretion systems have been identified in epidemic *B. cenocepacia* lineages. From two of these, an effector has been identified that has cytotoxic effects on eukaryotic cells, and at least one of these type IV secretion systems is able to translocate DNA substrates.

DEDICATION

To my husband Jason, and my daughter Jayna,

for the endless joy they bring to my life.

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I would especially like to thank my committee chair, Dr. Carlos F. Gonzalez for the infinite guidance, wisdom, and support which he has kindly given me over the past five years. I thank him for always making time for me and providing an intellectually stimulating and friendly environment for research. In every sense, none of this work would have been possible without him.

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lending a hand and helping me when I needed it most, without ever asking for anything but love in return. My deepest thanks goes to my wonderful husband Jason Engledow whose understanding, patience, encouragement, and love have comforted me in hard times and made the good times infinitely more pleasurable. For all this, he is my everlasting love. And finally, I would like to thank my daughter Jayna for bringing me joy each and every day, for she is my greatest accomplishment.

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CHAPTER I

GENERAL INTRODUCTION

Burkholderia cepacia was initially identified as the causative agent of soft rot of onion (Burkholder, 1950). Currently, this bacterium is a well-documented opportunistic pathogen of individuals with cystic fibrosis (CF) (Govan and Deretic, 1996, LiPuma, 1998), a notable bioremediation agent (Tomasek *et al.*, 1989), and an effective constituent in biological control practices of plant disease (Kang *et al.*, 1998). Recently, comprehensive polyphasic taxonomic reassessment has shown that several distinct genomic species exist among bacterial isolates previously identified as "*B. cepacia*" based solely on phenotypic analysis (Gillis *et al.*, 1995; Coenye *et al.*, 2001a,b,c; Vandamme *et al.*, 1997; Vandamme *et al.*, 2000; Vandamme *et al.*, 2002; Vandamme *et al.*, 2003; Vermis *et al.*, 2004). Each of the nine species in this group, which is now referred to collectively as the "*B. cepacia* complex" (Bcc), has received a formal binomial designation. Using 16S rDNA and *recA* directed species-specific PCR assays (LiPuma *et al.*, 1999; Mahenthiralingam *et al.*, 2000a), all nine Bcc species have been identified from sputum culture isolates recovered from approximately 1700 CF patients in the US (LiPuma *et al.*, 2001; LiPuma, unpublished). The distribution of species however, is quite disproportionate. Approximately one-half of infected patients harbor *B. cenocepacia*, whereas 38% are infected with *B. multivorans* (LiPuma *et al.*, 2001).

This dissertation follows the style of *Molecular Microbiology*.

In the mid-1980s, many CF treatment centers noted dramatically increasing rates of "*B. cepacia*" infection among their patients, with up to 45% of patients becoming infected in some centers. Studies by multiple laboratories employed genotyping analyses to identify the existence, in several treatment centers, of so-called "epidemic" strains that were common to multiple patients (LiPuma *et al.*, 1988). In 1994, Johnson *et al.* (1994) used multilocus enzyme electrophoresis to identify a *B. cepacia* strain (designated ET12) that was especially common among CF patients in Ontario, Canada. Through presumed interpatient transmission at an international CF summer camp, this strain spread to the United Kingdom, where it now accounts for nearly one-half of Bcc-infected CF patients (Pitt *et al.*, 1996). In another study, Kumar *et al.* (1997) used macro-restriction digestion with pulsed field gel electrophoresis (PFGE) analysis to demonstrate that 29 of 32 *B. cepacia* isolates from five CF centers in Michigan were the same strain type (since referred to as the Midwest clone). That same year, Mahenthiralingam *et al.* (1997) identified several other *B. cepacia* strains that also infected more than one CF patient. All of these studies preceded the appreciation that "*B. cepacia*" is actually comprised of multiple distinct species (as detailed above). Retrospective analyses of these 'epidemic' strains now indicate that all are *B. cenocepacia*. In contrast, relatively few 'epidemic' strains have been found in the other eight Bcc species.

Recently, another *B. cenocepacia* strain has been described, and designated PHDC, which is recovered from essentially all Bcc-infected CF patients in the mid-Atlantic region of the US (Chen *et al.*, 2001). In a retrospective analysis of stored isolates using various genotyping methods, including random amplified polymorphic DNA (RAPD)

typing, repetitive element PCR typing (rep-PCR) and PFGE, the 20-year persistence of strain PHDC has been demonstrated in a large CF treatment center, and has uncovered the spread of this strain to a second center, apparently via a patient who transferred care between centers (Chen *et al.*, 2001). Subsequently, this strain has been identified in agricultural soil from upstate New York onion fields (LiPuma *et al.*, 2002), as well as in CF patients receiving care in 24 US states (Liu *et al.*, 2003) and Europe (Coenye *et al.*, 2004).

Relatively little is known regarding specific virulence factors or pathogenic mechanisms of the Bcc (Hutchison and Govan, 2000). Postulated virulence determinants for *B. cenocepacia* include hemolysins (Hutchison *et al.*, 1998; Vasil *et al.*, 1990), siderophores (Sokol *et al.*, 1999), and cable pili (Sajjan *et al.*, 2000b). A hemolysin induces apoptosis and degranulation of phagocytes (Hutchison *et al.*, 1998), and siderophore production, regulated by quorum sensing (Lewenza *et al.*, 1999), may play a role in colonization of animal lung and spleen tissues (Sokol *et al.*, 1999).

Studies in our laboratory have established that in the plant pathogenic *B. cepacia* type strain ATCC 25416, a plasmid encoded pectate hydrolase (Peh), is a virulence factor necessary for maceration of onion tissue (Gonzalez *et al.*, 1997). 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 (Gonzalez *et al.*, 1997). Knockout mutants for the ptw phenotype cured of the Peh-encoding plasmid caused no

symptoms on plant tissue. Introduction of the cloned *pehA* into the ptw-negative, Peh-negative mutants failed to restore the plant tissue maceration phenotype, although Peh was expressed and secreted *in vitro*. These results indicate that Peh is a virulence factor, whereas the ptw phenotype is a pathogenicity factor since bacteria do not cause symptoms on plant tissue without this function. In a survey of Bcc species, we found that only *B. cepacia* and *B. cenocepacia* strains (and one *B. vietnamiensis*) produced the ptw phenotype, suggesting that members of these genomic species may produce a cytotoxic factor(s) that affects eukaryotic cell integrity (Engledow *et al.*, 2004).

In recent years, a body of research has substantiated the concept that bacterial pathogens share common secretion mechanisms for the delivery of virulence determinants (Christie and Vogel, 2000; Plano *et al.*, 2001). There are numerous examples of the importance of type III (Plano *et al.*, 2001) and type IV secretion systems (Christie, 2001) 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 (Macnab, 1999). The type IV secretion system from *Agrobacterium tumefaciens* was the first to be identified and is used to deliver oncogenic transfer DNA and effector proteins to plant cells during infection (Christie, 2001; Kumar and Das, 2002). 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 (Ding *et al.*, 2003). 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) (Nagai and Roy, 2003). *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 (Ding *et al.*, 2003; Dhar *et al.*, 2003).

Recently, a plasposon mutagenesis system was used to generate 5000 random mutants, of ET12 strain K56-2, which were screened for ptw activity using the plant tissue assay. Genomic analysis of the ptw-negative mutants identified a cluster of genes that encoded proteins with similarities to components of type IV secretion systems, and appeared to play a role in the expression of the ptw phenotype. Because of the known importance which translocation systems play in the delivery of virulence determinants it is important to determine if *B. cenocepacia* possesses a similar system, what substrate(s) that system is transporting, and whether or not it is necessary for pathogenicity in the plant and/or human systems.

CHAPTER II

INVOLVEMENT OF A PLASMID ENCODED TYPE IV SECRETION SYSTEM IN THE PLANT TISSUE WATERSOAKING PHENOTYPE OF *Burkholderia cenocepacia* AND THE STIMULATION OF IL-8 RELEASE FROM BRONCHIAL EPITHELIAL CELLS*

INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) consists of nine genomovars recently elevated to species status (Coenye *et al.*, 2001a, b, c; Vandamme *et al.,* 2002; Vandamme *et al.*, 2003; Vermis *et al.*, 2004). Members of the Bcc include plant and animal pathogens as well as catabolically active soil saprophytes (Tomasek *et al.*, 1989). Some Bcc members can cause life-threatening respiratory infections particularly in persons with cystic fibrosis (CF) (LiPuma, 2003). Studies indicate that 85 to 90% of strains isolated from infected CF patients are *B. cenocepacia* or *B. mulitvorans*, with other Bcc species infrequently isolated (LiPuma, 1998). Several epidemic clonal lineages of *B. cenocepacia* have been identified (Coenye and LiPuma, 2002), including: the ET12 lineage, which is responsible for infecting many CF patients in Canada and the

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* Part of the data reported in this chapter is reprinted with permission from "Involvement of a plasmid-encoded type IV secretion system in the plant tissue watersoaking phenotype of *Burkholderia cenocepacia*" by Engledow, A.S, Medrano E.G, Mahenthiralingam, E, LiPuma, J.J., and Gonzalez, C.F. (2004) *J Bacteriol* **186:** 6015-6024.

UK (Johnson *et al.*, 1994; Ledson *et al.*, 2002); the PHDC lineage, responsible for nearly all Bcc infections in the mid-Atlantic region of the USA (Chen *et al.*, 2001); and the Midwest lineage that is responsible for infecting numerous patients in CF centers in the mid-western region of the USA (LiPuma *et al.*, 2001). 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 (Hutchison *et al.*, 1998; Vasil *et al.*, 1990), siderophores (Sokol *et al.*, 1999), and cable pili (Sajjan *et al.*, 2000a). The hemolysin induces apoptosis and degranulation of phagocytes (Hutchison *et al.*, 1998), and siderophore production, regulated by quorum sensing (Lewenza *et al.*, 1999), plays a role in the primary colonization process of animal lung and spleen tissues (Sokol *et al.*, 1999). Cable pili are important in adherence to the respiratory mucosal blanket and epithelial cells (Sajjan *et al.*, 2000a).

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 (Gonzalez *et al.*, 1997). 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 (Frederick *et al.*, 2001). In a survey of isolates from the Bcc experimental strain panel (Mahenthiralingam *et al.*, 2000b), we found that all of the *B. cepacia* and *B. cenocepacia* strains tested and one *B. vietnamiensis* produced the ptw phenotype, suggesting that members of these genomic

species may produce a cytotoxic factor(s) that affects plant cell integrity (Medrano, 2002).

In recent years, a body of research has substantiated the concept that bacterial pathogens share common secretion mechanisms for the delivery of virulence determinants (Christie and Vogel, 2000; Plano *et al.*, 2001). There are numerous examples of the importance of type III (Plano *et al.*, 2001) and type IV secretion systems (Christie, 2001) 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 (Macnab, 1999). The type IV secretion system from *Agrobacterium tumefaciens* was the first to be identified and is used to deliver oncogenic transfer DNA and effector proteins to plant cells during infection (Christie, 2001; Kumar and Das, 2002). Currently, 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 (Ding *et al.*, 2003). 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) (Nagai and Roy, 2003). *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. (Ding *et al.*, 2003; Dhar *et al.*, 2003).

Recently, bacterial type IV secretion systems also have been implicated in playing a major role in inducing inflammatory responses from infected host tissues (Bauer *et al.*, 2005). For example, effector proteins translocated from bacterial to host cells via type IV secretion systems stimulated an intense inflammatory response from epithelium infected by *H. pylori* or *Bartonella henselae* (Bauer *et al.*, 2005; Schmid *et al.*, 2004). Of particular interest to our studies is the growing appreciation of the roles that type IV secretion systems play in the virulence of certain human respiratory pathogens, including *B. pertussis* and *L. pneumophilia* (Christie, 2001). Infection mediated lung inflammation is a major cause of morbidity and mortality in persons with CF (Chmiel and Davis, 2003; Hilliard *et al.*, 2002). Airway epithelium plays an important role in lung inflammation by expressing C-X-C chemokines, including interleukin 8 (IL-8), in response to invading pathogens (Strieter, 2002). Pathogens that are capable of stimulating high levels of chemokines from infected airway epithelium thereby likely add to the severity of the inflammation-induced lung disease that is a hallmark of CF.

This study reports on the presence of both a plasmid and a chromosomally encoded type IV secretion system in *B. cenocepacia* strain K56-2, describes the involvement of the plasmid encoded system in the export of a putative protein(s) responsible for the ptw phenotype, and shows that the Ptw type IV secretion system is involved in the induction of an IL-8 response of brochial epithelial cells as well as production of the ptw phenotype.

EXPERIMENTAL PROCEDURES

Media and growth conditions

Descriptions of plasmids and bacterial strains used in this study are listed in Tables 2.1 and 2.2, respectively. Luria Bertanni (LB) medium was used for routine maintenance of cultures. Minimal medium Vogel Bonner (Vogel and Bonner, 1965) amended with 1.0% glucose (VBG) was used in mating experiments. Minimal medium M9 (Sambrook *et al.*, 1989) containing 1.0% glucose was used for culturing bacteria for supernatant analysis. *Burkholderia cenocepacia* and *Escherichia coli* strains were grown at 37ºC. Media were supplemented with appropriate concentrations of antibiotics as needed for selection. Antibiotics were added to media at the following concentrations; 500μg/ml of chloramphenicol (Cm) and 500μg/ml of trimethoprim (Tp) for selection and maintenance of *B. cenocepacia*; and 10 μg/ml tetracycline (Tc), 10 μg/ml gentamicin (Gm), 40 μg/ml ampicillin and 100 μg/ml Tp for *E. coli*.

Table 2.1. Plasmids used in Chapter II.

Plasmid	Relevant Characteristics[a]	Reference
pK56-2	92 kb resident plasmid of *B. cenocepacia* strain K56-2 containing the *ptw* cluster.	This study
pDrive	Cloning Vector: Km^r, Ap^r, pUC origin, T7, SP6, *lacZ*α^+	Qiagen
pRK2013	Km^r, Tra^+, Mob^+, ColE1 replicon	Figurski and Helinski, 1979
pBR325	Tc^r, Ap^r, Cm^r, ColE1 replicon	Blank and Wilson, 1982
pR388	IncW, Su^r, Tp^r	Datta and Hedges, 1972
pTnMod-RTp'	Tn5, R6K *ori*R, Tp^r	Dennis and Zlystra, 1998
pScosBC1	cosmid with K56-2 genomic library	Sokol *et al.*, 1999
pSBC-9F5	pScosBC1 with K56-2 genomic DNA containing *ptwD4* and *ptwC*	This study
pSBC-3H3	pScosBC1 with K56-2 genomic DNA containing *ptwB4* and *ptwB10*	This study
pJQ200SK	Gm^r, *sac*B, Mob^+, P15A replicon	Quandt and Hines, 1993
pURF047	IncW, Amp^r, Gm^r, Mob^+, *lacZ*α^+, Par^+, derivative of pURF043	Swarup *et al.*, 1991
pASE101	pURF047 with 2.0 kb Tp^r cassette inserted at *Sca*I site of Ap^r cassette	This study
pASE102	pJQ200SK containing 10 kb *Bam*HI fragment (pSBC-9F5) containing *ptwD4* and *ptwC* inserted in the MCS	This study
pASE103	pJQ200SK containing 11 kb *Bam*HI fragment (pSBC-3H3) containing *ptwB4* and *ptwB10* inserted in the MCS	This study
pASE104	pASE101 containing 10 kb *Bam*HI fragment (pSBC-9F5) containing *ptwD4* and *ptwC* inserted in the MCS	This study
pASE105	pASE101 containing 11 kb *Bam*HI fragment (pSBC1-3H3) containing *ptwB4* and *ptwB10* inserted in the MCS	This study
pASE106	PASE102 with 1.9 kb Tc^r cassette from pBR325 inserted in the *EcoRV* site of *ptwD4*	This study
pASE107	pASE102 with 1.9 kb Tc^r cassette from pBR325 inserted in the *ScaI* site of *ptwC*	This study
pASE108	pASE103 with 1.9 kb Tc^r cassette from pBR325 inserted in the *Apa*I site of *ptwB4*	This study
pASE109	pASE103 with 1.9 kb Tc^r cassette from pBR325 inserted in the *Sac*I site of *ptwB10*	This study
pDrive-*virB6*	pDrive containing 1.5 kb PCR product containing *virB6*	This study
pDrive-*virB11*	pDrive containing 1.9 kb PCR product containing *virB11*	This study
pJQ200SK -*virB6*	pJQ200SK containing *virB6* from pASE110	This study
pJQ200SK – *virB11*	pJQ200SK containing *virB11* from pASE111	This study
pJQ200SK - *virB6*::Tc	pASE112 with 1.9 kb Tc^r cassette from pBR325 inserted into the *Sal*I site of *virB6*	This study
pJQ200SK - *virB11*::Tc	pASE113 with 1.9 kb Tc^r cassette from pBR325 inserted into the *Hind*III site of *virB11*	This study

[a] Ap^r, Cm^r, Km^r, Su^r, Tc^r, and Tp^r = resistance to ampicillin, chloramphenicol, kanamyacin, sulphonamide, tetracycline, and trimethoprim, respectively. Ptw=plant tissue watersoaking phenotype. + and – = positive and negative for phenotype, respectively.

Table 2.2. Bacterial strains used in Chapter II.

Strain	Relevant Characteristics[a, b]	Reference
Burkholderia cenocepacia		
K56-2	CF respritory isolate, ptw$^+$	Sokol *et al.*, 1999
GM237	K56-2 *ptwD4*::RTp', ptw$^-$	This study
GM238	K56-2::RTp', ptw$^+$	This study
GM241	K56-2::RTp', ptw$^-$	This study
GM242	K56-2 *ptwD4*::RTp', ptw$^-$	This study
GM243	K56-2::RTp', ptw$^-$	This study
AE307	K56-2 *ptwD4*::Tc, ptw$^-$	This study
AE320	K56-2 *ptwC*::Tc, ptw$^+$	This study
AE321	K56-2 *ptwB4*::Tc, ptw$^-$	This study
AE322	K56-2 *ptwB10*::Tc, ptw$^-$	This study
AE310	AE307 (pASE104), ptw$^+$	This study
AE323	AE321 (pASE105), ptw$^+$	This study
AE324	AE322 (pASE105), ptw$^+$	This study
AE360	K56-2 *virB6*::Tc, ptw$^+$	This study
AE361	K56-2 *virB11*::Tc, ptw$^+$	This study
Escherichia coli		
DH10B	F$^-$, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), *φ80dlacZΔM15*, Δ*lacX74*, *deoR*, *recA1*, *endA1*, *araD139*, Δ*(ara, leu)7697, galU*,*galK1*, *rpsL*, *nupG*	Life Technologies, Inc. (Gaithersburg, MD)
HB101	F$^-$, *recA13*	Boyer and Roulland-Dussoix, 1969
CC118λpir	Δ*lacX74*, *galE*, *galK*, *thi-1*, *rpsE*, *rpoB*, *recA1*, lysogenized with λ pir phage	Herrero *et al.*, 1990

[a] Tc^r, and Tp^r = resistance to tetracycline, and trimethoprim, respectively. Ptw=plant tissue watersoaking phenotype. + and – = positive and negative for phenotype, respectively.

Plant tissue assay and growth study

Plant tissue watersoaking (ptw) activity was determined as previously described (Gonzalez *et al.*, 1997). Onion cultivar 1015Y was used for plant tissue assays. Onion bulb scales were pierced and then inoculated, in triplicate, with 10 µl of an aqueous suspension of the isolate being tested as previously described (Gonzalez *et al.*, 1997). Aqueous bacterial suspensions were adjusted spectrophotometrically (A_{425} = 0.5) to yield a final concentration of ~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 37ºC. Plant tissue watersoaking activity was assessed at 24, 48, and 72 h post-inoculation 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 ± 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 post inoculation watersoaked zones were measured and tissue was processed to determine the bacterial population. Scales were commuted and crushed in 0.0125M phosphate buffer pH 7.1 containing Triton X-100 (0.01%) using a sterile mortar and pestle. A dilution series of each tissue slurry was

plated to LB agar amended with 10µg/ml of Gm and 10µg/ml of Tc and incubated at 37ºC for 48 h. Population data for each time interval was expressed as the average CFU/g of tissue ± SD for three independent experiments in triplicate. Tissue watersoaking data were reported as the average area of watersoaking ± SD for three independent experiments in triplicate.

Activity testing of culture supernatants

Cultures of strain K56-2, AE307 (K56-2 *ptwD4*-Tc), and AE310 (K56-2 *ptwD4*-Tc complement) were grown at 37ºC in M9 medium and harvested at late exponential phase (A_{425} = 0.9) by centrifugation (16,000 x g, 15 min at 5ºC). Supernatants (1 L) were filter sterilized using 0.22 µM filters (Pall) and concentrated to 3 ml using an Amicon ultrafiltration stirred cell (MWCO 10,000). Protein concentration of supernatant concentrates was determined by the method of Koch and Putnam (1971). Concentrated uninoculated medium (1L) 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 (Jones *et al.*, 1990). Young carrots were placed in a 10% sodium hypochlorite solution for 5 min and then 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% macerase (CalBiochem), and 0.75% bovine serum albumin (BSA) fraction V (Sigma) (Jones *et al.*, 1990). Tissue was digested in the dark for 5 h at 28ºC with gentle shaking (50 rpm). The carrot tissue was strained through nylon mesh and centrifuged (825 x g for 5 min at 25ºC). 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 using a hemacytometer (Hausser Scientific). On average, 1.8 x 10^6 protoplasts/ml were obtained.

The plasmolytic activity of supernatants was determined by using concentrates standardized to a protein concentration of 0.2 μg/ml in 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 100ºC for 10 min or, 80º, 65º, or 37ºC for 1 h. Concentrates

were also individually treated with 1 mg/ml final concentrations of: proteinase K (Boehringer Mannhein) dissolved in 0.05 M Tris-HCl, pH 8; DNase I (Sigma, 3200 units/mg) in 50 mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, and 50 µg/ml BSA; or RNase A (Sigma, 100 units/mg) in 0.25 M Tris-HCl, pH 6.8. All reactions were incubated at temperatures optimal for each enzyme's activity and were then adjusted to 10% mannitol before mixing 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 ± 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 (ANOVA). 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 x 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 x g for 10 min at 5ºC). The bacterial pellets were

suspended in phosphate buffer and dilution plated to VBG amended with 500 μg/ml of Tc and/or 500 μg/ml of Tp. Single colony isolates of individual transconjugants were obtained by streaking on selective media. Cultures were subjected to survey lysis followed by agarose gel electrophoresis (Gonzalez and Vidaver, 1979) to confirm plasmid transfer.

Plasposon mutagenesis

The plasposon pTnMod-RTp' was employed to obtain initial ptw-negative K56-2 mutants of strain K56-2 (Dennis and Zylstra, 1998). Transconjugants were selected on VBG supplemented with 500 μg/ml of Tp. After 48 h of incubation at 37ºC, isolated colonies were transferred to homologous media to confirm selection. Trimethoprim resistant transconjugants were evaluated for ptw activity 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 *Pst*I and self-ligated using T4 DNA ligase (New England Biolabs). The resulting plasmids were transformed into *E. coli* CC118 λ pir by $CaCl_2$ transformation (Sambrook *et al.*, 1989)

and selected on LB agar amended with 100 μg/ml of Tp. Survey lysis and agarose gel electrophoresis 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 2.3), 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 at 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/.

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 (Sokol *et al.*, 1999). 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 2.3) were designed based on sequence data from the *B. cenocepacia* Genome Project. Each PCR reaction (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 95ºC for 30 sec; annealing at 55, 52, 56.5, and 54ºC for 1 min for primer sets PD4, PC, PB4, and PB10

(Table 2.3) respectively; and extension at 72ºC for 1 min 15 sec. 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 *Bam*HI fragment from pSBC-9F5 and an 11 kb *Bam*HI fragment from pSBC-3H3 were individually inserted into the MCS of pJQ200SK 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 10μg/ml of Gm. Orientation of inserted DNA was determined by restriction enzyme digest analysis using *Sma*I. 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 (Blank and Wilson, 1982), into the *Eco*RV and *Sca*I sites of *ptwD4* and *ptwC,* respectively. Plasmids pASE108 and pASE109 were constructed by inserting the Tc-cassette into the *Apa*I and *Sac*I 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 10 μg/ml of Tc and 10 μg/ml of Gm.

Table 2.3. Primers used in Chapter II.

Primer[a]	Gene	Relevant Characteristics	Reference
RTp1	Tp^r cassette	5'-GGTACCGTCGACATGCATGG-3'	This study
RTp2	Tp^r cassette	5'-CAGTGCAAATTTATCCTGTG-3'	This study
PD4-1	*ptwD4*	5'-TGACTCAGCGAAGGAA-3'	This study
PD4-2	*ptwD4*	5'-ATCCGGTGGAAGCAA-3'	This study
PC-1	*ptwC*	5'-CACTCCGACATCGAAT-3'	This study
PC-2	*ptwC*	5'-AACTGTGTAGGACACT-3'	This study
PB10-1	*ptwB10*	5'-ATGCTTGTCGATGTTGCA-3'	This study
PB10-2	*ptwB10*	5'-TTCCGACGACTTCCAT-3'	This study
PB4-1	*ptwB4*	5'-AACTCAAGATGAATCAT-3'	This study
PB4-2	*ptwB4*	5'-AGAACGCATGGTGTTGA-3'	This study
VB1-1	*virB1*	5'-GACAGATCATGCGTGT-3'	This study
VB1-2	*virB1*	5'-AGGAGGAGACACAGGAGT-3'	This study
VB4-1	*virB4*	5'-ATCGCACTGTTGATCGTTG-3'	This study
VB4-2	*virB4*	5'-TCACGTTACGTGTGCATAC-3'	This study
VB6-1	*virB6*	5'-AAGGTGGTGTTCTATGAA-3'	This study
VB6-2	*virB6*	5'-ACAGTACGATATGCGTGAA-3'	This study
VB11-1	*virB11*	5'-TTCAAGTAACGATTGCTGA-3'	This study
VB11-2	*virB11*	5'-TGCAAATCGAGTTCTGGTA-3'	This study
VD4-1	*virD4*	5'-TGTTATGAGACAGATTGCA-3'	This study
VD4-2	*virD4*	5'-AGATAAGGATGGTCAGGT-3'	This study

[a] 1=forward primer; 2=reverse primer.

Insertion of the Tc-resistance cassette was confirmed by restriction enzyme digest analysis using *Bam*HI and PCR using primer sets PD4, PC, PB4, and PB10 (Table 2.3) 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 (plated to VBG amended with 500 μg/ml of Tc), to yield genetic exchange mutants. Tetracycline resistant transconjugants were single colony purified and retested for Tc^r. 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 2.3) were designed based on sequence data from the Sanger Centre. Each PCR reaction was performed as described above except that the cycle parameters were as follows: denaturation at 95˚C for 30 sec; annealing at 57.5 and 56˚C for 1 min for primer sets VB6 and VB11, respectively; and extension at 72˚C for 1 min 15 sec. PCR products were individually ligated into vector pDrive (Qiagen) and transformed into *E. coli* DH10B by electroporation followed by selection on LB agar amended with 40 μg/ml of Ap to yield pDrive-*virB6* and pDrive-*virB11*, respectively. Genes *virB6* and *virB11* were excised from pDrive-*virB6* and pDrive-*virB11*, respectively by double digestion using restriction enzymes *Pst*I and *Xho*I and individually inserted into the MCS of pJQ200SK to construct pJQ200SK-*virB6* and

pJQ200SK-*virB11*, respectively. Plasmids pJQ200SK-*virB6* and pJQ200SK-*virB11* were transformed into *E. coli* DH10B by electroporation followed by selection on LB agar amended with 10 μg/ml of Gm. Using pJQ200SK-*virB6* and pJQ200SK-*virB11*, plasmids pJQ200SK-*virB6*::Tc and pJQ200SK-*virB11*::Tc were constructed by inserting a Tc-resistance cassette, from pBR325, into the *Sal*I and *Hind*III sites of *virB6* or *virB11*, respectively. Constructs pJQ200SK-*virB6*::Tc and pJQ200SK-*virB11*::Tc were individually electroporated into *E. coli* DH10B and transformants were selected on LB agar amended with 10 μg/ml of Tc and 10 μg/ml of Gm. Insertion of the Tc-resistance cassette was confirmed by restriction enzyme digest analysis using *Bam*HI and PCR primer sets VB6 and VB11 to confirm a 1.9 kb increase in plasmid size and PCR product, respectively. Plasmids pJQ200SK-*virB6*::Tc and pJQ200SK-*virB11*::Tc 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^r. PCR using primer sets VB6 and VB11 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.

Complementation of disrupted genes

Complementation of the disrupted *ptwD4, ptwB4,* or *ptwB10* were accomplished *in trans*. Plasmid pURF047 (Swarup *et al.*, 1991) was amended by inserting a Tp-resistance cassette, obtained from R388 as a *Bam*HI fragment, into the *Sca*I site in the *bla* gene to obtain pASE101. A 10 kb *Bam*HI fragment from pSBC-9F5 that contained *ptwD4,* and an 11 kb *Bam*HI 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 100 μg/ml of Tp and 10 μg/ml of Gm. Orientation of inserted DNA was determined by restriction enzyme digest analysis using *Sca*I. 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 Tp^r 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 resident and complement plasmid as well as inserted and uninserted PCR products.

Epithelial cell cultures

The IB3 cell line is an adenovirus 12-SV40 hybrid-transformed human bronchial epithelial cell line derived from a CF patient (DF508/W1282X). The C38 cell line is derived from IB3, and stably expresses a transfected functional CF transmembrane conductance regulator protein (Zeitlin *et al.*, 1991). Both cell lines were grown in LHC-8 medium supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin.

The 16HBE14o$^-$ (16HBE) cell line is an SV-40 large T antigen transformed human bronchial epithelial cell line and was kindly provided by Dr. Dieter Gruenert (Cozens *et al.*, 1994). Cells were maintained in minimal essential medium containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). To obtain polarized 16HBE monolayers, cells were grown in Transwells (Costar) with 3 μ pore size membranes; transepithelial electrical resistance (TER) was measured as described previously (Kim *et al.*, 2005). 16HBE cell monolayers with TER values >350 Ω/cm2 were used in epithelial transmigration assays.

Stimulation of IL-8 secretion

IB3 and C38 cells (1 x 10^5 cells/well) seeded in 24 well culture plates were grown to 80-90% confluence in supplemented LHC-8 medium. Cells were shifted to serum- and antibiotic-free LHC-8 medium, incubated for 16 h and then infected with strain K56-2, AE307, or AE310 at a multiplicity of infection (MOI) of 0.1. For infection of bronchial epithelial cells, bacteria were grown in LB broth or LHC-8 media (Biosource International) for 16h at 37^{o}C in an orbital shaker, harvested by centrifugation, and suspended in PBS (Sambrook *et al.*, 1989) to the desired concentration. Cells incubated with cell culture media alone served as negative controls. After 24 h of incubation, an aliquot of media was collected, serially diluted, and plated to determine bacterial density. The remaining media was centrifuged and the amount of IL-8 in the supernatant was determined by ELISA (R&D systems).

Epithelial transmigration assays

Polarized 16HBE cell layers with a TER >350 Ω/cm2 were maintained in antibiotic free supplemented medium for 24 h prior to infection with selected bacterial strains. Cell cultures were infected with bacteria at an MOI of 1 and bacteria in the basolateral chambers were quantified at 4, 8, 12, and 24 h post infection. The TER was measured at the same time points.

RESULTS

Isolation and characterization of ptw-negative mutants

A total of 5000 transconjugants, of strain K56-2, were obtained using the plasposon mutagenesis system. Genomic DNA from 20 randomly selected transconjugants were digested with *Pst*I, which does not cleave the TnMod-RTp' insert. The DNA fragments were separated by agarose gel electrophoresis, and Southern blots were probed with the Tp-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.

From the pool of 5000 transconjugants, 56 mutants were verified as ptw-negative using the plant tissue assay (Fig. 2.1), and demonstrated no other apparent phenotypic differences when compared to the parental strain. The DNA flanking the plasposon insertion site of 10 ptw-negative mutants was cloned, sequenced, and a BLAST search was performed. The translated gene products disrupted in clone GM237 and GM242

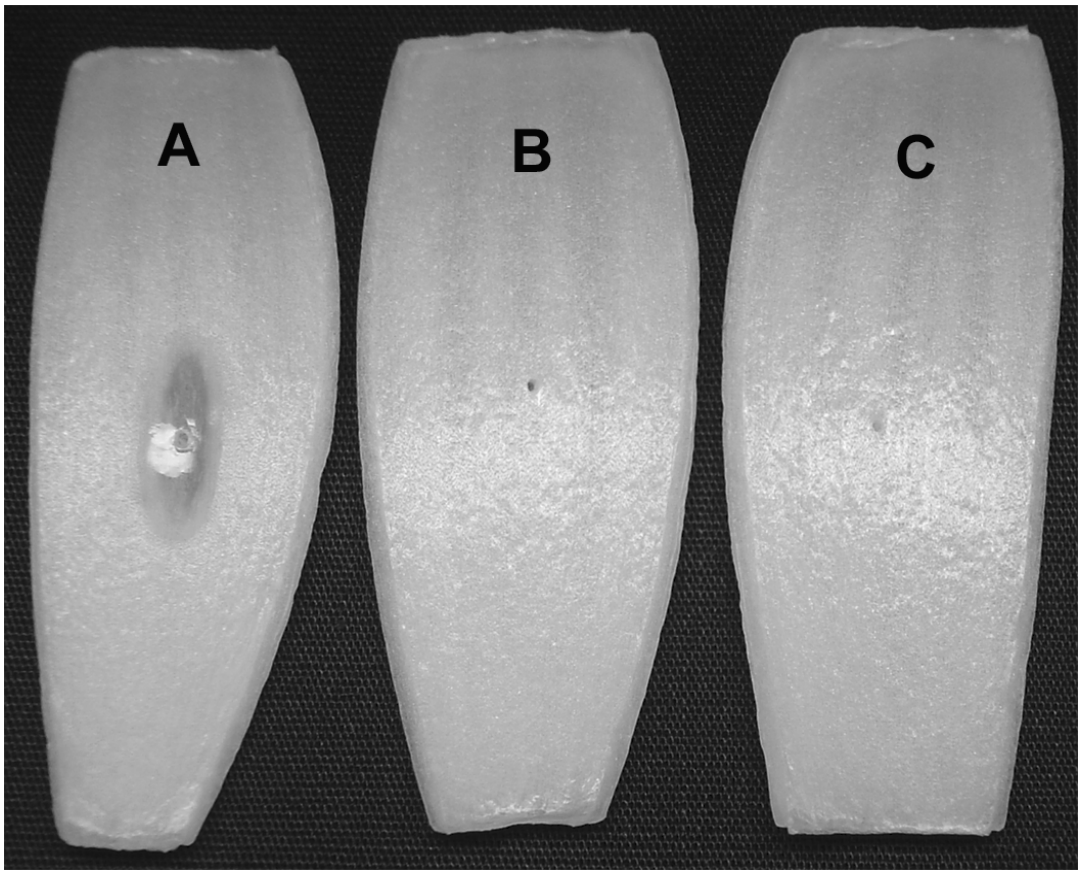

Fig. 2.1. Analysis of Ptw type IV secretion system using the plant tissue watersoaking assay. (A) Pierced onion bulb scale inoculated with *B. cenocepacia* strain K56-2 showing partial tissue collapse and translucence characteristic of the plant tissue watersoaking (ptw) phenotype. (B) Pierced onion 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 Fig. 2.1C.

(Table 2.2) showed homology toVirD4-like proteins, whereas GM241 and GM243 (Table 2.2) 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.

Using sequence data from the *B. cenocepacia* Genome Project, a 60 kb region proximal to the identified *virD4*-like 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 (Mahenthiranlingam *et al.*, 2000b), 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. 2.2). Southern analysis of K56-2 lysates 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. 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%.

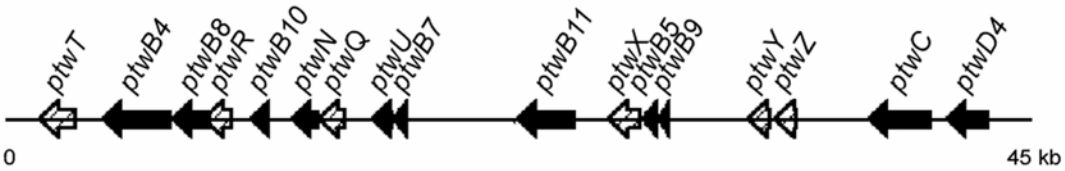

Fig. 2.2. A 45 kb segment from plasmid pK56-2 containing the *ptw* cluster. Designation of genes was based on homology to gene products of transfer and translocation related proteins and are depicted by solid arrows. ORFs having no homologs are illustrated by patterned arrows.

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 *A. tumefaciens*, *Salmonella 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 (Schröeder *et al.*, 2002; Schröeder and Lanka, 2003). PtwB4 and its homolog VirB4 of the VirB/D system may also exhibit NTPase activity (Baron *et al.*, 2002). Based on homology to R388 TrwC, PtwC may function as a component of a relaxosome (Llosa *et al.*, 1994). PtwB7, PtwB8, PtwB9, and PtwB10 were predicted to form complexes in the periplasm and/or membrane to create the pore (Yeo and Waksman, 2004). 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" (Planet *et al.*, 2001). PtwN as well as PtwU had no VirB/D 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 (Lawley *et al.*, 2003). 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 MW 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 (CDD), revealed a predicted Walker A (P-loop) and B site for nucleotide binding (Walker *et al.*, 1982) that is characteristic of type IV secretion ATP-binding proteins (Yeo and Waksman, 2004). 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 1013 amino acids in length, with a predicted MW 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 (Yeo and Waksman, 2004). Translation of the *ptwB10* sequence resulted in a protein that was 278 amino acids in length, with a predicted MW of 30 kDa and a pI of 5.2. Using rpsBLAST and the CDD, PtwB10 was shown to contain a well conserved C-terminal hydrophobic region (Yeo and Waksman, 2004). Translation of the ptwC sequence resulted in a protein that was 940 amino acids in length, with a predicted MW 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 1-200), PtwC and TrwC showed a 42% identity and signature motifs 2 and 3 that are conserved in proteins with DNA nicking activities (Llosa *et al.*, 1994). 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 (Hodgman, 1988).

Site-directed disruption of the *ptw* or *virB/D* 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. 2.1 and Table 2.2). Site-directed mutagenesis of *ptwD4*, *ptwC*, 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. 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 (Table 2.2).

Complementation of ptw-negative mutants was accomplished by mobilization of pASE104 into AE307, and pASE105 into AE321 or AE322 and selecting for Tc^r and Tp^r-transconjugants. All transconjugants tested were ptw-positive (Fig. 2.3). Plasmid pURF047 derivatives were used in complementation studies (Defeyer *et al.*, 1990). A survey of transconjugant lysates confirmed plasmid transfer. Representatives of ptw-

positive complemented *ptwD4*, *ptwB4,* and *ptwB10* mutants were designated AE310, AE323, and AE324, respectively (Fig. 2.3).

Genomic analysis of the J2315 sequence from the Sanger Centre identified a second cluster on chromosome II (designated VirB/D), which showed homology to the VirB/D4 type IV secretion system of *A. tumefaciens* (Fig. 2.4). Using PCR analysis, the presence of *virB1*, *virB4*, *virB6*, *virB11,* and *virD4* (Table 2.3), that span the entire VirB/D cluster, were confirmed in K56-2. 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 (Jakubowski *et al.*, 2003), and *virB11* is predicted to function as an NTPase, which provides the necessary energy for secretion (Yeo and Waksman, 2004). 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. Disruption of *virB6* or *virB11* did not result in loss of the ptw phenotype.

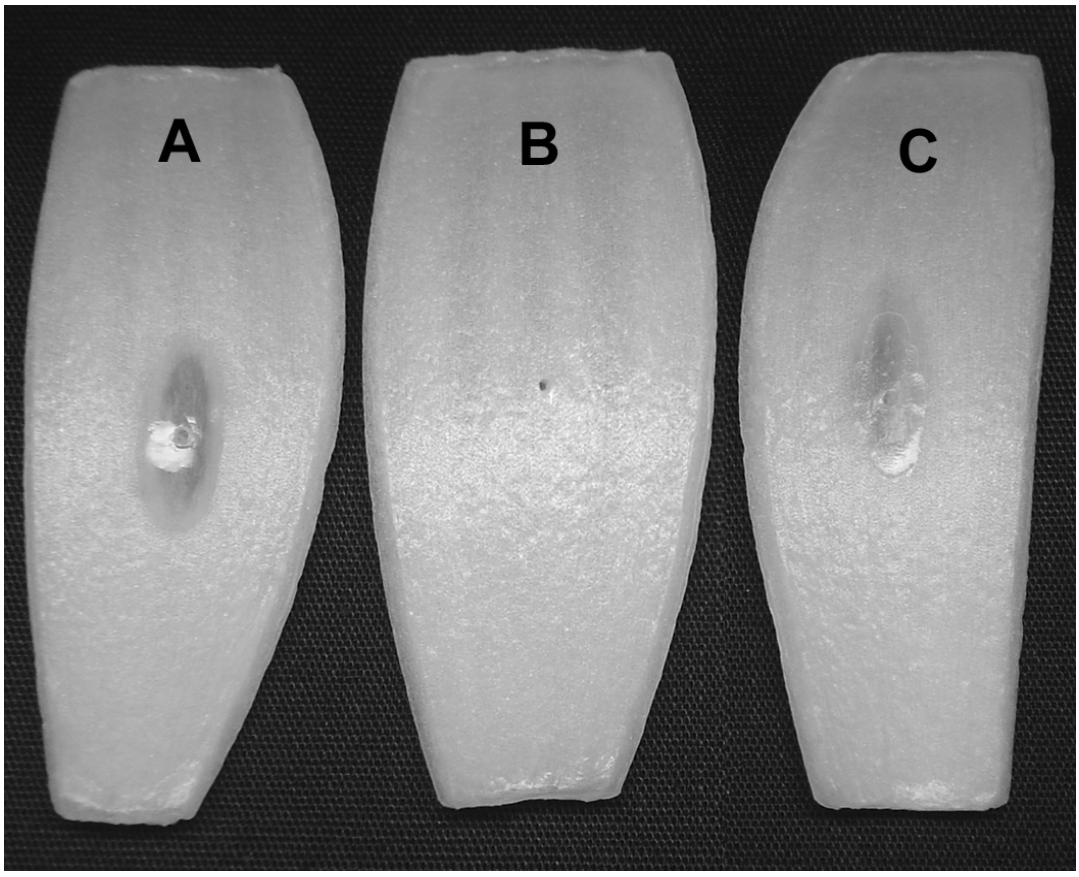

Fig. 2.3. Complementation of ptw-negative mutants. (A) Pierced onion bulb scale inoculated with strain K56-2 showing characteristic ptw activity. (B) Pierced onion bulb scale inoculated with *B. cenocepacia* ptw-negative mutant AE307 showing no ptw activity. (C) Pierced onion bulb scale inoculated with complemented AE307 (AE310) showing restored ptw activity.

Fig. 2.4. A 12 kb segment containing a *virB/D* like type IV secretion system cluster located on chromosome two 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.

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. 2.3). 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/g of tissue and an increase in the watersoaking area, whereas AE307 showed no watersoaking activity and an approximate 1000-fold decrease in CFU/g of tissue over the same period (Table 2.4).

Table 2.4. Growth and watersoaking activity of *B. cenocepacia* strain K56-2 and its derivatives in onion tissue.

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

[a] Average CFU/g tissue ± SD for three independent experiments in triplicate.
[b] Numbers in parentheses are the average area of tissue watersoaking (mm^2) ± SD for three independent experiments in triplicate. All strains were plated on LB amended with 10 µg/ml Tc and 10 µg/ml of Gm.

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, carrot protoplasts exposed to M9 medium concentrate did not experience significant plasmolysis when compared to the protoplast controls ($P>0.05$) (Table 2.5 and Fig. 2.5). However, significant ($P\leq0.05$) 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 as compared to 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 diluted 1:10 to a protein concentration of 0.02 µg/ml reduced activity to levels which approximated that of the control and AE307. Temperatures of 37º, 65º, and 80ºC for 1 h or 100ºC 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.

Table 2.5. Effect of supernatant concentrates from K56-2, *ptwD4* mutant (AE307) and complemented *ptwD4* mutant (AE310) on carrot protoplasts.

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

[a] All dilutions were adjusted to 10% mannitol
[b] Data are mean values ± SD of two replicates for three independent experiments.
[c] Values within the columns scored by the same letter were not statistically different at P=0.05.
[d] Undiluted concentrate adjusted to 0.2 μg/ml protein in 10% mannitol.

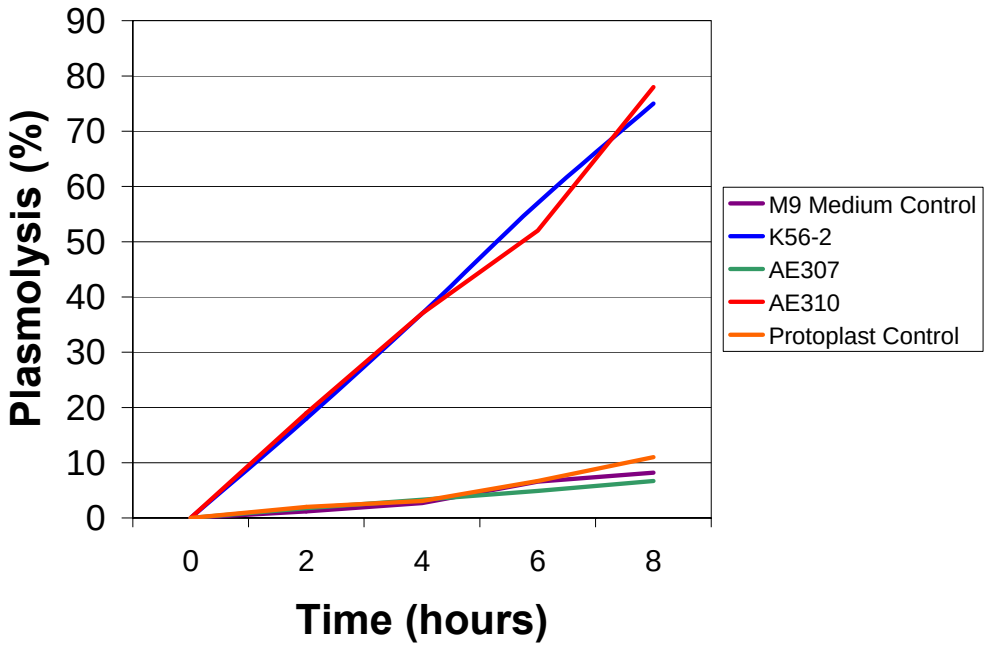

Fig. 2.5. Effect of supernatant concentrates from K56-2, *ptwD4* mutant (AE307) and complemented *ptwD4* mutant (AE310) on carrot protoplasts. Data are mean values of two replicates for three independent experiments.

Role of the Ptw type IV secretion system in stimulation of IL-8 from bronchial epithelial cells

IB3 cells were used to investigate the possible role of the Ptw type IV secretion system in contributing to IL-8 secretion by CF bronchial epithelial cells. In preliminary experiments, we found that *B. cenocepacia* strain K56-2 stimulated significantly greater IL-8 from IB3 (CF phenotype) cells than from C38 (corrected CF phenotype) cells *in vitro* (Fig. 2.6). This is similar to what is found with infection of well-differentiated CF and non-CF primary airway epithelial cells *in vitro* (Sajjan *et al.*, 2004). IB3 cells were grown in monolayers, and infected with *B. cenocepacia* K56-2, the ptw mutant (AE307) or the complement (AE310). After 24 h the amount of IL-8 secretion was measured. Compared to wild-type K56-2, IL-8 stimulation by all three ptw mutants was significantly attenuated (Fig. 2.7). Complementation of the ptw mutants *in trans* restored the IL-8 stimulatory activity.

To determine whether secreted bacterial products were involved in the stimulation of IL-8 from epithelial cells, we examined the effect of bacterial culture supernatants from wild-type or ptw mutants on IL-8 secretion. Supernatants from wild-type K56-2 stimulated maximum secretion of IL-8 at 6 h post-treatment. At this time point, supernatants from AE307 stimulated significantly ($p<0.05$) less IL-8 from IB3 cells compared to supernatant from wild-type K56-2. The pro-inflammatory activity of the mutant was restored by complementation of respective genes *in trans* (Fig. 2.8).

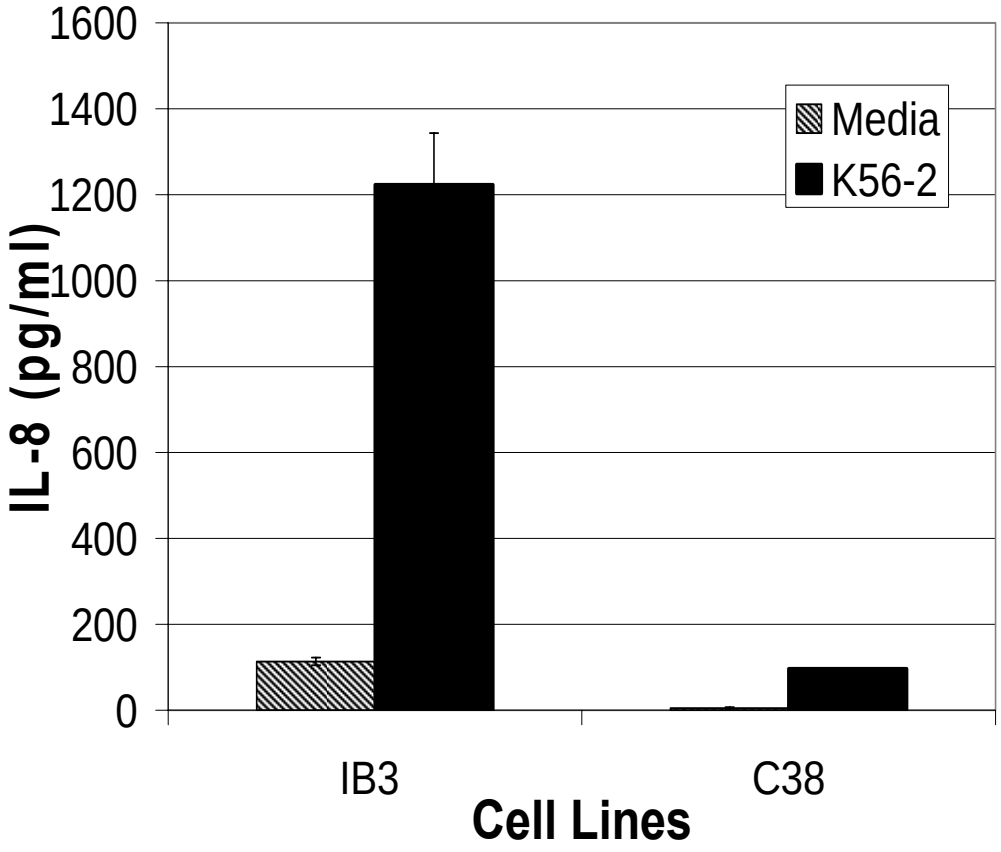

Fig. 2.6. Secretion of IL-8 from IB3 and C38 cells in response to infection with wild-type K56-2. Assays were performed in triplicate and results for mutants and complemented mutants are expressed as pg/ml of IL-8 (+/- SD).

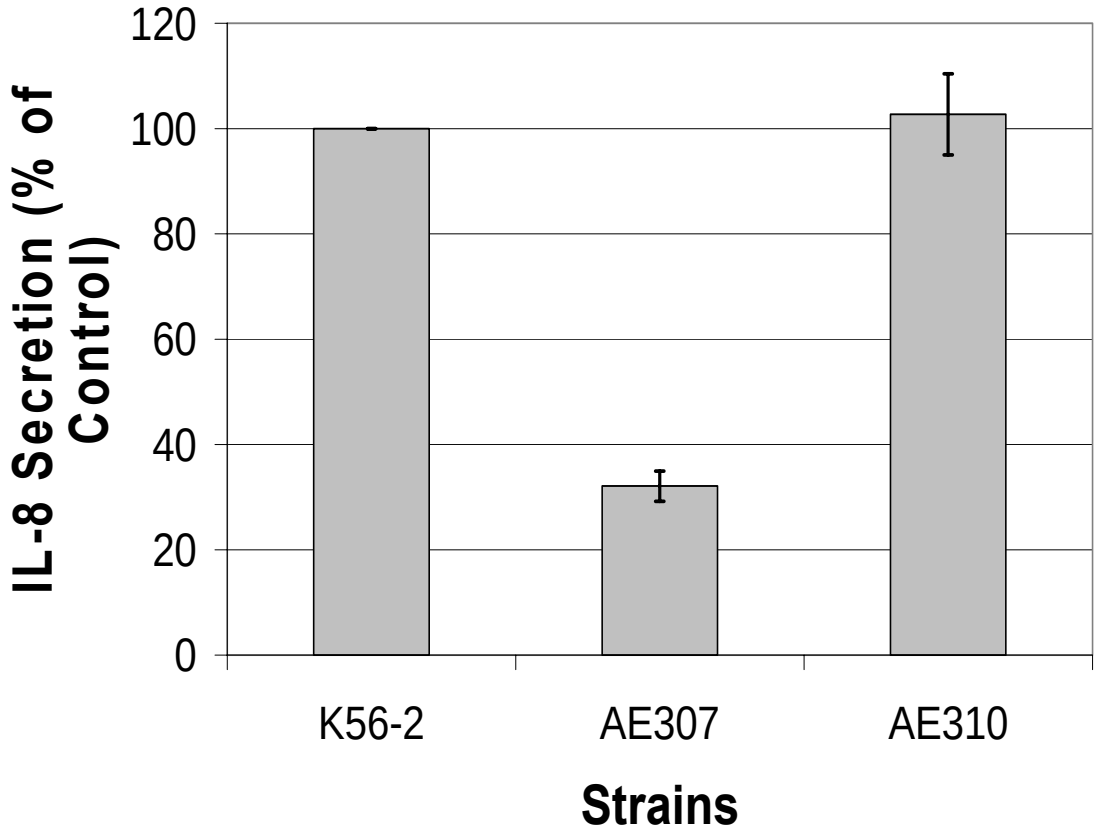

Fig. 2.7. Secretion of IL-8 from IB3 cells in response to infection with wild-type K56-2, AE307 (K56-2 *ptwD4*-Tc), and AE310 (K56-2 *ptwD4*-Tc complement). Assays were performed in triplicate and results for the mutant and complemented mutant are expressed as percent (+/- SD) of the K56-2 positive control.

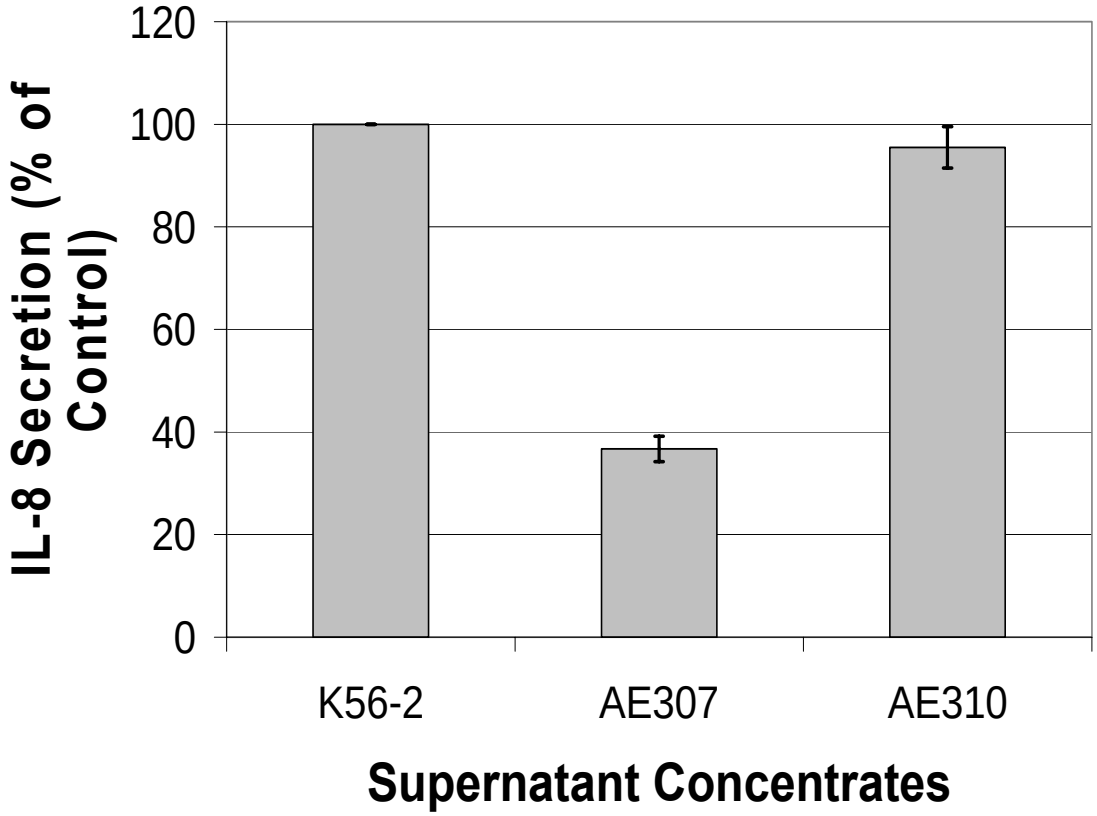

Fig. 2.8. Stimulation of IL-8 from IB3 cells in response to treatment with bacterial culture supernatants from wild-type K56-2, AE307 (K56-2 *ptwD4*-Tc), and AE310 (K56-2 *ptwD4*-Tc complement). Assays were performed in triplicate and results for the mutant and complemented mutant are expressed as percent (+/- SD) of the K56-2 positive control.

DISCUSSION

Previously, Gonzalez *et al.* (1997) investigated the role of a polygalacturonase (PehA) as a virulence factor in *B. cepacia* strain ATCC 25416. Derivatives cured of a resident plasmid that encodes for the PehA no longer macerated onion tissue; however, the derivatives still produced a phenotype described as plant tissue watersoaking. Of interest in the present study was the observation that all *B. cepacia* and *B. cenocepacia* isolates and a *B. vietnamiensis* from the Bcc experimental strain panel (Mahenthiralingam *et al.*, 2000b) produced the ptw phenotype in onion tissue (Medrano, 2002). 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 have evolved type IV secretion systems to transfer DNA or protein macromolecules to a wide array of target-cell types (Baron *et al.*, 2002; Christie, 2001; Nagai and Roy, 2003). Originally, the nomenclature referred to the VirB/D-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* (Christie, 2001). 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 (Christie, 2001; Grohmann *et al.*, 2003). However, it is important to distinguish functional translocation machines from mobile elements characteristically found in bacterial genomes. Ding *et al.,* (2003) have suggested that mutagenesis of a putative type IV system should yield a phenotype at least consistent with a translocation defect.

Christie (2001) has suggested sub-classification of type IV secretion systems based on ancestral lineage. Thus, the VirB/D-type has been designated IVA, and the ColIb-P9 Tra and *L. pneumophila* Dot-Icm types have been designated IVB. This sub-classification left open the possibility for 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.* (2003) 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 (Dillard and Seifert, 2001). 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 (Wilkins and Thomas, 2000). There is also a subset of these systems that can transfer DNA and protein substrates to a range of eukaryotic cell types, to include plant, fungal, and human (Bates *et al.*, 1998;

Ward and Zambryski, 2001; Waters, 2001). 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. pneumophilla*, *Brucella* spp., and *Bartonella* spp. (Burns, 2003). 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 translocators and are thought to be essential for infection (Ding *et al.*, 2003). The "DNA uptake and release" group, which presently contains three members, translocates DNA substrates across the cell envelope to or from the extracellular milieu (Ding *et al.*, 2003). *Neisseria gonorrhoeae* uses a system encoded by the gonococcal genetic island to export DNA (Dillard and Seifert, 2001; Hamilton *et al.*, 2001). 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 (Dhar *et al.*, 2003). The two other members of this subfamily, *Campylobacter jejuni* and *H. pylori*, translocate DNA in the opposite direction promoting genetic variation and cell survival (Bacon *et al.*, 2000; Hofreuter *et al.*, 2001).

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 (Ding *et al.*, 2003). The

ptw cluster contains homologs to components of multiple type IV secretion systems (Fig. 2.2). 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 fact 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 (Karlin, 2001).

The presence of a second type IV secretion system in *B. cenocepacia* is not unprecedented since many bacteria, such as *H. pylori* and *A. tumefaciens,* have been found to harbor multiple secretion systems (Ding *et al.*, 2003; Kersulyte *et al.*, 2003). The type IV secretion system located on chromosome II showed most similarity to that of the *A. tumefaciens* VirB/D translocation system with respect to arrangement and gene product similarity (Fig. 2.6). 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 (Karlin, 2001), 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 (Moncalián *et al.*, 1999; Schröeder and Lanka, 2003). It is thought to interact with the *oriT* bound relaxosome, which is made up of TrwC and TrwA, to facilitate DNA transfer (Fekete and Frost, 2000; Llosa *et al.*, 2003). In the Ptw system however, there is no TrwA homolog. The *ptwD4* product appears to have similar functions to Hp0524 of *H. pylori*. The *hp0524* product is critical for the transfer of CagA from the bacterium to epithelial cells (Fischer *et al.*, 2001). Further analysis 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 (Matson *et al.*, 1993; Matson *et al.*, 2001). Analysis of PtwC revealed that the N-terminus possessed signature motifs (2 and 3) conserved in proteins with DNA nicking activities (Llosa *et al.*, 1994) and the C-terminus possessed signature motifs (I, Ib, II, III, IV, V, and VI) conserved in DNA helicases (Hodgman, 1988); 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 (Yeo and Waksman, 2004). 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 (Yeo and Waksman, 2004). VirB4 is a large inner membrane protein with NTPase activity (Yeo and Waksman, 2004). The PtwB4 amino acid sequence was shown to possess four transmembrane regions that are characteristic of VirB4-like NTPases (Yeo and Waksman, 2004). 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 since successful secretion of the ptw effector protein(s) would likely involve: 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, a type IV secretion system has been identified that appears to export an effector molecule(s) that is proteinacious in nature and responsible for the activity. This activity plays an important role in the ability of *B. cenocepacia* strain K56-2 to cause watersoaking and grow in plant tissue (Fig. 2.2 and Table 2.4). 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. Currently, 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 (Bogdanove *et al.*, 1998). A disruption in *wtsE* renders *P. stewartii* subsp. *stewartii* incapable of eliciting an observable watersoaking phenotype and causing a systemic corn leaf infection (Frederick *et al.*, 2001). 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 (Nagai and Roy, 2003; Salmond, 1994). The first group alter 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 (Nagai and Roy, 2003). 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 multi-subunit structures that may include protein and/or DNA (Ding *et al.*, 2003). Thus, there are no universally conserved primary sequence motifs or physical characteristics that are readily discernible for secreted effectors.

Recently, there has been a growing appreciation of the roles that type IV secretion systems play in the virulence of certain human respiratory pathogens, including *B. pertussis* and *L. pneumophilia* (Christie, 2001). Type IV secretion systems have also been found to contribute to the induction of inflammatory responses from infected host tissues (Bauer, 2005). For example, effector proteins translocated from bacteria to host cells via type IV secretion systems stimulate an intense inflammatory response from gastric epithelium infected by *H. pylori* and *B. henselae* (Bauer *et al.*, 2005; Schmid *et al.*, 2004). Indeed, *H. pylori* mutant strains with a nonfunctional Cag type IV secretion system were recently shown to be significantly attenuated in their ability to stimulate IL-8 production from a variety of cultured mammalian cells (Bauer *et al.*, 2005).

It has been previously shown that *B. cenocepacia* strain K56-2 stimulates release of IL-8 from human respiratory epithelial cells (Sajjan *et al.*, 2004). Although a variety of bacterial components, such as LPS, flagella, and pili are likely involved in this process, the relative contributions of each of these components to immunostimulation of airway epithelial cells during respiratory infection in CF are not well understood. The results from this study indicate that the plasmid encoded Ptw type IV secretion system may also contribute to the stimulation of IL-8 from *B. cenocepacia* infected CF epithelial cells. Experiments using sterile broth culture supernatants showed similar results to that

observed with bacterial cells, suggesting that secreted effectors are involved in IL-8 stimulation.

In conclusion, this study (Chapter II) has identified a plasmid encoded type IV secretion system in *B. cenocepacia* strain K56-2 that was functionally an effector translocator. The Ptw type IV secretion system was found to be responsible for production of the ptw phenotype and at least partially responsible for the stimulation of release of IL-8 from human bronchial epithelial cells, caused by *B. cenocepacia* strain K56-2. Culture supernatant studies on plant protoplast and human bronchial epithelial cells indicate that an effector protein translocated into the extracellular milieu by the Ptw system is responsible for the toxicity on plant cells as well as the stimulation of IL-8 release in epithelial cells. The chromosomally located type IV secretion system showed modular similarity to the *A. tumefaciens* VirB/D4 translocation system; however, it was not responsible for the ptw phenotype in *B. cenocepacia* strain K56-2.

CHAPTER III

IDENTIFICATION OF AN EFFECTOR TRANSLOCATED BY THE *Burkholderia cenocepacia* PTW TYPE IV SECRETION SYSTEM

INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacterial species that are opportunistic pathogens capable of causing life-threatening respiratory infections, particularly in persons with cystic fibrosis (CF) (Coenye *et al.*, 2004). The Bcc consists of nine species (Coenye *et al.*, 2001a, b, c; Vandamme *et al.,* 2002; Vandamme *et al.*, 2003; Vermis *et al.*, 2004). Recent analyses indicate that although all nine species are capable of causing infection in CF patients, some are much more commonly involved than others. In the United States, approximately half of Bcc-infected CF patients harbor *Burkholderia cenocepacia* (LiPuma *et al.*, 1999; Reik *et al.*, 2005); in Canada and parts of Europe, the proportion can be as high as 83% (Speert *et al.*, 2002). There is extensive evidence for patient to patient spread and nosocomial transmission of certain *B. cenocepacia* strains (Coenye and LiPuma, 2003). However, the factors that account for the apparent enhanced capacity of some *B. cenocepacia* strains for pulmonary infection and/or interpatient spread in CF remain to be elucidated.

In recent years, extensive research has shown that bacterial pathogens share common secretion mechanisms for the delivery of virulence determinants (Plano *et al.*, 2001, Christie and Vogel, 2000). There are numerous examples of the importance of type III

(Plano *et al.*, 2001) and type IV secretion systems (Christie and Vogel, 2000) in the infection process for both plant and animal pathogens. It is hypothesized that type III secretion systems are derived from flagellar assembly constituents (Macnab, 1999), and that the type IV secretion systems share a common ancestry with bacterial conjugation systems. Type IV secretion systems are commonly used by bacteria to deliver macromolecular substrates to target cells, often for purposes associated with pathogenesis (Cascales and Christie, 2003; Llosa and O'Callaghan, 2004). The prototypical type IV secretion system is the *Agrobacterium tumefaciens* nucleoprotein T-DNA transfer system. Type IV secretion systems that are required for virulence have also been described in medically important pathogens, including the *Bordetella pertussis* Ptl (pertussis toxin) system (Farizo *et al.*, 2000), the Dot/Icm system of *Legionella pneumophila* (Zink *et al.*, 2002), and the *cag* pathogenicity island (PAI) of *Helicobacter pylori*.

The effector molecules secreted by type IV secretion systems have a variety of functions. The most well characterized effector is the pertussis toxin, which is a member of the AB_5 toxin family (Farizo *et al.*, 2000, Tamura *et al.*, 1982) and is directly transferred into the extracellular milieu (Tamura *et al.*, 1982), where it ultimately interferes with signaling pathways in the host cells (Malaisse *et al.*, 1984). The *L. pneumophila* Dot/Icm transporter is an example of a type IV secretion system that is capable of both conjugation and effector translocation (Nagai and Roy, 2003). Secreted effectors include RalF, which is a guanine nucleotide exchange factor for multiple ADP-ribosylation factor proteins (Nagai *et al.*, 2002), and LidA, for which the biochemical

activity is unknown (Conover *et al.*, 2003). *H. pylori* possesses two type IV secretion systems with differing functions: the Cag secretory apparatus (encoded for by the *cag* PAI) translocates CagA into host cells where it induces cellular changes (Censini *et al.*, 2001), and the Com system is used to uptake DNA to facilitate genetic variation and enhance cell survival (Dhar *et al.*, 2003, Ding *et al.*, 2003).

In this study (Chapter III) an effector protein, designated PtwE1 was identified, which was translocated via the previously identified (Chapter II) Ptw type IV secretion system of *B. cenocepacia* strain K56-2.

EXPERIMENTAL PROCEDURES

Bacterial strains, media, and culture conditions

Descriptions of plasmids and bacterial strains used in this study are listed in Tables 3.1 and 3.2, respectively. Luria Bertani (LB) medium was used for routine maintenance of

cultures. Minimal medium Vogel Bonner (Vogel and Bonner, 1965) amended with 1.0% glucose (VBG) was used in mating experiments. Minimal medium M9 (Sambrook *et al.*, 1989) containing 1.0% glucose was used for culturing bacteria for supernatant analysis and detection of glutathione *S*-transferase (GST) fusion proteins; and M9 containing 1.0% glycerol was used for β-galactosidase assays. Stability testing of pBCRLAR derivatives was accomplished using tryptone nutrient broth (TNB) (Hansen and Olsen, 1978) for liquid culture and VBG amended with 500 μg/ml of trimethoprim (Tp) and 500 μg/ml tetracycline (Tc) or VBG for selective and non-selective plating. *Burkholderia cenocepacia* and *Escherichia coli* strains were grown at 37ºC. Media were supplemented with appropriate concentrations of antibiotics as needed for selection. Antibiotics were added to media at the following concentrations: 500 μg/ml of chloramphenicol (Cm) and 500 μg/ml of trimethoprim (Tp) for selection and maintenance of *B. cenocepacia* isolates and derivatives; and 10 μg/ml tetracycline (Tc), 10 μg/ml gentamicin (Gm), 40 μg/ml ampicillin (Ap), and 100 μg/ml Tp for selection and maintenance of *E. coli*.

Table 3.1. Plasmids and primers used in Chapter III.

Plasmid	Relevant Characteristics[a, b]	Reference
pBR325	Tc^r, Ap^r, Cm^r, ColE1 replicon	Blank and Wilson, 1982
pDrive	Cloning Vector: Km^r, Ap^r, pUC origin, T7, SP6, *lacZ*α^+	Qiagen
pCR2.1	TA cloning vector	Invitrogen
pFUSE	*ori*R6K plasmid containing promoterless *lacZYA*	Baümler *et al.*, 1996
pGEX4T-1	GST-fusion vector	Amersham Biosiences
pJQ200SK	Gm^r, *sac*B, Mob^+, P15A replicon	Quandt and Hynes, 1993
pRK2013	Km^r, Tra^+, Mob^+, ColE1 replicon	Figurski and Helinski, 1979
pURFO47-Tp	pURF047 with 2.0-kb Tp^r cassette inserted at *Sca*I site of Ap^r cassette	Chapter II
pBCRLAR	Cm^r, Mob^+, *lacZ*α^+, pBBR1 replicon	LiPuma, Univ. of Michigan
pBCRLAR-Tp	pBCRLAR containing a blunted Tp^r cassette inserted at *Sca*I site of Cm^r cassette	This study
pDrive-*ptwD4*	pDrive containing a *ptwD4* PCR product	This study
pDrive-*ptwE1*	pDrive containing a *ptwE1* PCR product	This study
pDrive-*ptwE2*	pDrive containing a *ptwE2* PCR product	This study
pDrive-*ptwD4*::Cm	pDrive-*ptwD4* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *EcoR*V site of *ptwD4*	This study
pDrive-*ptwE1*::Cm	pDrive-*ptwE1* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *Hae*III site of *ptwE1*	This study
pDrive-*ptwE2*::Cm	pDrive-*ptwE2* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *Alu*I site of *ptwE2*	This study
pJQ200SK-*ptwD4*::Cm	pJQ200SK containing a *Bam*HI/*Sac*I *ptwD4*-Cm fragment from pDrive-*ptwD4*::Cm in the MCS	This study
pJQ200SK-*ptwE1*::Cm	pJQ200SK containing a *Bam*HI/*Sac*I *ptwE1*-Cm fragment from pDrive-*ptwE1*::Cm in the MCS	This study
pJQ200SK-*ptwE2*::Cm	pJQ200SK containing a *Bam*HI/*Sac*I *ptwE2*-Cm fragment from pDrive-*ptwE2*::Cm in the MCS	This study
pBCRLAR-Tp-*ptwD4*	pBCRLAR-Tp containing a *Bam*HI/*Sac*I *ptwD4* fragment from pDrive-*ptwD4* in the MCS	This study
pBCRLAR-Tp-*ptwE1*	pBCRLAR-Tp containing a *Bam*HI/*Sac*I *ptwE1* fragment from pDrive-*ptwE1* in the MCS	This study
pCR2.1-*ptwB4*	pCR2.1 containing a *ptwB4* PCR product	This study
pFUSE-*ptwB4*	pFUSE containing an *Xba*I/*Sm*aI *ptwB4* fragment from pCR2.1-*ptwB4* in the MCS	This study
pDrive-GST	pDrive containing a GST PCR product	This study
pDrive-*ptwE1*-GST	pDrive-GST containing a blunted *Bam*HI/*Sac*I *ptwE1* fragment from pDrive-*ptwE1* in the blunted *Kpn*I site	This study
pDrive-GST-*ptwE1*	pDrive-GST containing a blunted *Bam*HI/*Sac*I *ptwE1* fragment from pDrive-*ptwE1* in the blunted *Hind*III site	This study
pDrive-*ptwE1*::Cm-GST	pDrive-GST containing a blunted *Bam*HI/*Sac*I *ptwE1*::Cm fragment from pDrive-*ptwE1*::Cm in the blunted *Kpn*I site	This study
pDrive-GST-*ptwE1*::Cm	pDrive-GST containing a blunted *Bam*HI/*Sac*I *ptwE1*::Cm fragment from pDrive-*ptwE1*::Cm in the blunted *Hind*III site	This study

Table 3.1. Continued

Plasmid	Relevant Characteristics[a, b]	Reference
pJQ200SK-*ptwE1*-GST	pJQ200SK containing a *Pst*I/*Sac*I *ptwE1*-GST fragment from pDrive-*ptwE1*-GST in the MCS site	This study
pJQ200SK-GST-*ptwE1*	pJQ200SK containing a *Pst*I/*Sac*I GST-*ptwE1* fragment from pDrive-GST-*ptwE1* in the MCS site	This study
pJQ200SK-*ptwE1*::Cm-GST	pJQ200SK containing a *Pst*I/*Sac*I *ptwE1*::Cm-GST fragment from pDrive-*ptwE1*::Cm-GST in the MCS site	This study
pJS200SK-GST-*ptwE1*::Cm	pJQ200SK containing a *Pst*I/*Sac*I GST-*ptwE1*::Cm fragment from pDrive-GST-*ptwE1*::Cm in the MCS site	This study

[a] Ap^r, Cm^r, Gm^r, Km^r, Tc^r, and Tp^r = resistance to ampicillin, chloramphenicol, gentamicin, kanamyacin, tetracycline, and trimethoprim, respectively. GST= glutathione *S*-transferase. + and – = positive and negative for phenotype, respectively.

Table 3.2. Bacterial strains used in Chapter III.

Strain	Relevant Characteristics[a, b]	Reference
Burkholderia cenocepacia		
K56-2	cystic fibrosis isolate, ptw$^+$	Sokol *et al.*, 1999
AE349	K56-2 *ptwD4*::Cm, ptw$^-$	This study
AE350	K56-2 *ptwE1*::Cm, ptw$^-$	This study
AE351	K56-2 *ptwE2*::Cm, ptw$^+$	This study
AE352	AE349 (pBCRLAR-Tp-*ptwD4*), ptw$^+$	This Study
AE353	AE350 (pBCRLAR-Tp-*ptwE1*), ptw$^+$	This study
AE354	K56-2 *ptwB4-lacZYA*, ptw$^+$	This study
AE355	K56-2 *ptwD4*::Cm; *ptwB4-lacZYA*, ptw$^-$	This study
AE356	K56-2 *ptwE1*::Cm; *ptwB4-lacZYA*, ptw$^-$	This study
AE357	K56-2 *ptwE1*-GST (C-terminal fusion), ptw$^-$	This study
AE358	K56-2 GST-*ptwE1* (N-terminal fusion), ptw$^+$	This study
AE359	K56-2 *ptwD4*::Cm; *ptwE1*::Cm-GST (C-terminal fusion), ptw$^-$	This study
AE360	K56-2 *ptwD4*::Cm; GST-*ptwE1*::Cm (N-terminal fusion), ptw$^-$	This study
AE361	K56-2 *ptwE1*::Cm-GST (C-terminal fusion), ptw$^-$	This study
AE362	K56-2 GST-*ptwE1*::Cm (N-terminal fusion), ptw$^-$	This study
Escherichia coli		
DH5αMCR	F– *mcr* AΔ (*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1*rec*A1 *deo*R *thi*-1 *sup*E44λ–*gyr*A96 *rel*A1	Life Technologies, Inc. (Gaithersburg, MD)
ED8654	Met$^-$	Borck *et al.*, 1976
HB101	F$^-$, *recA13*	Boyer and Roulland-Dussoix, 1969

[a] Tp^r and Cm^r= resistance to trimethoprim and chloramphenicol, respectively. Ptw= plant tissue watersoaking phenotype. GST= glutathione *S*-transferase. + and – = positive and negative for phenotype, respectively.

Effector identification and partial purification

Culture supernatants were obtained as previously described (Chapter II). Briefly, cultures of strain K56-2 were grown at 37ºC and harvested at late exponential phase by centrifugation. Supernatants (1 L) were filter sterilized and concentrated by ultrafiltration using a membrane with a 10 kDa cut-off. The protein content of the supernatant concentrates was determined by the method of Koch and Putnam (1971). Supernatant concentrates (30 ml) were separated by preparative isoelectric focusing (IEF), using a Rotofor Cell (Bio-Rad Laboratories), and a pH 3-11 ampholyte (Fluka) gradient. The resulting fractions (1-2 ml) were dialyzed against 0.05 M Tris pH 7.0 and tested for plasmolytic activity using the carrot protoplast assay as previously described (Chapter II). Active fractions (pH 9-11) were combined and subjected to a second IEF separation using a narrow pH 7-11 ampholyte gradient. The active fractions were dialyzed against 0.05 M Tris pH 7.0, and tested for plasmolytic activity, and quantified for protein concentration. Samples were concentrated 10X using a speed vac (Savant), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized using a non-fixing sliver stain (Blum *et al.*, 1987).

Activity testing of IEF fractions

Protoplasts were obtained as described in Chapter II (Jones *et al.*, 1990). Briefly, the epidermis of young surface sterilized carrots was resected with a sterile scalpel and

discarded. Cortical and phloem tissues were obtained, diced into fragments and placed in a sterile petri dish containing 20 ml of a filter sterilized enzyme solution. Tissue was digested in the dark for 5 h at 28ºC with gentle shaking (50 rpm). The carrot tissue was strained through nylon mesh and centrifuged (825 x g for 5 min at 25ºC). The supernatant was removed using a pipette, and the protoplasts were washed with 20 ml of 10% mannitol by resuspending with gentle swirling followed by centrifugation. 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 using a hemacytometer (Hausser Scientific). On average, 1.8×10^6 protoplasts/ml were obtained.

The plasmolytic activity of culture supernatants was determined by using concentrates that were standardized to a protein concentration of 0.2 µg/ml in 10% mannitol. The standardized concentrates 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 4 h using a Zeiss inverted microscope. Each incubation duration was performed in duplicate in three independent experiments with an average of 50 protoplasts examined at 0, 2, and 4 h.

Plant tissue assay

Plant tissue watersoaking activity was determined as previously described in Chapter II. Briefly, pierced onion bulb scales were inoculated, in triplicate, with 10 µl of an aqueous suspension to yield a final concentration of ~10^6 CFU/scale. Sterile double distilled-deionized water was used as a negative control and strain AU1054 served as the positive control in all experiments. Scales were placed on a sheet of surface sterilized aluminum foil in containers layered with moistened paper towels, sealed, and incubated at 37ºC. Plant tissue watersoaking activity was assessed at 24, 48, and 72 h post-inoculation.

Triparental matings

Triparental matings were performed as previously described in Chapter II. Briefly, 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 x 15 mm LB agar petri dish. Following a 6 h incubation period, the cells from the mating and respective controls (e.g. donor, mobilizer, and recipient) were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 x g for 10 min at 5ºC). The bacterial pellets were suspended in phosphate buffer and dilution plated to VBG amended with 500 µg/ml of Cm and/or 500 µg/ml of Tp, depending on selection needed. Single colony

isolates of individual transconjugants were obtained by streaking on homologous selective media. Cultures were subjected to survey lysis followed by agarose gel electrophoresis (Gonzalez and Vidaver, 1979) to confirm plasmid transfer.

Site-directed mutagenesis

For construction of strains AE350 (K56-2 *ptwD4*::Cm) and AE351 (K56-2 *ptwE1*::Cm) (Table 3.2), products corresponding to *ptwD4* and *ptwE1,* were amplified by PCR using primer sets PD4 (PD4-1 and PD4-2), PE1 (PE1-1 and PE1-2), and PE2 (PE2-1 and PE2-2) (Table 3.3), respectively. Primer sets PD4, PE1, and PE2, (Table 3.3) were designed based on sequence data from the *B. cenocepacia* Genome Project (http://www.sanger.ac.uk/Projects/B cenocepacia/). Each PCR reaction (50 µl) was performed using a Taq PCR Master Mix (USB) and a GeneAmp 2700 (Applied Biosystems). Reactions were run for 29 cycles and parameters were as follows: denaturation at 95ºC for 1 min; annealing at 55 ºC for 1 min for both primer sets PD4 and PE1 and annealing at 60°C for PE2; and extension at 72ºC for 1 min.

Resulting products were analyzed by agarose gel electrophoresis and cloned into pDrive (Qiagen), yielding pDrive-*ptwD4,* pDrive-*ptwE1,* and pDrive-*ptwE2*, respectively. Constructs pDrive-*ptwD4,* pDrive-*ptwE1,* and pDrive-*ptwE2*, were transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 40 µg/ml of Ap, 50 µM of isopropyl-beta-D-thiogalactopyranoside (IPTG), and 80 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix A). Specifically, *ptwD4* insertions were determined using *Sma*I, and *ptwE1* and *ptwE2* insertions were determined using *Cla*I. Sequencing was conducted by the Norman E. Borlaug Center for Southern Crop Improvement, Laboratory for Plant Genome Technology at Texas A&M University.

The Cm-resistance cassette, obtained from pBR325 digested with *Bsa*AI and *Bcl*I, was blunted with T4 Polymerase and inserted into the *Eco*RV, *Hae*III, and *Alu*I sites of pDrive-*ptwD4,* pDrive-*ptwE1*, and pDrive-*ptwE2,* respectively to yield pDrive-*ptwD4*::Cm, pDrive-*ptwE1*::Cm, and pDrive-*ptwE2*::Cm, respectively. Constructs pDrive-*ptwD4*::Cm, pDrive-*ptwE1*::Cm, and pDrive-*ptwE2*::Cm were transformed into

Table 3.3. Primers used in Chapter III.

Primer[a]	Gene	Relevant Characteristics	Reference
PD4-1	*ptwD4*	5'-TGACTCAGCGAAGGAA-3'	Chapter II
PD4-2	*ptwD4*	5'-ATCCGGTGGAAGCAA-3'	Chapter II
PE1-1	*ptwE1*	5’-AGCCTTCACCCGGTAAGGT-3’	This study
PE1-2	*ptwE1*	5’-CTAGGTAGGTGAAAATCC-3’	This study
PE2-1	*ptwE2*	5’-ACGTGCCGAGATTCTTCAT-3’	This study
PE2-2	*ptwE2*	5’-AGGTTCAGATCGACACCGAG-3’	This study
PB4-1	*ptwB4*	5’-AACTCAAGATGAATCAT-3’	Chapter II
PB4-2	*ptwB4*	5’-AGAACGCATGGTGTTGA-3’	Chapter II
GST-1	*GST*	5’-ATGTCCCCTATACTAGGTTA-3’	This study
GST-2	*GST*	5’-TCAGTCACGATGCGGC-3’	This study

[a] 1=forward primer; 2=reverse primer

E. coli DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 40 μg/ml of Ap and 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix B). Specifically, *ptwD4*::Cm, *ptwE1*::Cm, and *ptwE2*::Cm insertions were determined using *Sca*I. Plasmids pDrive-*ptwD4*::Cm, pDrive-*ptwE1*::Cm, and pDrive-*ptwE2*::Cm were digested with *Bam*HI/*Sal*I to yield *ptwD4*::Cm, *ptwE1*::Cm, and *ptwE2*::Cm fragments, which were then ligated to pJQ200SK digested with the same restriction enzymes to yield pJQ200SK-*ptwD4*::Cm, pJQ200SK-*ptwE1*::Cm, and pJQ200SK-*ptwE2*::Cm, respectively. Plasmids pJQ200SK-*ptwD4*::Cm, pJQ200SK-*ptwE1*::Cm, and pJQ200SK-*ptwE2*::Cm were then transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 10 μg/ml of Gm and 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix C). Specifically, directionality of *ptwD4*::Cm insertions were determined using *Sal*I, and *ptwE1*::Cm and *ptwE2*::Cm insertions were determined using *Eco*RI. Plasmids pJQ200SK-*ptwD4*::Cm, pJQ200SK-*ptwE1*::Cm, and pJQ200SK-*ptwE2*::Cm were individually mobilized into K56-2 by utilizing pRK2013 in triparental matings to obtain allelic exchange mutants. Exchange mutants were selected on VBG amended with 500 μg/ml of Cm. Cm-resistant transconjugants were single colony purified and retested for Cm^r. Allelic exchange and loss of the suicide vector was confirmed by plasmid survey lysis. Single colony isolates of insertionally inactivated *ptwD4* and *ptwE1*, designated AE349, AE350, and AE351 (Table 3.2), respectively,

were tested for the ptw phenotype. Insertional inactivation of *ptwD4* and *ptwE1* in strains AE349 and AE350, respectively were confirmed by restriction enzyme digestion of genomic DNA with *Bam*HI, *Bam*HI/*Eco*RI, *Bam*HI/*Sca*I, and *Bam*HI/*Not*I followed by agarose gel electrophoresis and Southern blotting analysis using probes for the Cm^r-cassette, *ptwD4* and *ptwE1* (Appendix D).

Complementation of disrupted genes

Complementation of the disrupted *ptwD4* or *ptwE1* was accomplished *in trans*. Plasmid pBCRLAR (Appendix E) (LiPuma, unpublished) was amended by inserting a Tp-resistance cassette, obtained from R388 as a *Bam*HI fragment, blunted with T4 polymerase, into the *Sca*I site in the Cm^r cassette to obtain pBCRLAR-Tp. Plasmids pDrive-*ptwD4* and pDrive-*ptwE1* were digested with *Bam*HI/*Sac*I, to yield *ptwD4* or *ptwE1* fragments, which were then ligated to pBCRLAR-Tp digested with the same to yield pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1*, respectively. Plasmids pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1* were then transformed into *E. coli* ED8654, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on VBG agar amended with 50 μg/ml of Tp and 0.5 mM final concentration of methionine. Plasmids pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1* were then individually mobilized into AE349 or AE350, respectively to complement insertionally disrupted *ptw* genes. Chloramphenicol resistant and Tp^r transconjugants were single colony purified and retested for antibiotic resistance and designated AE352 and AE353

(Table 3.2), respectively. Plasmid survey lysis and PCR were used to confirm plasmid transfer, which was reflected by the presence of both the resident and complement plasmid as well as inserted and uninserted PCR products.

To assess the stability of the constructed plasmids (pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1*) in the absence of antibiotic selection, cultures (AE352 and AE353) were grown in TNB (initial O.D.= 0.05 at 600nm) and the generation time was followed spectrophotometrically. Serial transfers of the cultures were made to obtain actively growing cells for 30 generations. Samples were plated at 0, 10, 20, and 30 generations on selective (VBG amended with 500 μg/ml of Cm and 500 μg/ml of Tp) and nonselective media (VBG). The stability of each plasmid was determined by comparing the numbers of colonies present on selective and nonselective plates. Means from two independent experiments were calculated.

Construction of LacZYA and GST fusion proteins

For construction of strains AE354 (K56-2-*ptwB4-lacZYA*), AE355 (K56-2-*ptwD4*::Cm; *ptwB4-lacZYA*), and AE356 (K56-2-*ptwE1*::Cm; *ptwB4-lacZYA*) (Table 3.2), a fragment corresponding to *ptwB4* was amplified by PCR using primer set PB4 (PB4-1 and PB4-2) (Table 3.3) and cloned into pCR2.1 (Invitrogen), yielding pCR2.1-*ptwB4*. The *ptwB4* fragment was excised by digestion using *Xba*I and *Sma*I and ligated to pFUSE (Baümler *et al.*, 1996) digested with the same enzymes to yield pFUSE-*ptwB4*. Plasmid pFUSE-*ptwB4* was then transformed into *E. coli* DH5αMCR, by chemical transformation

(Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion with *Sal*I and sequencing analyses (Appendix F). Plasmid pFUSE-*ptwB4* was individually mobilized into K56-2, AE349, and AE350, and recombinants were designated AE354, AE355, and AE356, respectively, and tested for the ptw phenotype. Integration of LacZYA fusions were confirmed by PCR and Southern blot analysis.

To obtain the GST cassette, a fragment corresponding to bp 258 to 977 of pGEX4T-1 was amplified using primer set GST (GST-1 and GST-2) (Table 3.3). Each PCR reaction (50 μl) was performed using a Taq PCR Master Mix (USB) and a GeneAmp 2700 (Applied Biosystems). Reactions were run for 29 cycles and parameters were as follows: denaturation at 95ºC for 1 min; annealing at 57 ºC for 1 min for primer set GST; and extension at 72ºC for 1 min. Resulting products were analyzed by agarose gel electrophoresis and cloned into pDrive (Qiagen), yielding pDrive-GST. Plasmid pDrive-GST was transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 40 μg/ml of Ap, 50 μM of isopropyl-beta-D-thiogalactopyranoside (IPTG), and 80 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Directionality of the insertion was determined by restriction enzyme digestion with *Bam*HI and sequencing analyses (Appendix G). Sequencing was conducted by the Norman E. Borlaug Center for Southern Crop Improvement, Laboratory for Plant Genome Technology at Texas A&M University.

Fragments corresponding to *ptwE1* and *ptwE1*::Cm were obtained using *Bam*HI/*Sac*I from pDrive-*ptwE1* and pDrive-*ptwE1*::Cm, respectively. A *ptwE1* fragment was blunted with T4 polymerase and ligated individually to pDrive-GST digested with *Hind*III or *Kpn*I and then blunted with T4 polymerase, yielding pDrive-*ptwE1*-GST and pDrive-GST-*ptwE1*. A *ptwE1*::Cm fragment was blunted with T4 polymerase and ligated individually to pDrive-GST digested with *Hind*III or *Kpn*I and then blunted with T4 polymerase, yielding pDrive-*ptwE1*::Cm-GST and pDrive-GST-*ptwE1*::Cm. Directionality of the insertion was determined by restriction enzyme digestion with *Bsa*AI/*Xba*I and sequenced to confirm in frame fusion of GST with *ptwE1* or *ptwE1*::Cm (Appendix H).

Plasmids pDrive-*ptwE1*-GST, pDrive-GST-*ptwE1*, pDrive-*ptwE1*::Cm-GST, and pDrive-GST-*ptwE1*::Cm were digested with *Eco*RI to yield *ptwE1*-GST, GST-*ptwE1*, *ptwE1*::Cm-GST, and GST-*ptwE1*::Cm fragments, blunted with T4 polymerase, ligated to pJQ200SK digested with *Sma*I to yield pJQ200SK-*ptwE1*-GST, pJQ200SK-GST-*ptwE1*, pJQ200SK-*ptwE1*::Cm-GST, and pJQ200SK-GST-*ptwE1*::Cm, respectively. Plasmids pJQ200SK-*ptwE1*-GST, pJQ200Sk-GST-*ptwE1*, pJQ200SK-*ptwE1*::Cm-GST, and pJQ200SK-GST-*ptwE1*::Cm were then transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 10 μg/ml of Gm and/or 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion with *Cla*I/*Xba*I and sequencing analyses (Appendix I).

Plasmids pJQ200SK-*ptwE1*-GST and pJQ200SK-GST-*ptwE1* were individually mobilized into K56-2 and AE50 (K56-2 *ptwD4*::Cm) by utilizing pRK2013 in triparental matings. Transconjugants were selected on VBG or VBG amended with 500 μg/ml of Cm, respectively. Transconjugants were single colony purified and loss of the suicide vector was confirmed by PCR and plasmid survey lysis (Gonzalez and Vidaver, 1979). Single colony isolates were designated AE357 (K56-2 *ptwE1*-GST), AE358 (K56-2 GST-*ptwE1*), AE359 (K56-2 *ptwD4*::Cm; *ptwE1*-GST), and AE360 (K56-2 *ptwD4*::Cm; GST-*ptwE1*), respectively, and tested for the ptw phenotype. Plasmids pJQ200SK-*ptwE1*::Cm-GST and pJQ200SK-GST-*ptwE1*::Cm were individually mobilized into K56-2 by utilizing pRK2013 in triparental matings. Transconjugants were selected on VBG amended with 500 μg/ml of Cm. Cm-resistant transconjugants were single colony purified and loss of the suicide vector was confirmed by plasmid survey lysis (Gonzalez and Vidaver, 1979). Single colony isolates were designated AE361 (K56-2 *ptwE1*::Cm-GST), and AE362 (K56-2 GST-*ptwE1*::Cm), respectively, and tested for the ptw phenotype.

β-galactosidase assay

β-galactosidase assays were carried out with whole cells as described by Sambrook *et al.* (1989) with the modification that the reaction mixtures were centrifuged before the determination of A_{420}. β-galactosidase activity was expressed as Miller Units (A_{420}/volume). Minimal background β-galactosidase activity was detected from strain K56-2. Each β-galactosidase experiment was performed in triplicate in three independent experiments. Data were expressed as average Miller Units ± SD.

Detection of GST fusion proteins

Burkholderia cenocepacia cultures inoculated in 50 ml of M9-glucose to a starting optical density of 0.05 at 425 nm were incubated at 37ºC with shaking at 200 rpm for 20 h. Bacteria were pelleted and resuspended in 1× Laemmli sample buffer at a concentration of 10^{10} CFU/ml. Culture supernatants were lyophilized and resuspended in 1ml of 1× Laemmli sample buffer. Both bacterial pellet and supernatants were heated at 100ºC for 5 min, and the total protein from 10^8 CFU (0.01 ml) or 10 µl of 50× supernatant was separated by SDS-PAGE (Laemmli, 1970). Proteins were transferred to polyvinylidene difluoride membranes by electro-blotting and probed with polyclonal goat antibody to GST (Amersham Biosciences). The bound antibody was detected by using anti-goat IgG conjugated to horseradish peroxidase and HyGlo chemiluminescent HRP antibody detection reagent (Denville) using HyBlot CL autoradiography film.

RESULTS

Identification of putative effector(s)

Analysis of 10-kb regions flanking the Ptw gene cluster of *B. cenocepacia* K56-2 identified an ORF, designated *ptwE1* (Fig. 3.1). Translation of *ptwE1* sequence predicted a protein with a molecular mass of 14 kDa, and showed no significant sequence similarity to known proteins by BLAST analysis. An isoelectric point (PI) calculated using the ProtParam Tool (http://www.expasy.org/cgi-bin/protparam) was 11.6.

Previous studies indicate that an effector protein(s) is translocated by the Ptw type IV secretion system into the extracellular milieu (Engledow *et al.*, 2004). Proteins in concentrated *B. cenocepacia* K56-2 broth culture supernatants were separated by preparative IEF, which in the presence of a broad ampholyte gradient (pH 3-11) yielded three fractions with plasmolytic activity ($P>0.05$) corresponding to a PI ranging from 9-ll. To obtain further separation, an additional IEF, using a narrow ampholyte gradient (pH 7-11), was performed on the combined plasmolytically active fractions. Two fractions exhibited significantly more plasmolytic activity compared to controls or other fractions ($P> 0.05$). Isoelectric points of plasmolytically active fractions approximately correspond to the predicted PI of PtwE1.

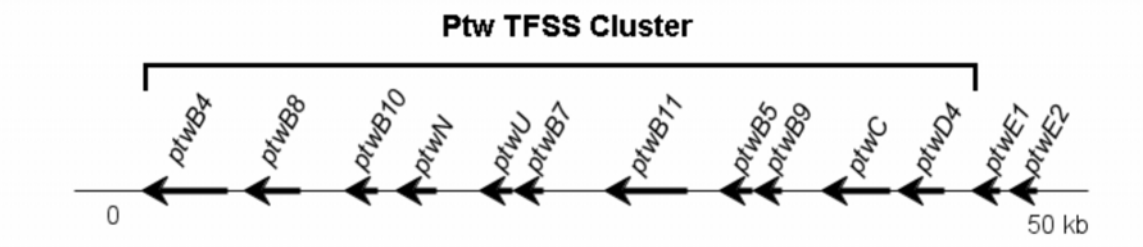

Fig. 3.1. A 50 kb segment from plasmid pK56-2 containing the Ptw type IV secretion system (TFSS) cluster and effector ORFs.

Since recent work indicates that effectors translocated by type IV secretion systems contain a conserved positively charged C-terminal signal that is necessary for translocation (Simone *et al.*, 2001, Vergunst *et al.*, 2000; Vergunst *et al.*, 2003; Vergunst *et al.*, 2005) we analyzed the C-terminal region of PtwE1 for amino acid content, net charge, and consensus sequences. The incidence of Arg residues among the C-terminal 20 amino acids for PtwE1, was 20%, which is higher than expected for the Arg composition of *B. cenocepacia* proteins (~7.2%), and the calculated net charge was +3. Alignment of the 30 C-terminal amino acids of PtwE1 showed similarity in position of Arg residues to known type IV secreted proteins (Fig. 3.2). One additional ORF, designated *ptwE2,* which was positioned upstream of the Ptw system, was identified; however, the C-terminal signal did not show homologous Arg residue positioning nor a significant increase in incidence of Arg residues, and an insertional mutation had no effect on the ptw phenotype.

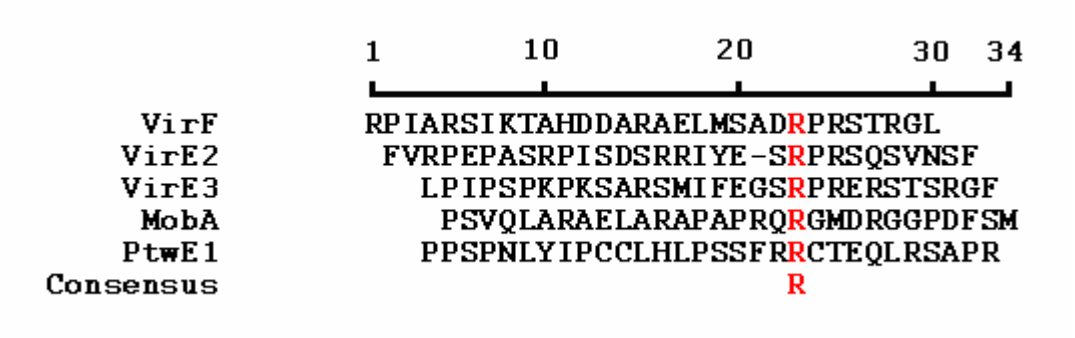

Fig 3.2. Alignment of the C-terminal amino acids of *A. tumefaciens* VirE2, VirE3, and VirF; RSF1010 MobA; and *B. cenocepacia* effector protein PtwE1.

Site-directed mutagenesis of Ptw effector and effect on the ptw phenotype

By combining site-directed mutagenesis of *ptwD4* and *ptwE1* with cytotoxic assays employing both plant tissue and protoplasts, we determined that effector protein PtwE1 was involved in production of the ptw phenotype. Complementation *in trans* of the mutants resulted in restoration of the ptw phenotype (Fig. 3.3). Stability testing of strains AE352 and AE353 showed that pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1* were stable for at least 30 generations under non-selective conditions, with an average retention rate of 89 % (Table 3.4). In order to determine whether an insertional mutation in either *ptwD4* or *ptwE1* exerted a polar effect on downstream *ptw* genes, strains containing a LacZYA fusion downstream of *ptwB4* [AE354 (K56-2 *ptwB4-lacZYA*), AE355 (K56-2 *ptwD4*::Cm; *ptwB4-lacZYA*), and AE356 (K56-2 *ptwE1*::Cm; *ptwB4-lacZYA*)] were constructed. Insertional mutations in either *ptwD4* or *ptwE1* did not result in any downstream affects as determined by β-galactosidase activity (Fig. 3.4). Strain AE354 (K56-2 *ptwB4::lacZYA*) remained capable of producing the ptw phenotype on onion tissue.

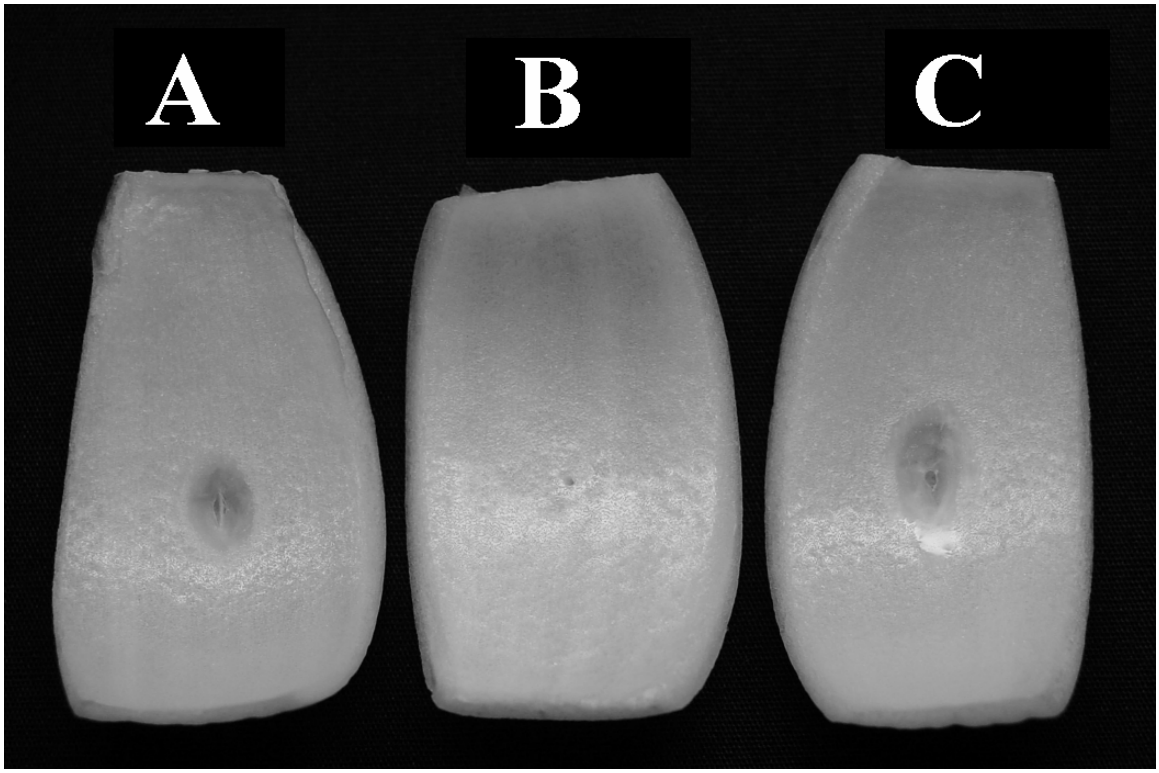

Fig. 3.3. Analysis of PtwE1 using the plant tissue watersoaking assay. (A) Pierced onion bulb scale inoculated with *B. cenocepacia* strain K56-2 showing partial tissue collapse and translucence characteristic of the plant tissue watersoaking (ptw) phenotype. (B) Pierced onion bulb scale inoculated with ptw-negative mutant (AE349 or AE350). (C) Pierced onion bulb scale inoculated with a complemented ptw-negative mutant (AE352 or AE353).

Table 3.4. Stability testing of plasmids pBCRLAR-Tp-*ptwD4* and pBCRLAR-Tp-*ptwE1* in complemented strains AE352 and AE353 of *ptwD4* and *ptwE1* mutant strains AE350 and AE351, respectively.

	% Plasmid Containing CFU [a, b]	
	Strain	
Generations	**AE352**	**AE353**
0	100	100
10	98	97
20	95	94
30	89	89

[a] Percent plasmid containing CFU are the average for two independent experiments.
[b] All strains were grown in TNB medium at 37°C while shaking.

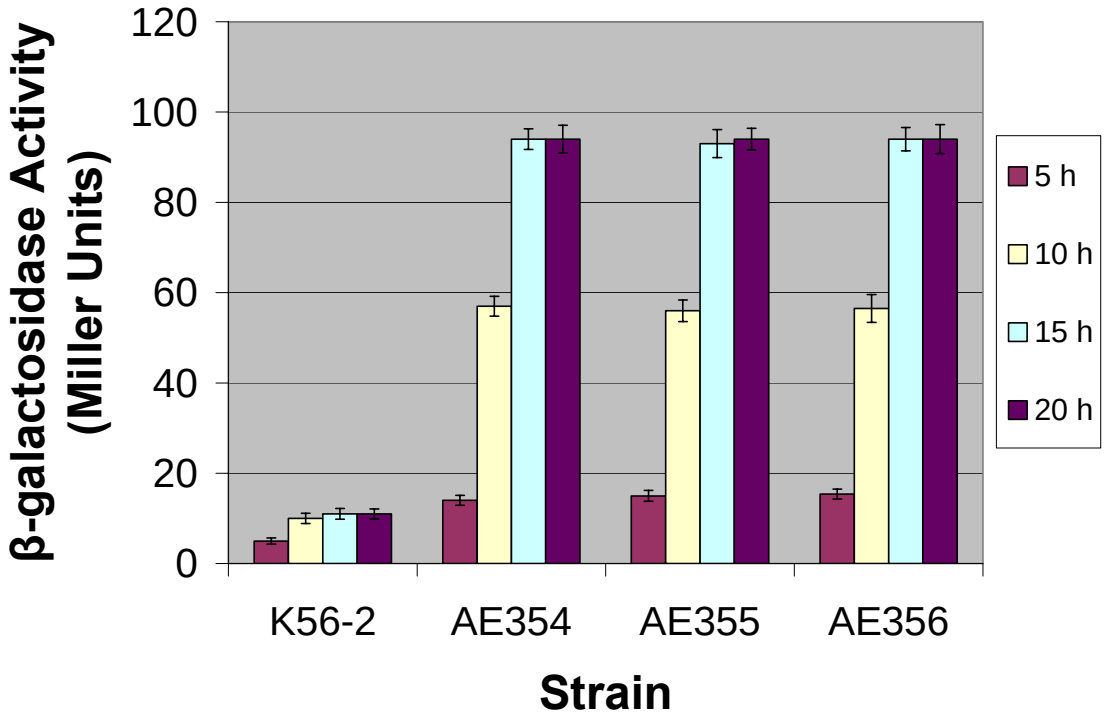

Fig 3.4. β-galactosidase activity of K56-2, AE354 (K56-2 *ptwB4-lacZYA*), AE355 (K56-2 *ptwD4*::Cm; *ptwB4-lacZYA*), and AE355 (K56-2 *ptwE*1::Cm; *ptwB4-lacZYA*). β-galactosidase activity of AE355 and AE256 is reflective of a nonpolar mutation. Data are expressed as the average of three independent experiments performed in triplicate ± SD.

Translocation of PtwE1 by the Ptw type IV secretion system

To further determine if the Ptw type IV secretion system was responsible for the translocation of PtwE1 into the extracellular milieu, and whether the C-terminal portion of PtwE1 was important for that translocation GST fusion strains AE357 (*ptwE1*-GST), AE358 (GST-*ptwE1*), AE359 (*ptwD4*::Cm; *ptwE1*-GST), AE360 (*ptwD4*::Cm; GST-*ptwE1*), AE361 (*ptwE1*::Cm-GST), and AE362 (GST-*ptwE1*::Cm) were evaluated for translocation of PtwE1. Results from GST experiments are presented in Table 3.5 and Figure 3.5. The GST fusion proteins were detectable in culture supernatants from strains AE358 and AE362, whereas they were not detectable in strains AE357, AE359, AE360, or AE362. The fusion proteins detected in supernatants of AE358 and AE362 were approximately 41 and 73 kDa, respectively, which is the predicted size for *ptwE1* (14kDa) fused with GST (27 kDa) and *ptwE1*::Cm (46 kDa) fused with GST (27 kDa). The GST fusion proteins were also detectable in bacterial pellets from strains AE357, AE358, AE359, AE360, AE361, and AE362. Strains AE357, AE358, AE359, and AE360 all showed a fusion protein of approximately 41 kDa, which is the predicted size for *ptwE1* (14 kDa) fused with GST (27 kDa), whereas strains AE361 and AE362 showed a fusion protein of about 73 kDa, which is the predicted size for *ptwE1*::Cm (46 kDa) fused with GST (27 kDa). Only strain AE358 remained capable of producing the ptw phenotype on onion tissue.

Table 3.5. GST fusion protein detection in bacterial culture supernatants and pellets. Strains AE357 (*ptwE1*-GST), AE358 (GST-*ptwE1*), AE359 (*ptwD4*::Cm; *ptwE1*-GST), AE360 (*ptwD4*::Cm; GST-*ptwE1*), AE361 (*ptwE1*::Cm-GST), and AE362 (GST-*ptwE1*::Cm) were used for GST fusion studies.

Strain	Strain Description	GST Fusion Detected in Supernatant	GST Fusion Detected in Pellet	Ptw Activity
AE357	*ptwE1*-GST (C-terminal fusion)	No	Yes	No
AE358	GST-*ptwE1* (N-terminal fusion)	Yes	Yes	Yes
AE359	*ptwD4*::Cm; *ptwE1*-GST (C-terminal fusion)	No	Yes	No
AE360	*ptwD4*::Cm; GST-*ptwE1* (N-terminal fusion)	No	Yes	No
AE361	*ptwE1*::Cm-GST (C-terminal fusion)	No	Yes	No
AE362	GST-*ptwE1*::Cm (N-terminal fusion)	Yes	Yes	No

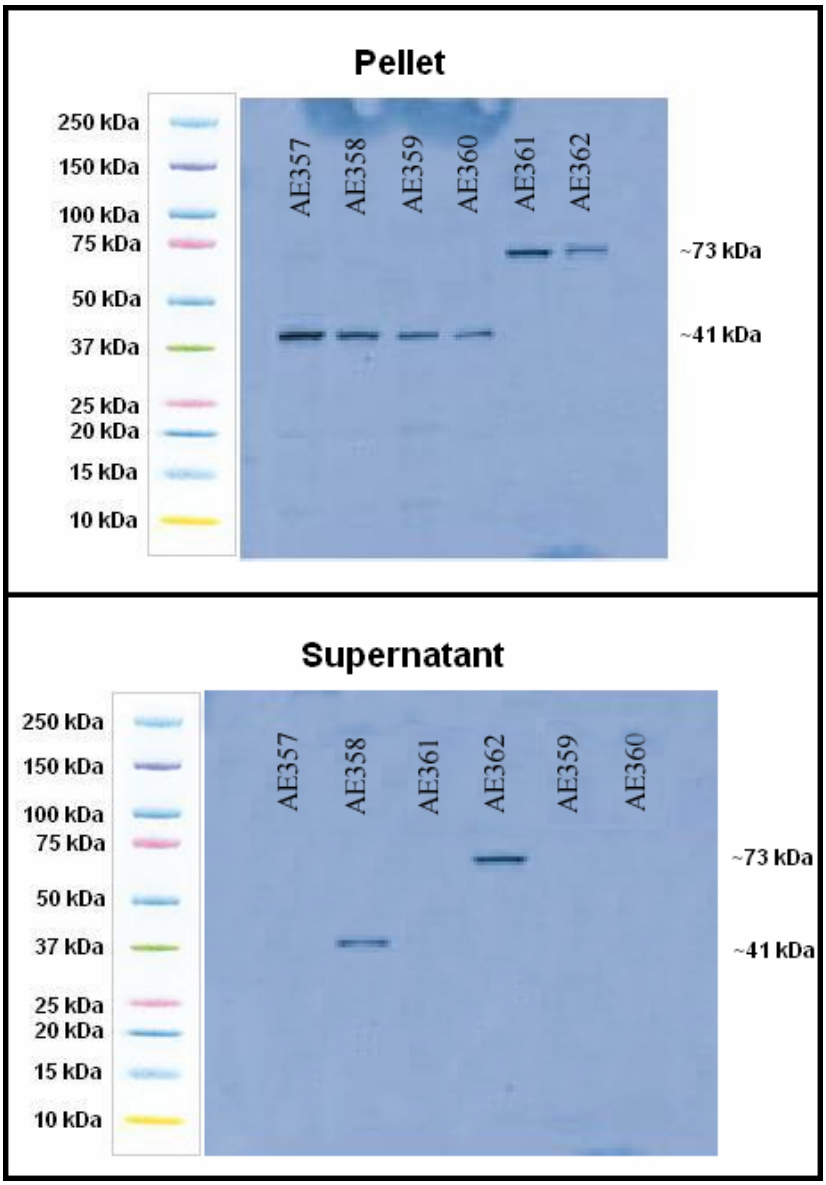

Fig. 3.5. Western blot analysis of supernatants and pellets from GST fusion strains. AE357 (*ptwE1*-GST), AE358 (GST-*ptwE1*), AE359 (*ptwD4*::Cm; *ptwE1*-GST), AE360 (*ptwD4*::Cm; GST-*ptwE1*), AE361 (*ptwE1*::Cm-GST), and AE362 (GST-*ptwE1*::Cm) were used for Southern blot analysis. Molecular masses of PtwE1 and PtwE1::Cm are 14 and 46 kDa, respectively; and molecular mass of GST is 27 kDa.

DISCUSSION

Previously, two type IV secretion systems were identified in *B. cenocepacia* of the epidemic ET12 lineage; one system is plasmid encoded (designated the Ptw type IV secretion system), whereas the other is chromosomally encoded (designated the VirB/D type IV secretion system) and shows homology to the *A. tumefaciens* VirB/D4 type IV secretion system (Engledow *et al.*, 2004). The plasmid encoded Ptw system is a chimeric type IV secretion system composed of VirB/D4-like elements and F-specific subunits. This system is responsible for production of the plant disease-associated ptw phenotype and the translocation of Ptw effector protein(s). Studies in our laboratory indicate that this phenotype is expressed by all *B. cenocepacia* strains included in the Bcc experimental strain panel described by Mahenthiralingam *et al.* (2000b). Studies on the chromosomally encoded VirB/D system using mutational analysis indicated that this system is not involved in production of the ptw phenotype.

Most bacterial protein secretion systems recognize their substrates through a signal in the N terminus. For example, there is a clear consensus in the cleavable N-terminal signal peptide sequences recognized by the Sec-dependent and twin Arg translocation systems (Dilks *et al.*, 2003; Cao and Saier, 2003). However, to date no consensus has been found to define the N-terminal signal of effectors secreted by type III or type IV secretion systems. Recent studies of *A. tumefaciens* and *Bartonella henselae* type IV secretion systems have shown that the interaction between the C-termini of effector proteins and VirD4 is a critical step in effector translocation. In these studies, a

positively charged C-terminal signal was present in several type IV secreted proteins including the VirE2 effector of *A. tumefaciens* and the MobA relaxase of RSF1010 (Vergunst *et al.*, 2005). In this study two ORFs designated *ptwE1* and *ptwE2* were identified that had no known homologs by BLAST analysis. The C-terminus of PtwE1 was positively charged and showed a higher incidence of Arg residues than expected for *B. cenocepacia* proteins, and an alignment of the 30 C-terminal amino acids showed similarity in the position of these residues. In contrast, Ptw2 did not show homologous Arg residue positioning, or a significant increase in incidence of Arg residues. Site-directed mutagenesis indicated that PtwE1 was involved in the expression of the ptw phenotype, whereas PtwE2 was not.

Studies utilizing GST fusion proteins showed that PtwE1 was translocated by the Ptw type IV secretion system and that the positively charged C-terminal region of PtwE1 is important for translocation. Strains with N-terminal fusions of GST to PtwE1 and a functioning type IV secretion system were able to translocate the GST-PtwE1 or GST-PtwE1-Cm to the extracellular milieu, whereas strains with the same fusions but a non-functional type IV secretion system coupling protein (PtwD4) did not. Strains with a C-terminal fusion of GST to PtwE1 were not able to translocated PtwE1 regardless of whether or not there was a functional type IV secretion system. However, GST fusions were detected in all cellular fractions of GST fusion strains showing that GST fusion proteins were being produced but not exported. A C-terminal GST fusion to PtwE1 (in the presence of a functioning type IV secretion system) resulted in loss of the ptw phenotype, since PtwE1 was no longer being translocated by the type IV secretion

system. Accumulating evidence suggests that it is the coupling protein that recruits both the protein substrates and the nucleoprotein complex to the type IV secretion system (Llosa and O'Callaghan, 2004; Cascales and Christie, 2004; Atmakuri *et al.*, 2003; Llosa *et al.*, 2002; Escudero *et al.*, 2003; Szpirer *et al.*, 2000). Cascales and Christie (2004) recently demonstrated that the coupling protein VirD4 is the first component of the type IV secretion system to interact with the T-DNA/VirD2 transfer intermediate. The VirE2 protein interacts at the cell poles of the bacterium with VirD4 (Atmakuri *et al.*, 2003), strongly supporting the model that VirD4 is the cytoplasmic component of the type IV secretion system that sorts nucleoprotein complexes and effector proteins for translocation and that type IV secretion systems are actually committed protein translocation systems (Regensburg-Tuïnk and Hooykaas, 1993; Llosa *et al.*, 2002). Translocation of the effector proteins VirF, VirE2, and VirE3 is also mediated by a C-terminal transport signal (Vergunst *et al.*, 2000; Atmakuri *et al.*, 2004; Vergunst *et al.*, 2003; Simone *et al.*, 2001); and recent studies show a correlation between a positively charged C-terminus and transport function (Vergunst *et al.*, 2005).

In conclusion, this study (Chapter III) has identified an effector protein which is involved in the production of the ptw phenotype in *B. cenocepacia* strain K56-2. The effector was also shown to be translocated by the previously identified (Chapter II) Ptw type IV secretion system. Mutational analysis of PtwE1 indicates that it may play a role in expression of the ptw phenotype. GST fusion strains indicate that PtwE1 is translocated into the extracellular milieu via the Ptw type IV secretion system and that the positively charged C-terminal portion of PtwE1 is involved in the translocation

process. Future studies using the bimolecular fluorescence complementation assay (Atmakuri *et al.*, 2003) will elucidate the interaction between PtwE1 and PtwD4.

CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF A GROUP IVA TYPE IV SECRETION SYSTEM AND EFFECTOR IN *Burkholderia cenocepacia* OF THE PHDC LINEAGE

INTRODUCTION

Organisms belonging to the *Burkholderia cepacia* complex (Bcc) can cause life threatening infections in patients with cystic fibrosis (CF) (Govan and Deretic, 1996; LiPuma, 1998). Although Bcc organisms only infect a relatively small proportion of these patients, they have a significant impact on survival and numerous reports have provided evidence for patient to patient spread and nosocomial transmission (Coenye and LiPuma, 2003). Isolates belonging to all nine Bcc species have been recovered from the sputum of patients with CF, but recent large scale surveys in several countries (Coenye and Vandamme, 2003) have shown that, although there are national differences, most patients are infected with *B. cenocepacia* (Vandamme *et al.*, 2003). Several epidemic *B. cenocepacia* lineages have been described, including the ET12 (Johnson *et al.*, 1994), PHDC (Chen *et al.*, 2001), and Midwest (Kumar *et al.*, 1997). Isolates belonging to the transatlantic ET12 lineage infect many patients in Canada and the UK, whereas isolates belonging to the PHDC lineage appear to be dominant among CF patients in the mid-Atlantic region of the USA (Chen *et al.*, 2001) and has also been reported in Europe (Coenye *et al.*, 2004). Molecular epidemiological studies have shown

that *B. cenocepacia* strains of the PHDC lineage can be isolated from agricultural soil in New York State (LiPuma *et al.,* 2002) and is much more commonly found in CF patients in the USA than was initially appreciated (Liu *et al.*, 2003). Isolates of the Midwest lineage are found in the Midwest region of the USA (Kumar *et al.*, 1997).

Several putative virulence determinants for *B. cenocepacia* including hemolysins (Hutchinson *et al.*, 1998; Vasil *et al.*, 1990), siderophores (Sokol, *et al.*, 1999), cable pili (Sajjan *et al.*, 2000a), and membrane active effector protein(s) (Engledow *et al.*, 2004), have been reported. *Burkholderia cenocepacia* strain K56-2, is part of the clinically problematic ET12 lineage. The strain produces plant tissue watersoaking (ptw) on onion tissue, which is a plant disease-associated trait (Engledow *et al.*, 2004). In Chapter II a plasmid-encoded type IV secretion system was identified, in strain K56-2, that is functionally an effector translocator and responsible for expression of the ptw phenotype; a chromosomally-encoded type IV secretion system was also identified for which the function is at this time is unknown (Engledow *et al.*, 2004). It has been determined that the ptw phenotype is common among isolates belonging to *B. cenocepacia* of distinct lineages. In this study (Chapter IV) it was determined that

isolates representative of the PHDC lineage contain a VirB/D-like type IV secretion system, designated BcVirB/D, and at least one putative effector that appears to be involved in the expression of the ptw phenotype. The BcVirB/D type IV secretion system was found to not only function as an effector translocator, but also as a conjugation system. Mutational analysis of the BcVirB/D system indicates that the BcVirB2 and BcVirD4 play a functional role in expression of the ptw phenotype and DNA transfer. Preliminary characterization of the transfer function indicates that the oncogenic suppression activity protein (Osa) can disrupt transfer of proteins via the BcVirB/D system.

EXPERIMENTAL PROCEDURES

Media and growth conditions

Descriptions of plasmids and bacterial strains used in this study are listed in Tables 4.1 and 4.2, respectively. Luria Bertanni (LB) medium was used for routine maintenance of

cultures. Minimal medium Vogel Bonner (Vogel and Bonner, 1965) amended with 1.0% glucose (VBG) was used in mating experiments. Minimal medium M9 (Sambrook *et al.*, 1989) containing 1.0% glucose was used for culturing bacteria for supernatant analysis. Stability testing of pBCRLAR derivatives was accomplished using tryptone nutrient broth (TNB) (Hansen and Olsen, 1978) for liquid culture and VBG amended with 500 μg/ml of trimethoprim (Tp) and 500 μg/ml tetracycline (Tc) or VBG for selective and non-selective plating. *Burkholderia cenocepacia* and *Escherichia coli* strains were grown at 37ºC. Media were supplemented with appropriate concentrations of antibiotics as needed for selection. Antibiotics were added to media at the following concentrations: 500 μg/ml of chloramphenicol (Cm), 500 μg/ml of trimethoprim (Tp), and 500 μg/ml of naldixic acid (Nal) for selection and maintenance of *B. cenocepacia* and *B. multivorans* isolates and derivatives; and 10 μg/ml tetracycline (Tc), 10 μg/ml gentamicin (Gm), 40 μg/ml ampicillin (Ap), and 100 μg/ml Tp for selection and maintenance of *E. coli*.

Table 4 .1. Plasmids used in Chapter IV.

Plasmid	Relevant Characteristics[a, b]	Reference
pDrive	Cloning Vector: Km^r, Ap^r, pUC origin, T7, SP6, *lac*$Z\alpha^+$	Qiagen
pRK2013	Km^r, Tra^+, Mob^+, ColE1 replicon	Figurski and Helinski, 1979
pBR325	Tc^r, Ap^r, Cm^r, ColE1 replicon	Blank and Wilson, 1982
pR388	IncW, Su^r, Tp^r	Datta and Hedges, 1972
pJQ200SK	Gm^r, *sac*B, Mob^+, p15A replicon	Quandt and Hynes, 1993
pBCRLAR	Cm^r, Mob^+, *lac*$Z\alpha^+$, pBBR1 replicon	LiPuma, University of Michigan
pML122	Gm^r, Km^r, IncQ, Mob^+, p15A replicon	Labes *et al.*, 1990
pKA165	$Carb^r$, *virB* promoter, *osa*	Berger and Christie, 1994
pURF047-Tp	pURF047 with 2.0 kb Tp^r cassette inserted at *Sca*I site of Ap^r cassette	Chapter II
pBCRLAR-Tp	pBCRLAR containing a blunted Tp^r cassette inserted at *Sca*I site of Cm^r cassette	This study
pDrive-*bcvirB2*	pDrive containing a *bcvirB2* PCR product	This study
pDrive-*bcvirD4*	pDrive containing a *bcvirD4* PCR product	This study
pDrive-*bcvirP3*	pDrive containing a *bcvirP3* PCR product	This study
pDrive-*bcvirB2*::Cm	pDrive-*bcvirB2* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *EcoR*V site of *bcvirB2*	This study
pDrive-*bcvirD4*::Cm	pDrive-*bcvirD4* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *Sca*I site of *bcvirD4*	This study
pDrive-*bcvirP3*::Cm	pDrive-*bcvirP3* containing a blunted *Bsa*AI/*Bcl*I Cm^r fragment from pBR325 in the *Bsa*AI site of *bcvirP3*	This study
pJQ200SK-*bcvirB2*::Cm	pJQ200SK containing a *Bam*HI/*Sac*I *bcvirB2*-Cm fragment from pDrive-*bcvirB2*::Cm in the MCS	This study
pJQ200SK-*bcvirD4*::Cm	pJQ200SK containing a *Kpn*I/*Hind*III *bcvirD4*-Cm fragment from pDrive-*bcvirD4*::Cm in the MCS	This study
pJQ200SK-*bcvirP3*::Cm	pJQ200SK containing a *Bam*HI/*Hind*III *bcvirP3*-Cm fragment from pDrive-*bcvirP3*::Cm in the MCS	This study
pBCRLAR-Tp-*bcvirB2*	pBCRLAR-Tp containing a *Bam*HI/*Sac*I *bcvirB2* fragment from pDrive-*bcvirB2* in the MCS	This study
pBCRLAR-Tp-*bcvirD4*	pBCRLAR-Tp containing a *Kpn*I/*Hind*III *bcvirD4* fragment from pDrive-*bcvirD4* in the MCS	This study
pBCRLAR-Tp-*bcvirP3*	pBCRLAR-Tp containing a *Bam*HI/*Hind*III *bcvirP3* fragment from pDrive-*bcvirP3* in the MCS	This study
pML122-Tp	pML122 containing a blunted *Bam*HI Tp^r cassette inserted at *Sma*I of the Gm^r cassette	This study
pURFO47-Tp-*osa*	pURF047-Tp containing blunted *Nde*I/*Xho*I *osa* fragment from pKA165 in the MCS (*Sma*I)	This study

[a] Ap^r, $Carb^r$, Cm^r, Gm^r, Km^r, Su^r, Tc^r, and Tp^r = resistance to ampicillin, carbenicillin, chloramphenicol, genamicin, kanamyacin, sulphonamide, tetracycline, and trimethoprim, respectively. + and – = positive and negative for phenotype, respectively.

Table 4.2. Bacterial strains used in Chapter IV.

Strain	Relevant Characteristics[a]	Reference
Burkholderia cenocepacia		
AU1054	CF respiratory isolate, PHDC lineage, ptw$^+$	LiPuma, J. J. (University of Michigan)
AE607	AU1054 *bcvirB2::*Cm, ptw$^-$	This study
AE608	AU1054 *bcvirD4::*Cm, ptw$^-$	This study
AE609	AU1054 *bcvirP3::*Cm, ptw$^-$	This study
AE610	AE607 (pBCRLAR-Tp-*bcvirB2*), ptw$^+$	This study
AE611	AE608 (pBCRLAR-Tp-*bcvirD4*), ptw$^+$	This study
AE612	AE609 (pBCRLAR-Tp-*bcvirP3*), ptw$^+$	This study
AE613	AU1054 (pURFO47-Tp)	This study
AE614	AE608 (pURFO47-Tp)	This study
AE615	AU1054 (pML122-Tp)	This study
AE616	AE608 (pML122-Tp)	This study
AE617	AU1054 (pURFO47-Tp-*osa*), ptw$^-$	This study
Burkholderia multivorans		
TL249-2	Soil isolate, plasmid-free, *lys*-2, Peh$^-$, ino$^+$, ita$^+$	Gaffney and Lessie, 1987
BCS11	TL249-2 (Nal^r)	This Study
Escherichia coli		
DH5αMCR	F-, *mcr*A, Δ (*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1*rec*A1 *deo*R *thi*-1 *sup*E44λ–*gyr*A96 *rel*A1	Life Technologies, Inc. (Gaithersburg, MD)
HB101	F$^-$, *recA13*	Boyer and Roulland-Dussoix, 1969
ED8654	Met$^-$	Borck *et al.*, 1976

[a] Nal^r, Tp^r, Cm^r= resistance to nalidixic acid, trimethoprim, and chloramphenicol, respectively. Ptw= plant tissue watersoaking phenotype. Peh = polygalacturonase phenotype. Ino and ita = ability to catabolize myo-inositol and itaconic acid. + and – = positive and negative for phenotype, respectively.

Plant tissue assay

Plant tissue watersoaking activity was determined as previously described in Chapter II. Briefly, pierced onion bulb scales were inoculated, in triplicate, with 10 µl of an aqueous suspension to yield a final concentration of ~10^6 CFU/scale. Sterile double distilled-deionized water was used as a negative control and strain AU1054 served as the positive control in all experiments. Scales were placed on a sheet of surface sterilized aluminum foil in containers layered with moistened paper towels, sealed, and incubated at 37ºC. Plant tissue watersoaking activity was assessed at 24, 48, and 72 h post-inoculation.

Activity testing of culture supernatants

Culture supernatants were obtained as previously described in Chapter II. Briefly, cultures of strain AU1054, AE608 (AU1054 *bcvirD4*::Cm), and AE611 [AU1054 *bcvirD4*::Cm (pBCRLAR-Tp-*bcvirD4*)] were grown in M9 medium at 37ºC and harvested at late exponential phase (A_{425} = 0.9) by centrifugation (16,000 x g, 15 min at 5ºC). Supernatants (1 L) were filter sterilized using 0.22 µM filters (Pall) and concentrated to 3 ml using an Amicon ultrafiltration stirred cell (MWCO 10,000). Protein concentration of supernatant concentrates was determined by the method of Koch and Putnam (1971). Concentrated uninoculated medium (1L) 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. Protoplasts were obtained as described in Chapter II (Jones *et al.*, 1990). Briefly, the epidermis of young surface sterilized carrots was resected with a sterile scalpel and discarded. Cortical and phloem tissues were obtained, diced into fragments and placed in a sterile petri dish containing 20 ml of a filter sterilized enzyme solution. Tissue was digested in the dark for 5 h at 28ºC with gentle shaking (50 rpm). The carrot tissue was strained through nylon mesh and centrifuged (825 x g for 5 min at 25ºC). The supernatant was removed using a pipette, and the protoplasts were washed with 20 ml of 10% mannitol by resuspending with gentle swirling followed by centrifugation. 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 using a hemacytometer (Hausser Scientific). On average, 1.8×10^6 protoplasts/ml were obtained.

The plasmolytic activity of supernatants was determined by using concentrates that were standardized to a protein concentration of 0.2 μg/ml in 10% mannitol. The standardized concentrates 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, proteinase K, RNase, and DNase on plasmolytic activity was tested as

previously described in Chapter II. 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 ± 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 (ANOVA). Statistical analysis was performed using SAS software and the general linear model procedure (SAS Institute Inc.).

Triparental and biparental matings

Triparental matings were performed as previously described in Chapter II. Briefly, 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 x 15 mm LB agar petri dish. Following a 6 h incubation period, the cells from the mating and respective controls (e.g. donor, mobilizer, and recipient) were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 x g for 10 min at 5ºC). The bacterial pellets were suspended in phosphate buffer and dilution plated to VBG amended with 500 µg/ml of Cm and/or 500 µg/ml of Tp, depending on selection needed. Single colony isolates of individual transconjugants were obtained by streaking on homologous

selective media. Cultures were subjected to survey lysis followed by agarose gel electrophoresis (Gonzalez and Vidaver, 1979) to confirm plasmid transfer.

For biparental matings, donor strains and recipient strain (BCS11) 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 (A600 = 0.5), mixed at an equal ratio (1:1), and transferred to a positively charged sterile membrane layered on a 100 x 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 x g for 10 min at 5ºC). The bacterial pellets were suspended in phosphate buffer and dilution plated to VBG amended with 0.5 mM final concentration of lysine and 500µg/ml of Tp. Single colony isolates of individual transconjugants were obtained by streaking on VBG amended with 0.05 mM final concentration of lysine and 500 µg/ml of Tp and/or 500 µg/ml Tc. DNA was isolated using a miniprep kit (Qiagen) followed by agarose gel electrophoresis to confirm plasmid transfer.

Site-directed mutagenesis

For construction of strains AE607 (AU1054 *bcvirB2*::Cm), AE608 (AU1054 *bcvirD4*::Cm), AE609 (AU1054 *bcvirP3*::Cm) (Table 4.2), products corresponding to *bcvirB2*, *bcvirD4*, and *bcvirP3,* were amplified by PCR using primer sets BCVB2 (BCVB2-1 and BCVB2-2), BCVD4 (BCVD4-1 and BCVD4-2), and BCVP3 (BCVP3-1

and BCVP3-2) (Table 4.3), respectively. Primer sets BCVB2, BCVD4, and BCVP3 (Table 4.3) were designed based on sequence data from the Joint Genome Institute *B. cenocepacia* AU1054 Genome Project (http://genome.jgi-psf.org/draft_microbes/burca/burca.home.html). Each PCR reaction (50 µl) was performed using a Taq PCR Master Mix (USB) and a GeneAmp 2700 (Applied Biosystems). Reactions were run for 29 cycles and parameters were as follows: denaturation at 95ºC for 1 min; annealing at 57, 56, or 56 ºC for 1 min for primer sets BCVB2, BCVD4, and BCVP3 respectively; and extension at 72ºC for 1 min. Resulting products were analyzed by agarose gel electrophoresis and cloned into pDrive (Qiagen), yielding pDrive-*bcvirB2*, pDrive-*bcvirD4*, or pDrive-*bcvirP3*, respectively. Constructs pDrive-*bcvirB2*, pDrive-*bcvirD4*, and pDrive-*bcvirP3*, were transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 40 µg/ml of Ap, 50 µM of isopropyl-beta-D-thiogalactopyranoside (IPTG), and 80 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix J). Specifically, *bcvirB2* insertions were determined using *Bam*HI/*Eco*RV, *bcvirD4* insertions were determined using *Bam*HI, and *bcvirP3* insertions were determined using *Sac*I. Sequencing was conducted by the Norman E. Borlaug Center for Southern Crop Improvement, Laboratory for Plant Genome Technology at Texas A&M University.

Table 4.3. Primers used in Chapter IV.

Primer[a]	Gene	Relevant Characteristics	Reference
BCVB2-1	*bcvirB2*	5'-GATGGAGTGCATTATGAAATCT-3'	This study
BCVB2-2	*bcvirB2*	5'- TTAGGCCGTCGGAATCA -3'	This study
BCVD4-1	*bcvirD4*	5'-CATGGTACGACGGCC-3'	This study
BCVD4-2	*bcvirD4*	5'-TCATTGAACACCACCACTTG-3'	This study
BCVP3-1	*bcvirP3*	5'-CATGGGATCGAGAAAC-3'	This study
BCVP3-2	*bcvirP3*	5'-TCAACGGCTCCATCG-3'	This study

[a] 1=forward primer; 2=reverse primer

The Cm-resistance cassette, obtained from pBR325 digested with *Bsa*AI and *Bcl*I, was blunted with T4 Polymerase and inserted into the *Eco*RV, *Sca*I, and *Bsa*AI sites of pDrive-*bcvirB2*, pDrive-*bcvirD4*, and pDrive-*bcvirP3,* respectively to yield pDrive-*bcvirB2*::Cm, pDrive-*bcvirD4*::Cm, and pDrive-*bcvirP3*::Cm, respectively. Constructs pDrive-*bcvirB2*::Cm, pDrive-*bcvirD4*::Cm, and pDrive-*bcvirP3*::Cm were transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 40 μg/ml of Ap and 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix K). Specifically, *bcvirB2::Cm* insertions were determined using *Bam*HI/*Eco*RV, *bcvirD4::Cm* insertions were determined using *Bam*HI, and *bcvirP3::Cm* insertions were determined using *Sac*I. Plasmids pDrive-*bcvirB2*::Cm, pDrive-*bcvirD4*::Cm, and pDrive-*bcvirP3*::Cm were digested with *Bam*HI/*Sac*I, *Kpn*I/*Hind*III, and *Bam*HI/*Hind*III respectively to yield *bcvirB2*::Cm, *bcvirD4*::Cm, and *bcvirP3*::Cm fragments, which were then ligated to pJQ200SK digested with the same restriction enzymes to yield pJQ200SK-*bcvirB2*::Cm, pJQ200SK-*bcvirD4*::Cm, and pJQ200SK-*bcvirP3*::Cm, respectively. Plasmids pJQ200SK-*bcvirB2*::Cm, pJQ200SK-*bcvirD4*::Cm, and pJQ200SK-*bcvirP3*::Cm were then transformed into *E. coli* DH5αMCR, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on LB agar amended with 10 μg/ml of Gm and 10 μg/ml of Cm. Directionality of the insertions was determined by restriction enzyme digestion and sequencing analyses (Appendix L). Specifically, directionality of *bcvirB2*::Cm insertions were determined using *Sma*I, *bcvirD4*::Cm insertions were

determined using *Bam*HI, and *bcvirP3*::Cm insertions were determined using *Sac*I. Plasmids pJQ200SK-*bcvirB2*::Cm, pJQ200SK-*bcvirD4*::Cm, and pJQ200SK-*bcvirP3*::Cm were individually mobilized into AU1054 by utilizing pRK2013 in triparental matings to obtain allelic exchange mutants. Exchange mutants were selected on VBG amended with 500 μg/ml of Cm. Cm-resistant transconjugants were single colony purified and retested for Cm^r. Allelic exchange and loss of the suicide vector was confirmed by PCR and plasmid survey lysis (Gonzalez *et al.*, 1997). Single colony isolates of insertionally inactivated *bcvirB2*, *bcvirD4*, and *bcvirP3*, designated AE607, AE608, and AE609 (Table 4.2), respectively, were tested for the ptw phenotype. Insertional inactivation of *bcvirB2*, *bcvirD4*, and *bcvirP3* in strains AE607, AE608, and AE609, respectively were confirmed by restriction enzyme digestion of genomic DNA with *Apa*I and *Apa*I/*Sca*I followed by agarose gel electrophoresis and Southern blot analysis using probes for the Cm^r-cassette, *bcvirB2*, *bcvirD4*, or *bcvirP3* (Appendix M).

Complementation of disrupted genes

Complementation of the disrupted *bcvirB2*, *bcvirD4*, or *bcvirP3* was accomplished *in trans*. Plasmid pBCRLAR (LiPuma, unpublished) was amended by inserting a Tp-resistance cassette, obtained from R388 as a *Bam*HI fragment, blunted with T4 polymerase, into the *Sca*I site in the Cm^r cassette to obtain pBCRLAR-Tp. Plasmids pDrive-*bcvirB2*, pDrive-*bcvirD4*, and pDrive-*bcvirP3* were digested with *Bam*HI/*Sac*I, *Kpn*I/*Hind*III, and *Bam*HI/*Hind*III, respectively, to yield *bcvirB2*, *bcvirD4*, and *bcvirP3*

fragments, which were then ligated to pBCRLAR-Tp digested with the same to yield pBCRLAR-Tp-*bcvirB2*, pBCRLAR-Tp-*bcvirD4*, and pBCRLAR-Tp-*bcvirP3*, respectively. Plasmids pBCRLAR-Tp-*bcvirB2*, pBCRLAR-Tp-*bcvirD4*, and pBCRLAR-Tp-*bcvirP3* were then transformed into *E. coli* ED8654, by chemical transformation (Sambrook *et al.*, 1989), and transformants were selected on VBG agar amended with 50 µg/ml of Tp and 0.5 mM final concentration of methionine. Plasmids pBCRLAR-Tp-*bcvirB2*, pBCRLAR-Tp-*bcvirD4*, and pBCRLAR-Tp-*bcvirP3* were then mobilized into AE607, AE608, or AE609, respectively to complement insertionally disrupted *bcvir* genes. Chloramphenicol resistant and Tp^r transconjugants were single colony purified and retested for antibiotic resistance and designated AE610, AE611, and AE612 (Table 4.2), respectively. Plasmid survey lysis and PCR were used to confirm plasmid transfer, which was reflected by the presence of both the resident and complement plasmid as well as inserted and uninserted PCR products.

To assess the stability of the constructed plasmids (pBCRLAR-Tp-*bcvirB2*, pBCRLAR-Tp*bcvirD4*, and pBCRLAR-Tp*bcvirP3*) in the absence of antibiotic selection, cultures (AE610, AE611, and AE612) were grown in TNB (initial O.D.= 0.05 at 600nm) and the generation time was followed spectrophotometrically. Serial transfers of the cultures were made to obtain actively growing cells for 30 generations. Samples were plated at 0, 10, 20, and 30 generations on selective (VBG amended with 500 µg/ml of Cm and 500 µg/ml of Tp) and nonselective media (VBG). The stability of each plasmid was determined by comparing the numbers of colonies present on selective and nonselective plates. Means from two independent experiments were calculated.

Mobilization assays

A Tp-resistance cassette, obtained as a *Bam*HI fragment from R388 was blunted with T4 polymerase. The Tp-resistance cassette was then individually inserted into the *Sma*I of the Gm-resistance cassette site of pML122 (Labes *et al.*, 1990). Plasmid pML122-Tp was then transformed into *E. coli* ED8654, using a chemical transformation, and transformants were selected for on VBG amended with 0.5 mM final concentration of methionine and 50 μg/ml Tp. Transformants were single colony purified and the presence of pML122-Tp was confirmed using minipreps (Qiagen) followed by agarose gel electrophoresis. Plasmids pURFO47-Tp and pML122-Tp were individually mobilized into strain AU1054 or AE608 by utilizing pRK2013. Transconjugants were selected for on VBG amended with 500μg/ml of Tp, single colony purified, and designated AE613 [AU1054 (pURFO47-Tp)], AE614 [AE608 (pURFO47-Tp)], AE615 [AU1054 (pML122-Tp)], and AE616 [AE608 (pML122-Tp)] (Table 4.2). Plasmid survey lysis confirmed the presence of plasmids pURFO47-Tp and pML122-Tp. Isolates harboring pURFO47-Tp or pML122-Tp were tested for the ptw phenotype. Mobilization of pURFO47-Tp or pML122-Tp, which are not self-transmissible, by strains AE613, AE614, AE615, and AE616 was tested using biparental matings with recipient strain BCS11. The conjugation frequency for each isolate was calculated as the number of CFU transconjugants per intial CFU of the recipient.

Oncogenic suppressor activity

The *osa* gene was obtained as a *Nde*I/*Xho*I fragment from pKA165 (Berger and Christie, 1994), blunted with T4 polymerase, and ligated to the *Sma*I site of pURFO47-Tp (Table 4.1). Plasmid pURFO47-Tp-*osa* was then transformed into *E. coli* ED8654, using a chemical transformation, and transformants were selected for on VBG amended with 0.5 mM final concentration of methionine and 50 μg/ml Tp. Plasmid pURFO47-Tp-*osa* was mobilized into AU1054 by utilizing pRK2013 in a triparental mating. Transconjugants were selected on VBG amended with 500 μg/ml of Tp. Single colony isolates of individual transconjugants were obtained by streaking on VBG amended with 500 μg/ml of Tp, single colony purified, and designated AE617 (Table 4.2). Cultures were subjected to survey lysis followed by agarose gel electrophoresis (Gonzalez and Vidaver, 1979) to confirm plasmid transfer.

RESULTS

DNA sequence and protein similarities of the *bcvirB/D* cluster

Preliminary evidence obtained using targeted PCR and Southern blot analyses indicated the presence of a type IV secretion system in strain AU1054. Using sequence data from the JGI *B. cenocepacia* strain AU1054 Genome Project (http://genome.jgi-psf.org/draft_microbes/burca/burca.home.html) and BLAST analysis, an entire gene cluster was confirmed, with homology to various translocation and/or conjugation related systems from other bacteria including *Agrobacterium tumefaciens*, *Xanthomonax axonopodis* pv. *citri, Brucella suis,* and *E. coli*. The gene cluster was designated *bcvirB/D* (Fig. 4.1), and was analyzed for similarities to other type IV secretion systems. The ProtParam Tool (http://www.expasy.ch/tools/protparam.html) was utilized to obtain predicted molecular weights (MW) and isoelectric points (PI) for the BcVirB/D proteins, and the ProtSite database of protein families and domains (http://www.expasy.ch/prosite/) was used to identify motifs.

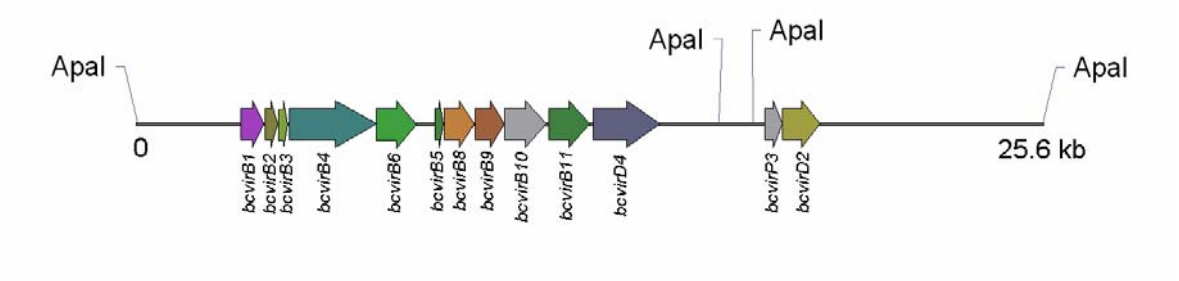

Fig. 4.1. A 25.6 kb segment containing the BcVirB/D like type IV secretion system cluster of *B. cenocepacia* strain AU1054. Designation of genes was based on homology to gene products of transfer and translocation related proteins.

BcVirB1 had a predicted MW of 22.9 kDa, an isoelectric point PI of 10.1, and showed similarity to VirB1-like proteins. VirB1-like proteins are members of a large family of subunits commonly associated with macromolecular surface structures, including the type IV secretion system. The signature of this protein family is a lysozyme-like structural fold (Koraimann, 2003; Mushegian *et al.*, 1996) which BcVirB1 possesses. BcVirB2 had a predicted MW of 12.6 kDa, a PI of 9.6, and showed similarity to VirB2-like proteins. VirB2 is the major pilin subunit of the *A. tumefaciens* VirB/D4 T pilus and an essential component of the secretion channel. Analysis of BcVirB2 showed that it is a small protein with a grand average hydropathicity index (GRAVY) of 1.008, indicating that it is hydrophobic in nature. BcVirB2 was also found to contain a long signal sequence and two hydrophobic stretches, both of which are common trait of VirB2-like proteins (Christie *et al.*, 2005). BcVirB3 had a predicted MW of 9.7 kDa, a PI of 7.7, and showed similarity to VirB3-like proteins. BcVirB3-like proteins are short polypeptides with two predicted transmembrane segments near the N-terminus. A Clustal W alignment of BcVirB3 with other VirB3-like proteins identify clusters of strongly conserved residues (e.g. (GAT)L(ST)RP) and P(VI)G motifs, before the predicted transmembrane segment. BcVirB4 had a predicted MW of 92.9 kDa, a PI of 6.6, and shows high similarity to other VirB4-like proteins. BcVirB4 is predicted to be a large inner membrane protein with a consensus Walker A motif (amino acids 456-463). A Clustal W alignment of BcVirB4 with other VirB4-like proteins revealed extensive sequence similarities. BcVirB5 had a predicted MW of 16.9 kDa, a PI of 8.6, and showed homology to VirB5-like proteins, which are exported to the periplasm and

localize extracellularly as components of the pilus (Schmidt-Eisenlohr *et al.*, 1999a, b). BcVirB6 had a predicted MW of 39.1 kDa, a PI of 5.9, and showed homology to VirB6-like proteins. BcVirB6 is a hydrophobic protein (GRAVY of 0.594) with three predicted transmembrane domains. BcVirB8 had a predicted MW of 31.6 kDa, a PI of 7.8, and showed similarity to VirB8-like proteins, which are associated with most type IVA type IV secretion systems (Christie *et al.*, 2005). These VirB8-like subunits are configured as bitopic inner membrane proteins with an N-proximal transmembrane segment (Das and Xie, 1998; Lawley *et al.*, 2003). A Clustal W alignment of BcVirB8 with other VirB8-like proteins confirms and extends an earlier identified sequence conservation between residues 100 and 143 and between 190 and 235 (Kumar *et al.*, 2000). BcVirB9 had a predicted MW of 31.0 kDa, a PI of 6.6, and showed similarity to VirB9-like proteins which are hydrophilic and predicted to localize in the periplasm or outer membrane (Christie *et al.*, 2005; Jakubowski *et al.*, 2005). BcVirB9 had a GRAVY of -0.208, as well as a predicted cell attachment site (amino acids 54-57). Similarities exist between the C-terminal and N-terminal regions of VirB9-like proteins, including BcVirB9. BcVirB10 had a predicted MW of 43.0 kDa, a PI of 5.4, and showed similarity to VirB10-like proteins, which are inner membrane proteins situated with the bulk of the protein in the periplasm (Das and Xie, 1998). BcVirB10 contains a transmembrane

segment, a Pro-rich region following the transmembrane segment, several coiled-coils near the transmembrane segment, and clusters of hydrophobic residues near the C-terminus; all of which are common features of VirB10 homologs (Chrisite *et al.*, 2005). BcVirB11 had a predicted MW of 50.5 kDa, a PI of 9.5, and showed similarity to VirB11-like proteins, which are members of a large family of ATPases associated with systems dedicated to secretion of macromolecules (Cao and Saier, 2001). The VirB11-like ATPases fractionate as cytoplasmic or peripheral inner membrane proteins (Krause *et al.*, 2000; Rashkova *et al.*, 1997). Sequence analysis of BcVirB11 showed that it contains a Walker-A site (amino acids 264-271) and that it is very similar to other VirB11-like proteins including HP0525 of *Helicobacter pylori*. BcVirD4 had a predicted MW of 70.3 kDa, a PI of 8.6, and showed similarity to VirD4-like proteins, which are also termed Coupling Proteins (CP) (Christie *et al.*, 2005). BcVirD4 is composed of an N-proximal periplasmic loop and a large C-terminal cytoplasmic domain (Das and Xie, 1998; Lee *et al.*, 1999), both common features of VirD4-like proteins. BcVirD2 had a predicted MW of 40.2 kDa, a PI of 10.4, and showed similarity to VirD2-like proteins, which are relaxases.

Site-directed disruption of the *bcvirB/D* type IV secretion system and the effect on the ptw phenotype

Translated products of the *bcvirB/D* cluster showed homology to proteins from known type IV secretion systems. Based on their predicted function, the role of *bcvirB2* and *bcvirD4* in the expression of the ptw phenotype was determined. Site-directed mutagenesis of *bcvirB2* and *bcvirD4* resulted in loss of the ptw phenotype and complementation *in trans* restored ptw activity (Fig. 4.2). Stability testing of AE610, AE611, and AE612 showed that pBCRLAR-Tp-*bcvirB2*, pBCRLAR-Tp*bcvirD4*, and pBCRLAR-Tp-*bcvirP3* are stable for at least 30 generations under non-selective conditions, with an average retention rate of 90 % (Table 4.4).

Based on analysis of the *bcvirB/D* 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, carrot

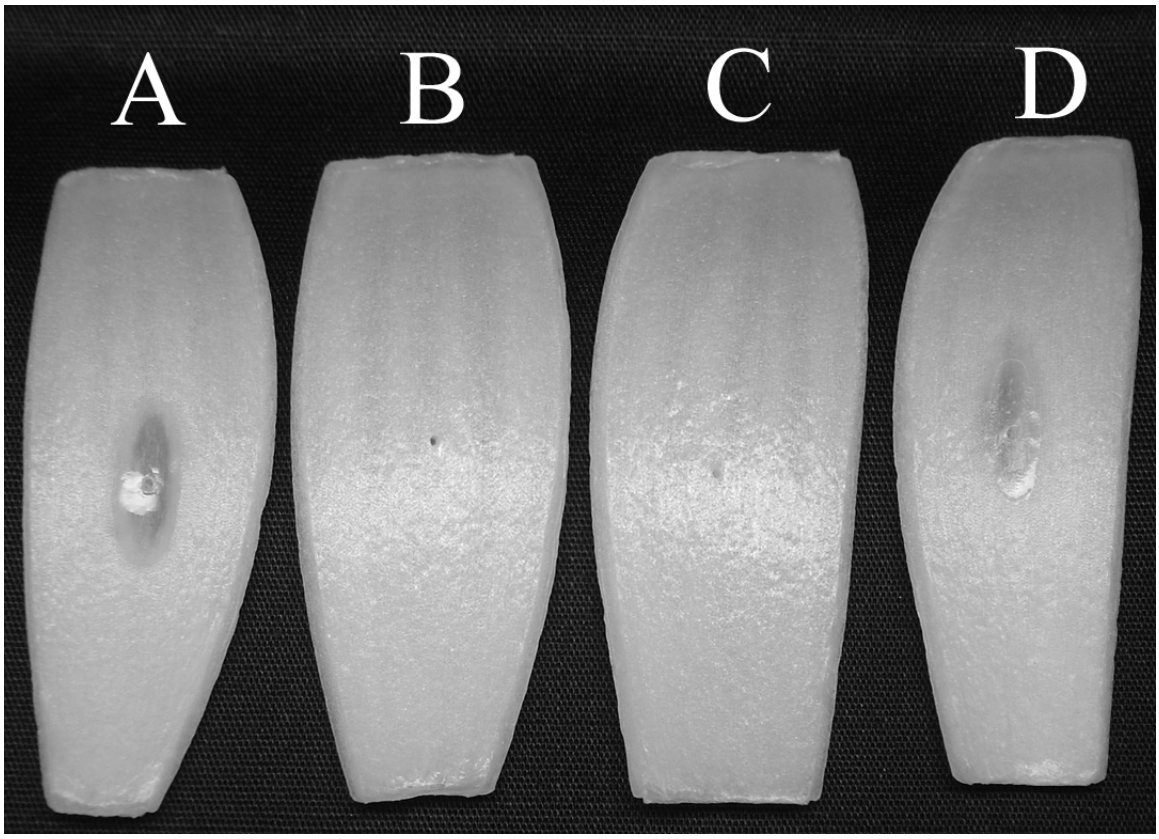

Fig. 4.2. Analysis of the BcVirB/D type IV secretion system using the plant tissue watersoaking assay. (A) Pierced onion bulb scale inoculated with *B. cenocepacia* strain AU1054 showing partial tissue collapse and translucence characteristic of the plant tissue watersoaking (ptw) phenotype. (B) Pierced onion bulb scale inoculated with sterile water (ptw-negative). (C) Pierced onion bulb scale inoculated with ptw-negative mutant (e.g. AE607, AE608, and AE609). (D) Pierced onion bulb scale inoculated with complemented ptw-negative mutant (e.g. AE610, AE611, and AE612) showing restored ptw activity.

Table 4.4. Stability testing of plasmids pBCRLAR-Tp-*bcvirB2,* pBCRLAR-Tp-*bcvirD4*, and pBCRLAR-Tp-*bcvirP3* in complemented strains AE610, AE611, and AE612 of *bcvirB2, bcvirD4,* and *bcvirP3* mutant strains AE607, AE608, and AE609.

	% Plasmid Containing CFU[a, b]		
	Strain		
Generations	**AE610**	**AE611**	**AE612**
0	100	100	100
10	98	97	97
20	95	94	95
30	91	90	90

[a] Percent plasmid containing CFU are the average for two independent experiments.
[b] All strains were grown in TNB medium at 37°C while shaking.

protoplasts exposed to M9 medium concentrate did not experience significant plasmolysis when compared to the protoplast controls ($P>0.05$) (Table 4.5 and Fig. 4.3). However, significant ($P\leq0.05$) plasmolysis was observed for protoplasts exposed to the AU1054 or complemented mutant (AE611) 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 as compared to medium controls. Concentrated supernatant from the *bcvirD4* mutant (AE608) caused 6.0% plasmolysis after 8 h, which was not statistically different ($P>0.05$) from plasmolysis resulting from treatment with medium controls. Dilution of the AU1054 and AE611 concentrates diluted 1:10 to a protein concentration of 0.02 μg/ml reduced activity to levels which approximated that of the control and AE608. Temperatures of 37º, 65º, and 80ºC for 1 h or 100ºC for 10 min did not affect the plasmolysis activity of the AU1054 concentrate. Complete inactivation of plasmolysis activity was observed when the AU1054 concentrate was treated with proteinase K; however, incubation with DNase I or RNase A showed no effect on activity.

Table 4.5. Effect of supernatant concentrates from AU1054, *bcvirD4* mutant (AE608) and complemented *bcvirD4* mutant (AE611) on carrot protoplasts.

Supernatant Concentrate	Concentrate Dilution[a]	% Plasmolysis[b, c]				
		0 h	2 h	4 h	6 h	8 h
M9 medium	0	0x	1.4 ± 1.7x	2.9 ± 1.2x	6.4 ± 1.7x	9.0 ± 1.9x
	1:1	0x	1.2 ± 1.1x	2.2 ± 1.5x	6.2 ± 1.1x	8.5± 2.6x
	1:5	0x	2.1 ± 1.0x	3.2 ± 1.1x	5.3 ± 2.0x	7.0 ± 2.2x
	1:10	0x	2.2 ± 1.7x	2.5 ± 1.1x	4.5 ± 2.1x	8.2 ± 2.6x
AU1054	0[d]	0x	19 ± 3.4y	37 ± 3.4y	59 ± 5.6y	77 ± 6.1y
	1:1	0x	20 ± 2.1y	39 ± 4.6y	57 ± 5.6y	75 ± 7.1y
	1:5	0x	19 ± 3.4y	37 ± 4.6y	59 ± 6.1y	76 ± 6.6y
	1:10	0x	1.4 ± 1.1x	3.2 ± 1.5x	4.9 ± 0.6x	6.9 ± 1.4x
AE608	0[d]	0x	2.0 ± 1.1x	3.2 ± 0.7x	4.5 ± 1.2x	6.4 ± 1.9x
	1:1	0x	2.3 ± 1.2x	3.9 ± 2.2x	5.5 ± 1.1x	7.2 ± 1.6x
	1:5	0x	2.2 ± 1.7x	3.2 ± 1.1x	6.0 ± 0.8x	8.2 ± 1.6x
	1:10	0x	2.2 ± 1.1x	4.2 ± 1.2x	6.2 ± 1.1x	6.7 ± 0.6x
AE611	0[d]	0x	20 ± 1.2y	39 ± 2.6y	58 ± 4.6y	79 ± 7.1y
	1:1	0x	18 ± 1.7y	38 ± 3.7y	54 ± 3.4y	77 ± 6.1y
	1:5	0x	19 ± 2.1y	40 ± 3.7y	57 ± 3.4y	70 ± 6.6y
	1:10	0x	2.0 ± 1.1x	2.0 ± 1.1x	6.4 ± 2.2x	7.0 ± 1.2x
None	0	0x	2.0 ± 1.4x	3.2 ± 7.1x	6.9 ± 1.6x	9.1 ± 2.6x

[a] All dilutions were adjusted to 10% mannitol
[b] Data are mean values ± SD of two replicates for three independent experiments.
[c] Values within the columns scored by the same letter were not statistically different at P=0.05.
[d] Undiluted concentrate adjusted to 0.2 µg/ml protein in 10% mannitol.

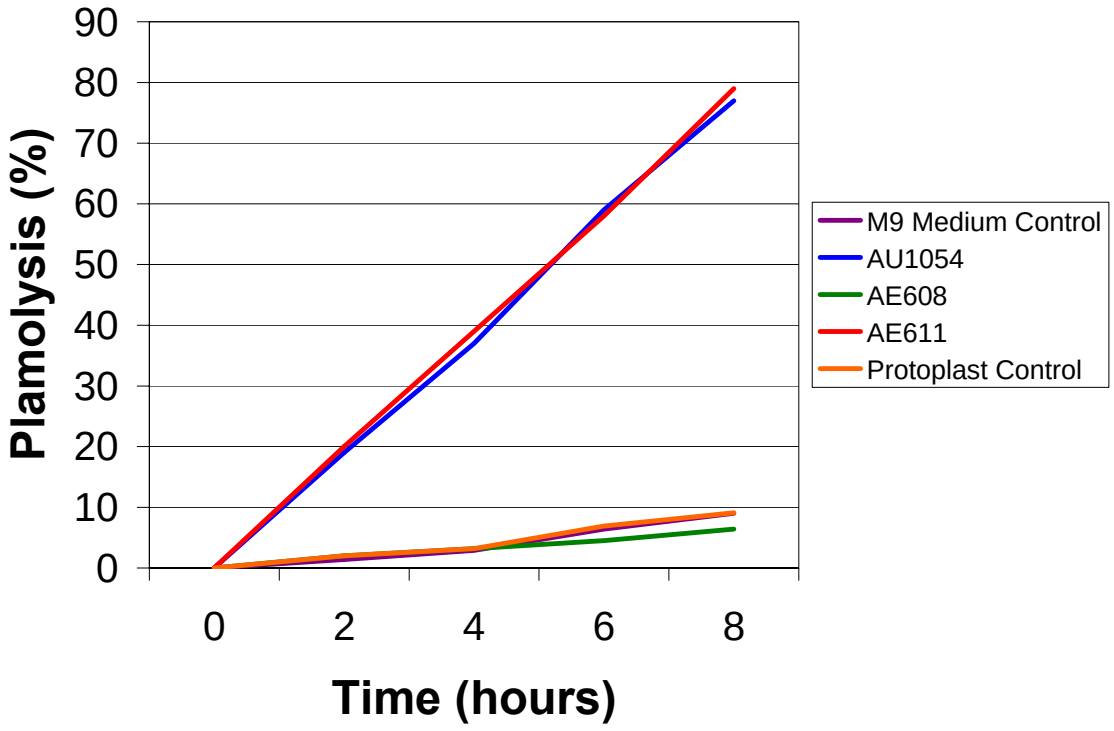

Fig. 4.3. Effect of supernatant concentrates from AU1054 *bcvirD4* mutant (AE608) and complemented *bcvirD4* mutant (AE611) on carrot protoplasts.

Identification of a putative effector translocated by the *bcvirB/D* type IV secretion system

Analysis of 10-kb regions flanking the *bcvirB/D* gene cluster of *B. cenocepacia* AU1054 identified a putative ORF, designated *bcvirP3* (Figure 4.1). Translation of the *bcvirP3* sequence predicted a protein with a MW of 19.0 kDa, a PI of 9.5, a GRAVY of -0.138, and showed no significant sequence similarity to know proteins by BLAST analysis. In

Since recent studies have shown the importance of a positively charged C-terminal transport signal in effectors translocated by type IV secretion systems (Simone *et al.*, 2001; Vergunst *et al.*, 2000; Vergunst *et al.,* 2003; Vergunst *et al.*, 2005) the C-terminal regions of *bcvirP3*was analyzed for amino acid content, net charge, and consensus sequences. The incidence of Arg residues among the C-terminal 20 amino acids, for *bcvirP3* was 15%, which is higher than expected for the Arg composition of *B. cenocepacia* AU1054 proteins (7%), and the calculated net charge was +2. Alignment of the 30 C-terminal amino acids of *bcvirP3* with other known type IV secreted effectors, such as VirD2, VirE2, VirE3, and VirF of *A. tumefaciens* as well as MobA from RSF1010, showed similarity in position of Arg residues to known type IV secreted proteins (Fig. 4.4).

By combining site-directed mutagenesis of *bcvirP3* with a cytotoxic assay employing plant tissue, it was determined that effector protein BcVirP3 was involved in the production of the ptw phenotype. Complementation *in trans* of the *bcvirP3* mutant resulted in restoration of the ptw phenotype (Fig. 4.2).

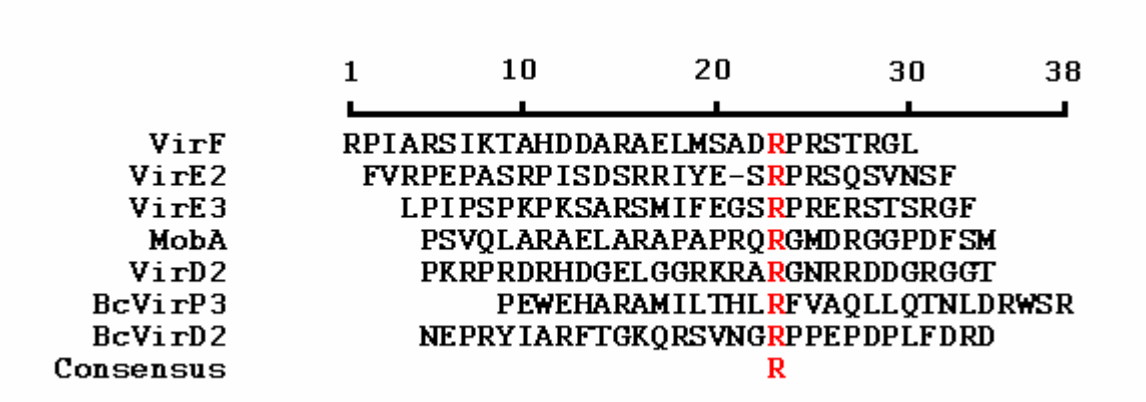

Fig. 4.4. Alignment of the C-terminal amino acids of *A. tumefaciens* VirD2, VirE2, VirE3, and VirF; RSF1010 MobA; and *B. cenocepacia* proteins BcVirD2 and BcVirP3.

An ORF which showed homology to VirD2-like proteins was identified during the analysis of regions flanking the BcVirB/D type IV secretion system. In *A. tumefaciens* VirD2 is the relaxase protein that is covalently bound to the 5' end of the ssDNA molecule and is thought to possess the type IV secretion system transport signal and thus act as a pilot for the complex. Therefore, we analyzed the BcVirD2 sequence. BcVirD2 had a predicted MW of 40.2 kDa, a PI of 10.4, a GRAVY of 0.638, and showed similarity to VirD2-like proteins, which are relaxases. ORF *bcvirD2* was analyzed for amino acid content, net charge, and consensus sequences. The incidence of Arg residues among the C-terminal 20 amino acids, for *bcvirD2*, was 15.8%, which is higher than expected for the Arg composition of *B. cenocepacia* AU1054 proteins (7%), and the calculated net charges was 0. Alignment of the 30 C-terminal amino acids of *bcvirD2* with other known type IV secreted effectors, to include VirD2, VirE2, VirE3, and VirF of *A. tumefaciens* as well as MobA from RSF1010, showed similarity in position of Arg residues to known type IV secreted proteins (Figure 4.4).

Translocation of DNA and protein substrates

Conjugative plasmids often encode factors that inhibit the transfer of co-resident plasmids (Olsen and Shipley, 1975; Yusoff and Stanisich, 1984). Such fertility inhibition factors appear to fall into two broad classes, those acting to repress expression of transfer (*tra*) genes (Gasson and Willetts, 1976; Tanimoto and Iino, 1983), and those functioning at a step in the translocation pathway subsequent to the synthesis and

assembly of the type IV secretion system (Yusoff and Stanisich, 1984; Winans and Walker, 1985). Little is known about the latter. In *A. tumefaciens*, two factors have been shown to render wild-type cells nearly or completely avirulent, a phenomenon termed 'oncogenic suppression' (Close and Kado, 1991; Ward *et al.*, 1991). One factor is the RSF1010 transfer intermediate composed of the MobA relaxase covalently bound to the 5' end of the transferred ssDNA (R-strand) (Ward *et al.*, 1991; Stahl *et al.*, 1998). The second is the Osa protein encoded by the IncW plasmid pSa (Close and Kado, 1991). Osa does not affect accumulation or membrane association of VirB proteins (Chen and Kado, 1994; Chen and Kado, 1996), but does share sequence similarity to FiwA, a fertility inhibition factor (Chen and Kado, 1994; Fong and Stanisich, 1989). It has been postulated that Osa represents a class of fertility inhibition factors acting after type IV secretion system machine biogenesis to block one or more steps of translocation (Cascales *et al.*, 2005).

Due to the high similarity of the AU1054 BcVirB/D type IV secretion system to the *A. tumefaciens* VirB/D4 type IV secretion system, the role of the BcVirB/D type IV secretion system in mobilization of DNA was tested using biparental matings. Strain AE613 [AU1054 (pURFO47-Tp)] was able to mobilize pURFO47-Tp, which is not self-transmissible, into strain BCS11; however, strain AE614 [AU1054 *bcvirD4*::Cm (pURFO47-Tp)] was not able to mobilize pURFO47-Tp to strain BCS11. Strain AE615 [AU1054 (pML122-Tp)] was able to mobilize pML122-Tp, which is also not self-transmissible, to BCS11; however, strain AE616 [AU1054 *bcvirD4*::Cm (pML122-Tp)] was not able to mobilize pML122-Tp to strain BCS11. The conjugation frequency for each isolate was calculated as the number of CFU transconjugants per initial CFU of input recipient (Table 4.6). In addition, strains harboring either pURFO47-Tp or pML122-Tp retained the ability to cause watersoaking on plant tissue.

It was also of interest to determine if an oncogenic suppressor such as Osa had any effect on other functions of the BcVirB/D type IV secretion system. Mobilization of pURFO47-Tp::*osa* into AU1054 (strain AE617) by utilizing a triparental mating resulted in the loss of the ptw phenotype. Indicating that Osa interferes with the translocation of the putative effector involved in the ptw phenotype.

Table 4.6. Mobilization frequencies of pML122-Tp or pURFO47-Tp by the AU1054 donor strains into BCS11 in biparental matings.

Donor	Recipient	Frequency[a]
AE613 [AU1054 (pURFO47-Tp)]	BCS11	1.1×10^{-5}
AE614 [AU1054 *bcvirD4*::Cm (pURFO47-Tp)]	BCS11	0
AE615 [AU1054 (pML122-Tp)]	BCS11	4.9×10^{-5}
AE616 [AU1054 *bcvirD4*::Cm (pML122-Tp)]	BCS11	0

[a] Mobilization frequencies are reported as the number of CFU transconjugants per intial CFU of recipient.

DISCUSSION

The identified gene products of the *bcvirB/D* cluster had homology to proteins from known type IV secretion systems. *A. tumefaciens* utilizes a VirB/D4 type IV secretion system to translocate DNA, and protein effector molecules to plant cells during infection (Zhu *et al.*, 2000; Cascales and Christie, 2003). The bacterial type IV secretion systems mediate the translocation of macromolecules across the cell envelopes of Gram-negative and Gram-positive bacteria (Cascales and Christie, 2003; Grohmann *et al.*, 2003). These systems are classified on the basis of an ancestral relatedness to bacterial conjugation machines (Christie, 2004; Lawley *et al.*, 2003). The conjugation machines are a large subfamily of type IV secretion systems that transmit DNA substrates among diverse species of bacteria, and some systems also deliver DNA to fungal, plant, and mammalian cells (Bates *et al.,* 1998; Bundock *et al.*, 1995; Schrammeijer *et al.*, 2003; Waters, 2001; Zhu *et al.*, 2000). Conjugation systems enable bacteria to adapt to changing environments through acquisition of fitness traits.

Although conjugation is usually depicted as a mechanism for intercellular DNA transfer, recent studies indicate that conjugation systems actually transmit the DNA as a nucleoprotein particle composed of a protein component, the relaxase, covalently bound to the 5' end of a molecule of ssDNA (Christie, 2004). In addition, DNA transfer is directional, from 5' to 3', further suggesting that the relaxase serves to pilot the ssDNA through the secretion channel. Conjugation machines might therefore correspond to ancestral protein translocation systems that evolved to recognize and translocate

relaxases as substrates and, only coincidentally, the "hitch-hiker" ssDNA. With this view, it is not surprising that conjugation machines also translocate protein substrates independently of associated DNA. Moreover, a second large subfamily of type IV secretion systems is now known to translocate only protein substrates to target cells (Cascales and Christie, 2003; Ding *et al.*, 2003; Nagai and Roy, 2003). Studies conducted over the past 10 years have identified numerous examples in which bacteria have appropriated type IV secretion systems for delivery of effector proteins into the cytosol of eukaryotic cells (Llosa and O'Callaghan, 2004). Through interkingdom protein transfer, these effector translocator systems induce a myriad of changes in host cell physiology to aid in the establishment of pathogenic or symbiotic relationships with the eukaryotic host (Cascales and Christie, 2003; Ding e*t al.*, 2003; Fischer *et al.*, 2002). Conjugation and most effector translocator systems transmit their substrates intercellularly by a cell-contact-dependent mechanism. Other type IV translocation systems deliver DNA substrates to or acquire them from the extracellular environment. In *Neisseria gonorrhoeae*, a type IV secretion machine highly related to the F plasmid conjugation system exports DNA substrates to the extracellular milieu (Dillard and Seifert, 2001). Conversely, in *H. pylori*, a competence system mediates uptake of DNA from the milieu (Hofreuter *et al.*, 2001; Hofreuter *et al.*, 2003). Such systems are called uptake and release systems (Cascales and Christie, 2003; Ding *et al.*, 2003). Besides a classification scheme based broadly on function, the type IV secretion systems have been grouped as type IVA, IVB, or "other" (Christie and Vogel, 2000). The type IVA systems are composed of subunits similar in composition and number to those of the

archetypal *A. tumefaciens* VirB/D4 system (Christie, 2004). The type IVB systems are assembled from subunits related to the archetypal *Legionella pneumophila* Dot/Icm system (Segal *et al.*, 2005). The "other" systems bear little or no discernible ancestral elatedness to the types IVA or IVB systems and will be named once their subunit compositions and phylogenies are better understood.

The archetypal VirB/D4 type IV secretion system is assembled from 11 VirB proteins, termed the mating pair formation (Mpf) proteins (Lessl *et al.*, 1993), and the VirD4 coupling protein (CP) (Ding *et al.*, 2003; Gomis-Ruth *et al*., 2004), also termed the substrate receptor (Atmakuri *et al.*, 2003; Cascales and Christie, 2004). This system is a conjugation apparatus, as shown by its capacity to deliver oncogenic transfer DNA (T-DNA) as well as the mobilizable plasmid RSF1010 (IncQ) to target cells through a cell-contact-dependent mechanism (Buchanan-Wollaston *et al.*, 1987; Fullner, 1998). The identified BcVirB/D type IV secretion system in *B. cenocepacia* of the PHDC lineage most closely resembles the VirB/D4 system of *A. tumefaciens*. The fact that the BcVirB/D system was involved in both the translocation of a protein as well as DNA, indicate that it is functionally a member of the IVA group of type IV secretion systems.

Identification of a cluster of genes that encoded proteins with similarities to components of a type IV secretion system allowed for strategic generation of AU1054 mutants to support the hypothesis that such a system was involved in expression of the ptw phenotype as well as conjugation. The genes selected for functional characterization were *bcvirB2* and *bcvirD4*. VirD4-like proteins (BcVirD4 homologs) are cytoplasmic membrane NTP-binding proteins that are essential for coupling the relaxosome to the

macromolecular transport system (Moncalián *et al.*, 1999; Schröeder and Lanka, 2003). Analysis of BcVirD4 revealed a P-loop which is characteristic of type IV secretion ATP-binding proteins. VirB2-like proteins (BcVirB2 homologs) are the major pilin subunit and are an essential component of the secretion channel (Berger and Christie, 1994; Jakubowski *et al.*, 2005; Lai and Kado, 1998). VirB2 homologs are components of most type IVA systems (Cao and Saier, 2001). Analysis of BcVirB2 revealed a small, hydrophobic protein. In general, VirB2-like proteins display low levels of sequence similarity, but a Clustal W alignment with BcVirB2 revealed several conserved Gly residues dispersed along the polypeptide. The ptw phenotype was not expressed by AU1054 derivatives with a disruption in *bcvirD4* or *bcvirB2*. These results correlate with the predicted function of the gene products since, successful secretion of an effector protein would likely involve: a protein that potentially couples the moiety to the secretion system and supplies the necessary energy for export (BcVirD4), and a protein that forms the pilus through which the moiety would be exported (BcVirB2).

By combining mutational analysis with cytotoxic assays employing both plant protoplasts, a type IV secretion system has been identified that appears to export an effector molecule that is proteinacious in nature and responsible for the activity. This activity plays an important role in the ability of *B. cenocepacia* strain AU1054 to cause watersoaking of plant tissue. Currently, the role of the putative virulence effector protein 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.

Most bacterial protein secretion systems recognize their substrates through a signal in the N terminus. For example, there is a clear consensus in the cleavable N-terminal signal peptide sequences recognized by the Sec-dependent and twin Arg translocation systems (Dilks *et al*., 2003; Cao and Saier, 2003). However, to date no consensus has been found to define the N-terminal signal of effectors secreted by type III or type IV secretion systems. Recent studies of *A. tumefaciens* and *B. henselae* type IV secretion systems have shown that the interaction between the C-termini of effector proteins and VirD4 is a critical step in effector translocation. In these studies, a positively charged C-terminal signal was present in several type IV secreted proteins including the VirD2 and VirE2 proteins of *A. tumefaciens* and the MobA relaxase of RSF1010 (Vergunst *et al.*, 2005). In strain AU1054 an ORF which showed similarity to VirD2 like proteins (relaxosomes) was identified. The ORF, designated *bcvirD2* was predicted to function as a relaxosome by ProtSite. Like VirD2 this ORF had a higher incidence of Arg residues than expected for *B. cenocepacia* proteins; however, the C-terminal 20 amino acids had a charge of 0 (Fig. 4.4). In strain AU1054, an ORF designated *bcvirP3* was identified that had no known homologs by BLAST analysis. The protein depicted by this ORF had a positively charged C-terminal region. The C-termini of BcVirP3 showed a higher incidence of Arg residues than expected for *B. cenocepacia* proteins, and an alignment of the 30 C-terminal amino acids showed similarity in the position of these residues (Fig. 4.4). Site-directed mutagenesis indicated that BcVirP3 was involved in the expression of the ptw phenotype.

It has been established in *A. tumefaciens* that two 'oncogenic suppressors' act on a common target, the VirD4 receptor, to block passage of the T-DNA and the VirE2 effector protein through the VirB/D4 type IV secretion system. One suppressor factor, RSF1010, is a promiscuous plasmid that parasitizes the VirB/D4 system as well as many other type IV secretion systems of Gram-negative bacteria (Rawlings and Tietze, 2001). The second suppressor factor is the Osa protein of pSa, which blocks VirD4 receptor activity not as a competing substrate, but rather through a mechanism shared by related fertility inhibition factors encoded by IncP, W, and N conjugative plasmids (Cascales *et al.*, 2005). By utilizing an RSF1010 derivative (pML122-Tp), it was shown that the BcVirB/D type IV secretion system was able to mobilize DNA substrates since, wild-type AU1054 was able to mobilize pML122-Tp and pURFO47-Tp, which are not self-transmissible, whereas a derivative of AU1054 with an insertionally inactivated BcVirD4 was not able to mobilize either the RSF1010 derivative or pURFO47-Tp. It was also shown that the Osa protein is able to render AU1054 avirulent in plant tissue as shown by the lack of watersoaking in an AU1054 derivative containing Osa. It is noteworthy, that RSF1010 blocks access of the VirE2 effector of *A. tumefaciens* to the VirD4 receptor, and thus inhibits T-DNA transfer. This was not the case in a *B. cenocepacia* AU1054 derivative harboring an RSF1010 derivative (pML122-Tp) since this strain remained capable of expressing the ptw phenotype on onion tissue. In *A. tumefaciens*, it is hypothesized that RSF1010 inhibits VirE2 translocation by a mechanism other than simple competitive inhibition (Cascales *et al.*, 2005).

In conclusion, this study (Chapter IV) has identified a group IVA type IV secretion system and a putative effector in *B. cenocepacia* strain AU1054 that is involved in the production of the ptw phenotype. The type IV secretion system was also shown to be responsible for mobilization of DNA indicating that it is a group IVA type IV secretion system. Mutational analysis of the BcVirB/D system indicates that BcVirB2 and BcVirD4 may play a role in expression of the ptw phenotype and DNA transfer. Culture supernatant studies on plant protoplasts indicate that an effector protein translocated into the extracellular milieu by the BcVirB/D system is involved in the observed toxicity to plant cells. Preliminary characterization of the transfer function indicates that Osa can disrupt transfer of proteins via the BcVirB/D system and future experiments will determine if Osa can disrupt DNA transfer as observed in *A. tumefaciens*.

CHAPTER V

CONCLUSION

The *Burkholderia cepacia* complex (Bcc) contains nine genetically distinct but phenotypically similar species (Mahenthiralingam *et al.*, 2005). Originally identified as phytopathogens (Burkholder, 1950), this group of organisms has become a focus of attention as human pathogens following isolation from the respiratory tracts of patients that are immunocompromised or have cystic fibrosis (CF) (Rosenstein and Hall, 1980; Isles *et al.*, 1984; Thomassen *et al.*, 1985). Although all nine species have been recovered from CF patients, *B. cenocepacia* and *B. multivorans* account for the great majority (~85%) of infection in patients in North America and Europe (Agodi *et al.*, 2001; LiPuma *et al.*, 2001; Speert *et al.*, 2002). Genotyping studies have also identified several epidemic strains that are common to multiple CF patients. Most epidemic strains identified to date, including strain ET12 (common among infected patients in Canada and UK) (Johnson *et al.*, 1994; Pitt *et al.*, 1996; Mahenthiralingam *et al.*, 2002) and strain PHDC (widespread in the United States) (Chen *et al.*, 2001; Reik *et al.*, 2005) are members of the *B. cenocepacia* species.

Although the pathogenic mechanisms involved in human infection by *B. cenocepacia* are largely unknown, several putative virulence factors have been described (Mahenthiralingam *et al.*, 2005). These include, cable pili and the associated adhesion (Sajjan *et al.*, 2002; Urban *et al.*, 2005), flagella (Tomich *et al.*, 2002; Urban *et al.*, 2004), lipopolysaccharide and surface exopolysaccharides (Shaw *et al.*, 1995; Chung *et*

al., 2003), type III secretion system (Tomich *et al.*, 2003), production of siderophores (Visser *et al.*, 2004), catalase and superoxide dismutase (Lefebre and Valvano, 2001), proteases and lipases (McKevitt *et al.*, 1989) and quorum sensing system (Sokol *et al.*, 2003). To date the role of these factors in contributing to lung disease has not been clearly elucidated. In this research type IV secretion systems, which are an important mechanism involved in pathogenesis of bacteria, were identified and studied.

In a CF clinical isolate of *B. cenocepacia*, strain K56-2 of the ET12 lineage, two type IV secretion systems were identified (Chapter II). One is a plasmid-encoded type IV secretion system, designated Ptw, which is a member of the effector translocator subfamily; and the other is a chromosomally encoded type IV secretion system, designated BcVirB/D, for which the function is at this time unknown. The Ptw system is a chimeric type IV secretion system containing homologs to components of multiple type IV secretion systems. These studies have shown that the Ptw system is responsible for the production of the ptw phenotype and at least partially responsible for the stimulation of release of IL-8 from human bronchial epithelial cells, by strain K56-2.

An effector, designated PtwE1, involved in production of the ptw phenotype in *B. cenocepacia* strain K56-2 was identified (Chapter III). PtwE1 was also shown to be translocated into the extracellular milieu via the Ptw type IV secretion system. A positively charged C-terminal transport signal as well as a functional Ptw system was also found to be necessary for the translocation process. Future studies will involve elucidation of the of the interaction between PtwE1 and the Ptw type IV secretion

system as well as experiments to determine the role of PtwE1 in the stimulation of release of IL-8 from human bronchial epithelial cells.

In a CF clinical isolate of *B. cenocepacia*, strain AU1054 of the PHDC lineage, a chromosomally encoded type IV secretion system was identified which showed homology to the VirB/D4 system of *A. tumefaciens*. This system, designated BcVirB/D, was shown to be involved in production of the ptw phenotype. The BcVirB/D system was able to mobilize DNA and effector proteins, and therefore is a member of the group IVA type IV secretion systems. Studies indicated that an effector protein translocated into the extracellular milieu by the BcVirB/D system is involved in the observed toxicity to plant cells. Preliminary characterization of the transfer function indicates that an oncogenic suppression activity protein (Osa) can disrupt transfer of effector proteins via the BcVirB/D system. Future studies will determine if Osa can disrupt DNA transfer and elucidate the interaction between BcVirP3 and the BcVirB/D system.

The studies conducted during my dissertation research have established a beginning point to determine the role of type IV secretion systems in pathogenesis caused by *B. cenocepacia*.

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APPENDIX A

Restriction enzyme and sequence analyses of pDrive-*ptwD4*

Predicted and obtained results for digestion of pDrive-*ptwD4* with *Sma*I

Correct insertion yields fragments of sizes 2.1 and 3.7 kb
Incorrect insertion yields fragments of sizes 4.0 and 1.8 kb

Sequencing results obtained from pDrive-*ptwD4*

tgactcagcgaaggaacgagctaccgagcttttgcggggtagccagaaatgacgggaaaa
aagccaaacgggctcttccaggatttttccaccggctcggaaatctggactcaccgtgtc
gcgctggtgctcgtcgcgatccgaaatatcctgctgctctctttgtttctcggtctggcg
gccgggttcatctacgccgcattcttcgtcgaccgcgacatctggcgtccagcagtcgcg
aacgttatcgcgcatcttcggtcggtcgtcatgatgacggcggtaccgatggaatacgaa
gtcaacggccagaccgtgcgacttcccgtcgatcaagttgtcgccctgacggacccagtg
tgggatgtggcttatttctttttgcggcgattcctgatcattgtcgcaaccgtcacggca
tgtacatgcgtctcgatcgctgcctactggtatgagttcggtcgcagcacgatggccgac
aaggatctgcgtggcgcaacgctcgtcaacgggaagcaactggcgaagatgattcggcaa
gacgatatggagagcccgtattcgatagcgggcgtgccgatgctgcgcaacgcggagacc
ctgcataccctggtggtcggcgcacagggtacgggtaagtctcagcagttcttcgatcgg
ctcaggcaagtccgcgcccgtgggaagcgcgcgatcgtatacgatccgtcgggcgaattt
gcggaggaatttttccgggaaggcaaggacgtcatgatgaatccgttcgacgaccgttcg
ccaaactggaacatcaagtatgagatcgaggaggagtaccactatgacagcatggcaacc
gcgctgattcccgatccgcgcgaagccgatccattttggtcattggcggggcgcgaggtg
tttcgagatgtcgcgcgtacgttgcacagagagggcaatctgacgaaccgcaatctttac
aattcgatcgcaatgagcaacctcgacgcgatctaccagttgctgcgcaataccgccggc
gcgtcgtacgtcgatcccaagaccgagcgcacgggtatgtcgttgaagatgaccgtccag
aaccagctttcctctttcaggtatctccgtgatgatggaaagccgttctcgatccgtcag
tggatttacaacgagagtgattcgtggatgttcatctcgacccgtgaggaaatgctcgag
gcgatcaagccaatcatgtcgctctggatcgacatcgcgatcaaagcggtgctcaacctc
acaccgattcatcgcgagcgactgcacttctcgatcgacgaagtcccgaccctgcagaag
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ggtgcgcaggatatgccgcagttcctcgacatctacggggaatacctcgcgcggacgatt
atcaacgggtgtcaaaccaagctgttgttgcgcgtaaccgacgcagaagccgctgagatg
ttctcgaaaatcatcgggcagacggaagtcgaggaaaaggacgagtcgctgtcgtttggt
gtgaactctcagcgggacggattcagcatcgcgaagcgtcggctgctgcgcgacctagcg
ttgccctcacagatattgcgactgcgggacatgacaggctatttggtctatcccggcgaa
tacccgatcgctcgcgtgaagtacgggtttgtcaaaccgcggaaaaacgcacctgctttt
atcaagcgtcaggcattctccgaactattcgcgccggcggccggctcgccggcaaccgct
gccgcttcggaatccgtagtcgctggcccatcacctgactggatcgcgcacccggagata
ctcgtcgcggcgagcggcggcgtcgatccggaaactggcgaaatcctgccaccccgcccg
ggcgcctggcgctggggcgacggcaatcaacgaggagaccatgcatgaaggccctttgtc
gtgttttgtccaccgccctttttcttgcggtgcccgccgtgagcgcattcgcgcagtctg
cgcccgggttgcttccaccggat

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*ptwE1*

Predicted and obtained results for digestion of pDrive-*ptwE1* with *Cla*I

Correct insertion yields fragments of sizes 2.5 and 1.9 kb
Incorrect insertion yields fragments of sizes 2.2 and 2.2 kb

Sequencing results obtained from pDrive-*ptwE1*

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattc
cgcaaatcttcgactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcaca
cgcccaaaaatttcccgacgccgcattgaccacagatccaccgcgccccgcgctcacacc
ggtgttgccacacactgacgtgtggacaacaccgcgcggcgaccggctccgtgatcttta
tcgatcacggccgttggtccggcaccgtggatatgtggacaatgctcaccggtccaccac
cgcgtcaccttctgctactgtcctgcccacactaccgccgtccccgaacctgtatattcc
ctgctgcctccacctcccttcttccttccgacgttgcactgaacagctccgctccgctcc
gaggtgaagatggcgaaaatccaccgctcacgatccaaaccacgccgtcgcttttcccc
gattacgccgattcccttgagcgggaaccgccgtaccttaccgggtgaaggct

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed.

APPENDIX B

Restriction enzyme and sequence analyses of pDrive-*ptwD4*::Cm

Predicted and obtained results for digestion of pDrive-*ptwD4*::Cm with *Sca*I

Correct insertion yields fragments of sizes 3.3 and 3.5 kb
Incorrect insertion yields fragments of sizes 4.4 and 2.4 kb

Sequencing results obtained from pDrive-*ptwD4*::Cm

tgactcagcgaaggaacgagctaccgagcttttgcggggtagccagaaatgacgggaaaaaagccaaacgggctcttcc
aggatttttccaccggctcggaaatctggactcaccgtgtcgcgctggtgctcgtcgcgatccgaaatatcctgctgct
ctctttgtttctcggtctggcggccgggttcatctacgccgcattcttcgtcgaccgcgacatctggcgtccagcagtc
gcgaacgttatcgcgcatcttcggtcggtcgtcatgatgacggcggtaccgatggaatacgaagtcaacggccagaccg
tgcgacttcccgtcgatcaagttgtcgccctgacggacccagtgtgggatgtggcttatttctttttgcggcgattcct
gatcattgtcgcaaccgtcacggcatgtacatgcgtctcgatcgctgcctactggtatgagttcggtcgcagcacgatg
gccgacaaggatctgcgtggcgcaacgctcgtcaacgggaagcaactggcgaagatgattcggcaagacgatatggaga
gcccgtattcgatagcgggcgtgccgatgctgcgcaacgcggagaccctgcataccctggtggtcggcgcacagggtac
gggtaagtctcagcagttcttcgatcggctcaggcaagtccgcgcccgtgggaagcgcgcgatcgtatacgatccgtcg
ggcgaatttgcggaggaatttttccgggaaggcaaggacgtcatgatgaatccgttcgacgaccgttcgccaaactgga
acatcaagtatgagatcgaggaggagtaccactatgacagcatggcaaccgcgctgattcccgatccgcgcgaagccga
tccattttggtcattggcggggcgcgaggtgtttcgagatgtcgcgcgtacgttgcacagagagggcaatctgacgaac
cgcaatctttacaattcgatcgcaatgagcaacctcgacgcgatctaccagttgctgcgcaataccgccggcgcgtcgt
acgtcgatcccaagaccgagcgcacgggtatgtcgttgaagatgaccgtccagaaccagctttcctctttcaggtatct
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gaggaaatgctcgaggcgatcaagccaatcatgtcgctctggatcgacatcgcgatcaaagcggtgctcaacctcacac
cgattcatcgcgagcgactgcacttctcgatcgacgaagtcccgaccctgcagaagctcgatgtaagaggttccaactt
tcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagattttcaggagctaaggaagctaaaatg
gagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcag
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gttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatggcaatgaaagacggt
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gtgaataccacgacgatttccggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggccta
tttccctaaagggtttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaac
gtggccaatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctgatgc
cgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactg
cgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataa
gtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaatgcctgag
gccagtttgctcaggctctccccgtggaggtaataattgacgatatatcatgaagctcgcggtgagcaatacgcggaag
tacggcttatgcatgatgctcggtgcgcaggatatgccgcagttcctcgacatctacggggaatacctcgcgcggacga
ttatcaacgggtgtcaaaccaagctgttgttgcgcgtaaccgacgcagaagccgctgagatgttctcgaaaatcatcgg
gcagacggaagtcgaggaaaaggacgagtcgctgtcgtttggtgtgaactctcagcgggacggattcagcatcgcgaag
cgtcggctgctgcgcgacctagcgttgccctcacagatattgcgactgcgggacatgacaggctatttggtctatcccg
gcgaatacccgatcgctcgcgtgaagtacgggtttgtcaaaccgcggaaaaacgcacctgcttttatcaagcgtcaggc
attctccgaactattcgcgccggcggccggctcgccggcaaccgctgccgcttcggaatccgtagtcgctggcccatca
cctgactggatcgcgcacccggagatactcgtcgcggcgagcggcggcgtcgatccggaaactggcgaaatcctgccac
cccgcccgggcgcctggcgctggggcgacggcaatcaacgaggagaccatgcatgaaggccctttgtcgtgttttgtcc
accgccctttttcttgcggtgcccgccgtgagcgcattcgcgcagtctgcgcccgggttgcttccaccggat

Note: Primers, start codon, and Cm gene are highlighted
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*ptwE1*::Cm

Predicted and obtained results for digestion of pDrive-*ptwE1*::Cm with *Sca*I

Correct insertion yields fragments of sizes 2.5 and 2.9 kb
Incorrect insertion yields fragments of sizes 2.0 and 3.3 kb

Sequencing results obtained from pDrive-*ptwE1*::Cm

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
gcgcggcgaccggctccgtgatctttatcgatcacgggtaagaggttccaactttcaccataatgaaataa
gatcactaccgggcgtatttttgagttatcgagattttcaggagctaaggaagctaaaatggagaaaaaa
atcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcagt
tgctcaatgtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaata
agcacaagttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatg
gcaatgaaagacggtgagctggtgatatgggatagtgttcacccttgttacaccgttttccatgagcaaac
tgaaacgttttcatcgctctggagtgaataccacgacgatttccggcagtttctacacatatattcgcaag
atgtggcgtgttacggtgaaaacctggcctatttccctaaagggtttattgagaatatgtttttcgtctca
gccaatccctgggtgagtttcaccagttttgatttaaacgtggccaatatggacaacttcttcgcccccgt
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ccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactgcgatgagtggcagggc
ggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataagtgataat
aagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaa
tgcctgaggccagtttgctcaggctctccccgtggaggtaataattgacgatatccgttggtccggcaccg
tggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgc
cgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctc
cgctccgctccgaggtgaagatggcgaaaatccaccgctcacgatccaaaccacgccgtcgcttttttcccc
gattacgccgattcccttgagcgggaaccgccgtaccttaccgggtgaaggct

Note: Primers, start codon, and Cm gene are highlighted
Note: Vector sequence has been removed.

APPENDIX C

Restriction enzyme and sequence analyses of pJQ200SK-*ptwD4*::Cm

Predicted and obtained results for digestion of pJQ200SK-*ptwD4*::Cm with *Sal*I

Correct insertion yields fragments of sizes 0.2 and 5.3 kb
Incorrect insertion yields fragments of sizes 2.8 and 2.7 kb

Sequencing results obtained from pJQ200SK-*ptwD4*::Cm

tgactcagcgaaggaacgagctaccgagcttttgcggggtagccagaaatgacgggaaaaaagccaaacgggctcttcc
aggatttttccaccggctcggaaatctggactcaccgtgtcgcgctggtgctcgtcgcgatccgaaatatcctgctgct
ctctttgtttctcggtctggcggccgggttcatctacgccgcattcttcgtcgaccgcgacatctggcgtccagcagtc
gcgaacgttatcgcgcatcttcggtcggtcgtcatgatgacggcggtaccgatggaatacgaagtcaacggccagaccg
tgcgacttcccgtcgatcaagttgtcgccctgacggacccagtgtgggatgtggcttatttctttttgcggcgattcct
gatcattgtcgcaaccgtcacggcatgtacatgcgtctcgatcgctgcctactggtatgagttcggtcgcagcacgatg
gccgacaaggatctgcgtggcgcaacgctcgtcaacgggaagcaactggcgaagatgattcggcaagacgatatggaga
gcccgtattcgatagcgggcgtgccgatgctgcgcaacgcggagaccctgcataccctggtggtcggcgcacagggtac
gggtaagtctcagcagttcttcgatcggctcaggcaagtccgcgcccgtgggaagcgcgcgatcgtatacgatccgtcg
ggcgaatttgcggaggaatttttccgggaaggcaaggacgtcatgatgaatccgttcgacgaccgttcgccaaactgga
acatcaagtatgagatcgaggagcagtaccactatgacagcatggcaaccgcgctgattcccgatccgcgcgaagccga
tccattttggtcattggcggggcgcgaggtgtttcgagatgtcgcgcgtacgttgcacagagagggcaatctgacgaac
cgcaatctttacaattcgatcgcaatgagcaacctcgacgcgatctaccagttgctgcgcaataccgccggcgcgtcgt
acgtcgatcccaagaccgagcgcacgggtatgtcgttgaagatgaccgtccagaaccagctttcctctttcaggtatct
ccgtgatgatggaaagccgttctcgatccgtcagtggatttacaacgagagtgattcgtggatgttcatctcgacccgt
gaggaaatgctcgaggcgatcaagccaatcatgtcgctctggatcgacatcgcgatcaaagcggtgctcaacctcacac
cgattcatcgcgagcgactgcacttctcgatcgacgaagtcccgaccctgcagaagctcgatgtaagaggttccaactt
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gagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcag
ttgctcaatgtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaataagcacaa
gttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatggcaatgaaagacggt
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gtgaataccacgacgatttccggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggccta
tttccctaaagggtttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaac
gtggccaatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctgatgc
cgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactg
cgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataa
gtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaatgcctgag
gccagtttgctcaggctctccccgtggaggtaataattgacgatatatcatgaagctcgcggtgagcaatacgcggaag
tacggcttatgcatgatgctcggtgcgcaggatatgccgcagttcctcgacatctacggggaatacctcgcgcggacga
ttatcaacgggtgtcaaaccaagctgttgttgcgcgtaaccgacgcagaagccgctgagatgttctcgaaaatcatcgg
gcagacggaagtcgaggaaaaggacgagtcgctgtcgtttggtgtgaactctcagcgggacggattcagcatcgcgaag
cgtcggctgctgcgcgaccatgcgttgccctcacagatattgcgactgcgggacatgacaggctatttggtctatcccg
gcgaatacccgatcgctcgcgtgaagtacgggtttgtcaaaccgcggaaaaacgcacctgcttttatcaagcgtcaggc
attctccgaactattcgcgccggcggccggctcgccggcaaccgctgccgcttcggaatccgtagtcgctggcccatca
cctgactggatcgcgcacccggagatactcgtcgcggcgagcggcggcgtcgatccggaaactggcgaaatcctgccac
cccgcccgggcgcctggcgctggggcgacggcaatcaacgaggagaccatgcatgaaggccctttgtcgtgttttgtcc
accgccctttttcttgcggtgcccgccgtgagcgcattcgcgcagtctgcgcccgggttgcttccaccggat

Note: Primers, start codon, and Cm gene are highlighted
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-*ptwE1*::Cm

Predicted and obtained results for digestion of pJQ200SK-*ptwE1*::Cm with *Eco*RI

Correct insertion yields fragments of sizes 0.6 and 3.5 kb
Incorrect insertion yields fragments of sizes 1.0 and 3.1 kb

Sequencing results obtained from pJQ200SK-*ptwE1*::Cm

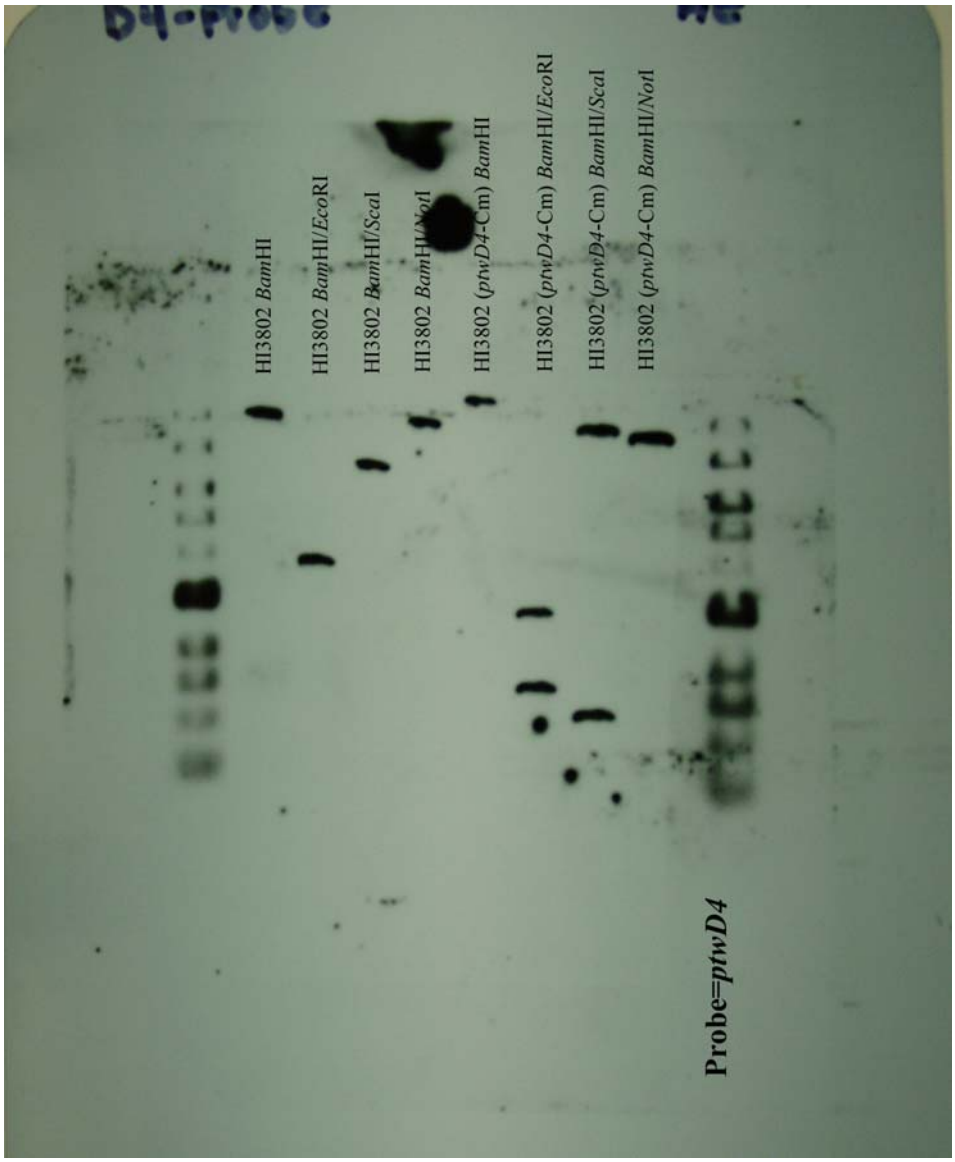
ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
gcgcggcgaccggctccgtgatctttatcgatcacgggtaagaggttccaactttcaccataatgaaataa
gatcactaccgggcgtatttttgagttatcgagattttcaggagctaaggaagctaaaatggagaaaaaa
atcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcagt
tgctcaatgtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaata
agcacaagttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatg
gcaatgaaagacggtgagctggtgatatgggatagtgttcacccttgttacaccgttttccatgagcaaac
tgaaacgttttcatcgctctggagtgaataccacgacgatttccggcagtttctacacatatattcgcaag
atgtggcgtgttacggtgaaaacctggcctatttccctaaagggtttattgagaatatgtttttcgtctca
gccaatccctgggtgagtttcaccagttttgatttaaacgtggccaatatggacaacttcttcgcccccgt
tttcaccatgggcaaatattatacgcaaggcgacaaggtgctgatgccgctggcgattcaggttcatcatg
ccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactgcgatgagtggcagggc
ggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataagtgataat
aagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaa
tgcctgaggccagtttgctcaggctctccccgtggaggtaataattgacgatatccgttggtccggcaccg
tggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgc
cgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctc
cgctccgctccgaggtgaagatggcgaaaatccaccgctcacgatccaaaccacgccgtcgcttttttcccc
gattacgccgattcccttgagcgggaaccgccgtaccttaccgggtgaaggct

Note: Primers, start codon, and Cm gene are highlighted
Note: Vector sequence has been removed.

APPENDIX D

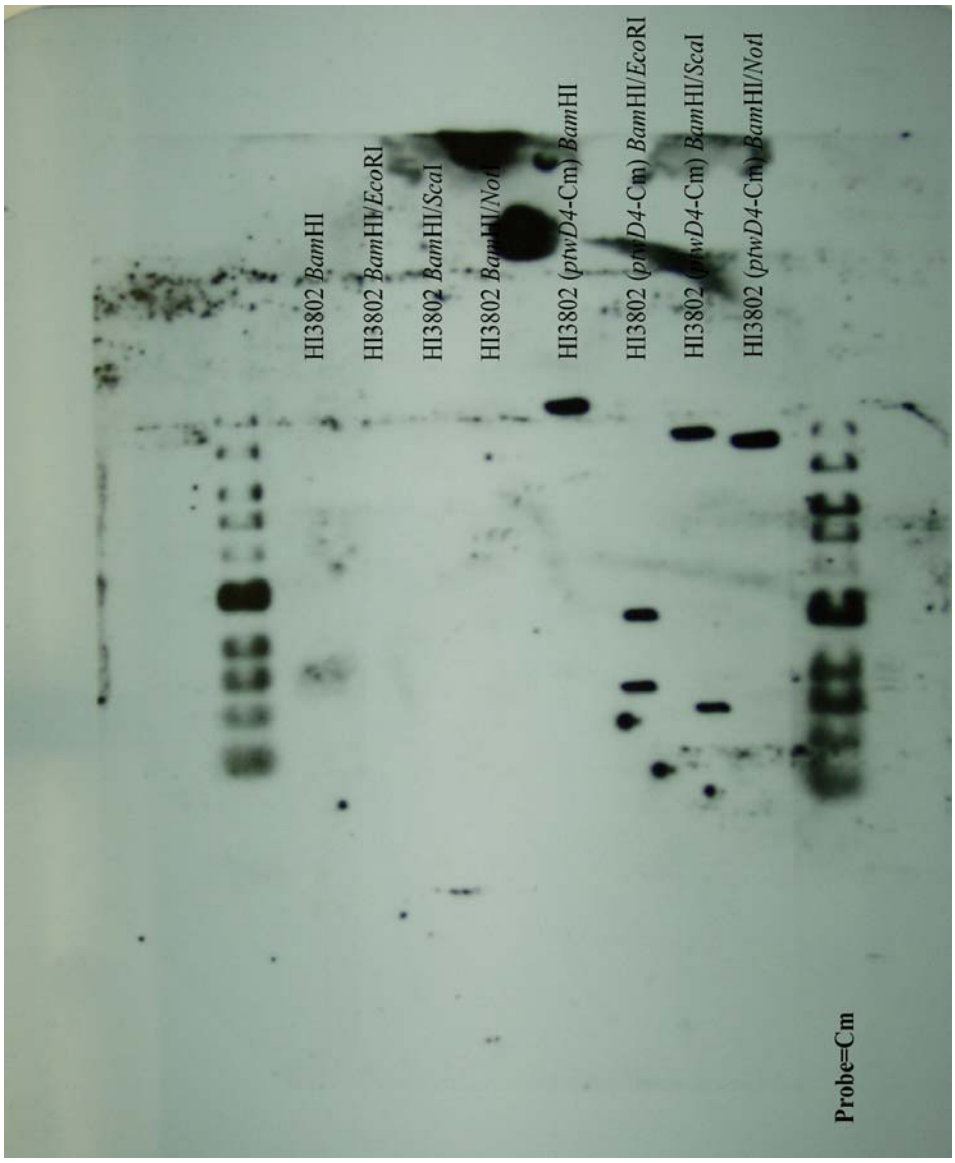
Predicted and obtained results of restriction enzyme digests and Southern blot analysis of HI3802 and HI3802 (*ptwD4*::Cm) based on sequence data obtained from the Sanger Centre J2315 sequencing project.

Probe=*ptwD4* PCR Product		
	HI3802	**HI3802 (*ptwD4*::Cm)**
***Bam*HI**	10 kb	11 kb
***Bam*HI/*Eco*RI**	4 kb	1.7 kb & 3.2 kb
***Bam*HI/*Sca*I**	7.8 kb	1.3 kb & 9.7 kb
***Bam*HI/*Not*I**	10 kb	8.8 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

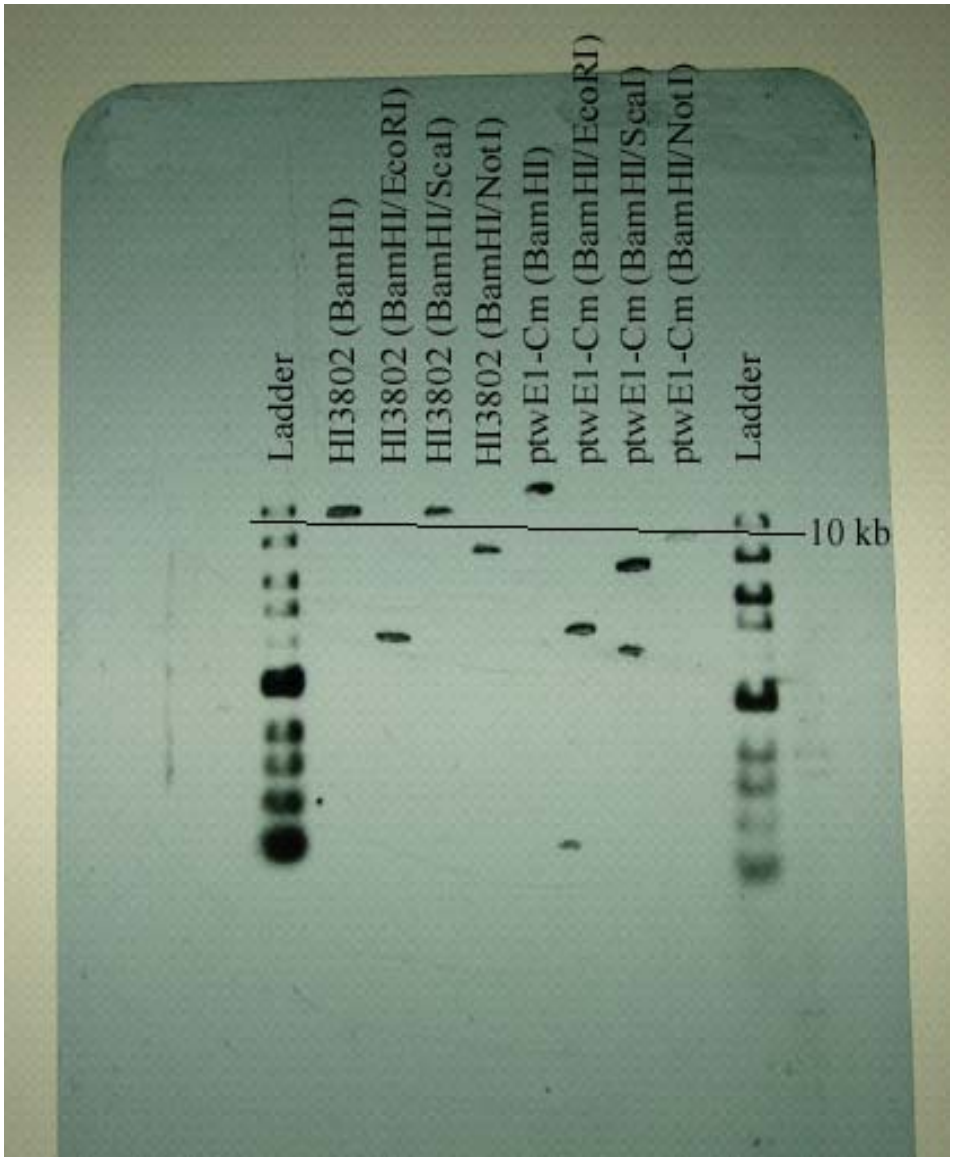
Probe=Cm PCR Product		
	HI3802	**HI3802 (*ptwD4*::Cm*)***
***Bam*HI**	N/A	11 kb
***Bam*HI/*Eco*RI**	N/A	1.7 kb & 3.2 kb
***Bam*HI/*Sca*I**	N/A	1.3 kb & 9.7 kb
***Bam*HI/*Not*I**	N/A	8.8 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

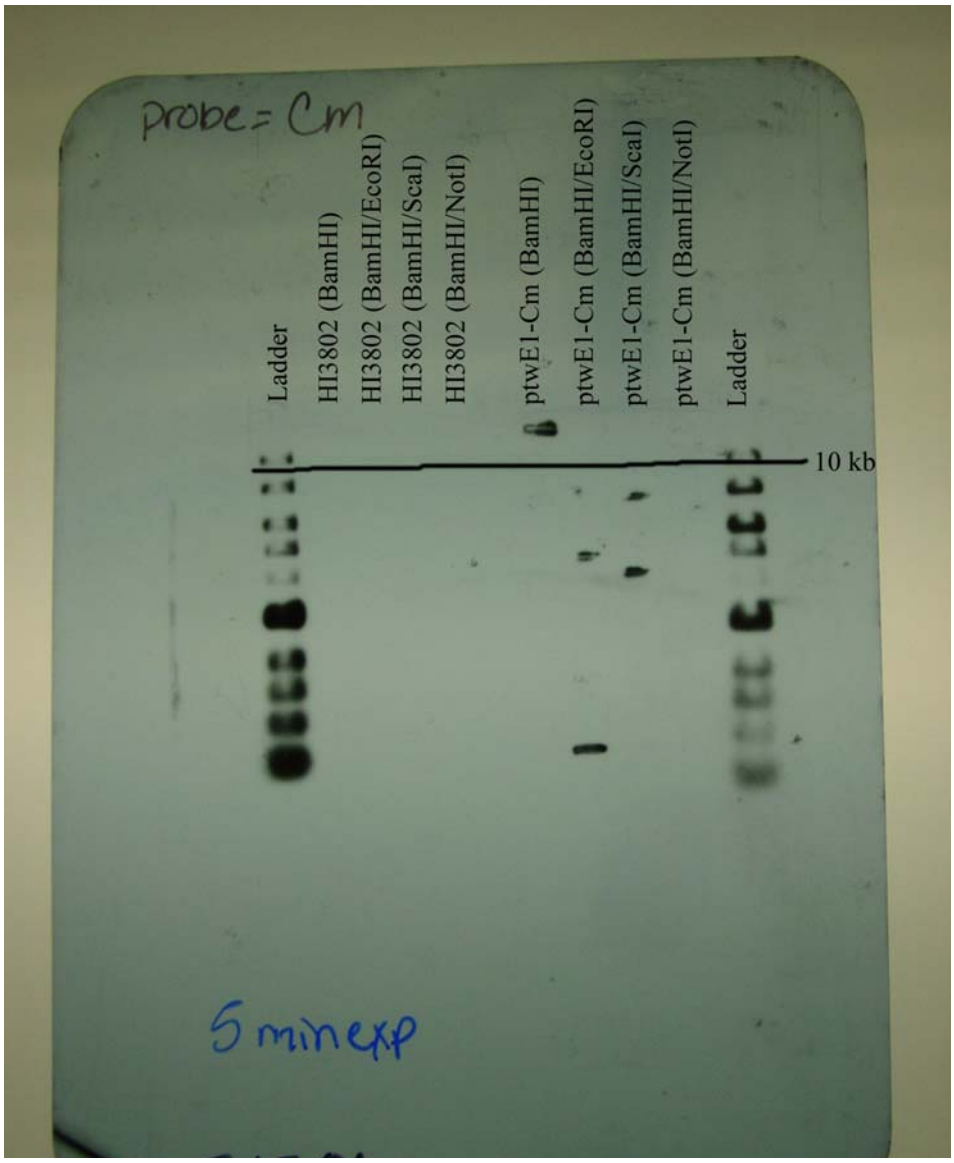
Predicted and obtained results of restriction enzyme digests and Southern blot analysis of HI3802 and HI3802 (*ptwE1*::Cm) based on sequence data obtained from the Sanger Centre J2315 sequencing project.

Probe=*ptwE1* PCR Product		
	HI3802	**HI3802 (*ptwE1*::Cm)**
***Bam*HI**	10 kb	11 kb
***Bam*HI/*Eco*RI**	4 kb	0.57 kb & 4.3 kb
***Bam*HI/*Sca*I**	10 kb	3.9 kb & 7.0 kb
***Bam*HI/*Not*I**	7.75 kb	8.76 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

Probe=Cm PCR Product		
	HI3802	**HI3802 (*ptwE1*::Cm)**
***Bam*HI**	N/A	11 kb
***Bam*HI/*Eco*RI**	N/A	0.57 kb & 4.3 kb
***Bam*HI/*Sca*I**	N/A	3.9 kb & 7.0 kb
***Bam*HI/*Not*I**	N/A	8.76 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

APPENDIX E

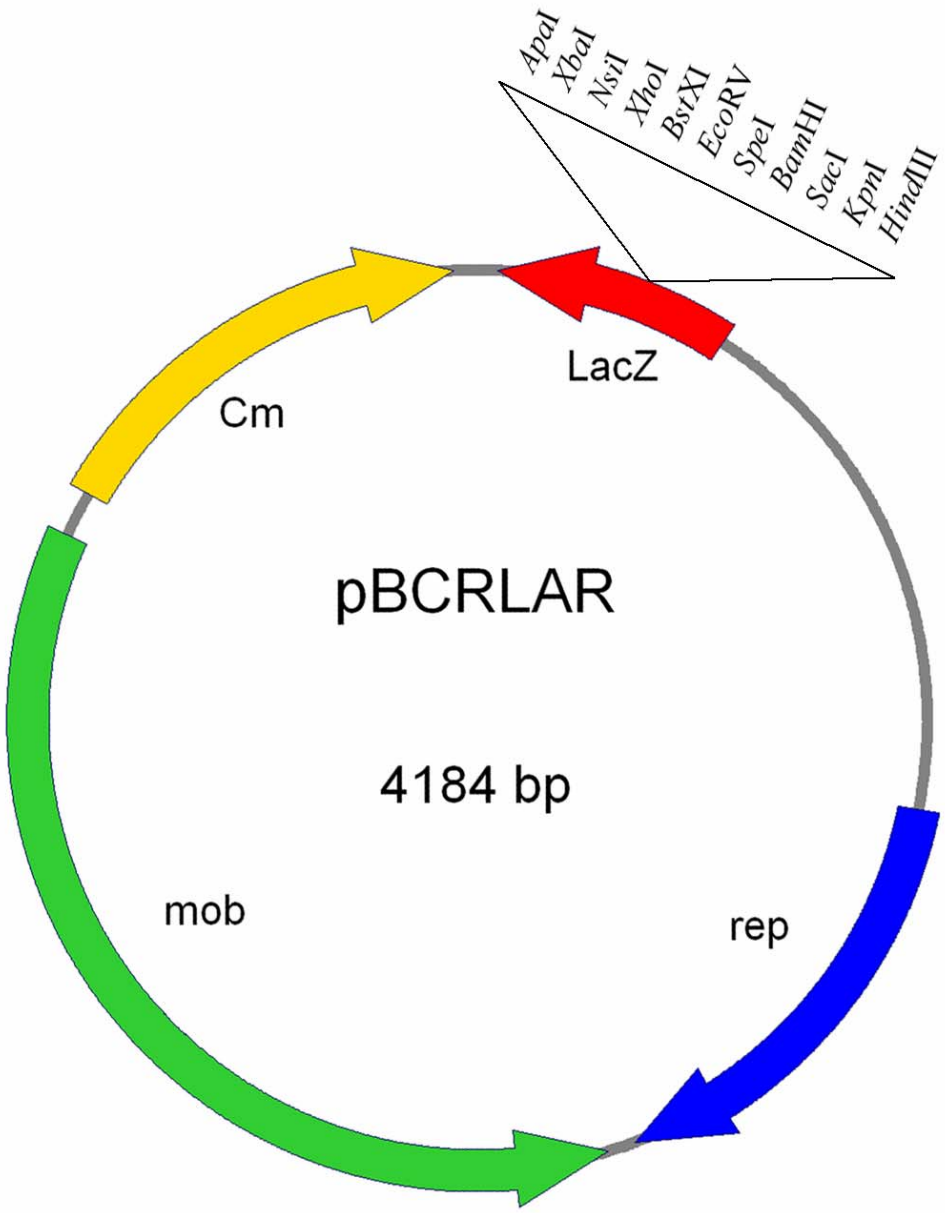


Fig. E-1. Cloning vector pBCRLAR. See Table 3.1 for description.

APPENDIX F

Restriction enzyme and sequence analyses of pFUSE-*ptwB4*

Predicted and obtained results for digestion of pFUSE-*ptwB4* with *Sal*I

Correct insertion yields fragments of sizes 7.2 and 5.2 kb
Incorrect insertion yields fragments of sizes 5.8 and 6.6 kb

Sequencing results obtained from pFUSE-*ptwB4*

tatgaacatcgagatccagacctactggaacgtcgaaacgctctactacgtgctcaacgccgtcgcatcgg
tgatgacgagcggcgggtggcccggcttgatcaagttcgtcttcctcgttgcgttgctcatcgcgatgttc
gcgtacatggggcgcccacggcgacatggcgatgtggttcattcaggcgttcgcgtttgtcacgattctcaa
tatgccgatcgcgcgagtgatgctttccgatcgcacggacctccagccccctcggcaggtcgatcatgtgc
cggtggcgcttgccgcaatcgcgcaggcaagctcgctttcgttcgggttcctgacgcgcgcgtacgaaacc
gcgttcaatgttccggatgatctcggtctcgcgaagggtgacgtaggctttggtcaccgaattctgcggca
ggtgaattccgcagtggtacgcgatcctgcgctccgcgccgacctgatgcagttcttcaaggagtgtacga
agtacgacatcctggacggggcgatcgcgcccagccagatcgtcggtgcgacggacacgtggaacacgatc
ttcagcaataccagccctgcccgcttcgttacatacaacgtgctgacggctcaacccgtcacggatacgtg
ccagaacgtggcgctgcttctcaagccacgagtggacgctgccgtcgcggacgcacaagcattttatggcc
gcaaggcgtttccgcaggtgagctcggacgggatcgctcagcagatgttcctctcgaccgtctcgacgtcc
tactcgtggctcctcgacgcatcgcagtcagcctcggatgccatgaagcaggcgatgttcaacaacatctg
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tcgcggaggcagaagcggcgcgccaggcgaacgggtcgaatagcgccctaagcctgctcggccaagaaacg
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cgtggtaccgcaggagaaggcgaagtacgtcctcggcggctatttcatgatcctcgtgtggatcgggttgt
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ctgtcggccggcgtgccgttccagctctcctcggcgttcgattcgactctggtcgacgagcaggcgatgat
cggatacatggtggtgctcgtgccgttcatcgcgggcgcaatcgtcaagatgggcgacggcgcgatcttcg
gcctggccgatcgagctttgagtggcttcgccaccgccggttcgcgggccggcgccgcaattgcgtccggc
aactacagcatgggacaggccggccttgacaccgcatcggtcaacactacgacgatgcagaagttcgacgg
caacatgctgatgaattccggcatgaagacggtccagctttcggacggttccacgatgtcggtatccagca
acggccgcgctgcgtatcagaagttcgcgaaccaccttctgacgtcgatgcagctcgaagatgcccagaac
tctggtcacagtttcgaccggtttagcggtacaacatcgacgtcgggctggagcagttcgacgcgttcggg
aagttcggtcggcagcagcgagagtttcgggcacgcgtccgagcgcggtgcgactcagagcatcgggtcgg
aaactgcgacgacgatgtccggcggtaccagcggccgcgcaatgcacgacgagagcgtcggacaaaccaat
cagatcgggacacggtttggtacgggcatcggtgccaccgacagtattcaacttcacgccggcgggagtgg
tcaccttggaatcgatgcttcccagggcggcgccactccgcgcgcaccgggcgccggcgctagtgaccgcg
acgcgcgtcgggttgccaacgctatgaagcaaggggcgcgtcggcagagcaaatcgattccgcgatgcag
aacatgcgcggcaaccaagcggcggcggaaggcgcaccttctgtccggcgcggcggtggcgcttcgctgag
ccttggggcgcaagtaaccggtattcgaaactacacggccggccacgatcgcgactggtcgtcgcaatcgg
accatcgcactggtgattcggtatcgtcgggcagcgaagttagccaaacgggttcgtccagcacacgtaac
gcaactggtacccaatcgtcgcaatcggatcgccatgattcgtcggcaactcggtctagcttttcggaaag
cgcgttccagcgcgagcatggcgtgcacagcgaggctggctcgcgttccacttcgtcagacggcaattcga
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gtttcggcgacgcgcatgtggcagatgtccgaggacaaggtaatcggcatggccgccgagtatctggactg
gaaagcgatggcgcagaacatgcgcggcccacaaggagccggagccaatgacgatgccgtcgcccccaagt
tggcccacgactttggatcgtcgtcgcgtggtcgagctggtgccggccacacctccgctggcgccggcgat
ttcggaggccgggtcaaggcaacgggcttctcctcgaccgatccggtcaagccgaacatgacgtcgccgac
cgaaatctcggcagcacagcacgcgcttgatcaaggtcgcggcatcggccaacgcgcgaaggattttgacg
cggacgtgcacaagcacgcgagcccggaccgggcgcttggcgaggggcgcgtgacccagaccgaggtgaat
gcggcgaacgaggatcgggatcttgccgataccgtccgccggtcatggaatgccccgcgccgggcgctcgg
gatgaaggaatacgacgactacgacacgaaggatgtcccgccggagatcaagaaatga

Note: Primers and start codon are highlighted
Note: Vector sequence has been removed.

APPENDIX G

Restriction enzyme and sequence analyses of pDrive-GST

Predicted and obtained results for digestion of pDrive-GST with *Bam*HI

Correct insertion yields fragments of sizes 0.7 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.05 and 4.5 kb

Sequencing results obtained from pDrive-GST

atgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatct
tgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaat
tgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatc
atacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgct
tgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctca
aagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatat
ttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatgga
cccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattg
ataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggc
gaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgca
tcgtgactga

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed.

APPENDIX H

Restriction enzyme and sequence analyses of pDrive-*ptwE1*-GST

Predicted and obtained results for digestion of pDrive-*ptwE1*-GST with *Bsa*AI/*Xba*I

Correct insertion yields fragments of sizes 1.1 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.8 and 4.2 kb

Sequencing results obtained from pDrive-*ptwE1*-GST

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
gcgcggcgaccggctccgtgatctttatcgatcacggccgttggtccggcaccgtggatatgtggacaatg
ctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgccgtccccgaacctgtat
attccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctccgctccgctccgaggat
gtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttctttttggaatatcttg
aagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaattg
ggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatcat
acgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgcttg
aaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctcaaa
gttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatattt
aaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatggacc
caatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattgat
aagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggcga
ccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgcatc
gtgactga

Note: Start codon is highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-GST-*ptwE1*

Predicted and obtained results for digestion of pDrive-GST-*ptwE1* with *Bsa*AI/*Xba*I

Correct insertion yields fragments of sizes 0.4 and 4.6 kb
Incorrect insertion yields fragments of sizes 0.04 and 5.0 kb

Sequencing results obtained from pDrive-GST-*ptwE1*

atgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatct
tgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaat
tgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatc
atacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgct
tgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctca
aagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatat
ttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatgga
cccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattg
ataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggc
gaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgca
tcgtgacatgcattccgcaaatcttcgactccccgcccgacaccgacgtcgagcggcacaccgaccgtctg
cacacgcccaaaaatttcccgacgccgcattgaccacagatccaccgcgccccgcgctcacaccggtgttg
ccacacactgacgtgtggacaacaccgcgcggcgaccggctccgtgatctttatcgatcacggccgttggt
ccggcaccgtggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgccca
cactaccgccgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcact
gaacagctccgctccgctccgaggtgaagatggcgaaaatccaccgctcacgatccaaaccacgccgtcgc
ttttccccgattacgccgattcccttgagcgggaaccgccgtaccttaccgggtgaaggct

Note: Start codon is highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*ptwE1*::Cm-GST

Predicted and obtained results for digestion of pDrive-*ptwE1*::Cm-GST with *Bsa*AI/*Xba*I

Correct insertion yields fragments of sizes 2.1 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.8 and 5.2 kb

Sequencing results obtained from pDrive-*ptwE1*::Cm-GST

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
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tgctcaatgtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaata
agcacaagttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatg
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tgaaacgttttcatcgctctggagtgaataccacgacgatttccggcagtttctacacatatattcgcaag
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gccaatccctgggtgagtttcaccagttttgatttaaacgtggccaatatggacaacttcttcgcccccgt
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ccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactgcgatgagtggcagggc
ggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataagtgataat
aagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaa
tgcctgaggccagtttgctcaggctctccccgtggaggtaataattgacgatatccgttggtccggcaccg
tggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgc
cgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctc
cgctccgctccgaggatgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgact
tcttttggaatatcttgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaa
acaaaaagtttgaattgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaaca
cagtctatggccatcatacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgc
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actttgaaactctcaaagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgttta
tgtcataaaacatatttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgt
tgttttatacatggacccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaacgtattgaag
ctatcccacaaattgataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagcc
acgtttggtggtggcgaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcg
actcgagcggccgcatcgtgactga

Note: Start codon and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-GST-*ptwE1*::Cm

Predicted and obtained results for digestion of pDrive-GST-*ptwE1*::Cm with *Bsa*AI/*Xba*I

Correct insertion yields fragments of sizes 1.4 and 4.6 kb
Incorrect insertion yields fragments of sizes 0.04 and 6.0 kb

Sequencing results obtained from pDrive-GST-*ptwE1*::Cm

atgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatct
tgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaat
tgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatc
atacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgct
tgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctca
aagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatat
ttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatgga
cccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattg
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tcgtgacatgcattccgcaaatcttcgactccccgcccgacaccgacgtcgagcggcacaccgaccgtctg
cacacgcccaaaaatttcccgacgccgcattgaccacagatccaccgcgccccgcgctcacaccggtgttg
ccacacactgacgtgtggacaacaccgcgcggcgaccggctccgtgatctttatcgatcacgggtaagagg
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ctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaaa
gaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatattacggc
ctttttaaagaccgtaaagaaaaataagcacaagttttatccggcctttattcacattcttgcccgcctga
tgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgttcaccct
tgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaataccacgacgatttccg
gcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaaagggt
ttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaacgtggcc
aatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctgat
gccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaattac
aacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcct
ggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtcg
tcggttcagggcagggtcgttaaatagccgcttatgtctattgctggtttaccggtttattgactaccgga
agcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttgctcaggctctccccgtggaggtaataat
tgacgatatccgttggtccggcaccgtggatatgtggacaatgctcaccggtccaccaccgcgtcaccttc
tgctactgtcctgcccacactaccgccgtccccgaacctgtatattccctgctgcctccacctcccttctt
ccttccgacgttgcactgaacagctccgctccgctccgaggtgaagatggcgaaaatccaccgctcacgat
ccaaaccacgccgtcgctttttccccgattacgccgattcccttgagcgggaaccgccgtaccttaccggg
tgaaggct

Note: Start codon and Cm gene are highlighted.
Note: Vector sequence has been removed.

APPENDIX I

Restriction enzyme and sequence analyses of pJQ200SK-*ptwE1*-GST

Predicted and obtained results for digestion of pJQ200SK-*ptwE1*-GST with *Cla*I/*Xba*I

Correct insertion yields fragments of sizes 0.2 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.9 and 3.2 kb

Sequencing results obtained from pJQ200SK -*ptwE1*-GST

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
gcgcggcgaccggctccgtgatctttatcgatcacggccgttggtccggcaccgtggatatgtggacaatg
ctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgccgtccccgaacctgtat
attccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctccgctccgctccgaggatg
gtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatcttg
aagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaattg
ggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatcat
acgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgcttg
aaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctcaaa
gttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatattt
aaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatggacc
caatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattgat
aagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggcga
ccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgcatc
gtgactga

Note: Start codon is highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-GST-*ptwE1*

Predicted and obtained results for digestion of pJQ200SK-GST-*ptwE1* with *Cla*AI/*Xba*I

Correct insertion yields fragments of sizes 0.9 and 3.3 kb
Incorrect insertion yields fragments of sizes 0.3 and 3.9 kb

Sequencing results obtained from pJQ200SK-GST-*ptwE1*

atgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatct
tgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaat
tgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatc
atacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgct
tgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctca
aagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatat
ttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatgga
cccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattg
ataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggc
gaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgca
tcgtgacatgcattccgcaaatcttcgactccccgcccgacaccgacgtcgagcggcacaccgaccgtctg
cacacgcccaaaaatttcccgacgccgcattgaccacagatccaccgcgccccgcgctcacaccggtgttg
ccacacactgacgtgtggacaacaccgcgcggcgaccggctccgtgatctttatcgatcacggccgttggt
ccggcaccgtggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgccca
cactaccgccgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcact
gaacagctccgctccgctccgaggtgaagatggcgaaaatccaccgctcacgatccaaaccacgccgtcgc
ttttccccgattacgccgattcccttgagcgggaaccgccgtaccttaccgggtgaaggct

Note: Start codon is highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-*ptwE1*::Cm-GST

Predicted and obtained results for digestion of pJQ200SK-*ptwE1*::Cm-GST with *Cla*AI/*Xba*I

Correct insertion yields fragments of sizes 0.2 and 4.9 kb
Incorrect insertion yields fragments of sizes 1.9 and 3.2 kb

Sequencing results obtained from pJQ200SK-*ptwE1*::Cm-GST

ctgttaggtgaaaatccggcgcgcgggaaagtcacacgtgtttgcggaatgcatgcattccgcaaatcttc
gactccccgcccgacaccgacgtcgagcggcacaccgaccgtctgcacacgcccaaaaatttcccgacgcc
gcattgaccacagatccaccgcgccccgcgctcacaccggtgttgccacacactgacgtgtggacaacacc
gcgcggcgaccggctccgtgatctttatcgatcacgggtaagaggttccaactttcaccataatgaaataa
gatcactaccgggcgtatttttgagttatcgagattttcaggagctaaggaagctaaaatggagaaaaaa
atcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcagt
tgctcaatgtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaata
agcacaagttttatccggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatg
gcaatgaaagacggtgagctggtgatatgggatagtgttcacccttgttacaccgttttccatgagcaaac
tgaaacgttttcatcgctctggagtgaataccacgacgatttccggcagtttctacacatatattcgcaag
atgtggcgtgttacggtgaaaacctggcctatttccctaaagggtttattgagaatatgtttttcgtctca
gccaatccctgggtgagtttcaccagttttgatttaaacgtggccaatatggacaacttcttcgcccccgt
tttcaccatgggcaaatattatacgcaaggcgacaaggtgctgatgccgctggcgattcaggttcatcatg
ccgtttgtgatggcttccatgtcggcagaatgcttaatgaattacaacagtactgcgatgagtggcagggc
ggggcgtaatttttttaaggcagttattggtgcccttaaacgcctggtgctacgcctgaataagtgataat
aagcggatgaatggcagaaattcgaaagcaaattcgacccggtcgtcggttcagggcagggtcgttaaata
gccgcttatgtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaa
tgcctgaggccagtttgctcaggctctccccgtggaggtaataattgacgatatccgttggtccggcaccg
tggatatgtggacaatgctcaccggtccaccaccgcgtcaccttctgctactgtcctgcccacactaccgc
cgtccccgaacctgtatattccctgctgcctccacctcccttcttccttccgacgttgcactgaacagctc
cgctccgctccgaggatgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgact
tcttttggaatatcttgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaa
acaaaaagtttgaattgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaaca
cagtctatggccatcatacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgc
agagatttcaatgcttgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaag
actttgaaactctcaaagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgttta
tgtcataaaacatatttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgt
tgttttatacatggacccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaag
ctatcccacaaattgataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagcc
acgtttggtggtggcgaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcg
actcgagcggccgcatcgtgactga

Note: Start codon and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-GST-*ptwE1*::Cm

Predicted and obtained results for digestion of pJQ200SK-GST-*ptwE1*::Cm with *Cla*AI/*Xba*I

Correct insertion yields fragments of sizes 0.9 and 4.3 kb
Incorrect insertion yields fragments of sizes 1.3 and 3.9 kb

Sequencing results obtained from pJQ200SK-GST-*ptwE1*::Cm

atgtcccctatactaggttattggaaaattaagggccttgtgcaacccactcgacttcttttggaatatct
tgaagaaaaatatgaagagcatttgtatgagcgcgatgaaggtgataaatggcgaaacaaaaagtttgaat
tgggtttggagtttcccaatcttccttattatattgatggtgatgttaaattaacacagtctatggccatc
atacgttatatagctgacaagcacaacatgttgggtggttgtccaaaagagcgtgcagagatttcaatgct
tgaaggagcggttttggatattagatacggtgtttcgagaattgcatatagtaaagactttgaaactctca
aagttgattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaaacatat
ttaaatggtgatcatgtaacccatcctgacttcatgttgtatgacgctcttgatgttgttttatacatgga
cccaatgtgcctggatgcgttcccaaaattagtttgttttaaaaaacgtattgaagctatcccacaaattg
ataagtacttgaaatccagcaagtatatagcatggcctttgcagggctggcaagccacgtttggtggtggc
gaccatcctccaaaatcggatctggttccgcgtggatccccggaattcccgggtcgactcgagcggccgca
tcgtgacatgcattccgcaaatcttcgactccccgcccgacaccgacgtcgagcggcacaccgaccgtctg
cacacgcccaaaaatttcccgacgccgcattgaccacagatccaccgcgccccgcgctcacaccggtgttg
ccacacactgacgtgtggacaacaccgcgcggcgaccggctccgtgatctttatcgatcacgggtaagagg
ttccaactttcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagattttcaggag
ctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaaa
gaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatattacggc
ctttttaaagaccgtaaagaaaaataagcacaagttttatccggcctttattcacattcttgcccgcctga
tgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgttcaccct
tgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaataccacgacgatttccg
gcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaaagggt
ttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaacgtggcc
aatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctgat
gccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaattac
aacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcct
ggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtcg
tcggttcagggcagggtcgttaaatagccgcttatgtctattgctggtttaccggtttattgactaccgga
agcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttgctcaggctctccccgtggaggtaataat
tgacgatatccgttggtccggcaccgtggatatgtggacaatgctcaccggtccaccaccgcgtcaccttc
tgctactgtcctgcccacactaccgccgtccccgaacctgtatattccctgctgcctccacctcccttctt
ccttccgacgttgcactgaacagctccgctccgctccgaggtgaagatggcgaaaatccaccgctcacgat
ccaaaccacgccgtcgctttttccccgattacgccgattcccttgagcgggaaccgccgtaccttaccggg
tgaaggct

Note: Start codon and Cm gene are highlighted.
Note: Vector sequence has been removed.

APPENDIX J

Restriction enzyme and sequence analyses of pDrive-*bcvirB2*

Predicted and obtained results for digestion of pDrive-*bcvirB2* with *Bam*HI/*Eco*RV

Correct insertion yields fragments of sizes 0.3 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.1 and 4.1 kb

Sequencing results obtained from pDrive-*bcvirB2*

gatggagtgcattatgaaatctgtattcctaattagcataagcgacgtgagactgagaaaaacgtgtgttg
cgtggatctcgtcgaattggcgccgcattgcagtgctcggcgtgctcgtcatggcaacccctccggcatct
gcacaactcagtcaggtcaataccttgctgatcactattcagaacacgttgctcggtgtgggcggggtggt
gtgttcgatttccatcatctgggccggcttcaaaatgatgtttcagcacgcccggtttggcgatatcgcgc
atgttttcgtcggtggccttttcgttggctgcgcaacggtcattgctggcatgttgattccgacggcctaa

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*bcvirD4*

Predicted and obtained results for digestion of pDrive-*bcvirD4* with *Bam*HI

Correct insertion yields fragments of sizes 1.7 and 4.1 kb
Incorrect insertion yields fragments of sizes 0.2 and 5.6 kb

Sequencing results obtained from pDrive-*bcvirD4*

catggtacgacggccgttttccgaatatggggagacgtgtggccggtgcgggcctcatggggttgcttgtc
gccttcggcggccctgcgcttgccgtgtatacgtggctggaacaagcaggccgcagacccttgtatggcga
cgcgcgctttgcgaacgatgcggagattcgtcgggcggggctgttatgagacagattgcacccgcgaagtc
tcaaccgacgcttgtggtgggccgttggcgtggccgctatctgcgctttgccggtcagcaattcgtgctac
ttgccgcgcccgctcgttcaggaaagggcgtggggattgtgatcccgaatctgctgagctattccgactcc
gtggtcgtgcttgatatcaaacaggaaaattttcgtctcacggcaggctttcggcgcgcgcacggacaagc
cgtgtacctgttcaatccgtttgcagaagatggctgcacgcaccgctacaacccgttgtcggtcattgcag
acgacatgtttcgcgtcggcgacattctggcgatcggttatgtgttgtatccggccggcgggcatgacgaa
ttctggaaagaccaggcacgcaaccttctgttgggactggtcctgctgctgtgcgatttgcgtgaggcgcg
tcgtgctggcgcgagcggggtgcccgactacccgatcacaatgggcgaggtattgcggcaatcgtcgggaa
atggtcagccggtcaaaacctacttgaaccggatgcttatacagcatcgacagtacttgagccgcgcctgt
attgatgcactgaatcgattcctcgcgaatgacgacaaggtcctggcgagcattctggccacttttaatgc
gccactgacgatctgggccaacccgattgttgacgcggcgacgagcgcgaacgactttgatctgcgagacg
tcagacgccgcaagatgtccgtatatcttggcgtgacgcccgatcacttaagtgaggcggcgatactgatg
aatctgatgttctcgcagctcgtgaatctgaacacgaaggagttggcagaggacaatccggcattgaagta
tcaatgcctgctgctgctcgacgagatgacggcaatcggaaaaatccagattatcgcgcgtgcggtggcct
atatggcgggctacaacctgcgcctactctcgatcgtgcagtccgactcgcagctcgagtctgtgtatggc
cgagctgacgcgaggaccattgtcacgaatcatgcgatgcagattcttttcgccccgcgcgaacagaagga
cgccaatgcctactccgagatgctcggcacgcgcacggagcgatcgcgcaccacgagccgatcgaatggca
tgttcggtgcgcgcggcggtgcgagcgagagcttttccgatcaacgtcgtgcgctgatgttgccgcaggag
atcaaggagctcgcgcgcgacaaggaaatcattcttctcgaaaataccaagccgattttggcagaccggat
ctgctattggcgcgatcgtgcatttacttcacgggtaatggacgcgccagcggtgcctgcactcgatctcg
tgagatttggtgcacaaatcgagcagcggctgcgcgaactgagcgatgacgacgtcgatgaacagacgggc
gagctgacgcatgtgcgagcggactatctggaacttgtacacgcctgggatccgcgcgagctgccgcaggc
gctggacaatgtcagttgcgaggaggcagccgcgtatgtggatcggcatttcacgctgctgggcgtgccgg
ccgaggacgtagcgcgcgccggacgtcggttggcgcgcggcgaagatggcatgactgatactgagacgggc
gcgcgcaagcgcgacccgagccccgtcgcccgggcaagtggtggtgttcaatga

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed

Restriction enzyme and sequence analyses of pDrive-*bcvirP3*

Predicted and obtained results for digestion of pDrive-*bcvirP3* with *Sac*I

Correct insertion yields fragments of sizes 0.1 and 4.3 kb
Incorrect insertion yields fragments of sizes 0.4 and 4.0 kb

Sequencing results obtained from pDrive-*bcvirP3*

catgggatcgcgaaaccgagcgcgcattgcggtcgaactgggtgcgctaaagcctttgtggcaggcacgat
gtgcgcagaacggggtgagcgccggtgagggcatcaggcggctcgtggccgaagcactggacattgcccat
gacccgccggacacggccgcaatactttcgacgccgagcgagccgtttgtacggatcggcgtgggtctgat
gccggccgagttggaatgtatcaaagggctggcacatgtgcaggggttcaccgccaatcgatggattgctg
cgttaattcgcgcccatctgaccggagaaccgcaactgggaaatcgggagatgatgctgcttgcggaatcg
aatcggcagcttgctgcgattcgtacgcggctgggtgagctcgcgcgcaacacgggcgagggccaccacgt
gcccgagtgggagcacgcgcgcgccatgatccttacacatctgcgttttgtcgcacagttgctgcaaacca
acctggaccgatggagccgttga

Note: Primers and start codon are highlighted.
Note: Vector sequence has been removed.

APPENDIX K

Restriction enzyme and sequence analyses of pDrive-*bcvirB2*::Cm

Predicted and obtained results for digestion of pDrive-*bcvirB2*::Cm with *Bam*HI/*Eco*RV

Correct insertion yields fragments of sizes 1.3 and 3.9 kb
Incorrect insertion yields fragments of sizes 0.1 and 5.1 kb

Sequencing results obtained from pDrive-*bcvirB2*::Cm

gatggagtgcattatgaaatctgtattcctaattagcataagcgacgtgagactgagaaaaacgtgtgttg
cgtggatctcgtcgaattggcgccgcattgcagtgctcggcgtgctcgtcatggcaacccctccggcatct
gcacaactcagtcaggtcaataccttgctgatcactattcagaacacgttgctcggtgtgggcggggtggt
gtgttcgatttccatcatctgggccggcttcaaaatgatgtttcagcacgcccggtttggcgatgtaagag
gttccaactttcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagattttcagga
gctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaa
agaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatattacgg
cctttttaaagaccgtaaagaaaaataagcacaagttttatccggcctttattcacattcttgcccgcctg
atgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgttcaccc
ttgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaataccacgacgatttcc
ggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaaaggg
tttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaacgtggc
caatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctga
tgccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaatta
caacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcc
tggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtc
gtcggttcagggcagggtcgttaaatagccgcttatgtctattgctggtttaccggtttattgactaccgg
aagcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttgctcaggctctccccgtggaggtaataa
ttgacgatatatcgcgcatgttttcgtcggtggccttttcgttggctgcgcaacggtcattgctggcatgt
tgattccgacggcctaa

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*bcvirD4::*Cm

Predicted and obtained results for digestion of pDrive-*bcvirD4*::Cm with *Bam*HI

Correct insertion yields fragments of sizes 2.7 and 4.1 kb
Incorrect insertion yields fragments of sizes 0.2 and 6.6 kb

Sequencing results obtained from pDrive-*bcvirD4*::Cm

catggtacgacggccgttttccgaatatggggagacgtgtggccggtgcgggcctcatggggttgcttgtcgccttcgg
cggccctgcgcttgccgtgtatacgtggctggaacaagcaggccgcagacccttgtatggcgacgcgcgctttgcgaac
gatgcggagattcgtcgggcggggctgttatgagacagattgcacccgcgaagtctcaaccgacgcttgtggtgggccg
ttggcgtggccgctatctgcgctttgccggtcagcaattcgtgctacttgccgcgcccgctcgttcaggaaagggcgtg
gggattgtgatcccgaatctgctgagctattccgactccgtggtcgtgcttgatatcaaacaggaaaattttcgtctca
cggcaggctttcggcgcgcgcacggacaagccgtgtacctgttcaatccgtttgcagaagatggctgcacgcaccgcta
caacccgttgtcggtcattgcagacgacatgtttcgcgtcggcgacattctggcgatcggttatgtgttgtatccggcc
ggcgggcatgacgaattctggaaagaccaggcacgcaaccttctgttgggactggtcctgctgctgtgcgatttgcgtg
aggcgcgtcgtgctggcgcgagcggggtgcccgactacccgatcacaatgggcgaggtattgcggcaatcgtcgggaaa
tggtcagccggtcaaaacctacttgaaccggatgcttatacagcatcgacagtgtaagaggttccaactttcaccataa
tgaaataagatcactaccgggcgtatttttgagttatcgagatttcaggagctaaggaagctaaaatggagaaaaaa
atcactggatataccaccgttgatatatcccaatggcatcgtaaagaacattttgaggcatttcagtcagttgctcaat
gtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaataagcacaagttttatcc
ggcctttattcacattcttgcccgcctgatgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtg
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acgacgatttccggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaa
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cgcggcgacgagcgcgaacgactttgatctgcgagacgtcagacgccgcaagatgtccgtatatcttggcgtgacgccc
gatcacttaagtgaggcggcgatactgatgaatctgatgttctcgcagctcgtgaatctgaacacgaaggagttggcag
aggacaatccggcattgaagtatcaatgcctgctgctgctcgacgagatgacggcaatcggaaaaatccagattatcgc
gcgtgcggtggcctatatggcgggctacaacctgcgcctactctcgatcgtgcagtccgactcgcagctcgagtctgtg
tatggccgagctgacgcgaggaccattgtcacgaatcatgcgatgcagattcttttcgccccgcgcgaacagaaggacg
ccaatgcctactccgagatgctcggcacgcgcacggagcgatcgcgcaccacgagccgatcgaatggcatgttcggtgc
gcgcggcggtgcgagcgagagcttttccgatcaacgtcgtgcgctgatgttgccgcaggagatcaaggagctcgcgcgc
gacaaggaaatcattcttctcgaaaataccaagccgattttggcagaccggatctgctattggcgcgatcgtgcattta
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cgaactgagcgatgacgacgtcgatgaacagacgggcgagctgacgcatgtgcgagcggactatctggaacttgtacac
gcctgggatccgcgcgagctgccgcaggcgctggacaatgtcagttgcgaggaggcagccgcgtatgtggatcggcatt
tcacgctgctgggcgtgccggccgaggacgtagcgcgcgccggacgtcggttggcgcgcggcgaagatggcatgactga
tactgagacgggcgcgcgcaagcgcgacccgagccccgtcgcccgggcaagtggtggtgttcaatga

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pDrive-*bcvirP3*::Cm

Predicted and obtained results for digestion of pDrive-*bcvirP3*::Cm with *Sac*I

Correct insertion yields fragments of sizes 0.1 and 5.3 kb
Incorrect insertion yields fragments of sizes 1.4 and 4.0 kb

Sequencing results obtained from pDrive-*bcvirP3*::Cm

catgggatcgcgaaaccgagcgcgcattgcggtcgaactgggtgcgctaaagcctttgtggcaggcacgat
gtgcgcagaacggggtgagcgccggtgagggcatcaggcggctcgtggccgaagcactggacattgcccat
gacccgccggacacggccgcaatactttcgacgccgagcgagccgtttgtacggatcggcgtgggtctgat
gccggccgagttggaatgtatcaaagggctggcacatgtgcaggggttcaccgccaatcgatggattgctg
cgttaattcgcgcccatctgaccggagaaccgcaactgggaaatcgggagatgatgctgcttgcggaatcg
aatcggcagcttgctgcgattcgtacgcggctgggtgagctcgcgcgcaacacgggcgagggccaccacgt
aagaggttccaactttcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagatttt
caggagctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcat
cgtaaagaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatat
tacggccttttaaagaccgtaaagaaaaataagcacaagttttatccggcctttattcacattcttgccc
gcctgatgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgtt
cacccttgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaataccacgacga
tttccggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttcccta
aagggtttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaac
gtggccaatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggt
gctgatgccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatg
aattacaacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaa
acgcctggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacc
cggtcgtcggttcagggcagggtcgttaaatagccgcttatgtctattgctggtttaccggtttattgact
accggaagcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttgctcaggctctccccgtggaggt
aataattgacgatatgtgcccgagtgggagcacgcgcgcgccatgatccttacacatctgcgttttgtcgc
acagttgctgcaaaccaacctggaccgatggagccgttga

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

APPENDIX L

Restriction enzyme and sequence analyses of pJQ200SK-*bcvirB2*::Cm

Predicted and obtained results for digestion of with pJQ200SK-*bcvirB2*::Cm with *Sma*I

Correct insertion yields fragments of sizes 0 and 5.9 kb
Incorrect insertion yields fragments of sizes 2.9 and 3.0 kb

Sequencing results obtained from pJQ200SK-*bcvirB2*::Cm

gatggagtgcattatgaaatctgtattcctaattagcataagcgacgtgagactgagaaaaacgtgtgttg
cgtggatctcgtcgaattggcgccgcattgcagtgctcggcgtgctcgtcatggcaacccctccggcatct
gcacaactcagtcaggtcaataccttgctgatcactattcagaacacgttgctcggtgtgggcggggtggt
gtgttcgatttccatcatctgggccggcttcaaaatgatgtttcagcacgcccggtttggcgatgtaagag
gttccaactttcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagattttcagga
gctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcatcgtaa
agaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatattacgg
cctttttaaagaccgtaaagaaaaataagcacaagttttatccggcctttattcacattcttgcccgcctg
atgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgttcaccc
ttgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaataccacgacgatttcc
ggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaaaggg
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caatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctga
tgccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatgaatta
caacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaaacgcc
tggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacccggtc
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aagcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttgctcaggctctccccgtggaggtaataa
ttgacgatatatcgcgcatgttttcgtcggtggccttttcgttggctgcgcaacggtcattgctggcatgt
tgattccgacggcctaa

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-*bcvirD4*::Cm

Predicted and obtained results for digestion of with pJQ200SK-*bcvirD4*::Cm with *Bam*HI

Correct insertion yields fragments of sizes 2.7 and 3.2 kb
Incorrect insertion yields fragments of sizes 0.2 and 5.7 kb

Sequencing results obtained from pJQ200SK-*bcvirD4*::Cm

catggtacgacggccgttttccgaatatggggagacgtgtggccggtgcgggcctcatggggttgcttgtcgccttcgg
cggccctgcgcttgccgtgtatacgtggctggaacaagcaggccgcagacccttgtatggcgacgcgcgctttgcgaac
gatgcggagattcgtcgggcggggctgttatgagacagattgcacccgcgaagtctcaaccgacgcttgtggtgggccg
ttggcgtggccgctatctgcgctttgccggtcagcaattcgtgctacttgccgcgcccgctcgttcaggaaagggcgtg
gggattgtgatcccgaatctgctgagctattccgactccgtggtcgtgcttgatatcaaacaggaaaattttcgtctca
cggcaggctttcggcgcgcgcacggacaagccgtgtacctgttcaatccgtttgcagttgatggctgcacgcaccgcta
caacccgttgtcggtcattgcagacgacatgtttcgcgtcggcgacattctggcgatcggttatgtgttgtatccggcc
ggcgggcatgacgaattctggaaagaccaggcacgcaaccttctgttgggactggtcctcgtgctgtgcgatttgcgtg
aggcgcgtcgtgctggcgcgagcggggtgcccgactacccgatcacaatgggcgaggtattgcggcaatcgtcgggaaa
tggtcagccggtcaaaacctacttgaaccggatgcttatacagcatcgacagtgtaagaggttccaactttcaccataa
tgaaataagatcactaccgggcgtatttttgagttatcgagatttcaggagctaaggaagctaaaatggagaaaaaa
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gtacctataaccagaccgttcagctggatattacggccttttaaagaccgtaaagaaaaataagcacaagttttatcc
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atatgggatagtgttcacccttgttacaccgttttccatgagcaaactgaaacgttttcatcgctctggagtgaatacc
acgacgatttccggcagtttctacacatatattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaa
agggtttattgagaatatgtttttcgtctcagccaatccctgggtgagtttcaccagttttgatttaaacgtggccaat
atggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggtgctgatgccgctggcga
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gtctattgctggtttaccggtttattgactaccggaagcagtgtgaccgtgtgcttctcaaatgcctgaggccagtttg
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cgaatgacgacaaggtcctggcgagcattctggccacttttaatgcgccactgacgatctgggccaacccgattgttga
cgcggcgacgagcgcgaacgactttgatctgcgagacgtcagacgccgcaagatgtccgtatatcttggcgtgacgccc
gatcacttaagtgaggcggcgatactgatgaatctgatgttctcgcagctcgtgaatctgaacacgaaggagttggcag
aggacaatccggcattgaagtatcaatgcctgctgctgctcgacgagatgacggcaatcggaaaaatccagattatcgc
gcgtgcggtggcctatatggcgggctacaacctgcgcctactctcgatcgtgcagtccgactcgcagctcgagtctgtg
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ccaatgcctactccgagatgctcggcacgcgcacggagcgatcgcgcaccacgagccgatcgaatggcatgttcggtgc
gcgcggcggtgcgagcgagagcttttccgatcaacgtcgtgcgctgatgttgccgcaggagatcaaggagctcgcgcgc
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cttcacgggtaatggacgcgccagcggtgcctgcactcgatctcgtgagatttggtgcacaaatcgagcagcggctgcg
cgaactgagcgatgacgacgtcgatgaacagacgggcgagctgacgcatgtgcgagcggactatctggaacttgtacac
gcctgggatccgcgcgagctgccgcaggcgctggacaatgtcagttgcgaggaggcagccgcgtatgtggatcggcatt
tcacgctgctgggcgtgccggccgaggacgtagcgcgcgccggacgtcggttggcgcgcggcgaagatggcatgactga
tactgagacgggcgcgcgcaagcgcgacccgagccccgtcgcccgggcaagtggtggtgttcaatga

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

Restriction enzyme and sequence analyses of pJQ200SK-*bcvirP3*::Cm

Predicted and obtained results for digestion of pJQ200SK-*bcvirD4*::Cm with *Sac*I
Correct insertion yields fragments of sizes 0.1 and 4.4 kb
Incorrect insertion yields fragments of sizes 1.4 and 3.1 kb

Sequencing results obtained from pJQ200SK-*bcvirD4*::Cm

catggatcgcgaaaccgagcgcgcattgcggtcgaactgggtgcgctaaagcctttgtggcaggcacgat
gtgcgcagaacggggtgagcgccggtgagggcatcaggcggctcgtggccgaagcactggacattgcccat
gacccgccggacacggccgcaatactttcgacgccgagcgagccgtttgtacggatcggcgtgggtctgat
gccggccgagttggaatgtatcaaagggctggcacatgtgcaggggttcaccgccaatcgatggattgctg
cgttaattcgcgcccatctgaccggagaaccgcaactgggaaatcgggagatgatgctgcttgcggaatcg
aatcggcagcttgctgcgattcgtacgcggctgggtgagctcgcgcgcaacacgggcgagggccaccacgt
aagaggttccaactttcaccataatgaaataagatcactaccgggcgtatttttgagttatcgagatttt
caggagctaaggaagctaaaatggagaaaaaaatcactggatataccaccgttgatatatcccaatggcat
cgtaaagaacattttgaggcatttcagtcagttgctcaatgtacctataaccagaccgttcagctggatat
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gcctgatgaatgctcatccggaattccgtatggcaatgaaagacggtgagctggtgatatgggatagtgtt
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gtggccaatatggacaacttcttcgcccccgttttcaccatgggcaaatattatacgcaaggcgacaaggt
gctgatgccgctggcgattcaggttcatcatgccgtttgtgatggcttccatgtcggcagaatgcttaatg
aattacaacagtactgcgatgagtggcagggcggggcgtaatttttttaaggcagttattggtgcccttaa
acgcctggtgctacgcctgaataagtgataataagcggatgaatggcagaaattcgaaagcaaattcgacc
cggtcgtcggttcagggcagggtcgttaaatagccgcttatgtctattgctggtttaccggtttattgact
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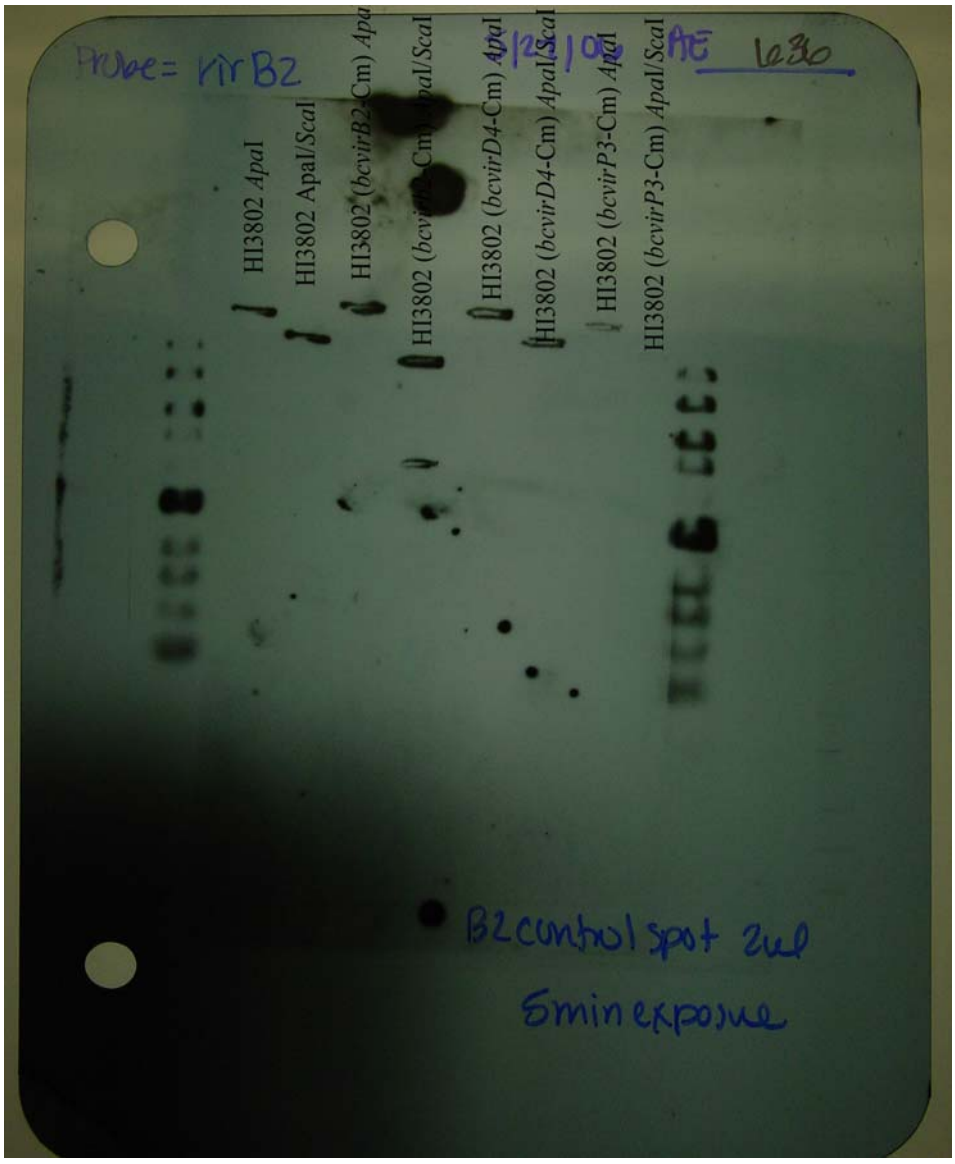
aataattgacgatatgtgcccgagtgggagcacgcgcgcgccatgatccttacacatctgcgttttgtcgc
acagttgctgcaaaccaacctggaccgatggagccgttga

Note: Primers, start codon, and Cm gene are highlighted.
Note: Vector sequence has been removed.

APPENDIX M

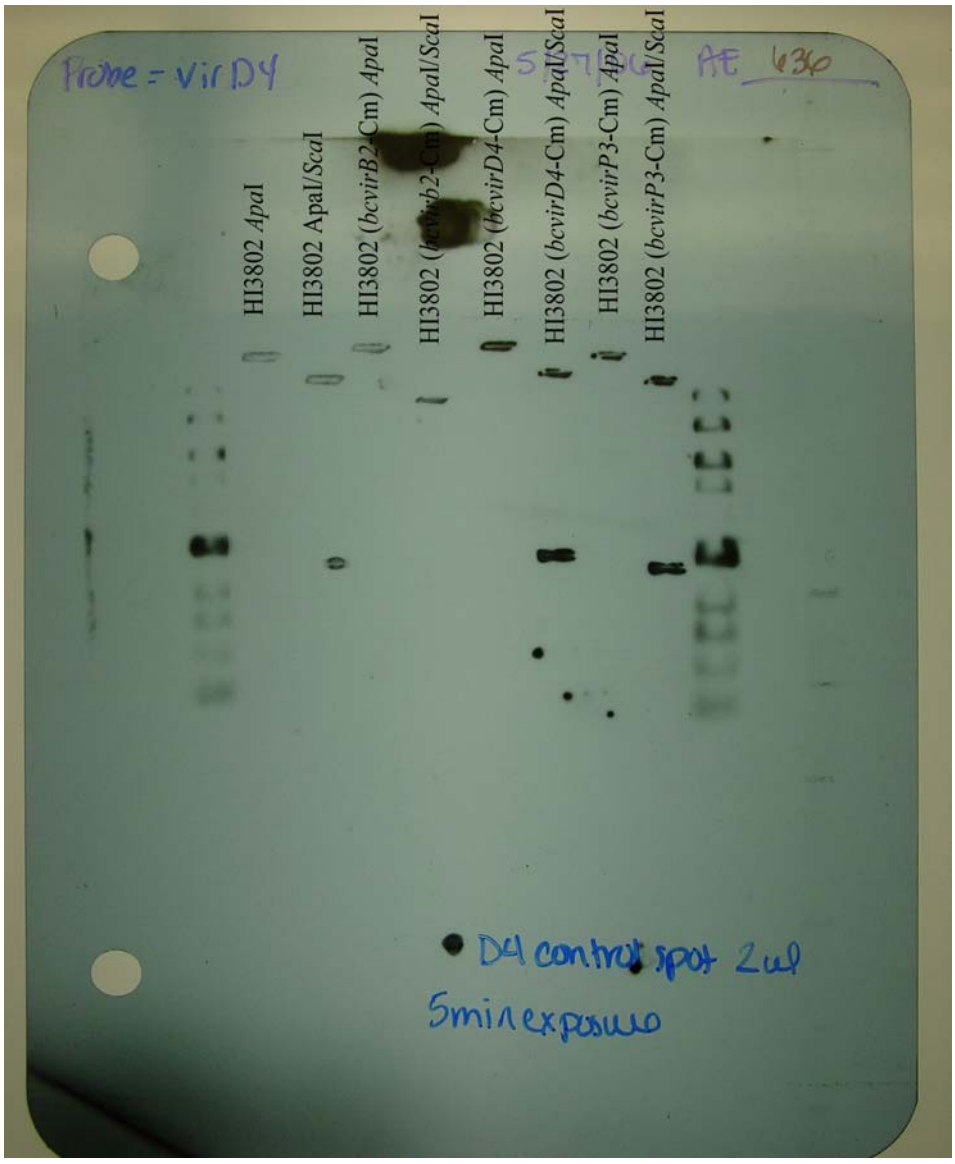
Predicted and obtained results of restriction enzyme digests follow by Southern blot analysis of AU1054, AU1054 (*bcvirB2*::Cm), AU1054 (*bcvirD4*::Cm), and AU1054 (*bcvirP3*::Cm) based on sequence data obtained from the JGI AU1054 sequencing project.

Probe=*bcvirB2* PCR Product				
	AU1054	**AU1054 (*bcvirB2*::Cm)**	**AU1054 (*bcvirD4*::Cm)**	**AU1054 (*bcvirP3*::Cm)**
ApaI	16.4 kb	17.5 kb	17.5 kb	16.4 kb
***Apa*I/*Sca*I**	13.7 kb	10 & 4.6 kb	14.4 kb	13.7 kb



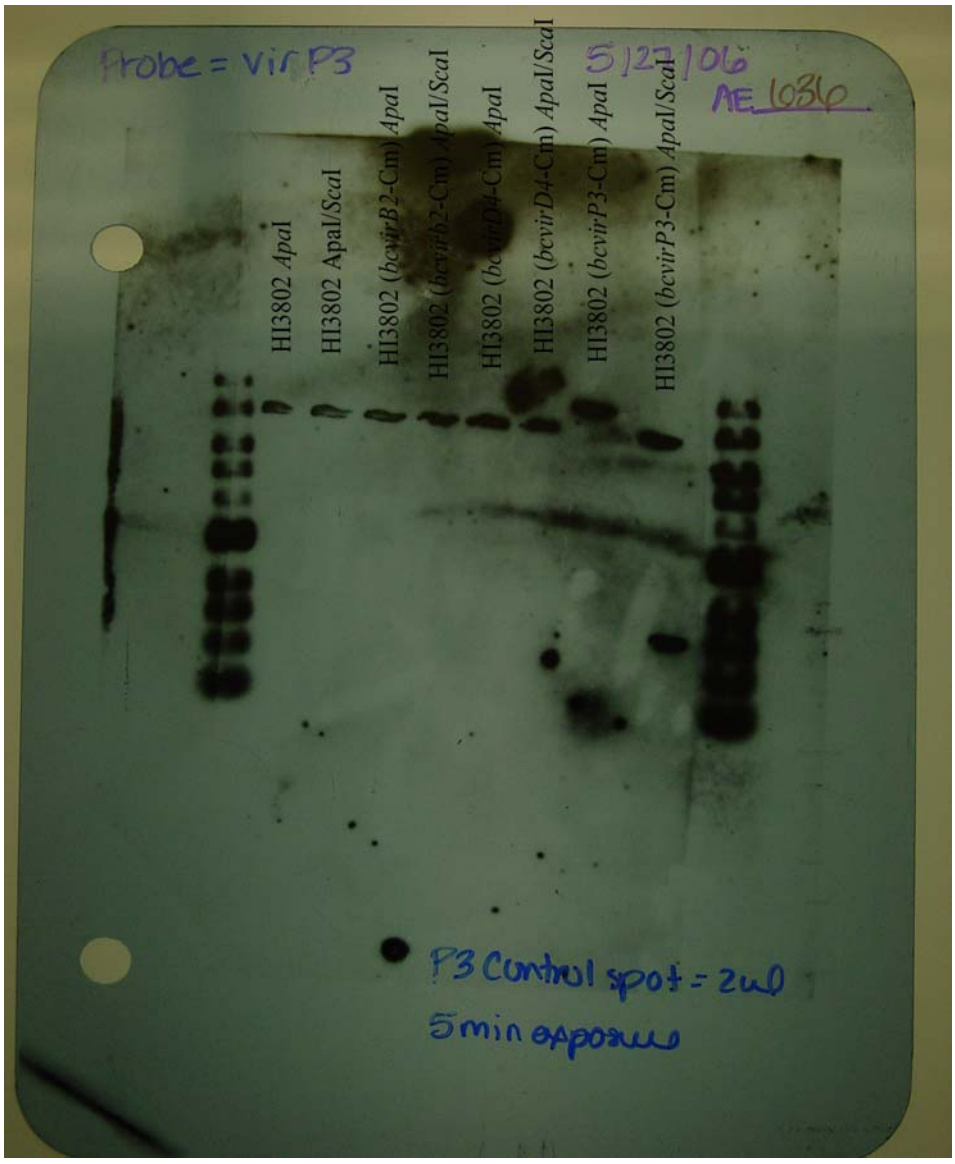
Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

Probe=*bcvirD4* PCR Product				
	AU1054	**AU1054 (*bcvirB2*::Cm)**	**AU1054 (*bcvirD4*::Cm)**	**AU1054 (*bcvirP3*::Cm)**
ApaI	16.4 kb	17.5 kb	17.5 kb	16.4 kb
***Apa*I/*Sca*I**	13.7 & 2.8 kb	10 kb	14.4 & 3.1 kb	13.7 & 2.8 kb



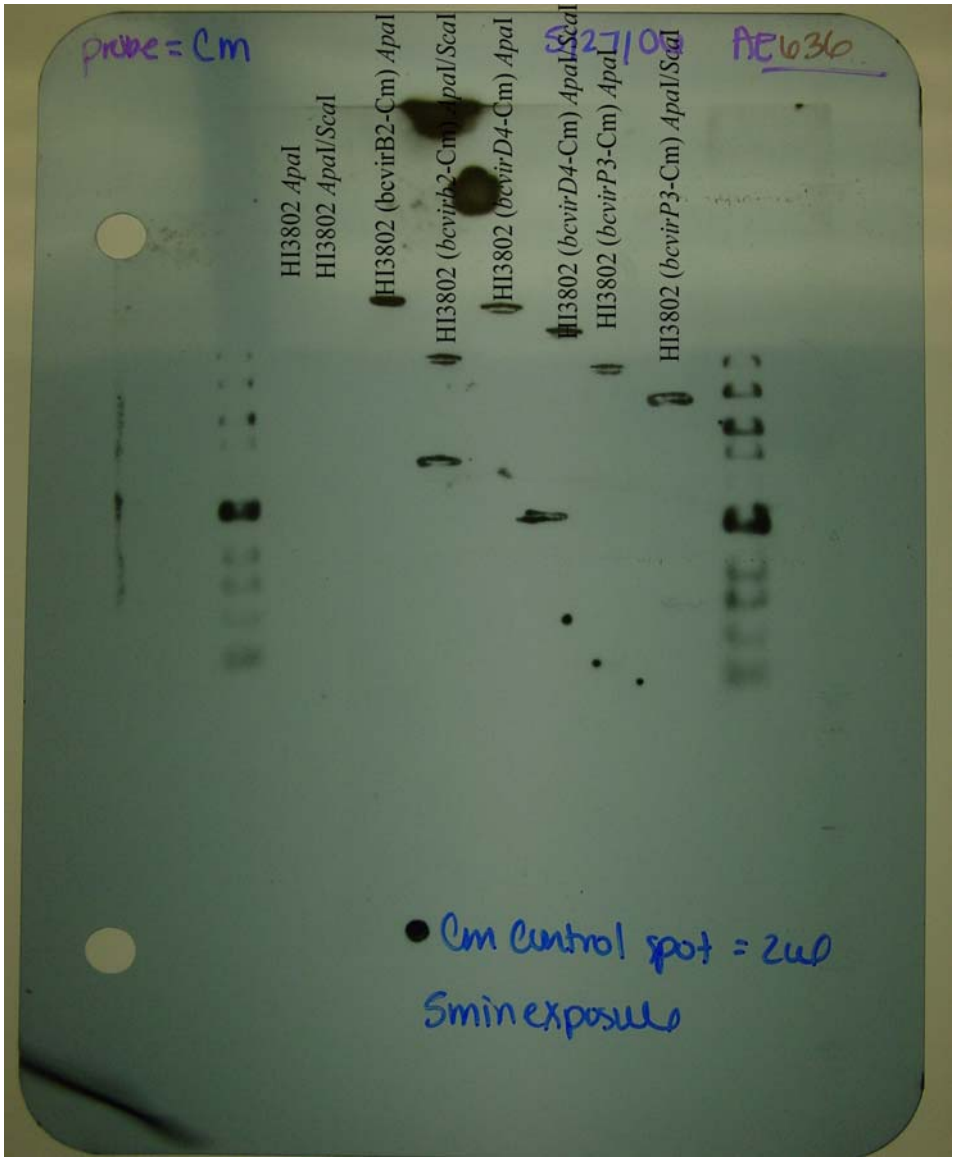
Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

Probe=*bcvirP3* PCR Product				
	AU1054	**AU1054 (*bcvirB2*::Cm)**	**AU1054 (*bcvirD4*::Cm)**	**AU1054 (*bcvirP3*::Cm)**
ApaI	8.2 kb	8.2 kb	8.2 kb	9.2 kb
***Apa*I/*Sca*I**	8.2 kb	8.2 kb	8.2 kb	7.7 & 1.5 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

Probe=Cm PCR Product				
	AU1054	**AU1054 (*bcvirB2*::Cm)**	**AU1054 (*bcvirD4*::Cm)**	**AU1054 (*bcvirP3*::Cm)**
ApaI	N/A	17.5 kb	17.5 kb	9.2 kb
***Apa*I/*Sca*I**	N/A	10 & 4.6 kb	14.4 & 3.1 kb	7.7 & 1.5 kb



Ladder 10, 8 ,6 ,5 ,4 ,3 ,2 ,1.5 ,1, and 0.5 kb

VITA

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