Two type IV secretion systems with different functions in *Burkholderia cenocepacia* K56-2

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Bacterial type IV secretion systems (T4SS) perform two fundamental functions related to pathogenesis: the delivery of effector molecules to eukaryotic target cells, and genetic exchange. Two T4SSs have been identified in *Burkholderia cenocepacia* K56-2, a representative of the ET12 lineage of the *B. cepacia* complex (Bcc). The plant tissue watersoaking (Ptw) T4SS encoded on a resident 92 kb plasmid is a chimera composed of VirB/D4 and F-specific subunits, and is responsible for the translocation of effector(s) that have been linked to the Ptw phenotype. The bc-VirB/D4 system located on chromosome II displays homology to the VirB/D4 T4SS of *Agrobacterium tumefaciens*. In contrast to the Ptw T4SS, the bc-VirB/D4 T4SS was found to be dispensable for Ptw effector(s) secretion, but was found to be involved in plasmid mobilization. The fertility inhibitor Osa did not affect the secretion of Ptw effector(s) via the Ptw system, but did disrupt the mobilization of a RSF1010 derivative plasmid.

INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) consists of at least 17 phenotypically similar but genotypically distinct species of non-fermenting, Gram-negative bacteria that are found in a diverse set of niches (Coenye *et al.*, 2001; Vanlaere *et al.*, 2005). These species have a high degree of 16S rDNA gene (98–100 %) and *recA* (94–95 %) sequence similarity, and moderate levels of DNA–DNA hybridization (30–50 %) (Coenye & Vandamme, 2003). Members of the Bcc are opportunistic pathogens, capable of causing disease in plants, animals and particularly causing life-threatening respiratory infections in persons with cystic fibrosis (Berriatua *et al.*, 2001; Gonzalez *et al.*, 1997; LiPuma, 2003). Strains isolated from infected cystic fibrosis patients indicate that the distribution of species is quite disproportionate, with *B. cenocepacia* and *Burkholderia multivorans* accounting for 85–90 % of infection in this patient population (LiPuma, 1998a, b).

Secretion of proteins across bacterial membranes is of fundamental importance in bacterial virulence and occurs through a variety of mechanisms, from simple one-component systems to complex multi-component machineries. Secretion pathways have been classified into

seven major evolutionarily and functionally related groups, termed types I–VII (Abdallah *et al.*, 2007; Kostakioti *et al.*, 2005; Schell *et al.*, 2007; Thanassi & Hultgren, 2000). There are numerous examples of the importance of type III (Plano *et al.*, 2001) and type IV secretion systems (T4SSs) (Christie, 2001) in the infection process for both plant and animal pathogens, with evidence that type III secretion systems are derived from flagella assembly constituents modified to function as a transport mechanism for virulence factors (Macnab, 1999). T4SSs originally evolved from bacterial conjugation systems and are functionally diverse, in terms of both the transported substrate (proteins or DNA–protein complexes) and the intended recipients, which range from micro-organisms of the same or different species to inter-kingdom transfer to fungi, plants or mammalian target cells (Cascales & Christie, 2003).

The T4SSs have been classified into four subgroups, each of which is specialized for a specific function and contributes in a unique manner to pathogenesis: effector molecule translocation into host target cells; conjugation of chromosomal and plasmid DNA; DNA uptake and transformation; and DNA release into the extracellular milieu (Backert & Meyer, 2006). *Agrobacterium tumefaciens* has three T4SSs: the *virB/D4* system encoded by the Ti plasmid is the paradigm of T4SS and transfers T-DNA into plant cells, resulting in the development of crown-gall tumours (Christie *et al.*, 2005); the *trb* system, required for the conjugal transfer of the Ti plasmid between cells of *A. tumefaciens* (Li *et al.*, 1998, 1999); and the *avhB* system,

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Abbreviations: T4SS, type IV secretion system; Bcc, *Burkholderia cepacia* complex; Ptw, plant tissue watersoaking; VBG, Vogel–Bonner minimal medium amended with 1.0 % glucose.

which mediates the conjugal transfer of the pAtC58 cryptic plasmid (Chen *et al.*, 2002). AvhB also promotes the conjugal transfer of the IncQ plasmid RSF1010. *Helicobacter pylori* possesses two T4SSs with different roles. The Cag secretory apparatus functions in the translocation of CagA into host cells, whereas the *com* system functions to take up DNA to facilitate genetic variation (Dhar *et al.*, 2003; Ding *et al.*, 2003). *Legionella pneumophila* has a functional T4SS designated *dot/icm* that is essential for pathogenesis and plasmid DNA conjugal transfer (Segal *et al.*, 1998; Segal & Shuman, 1998; Vogel *et al.*, 1998). In addition, this pathogen has another T4SS distinct from *dot/icm*, designated the *lvh* system, which is dispensable for intracellular growth in its host, only partially required for RSF1010 conjugation, and can substitute for some components of the *dot/icm* system in plasmid conjugation (Segal *et al.*, 1999). Other pathogens, such as *Brucella* and *Bartonella* spp., require T4SSs to persist in the intracellular environments of their mammalian hosts (O'Callaghan *et al.*, 1999; Schulein & Dehio, 2002), whereas the closely related plant symbiont *Sinorhizobium meliloti* 1021 exploits a *virB*-like T4SS to transfer DNA (Jones *et al.*, 2007).

Two T4SSs have been identified in *B. cenocepacia* K56-2. The plant tissue watersoaking (Ptw) T4SS, which is encoded on a resident 92 kb plasmid, is a chimera composed of VirB/D4 and F-specific subunits, and is responsible for the secretion of a plant cytotoxic protein(s) that causes Ptw (Engledow *et al.*, 2004). The second T4SS, designated bc-VirB/D4, is located on chromosome II and bears homology to the VirB/D4 T4SS of *A. tumefaciens*, but its function is still unknown. In this report, we elucidated the functions of the two T4SSs in *B. cenocepacia* K56-2.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was used for routine maintenance of cultures. Vogel-Bonner minimal medium (Vogel & Bonner, 1956) amended with 1.0% glucose (VBG) was used in mating experiments. *B. cenocepacia* and *Escherichia coli* strains were grown at 37 °C. Antibiotics were added to media at the following concentrations: 100 µg trimethoprim (Tp) ml⁻¹, 20 µg tetracycline (Tc) ml⁻¹, 30 µg chloramphenicol (Cm) ml⁻¹, and 30 µg kanamycin (Km) ml⁻¹ for *E. coli*; 200 µg Tp ml⁻¹, 200 µg Tc ml⁻¹ and 300 µg Cm ml⁻¹ for *B. cenocepacia* K56-2. For *B. multivorans* TL249-Nal, VBG was amended with 0.05 mM lysine, 500 µg nalidixic acid ml⁻¹ and 50 µg Tc ml⁻¹ for selection of plasmid transfer. The complex medium TN broth (TNB) was used for liquid cultures in plasmid-curing studies (Hansen & Olsen, 1978). Solid medium was identical except it lacked KNO₃ and was supplemented with 20 g agar l⁻¹.

DNA manipulations. Genomic DNA was extracted using a DNeasy kit (Qiagen), small-scale plasmid preparation was done using the Miniprep kit (Qiagen), and gel extraction and PCR product purification were conducted using the Qiaquick gel extraction kit and Qiaquick PCR purification kit, respectively (Qiagen). All restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. Oligonucleotide primers were synthesized by Operon Biotechnologies Inc. A Gene Pulser (Bio-

Rad) was used for electroporation. Southern blot analysis was conducted using the DIG High Prime DNA Labelling and Detection Starter kit, according to the manufacturer's instructions (Roche Applied Science), DNA sequences were determined in the DNA sequencing laboratory of the Institute of Plant Genomics & Biotechnology, Texas A&M University. Sequence analysis and alignments were conducted using Vector NTI software (Invitrogen).

Plasmid construction for allelic exchange. Since *B. cenocepacia* strain K56-2 is clonally related to the sequenced strain J2315 (Mahenthiralingam *et al.*, 2000; Holden *et al.*, 2009), PCR primers for regions flanking the *bcvirD4* locus in strain K56-2 were designed based on J2315 sequences BCA0334 and BCA0336 (www.sanger.ac.uk/Projects/B_cenocepacia/). To construct plasmid pEX18Tc- Δ *bcvirD4*::Tp used to delete *bcvirD4* from K56-2, a 1.7 kb fragment upstream of *bcvirD4* was amplified with primers BcvirD4-up-forward (5'-CAACATCAGGGTACCGAGTT-3', *KpnI* site underlined) and BcvirD4-up-reverse (5'-GTACGGATCCTAGCACGAATTGCTGA-3', *BamHI* site underlined), and the PCR product was cloned into plasmid pEX18Tc, digested with *KpnI* and *BamHI*, to obtain plasmid pEX18Tc:: Δ *bcvirD4*up. The 1.7 kb fragment downstream of *bcvirD4* was amplified using primers BcvirD4-down-forward (5'-CATGTGCGAGCGGACTATCTGGAA-3', located next to an endogenous *BamHI* site) and BcvirD4-down-reverse (5'-GTTCTAGACCGACACGAATG-3', the introduced *XbaI* site is underlined). The PCR product was cloned into pEX18Tc:: Δ *bcvirD4*up and digested with *BamHI* and *XbaI* to obtain pEX18Tc:: Δ *bcvirD4*. The Tp cassette from plasmid R388 was amplified with primers Tp-forward (5'-GTGGATCCGCTTTCTGATAG-3', *BamHI* site underlined) and Tp-reverse (5'-GAGGATCCGATTGTGAGCG-3', *BamHI* site underlined). The PCR product was cloned into the *BamHI* site of plasmid pEX18Tc:: Δ *bcvirD4*, to obtain pEX18Tc- Δ *bcvirD4*::Tp, which was used for allelic exchange to delete the *bcvirD4* gene and introduce a selectable marker.

Genomic sequences of strain J2315 (www.sanger.ac.uk/Projects/B_cenocepacia/) were again used to design PCR primers to amplify *ptwD4* from strain K56-2. To construct plasmid pEX18Tc- Δ *ptwD4*::Tp, used to obtain a deletion in *ptwD4*, a 1.8 kb fragment upstream of *ptwD4* was amplified with primers PtwD4-up-forward (5'-GCGCGAATTTTCGTCCAGTT-3') and PtwD4-up-reverse (5'-ACAACCTGATCGACGGGAAG-3'). The PCR product had *EcoRI* and *KpnI* sites on the 5' and 3' ends, respectively, and was cloned into plasmid pEX18Tc digested with *EcoRI* and *KpnI* to obtain pEX18Tc:: Δ *ptwD4*up. The 1.8 kb fragment downstream of *ptwD4* was amplified with primers PtwD4-down-forward (5'-GTCTCATATGCCGCTTCGGAA-3') and PtwD4-down-reverse (5'-GTC-TCTAGATCAGACTTGTCAACC-3'; the introduced *XbaI* site is underlined). The PCR product was first cloned into plasmid pDrive (Qiagen) to obtain plasmid pDrive:: Δ *ptwD4*down, then double-digested with *KpnI* and *XbaI*. The fragment was cloned into pEX18Tc:: Δ *ptwD4*up double-digested with *KpnI* and *XbaI* to obtain pEX18Tc:: Δ *ptwD4*. The Tp cassette was obtained by *KpnI* digestion of plasmid p34S-Tp and inserted into the *KpnI* site of pEX18Tc:: Δ *ptwD4* to obtain pEX18Tc- Δ *ptwD4*::Tp. All the constructed plasmids were confirmed by sequence analyses. All plasmid constructs were performed using *E. coli* DH5 α MCR as host. Plasmid constructs were introduced by electroporation (25 µF, 1.5 kV, 200 Ω) followed by selection on LB agar amended with appropriate antibiotic(s).

Site-directed mutagenesis. Plasmids pEX18Tc- Δ *virD4*::Tp and pEX18Tc- Δ *ptwD4*::Tp were individually introduced into *B. cenocepacia* K56-2 by conjugation using triparental matings with pRK2013 as the mobilizing plasmid, and selection on VBG agar plates containing 200 µg Tp ml⁻¹ for single-crossover events. It is our experience that *sacB* gene selection to screen the double-crossover

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics*	Reference or source
<i>E. coli</i>		
DH5 α MCR	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>recA1 endA gyrA96 thi-1 hsdR17 supE44 relA1 deoR U169</i>	Invitrogen
<i>B. multivorans</i>		
TL249	Soil isolate, plasmid-free, <i>lys</i> -2	Gaffney & Lessie (1987)
TL249-Nal ^r	Soil isolate, plasmid-free, <i>lys</i> -2, Nal ^r	This study
<i>B. cenocepacia</i>		
K56-2	Cystic fibrosis respiratory isolate, Ptw ⁺	Sokol <i>et al.</i> (1999)
RZ101	K56-2 Δ <i>bcvirD4</i> ::Tp, Tp ^r	This study
RZ102	K56-2(pML122Tc)	This study
RZ103	RZ101(pML122Tc)	This study
RZ104	RZ101(pML122Tc, pBcLaR)	This study
RZ105	RZ101(pML122Tc, pBcLaR::bcvirD4)	This study
RZ072	K56-2 Δ <i>ptwD4</i> ::Tp, Tp ^r	This study
RZ073	RZ072(pBcLaR)	This study
RZ074	RZ072(pBcLaR::ptwD4)	This study
RZ075	RZ072(pML122Tc)	This study
RZ106	K56-2(pBcRLaR::osa)	This study
RZ107	K56-2(pML122Tc, pBcLaR)	This study
RZ108	K56-2(pML122Tc, pBcLaR::osa)	This study
Plasmids		
pEX18Tc	Suicide vector, <i>sacB</i> , Tc ^r	Hoang <i>et al.</i> (1998)
pDrive	Cloning of PCR products, Ap ^r Km ^r	Qiagen
pBR325	Source of Tc cassette, Tc ^r	Laboratory stock
pRK2013	Tra ⁺ Mob ⁺ ColE1 replicon, Km ^r	Laboratory stock
p34S-Tp	Source of Tp resistance cassette	Dennis & Zylstra (1998)
pEX18Tc- Δ <i>virD4</i> ::Tp	pEX18Tc with <i>virD4</i> upstream and downstream fragments joined by Tp cassette, Tc ^r Tp ^r	This study
pEX18Tc- Δ <i>ptwD4</i> ::Tp	pEX18Tc with <i>ptwD4</i> upstream and downstream fragments joined by Tp cassette, Tc ^r Tp ^r	This study
pBcRLaR	Mob ⁺ <i>lacZx</i> pBBR1 replication, Cm ^r	Sajjan <i>et al.</i> (2008)
pML122	IncQ Mob ⁺ p15A replication, Km ^r Gm ^r	Labes <i>et al.</i> (1990)
pML122Tc	Tc cassette inserted into <i>EcoRI</i> site of pML122, Km ^r , Tc ^r	This study
pKA165	Source of <i>osa</i> gene, Cb ^r	Cascales <i>et al.</i> (2005)
pBcRLaR::osa	<i>osa</i> gene cloned into pBcRLaR, Cm ^r	This study
pDrive::bcvirD4	<i>bcvirD4</i> gene cloned into pDrive, Ap ^r Km ^r	This study
pBcRLaR::bcvirD4	<i>bcvirD4</i> gene from pDrive- <i>bcvirD4</i> cloned into pBcRLaR, Cm ^r	This study
pDrive::ptwD4	<i>ptwD4</i> gene cloned into pDrive, Ap ^r Km ^r	This study
pBcRLaR::ptwD4	<i>ptwD4</i> gene from pDrive:: <i>ptwD4</i> cloned into pBcRLaR, Cm ^r	This study

*Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nal, nalidixic acid; Tc, tetracycline; Tp, trimethoprim.

transconjugants does not function for *B. cenocepacia* K56-2. To screen for double-crossover events in transconjugants that had lost the vector sequence, Tp^r transconjugants were picked onto both VBG agar plates containing 200 μ g Tp ml⁻¹ and VBG agar plates containing 200 μ g Tc ml⁻¹. Transconjugants that were Tp^r Tc^s were selected as target gene deletion mutants and confirmed by PCR using multiple primer set combinations, both internal and external to the target gene deletion. Deletion mutants were also confirmed by Southern blot analysis using multiple probes, including the Tp cassette, the deleted sequence and the amplified sequence adjacent to the target deletion.

Complementation experiments. The *bcvirD4* gene was amplified using primers K56bcvirD4-forward (5'-GCTCTAGAAATGAGAC-AGATTGC-3') and K56bcvirD4-reverse (5'-TGAAGTGCAGCTCA-

TTGAACACC-3'), and cloned into pDrive to obtain pDrive::bcvirD4. The orientation of the inserted *bcvirD4* gene was determined by sequencing the insert to confirm the in-frame orientation of *bcvirD4*. Plasmid pDrive::bcvirD4 was then double-digested with *KpnI* and *HindIII*, and the fragment containing *bcvirD4* was gel-extracted and cloned into plasmid pBcRLaR digested with *KpnI* and *HindIII* to construct pBcRLaR::bcvirD4. Plasmids pBcRLaR or pBcRLaR::bcvirD4 were individually mobilized into isolate RZ101 (*bcvirD4* deletion mutant) as vector control and complementation plasmid, respectively.

ptwD4 was amplified using primers PD4-1 (5'-TGAAGTCA-GCGAAGGAA-3') and PD4-2 (5'-ATCCGGTGGGAAGCAA-3'), and the PCR product was cloned into plasmid pDrive to obtain plasmid pDrive::ptwD4. The orientation of the inserted *ptwD4* gene was

determined by sequencing the insert to confirm the in-frame orientation of *ptwD4*. Plasmid pDrive::*ptwD4* was then double-digested with *Bam*HI and *Hind*III, and the fragment containing *ptwD4* was gel-extracted and cloned into pBcRLaR double-digested with *Bam*HI and *Hind*III to construct pBcRLaR::*ptwD4*. Plasmids pBcRLaR or pBcRLaR::*ptwD4* were individually mobilized into isolate RZ072 (*ptwD4* deletion mutant) as vector control and complementation plasmid, respectively.

Construction of pML122Tc and pBcRLaR::*osa*. To construct pML122Tc, the Tc cassette from pBR325 was amplified using primers TET-1 (5'-GCCATGTTTGACAGCTTATC-3') and TET-2 (5'-TGCGCATTCACAGTTCTCCG-3'), and cloned into pDrive. The Tc cassette was excised from pDrive by *Eco*RI digestion, gel-purified and cloned into the *Eco*RI sites of the RSF1010 derivative plasmid pML122, to obtain pML122Tc. To construct pBcRLaR::*osa*, plasmid pKA165 was double-digested with *Bam*HI and *Xho*I to obtain the *osa* fragment. The *osa* fragment was gel-extracted, and ligated into pBcRLaR double-digested with *Bam*HI and *Xho*I to obtain pBcRLaR::*osa*.

Mobilization of plasmid pML122Tc. Mobilization of plasmid pML122Tc by strain K56-2 and derivatives was determined using *B. multivorans* TL249-Nal as the recipient in biparental and triparental (using pRK2013 as helper) matings. Donor, helper and recipient strains were grown overnight at 37 °C with appropriate selection. Bacterial suspensions in LB broth were adjusted spectrophotometrically ($OD_{600}=0.5$), mixed at an equal ratio, and transferred to a positively charged sterile membrane layered on a 100 × 15 mm LB Petri dish containing LB agar and incubated at 37 °C. Following an 18 h incubation period, the cells from the matings and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12 096 g for 10 min at 5 °C). The bacterial pellets were resuspended in phosphate buffer and dilution-plated onto VBG plates amended with 0.05 mM lysine, 500 µg nalidixic acid ml⁻¹ and 50 µg Tc ml⁻¹. After 48 h incubation at 37 °C, all plates were evaluated. To determine the donor input (c.f.u. ml⁻¹), dilutions of donor control cell suspension were plated onto VBG plates with appropriate antibiotics. Single-colony isolates of individual transconjugants were obtained by streaking on selective media. Cultures were subjected to survey lysis followed by agarose gel electrophoresis to confirm plasmid transfer.

Ptw assay. The Ptw assay was conducted as described by Engledow *et al.* (2004). Onion cultivar 1015Y was used throughout the study. Bacterial suspensions to be tested were adjusted to $OD_{600}=0.5$, individual onion scales were wounded on the abaxial (inner) surface with a sterile toothpick and 10 µl bacterial suspension (10⁶ c.f.u. per scale) was inoculated into the wound. Sterile double-distilled deionized water was used as negative control. Onion scales were placed on a sheet of aluminium foil that had been surface sterilized with 70% ethanol in containers containing paper towels pre-moistened with sterile distilled water, sealed, and incubated at 37 °C. Ptw activity was assessed at 24 h post-inoculation by measuring the vertical and horizontal diameters of the zones.

Plasmid curing. Plasmid-curing studies were conducted essentially as described by Gonzalez *et al.* (1997). Briefly, cultures were grown for 18 h at 37 °C in TN broth with shaking (200 r.p.m.). Bacterial cells were subcultured into prewarmed TN broth (42 or 44 °C) to a final concentration of 10⁴ c.f.u. ml⁻¹ and grown with shaking (200 r.p.m.) in a water bath for 18 h. Temperature-treated cultures were diluted and plated onto TN agar. Individual colonies were transferred onto TN agar (stock culture) and stab-inoculated onto onion scales to test for Ptw activity as described above. Isolated colonies expressing a Ptw⁻ phenotype were purified by streaking on TN agar, and individual colonies were retested. Colonies reconfirmed as Ptw⁻

were subjected to plasmid survey lysis as described by Gonzalez *et al.* (1997).

RESULTS

The bc-VirB/D4 system promotes the mobilization of plasmid pML122Tc

We previously reported that the Ptw T4SS encoded on a resident 92 kb plasmid (pK56-2) is involved in the translocation of an effector(s) molecule that is responsible for the Ptw phenotype expressed by *B. cenocepacia* K56-2 (Engledow *et al.*, 2004). We also identified a chromosomally encoded T4SS, which we designated the bc-VirB/D4 system (Engledow *et al.*, 2004). Sequence analysis of this region revealed a lower G+C content (63 mol%) compared with the G+C content of the entire genome (66.9 mol%). Two genes, designated with the genome ORF numbers BCAM0322 and BCAM0323, upstream of the bc-VirB/D4 system showed homology to a two-component regulatory system (Fig. 1). A gene designated *bcvirD4* showed homology to other T4SS coupling proteins, which serve as active motors necessary for substrate transfer through the T4SS (Atmakuri *et al.*, 2003; Christie *et al.*, 2005). To investigate the role of the chromosome-encoded bc-VirB/D4 system in *B. cenocepacia* K56-2, a deletion in *bcvirD4* with insertion of the Tp^r cassette was constructed.

Because *sacB* gene selection to screen for double-crossover transconjugants does not function in *B. cenocepacia* (Barrett *et al.*, 2008; Flannagan *et al.*, 2008), only Tp^r Tc^s transconjugants were considered to be putative double-crossover mutants. The target gene deletion was confirmed by using multiple PCR set combinations, both internal and external to the target deletion, and by Southern blot analysis using the Tp cassette and PCR products of sequence immediately downstream of *bcvirD4* as probes (data not shown). The *bcvirD4* deletion mutant, RZ101, caused Ptw in onion tissue-producing zones equal to that observed for K56-2 when inoculated at the same concentration (Fig. 2).

Since T4SSs play a role in recruiting and transferring plasmid DNA (Chen *et al.*, 2002; Jones *et al.*, 2007; Segal *et al.*, 1998; Segal & Shuman, 1998), we sought to determine if the bc-VirB/D4 system was involved in these functions. Plasmid pML122Tc, a derivative of the IncQ plasmid RSF1010, was introduced into K56-2 and RZ101 to obtain the strains RZ102 and RZ103, respectively. In control matings, plasmid pML122Tc was mobilized at a frequency of 1.23×10^{-5} transconjugants per donor using donor strain RZ102, mobilizing plasmid pRK2013 and TL249-Nal as the recipient in triparental matings. In biparental matings, using only RZ102 as the donor, the mobilization of plasmid pML122Tc into the same recipient was reduced by two logs (Table 2). When the *bcvirD4* deletion mutant (RZ103) was used as the donor strain, no detectable ($<3.57 \times 10^{-10}$ transconjugants per donor)

Fig. 1. Schematic representation of the bc-VirB/D4 T4SS based on the T4SS located on chromosome II of *B. cenocepacia* strain J2315. Designation of genes was based on homology to gene products of transfer- and translocation-related proteins. Genes are represented as solid boxes with arrowheads indicating their orientation.

transfer of pML122Tc was observed in biparental matings with TL249-Nal as the recipient. The complementation *in trans* of RZ103 with pBcRLaR::bcvirD4 (RZ105) restored the ability to mobilize pML122Tc in biparental matings to approximately parental levels (Table 2). In triparental matings, pML122Tc was mobilized from strain RZ105 at parental levels (Table 2).

Ptw T4SS does not mobilize plasmid pML122Tc

We have previously reported that a functional coupling protein, PtwD4, and several other genes of the Ptw T4SS were essential to the functioning of the system involved in producing Ptw of onion tissue (Engledow *et al.*, 2004). In the present study, we constructed a *ptwD4* gene deletion mutant that circumvented potential polar effects. The deletion mutant was constructed by introducing plasmid pEX18Tc- Δ *ptwD4*::Tp into K56-2 and selecting for double-crossover events. A deletion mutant, designated RZ072, was confirmed by using multiple PCR combinations both internal and external to the target deletion and by Southern blot analysis using the Tp cassette and PCR

products of sequence immediately downstream of the deleted *ptwD4* as probes (data not shown). Strain RZ072 exhibited a Ptw⁻ phenotype, reconfirming the role of the Ptw T4SS in the expression of the phenotype on onion tissue. Complementation of RZ072 *in trans* with pBcRLaR::*ptwD4* (RZ074) resulted in the production of Ptw zones in plant tissue equivalent to that produced by the parental strain, K56-2 (Fig. 3).

Additionally, to determine the potential role for the Ptw T4SS in plasmid mobilization, strain RZ072 was evaluated for its ability to mobilize plasmid pML122Tc by introducing the plasmid into strain RZ072 (strain RZ075), and conducting biparental matings. The mobilization frequency of pML122Tc using donor strain RZ075 was similar to that observed for parental strain K56-2 (2.42×10^{-7} vs 2.44×10^{-7}).

Since it would have been desirable to obtain a plasmid-free derivative of strain K56-2 to confirm the results observed with the *ptwD4* deletion mutant, experiments to cure plasmid pK56-2 were conducted. Over 5000 colonies from heat-treated cultures were stab-inoculated onto onion scales to evaluate their ability to cause Ptw. Growth of K56-2 at temperatures that were successful in curing a 200 kb PehA-encoding plasmid harboured by *B. cepacia* strain ATCC 25416 (Gonzalez *et al.*, 1997) did not result in loss of pK56-2 from strain K56-2 (data not shown).

Fig. 2. Onion Ptw assay. Onion slices were wounded, inoculated with 10 μ l of different bacterial suspensions (10^6 c.f.u. per scale), and incubated at 37 °C for 24 h. (a) Strain K56-2; (b) strain RZ101; (c) 10 μ l sterile double-distilled deionized water as negative control.

Table 2. Mobilization of plasmid pML122Tc by K56-2 and derivatives

B. multivorans TL249-Nal was used as recipient in all assays. The results are the means of three independent experiments.

Donor strains	Transfer frequency for triparental mating per transconjugants donor*	Transfer frequency for biparental mating per transconjugant donor
RZ102	1.23×10^{-5}	2.44×10^{-7}
RZ103	0.76×10^{-5}	$<3.57 \times 10^{-10}$
RZ104	0.60×10^{-5}	$<4.17 \times 10^{-10}$
RZ105	1.01×10^{-5}	1.95×10^{-7}

*Helper plasmid pRK2013 was used in triparental matings.

Fig. 3. Onion Pt看 assay. Onion slices were wounded, inoculated with 10 µl of different bacterial suspensions (10^6 c.f.u. per scale), and incubated at 37 °C for 24 h. (a) Strain K56-2; (b) strain RZ072; (c) strain RZ073; (d) strain RZ074; (e) 10 µl sterile double-distilled deionized water as negative control.

Effect of pML122Tc and Osa on effector molecule translocation via the Pt看 T4SS system

Plasmid RSF1010 has been shown to interrupt T-DNA translocation via the VirB/D4 secretion channel and in the *L. pneumophila* *icm/dot* system (Cascales *et al.*, 2005; Segal & Shuman, 1998). To determine if the RSF1010 derivative plasmid pML122Tc could interfere with the secretion of effector protein(s) translocated via the Pt看 system and attenuate the virulence of K56-2 in onion tissue, we compared the amount of Pt看 of strain RZ102 (K56-2 harbouring pML122Tc) and wild-type K56-2. Both strains produced Pt看 zones of similar diameter when inoculated on onion tissue at the same rate (data not shown).

Osa, encoded on plasmid pSa (IncW) and related in sequence to the FiwA fertility inhibition factor of plasmid RP1 (IncP α), is sufficient for suppressing oncogenicity of *A. tumefaciens*. Osa competes with VirB/D4 system substrates, such as T-DNA and VirE2, to bind to the coupling protein (Cascales *et al.*, 2005; Lee *et al.*, 1999; Lee & Gelvin, 2004).

To determine the effect of Osa on the translocation of Pt看 protein(s) by the Pt看 system, we cloned *osa* into pBcRLaR, and introduced pBcRLaR::*osa* into K56-2 to obtain strain RZ106. Strain RZ106 produced Pt看 zones on onion tissue similar to that observed for the parental strain K56-2 control (data not shown).

Effect of Osa on bc-VirB/D4 system-dependent mobilization of pML122Tc

Osa interferes with transfer of IncQ and IncW plasmids when co-resident (Chen & Kado, 1994; Fong & Stanisich, 1989). More recently, it has been determined that the RSF1010 transfer intermediate and Osa suppress *A. tumefaciens* oncogenesis specifically by interfering with T-DNA and VirE2 substrate binding to the VirD4 receptor (Cascales *et al.*, 2005). The similarity between the *A. tumefaciens* VirB/D4 system and the chromosomally encoded bc-VirB/D4 system prompted us to determine if Osa could interfere with the mobilization of pML122Tc in *B. cenocepacia*. Co-residency of pBcRLaR::*osa* and pML122Tc in strain K56-2 (donor strain RZ108) resulted in no detectable mobilization ($<2.34 \times 10^{-10}$ transconjugants per donor) of pML122Tc in biparental matings using TL249-Nal as the recipient, whereas mobilization of pML122Tc was observed at slightly lower levels (0.73×10^{-7} transconjugants per donor) for donor strain RZ107 harbouring the vector control pBcRLaR compared to that observed for K56-2 harbouring pML122Tc in biparental matings (2.44×10^{-7} transconjugants per donor).

DISCUSSION

Previously, we investigated the *B. cenocepacia* plasmid-encoded Pt看 T4SS (Engledow *et al.*, 2004). In that study, we determined that the Pt看 T4SS, a chimera composed of VirB/D4 and F-specific subunits, is responsible for the production of the plant-disease-associated Pt看 phenotype and the translocation of Pt看 effector protein(s). The observations that the Pt看 T4SS is involved in the translocation of a protein(s), does not contain the components necessary to support conjugation, and contains no *oriT* homologue indicate that functionally it is a member of the effector–translocator subfamily of T4SSs.

In that same study we identified a second gene cluster on chromosome II that showed homology to the VirB/D4 T4SS of *A. tumefaciens* with respect to arrangement and gene product similarity (Fig. 1) (Engledow *et al.*, 2004). The presence of a second T4SS in *B. cenocepacia* is not unprecedented, since other species, such as *H. pylori*, *A. tumefaciens* and *Bartonella henselae*, have been found to harbour multiple secretion systems (Chen *et al.*, 2002; Dhar *et al.*, 2003; Li *et al.*, 1998, 1999; Schröder & Lanka, 2005). Our results indicate that the two T4SSs present in *B. cenocepacia* K56-2 do not appear to have redundant functions. Analyses of the available annotated sequenced

genomes of *B. cenocepacia* AU1054 and HI2424, *Burkholderia vietnamiensis* G4 and *Burkholderia xenovorans* LB 400 did not reveal the presence of a Ptw-like T4SS in these strains. However, each of these strains contained a VirB/D4-like T4SS (accession numbers CP000379, CP000459, CP000616 and CP000272, respectively).

T4SSs are used by bacteria to translocate DNA and protein macromolecules to a diverse range of bacterial and eukaryotic cells (Christie *et al.*, 2005). T4SSs also mediate horizontal gene transfer, and contribute to genome plasticity and the evolution of pathogens through dissemination of antibiotic resistance and virulence genes (Juhas *et al.*, 2007). The *A. tumefaciens* VirB/D4 T4SS that transfers oncogenic T-DNA to various eukaryotic cells, as a derivative of plasmid conjugation systems, can also transfer certain mobilizable plasmids and bacterial proteins like VirE2 and VirF (Christie *et al.*, 2005; Guo *et al.*, 2007). In *A. tumefaciens*, the RSF1010 transfer intermediate and the Osa protein of plasmid pSa (IncW) render *A. tumefaciens* host cells nearly avirulent by interfering specifically with VirD4 receptor function by inhibiting docking of DNA and protein substrates to the translocation apparatus (Cascales *et al.*, 2005). Our results indicate that neither the RSF1010 derivative plasmid pML122Tc nor the Osa protein affects effector protein translocation via the Ptw T4SS. The VirD4 homologue PtwD4 is the coupling protein in the Ptw T4SS, and insertions (Engledow *et al.*, 2004) or deletions in *ptwD4* (present study) disrupt translocation of the Ptw effectors. Since pML122 transfer intermediates and the Osa protein specifically interfere with VirD4 receptor function in the *A. tumefaciens* VirB/D4 T4SS, they are unlikely to be substrates for the PtwD4 coupling protein. This is most probably due to specificity of the coupling protein for the Ptw effector(s) as the coupling protein functions as a gate to mating-pair formation channels (Schröder & Lanka, 2005). Several studies indicate that the T4SS coupling protein (i.e. VirD4 and TraG-like proteins) is the primary factor that determines the specificity of the secretion system for a particular substrate (Cabezón *et al.*, 1994; Hamilton *et al.*, 2000; Llosa *et al.*, 2003). A C-terminal signal mediating the translocation of T4SS protein substrates has been identified in the VirB/VirD4 systems of *A. tumefaciens* and *B. henselae*, and in the Dot/Icm system of *L. pneumophila* (Atmakuri *et al.*, 2003; Nagai *et al.*, 2005; Schulein *et al.*, 2005; Simone *et al.*, 2001; Vergunst *et al.*, 2005). It has been proposed that these secretion systems evolved from conjugation systems and that the C-terminal signal of their secreted protein substrates originates from a relaxase ancestor (Schulein *et al.*, 2005). It is highly likely that co-evolution of the PtwD4 coupling protein and the T4SS signal has occurred and that a specific signal mediates protein-protein interaction to initiate transfer of the Ptw effector(s) by the Ptw T4SS.

While a deletion in *ptwD4* resulted in the loss of the Ptw phenotype in K56-2, it did not disrupt the ability to mobilize plasmid pML122Tc, since mobilization frequencies of the plasmid using donor strain RZ075 were similar to that observed for parental strain K56-2 (2.42×10^{-7} vs

2.44×10^{-7}) in biparental matings. The bc-VirB/D4 T4SS in *B. cenocepacia* K56-2 exhibited the ability to promote the transfer of the IncQ plasmid RSF1010 derivative pML122Tc (Table 2). Osa inhibits conjugative transfer of pML122 to agrobacterial recipients by interfering with pML122 binding to the VirD4 receptor without further negative effects on substrate binding to the VirB subunits (Cascales *et al.*, 2005; Lee & Gelvin, 2004). The observed inhibitory effect of Osa with agrobacterial recipients was approximately one log reduction in conjugative transfer frequency and 65 % reduction in pML122 binding to the VirD4 receptor (Cascales *et al.*, 2005). In the *Burkholderia* system, the presence of Osa reduced the transfer of pML122Tc below a detectable level ($<2.34 \times 10^{-10}$ trans-conjugants per donor) when supplied *in trans* by plasmid pBcRLaR::osa, therefore reducing the mobilization frequency by over two logs. We observed that the mobilization frequency of pML122Tc was reduced slightly when donor strain RZ107 harbouring the vector control pBcRLaR was used in biparental matings. No detectable mobilization of pBcRLaR was observed in biparental matings.

We have reported previously that the plasmid-encoded Ptw T4SS plays a role in the intracellular survival of *B. cenocepacia* in both professional and non-professional phagocytes (Sajjan *et al.*, 2008). A functional plasmid-encoded T4SS contributes to the ability of *B. cenocepacia* to evade endocytic degradation, and to survive and replicate in both airway epithelial cells and monocyte-derived macrophages. In *B. cenocepacia* K56-2, it is unknown if gene products from the Ptw system substitute for proteins in the bc-VirB/D4 system, as has been observed in *L. pneumophila* where components of the dot/icm and lvh T4SSs are able to interact with one another, with *lvh* gene products being able to substitute for some components of the dot/icm system for RSF1010 conjugation (Segal *et al.*, 1999). We do, however, know that the *ptwD4* deletion was not complemented by *bcvirD4* and vice versa, and that the Ptw system is not involved in mobilization of pML122-Tc. Although both coupling proteins contain P-loop and Walker B sites, which are characteristic of T4 secretion ATP-binding proteins (Walker *et al.*, 1982), they show low protein sequence similarity. Further studies will identify the structural gene(s) for the Ptw system watersoaking effector protein(s) and the substrate for the bcVirB/D4 involved in DNA mobilization.

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