Functional interactions between type IV secretion systems involved in DNA transfer and virulence

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This paper reports an analysis of the functional interactions between type IV secretion systems (T4SS) that are part of the conjugative machinery for horizontal DNA transfer (cT4SS), and T4SS involved in bacterial pathogenicity (pT4SS). The authors' previous work showed that a conjugative coupling protein (T4CP) interacts with the VirB10-type component of the T4SS in order to recruit the protein–DNA complex to the transporter for conjugative DNA transfer. This study now shows by two-hybrid analysis that conjugative T4CPs also interact with the VirB10 element of the pT4SS of *Agrobacterium tumefaciens* (At), *Bartonella tribocorum* (Bt) and *Brucella suis* (Bs). Moreover, the VirB10 component of a cT4SS (protein TrwE of plasmid R388) could be partially substituted by that of a pT4SS (protein TrwE of Bt) for conjugation. This result opens the way for the construction of hybrid T4SS that deliver DNA into animal cells. Interestingly, in the presence of part of the Bs T4SS the R388 T4SS protein levels were decreased and R388 conjugation was strongly inhibited. Complementation assays between the Trw systems of R388 and Bt showed that only individual components from the so-called 'core complex' could be exchanged, supporting the concept that this core is the common scaffold for the transport apparatus while the other 'peripheral components' are largely system-specific.

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INTRODUCTION

Type IV secretion systems (T4SS) constitute a family of macromolecular transporters involved in bacterial functions as different as conjugative DNA transfer and virulence (Cascales & Christie, 2003). Conjugative T4SS (cT4SS) are part of the bacterial machinery involved in horizontal DNA transfer among bacteria. Pathogenic T4SS (pT4SS) are required by many pathogens to infect their eukaryotic hosts; in many instances they have been shown to be the transport channels to eukaryotic cells of essential virulence factors for establishment of the donor bacterium in the eukaryotic host. In the case of *Agrobacterium tumefaciens* (At), its T4SS can be considered a cT4SS as well as a pT4SS, since it is required for T-DNA transfer into the plant host cell.

In spite of the differences between cT4SS and pT4SS

in the nature of the secreted substrate (nucleoprotein vs protein complex), in their biological role (DNA transfer vs virulence), and in the target cell (prokaryotic vs eukaryotic), there is homology among them. Most T4SS are formed by 11 proteins, named VirB1 to VirB11 for the components of the prototypical At T4SS. The overall architecture of the transporter is conserved in the family, as shown by a common gene organization (see Fig. 1), the same membrane topology of each component, and conservation of protein–protein interactions between the T4SS components. For instance the VirB7–VirB9 interaction has been described for the T4SS of At (Baron *et al.*, 1997; Das *et al.*, 1997; Spudich *et al.*, 1996), *Bordetella pertussis* (Farizo *et al.*, 1996), *Bartonella henselae* (Shamaei-Tousi *et al.*, 2004) and *Xanthomonas axonopodis* (Alegria *et al.*, 2005).

Several lines of evidence suggest that there is a 'core' complex made up of proteins VirB7, VirB8, VirB9 and VirB10 that would make up the central transmembrane channel. Topology studies indicate that these proteins are located mostly in the periplasm. VirB8, VirB9 and VirB10 were shown to interact with each other (Beaupre *et al.*, 1997; Das & Xie, 2000), in addition to the VirB7–VirB9 interaction already mentioned. All four proteins can be extracted

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Abbreviations: At, *Agrobacterium tumefaciens*; Bs, *Brucella suis*; Bt, *Bartonella tribocorum*; Ec, *Escherichia coli*; T4SS, type IV secretion system(s); cT4SS, T4SS involved in bacterial conjugation; pT4SS, T4SS involved in pathogenicity; T4CP, coupling protein.

Fig. 1. Scheme of T4SS genetic determinants. Representation of the gene arrays that code for the T4SS studied in this work: *At*-Vir (Berger & Christie, 1994), *Bs*-VirB (O'Callaghan *et al.*, 1999), *Bt*-Trw (Seubert *et al.*, 2003) and R388-Trw (this work). Homologous genes are represented in the same colour. Dashed lines represent genetic discontinuities. The prefix of the genes (*vir* or *trw*, as indicated) has been omitted for clarity. In the maps of R388 and *Bt* T4SS, postulated transcriptional units are indicated with black arrows that start at the proposed promoter sites and point in the direction of transcription. *kor* boxes are indicated by vertical dashed lines. In the *Bt*-Trw region, the seven *trwL* repetitions and the five *trwJ*–*trwI*–*trwH* repetitions are designated as in Seubert *et al.* (2003). The R388 insertion mutants used in this work are indicated with triangles at the site of the transposon insertion and the name of the plasmid, without the pSU prefix. The dark triangle indicates the Ω insertion in pSU4035. Grey rectangles show the extent of the *trw* fragments cloned in plasmids pHP109 and pHP111.

from the membrane as a complex (Krall *et al.*, 2002). Furthermore, there is functional dependence of one protein upon another: VirB9 and VirB10 form clusters in the bacterial envelope only if VirB8 is present (Kumar *et al.*, 2000), VirB8 and VirB9 sequentially bind the substrate DNA during its exit pathway through the *At* T4SS (Cascales & Christie, 2004a), VirB10 undergoes an energy-dependent conformational change to interact with the VirB7–VirB9 complex (Cascales & Christie, 2004b), and VirB9–VirB10 complex formation is required for substrate passage through the secretion channel (Cascales & Christie, 2004a, b).

The other components that make up a T4SS can be summarized as follows (for a review and update, see Cascales & Christie, 2003; Llosa & O'Callaghan, 2004): two highly conserved inner-membrane-bound NTPases, VirB11 and VirB4, are involved in early substrate transfer reactions

(Atmakuri *et al.*, 2004); VirB2 and VirB5 are pilus components (Lai & Kado, 1998; Schmidt-Eisenlohr *et al.*, 1999); VirB6 is an integral membrane protein required for T4SS assembly and function (Hapfelmeier *et al.*, 2000; Jakubowski *et al.*, 2004); VirB1 is a lytic transglycosylase required for T4SS early assembly (Koraimann, 2003), although not an essential component in all systems; finally, the function of the outer-membrane associated protein VirB3 is unknown.

Many T4SS, including all cT4SS, have an associated coupling protein (T4CP). T4CPs are proteins anchored to the inner membrane through their N-terminus, so named because they interact both with the secretion substrate and with the secretion machinery (Cabezón *et al.*, 1997; Llosa *et al.*, 2003). Besides their coupling role, they may act as DNA pumps during conjugation (Gomis-Rüth *et al.*, 2001;

Tato *et al.*, 2005). Specific protein–protein interactions between the T4CP and cT4SS components have now been described. TrwB, the T4CP of conjugative plasmid R388, interacts with proteins TrwC and TrwA, which bind to the substrate DNA, and with protein TrwE, a VirB10 homologue (Llosa *et al.*, 2003). Moreover, T4CPs interact with VirB10 homologues from heterologous cT4SS so that they can deliver their substrate DNA through the heterologous transporter, with efficiencies that correlate with the strength of the corresponding T4CP–VirB10 interaction (Llosa *et al.*, 2003). Interactions of the *At* T4CP have been described with a protein substrate, protein VirE2, and with the T4SS NTPases VirB4 and VirB11 (Atmakuri *et al.*, 2003, 2004).

Functional complementation between homologues from different T4SS has been reported in a few instances: the VirB5 homologues of two cT4SS, TraC protein of conjugative plasmid pKM101 and VirB5 of *At* T4SS (Schmidt-Eisenlohr *et al.*, 1999); and some VirB1 homologues, but not others, could be exchanged (Hoppner *et al.*, 2004). The most related T4SS systems described to date are the Trw systems found in the conjugative plasmid R388 and in *Bartonella* spp. [*B. henselae* and *B. tribocorum* (*Bt*)], which are a cT4SS and a pT4SS respectively. Identities among the Trw components of each system range from 25 to 80 %; the ‘core’ components share more than 50 % identity. Functional complementation was observed between the TrwD and TrwH components of both systems (Seubert *et al.*, 2003), underscoring the close relationships between T4SS

even when their biological role involves very different processes. Thus, the Trw T4SS of R388 and *Bartonella* spp. are probably the best candidates to obtain a hybrid c/pT4SS which ideally could be used to deliver DNA into the eukaryotic host cells (Llosa & de la Cruz, 2005).

In this work we undertook an extended analysis of T4CP–T4SS interactions and showed that T4CPs also interact with the VirB10-like component of several pT4SS. This interaction reflects a functional interaction in the case of the Trw T4SS, as shown by functional complementation between the respective T4SS components. We performed a complementation analysis between these two systems in order to obtain information about the building blocks of a T4SS that can be exchanged. Our results support the concept of a ‘core complex’ of highly conserved components that can be substituted, while the ‘peripheral’ components are more specific for their host/function.

METHODS

Bacterial strains. These are listed in Table 1. The *Escherichia coli* (*Ec*) *lacI^q* strain D1210 was used for *Plac*-driven expression. For conjugation experiments, strains D1210, HB101 or DH5 α were used as donor and recipient strains, as indicated. Strain DHM1 was used as a host in two-hybrid assays. Strain JC7623 was used to transfer mutations to R388 by homologous recombination. *At* genomic DNA was obtained from wild-type nopaline *At* strain C58. *Brucella suis* (*Bs*) 1330 is a virulent wild-type strain. *Bs* 1330 *virB5::Kan* is a mini-Tn5-km2 *virB5* mutant.

Table 1. Bacterial strains

Strain	Genotype*	Source or reference
<i>Agrobacterium tumefaciens</i> (<i>At</i>)		
C58	Wild-type nopaline	Patricia Zambryski, University of California at Berkeley
<i>Brucella suis</i> (<i>Bs</i>)		
1330	Wild-type	David O’Callaghan, INSERM, France
1330 <i>virB5::Kan</i>	Km ^R <i>virB5</i> polar mutant	O’Callaghan <i>et al.</i> (1999)
<i>Escherichia coli</i> (<i>Ec</i>)		
D1210	Sm ^R <i>proA2 leuB6 thi-1 ara-14 lacY1 galK2 xyl5 mtl-1 recA13 hsdR hsdM rpsL lacI^q</i>	Sadler <i>et al.</i> (1980)
DH5 α	Nx ^R <i>endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(argF–lacZYA)U169 ϕ80dlacZΔM15</i>	Grant <i>et al.</i> (1990)
DHM1	Nx ^R <i>cya-854 recA1 endA1 gyrA96 thi-1 hsdR17 spoT1 rfbD1 glnV44(AS)</i>	Karimova <i>et al.</i> (2005)
HB101	Sm ^R <i>thi-1 hsdS20 (r_B[–] m_B[–]) recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</i>	Boyer & Roulland-Dussoix (1969)
JC7623	Sm ^R <i>recB21 recC22 sbcB15 sbcC arg thi thr leu pro his strA</i>	Kushner <i>et al.</i> (1971)

*Km^R, Sm^R and Nx^R, resistant to kanamycin, streptomycin and nalidixic acid, respectively.

Table 2. Plasmids

Plasmid	Description	Reference
pAB2	pLAFR5-Km:: <i>Bt trw</i> region	Seubert <i>et al.</i> (2003)
pFJS134	pRL662::MobW (<i>oriT</i> + <i>trwABC</i>)	This work
pFJS193	pRL662::Mob (CloDF13)	This work
pHG327	Ap ^R Cloning vector, Rep (pMB1)	Stewart <i>et al.</i> (1986)
pHG329	Ap ^R Cloning vector, Rep (pMB1)	Stewart <i>et al.</i> (1986)
pHP100	pSU24:: <i>trwE</i> (<i>Bt</i>)	This work
pHP101	pSU24:: <i>virB10</i> (<i>Bs</i>)	This work
pHP102	pSU24:: <i>trwE</i> (R388)	This work
pHP109	pSU18:: <i>trwH-D</i> + <i>korB</i>	This work
pHP111	pHG329:: <i>trwL-I</i>	This work
pMTX513	pUT18C:: <i>trwB</i>	Llosa <i>et al.</i> (2003)
pMTX514	pT25:: <i>trwB</i>	Llosa <i>et al.</i> (2003)
pMTX631	pUT18C:: <i>trwE</i>	Llosa <i>et al.</i> (2003)
pMTX632	pT25:: <i>trwE</i>	Llosa <i>et al.</i> (2003)
pMTX644	pUT18C:: <i>traJ</i>	Llosa <i>et al.</i> (2003)
pMTX651	pT25:: <i>traJ</i>	Llosa <i>et al.</i> (2003)
pMTX667	pUT18C:: <i>traF</i>	Llosa <i>et al.</i> (2003)
pMTX671	pUT18C:: <i>virB10</i> (<i>At</i>)	This work
pMTX672	pUT18C:: <i>virD4</i> (<i>At</i>)	This work
pMTX673	pT25:: <i>virB10</i> (<i>At</i>)	This work
pMTX674	pT25:: <i>traF</i>	Llosa <i>et al.</i> (2003)
pMTX677	pUT18C:: <i>pilX10</i>	Llosa <i>et al.</i> (2003)
pMTX679	pUT18C:: <i>taxB</i>	Llosa <i>et al.</i> (2003)
pMTX680	pT25:: <i>taxB</i>	Llosa <i>et al.</i> (2003)
pMTX682	pT25:: <i>pilX10</i>	This work
pMTX687	pT25:: <i>virD4</i> (<i>At</i>)	This work
pMTX688	pT25:: <i>virB10</i> (<i>Bs</i>)	This work
pMTX689	pUT18C:: <i>virB10</i> (<i>Bs</i>)	This work
pMTX697	pUT18C:: <i>trwE</i> (<i>Bt</i>)	This work
pMTX698	pT25:: <i>trwE</i> (<i>Bt</i>)	This work
pRL662	Gm ^R broad host-range cloning vector	Vergunst <i>et al.</i> (2000)
pSU18	Cm ^R cloning vector, Rep (p15A)	Bartolomé <i>et al.</i> (1991)
pSU24	Cm ^R cloning vector, Rep (p15A)	Bartolomé <i>et al.</i> (1991)
pSU1423	pSU18:: <i>oriT</i> + <i>trwABC</i>	Bolland <i>et al.</i> (1990)
pSU1425	R388 without <i>EcoRI</i> site	Llosa <i>et al.</i> (1994)
pSU2007	R388 Km ^R	Martínez & de la Cruz (1988)
pSU1087	pUB2413 (Cm ^R , Rep (p15A)):: <i>trwA-trwN</i>	Bolland <i>et al.</i> (1990)
pSU4035	pSU1087::Ω insertion in <i>trwG</i>	Bolland <i>et al.</i> (1990)
pSU4051	pHG327:: <i>oriT</i> + <i>trwABC</i>	Bolland <i>et al.</i> (1990)
pSU4058	pHG327:: <i>trwL-trwD</i>	Bolland <i>et al.</i> (1990)
pSU4063	pSU4058::Tn5tac1 in <i>trwD</i>	This work (Table 4)
pSU4064	pSU4058::Tn5tac1 in <i>trwK</i>	This work (Table 4)
pSU4065	pSU4058::Tn5tac1 in <i>trwL</i>	This work (Table 4)
pSU4067	pSU4058::Tn5tac1 in <i>trwE</i>	This work (Table 4)
pSU4070	pSU4058::Tn5tac1 in <i>trwF</i>	This work (Table 4)
pSU4078	pSU4058::Tn5tac1 in <i>trwH</i>	This work (Table 4)
pSU4082	pSU4058::Tn5tac1 in <i>trwJ</i>	This work (Table 4)
pSU4089	pSU4058::Tn5tac1 in <i>trwI</i>	This work (Table 4)
pSU4105	pSU4058::Tn5tac1 between <i>trwH</i> and <i>trwI</i>	This work
pSU4130	pSU1425::Tn5tac1 in <i>trwL</i>	This work (Table 4)
pSU4131	pSU1425::Tn5tac1 in <i>trwH</i>	Seubert <i>et al.</i> (2003)
pSU4132	pSU1425::Tn5tac1 in <i>trwD</i>	Rivas <i>et al.</i> (1997)
pSU4133	pSU1425::Tn5tac1 in <i>trwK</i>	Llosa <i>et al.</i> (2003)
pSU4134	pSU1425::Tn5tac1 in <i>trwE</i>	Llosa <i>et al.</i> (2003)

Table 2. cont.

Plasmid	Description	Reference
pSU4135	pSU1425::Tn5tac1 in <i>trwF</i>	This work (Table 4)
pSU4136	pSU1425::Tn5tac1 in <i>trwJ</i>	This work (Table 4)
pSU4137	pSU1425::Tn5tac1 in <i>trwI</i>	This work (Table 4)
pT25	Cm ^R vector for T25 fusions, Rep (p15A)	Karimova <i>et al.</i> (1998)
pT25zip	Positive control for two-hybrid assays	Karimova <i>et al.</i> (1998)
pUT18C	Ap ^R vector for T18 fusions, Rep (pMB1)	Karimova <i>et al.</i> (2001)
pUT18Czip	Positive control for two-hybrid assays	Karimova <i>et al.</i> (2001)

Plasmid constructions. Plasmids used are shown in Table 2. Plasmids were constructed by using standard recombinant DNA technology (Sambrook *et al.*, 1989). Inserts were obtained by restriction digestion or PCR amplification with specific oligonucleotides which created the appropriate restriction sites for cloning into the same sites of the corresponding vector. All constructions involving PCR were confirmed by DNA sequencing. Table 3 shows the oligonucleotides used, with the restriction sites introduced underlined. As template DNA for amplification, we used plasmid minipreps of R388 derivatives for R388 genes, total genomic DNA obtained as described by Bruce & Jordens (1991) for *Bs* genes, and miniprep DNA from cosmid pAB2 for *Bt* genes.

To obtain fusion proteins with the T18 and T25 domains of adenylate cyclase for two-hybrid assays, ORFs of the test proteins were placed in-frame with T18 and T25 in plasmids pUT18C and pT25 using *Bam*HI and *Kpn*I restriction sites. The only exception is *At*-VirD4, since the gene includes a *Bam*HI site, so the oligonucleotides introduced instead a *Xba*I site (T18 fusion) or *Sma*I site (T25 fusion).

To produce wild-type proteins R388-TrwE (pHP102), *Bt*-TrwE (pHP100) and *Bs*-VirB10 (pHP101), the corresponding genes were amplified with oligonucleotides that introduced *Sal*I and *Eco*RI sites

and the digested products were inserted into the same sites of vector pSU24. Expression of the inserted genes was dependent on the vector *lac* promoter.

Plasmid pFJS134 was constructed by insertion of an *Eco*RI–*Hind*III fragment from pSU4051 carrying the R388 *oriT*–*trwABC* region into the same sites of broad-host-range vector pRL662. Plasmid pFJS193 contains a 3.6 kb fragment from plasmid pSU4814 (Núñez & de la Cruz, 2001) cloned into the *Eco*RI–*Hind*III sites of pRL662; this fragment contains the mobilization region of CloDF13.

To obtain separately the two putative R388 *trw* operons present in pSU4058, we used plasmid pSU4105 (Table 2), which carries a Tn5tac1 insertion at coordinate 4101 (GenBank sequence X81123), in the intergenic region between the two proposed transcripts. The insertion lies between *trwH* and *trwI* and does not affect any *trw* gene, and in fact it was the only mutant obtained that retained sensitivity to the pilus-specific phage PRD1 (data not shown). Since the transposon has several restriction sites close to both ends and pSU4058 has *Hind*III sites at both ends of the R388 *trw* region, the 5.5 kb *Eco*RI–*Hind*III fragment and the 4 kb *Bgl*II–*Hind*III fragment from pSU4105 contained the *korA*–*trwI* and *trwH*–*trwD* regions of R388, respectively. Plasmid pHP111 was constructed by insertion of the *Eco*RI–*Hind*III fragment

Table 3. Oligonucleotides

Name	Used for construct	Sequence (5'–3')*
CYABam-B10	pT25/pUT18C: <i>At</i> -virB10	CCAGGATCCCATGAACGACGACAATCAGCA
B10stop-Kpn	pT25/pUT18C: <i>At</i> -virB10	CCAGGTACCTTATGGACGGCGATCCC
CYAXba-D4	pUT18C: <i>At</i> -virD4	CCATCTAGAGATGAATTCGAGCAAGACTAC
CYA-Sma-D4	pT25: <i>At</i> -virD4	CCACCCGGGTAATGAATTCGAGCAAGACTAC
D4stop-Kpn	pT25/pUT18C: <i>At</i> -virD4	CCAGGTACCTCATTTCACCGAGTGCGA
CYABam-BarTrwE2	pT25/pUT18C: <i>Bt</i> -trwE	CCAGGATCCAATGTTTGACAATAAGGAAGGAGA
BarTrwEstop-Kpn	pT25/pUT18C: <i>Bt</i> -trwE	CCAGGTACCTAACGTGTGACGAGACTG
CYABam-BruB10	pT25/pUT18C: <i>Bs</i> -virB10	CCAGGATCCAATGACACAGGAAAACATTC
BruB10 stop-Kpn	pT25/pUT18C: <i>Bs</i> -virB10	CCAGGTACCTCACTTCGGTTTGACATCA
SalSD-trwE	pSU24: <i>trwE</i>	CCAGTCGACAAGGAGGCAACTAATGTTCCGG
trwEstop-Eco	pSU24: <i>trwE</i>	CCAGAATTCCGGTCCCGCTACTTAGT
SalSD-BarTrwE	pSU24: <i>Bt</i> -trwE	CCAGTCGACGGAGGAAAATAATGTTTGACA
BarTrwEstop-Eco	pSU24: <i>Bt</i> -trwE	CCAGAATTCTCTTTTGATATAGGGTTAAC
SalSD-BruB10	pSU24: <i>Bs</i> -virB10	CCAGTCGACGGGGAGAACCCTGCAATGAC
BruB10stop-Eco	pSU24: <i>Bs</i> -virB10	CCAGAATTCTCACTTCGGTTTGACATCA
Eco-KorA	pSU18: <i>korA</i>	CCAGAATTCAAGGGCCAACCAAAATG
KorAstop-Bam	pSU18: <i>korA</i>	CCAGGATCCGTTAGCTTTCGCCCGGA

*New restriction sites are underlined.

from pSU4105 into the same sites of vector pHG329. Plasmid pH109 carries the R388 *trwH*–*trwD* operon in vector pSU18. Since this operon includes a *Kil* function (Bolland *et al.*, 1990), the plasmid was constructed in two steps: first, the *korA* gene from R388 was PCR-amplified and cloned into the *EcoRI*–*BamHI* sites of vector pSU18, selecting for the orientation that allows *KorA* expression from the vector lactose promoter; second, the *BglII*–*HindIII* fragment from pSU4105 was inserted into the *BamHI*–*HindIII* sites of the previous construction. These plasmids were maintained in strain D1210, which has a chromosomal *lacI^d* gene, to avoid toxicity by expression of the *trw* genes from the vector *lac* promoter.

Two-hybrid assay. Strain DHM1 was co-transformed with plasmids bearing a T25 and a T18 fusion. Three independent transformants were grown together overnight in liquid medium at 30 °C, then 10 µl samples of these cultures were spread on sectors of X-Gal-containing plates to observe and compare the blue colour. β -Galactosidase levels were measured on 100 µl samples as described by Miller (1992). All experiments included positive and negative controls. Plasmid pSU4111 (Moncalián *et al.*, 1997), which carries *lacZ* under the control of the lactose promoter, produced about 6000 Miller units in this system.

Quantitative mating assays. Samples (100 µl, or 1 ml in the case of matings involving *Bs* strains) of overnight cultures of donor and recipient strains were mixed; cells were collected and placed on 0.22 µm filters on prewarmed agar plates for 1 h at 37 °C. When indicated, IPTG (0.5 mM) was added to the agar plate. To induce expression of *Bs virB* genes by low pH (Boschiroli *et al.*, 2002), the *Bs* pellets were resuspended in 1 ml MM broth at pH 4.5 (Rouot *et al.*, 2003), and incubated for 4 h at 37 °C with shaking prior to mating. Plating was done in selective medium for both donor cells and transconjugants. Transfer frequencies are expressed as the number of transconjugants per donor cell. The data reported are the mean of at least two independent assays. Mean values were calculated as simple means of the logarithm of the frequencies obtained, followed by calculation of the anti-logarithm of this mean value.

Transposon mutagenesis. *In vivo* insertional mutagenesis with transposon Tn5tac1 was carried out as described by Chow & Berg (1988) on plasmid pSU4058. Selected transposon insertions were then transferred to plasmid pSU1425 by homologous recombination using strain JC7623, as previously described (Llosa *et al.*, 1994).

Bacteriophage PRD1 sensitivity assays. Sensitivity to the pilus-specific phage PRD1 was assayed as previously described (Bolland *et al.*, 1990).

Cell extracts for protein analysis. Bacterial cultures (50 ml) in early stationary phase were harvested at 4500 r.p.m. for 15 min. Bacterial pellets were resuspended in 500 µl PBS buffer containing 1 mM EDTA, 1 mM PMSF and 10 mM benzamidine. The suspensions were transferred to chilled FASTPREP tubes containing glass beads (lysing matrix B) and cells lysed in a FASTPREP FP120 instrument (Bio 101 Thermo Savant) at a speed of 6.0 for two cycles of 30 s. Cell supernatants were harvested by centrifugation at 13 000 r.p.m. for 15 min at 4 °C. Protein concentrations were quantified by the Bradford method using decimal dilutions of BSA as a standard, and kept frozen at –80 °C.

Immunoblot analysis. Samples (20 µg) of total protein from each of the *Bs* cell lysates were run in 12 % (w/v) SDS-PAGE. After the run, protein samples were transferred from the SDS-PAGE gel to a PVDF membrane (Bio-Rad) (Towbin *et al.*, 1979), and blocked using 3 % (w/v) bovine serum albumin (Sigma) in TBST. Incubation with primary antibody was performed for 1 h at room temperature using rabbit polyclonal antibodies at the following dilutions: anti-TrwC (Grandoso *et al.*, 1994), 1:10 000; anti-TrwD

(Rivas *et al.*, 1997), 1:5000; anti-TrwF (Sastre, 1996; Seubert *et al.*, 2003), 1:20 000. The secondary antibody was goat anti-rabbit IgG horseradish peroxidase conjugate (1:2000 dilution) (Pierce). Blots were then developed with SuperSignal West Dura Extended Duration Substrate (Pierce), and either exposed to Hyperfilm MP (Amersham), or quantified by using the Chemidoc system and Quantity One software (Bio-Rad).

RESULTS

Protein–protein interactions between T4CP and pT4SS

Previous work showed that the strength of a T4CP–VirB10-like interaction correlates with the ability of a given cT4SS to serve as a conduit for the heterologous substrates driven by the T4CP (Llosa *et al.*, 2003). Using the bacterial two-hybrid assay (Karimova *et al.*, 1998), we tested for protein–protein interactions between T4CPs and VirB10-like elements of different pT4SS. To the elements previously analysed from the cT4SS of plasmids R388, pKM101 and R6K (Llosa *et al.*, 2003), we added those of the pT4SS VirB of *At*, Trw of *Bt* and VirB of *Bs*. The different T4CPs and VirB10s obtained from the pT4SS were expressed as fusion proteins with the T18 and T25 domains of adenylate cyclase (*Cya*). *Cya* domains were always placed at the N-terminus, since both T4CPs and VirB10 homologues are expected to have their N-termini in the cytoplasm, where the combined T18 + T25 can form an active *Cya*. Two-hybrid assays were performed with all pairs of proteins. Protein interactions, detected by development of blue colour on X-Gal-containing plates, were confirmed by measuring β -galactosidase activity.

Fig. 2 shows a summary of the results obtained. All interactions were made reciprocal (with each protein fused to the T25 or the T18 *Cya* domains) and no significant differences were found in any of the pairs (data not shown). The T4CP protein *At* VirD4 did not interact with any of the other fusion proteins. We do not have a way of testing the integrity of the protein, so no conclusions can be drawn from this negative result. The remaining constructions tested showed interactions with one another. VirB10 homologues interacted with themselves and with each other, as previously observed for the VirB10 members of cT4SS (Llosa *et al.*, 2003). The main result was the existence of interactions between the conjugative T4CPs (TrwB, TraJ and TaxB) and the VirB10 components of pT4SS (*At*, *Bt* and *Bs*). Interactions with their cognate VirB10 homologues (TrwE, TraF and PilX10) are also shown for comparison, although in the absence of protein quantification the results are considered only qualitative.

We performed complementation tests of an R388 *trwE* mutant that is transfer-deficient (pSU4134; Table 2) with the various VirB10-like proteins used in the two-hybrid assays to further confirm the interactions detected in Fig. 2. Plasmids were constructed that carry the R388-*trwE*, *Bt*-*trwE* and *Bs*-*virB10* wild-type genes with appropriate expression signals under the control of the vector *lac*

Fig. 2. Two-hybrid interactions among T4CP and VirB10 homologues of various T4SS. Strain *Ec* DHM1 (*cya*) was transformed with plasmids encoding T25 and T18 fusions. Co-transformants were grown at 30 °C and spread on sections of LB plates supplemented with antibiotics and X-Gal for colour development. β -Galactosidase activity was calculated in Miller units and is summarized as follows: + + +, >1000 units; + +, 100–1000 units; +, 20–100 units; –, <20 units. Key plasmid pairs are shown in the figure and labelled in the format T18:T25 fusions with the following abbreviations: At, At-VirB10; Bs, Bs-VirB10; Bt, Bt-TrwE; B, R388-TrwB; E, R388-TrwE; J, pKM101-TraJ; F, pKM101-TraF; Tx, R6K-TaxB; X, R6K-PilX10; C+ and C–, positive and negative controls (T18zip:T25zip and T18:T25zip, respectively).

promoter (plasmids pHP102, pHP100 and pHP01; Table 2). The R388 *trwE* mutant was complemented by R388-TrwE *in trans* although the conjugation efficiency decreased more than one log compared to wild-type R388. The non-polar effect of the *trwE* insertion mutation was confirmed by complementation by a *trwD* mutant (pSU4063) to wild-type levels. The same frequency was obtained when matings were done in the presence of IPTG to increase *trwE* expression (data not shown). Since TrwE forms part of a multiprotein complex it might need to be co-expressed with other components of the complex for optimal efficiency. Requirement for tight regulation of the *Bt-trwE* copy number has been reported (Seubert *et al.*, 2003).

With respect to heterologous complementations, we did not detect significant complementation with Bs-VirB10. However, there was a low but consistent complementation by Bt-TrwE, the closest homologue to R388-TrwE. The transfer efficiency of the R388 *trwE* mutant when complemented with Bt-TrwE was 100 times lower than when complemented with R388-TrwE. Similar frequencies were obtained in the presence of IPTG (not shown). Thus, the interaction observed between R388-TrwB and Bt-TrwE could reflect a functional interaction since Bt-TrwE can partially substitute for R388-TrwE.

Analysis of the R388 Trw region

The DNA sequence of the R388 T4SS genetic region (GenBank accession no. X81123) includes 11 genes, named

trwN to *trwD*, with homology to *virB1* to *virB11* respectively, plus four additional ORFs presumably involved in entry exclusion (*eex*) and regulation functions (*korA*, *orf34* and *korB*) (Bolland *et al.*, 1990 and our unpublished results). Fig. 1 shows a detailed map of this region. The role of *trwN* in R388 conjugation remains to be determined, since full transfer efficiency was obtained without this gene (Bolland *et al.*, 1990). Analysis of the DNA sequence suggested that this region is organized in four operons: *korB* (which is transcribed in the opposite direction to the rest of the genes), *trwN-orf34*, *korA-trwI*, and *trwH-trwD*. This assumption is based first on the arrangement of the genes within each proposed transcript, suggesting translational coupling, while two intergenic regions of more than 100 bp are found between each proposed transcript (225 bp between *trwN* and *korA*, and 112 bp between *trwI* and *trwH*); second, on the presence of sequences with homology to the consensus promoter 5' of each proposed transcript; and third, the three putative promoter regions are defined by the presence of 'kor boxes' (shown as dashed vertical lines in Fig. 1), presumed transcription control sites by the Kor proteins (R. Fernández, C. Revilla, M. P. Garcillán & F. de la Cruz, unpublished), that are shared by the homologous T4SS of plasmid pKM101 and Bt-Trw (More *et al.*, 1996; Seubert *et al.*, 2003). From now on we will refer to the two putative operons containing the R388 T4SS genes as 'trw region 1' (*korA-trwI*) and 'trw region 2' (*trwH-trwD*).

In order to analyse this R388 region in detail, we obtained Tn5tac1 insertion mutants along the R388 DNA segment

Table 4. Selected Tn5tac1 insertion mutants in the R388 T4SS genetic region

Insertion in pSU4058 (<i>trwL</i> – <i>trwD</i>)	Disrupted gene	Coords.*	Same insertion in R388†	Transfer frequency with helper plasmid‡				
				None	pSU4058 (<i>trwL</i> – <i>D</i>)	pHP109 (<i>trwH</i> – <i>D</i>)	pHP111 (<i>trwL</i> – <i>I</i>)	pHP109 + pHP111 (<i>trwL</i> – <i>I</i> + <i>trwH</i> – <i>D</i>)
pSU4065	<i>trwL</i>	8931	pSU4130	$< 10^{-6}$	2.6×10^{-1}	ND	8.6×10^{-5}	1.0×10^{-2}
pSU4064	<i>trwK</i>	7271	pSU4133	$< 10^{-6}$	8.0×10^{-2}	ND	3.2×10^{-4}	8.4×10^{-3}
pSU4082	<i>trwJ</i>	5920	pSU4136	$< 10^{-6}$	9.0×10^{-2}	ND	5.0×10^{-2}	1.0×10^{-2}
pSU4089	<i>trwI</i>	4686	pSU4137	$< 10^{-6}$	3.3×10^{-1}	ND	2.3×10^{-3}	1.8×10^{-2}
pSU4078	<i>trwH</i>	3974	pSU4131	5.2×10^{-5}	1.5×10^{-1}	1.6×10^{-3}	ND	1.6×10^{-2}
	<i>trwG</i>	3800	pSU4035§	$< 10^{-6}$	5.1×10^{-2}	ND	ND	ND
pSU4070	<i>trwF</i>	2897	pSU4135	$< 10^{-6}$	3.0×10^{-1}	7.0×10^{-3}	ND	2.2×10^{-2}
pSU4067	<i>trwE</i>	1622	pSU4134	$< 10^{-6}$	1.8×10^{-1}	7.9×10^{-3}	ND	9.8×10^{-3}
pSU4063	<i>trwD</i>	622	pSU4132	$< 10^{-6}$	1.2×10^{-1}	9.1×10^{-3}	ND	3.5×10^{-2}

*Coordinates at the site of Tn5tac1 insertion (GenBank X81123). The transposon duplicates 9 bp upon insertion. The site of insertion has been considered 3' to the 9 bp repeat closer to the 5' end of the gene.

†Mutations were transferred to the R388 derivative pSU1425 by homologous recombination, as explained in Methods.

‡Each R388 insertion mutant was introduced into *Ec* D1210 with the indicated helper plasmids for complementation, and mated with *Ec* DH5 α . Plasmid pSU4058 carries the complete *trw* region, and plasmids pHP109 and pHP111 carry putative operons 1 and 2, respectively (see text for further details). Transfer frequencies are expressed as number of transconjugants per donor cell; ND, not determined.

§pSU4035 is a Ω insertion mutant of pSU1087 (Table 2); the exact coordinates of the insertion were not determined. pSU4035 could not be assayed for complementation by pHP109 since both plasmids have a p15A replicon.

present in plasmid pSU4058, which includes *trw* regions 1 and 2. Tn5tac1 insertions have been shown to be non-polar (Llosa *et al.*, 1991), so mutations are expected to affect only the target ORF, unless high levels of expression of the gene are required for its normal functioning. Insertions were obtained in all *trw* genes except for *trwG* and *trwM*. A *trwG* mutant was obtained from a previous collection of mutants by insertion of interposon Ω along the *trw* region (Bolland *et al.*, 1990). All insertions affecting a *trw* gene conferred resistance to the pilus-specific phage PRD1. Selected insertions mapping in each gene (shown in Fig. 1) were transferred to R388 by homologous recombination to test for their conjugation frequency (see relevant plasmid pairs in Table 4). The *trwG* mutation was already in a plasmid containing the whole R388 transfer region. The nine mutants with insertions in different *trw* genes were transfer-deficient, except the mutant in *trwH*, which conjugated about 1000-fold less efficiently than wild-type (Table 4). All were complemented to wild-type frequencies by pSU4058, which provides the whole *korA*–*trwD* region (Table 4). We confirmed the non-polar character of these mutants by showing full complementation of mutant pairs in adjacent genes in all cases (e.g. pSU4130 + pSU4064, see Table 4; data not shown).

We next dissected the T4SS region into two segments corresponding to *trw* regions 1 and 2 (plasmids pHP111 and pHP109, respectively; Fig. 1). In order to check for the integrity and functionality of both putative operons when expressed separately, we performed mobilization assays of plasmid pFJS134, containing the R388 *oriT*–*trwABC* region in a compatible vector. pFJS134 was mobilized from strain

Ec D1210 by plasmid pSU4058 (containing the *korA*–*trwD* region) at a frequency of 1.1×10^{-1} transconjugants per donor and it was mobilized with a similar frequency in the presence of plasmids pHP109 + pHP111, providing *trw* regions 1 and 2 (2.1×10^{-1} transconjugants per donor), thus providing evidence that a functional T4SS is assembled when the two putative operons are expressed separately.

We also assayed complementation of the R388 insertion mutants by each putative *trw* operon separately, as shown in Table 4. All mutants were complemented to some extent; however, complementation levels were significantly lower than when complemented by pSU4058. When both pHP109 and pHP111 plasmids were present, complementation levels were higher, but still about 10 times lower than when complemented by pSU4058.

Functional interactions between R388 cT4SS and *Bs* pT4SS

In order to detect possible functional relationships between the R388 and *Brucella* T4SS, a series of matings were performed using *Bs* strains as donors harbouring R388 or its mobilizable derivatives. R388 conjugated from *Bs* as efficiently as from *Ec* (about 10^{-1} transconjugants per donor). None of the R388 insertion mutants was mobilized from *Bs* significantly better than from *Ec* donors (data not shown), suggesting that individual T4SS components cannot be exchanged between the two T4SS. We did not detect conjugative transfer of a plasmid carrying the R388 *oriT*–*trwABC* region (pFJS134) through the intact *Bs* T4SS. We assayed mobilization of pFJS193, a plasmid containing

Fig. 3. Immunoblots showing Trw protein levels in cell extracts from *Bs* 1330 (sample 1), *Bs* 1330 (pSU2007) (sample 2) and *Bs* 1330 *virB5::Kan* (pSU2007) (sample 3). Sample preparation and Western blotting were performed as described in Methods. The primary antibody used is indicated at the bottom of each blot. Arrows indicate the expected size of the corresponding Trw protein.

the mobilization region of plasmid CloDF13, in order to test if *Bs*-T4SS could be used by a mobilizable plasmid rather than a conjugative system, since the former are more flexible in the use of T4SS. pFJS193 was not mobilized by the *Bs* T4SS either.

We assayed a *Bs virB5* mutant that contains a polar mutation in *virB5* (Boschirola *et al.*, 2002; O'Callaghan *et al.*, 1999). Thus, in this strain presumably only the VirB1 to VirB4 proteins are produced and no functional T4SS is assembled. Consequently, the strain is avirulent in cellular models of infection (O'Callaghan *et al.*, 1999). When R388 was mobilized from this strain, the transfer frequency dropped drastically to 10^{-6} transconjugants per donor. We determined R388 Trw protein levels in both wild-type and *virB5* *Bs* strains harbouring R388. Fig. 3 shows immunoblots of cell extracts from *Bs* strains probed with anti-TrwC, anti-TrwD and anti-TrwF antibodies. It can be observed that, while the amount of TrwC (the conjugative relaxase) remains constant in both wild-type and *virB5* strains, the levels of the T4SS components TrwD and TrwF are diminished significantly. We calculated a 17-fold decrease in the amount of TrwD in the *Bs virB5* mutant compared to wild-type. Quantification of TrwF was not accurate due to non-specific binding of the anti-TrwF antibody to proteins of a size similar to TrwF (Fig. 3).

Heterologous complementation between components of the R388 and *Bt* T4SS

The Trw T4SS of R388 and *Bt* are the closest family members according to DNA sequence identity, yet they are a cT4SS and a pT4SS, respectively. Analyses of the functional relationships between these two T4SS are thus especially relevant. In previous sections we have shown that *Bt-trwE* is the only VirB10 homologue capable of complementing a R388 *trwE* mutant. We extended this analysis with a series of complementation assays using plasmid pAB2, which carries the whole *trw* locus from *Bt* on a cosmid that can replicate in *Ec*, and the R388 insertion mutants described in Table 4. Results are shown in Table 5. R388 derivatives with mutations in *trwD*, *trwE*, *trwF*, *trwG* and *trwH* showed a

Table 5. Complementation of R388 T4SS mutants

Plasmids in the first column were introduced in *Ec* D1210 and mated with *Ec* DH5 α . Plasmids pSU4130 to 4137 are described in Table 4.

Plasmids in donor	R388 Trw	<i>Bt</i> T4SS	Transfer frequency
pSU4130	All but TrwL	–	$<10^{-6}$
pSU4130+pAB2	All but TrwL	+	$<10^{-6}$
pSU4133	All but TrwK	–	$<10^{-6}$
pSU4133+pAB2	All but TrwK	+	$<10^{-6}$
pSU4136	All but TrwJ	–	$<10^{-6}$
pSU4136+pAB2	All but TrwJ	+	$<10^{-6}$
pSU4137	All but TrwI	–	$<10^{-6}$
pSU4137+pAB2	All but TrwI	+	$<10^{-6}$
pSU4131	All but TrwH	–	5.2×10^{-5}
pSU4131+pAB2	All but TrwH	+	1.6×10^{-4}
pSU4035	All but TrwG	–	$<10^{-7*}$
pSU4035+pAB2	All but TrwG	+	2.9×10^{-6}
pSU4135	All but TrwF	–	$<10^{-6}$
pSU4135+pAB2	All but TrwF	+	1.7×10^{-5}
pSU4134	All but TrwE	–	$<10^{-6}$
pSU4134+pAB2	All but TrwE	+	1.1×10^{-5}
pSU4132	All but TrwD	–	$<10^{-6}$
pSU4132+pAB2	All but TrwD	+	2.1×10^{-2}
pSU1423†+pSU4058	TrwABC+TrwL-D	–	4×10^{-1}
pSU1423†+pAB2	TrwABC	+	$<10^{-6}$
pSU1423†+	TrwABC+TrwL-I	+	$<10^{-6}$
pHP111+pAB2			
pSU4051†+	TrwABC+TrwH-D	+	$<10^{-6}$
pHP109+pAB2			

*Matings were scaled up to better discriminate between a low transfer frequency and the negative control.

†Plasmids pSU1423 and pSU4051 contain the same R388 region, but in different replicons (see Table 2).

significant increase in transfer efficiency in the presence of plasmid pAB2, while those with mutations in *trwI*, *trwJ*, *trwK* and *trwL* remained transfer-deficient. In order to rule out the possibility that recombination had taken place between the two *trw* regions, transconjugants from the above matings were checked for tetracycline sensitivity and were also used as donors in a new round of conjugations; the results confirmed that transconjugants remained transfer-deficient.

We next tried to mobilize an R388 derivative by the *Bt*-T4SS (Table 5). Plasmid pSU1423 carries R388 *oriT*+*trwABC* and could be readily mobilized by a plasmid containing *trwL* to *trwD* (pSU4058). However, we could not detect mobilization of plasmid pSU1423 by the *Bt-trw* locus present in plasmid pAB2. Since complementation of R388 single mutants by pAB2 was observed only for mutations lying in the *trwH*–*trwD* putative operon, plasmids containing *oriT*+*trwABC* were assayed for mobilization by pAB2 in the presence of an additional plasmid carrying one of the

two R388 *trw* regions (either pHP111 or pHP109). The pAB2 plasmid did not provide the remaining functions in either case (Table 5).

DISCUSSION

In this work we have analysed the functional interactions between cT4SS and pT4SS in order to better understand their constituent genetic modules and the level of functional conservation among their individual protein components.

Our model cT4SS is the R388 Trw system. Its T4SS genetic determinant comprises genes *trwL*–*trwD* (Fig. 1). The adjacent gene *trwN* is a homologue of the VirB1-type components of other T4SS; however, to date there is no evidence that *trwN* is required for R388 conjugal transfer (Bolland *et al.*, 1990), so we have excluded this gene from our analysis. Non-polar insertion mutations proved that genes *trwD*, *E*, *F*, *G*, *I*, *J*, *K* and *L* are essential for R388 conjugation; an insertion in *trwH* renders a plasmid that can self-transfer with very low efficiency (Table 4). Analysis of the DNA sequence strongly suggests that the *trwL*–*D* genes are expressed from two transcriptional units: *korA*–*trwI* and *trwH*–*trwD*. The functionality of the promoter upstream of *trwH* has been shown for the homologous regions in *Bt*-Trw and conjugative plasmid pKM101 (More *et al.*, 1996; Seubert *et al.*, 2003). We have shown that expression of each putative operon from different replicons allows the assembly of a functional T4SS, as tested by high-efficiency mobilization of a plasmid carrying R388 *oriT*–*trwABC* (pFJS134).

It was previously reported that conjugative T4CPs could interact with VirB10 homologues from other cT4SS, and moreover the strength of this interaction affected the efficiency of DNA mobilization (Llosa *et al.*, 2003). We extended the analysis of this interaction to the pT4SS of *At*, *Bt* and *Bs*. By two-hybrid analysis we have shown that conjugative T4CPs interact similarly with VirB10 homologues from pT4SS. VirB10 proteins interact with themselves and with each other, from both pT4SS and cT4SS. Thus, these interactions are conserved even in T4SS that do not have any known T4CP, such as *Bt* and *Bs*. In the case of *Bt*, it cannot be excluded that the T4CP from the VirB T4SS is being used by both coexisting T4SS (Schröder & Dehio, 2005).

The interaction detected between TrwB and *Bt*-TrwE (the closest TrwE homologue according to the amino acid sequence) may also reflect a functional interaction since *Bt*-TrwE could partially complement an R388-*trwE* mutation. This complementation adds evidence to previous work showing that *Bt*-*trwD* and *Bt*-*trwH* could complement *trwD* and *trwH* mutations in R388 (Seubert *et al.*, 2003), underscoring the functional similarity between the R388- and *Bt*-Trw T4SS. This prompted us to extend the complementation analysis between these two systems. As shown in Table 5, several R388 *trw* mutants could conjugate at low efficiency in the presence of pAB2, a cosmid providing the whole *Bt*-*trw* region. It is noteworthy that complementation is observed only for genes belonging to R388 *trw*

region 2. The conservation of gene synteny is frequently due to the need for co-expression of gene products that strongly depend on each other for function. Region 2 encodes the more conserved elements of the T4SS apparatus, the ‘core’ components, which may play a similar function in both systems and could thus be exchanged to a certain extent. On the other hand, the T4SS components encoded in region 1 may be system-specific. For instance, the pilus components (TrwL and TrwJ) could be responsible for specific interactions with the recipient cells.

Possible functional relationships between R388-Trw and *Bs*-VirB T4SS were also addressed. *Bs* T4SS did not complement any of the R388 T4SS individual mutants, not surprisingly considering that the similarity between these two T4SS is significantly lower than that between the R388- and *Bt*-Trw T4SS. However, when a *Bs* *virB5* polar mutant was used as a donor, a strong dominant negative effect was exerted on R388 transfer. Dominant negative effects are typical of proteins that make oligomers. The results suggest that when components of the *Bs*-T4SS cannot assemble into their own T4SS, they interact with the related R388 Trw proteins, resulting in non-functional heteromultimers. The unassembled Trw proteins are probably destabilized, leading to diminished cellular levels of the R388 T4SS proteins (Fig. 3). The poisoning effect could be mediated for example by VirB2, the homologue to the major pilus component. Pili are made up of a high number of pilin subunits and interference by a low number of heterologous pilin subunits could impede T4SS assembly, thus explaining the strong interference observed.

The existence of interactions between cT4SS and pT4SS components opens up the attractive possibility that DNA substrates recruited by the T4CPs could be coupled to pT4SS, so pathogens could be used as intracellular DNA delivery tools. So far, our attempts to use the pT4SS of both *Bs* and *Bt* to mobilize R388 derivatives lacking their cognate T4SS have had no success. Substrate selection by T4SS probably depends on more than a single protein–protein interaction. Each T4SS component interacts with several other components of the secretion machinery (Ward *et al.*, 2002). Recent results on the *At* T4SS show that formation of the VirB9–VirB10 complex is essential for T-DNA substrate selection and translocation through the distal portion of the secretion channel (Cascales & Christie, 2004a, b; Jakubowski *et al.*, 2005). The partial functional exchanges found between the R388 and *Bt* T4SS open up the way for mutagenesis experiments in order to obtain mutant T4CPs that better interact with selected pT4SS components or subassemblies. The study of these two highly related T4SS will also help in understanding the similarities and differences between cT4SS and pT4SS.

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