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., 1996), Bordetella pertussis (Farizzo 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
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-Ruth et al., 2001;
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 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 strain D1210 was used for P<sub>lac</sub>-driven expression. For conjugation experiments, strains D1210, HB101 or DH5α 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>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium tumefaciens</em> (At)</td>
<td>C58</td>
<td>Wild-type nopaline</td>
</tr>
<tr>
<td><em>Brucella suis</em> (Bs)</td>
<td>1330</td>
<td>Wild-type</td>
</tr>
<tr>
<td></td>
<td>1330 virB5::Kan</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt; virB5 polar mutant</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (Ec)</td>
<td>D1210</td>
<td>Sm&lt;sup&gt;α&lt;/sup&gt; proA2 leuB6 thi-1 ara-14 lacY1 galK2 xyl5 mtl-1 recA13 hsdR hsdM rpsL lacI</td>
</tr>
<tr>
<td></td>
<td>DH5α</td>
<td>N&lt;sup&gt;x&lt;/sup&gt; endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(argF-lacZYA)U169 φ80lacZAM15</td>
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<td>DHM1</td>
<td>N&lt;sup&gt;x&lt;/sup&gt; cya-854 recA1 endA1 gyrA96 thi-1 hsdR17 spoT1 rfbD1 glnV44(AS)</td>
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<td></td>
<td>HB101</td>
<td>Sm&lt;sup&gt;β&lt;/sup&gt; thi-1 hsdS20 (r&lt;sub&gt;β&lt;/sub&gt; m&lt;sub&gt;β&lt;/sub&gt;) recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</td>
</tr>
<tr>
<td></td>
<td>JC7623</td>
<td>Sm&lt;sup&gt;β&lt;/sup&gt; recB21 recC22 sbcB15 sbcC arg thi thr leu pro his strA</td>
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</table>

*Km<sup>R</sup>, Sm<sup>R</sup> and N<sup>x</sup>, resistant to kanamycin, streptomycin and nalidixic acid, respectively.
### Table 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pAB2</td>
<td>pLAFR5-Km :: Bt trw region</td>
<td>Seubert et al. (2003)</td>
</tr>
<tr>
<td>pFJS134</td>
<td>pRL662 :: MobW (oriT + trwABC)</td>
<td>This work</td>
</tr>
<tr>
<td>pFJS193</td>
<td>pRL662 :: Mob (CloDF13)</td>
<td>This work</td>
</tr>
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<td>Ap&lt;sup&gt;R&lt;/sup&gt; Cloning vector, Rep (pMB1)</td>
<td>Stewart et al. (1986)</td>
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<td>pHG329</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; Cloning vector, Rep (pMB1)</td>
<td>Stewart et al. (1986)</td>
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<td>pSU24 :: trwE (Bt)</td>
<td>This work</td>
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<td>pHPI01</td>
<td>pSU24 :: virB10 (Bs)</td>
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</tr>
<tr>
<td>pHPI02</td>
<td>pSU24 :: trwE (R388)</td>
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<tr>
<td>pHPI09</td>
<td>pSU18 :: trwH–D+ korB</td>
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<td>pHG329 :: trwL–I</td>
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<td>pUT18C :: trwB</td>
<td>Llosa et al. (2003)</td>
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<td>Llosa et al. (2003)</td>
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<td>pT25 :: traI</td>
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<td>pUT18C :: tr,F</td>
<td>Llosa et al. (2003)</td>
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<td>pUT18C :: virD4 (At)</td>
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<td>pT25 :: virB10 (Bs)</td>
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<td>R388 without EcoRI site</td>
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<td>pSU2007</td>
<td>R388 Km&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pSU4063</td>
<td>pSU4058 :: Tn5StaCl in trwD</td>
<td>This work (Table 4)</td>
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<td>pSU4064</td>
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<td>This work (Table 4)</td>
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<td>pSU4058 :: Tn5StaCl in trwL</td>
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<td>pSU4067</td>
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<td>pSU4070</td>
<td>pSU4058 :: Tn5StaCl in trwF</td>
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<td>pSU4078</td>
<td>pSU4058 :: Tn5StaCl in trwH</td>
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<td>pSU4082</td>
<td>pSU4058 :: Tn5StaCl in trwf</td>
<td>This work (Table 4)</td>
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<td>pSU4089</td>
<td>pSU4058 :: Tn5StaCl in trwl</td>
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<td>pSU4105</td>
<td>pSU4058 :: Tn5StaCl between trwH and trwl</td>
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<td>pSU4130</td>
<td>pSU1425 :: Tn5StaCl in trwL</td>
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<td>Rivas et al. (1997)</td>
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<td>pSU1425 :: Tn5StaCl in trwK</td>
<td>Llosa et al. (2003)</td>
</tr>
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<td>pSU4134</td>
<td>pSU1425 :: Tn5StaCl in trwE</td>
<td>Llosa et al. (2003)</td>
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Table 2. cont.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
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<td>This work (Table 4)</td>
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<td>pSU1437</td>
<td>pSU1425::Tn.5stcl in trwI</td>
<td>This work (Table 4)</td>
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<td>pT25</td>
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<td>Karimova et al. (1998)</td>
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<td>pT25zip</td>
<td>Positive control for two-hybrid assays</td>
<td>Karimova et al. (1998)</td>
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<td>ApR vector for T18 fusions, Rep (pMB1)</td>
<td>Karimova et al. (2001)</td>
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<td>pUT18Czip</td>
<td>Positive control for two-hybrid assays</td>
<td>Karimova et al. (2001)</td>
</tr>
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</table>

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 BamHI and KpnI restriction sites. The only exception is At-VirB10, since the gene includes a BamHI site, so the oligonucleotides introduced instead a XbaI site (T18 fusion) or SmaI 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 introducedSalI and EcoRI 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 EcoRI–HindIII fragment from pSU4051 carrying the R388 ort–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 EcoRI–HindIII 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 Tn5stcl 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 HindIII sites at both ends of the R388 trw region, the 5-5 kb EcoRI–HindIII fragment and the 4 kb BglII–HindIII fragment from pSU4105 contained the korA–trwI and trwH–trwI regions of R388, respectively. Plasmid pHP111 was constructed by insertion of the EcoRI–HindIII fragment.

Table 3. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Used for construct</th>
<th>Sequence (5′–3′)*</th>
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<tbody>
<tr>
<td>CYABam-B10</td>
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<td>CACGATCCCAATGACGAGACGAGAAATTCAGCA</td>
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<td>B1stop-Kpn</td>
<td>pT25/pUT18C:At-virB10</td>
<td>CACGATCCCAATGACGAGACGAGAAATTCAGCA</td>
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<tr>
<td>CYAXba-D4</td>
<td>pUT18C:At-virD4</td>
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<tr>
<td>CYA-Sma-D4</td>
<td>pT25:At-virD4</td>
<td>CACCCCGGGTAATGATGATCGAGCAAGACTAC</td>
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<tr>
<td>D4stop-Kpn</td>
<td>pT25/pUT18C:At-virD4</td>
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<td>BarTrwEstop-Kpn</td>
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<td>CYABam-BruB10</td>
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<td>BruB10 stop-Kpn</td>
<td>pT25/pUT18C:Bs-virB10</td>
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<td>BarTrwEstop-Eco</td>
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<td>CACGATCCCAATGACGAGACGAGAAATTCAGCA</td>
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<tr>
<td>SalSd-BruB10</td>
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<td>Eco-KorA</td>
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<td>KorAstop-Bam</td>
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</table>

*New restriction sites are underlined.
from pSU4105 into the same sites of vector pHG329. Plasmid pHPI09 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 constructions. These plasmids were maintained in strain D1210, which has a chromosomal lac^P 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 (Boschirol 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 TnStac1 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 rpm 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 rpm 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 (Grandozo et al., 1994), 1:10000; 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) (Fierce). Blots were then developed with SuperSignal West Dura Extended Duration Substrate (Fierce), 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 Plx10) 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...
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; 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 Tn5tat1 insertion mutants along the R388 DNA segment.
Table 4. Selected TnStac1 insertion mutants in the R388 T4SS genetic region

<table>
<thead>
<tr>
<th>Insertion in pSU4058 (trwL→trwD)</th>
<th>Disrupted gene</th>
<th>Coords.*</th>
<th>Same insertion in R388†</th>
<th>Transfer frequency with helper plasmid‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSU4065</td>
<td>trwL</td>
<td>8931</td>
<td>pSU4130</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4064</td>
<td>trwK</td>
<td>7271</td>
<td>pSU4133</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4082</td>
<td>trwJ</td>
<td>5920</td>
<td>pSU4136</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4089</td>
<td>trwI</td>
<td>4686</td>
<td>pSU4137</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4078</td>
<td>trwH</td>
<td>3974</td>
<td>pSU4131</td>
<td>5.2×10^{−5}</td>
</tr>
<tr>
<td>pSU4070</td>
<td>trwG</td>
<td>3800</td>
<td>pSU4035§</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4079</td>
<td>trwE</td>
<td>2897</td>
<td>pSU4135</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4069</td>
<td>trwD</td>
<td>1622</td>
<td>pSU4134</td>
<td>&lt;10^−6</td>
</tr>
<tr>
<td>pSU4063</td>
<td></td>
<td>622</td>
<td>pSU4132</td>
<td>&lt;10^−6</td>
</tr>
</tbody>
</table>

*Coordinates at the site of TnStac1 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 DH5a. Plasmid pSU4058 carries the complete trw region, and plasmids pH109 and pH111 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 pH109 since both plasmids have a p15A replicon.

Functionality of the trw region

The trw region of R388 contains a series of genes encoding components of the T4SS that mediate conjugative transfer of the plasmid. Insertions of TnStac1 were used to disrupt specific genes within the trw region. Table 4 summarizes the results of these insertions, showing the disrupted genes, the coordinates of the insertions, and the transfer frequencies observed with different helper plasmids.

Results indicate that trw genes are necessary for efficient conjugative transfer. Mutants in trw genes resulted in decreased conjugation frequencies in comparison to the wild-type strain. For example, the insertion mutant pSU4065, which disrupts trwL, showed a transfer frequency of 10^−6, significantly lower than the wild-type strain.

It was observed that mutations in trw genes affected the efficiency of conjugative transfer in a polar manner. This suggests that these genes are essential for proper function of the T4SS.

We next dissected the T4SS region into two segments corresponding to trw regions 1 and 2 (plasmids pH111 and pH109, 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 pH109 + pH111, 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 pH109 and pH111 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 conjugal transfer of a plasmid carrying the R388 oriT–trwABC region (pFJS134) through the intact Bs T4SS.

We assayed mobilization of pFJS193, a plasmid containing...
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 (Boschiroli 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 significant increase in transfer efficiency in the presence of plasmid pAB2, while those with mutations in trwI, trwf, 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

### Table 5. Complementation of R388 T4SS mutants

Plasmids in the first column were introduced in Ec D1210 and mated with Ec DH5α. Plasmids pSU4130 to 4137 are described in Table 4.

<table>
<thead>
<tr>
<th>Plasmids in donor</th>
<th>R388 Trw</th>
<th>Bt T4SS</th>
<th>Transfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSU4130</td>
<td>All but TrwL</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4130 + pAB2</td>
<td>All but TrwL</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4133</td>
<td>All but TrwK</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4133 + pAB2</td>
<td>All but TrwK</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4136</td>
<td>All but TrwJ</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4136 + pAB2</td>
<td>All but TrwJ</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4137</td>
<td>All but TrwI</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4137 + pAB2</td>
<td>All but TrwI</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4131</td>
<td>All but TrwH</td>
<td>–</td>
<td>$5.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>pSU4131 + pAB2</td>
<td>All but TrwH</td>
<td>+</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>pSU4035</td>
<td>All but TrwG</td>
<td>–</td>
<td>$&lt;10^{-7}$</td>
</tr>
<tr>
<td>pSU4035 + pAB2</td>
<td>All but TrwG</td>
<td>+</td>
<td>$2.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>pSU4135</td>
<td>All but TrwF</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4135 + pAB2</td>
<td>All but TrwF</td>
<td>+</td>
<td>$1.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>pSU4134</td>
<td>All but TrwE</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4134 + pAB2</td>
<td>All but TrwE</td>
<td>+</td>
<td>$1.1 \times 10^{-5}$</td>
</tr>
<tr>
<td>pSU4132</td>
<td>All but TrwD</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4132 + pAB2</td>
<td>All but TrwD</td>
<td>+</td>
<td>$2.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>pSU1423 + pSU4058</td>
<td>TrwABC+TrwL-D</td>
<td>–</td>
<td>$4 \times 10^{-1}$</td>
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<tr>
<td>pSU1423 + pAB2</td>
<td>TrwABC+TrwL-I</td>
<td>–</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pH111 + pAB2</td>
<td>TrwABC+TrwH-D</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
<tr>
<td>pSU4051 + pAB2</td>
<td>TrwABC+TrwH-D</td>
<td>+</td>
<td>$&lt;10^{-6}$</td>
</tr>
</tbody>
</table>

*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).
two R388 trw regions (either pH111 or pH109). 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–trwl 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 Bs and Bt. 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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