Bacterial conjugation: a two-step mechanism for DNA transport

Matxalen Llosa, F. Xavier Gomis-Rüth, Miquel Coll and Fernando de la Cruz

Departamento de Biología Molecular (Unidad Asociada al CIB-CSIC), Universidad de Cantabria, C. Herrera Oria s/n, 39011 Santander, Spain.
Institut de Biologia Molecular de Barcelona, CSIC, Jordi Girona, 18–26, 08034 Barcelona, Spain.

MicroReview

Summary

Bacterial conjugation is a promiscuous DNA transport mechanism. Conjugative plasmids transfer themselves between most bacteria, thus being one of the main causal agents of the spread of antibiotic resistance among pathogenic bacteria. Moreover, DNA can be transferred conjugatively into eukaryotic host cells. In this review, we aim to address several basic questions regarding the DNA transfer mechanism. Conjugation can be visualized as a DNA rolling-circle replication (RCR) system linked to a type IV secretion system (T4SS), the latter being macromolecular transporters widely involved in pathogenic mechanisms. The scheme ‘replication + secretion’ suggests how the mechanism would work on the DNA substrate and at the bacterial membrane. But, how do these two parts come into contact? Furthermore, how is the DNA transported? T4SS are known to be involved in protein secretion in different organisms, but DNA is a very different macromolecule. The so-called coupling proteins could be the answer to both questions by performing a dual role in conjugation: coupling the two main components of the machinery (RCR and T4SS) and actively mediating DNA transport. We postulate that the T4SS is responsible for transport of the pilot protein (the relaxase) to the recipient. The DNA that is covalently linked to it is initially transported in a passive manner, trailing on the relaxase. We speculate that the pilus appendage could work as a needle, thrusting the substrate proteins to cross one or several membrane barriers into the recipient cytoplasm. This is the first step in conjugation. The second step is the active pumping of the DNA to the recipient, using the already available T4SS transport conduit. It is proposed that this second step is catalysed by the coupling proteins. Our ‘shoot and pump’ model solves the protein–DNA transport paradox of T4SS.

Conjugation as the merging of two ancient bacterial systems

DNA replication and macromolecule transport across membranes are basic processes of life. If linked, they could form the basis for a new mechanism, DNA secretion, by simply driving the displaced replicating DNA strand to the macromolecular transporter in the membrane. It is tempting to imagine that bacterial conjugation once arose in this way. For this to happen, some DNA-replicating enzymes on one side and protein transporters on the other would have to adjust to new substrates. In addition, a new protein is needed to bring the displaced DNA in contact with the transporter: a coupling protein. With this one and only new protein (and a few adjustments), bacteria could have acquired the basics for conjugation, a mechanism that provides them with a unique means for genetic exchange and a powerful source of genetic variability.

Indeed, bacterial conjugation systems (including the related Vir system that Agrobacterium tumefaciens uses to transfer DNA to plants) show striking similarities to both DNA replication and macromolecular transport systems. Conjugative DNA-processing enzymes and their substrate DNA sequence (components of the so-called relaxosome) show extended sequence similarity to rolling-circle replication (RCR) systems (Waters and Guiney, 1993). Moreover, the set of conjugative proteins that assembles the membrane transporter belong to the type IV secretion system (T4SS) family (Christie, 2001). In addition to proteins with similarity to RCR or T4SS, all conjugative systems include one protein that has no obvious counterpart in either system. This is considered to be the coupling protein that connects the relaxosome with the membrane transporter. The evidence for this role will be discussed below.
Based on the ‘replication + secretion’ scheme, we can construct a first picture of the conjugative transfer process. (i) In the cytoplasm, an RCR-like system nicks the transferred strand (T-strand) at the origin of transfer (oriT), unwinds the nicked strand, replicates the remaining strand from the free 3'–OH end and, once a full-length T-strand has been displaced, rejoins both ends. Biochemical evidence supports the similarity of the DNA-processing reactions in both conjugation and RCR (Lanka and Wilkins, 1995). (ii) The coupling protein guides this protein–DNA complex to the T4SS at the membrane, where the T-DNA gets out of the bacteria.

But does DNA exit through the T4SS? T4SS have been shown to be directly responsible for protein transport in several systems (see below). It is difficult to imagine how such a different macromolecule as DNA would use the same transporter. We propose that the coupling protein, in addition to its coupling role, serves as an adaptor for the T4SS to a new substrate, DNA instead of protein, working as a pushing molecular motor that would confer processivity to the secretion process.

Finally, the mechanism of entering the recipient cell remains to be determined. Conjugation implies the crossing of at least one recipient lipidic barrier and usually more than that. Our proposal is that the pilus assembly opens the way into the recipient through all barriers.

**Coupling proteins**

Coupling proteins are present in all conjugative systems. The best characterized are proteins TrwB of IncW plasmid R388, TraD of IncF plasmids, TraG of IncP plasmids and VirD4 of *A. tumefaciens* Ti plasmids. There are no coupling proteins in mobilizable plasmids, such as RSF1010 or ColE1, with the notable exception of plasmid CloDF13 (Núñez and de la Cruz, 2001). These proteins share the following features:

(i) They are not involved in DNA processing reactions (Pansegrau et al., 1990; Howard et al., 1995; Llosa et al., 1995; Scheiffele et al., 1995; Tinland et al., 1995) or in pilus production (Bolland et al., 1990; Waters et al., 1992; Lai et al., 2000). They are needed after pilus assembly and contact formation (Panicker and Minkley, 1985). Interestingly, *traD* mutants of plasmid F inhibit the progression of transfer, but not its start: they prevent DNA transport to the recipient cell, but not conjugal DNA synthesis in the donor cell (Kingsman and Willetts, 1978).

(ii) They have several (usually two) transmembrane α-helices in their N-terminal region that mediate anchoring to the inner membrane and a main cytoplasmic C-terminal domain (Llosa et al., 1994; Das and Xie, 1998; Lee et al., 1999; Grahn et al., 2000). Thus, their location is adequate to be the link between a cytoplasmic system and a membrane complex.

(iii) They include conserved Walker motifs in their sequence. No NTPase activity has been reported so far, but the presence of these motifs raises the possibility that they use ATP hydrolysis as an energy source to work as motors. A soluble domain of protein TrwB (TrwBΔN70) binds NTPs (Moncalián et al., 1999), and binary complexes with different NTPs or analogues have been crystallized (Gomis-Rüth et al., 2002). Mutants in putative NTP-binding residues are transfer deficient (Balzer et al., 1994; our unpublished results).

(iv) Protein TrwBΔN70 is able to bind both single- and double-stranded DNA non-specifically (Moncalián et al., 1999); TraD and TraG may bind DNA too (Panicker and Minkley, 1992; E. Lanka, cited by Baron et al., 2002). This is a key aspect of coupling proteins. They appear to be mostly associated with systems known to transfer DNA (see below), suggesting a possible specific role in DNA transport.

(v) Unlike relaxosome components, which are specific for their cognate oriT sequences, coupling proteins show some level of interchangeability (Cabezón et al., 1994). There is indirect genetic data that coupling proteins may contact both the relaxosome and the transporter: using pieces from different conjugative systems, it was shown that the efficiency of coupling proteins in conjugation varies depending on both the relaxosome and the T4SS components used (Cabezón et al., 1997; Hamilton et al., 2000). In addition, direct interactions of coupling proteins with relaxosome components have been reported, both *in vitro* and *in vivo* (Disque-Kochem and Dreiseikelmann, 1997; Sastre et al., 1998; Szpirer et al., 2000; F. de la Cruz and E. Lanka, cited by Baron et al., 2002). No direct proof exists so far that these proteins interact with the T4SS but, as T4SS form transmembrane complexes, interactions may be more difficult to assess.

How would a coupling protein work? Their location and interactions strongly suggest that these proteins are the specific factors needed to make the contact between the relaxosome and the secretion machinery. Also, the binding of NTP and DNA suggests that the proteins use the triphosphate energy to pump DNA into the T4SS. Their role could be to thread the T-DNA towards the transmembrane pore and/or motor the DNA through it. As a main difference between a polynucleotide and a polypeptide is the length of the former, it seems that a motor would be needed to confer processivity to the transporter.

Sequence similarities further support this role. A comparison of coupling proteins against protein databases shows similarities to a family of bacterial proteins involved...
in DNA transport (Errington et al., 2001). This family includes proteins FtsK and SpoIIIE, involved in chromosome segregation at cell division and sporulation respectively. Both proteins locate to the septum, anchored to the membrane by their N-terminal domain, and have a main catalytic cytoplasmic domain, like conjugative coupling proteins. FtsK is an Escherichia coli division protein responsible for the specific partition of chromosome dimers at the septum at a late stage in cell division. It has been shown recently that FtsK is a DNA motor protein: it forms multimers that ring around DNA using ATP hydrolysis to translocate along it (Aussel et al., 2002). These authors also show that FtsK activates dimer resolution by an interaction with XerCD recombinases. Protein SpoIIIE of Bacillus subtilis is required for chromosome segregation into the prespore compartment. SpoIIIE has recently been characterized as a DNA exporter (Sharp and Pogliano, 2002). Its cytoplasmic domain is a DNA-dependent ATPase capable of tracking along DNA in the presence of ATP (Bath et al., 2000). Thus, during sporulation, SpoIIIE appears to act as a DNA pump that actively moves one of the replicated pair of chromosomes into the prespore. Finally, a group of proteins from Streptomyces called Tra proteins also belongs to the FtsK/SpoIIIE family; these proteins are responsible for intermycelial transfer of plasmids. All these similarities suggest that coupling proteins are DNA transporters that could work in a similar way to FtsK and SpoIIIE, by tracking along the DNA and thus pushing it through the T4SS. It has to be noted that FtsK and SpoIIIE act on double-stranded DNA, whereas coupling proteins would presumably track on single-stranded DNA.

The only known three-dimensional structure in the FtsK/SpoIIIE/coupling protein superfamily is that of the cytoplasmic domain of TrwB (Gomis-Rüth et al., 2001; 2002). This structure has provided valuable information on possible mechanisms of action for the coupling protein family. The protein has three structural domains: an N-terminal trans-membrane domain (TMD) that crosses the membrane twice and was deleted for structural work; a nucleotide-binding domain (NBD) that includes the Walker motifs and similarities with FtsK/SpoIIIE; and an all-alpha domain (AAD) facing the cytoplasmic side. The AAD shows significant structural similarity with XerD recombinase. The NBD resembles the RecA core structural fold, present in proteins such as the F1 subunit of the ATPase complex and in DNA ring helicases.

In view of the structural analogy, it is reasonable to deduce that TrwB could work as either a DNA helicase or a rotary DNA-tracking protein. A DNA helicase would drive the displaced DNA strand to the transporter as a result of its unwinding activity. However, TrwB has not yet been shown to possess DNA-dependent ATPase activity, a signature of DNA helicases. Alternatively, TrwB could work in an analogous way to F1 ATPase, pushing DNA directly into the transmembrane pore through its rotational tracking along DNA. Sequence similarity to SpoIIIE/FtsK reinforces this hypothesis.

DNA transport versus protein transport

T4SS are versatile macromolecular transporters. They are part of varied bacterial processes such as conjugation, T-DNA transfer to plants and toxin secretion. Lately, they have become the centre of much attention because of the finding that many human pathogens, such as Helicobacter pylori, Brucella or Bartonella, code for T4SS that are directly involved in virulence (recently reviewed by Christie, 2001). These processes imply protein and/or DNA secretion. However, DNA and proteins are very different macromolecules. How could they both be transported in a similar way? Is there evidence that both proteins and DNA are substrates for T4SS?

T4SS are protein secretors in many systems. The Ptl T4SS of Bordetella pertussis is responsible for secretion of the pertussis holotoxin (Farizo et al., 2000). Most of the newly discovered T4SS in pathogens are thought to be protein transporters, but the actual substrate is unknown, except in H. pylori, which uses a T4SS to translocate protein CagA into eukaryotic cells (Odenbreit et al., 2000). In conjugative systems, DNA is known to be transported into the recipient bacteria. Proteins may be translocated too: in certain conjugation systems, it has been shown that a DNA primase gets into the recipient cell (Rees and Wilkins, 1990; Wilkins and Thomas, 2000). We hypothesize that the protein that initially nicks the T-strand, the relaxase, is translocated with the DNA as a pilot protein into the recipient cell where it would perform the final strand-transfer step. Then, the relaxase would be the true substrate of conjugative T4SS. In the case of the Ti plasmid of A. tumefaciens, the function of VirD2 as a pilot protein is clear, and other Vir proteins are transported into plant cells (Vergunst et al., 2000) in addition to the T-DNA. Although no conjugative relaxase has yet been shown to be transported, this could be due to experimental difficulty, as presumably a single relaxase molecule would get into the recipient, whereas in the donor cell there can be hundreds of them (Abdel-Monem et al., 1977; G. Grandoso and F. de la Cruz, unpublished results).

Our hypothesis is that, after relaxase transport, the coupling protein would be the specific factor that adapts a T4SS to DNA secretion. A strong argument in support of this idea is the already mentioned similarity between coupling proteins and FtsK/SpoIIIE, which are known DNA transporters. Other arguments are that, on the one hand, no coupling protein is found associated with the Ptl system, which strongly suggests that a T4SS may be able to secrete proteins without a coupling protein. On the
other hand, no DNA secretion has been shown so far in the absence of a coupling protein.

Against this hypothesis, mutational analysis showed that coupling factors are required for protein secretion in both *H. pylori* (for CagA translocation; Fischer *et al.*, 2001) and the Ti plasmid Vir system (for VirF and VirE2 secretion; Vergunst *et al.*, 2000). However, this does not rule out a main role for coupling proteins as DNA transporters; if the couplers were originally recruited to secrete DNA by processes that need to secrete proteins too, their posterior involvement in protein secretion could help in the synchronization of the overall process. Besides, it is noteworthy that the coupling protein of the *H. pylori* T4SS binds DNA (E. Lanka, cited by Baron *et al.*, 2002). In addition, two putative DNA relaxases have been found in the *H. pylori* genome (Backert *et al.*, 1998). Thus, DNA translocation (present or past) cannot be ruled out in *H. pylori*.

**A two-step model for DNA transport**

We would like to present a model for conjugal DNA transport based on the above proposed roles for coupling proteins and pilot proteins, using the plasmid R388 transfer system as a paradigm. In R388, the coupling protein is TrwB, of known three-dimensional structure, and the pilot protein is TrwC, which has relaxase and DNA helicase activities (Grandoso *et al.*, 1994; 2000; Llosa *et al.*, 1995; 1996). We propose that the T-strand is transported in two mechanistically distinct steps (Fig. 1). In the first step, the DNA would be transported through the T4SS in a passive form, as a tail that is covalently linked to the pilot protein TrwC, the active substrate for the T4SS. After this step, the situation would be similar to that of the septum formed in division/sporulation. In a second step, TrwB would pump the T-DNA processively into the T4SS, in much the same way as FtsK or SpoIIIE pump the DNA to the dividing cell or the spore respectively. This two-step mechanism explains the need for the coupling protein late in the conjugation process, as well as most of the features of coupling proteins outlined above.

The overall process could be viewed as follows: a TrwC dimer binds oriT; one monomer nicks the T-strand and remains covalently bound to the 5’ end. The relaxosome is in close contact with the transporter through TrwB. Upon detection of a mating signal (that might come through the membrane, inducing a change in TrwB or in any of the relaxosome components), TrwC starts unwinding and the dimer dissociates; the T-DNA-bound monomer exits through the T4SS, carrying the T-strand along; the TrwC monomer remaining in the donor continues to unwind the DNA and performs the second strand-transfer reaction that liberates the T-strand from the donor molecule upon reaching the nic site for a second time. The displaced DNA strand, at first passively stuck within the T4SS that has actively transported the relaxase, is then pumped out by

---

**Fig. 1.** A two-step model for conjugal DNA transport. Horizontal thick black lines represent bacterial membranes, traversed by grey cylinders that represent the T4SS. TrwC is represented as the two-domain circle + oval (relaxase + helicase) shape; TrwB is represented as a hexamer, with an orange-like shape, anchored to the inner membrane. DNA is represented by a thin black line; newly replicated DNA, by a dashed arrow. The vertical arrowhead represents the nic site. Curved arrows indicate postulated motion forces required for DNA movement.

A. TrwB is coupling the T4SS and the relaxosome; a TrwC monomer covalently linked to the nicked T-strand is the substrate for T4SS secretion.

B. TrwB is pumping out the T-strand as it is displaced from the donor plasmid. Upon reaching the nic site for the second time, the TrwC monomer in the donor would perform a second strand-transfer reaction, thus liberating the T-strand. The translocated TrwC monomer would rejoin the two T-strand ends by a reverse cleavage reaction.
TrwB, which tracks along it; this is the motive force that sends the DNA strand through the transporter and into the recipient. In view of the recent evidence on the homologue FtsK (Aussel et al., 2002), the most plausible scenario is that the DNA goes through the central channel in the TrwB hexamer. Once the T-strand is completely transferred, the TrwC monomer in the recipient, bound to the 5′ end, would transfer it to the free 3′ end.

Other alternatives cannot definitely be ruled out at the present time. It remains possible that the T4SS is strictly a protein transporter and DNA moves through a separate pathway. After all, no direct interaction has ever been reported between coupling proteins and T4SS, and there is no definite proof for DNA going through it. In fact, protein secretion through the T4SS has been shown to occur independently of DNA transport in both A. tumefaciens (Vergunst et al., 2000) and conjugation (Wilkins and Thomas, 2000).

Another possibility would be the existence of a periplasmic step in secretion, which has been proposed for the Ptl system based on the recent finding (Farizo et al., 2002) that the holotoxin must be assembled in the periplasm for efficient secretion. Ti plasmid Vir proteins secreted through their cognate T4SS have also been detected in small amounts in the periplasm (Chen et al., 2000).

Finally, our model is based on coupling proteins working as motors. However, it is also possible that these proteins do not hydrolyse NTPs as an energy source to pump DNA. In R388, TrwC has an ATPase activity that is used to unwind DNA. This could be an alternative driving force to push the T-strand into the transporter. In this case, the role of the coupling protein would be threading the DNA into the T4SS rather than actively pumping it.

**Crossing all barriers**

Most research efforts in bacterial conjugation have been addressed to finding out how DNA is first processed in the cytoplasm and then exits the donor bacterium. Almost nothing is known about it entering the recipient cell. In conjugation-like mechanisms, there are two to four lipidic barriers to cross, apart from other barriers such as lipopolysaccharides or peptidoglycan cell walls. How are they all trespassed?

Conjugation was ‘designed’ to work among bacteria. Curiously, however, the same transfer system that operates between two bacteria works to get T-DNA into other cell types as different as yeast, plant or mammalian cells (Buchanan-Wollaston et al., 1987; Heinemann and Sprague, 1989; Waters, 2001). Thus, the mechanism of entry cannot be host specific. It appears that conjugation can transport DNA almost regardless of the number and sorts of barriers in its way. Maybe we should envisage this process as more aggressive than we traditionally think, that is working as a needle that can perforate all kinds of biological membranes.

Conjugative systems (including the Ti plasmid Vir system) form a filamentous appendage called pilus. Traditionally, the pilus was believed to play a role in attachment to the recipient. There was a long debate regarding the possibility that the pilus itself worked as a channel inside which the DNA would travel from one cell to the other. This possibility has not been completely ruled out, although it is not favoured by most experts. The recent finding that there are Ti plasmid Vir mutants that do not produce pili, yet they transfer DNA (Sagulenko et al., 2001), argues against a role for the pilus as a DNA channel.

Not all T4SS have been shown to be associated with a pilus. The only known T4SS that does not imply cell-to-cell transport, the Ptl system for pertussis toxin secretion into the milieu, does not have a pilus. No pili have been found so far associated with T4SS present in bacteria that infect animal cells. On the other hand, pili have been detected associated with type III secretion systems (T3SS), specifically in those that plant pathogens use to deliver virulence proteins into plant cells. These pili are not involved in attachment to plant cells but are the actual channels for protein transport (Jin and He, 2001). However, no pili have been detected associated with other T3SS such as that of *Yersinia*, intended to inject virulence proteins into animal cells. These systems form other much shorter structures that have been called ‘needles’, which are the actual conduits for protein transport into the recipient animal cell (Hoiczyk and Blobel, 2001).

Regardless of the type of secretion system, the secreted substrate or other possible functions in attachment or transport, it seems that pili are associated with systems intended to reach the cytoplasm of either plants or Gram-negative bacterial cells, that is recipients that present additional barriers in their envelopes when compared with the single lipidic bilayer present in animal cells. As suggested previously (Llosa and Zambrayski, 1998), pili could be required for opening the way through whatever additional barriers/envelopes are present in the recipient, thus allowing the entrance of DNA and/or proteins through a bacterial secretion system. T3SS or T4SS would be the syringes for macromolecular injection, and pili would work as the needles. Another possibility, either as an alternative to the idea that the pilus opens the way by brute force or in addition to it, is that the tip of the pilus would have some enzymatic activity that could digest peptidoglycan or plant cell walls.

Apart from opening the way, pili could inject proteins directly into the recipient cell by their sheer assembly force. Proteins could be carried at the pilus surface as it
grows, as in the 'external conveyor belt' model proposed for the Hrp pilus (Jin et al., 2001). In the Ptl system, although there are not assembled pili, there is a pilin homologue; it has been suggested (D. Burns, personal communication) that this pilin could push out the holotoxin, as in the beginning of a growing pilus. This could also be the mechanism for protein translocation into other cells, but with the help of the assembled rigid pilus to penetrate the recipient. A similar mechanism has been proposed for T2SS (Sandkvist, 2001). The appendage needed to penetrate a recipient animal cell would be much shorter than the one needed to penetrate a bacterium or a plant cell, thus appendages of different lengths could be assembled in each case, like the needles and pili found associated with T3SS in animal- and plant-associated bacteria respectively. In conjugation, the pilot protein could be injected directly into the recipient's cytoplasm with the covalently linked T-strand. The coupling protein could be injected directly into the recipient's cytoplasm with the covalently linked T-strand. The coupling protein molecular motor. This 'shoot and pump' model solves the protein–DNA transport paradox of T4SS. The DNA that lags behind to the recipient, perforating one drama. In the first act, a T4SS shoots the pilot protein and the DNA is pumped to the recipient cell by the coupling protein molecular motor. This 'shoot and pump' model solves the protein–DNA transport paradox of T4SS. Besides, we propose specific functions for T4SS and coupling proteins in conjugation-like mechanisms.

Acknowledgements

This work was supported by the Ministerio de Ciencia y Tecnología (Spain) (grants PB98-1106 to F.C., PB98-1631 and 2FD97-0518 to M.C. and BIO2000-1659 to F.X.G.-R.), and the Generalitat de Catalunya (grant 1999SGR188 to M.C.).

References


