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Adaptation of a conjugal transfer system for the export of pathogenic macromolecules

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Abstract

Conjugal transfer of bacterial plasmids requires a pore through which DNA can traverse the envelopes of the donor and recipient cells. Recent studies indicate that these pores, which are composed of approximately ten proteins, are evolutionarily related to the transport systems required for the transfer of oncogenic T-DNA from *Agrobacterium tumefaciens* to plant cells and for toxin secretion from *Bordetella pertussis*.

Nature provides countless examples of the appropriation and adaptation of familiar proteins or groups of proteins to satisfy newly arising needs. One example is the recently discovered adaptation of a conjugal transfer system by two unrelated pathogenic bacteria. Bacterial conjugation, the horizontal transfer of plasmid DNA by cell-to-cell contact, is found in many or perhaps most prokaryotes¹. It has recently become apparent that the plant pathogen *Agrobacterium tumefaciens* and the human pathogen *Bordetella pertussis* have independently coopted a similar set of *tra* (conjugal transfer system) genes for the export of macromolecules. *Agrobacterium tumefaciens* uses a conjugal transfer-like system to transfer oncogenic T-DNA to plants, whereas *B. pertussis* uses a related set of proteins to export one of its important virulence factors, pertussis toxin. Thus, similar systems are used by bacteria to transport both DNA and proteins across bacterial membranes.

Self-transmissible plasmids

The conjugal transfer systems of several self-transmissible plasmids that colonize Gram-negative bacteria have been studied. Plasmids of the N incompatibility (IncN) group direct the synthesis of conjugal pili. Unlike the thick flexible pili of the F plasmid, IncN pili are thin (approximately 10 nm) and brittle, and have pointed tips and basal knobs^{2,3}. They are readily detached from bacterial cells and are found predominantly in the culture

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supernatant². Plasmids producing this type of pilus require a solid substrate for efficient conjugation. The role of these pili in conjugation is unknown. These pili also provide attachment sites for a variety of donor-specific bacteriophages³, and the genes that confer sensitivity to these phages are generally thought to be required for the synthesis or function of the conjugal mating pore.

The genetic organization of the conjugation system of the IncN plasmid pKM101 has been elucidated. Mutants of pKM101 that are both Tra negative and resistant to donor-specific bacteriophages have been divided into seven complementation groups (one of which contains two genes, *traE* and *traO*). Mutations in another gene (*traL*) cause a 10–100-fold decrease in conjugation, but do not affect phage sensitivity. The DNA sequence of this region predicts the existence of two additional genes (*traM* and *traN*), bringing the total number of genes in this cluster to 11 (Fig. 1). All these genes are transcribed in the same direction, and are expressed from two promoters, one just upstream of *traL* and a second just upstream of *traN* (Ref. 4). Four additional *tra* genes (*traH*, *traI*, *traJ*, and *traK*) are required for conjugation, but not for pilus biosynthesis⁵. Therefore, efficient conjugation of pKM101 requires only 15 genes, making it one of the simplest conjugation systems yet characterized.

Little is known about the functions of the individual Tra proteins. It was originally believed that *traC* might encode a structural subunit of the pilus (pilin)⁵. This hypothesis was based on the observation that *traC* mutations can be complemented intercellularly by a strain that expresses all the genes required for pilus synthesis⁵. Mutants in *traC* were proposed to conjugate using pilin protein released from the helper strain. However, sequence analysis suggests that pilin may be encoded by *traM* (Ref. 6). If so, it remains possible that *traC* could encode a pilus-associated protein. Both TraB and TraG have nucleotide-binding motifs⁷, suggesting that they might provide energy for the export either of plasmid DNA or of other Tra proteins (Table 1).

Some information is available about the subcellular localization of the Tra proteins (Table 1). Except for TraB and TraG, the hydropathy profiles of these proteins suggest that they are either exported or have a membrane-spanning topology. Active *pboA* fusions to *traL*, *traM*, *traC*, *traD*, *traE*, *traO* and *traF* (57 independent fusions in all) have been obtained, suggesting that these proteins are exported^{4,8}. No active fusions have been isolated for *traA*, *traB*, *traN* or *traG*; while *traA* and *traN* provide small targets for transposon mutagenesis, *traB* and *traG* provide large targets, suggesting that their products may be cytoplasmically localized. Figure 2 shows a model of the possible localizations of and interactions between the Tra proteins.

The Tra proteins encoded by pKM101 are similar to other conjugal transfer proteins. For example, ten of the IncN Tra proteins are similar to the IncW Trw proteins (these sequences have not been published, but are described in Ref. 6) and their corresponding genes are colinear. There are lower levels of sequence similarity to the IncP Trb proteins and to the Tra proteins of the F plasmid^{1,9}. Six of the IncN Tra proteins are similar to the IncP Trb proteins, and several of the IncN Tra proteins are similar in sequence to the Tra proteins of the F plasmid.

T-DNA transfer

The transfer of oncogenic T-DNA from *Agrobacterium* species to the nuclei of infected plant cells requires approximately 20 proteins, termed Vir proteins, encoded within six operons. The largest of these operons is the *virB* operon, which contains 11 genes that encode proteins thought to form the channel in the bacterial membrane through which the T-DNA passes.

Evidence accumulated over several years suggests that Vir proteins are functionally similar to the conjugal transfer proteins of a variety of plasmids in Gram-negative bacteria. First, there are many similarities in the processing of DNA before transfer. Both modes of transfer originate at a cis-acting site, termed a 'border sequence' in *A. tumefaciens* and a 'transfer origin' in conjugal plasmids. In both cases, a specific Vir or Tra protein causes a site- and strand-specific nick at these sites, and remains covalently attached to the 5' end of the nicked strand^{10,11}. The sequence and the position of this nick are similar in T-DNA borders and in the transfer origin of IncP plasmids¹². In both cases, DNA is thought to be transferred in a single-stranded linear form^{13,14}. Interestingly, the product of the RP4 plasmid *traI* gene, which is involved in conjugal DNA processing, is similar to the product of the *A. tumefaciens virD2* gene, which processes T-DNA before transfer. Finally, both forms of transfer are thought to require a multiprotein conjugal pore through which T-DNA can traverse the envelopes of the donor bacterium and the plant or bacterial recipient. Direct evidence that Vir proteins are functionally similar to conjugal transfer proteins has been provided by the discovery that Vir proteins can mobilize derivatives of the RSF1010 plasmid for transfer to other bacteria¹⁵ or to plant cells¹⁶. These observations have led to the hypothesis that conjugation and T-DNA transfer have a common ancestry.

Additional support for this hypothesis comes from sequence analysis of the conjugal transport operons encoded by plasmids of incompatibility groups N, P, W and others^{5,17–21}. The *virB* genes of *A. tumefaciens* have significant similarity with several conjugal transport systems. A very close evolutionary similarity has been demonstrated between the *A. tumefaciens virB* genes and the *tra* genes of IncN plasmids⁴. All 11 *tra* genes of the pKM101 plasmid required to make the mating pore are similar to the 11 genes of the *virB* operon. The *tra* genes of pKM101 are arranged in the same order as their *virB* homologs (Fig. 1). In addition, the *trb* operon of the IncP plasmid RP4 appears to be related to the *virB* genes. Of the 11 *trb* genes thought to direct synthesis of a conjugal pore, six are similar to genes found in the *virB* operon, which is thought to encode a pore for T-DNA transfer⁹. Ten of the *virB* genes of *A. tumefaciens* are also similar to ten of the *trw* genes of the IncW plasmid R388.

Even before their similarities with conjugal pores were discovered, *A. tumefaciens* VirB proteins were hypothesized, on the basis of their hydropathy patterns, to form a channel for T-DNA export. Cell fractionation has shown that eight VirB proteins are membrane proteins^{22–24}, which is consistent with a role as a channel. The *virB1*, *virB2*, *virB5*, *virB6*, *virB7* and *virB10* genes have all been shown to encode exported products^{24–26} (Table 1).

The functions of individual VirB proteins are only beginning to be elucidated. Of the 11 *virB* genes with similarities to IncN *tra* genes, ten are essential for crown-gall tumorigenesis of

plants, while *virB1* (like its homolog *traL*) is dispensable²⁷. Like their Tra protein homologs, VirB4 and VirB11 contain nucleotide-binding motifs (Table 1), and purified VirB4 and VirB11 have been reported to hydrolyze ATP^{23,27–29}. Site-directed mutations in the nucleotide-binding motifs abolish or severely impair tumorigenesis, suggesting that these proteins provide energy for the export of other macromolecules or act as signaling proteins that control the opening and closing of a gate or channel. VirB2 has weak sequence similarity to the pilin protein of the F plasmid (encoded by *traA*), and both proteins are proteolytically processed during export from 12 kDa precursors to 7 kDa mature forms²². However, there is no direct evidence that the VirB operon directs the synthesis of a pilus.

Genetic evidence suggests that VirB proteins, in addition to transferring DNA, can transfer at least one protein. The VirE2 protein, which binds single-stranded DNA and may be involved in importing T-DNA into plant nuclei^{30,31}, appears to be exported from the bacterial cytoplasm by VirB proteins, even by mutant strains that cannot transfer T-DNA. This was discovered by finding that an avirulent helper strain (which has an intact *vir* regulon but lacks T-DNA) can restore tumorigenesis to a *virE* mutant by intercellular complementation³⁰. The rescuing strain must express all the *vir* genes except *virC1*, *virC2*, *virD1* and *virD2*, and must also express the genes required for binding to plant cells²⁸. This phenomenon is at least superficially similar to the complementation of pKM101 *traC* mutants described previously. While the mechanism of VirE2 export is not understood, the 7 kDa VirE1 protein may be involved because mutations in *virE2* can also be complemented by expressing VirE2 in transgenic plants³¹. In this case, *virE1* is not required, suggesting that VirE2 may function within the plant cell and that VirE1 may be needed only to export VirE2.

Pertussis toxin secretion system

The products of the *ptl* genes of *B. pertussis* are required to export the pertussis toxin³², which is composed of six subunits with a total molecular mass of 105 kDa (Ref. 33). Eight *ptl* genes (*ptlA–ptlH*) have been described^{32,34}, and inspection of the sequence between *ptlD* and *ptlE* suggests the existence of an additional gene (tentatively designated *ptlI* in this article). This suggests that nine proteins may be required for the export of pertussis toxin. Surprisingly, the *ptl* genes of *B. pertussis* are closely related to and colinear with both the *tra* genes of pKM101 and the *virB* genes of *A. tumefaciens* (Fig. 1). The Ptl proteins include homologs of every essential Tra protein of pKM 101, except for TraC, and of every essential VirB protein, except for VirB5. Thus, these three export systems form a subfamily, while the IncP-type and IncF-type conjugal transfer systems are more distantly related.

The *ptl* genes are localized immediately downstream from five *ptx* genes, which encode subunits of pertussis toxin. Although one report has provided evidence for a promoter just upstream of *ptlB*, more-recent experiments indicate that all *ptx* and *ptl* genes are expressed from a single promoter³⁵.

The Ptl proteins may form a gate or channel through which pertussis toxin leaves the bacterial cell. At least three Ptl proteins, PtlE, PtlF and PtlG, have been localized to the membrane fraction³⁶ (Table 1). Like the Tra and VirB systems, the Ptl system contains two

proteins, PtlC and PtlH, that have sequence motifs characteristic of nucleotide-binding sites³⁷. Thus, a potential function of one or both of these Ptl proteins may be to provide energy, via ATP hydrolysis, for the transport process. Although PtlA is weakly similar to TraA of the F plasmid, there is no evidence that the Ptl system encodes a pilus-like protein.

A major question about the function of the Ptl proteins concerns the specific steps involved in toxin export. Each pertussis toxin subunit is synthesized as a preprotein containing a signal sequence and may therefore be exported into the periplasmic space by the general export pathway³⁸. If this is a normal step in toxin export in *B. pertussis*, then Ptl proteins would be required only for export across the outer membrane. The localization of pertussis toxin subunits in the periplasmic fraction of *B. pertussis* supports this idea³⁹. However, the similar Tra and Vir proteins export substrates from the cytoplasm, rather than the periplasm. It is therefore unclear whether toxin transport occurs in two steps (with Ptl proteins required only for transfer across the outer membrane) or in a single Ptl-mediated step across both membranes. One report indicates that toxin export requires the S1 subunit of the pertussis toxin³⁹, suggesting that transport across at least one membrane requires assembly into the holotoxin form.

Divergent and convergent evolution

The evolution of Ptl proteins from Tra proteins is a remarkable example of divergent evolution from a common ancestor, as a DNA-transfer system has evolved into a protein-secretion system. The Ptl system is also an equally remarkable example of convergent evolution of two unrelated groups of proteins. A family of related export systems has recently been described that includes the *pul* operon of *Klebsiella oxytoca*³⁸, the *out* operon of *Erwinia* species⁴⁰ and the *xcp* operon of *Pseudomonas aeruginosa*⁴¹. These proteins export their substrates across only the outer membrane, and rely on the general export pathway for export across the inner membrane, which may also be the route for Ptl-mediated transport. However, these proteins did not evolve from a conjugal pore. Rather, they show striking similarities to the adhesive pili of various bacterial pathogens³⁸. Each export system of the Pul family contains subunits known as ‘pseudopilins’ that are similar to monomers of type 4 pili, and each system also contains a signal peptidase that cleaves and *N*-methylates these pseudopilins in a manner similar to the prepilin peptidase of type 4 pili. As these pseudopilins do not form pili, the existence of pilin homologs in the *virB* and *ptl* operons does not necessarily mean that pili are produced.

The Tra, VirB and Ptl systems each have several unique features. For example, the *tra* operon contains the *eex* gene, the product of which prevents the conjugal entry of other IncN plasmids⁴². The other two systems do not need to ward off incoming plasmids and, accordingly, lack *eex* homologs. Similarly, a *traL* homolog is found in *virB* operon, but not in the *ptl* region. TraL and VirB1 are not required for DNA transfer^{4,27}, and it appears that the Ptl system lacks a homologous protein. Interestingly, the IncW plasmid R388 also lacks a TraL homolog⁶, while F-like plasmids encode a TraL homolog, mutations in which decrease conjugal efficiencies to about 10% of the normal level⁴³.

The Ptl system also lacks a protein that is similar to TraC and VirB5. TraC mutants, although they have very low conjugal efficiencies, retain detectable levels of conjugation⁵, while VirB5 mutants are apparently completely avirulent. As described previously, *traC* mutations can be rescued by intercellular complementation, suggesting that TraC may be a pilus-associated protein. In contrast, *virB5* mutations are not complemented intercellularly. The fact that the two systems that contact recipient cells require this function, while the one that does not contact recipient cells does not require it, suggests that TraC and VirB5 could be involved in contacting recipient cells.

One additional distinguishing feature of these systems concerns their transcriptional regulation. pKM101 *tra* genes are repressed by the products of the *korA* and *korB* genes, which are themselves negatively auto-regulated⁴. However, at a phenotypic level, this transfer system is expressed constitutively, indicating either that KorA and KorB do not respond to any environmental stimulus, or that the stimulus to which they respond is supplied during laboratory conjugation experiments. IncP *tra* genes are also negatively regulated by two proteins¹⁸, while the *tra* genes of F-like plasmids are negatively regulated by a protein and a regulatory RNA⁴⁴. There are no similarities between these regulatory proteins. In contrast, the *ptx-ptl* operon and the *virB* operon are positively regulated by proteins of the two-component signal transduction family. The *ptx-ptl* operon is activated by BvgS (a membrane-spanning sensory protein kinase) and BvgA (a response regulator, see Ref. 45). These proteins presumably sense an uncharacterized stimulus found in the human upper respiratory tract, although, in the laboratory, they respond to a combination of nicotinamide and MgSO₄. The VirA and VirG proteins activate *vir* operons in response to a combination of substituted phenolic compounds, acidic pH and monosaccharides, all of which are found at plant wound sites⁴⁶. VirA and VirG are only weakly similar to the BvgS and BvgA proteins, suggesting that regulation of *virB* and *ptl* genes by two-component signal transduction proteins may have arisen separately by convergent evolution.

Questions for future research

- Are the Tra, VirB and Ptl proteins functionally interchangeable, either individually or in groups?
- Can these systems export heterologous macromolecules? For example, can Tra proteins export T-DNA, VirE2 protein or pertussis toxin? If not, which subunits recognize the correct substrate?
- Do the VirB and Ptl subunits include a pilus? If so, which proteins are found in the pilus, and what role do they play in export?
- Does the Ptl system export pertussis toxin from the periplasm or directly from the cytoplasm?
- Why are the TraC and VirB5 proteins essential components of their respective export systems, while the Ptl system appears not to require a homologous protein?

It is tempting to speculate about which system came first. Pathogenesis of plants or animals cannot predate the evolution of these hosts, so the *virB* and *ptl* systems must have evolved well after the Cambrian explosion. In contrast, conjugal transfer is ubiquitous among eubacteria and probably evolved during the Precambrian age. It is therefore highly probable that conjugal transfer systems were the ancestors of the *virB* and *ptl* systems, and that *A. tumefaciens* and *B. pertussis* subsequently adapted these genes to their needs.

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Fig. 1.
Alignments of the pKM101 pilus cluster genes with the *virB* operon of *Agrobacterium tumefaciens* and the *ptl* region of *Bordetella pertussis*.

Fig. 2.
A model describing the possible localizations of and interactions between 11 IncN plasmid Tra proteins. The colors used match those of Fig. 1.

Table 1

Subcellular localization and recognizable motifs of the Tra, VirB and Ptl proteins

Protein ^a	Subcellular localization ^b	Recognizable motif
TraL	Exported	–
VirB1	Membrane	Signal sequence, similarity with bacterial transglycosylases ⁴³
TraM	Exported	Signal sequence, pilin homolog
VirB2	Membrane	Signal sequence, pilin homolog
PtlA	Unknown	–
TraA	Unknown	–
VirB3	Membrane	–
PtlB	Unknown	–
TraB	Cytoplasmic/inner membrane	Nucleotide-binding motif
VirB4	Inner membrane	Nucleotide-binding motif
PtlC	Unknown	Nucleotide-binding motif
TraC	Exported	Signal sequence
VirB5	Exported	Signal sequence
TraD	Transmembrane	Membrane-spanning domains
VirB6	Transmembrane	Membrane-spanning domains
PtlD	Unknown	Membrane-spanning domains
TraN	Unknown	Lipid attachment site
VirB7	Membrane	Lipid attachment site
PtlI	Unknown	–
TraE	Transmembrane	Membrane-spanning domain
VirB8	Inner membrane	–
PtlE	Membrane	–
TraO	Exported	Signal sequence
VirB9	Membrane	Signal sequence
PtlF	Membrane	Signal sequence
TraF	Transmembrane	Membrane-spanning domain
VirB10	Inner membrane/exported	–
PtlG	Membrane	–
TraG	Cytoplasmic/inner membrane	Nucleotide-binding motif
VirB11	Cytoplasmic/membrane	Nucleotide-binding motif
PtlH	Unknown	Nucleotide-binding motif

^aSimilar proteins are grouped together. The Tra system is the conjugal transfer system of the IncN plasmid pKM101; the VirB system is involved in the transfer of T-DNA in *Agrobacterium tumefaciens*; the Ptl system is involved in the export of pertussis toxin in *Bordetella pertussis*.

^bThe localization ‘exported’ is defined as at least some of the protein having crossed at least one membrane, such that the protein is exposed to either the periplasmic space or the extracellular milieu.