MiniReview

F factor conjugation is a true type IV secretion system

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Abstract

The F sex factor of Escherichia coli is a paradigm for bacterial conjugation and its transfer (tra) region represents a subset of the type IV secretion system (T4SS) family. The F tra region encodes eight of the 10 highly conserved (core) gene products of T4SS including TraAF (pilin), the TraBF,-KF (secretin-like), -VF (lipoprotein) and TraCF (NTPase), -EF,-LF and TraGF (N-terminal region) which correspond to TrbCP,-IP,-GP,-HP,-EP,-JP,D P and TrbLP, respectively, of the P-type T4SS exemplified by the IncP plasmid RP4. F lacks homologs of TrbBP (NTPase) and TrbFP but contains a cluster of genes encoding proteins essential for F conjugation (TraFF,-HF,-UF,-WF, the C-terminal region of TraGF, and TrbCF) that are hallmarks of F-like T4SS. These extra genes have been implicated in phenotypes that are characteristic of F-like systems including pilus retraction and mating pair stabilization. F-like T4SS systems have been found on many conjugative plasmids and in genetic islands on bacterial chromosomes. Although few systems have been studied in detail, F-like T4SS appear to be involved in the transfer of DNA only whereas P- and I-type systems appear to transport protein or nucleoprotein complexes. This review examines the similarities and differences among the T4SS, especially F- and P-like systems, and summarizes the properties of the F transfer region gene products.

Keywords: Conjugation; Plasmid; Type IV secretion; Pili; Membrane complex

1. Introduction

In 1946, Joshua Lederberg proposed that a “cell fusion would be required” to facilitate the transfer of F factor DNA, integrated in the chromosome of the donor cell, into recipient Escherichia coli [1]. We now know that this cell fusion is constructed by the type IV secretion system (T4SS) encoded on Gram-negative conjugative elements. T4SS, also known as the mating pair formation (Mpf) apparatus, are central to the dissemination of numerous genetic determinants between bacteria, as highlighted by the spread of antibiotic resistance among pathogens [2,3]. T4SS are cell envelope-spanning complexes (11–13 core proteins) that are believed to form a pore or channel through which DNA and/or protein travels from the cytoplasm of the donor cell to the cytoplasm of the recipient cell. T4SS have also been found to secrete virulence factor proteins directly into host cells as well as take up DNA from the medium during natural transformation, revealing the versatility of this macromolecular secretion apparatus [4,5]. Despite the clinical and evolutionary importance of T4SS, the general mechanism by which they secrete or take up macromolecules remains unknown.

The F factor remains a paradigm for understanding the mechanism by which T4SS transfer macromolecules across the membranes of Gram-negative bacteria [6–8]. DNA transfer occurs within the tightly appressed cell envelopes of mating cells, which are referred to as conjugation Junctions [9–11]. These junctions form in the presence of the Mpf or T4SS proteins; the same proteins that assemble pilus (Figs. 1 and 2; Table 1) and transfer DNA. Conjugation is thought to be initiated by contact between the F-pilus and a suitable recipient resulting in pilus retraction [12] and stable mating pair or aggregate formation [9]. Prior to the initiation of DNA transfer, the relaxosome, consisting of proteins bound to the origin of transfer (oriT), resides within the cytoplasm of donor cells [13]. A mating signal, possibly generated by contact between the pilus and recipient cell, appears to result in a specific interaction between the relaxosome and the coupling protein, or nucleic acid pump, at the inner face of the con-
jugative pore [14,15]. Coupling protein–relaxosome contact could lead to DNA unwinding, generating a single strand of DNA that is then transferred to the recipient in a 5' to 3' direction [16–18]. This two-step mechanism has been proposed to result in the transport of the relaxase, covalently bound to the 5' end of the transferring strand (T-strand), into the recipient through the T4SS conjugative pore [19]. The detection of the relaxase in the recipient has as yet not been successful, however, the topological constraints of DNA transfer combined with the role of the relaxase in termination makes this highly probable. Considerable circumstantial evidence supports the transfer of a pilot protein, such as the relaxase, along with the DNA. The most compelling is the indirect evidence for transport of a primase, encoded as a domain of the relaxase protein by the IncQ mobilizable plasmid R1162, that could initiate replacement DNA strand synthesis in the new transconjugant [20]. Interestingly, the IncP and I conjugative systems also transport primase molecules either alone or in conjunction with the DNA [21,22], suggesting an evolutionary relationship with the IncQ system. The transport of the VirD2–T-DNA com-

Fig. 1. Comparison of F-like T4SS with each other and with P- and I-like T4SS. Transfer genes are presented with color/pattern, with the same color/pattern representing homologous gene products (see Table 1), while non-essential transfer genes are white. Light gray genes represent transfer gene products with no shared homology to other T4SS subfamilies. Lipo = lipoprotein motif; band within arrow = Walker A motif; upper case gene names = Tra; lower case gene names = Trb (F, pNL1 and RP4) or Trh (R27). Double slash indicates non-contiguous regions. The gene sizes are relative to each other. Maps were produced using the indicated GenBank accession numbers: F-NC_002483; pED208-AY046069; R27-NC_002305; Rts1-NC_003905; pNL1-NC_002033; R391-AY090559; SXT-AY055428; RP4-NC_001621; R64-AB027308. See text for details and references.
plex from *Agrobacterium tumefaciens* to wounded plant tissue to initiate crown gall formation is another example of a relaxase-like protein bound to the 5’ end of a single-stranded DNA molecule being transported via a T4SS [23–25]. Thus it is not impossible to think of conjugative DNA transfer as a protein transport system that has been modified to transfer DNA along with a protein substrate.

The core T4SS proteins in F, TraAF (pilin), -LF, -EF, -KF, -BF, -VF, -CF and -GF (N-terminal domain), also require the auxiliary, essential gene products TraFF, -GF (C-terminal domain), -HF, -NF, -UF, -WF and TrbCF for pilus assembly and mating pair stabilization. Additional essential gene products in the F conjugative system include the coupling protein, TraDF, and the members of the relaxosome, TraIF, a relaxase–helicase bifunctional protein, TraMF, and TraYF that are required for DNA transfer. TraBF along with TraCF are the quintessential T4SS proteins and are the easiest to find homologs for in BLAST searches. Similarly, the coupling protein (e.g. TraDF) is the signature homolog of conjugative T4SS systems capable of nucleic acid transport [19], whereas TrbBp/VirB11T/FrcaJ homologs are indicative of P-type/ Ti/I-type systems [26]. The auxiliary genes present in F (encoding TraFF, -GF (C-terminal domain), -HF, -NF, -UF, -WF and TrbCF) are conserved throughout F-type systems and serve as hallmarks of this family. These gene products are essential for F transfer and appear to be involved in pilus retraction and mating pair stabilization, which are critical factors for efficient F conjugation in liquid media. The conjugative ability of P-type systems, which lack these homologs, is lower in liquid media than on solid media and may reflect the different ecological niches inhabited by bacteria carrying the F- and P-type transfer systems [27].

The proteins involved in conjugal DNA metabolism as well as those involved in the regulation of gene expression or the prevention of conjugation between donor cells (surface and entry exclusion, TraTF and -SF, respectively) will not be discussed here. The interested reader is directed to reviews by Lanka and Wilkins [28], Lawley et al. [8], Llosa et al. [19] and Zechner et al. [18]. This review will discuss the essential T4SS proteins in F-type systems (IncF, IncH, IncJ, IncT and the SXT element, among others), which differ in significant ways from P-type systems such as that of RP4 (IncP), Ptl (*Bordetella pertussis* toxin excretion system) and VirB (Ti plasmid tumorigenesis system of *A. tumefaciens*) T4SS [2,4] (see below). A third system, the I-type, about which relatively little is known, is exemplified by the IncI plasmid T4SS that has significant homology to the virulence factor transport systems of *Legionella pneumophila* [29–31].

### 2. F-like T4SS components

The essential components of the F-like T4SS are defined as those Mpf proteins that are essential for conjugation, as determined by mutagenesis and complementation experiments of both the F factor and the IncHI1 plasmid R27 [7,32–34]. Results obtained from investigations into individual Mpf proteins from both the F factor and the R27 T4SS are combined to create an F transfer protein family.
in this overview, as it is likely that homologs are functionally equivalent. Based on work on the F factor, the T4SS proteins are organized according to three proposed functions: (1) pilin and pilin processing, (2) pilus tip formation and pilus extension and (3) mating pair stabilization [7] (Figs. 1 and 2; Table 1). Other non-essential components of F-like T4SS are TraP, a protein that stabilizes the extended pilus; TrbB, a putative thioredoxin homolog; TraI, a protein which promotes DNA transport and has homology to the FliK flagellum assembly protein (L.S. Frost, unpublished results); and Orf169, a lytic transglycosylase with homologs in P- and I-like systems.

2.1. F-like propilin processing

The propilin subunits from F-like T4SS ranges in size from 112 to 128 aa (Table 1). The pilin subunit is poorly conserved among T4SS, for example the pilin subunit of the R27 (TrhA9) shares more similarity with the IncP pilin (TrbCp) than with the IncF pilin (TraAp) [34]. All F-like propilin subunits contain a long leader sequence that is either known or predicted to be cleaved by the host leader peptidase, LepB, to produce a peptide of 68–78 aa [35,36]. After removal of the signal sequence, the F-pilin subunit is oriented in the inner membrane with its N- and C-termini positioned in the periplasm [37,38]. Indeed, all pilin subunits of the F-type T4SS appear to contain two hydrophobic regions that serve as transmembrane regions. The correct insertion and accumulation of F-pilin in the inner membrane require the chaperone-like inner membrane protein, TraQ, which is present only in T4SS closely related to F itself [39]. Pilin subunits typically undergo an additional processing reaction, which has been identified as acetylation by TraX F in F-like pilins (F, R1, R100-1, pED208) [39–41] or cyclization by the peptidase TraFP in P-like pilins (RP4 and Ti) [42]. The propilin subunits of R27, RtSl, R391, SXT and pNL1, which are encoded by F-like T4SS, are more similar to P-like pilins. They are likely cleaved at the C-terminus and possibly cyclized by a transfer peptidase/cyclase, although this has yet to be demonstrated.

Pilin insertion into the membrane and maturation are

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<th>Protein</th>
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<th>I-type homolog</th>
<th>Size range (aa)</th>
<th>Signal sequence</th>
<th>Motifs</th>
<th>Proposed function</th>
<th>Interacting partners in F- and P-like T4SS</th>
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<td>N</td>
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<td>Pore</td>
<td>TraC&lt;sub&gt;H&lt;/sub&gt;, TraC&lt;sub&gt;H&lt;/sub&gt;</td>
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<td>Y</td>
<td>IM/P</td>
<td>Pore</td>
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<td>Coiled-coil</td>
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<td>TraC&lt;sub&gt;F&lt;/sub&gt;</td>
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Components in bold indicate homology to P-like T4SS.

<sup>a</sup>Nomenclature according to the F system except for the peptidase TrhP which is named according to R27 nomenclature. The R27 transfer protein nomenclature is Trb [33,34]. The P- and I-type nomenclature is according to Christie [2].

<sup>b</sup>R27, RtSl, R391, SXT and pNL1 systems contain a peptidase with the peptidase of pNL1 containing an N-terminal fusion to TrbI, suggesting a coupled function. F and pED208 do not contain peptidases.

<sup>c</sup>In R27, RtSl, R391 and SXT systems TrbC is fused to the N-terminus of TraW, suggesting a coupled function, whereas they are separate proteins in F and pED208.

<sup>d</sup>Homology deduced based on similarity identified with PSI-BLAST analysis or functional analogy.

<sup>e</sup>Range is determined by comparing homologs in F, pED208, R27, RtSl, R391, SXT and pNL1.

<sup>f</sup>Signal sequence predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP).

<sup>g</sup>Inner membrane (IM), periplasm (P), outer membrane (OM) and extracellular (E).


<sup>i</sup>Transfer system in which direct or indirect interaction identified is indicated with subscript.
the first steps in pilus production. Assembly of conjugative F-like pili on the bacterial surface requires the remainder of the T4SS and the auxiliary gene products, except for the C-terminal domain of TraG, TraN and TraU. F-pilin subunits are stored as a pool in the inner membrane prior to assembly on the cell surface [43]. Pili are assembled by addition of pilin subunits to the base of the pilus, as demonstrated by H-pili of R27 [44]. In response to contact with a suitable recipient, pilus retraction appears to proceed in an energy-independent manner [40], which is the reverse of assembly, whereby the pilin subunits return to the membrane and possibly serve to stabilize the mating pair or be a part of the conjugative pore.

Homology studies have revealed that the pilin gene appears to have been shuffled among various T4SS during evolution. For example, the IncHI1 plasmid, R27, has an F-like T4SS except for a P-like pilin protein and corresponding peptidase/cyclase [34]. The lack of sequence conservation in pilin could be due to: (1) rapid evolution of the pilin subunits in response to strong selective forces of extracellular factors such as phage and receptors on recipients and (2) lateral gene transfer of F-, P- and I-like propilin and processing genes between T4SS subfamilies. In fact, the cassette-like nature for the development of the T4SS is striking and suggests that there has been considerable opportunity for ‘mix and match’ during evolution.

2.2. F-like T4SS pilus assembly

Mutations in traL, -E, -K, -B, -V, -C, -W, -F, -H, and the 5’ end of traG have broadly similar phenotypes, which include the inability to assemble pili and transfer DNA [7]. Using a sensitive M13K07 transducing phage assay, Anthony et al. [32] identified two mutant subgroups that are consistent with two steps in pilus assembly: (a) those mutations that prevent pilus tip formation on the cell surface (in traL, -E, -K, -C, -G) and (b) those that allow tip formation but block pilus extension (traB, -V, -W, -F, -H). These results provided the first example in any T4SS of a differentiation of roles for Mpf proteins. The F-like T4SS components will be organized according to these results.

2.2.1. Pilus tip formation

2.2.1.1. TraLF. Members of the TraLF family range in size from 93 to 105 aa and are homologous to TrbDP (103 aa) and VirB3TT (108 aa) [45]. TraL is predicted to localize to the inner membrane, as is TrbDP [46]. In F, TraLF has never been visualized, suggesting it could be the limiting factor determining the number of F-pili per cell. TrhLH, along with TrhE and TrhBH, of R27 (IncHI1) was shown to be essential for the formation of TrhCH complexes, indicating either a direct or an indirect interaction between TrhLH and TrhCH [47].

2.2.1.2. TraEF. TraEF family members range in size from 130 to 261 aa and are homologous to TrbIP (258 aa) and VirB5TT (220 aa) [7]. TraEF and TrbIP are predicted to be located in the inner membrane (RP4) [7,46] whereas VirB5TT is thought to be a minor component of the T-pilus [48].

2.2.1.3. TraKF. The TraKF family of proteins range in size from 299 to 410 aa and are homologous to TrbGP (297 aa), VirB9TT (293 aa) and TraN1 (327 aa) [7]. TraK-like proteins are predicted to be located in the periplasm or outer membrane [7,46,49]. This protein family shares similarity to secretin proteins, especially the HrcC subgroup of the type III secretion system (T3SS) encoded by Pseudomonas syringae [50] (Fig. 3). The C-terminal regions of TraKF proteins are conserved in both the β-domain and S-domain of the prototypical secretin PulD of Klebsiella oxytoca [34]. The β-domain is present in all secretins and is proposed to be embedded within the outer membrane to form the ring structure typical of secretins. The S-domain is a region of 60 aa that binds to a lipoprotein which serves as a periplasmic chaperone [51]. The C-terminus of TraKF has been shown to interact with TraVF, a lipoprotein, and the N-terminus of TraKF interacts with TraBF, an inner membrane protein [49]. The TraBF-TraKF-TraVF complex likely forms an enveloping structure similar to that of VirB10-VirB9-VirB7 of the Ti plasmid T4SS [52]. Although TraKF is a periplasmic protein, it associates with the outer membrane in the presence of the F T4SS [49]. The presence of a putative secretin within the T4SS suggests a mechanism by which both the pilus and DNA could transverse the outer membrane.

2.2.1.4. TraCF. Members of the TraCF family of proteins range in size from 799 to 893 aa and are homologous to TrbEP (852 aa), VirB4TT (788 aa) and TraU1 (1014 aa). TraCF is predicted to be a peripheral inner membrane protein whose localization is dependent upon the presence of the T4SS, specifically TraLF [47,53]. All members of this protein family contain both Walker A and Walker B motifs, which energize pilus assembly [54,55]. A point mutation in traCF, traC1044, is a temperature-sensitive mutation that blocks pilus assembly [56]. Using TrhCF-GFP fusions, TrhCH of R27 was shown to form complexes in the inner membrane, possibly containing other transfer proteins. The formation of TrhCF-GFP complexes was dependent on the presence of TrhBH, -E and -H, suggesting either a direct or an indirect interaction between these proteins [47].

2.2.1.5. TraGF. TraGF proteins range in size from 913 to 1329 aa. TraGF proteins have two roles in conjugation: the N-terminal region is involved in pilus tip formation and pilus assembly whereas the entire protein is involved in mating pair stabilization [57] (see below). The N-terminal 500–600 aa is proposed to be localized to the inner
 membrane and contains six to eight transmembrane regions whereas the remaining C-terminal region is located within the periplasmic space (unpublished results). The N-terminal domain is homologous with TraBp (528 aa) whereas the remaining C-terminal region is located within the periplasmic space (unpublished results). The regions whereas the remaining C-terminal region is located within the periplasmic space are conserved in sequences with very little divergence were discarded and the multiple alignment was imported into MacClade v4.0 to generate a * nexus file. Phylogenetic analysis was carried out with the PAUP 4.0 beta 8/10 software package using parsimony and a heuristic search of 100 replicates with the PAM250 matrix character type. Once a final tree was selected, it was bootstrapped through 100 replicates to give the consensus values shown. Therefore, it is interesting to note that several TraSS components localizing to the periplasm contain multiple, conserved cysteine residues including homologs of TraFp and TrbBp-like proteins are key to the thioredoxin superfamily, characterized by the C-X-X-C motif and the thioredoxin fold (Elton et al., in preparation). In light of these observations, perhaps TraFp- and TrbBp-like proteins are key to the disulfide bond chemistry in F-like TraSS assembly.

2.2.2.3. TraHF. TraHF-like proteins range in size from 453 to 501 aa and are unique to the F-like TraSS subfamily. Members of the TraHF protein family are localized to the periplasm/outer membrane [63] and contain C-terminal coiled-coil domains, suggesting the formation of higher order structures, either with other TraHF molecules or with other components of the TraSS.

2.2.2.4. TraWF-TrbCF. Members of the TraWF protein family range in size from 257 to 363 aa and shares homology to TraBF (a non-essential, conserved, F-like TraSS component) and TraBp of R27 was recently shown to interact with itself and with the coupling protein TraGH [59] providing exciting evidence that the TraSS and the coupling protein (in F, TraDF) do, indeed, ‘couple’, linking the relaxosome to the TraSS.
minus of TraW in R27, Rts1, R391 and SXT, whereas TraW and TrbC are separate proteins in F, pED208 and pNLT. The fusion of TrbCF to TraWF suggests that the functions of these proteins are linked. Both proteins are proposed to be localized to the periplasmic space. TrbCF is correctly processed in the presence of TraNF, suggesting a relationship between these two proteins, which are encoded on adjacent genes in the F tra operon [64].

2.2.2.5. TraVF. TraVF-like proteins range in size from 171 to 316 aa and are lipoproteins with a signature cysteine at the processing site [65]. Although TraVF proteins share little similarity to TrbHP (160 aa), VirB7T1 (55 aa) or TraI1 (272 aa) beyond two conserved cysteines thought to be involved in multimerization, they appear to be functional analogs that interact with secretin-like proteins such as TraKp and VirB9T1. Indeed, TraVF has been shown to interact with TraKp, a putative secretin [49], and VirB7T1 is known to interact with VirB9T1 [66, 67].

2.2.3. Mating pair stabilization

Mating pair stabilization is a unique feature of F-like T4SS and is believed to be at least partially responsible for facilitating DNA transfer in liquid environments. Based on the experimental evidence of Kingsman and Willetts [68] and recent evidence involving TraGF in recognition of the TraSF entry exclusion protein (L.S. Frost, unpublished results), mating pair stabilization might involve building a structure between the two cells that ‘staples’ them together. Mating pairs are difficult to break apart prematurely and require significant force to do so. However, about 30 min after the start of F plasmid transfer, the cells spontaneously separate suggesting an active mechanism involving the expression of genes in the new transconjugant, previously identified as being in the distal part of the F tra operon [69]. Candidates for mating pair separation include the entry and surface exclusion proteins TraSF and TraTF as well as the relaxase (TraI) and coupling protein (TraDF), which might generate a break in DNA transport signalling the termination of conjugation.

2.2.3.1. TraGF. The whole of TraGF, but especially the C-terminal region, is involved in mating pair stabilization. In F T4SS, the C-terminal region is fused to a homolog of TrbLP/VirB6T1 suggesting that these homologs might be involved in forming a conjugative pore with varying degrees of sturdiness. This region is predicted to be located within the periplasmic space and has been proposed to interact with TraNF to stabilize mating pairs [57]. A second C-terminal product TraG*, which is believed to be a cleavage product of the full-length protein, has been detected in the periplasm [57] although its importance in transfer is in doubt (L.S. Frost, unpublished results). TraGF is involved in entry exclusion, a process by which DNA synthesis and transport from the donor cell is blocked by TraSF in the inner membrane of the recipient cell. TraGF could be translocated to the recipient cell where it would interact with TraSF instead of its true receptor. TraGF is plasmid-specific for TraSF and this specificity maps to a central C-terminal domain of TraGF (L.S. Frost, unpublished observation). If homologs of TraGF are involved in mating pair junction formation, it suggests that the periplasmic space of the donor cell contracts bringing the inner and outer membrane together. In P-type systems, the TrbLP/VirB6T1 homologs might not be able to penetrate the cell envelope of the recipient cell, a function of the pilus, whereas the C-terminal domain of TraGF homologs reaches all the way to the inner membrane of the recipient to stabilize the pilus penetration event.

2.2.3.2. TraNF. TraNF-like proteins are 602–1230 aa and are unique to F-like T4SS; they are signature proteins for the auxiliary class of T4SS that define the F-like subfamily [70]. This family of proteins appear to act as ‘adhesins’ based on evidence for TraNF which is present in the outer membrane of donor cells. TraNF of the F plasmid interacts with the major outer membrane protein OmpA in recipient cells to stabilize the mating pairs prior to DNA transfer. Other F-like TraNF proteins do not necessarily interact with OmpA, for instance, TraNR100 of the F-like R100 plasmid does not share this receptor. The N- and C-terminal regions of TraNF proteins are highly conserved whereas the central region displays extensive divergence. It is this central region that is involved in OmpA recognition by TraNF as well as TraNF multimerization [71]. Preliminary evidence suggests that TraNF and TraVF interact since some mutants of tranF are destabilized in the absence of traV [70].

2.2.3.3. TraUF. Members of the TraUF protein family range in size from 330 to 358 aa and are unique to the F-like T4SS subfamily. TraUF is a periplasmic protein that is essential for DNA transfer but not formation of conjugative pili, as 20% of donors containing F traU mutations produce pili. TraUF is therefore proposed to be primarily involved in DNA transfer perhaps by aiding mating pair stabilization and conjugative pore formation since mutations in traUF, -G and -N have the same phenotype [72].

3. Relationships between F- and P-type T4SS

It has been long been recognized that there are two types of conjugative pili: long, flexible pili and short, rigid pili [27]. It is now evident that long, flexible pili are encoded by F-type T4SS (IncF, -H, -T, -J) whereas short, rigid pili are encoded by P-type T4SS (IncP, -N, -W, -I). The long, flexible pili produced by F-like T4SS measure 2–20 μm and have a diameter of 8 nm with a central lumen measuring 2 nm. The pilin subunits are arranged as a he-
F-pili are easily seen attached to cells and appear flexible in electron micrographs. The short, rigid pili produced by P-like T4SS are seldom seen attached to donors. They measure 8–12 nm in diameter [42] and are usually under 1 μm in length. No information on the arrangement of the circular subunits in the assembled pilus is currently available for P-like pili. The differences in pilus structure are not likely dictated by differences in pilin processing, such as acetylation or cyclization, since acetylase and transfer peptidase coding regions can be present in

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both F-like and P-like T4SS. Instead, the differences prob-
ably lie with the auxiliary genes in F-like systems or TrbBP
in P-like systems, which define these two groups (see be-
low).

Long, flexible pili allow donors to mate in liquid and on
solid media with approximately equal efficiencies whereas
short, rigid pili result in a surface-preferred mating pheno-

and IncI plasmids are also known to transfer the DNA
primases, TraC and Sog, respectively, from donor to re-

both the accessory genes in F-like systems or TrbBP
in P-like systems, which define these two groups (see be-
low).

Long, flexible pili allow donors to mate in liquid and on
solid media with approximately equal efficiencies whereas
short, rigid pili result in a surface-preferred mating pheno-
type [27,73]. Long, flexible pili likely retract and allow
mating pair stabilization thereby facilitating mating in liq-
uid media, a property not available to systems with short,
rigid pili. Retraction [12,74,75] is reminiscent of type IV
pili encoded by type II secretion systems [76,77] and is

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ize number of macromolecules. For example, IncP and
IncL plasmids are also known to transfer the DNA
primases, TraC and Sog, respectively, from donor to re-

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the pilus tip when it contacts a suitable recipient cell.

Other conjugative elements which contain type F T4SS
include, besides the F factor (E. coli) [7], R100 (IncFI)
[32], pED208 (IncFV; S. typhii) [39], R27 (IncH11; S. ty-
phi) [78], Rts1 (IncT; Proteus vulgaris) [79], R391 (IncJ;
Providencia rettgeri) [80], SXT element (Vibrio cholere-
ae) [81] and pNLL (Novosorphingomonas aromaticivorans)
[82] (Fig. 1). Neisseria gonorrhoeae contains an F-type
T4SS that is not used for conjugation, but rather for the
secretion of DNA [83]. It is interesting that no F-type
T4SS have yet been shown to secrete virulence factors.
In fact, no F-type T4SS to date has been shown to secrete
proteins [21].

Conjugative elements that contain P-type T4SS include
RP4 (IncPp; Pseudomonas aeruginosa) [84], R751 (IncPp;
Klebsiella aerogenosa) [85], pKM101 (IncN; Salomonella
typhimurium) [86] and R388 (IncW; E. coli; accession num-
ber X81123) (see [4]). In many respects, P-type T4SS ap-
pear to be capable of transferring/secreting/taking up a
broader repertoire of macromolecules. For example, IncP
and IncL plasmids are also known to transfer the DNA
primases, TraC and Sog, respectively, from donor to re-
cipient cells [21] even in the absence of DNA [22]. As
noted earlier, Helicobacter pylori utilizes a subset of the
P-type T4SS for DNA uptake [5]. Many pathogens use
P-type T4SS to secrete virulence factors into hosts as pro-
teins or nucleoprotein complexes, such as the T-DNA of
the Ti plasmid [87], CagA of H. pylori [88–90] and pertuss-
sis toxin of B. pertussis [91,92]. It is noteworthy that con-
jugative plasmids containing the P-type T4SS are broad-
host-range (IncP, W and N) [93] whereas F/H-type sys-
tems are narrow-host-range.

The nature of the conjugative pore

The nature of the conjugative pore is the central ques-
tion in conjugation, as well as in the biology of T4SS.
Only recently have we begun to understand how single-
stranded DNA can traverse the cell envelopes of both
donor and recipient cells (Fig. 2). At the inner face of
the conjugative pore are coupling proteins, which are
present in all conjugative transfer systems [19]. Coupling
proteins are inner membrane proteins that are thought
to recruit the cytoplasmic relaxosome complex to the
membrane-associated T4SS [94,95] with direct interactions
between relaxosomes and coupling proteins having recently

"Fig. 4. Alignment of a portion of TraG homologs to illustrate the evolutionary relationship between the N-terminal region of TraGp and members of the
VirB6 family. A PSI-BLAST search was performed as described in Fig. 3. Each sequence is labeled as follows: three-letter bacterial species abbrevi-

been demonstrated [14,15]. The hexameric coupling protein is anchored in the inner membrane with the cytoplasmic domain forming a channel that measures 22 Å in diameter, which could easily accommodate a single strand of DNA (≈ 10 Å). The coupling protein is thought to use ATP hydrolysis to energize the ‘pumping’ of DNA through the coupling protein channel [19,96].

Recently, the coupling protein of R27, TraG11, an
F-type system has been shown to interact with the N-terminus of TrbB1, a member of the TraB family. TrbB1 was also shown to form multimers, possibly forming a ring structure that could extend the pore of the coupling protein into the periplasmic space [59]. TraB of the F factor also interacts with TraK, which in turn interacts with TraV, a lipoprotein that could stabilize the secretin-like TraK protein [49]. Secretins are known to form gated, outer membrane rings that allow the passage of macromolecules in response to a signal that opens the pore [97,98]. A TrA secretin-like structure, anchored by TraV, could, therefore, extend the conjugative pore from the coupling protein through to the outer membrane, via TraB1. Although there is evidence for such a structure in other secretion systems, this needs to be demonstrated experimentally for the T4SS.

Consistent with the idea of TraB1, -K and -V forming the core of the pore which transfers DNA, expression of VirB3Ti, -B, -B7Ti, -B8Ti, -B9Ti and -B10Ti of the Ti plasmid in recipient cells increases the efficiency of RS1 transfers [99]. This suggests that the presence of these proteins within recipient aids in the transfer of DNA into the cytoplasm. Since all of these VirB proteins, except VirB8Ti, have a homolog/analog in F-like T4SS, including the scaffolding proteins of the putative pore (TraB1, -K and -V; Table 1), it is likely that the pore extends from the donor inner membrane to the recipient cytoplasm. Consistent with this proposal, homologs of VirB7Ti and -B10Ti have been shown to be responsible for DNA uptake by H. pylori [5], illustrating that these proteins likely represent the minimal membrane-spanning pore for DNA transfer.

Although these observations suggest a mechanism by which DNA could cross the donor envelope, the mechanism by which the DNA traverses the recipient envelope to gain access to the cytoplasm remains a key question. Some evidence is available that suggests the T4SS system of F penetrates the recipient cell. TraGF has been implicated in entry exclusion involving protein–protein interactions between TraGF and TraSF, the entry exclusion protein, located in the donor and recipient cells, respectively [32]. This suggests that TraGF is translocated into the recipient cell and interacts with TraSF to block DNA transfer. Also, 5S-labelled TraNp and possibly TraUF are found in the recipient cell after separation of the donor and recipient cells using magnetic bead technology (L.S. Frost, unpublished results). Since the net outcome of F-, P- and I-type conjugative systems is the same, there must be an underlying mechanism common to all T4SS, which do differ somewhat in their repertoire of proteins that promote pilus assembly and DNA transport. Does the F-pilus retract, and if so, does the P- and I-type pili also retract? Do P-type systems also translocate proteins into the recipient cell to form a stable mating junction? Does the DNA transfer through the pilus, situated within the conjugative pore, with the pilus penetrating the recipient cell envelope and depositing the DNA directly within the recipient cytoplasm, much like a phase tube within the contractile tails of T-even phages injects DNA? The idea that pili can be used to transport macromolecules is supported by the findings of Jin and He [100,101], who visualized protein secretion from the tips of type III secretion system pili. This observation implies that pili can indeed serve as a conduit for macromolecular trafficking.

5. Relationships between T4SS, T3SS and T2SS

Gram-negative bacteria possess multiple pathways for secreting macromolecules across the outer membrane [102], with conjugation via T4SS being one of the more complex pathways [8]. Secretion pathways with interesting similarities to T4SS are the type II secretion systems (T2SS; 12–16 proteins) and the type III secretion systems (T3SS; 30–40 proteins).
T3SS; 20 proteins). T2SS are one of the terminal branches of the general secretory pathway, which is responsible for secreting a wide range of extracellular toxins and enzymes by Gram-negative bacteria [103]. T2SS are also closely related to secretion pathways for the biosynthesis of type IV pili [104]. Among the T3SS are molecular syringses that inject virulence effector proteins directly into the cytoplasm of host cell [105]. T3SS also share both sequence and structural similarities with flagellar basal bodies [106,107].

Based on in silico analysis, the homology between T4SS, T3SS and T2SS is quite limited. However, each system does contain a secretin protein and an associated stabilizing lipoprotein, which together could function as a gated outer membrane channel that allows the passage of macromolecules. Each system also contains one or two NTPases that likely energize either assembly of the secretion apparatus or macromolecule secretion. NTPases contained within the T2SS (GspE) are homologous to the NTPases from P- and I-type T4SS (i.e. TrbBP/VirB11Ti and TraJ), but not NTPases from F-like T4SS [26]. How energy is utilized in these systems will be key to understanding their differences. Structural determination of key transfer proteins will undoubtedly provide valuable insight into the nature of T4SS that cannot be obtained from database searches. For example, the crystal structure of the coupling protein TrwB identified structural homologies to DNA ring helicases and therefore suggested a mechanism by which single-stranded DNA could be actively pumped through the conjugative pore. It will be interesting to determine if any homology exists between T4SS, T2SS and T3SS that allows the passage of macromolecules and whether the theme of interacting proteins assembled into multimeric rings is common to many secretion systems.

From a mechanistic and anatomical standpoint, there are striking similarities between T4SS, T3SS and T2SS. Each secretion system is a multi-protein, membrane-associated complex that can assemble filamentous appendages, such as pili or flagella, on the bacterial cell surface and are involved in macromolecular transport. Many type II and IV systems share the properties of retractile pili [12,76] and sensitivity to pilus-specific bacteriophages [75], which presumably take advantage of pilus retraction for entry into the host. Some type III and IV systems share an ability to trigger macromolecular transport in response to contact with host eukaryotic cells [108] or bacterial cells [68], respectively. Although the molecular mechanisms for each of these processes are not yet fully understood, various aspects of these secretion pathways appear to be conserved, possibly reflecting a common evolutionary origin of either complete systems or modular components of each system.

Interesting parallels exist between the substrates secreted by T4SS and T2SS. For example, natural transformation, or DNA uptake, can be mediated by either T2SS [109] or T4SS [5]. Also, secretion of structurally similar toxins can occur by either a T2SS (cholera toxin) [103] or a T4SS (pertussis toxin) [91,92]. Another interesting comparison involves DNA transfer mediated by the F T4SS that shares mechanistic similarities to filamentous phage (M13 and f1) replication and packaging, which uses a secretin/lipoprotein channel, thioredoxin and an NTPase [104]. Both systems use an evolutionarily related mechanism to produce a single-stranded DNA intermediate via rolling circle replication [110,111], which is either transferred to a recipient or packaged upon phage extrusion. The T4SS gene products assemble the conjugative pilus, a structure that is structurally related to class I filamentous phages, which consists of a helical array of proteins around a circular, single-stranded DNA molecule [112], possibly providing insight into the transport of DNA during conjugation.

The secretion system classification scheme (T2–T4SS) conveniently divides important pathways into logical categories, which has greatly facilitated the study and understanding of these systems [102]. However, the expanding genome databases and the molecular dissection of several model secretion systems has revealed both the diversity within and the shared relationships between secretion system categories. From an evolutionary perspective, these observations make it tempting to speculate that numerous variations of secretion pathways exist that are built on a finite array of central modular components.

6. Future studies on T4SS

Identification by genetic and computer-based methods of the essential components of T4SS provides a foundation to ask more detailed questions about the mechanism of macromolecular secretion, in general. Careful biochemical and genetic analysis of individual transfer proteins will continue to provide valuable insight into the mechanics of secretion. Methods to determine protein–protein interactions will be central to constructing a detailed model of the T4SS apparatus since microscopic analyses, so far, have proven uninformative. Such examples include the identification of the TraBf, -Kpf, -Vpf envelope-spanning structure [49] and identification of an interaction between TrhBf and the coupling protein TraGf [47] and the many examples in the VirBf literature [2]. Identifying how macromolecules access the pore and initiate the transfer process as well as their effect on the recipient as they enter the cytoplasm will also be key questions [10].

Bacterial conjugation provides a model system for studying bacterial signaling as the nature of the ever elusive mating signal remains unknown. It is anticipated that an external cue, possibly involving contact between donor and recipient, is transferred via the pilus, through the membrane-associated T4SS and coupling protein to the cytoplasmic relaxosome. This process appears to involve
pilus retraction, an as yet poorly understood phenomenon. Several T4SS components contain features of signaling molecules. For example, coiled-coil domains, such as those in TraB1 and TraH1, which can undergo modification, have been implicated in molecular signaling [113] and modulation of binding through changes in the local cellular environment [114]. This signal could then trigger events that resemble phage infection and injection of DNA or the injection of proteins in a contact-mediated manner as seen in T3SS.

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