A Specific Protease Encoded by the Conjugative DNA Transfer Systems of IncP and Ti Plasmids Is Essential for Pilus Synthesis

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TraF, an essential component of the conjugative transfer apparatus of the broad-host-range plasmid RP4 (IncP), which is located at the periplasmic side of the cytoplasmic membrane, encodes a specific protease. The traF gene products of IncP and Ti plasmids show extensive similarities to prokaryotic and eukaryotic signal peptidases. Mutational analysis of RP4 TraF revealed that the mechanism of the proteolytic cleavage reaction resembles that of signal and LexA-like peptidases. Among the RP4 transfer functions, the product of the Tra2 gene, trbC, was identified as a target for the TraF protease activity. TrbC is homologous to VirB2 of Ti plasmids and thought to encode the RP4 prepilin. The maturation of TrbC involves three processing reactions: (i) the removal of the N-terminal signal peptide by Escherichia coli signal peptide 1 (Lep), (ii) a proteolytic cleavage at the C terminus by an as yet unidentified host cell enzyme, and (iii) C-terminal processing by TraF. The third reaction of the maturation process is critical for conjugative transfer, pilus synthesis, and the propagation of the donor-specific bacteriophage PRD1. Thus, cleavage of TrbC by TraF appears to be one of the initial steps in a cascade of processes involved in export of the RP4 pilus subunit and pilus assembly mediated by the RP4 mating pair formation function.

Conjugation is a widespread plasmid- or transposon-encoded process by which DNA is transmitted from a bacterial donor into a recipient cell. One of the most intensively studied DNA transfer systems is that of the broad-host-range IncP plasmid RP4 (45, 46). Conjugation has gained increasing interest, since it was found that the T-DNA transfer of Ti plasmids from Agrobacterium spp. into plant cells, a transkingdom DNA transfer process, is accomplished by a conjugative mechanism (28, 34, 55, 68). Analogies between T-DNA transfer and RP4-mediated conjugation extend to gene organization and similarities of the gene products encoded by the Ti plasmid virulence operons (VirB, VirC, and VirD) and the RP4 transfer regions (Tra1 and Tra2) (32, 35, 44). In addition, the Ti plasmids are transferred within an Agrobacterium population by a second Ti-encoded transfer system, Tra, which is more similar to the RP4 transfer system than to the virulence functions on the same plasmid (1, 16).

According to the current model for conjugative DNA transfer, the essential intimate contact between donor and recipient cells is established by the mating pair formation (Mpf) system. Mpf functions of various transfer systems including the virulence system of the Ti plasmids encode conjugative pili, which are thought to initiate the cell-cell contact. Although conjugative pili of the F plasmid are the best characterized (for a recent review, see reference 54), little is known about the mechanism of the pilus assembly process. For RP4, 10 of 11 Tra2 genes and the Tra1 gene traF were found to be essential for pilus synthesis (21, 22). The assembly of Ti Vir-encoded pili depends on the 11 gene products of the VirB region, on virD4, and on the regulatory functions of virA and virG (17). For their weak similarity to TraA, the F-pilus pilin, TrbC of RP4, and VirB2 of the Ti plasmid were proposed to function as the respective pilus subunit (2, 51), although so far no experimental evidence has been published to support this hypothesis. The great number of functions required for pilus synthesis suggests that several consecutive steps are involved in the assembly process. Here we show that synthesis of RP4 pili comprises a proteolytic cleavage reaction carried out by TraF, the only component of the Tra1 region of RP4 that is part of the Mpf system. TraF is known to be essential not only for conjugative transfer but also for mobilization of the non-self-transmissible IncQ plasmid RSF1010, for pilus synthesis, and for donor-specific phage propagation (22, 33, 65).

MATERIALS AND METHODS

Strains, phage, and plasmids. Escherichia coli K-12 strains used in this study were SCS1 (a DH1 derivative [23]) and HB101 (8) as hosts for plasmids, the nalidixic acid-resistant derivative HB101 N’ as the recipient in conjugation experiments, XL-1 Blue (10) as the host for M13mp18 and M13mp19 derivatives (37, 67) and the nonpiliated strain JE2571 (ter thr thi lacY thy pil) (9) for phage sensitivity and adsorption assays and electron microscopy. Cells were grown in YT medium (39) buffer with 25 mM 3-(N-morpholino)propanesulfonic acid (sodium salt, pH 8.0) and supplemented with 0.1% glucose and 25 µg of thiamine hydrochloride per ml. When appropriate, antibiotics were added as follows: ampicillin (sodium salt), 100 µg/ml; chloramphenicol, 10 µg/ml; tetracycline hydrochloride, 10 µg/ml; and nalidixic acid (sodium salt), 30 µg/ml. Phage PRD1 (5, 41) was propagated as described previously (56). Plasmids used in this study are listed in Table 1.

DNA techniques. Standard molecular cloning techniques were performed by the method of Sambrook et al. (47). Plasmid pDB129, a derivative of pDB126 (3) lacking traF, was obtained by deletion of a 2.5-kb SpaI-PstI fragment comprising all of traF and part of traG. A DNA fragment was PCR by using the oligonucleotide primers CGCAATCTGACGGCTCTATCGTGGATCC and CGCTGACCATATTTGCGCCATCTTCC, which contained recognition sites for SpaI and PstI, respectively, was used to reconstitute the traG reading frame. Plasmid pWP471L1 was constructed by introducing a PCR-generated DNA fragment (oligonucleotide primers were CGACGACTCATAAGAACCCACAAAGACCATCCA and GCCGATCACTGCAGCTCGGTTCCTTAGTCA GCCGACCCAGCCCTCGCAGCCATCCTCCTGCTAGA GCCGCGGCGGACCCATTCAACCCACCTGAGGCACCAAGAAGACCATCCGCG) (corresponding to RP4 coordinates 46.455 to 46.474) (46) was used to change serine-37 to alanine in mutant traFS37A. Accordingly, the oligonucleotide CATGATGCGAATGCTTAGTCA TACCGGTTCCTTAGTCA GCCGACCCAGCCCTCGCAGCCATCCTCCTGCTAGA GCCGCGGCGGACCCATTCAACCCACCTGAGGCACCAAGAAGACCATCCGCG (corresponding to RP4 coordinates 46.296 to 46.315) (46) was used to change lysine-89 to glutamine in mutant traFK90. Exchanged bases are shown in boldface type. Following mutagenesis, the nucleo...
RESULTS

Similarities of TraF analogs encoded by IncP and Ti plasmids with bacterial signal or leader peptidases. Analogs to the RP4 gene product of traF have been described elsewhere for the IncPβ plasmid R751 (16) and for the conjugal transfer regions of different Ti plasmids (1, 16). Interestingly, there is also a TraF homolog among the virulence functions of the Ti plasmid, i.e., open reading frame 2 (ORF2) outside the virF region (16, 36). Database searches using the University of Wisconsin Genetics Computer Group program BLAST (14) with RP4 TraF as the query sequence revealed similarities not only to the TraF analogs but also to bacterial signal or leader peptidases, in particular to those from Staphylococcus aureus (11) and Phormidium laminosum (42). By using Lep, the E. coli signal peptidase, as the query sequence, TraF of the Ti plasmids was reported to be a related gene product. Multiple sequence alignments produced by PILEUP (14) clearly show extensive sequence similarities between the various traF gene products and signal or leader peptidases of different bacterial origin (Fig. 1). Each of the proteins compared contains at least one potential transmembrane helical segment (Fig. 1) which facilitates the integration of these proteins into the cytoplasmic membrane. For the signal peptidases of E. coli and Salmonella typhimurium, additional membrane-spanning regions have been located at their N termini (Fig. 1) (13). Several distinct domains which are present in each of the protein sequences can be identified and are shown in Fig. 1. These domains can also be found in eukaryotic signal peptidases from the inner membrane of mitochondria and the lumen of the endoplasmic reticulum (13, 61). Three of these domains, domains I, II, and III, contain catalytic amino acid residues identified for E. coli and B. subtilis signal peptidases (Fig. 1) (6, 59, 62). These residues are conserved not only throughout other signal peptidases but also in each of the TraF analogs (Fig. 1).

Despite the similarities of RP4 TraF to the E. coli signal peptidase encoded by lep, TraF could not complement the temperature-sensitive lep mutant in strain IT89 (26). TraF did not support cell growth of strain IT89 at the nonpermissive temperature (42°C). In addition, the nonprocessed form of the RP4 Tra2 gene product, TrbG, which was shown to contain a cleavable signal peptide (22), accumulated in extracts of IT89 at 42°C in the presence and absence of traF (data not shown). This suggests that the substrate specificity of TraF differs from that of Lep (64). Thus, we conclude that TraF of RP4 and its homologous proteins build a new class of plasmid-encoded
peptidases catalyzing a specific reaction which is important for conjugative DNA transfer.

Analogous to chromosome-encoded signal peptidases, amino acid residues Ser-37 and Lys-89 as well as the N-terminal transmembrane segment of RP4 TraF are important for function. To support the idea that TraF is indeed a RP4-specific peptidase and to begin functional analysis, we constructed three mutants. The potential catalytically active amino acid residues of RP4 TraF, serine-37 and lysine-89 (Fig. 1), were replaced by alanine and glutamine, respectively. As demonstrated by polyacrylamide gel electrophoresis, the resulting mutants, TraFS37A and TraFK89Q, were equally stable and had an electrophoretic mobility identical to that of the wild-type protein (data not shown). It should be noted here that the predicted first nine amino acid residues, (M)SRFQRLTK, of the overexpressed traF gene product (65) were confirmed by N-terminal protein sequencing. For an additional mutant, we constructed a mutant with a deletion of traF, lacking the first 32 codons. Thus, the resulting mutant protein was stable, i.e., we could overproduce it in large quantities without loss by proteolysis (data not shown). All three mutants were tested to determine whether they could complement a traF mutant derivative for different phenotypes. Conjugative transfer frequencies were determined by using a two-plasmid system in test strain HB101, consisting of plasmid pDB129 (Cmr, ColD replicon) (see Table 1 and Materials and Methods), a derivative of pDB126 (3), which contains all essential components for transfer of RP4 with the exception of traF. Wild-type or mutant traF was provided on the second plasmid, pWP471 (Ap r, pMB1 replicon) (65), in trans. While wild-type traF on plasmid pWP471 fully restored the transfer ability in strain HB101(pDB129, pWP471), transfer frequencies obtained with mutant strains containing pWP471S37A or pWP471K89Q were markedly reduced by 2 to 3 orders of magnitude (Table 2). The traF deletion mutant of strain HB101(pDB129, pWP471 D1) was completely transfer deficient (Table 2).

Another previously established two-plasmid system (22, 33) was used to analyze whether the traF mutants could support pilus synthesis and propagation of the donor-specific phage PRD1. The nonpiliated strain JE2571 contained the plasmid...
pML123 (Cm<sup>+</sup>, ColD replicon) (Table 1) carrying the entire RP4 Tra2 region and the traF plasmid pWP471 or one of its mutant derivatives. With none of the mutant plasmids, plaque formation was observed upon infection with PRD1 (Table 3). In addition, the numbers of phage particles adsorbed to the cell surfaces of the mutant strains were similar to background values obtained for JE2571 strains carrying no plasmid (Table 3). Electron microscopic examination revealed that no pili were produced when mutant traF<sup>+</sup> gene products were present in the test strain (Table 3). In contrast, a large number of pili was seen with cells that contain the wild-type traF as also described previously (22).

The obtained results indicate that serine-37 and lysine-89 are indeed important for TraF function, suggesting that the role of these amino acid residues in TraF may be analogous to that in the host-encoded signal peptidases. The exchange of either one of the residues Ser-37 and Lys-89 of TraF resulted in a gene product with severely reduced or no activity. This affects not only RP4 transfer but also the synthesis of conjugative pili and exposure of the PRD1 receptor at the cell surface. Deletion of the N-terminal 32 codons which contain the predicted transmembrane helix results in a complete inactivation of TraF. We found that TraF localizes predominantly to the cytoplasmic membrane (20). The integration of the protein into the cytoplasmic membrane and the translocation of the catalytic domain into the periplasmic space are probably enabled by the N-terminal hydrophobic segment. The proper targeting is certainly essential for TraF function.

**The TraF peptidase activity is involved in the maturation of RP4 Tra2 protein TrbC.** The question arises as to which polypeptide is the target for the peptidase activity of TraF. Studying the expression of the RP4 Tra2 gene products, we found that TrbC was not detectable in SDS extracts of SCS1 cells containing traF in addition to all or parts of the Tra2 region by solid-phase immunoassay with TrbC-specific polyclonal antiserum (data not shown). Thus, we investigated the effect of TraF on TrbC independent of any other RP4 function. When trbC is expressed alone or together with trbB, one major band corresponding to a polypeptide of 8.5 kDa and a weak band corresponding to an approximately 11-kDa polypeptide are obtained by a solid-phase immunoassay (Fig. 2, lanes b to d) (22). The amino terminus of the major 8.5-kDa polypeptide was determined to be SEGTTGGLS. This coincides with the predicted cleavage site for signal peptidase I (Lep) of *E. coli* (32), indicating that TrbC is indeed cleaved by this enzyme. Upon coexpression with wild-type traF, the TrbC-specific bands were no longer detectable (Fig. 2, lane f). However, when trbC is expressed together with one of the mutants described in the previous paragraph, traF<sup>S37A</sup> and traF<sup>K89Q</sup>, the TrbC bands are not affected (Fig. 2, lanes g and h). We suggest that TraF specifically processes TrbC and that amino acid residues Ser-37 and Lys-89 of TraF are important for the cleavage reaction.

Since we could not detect a TraF-dependent product of TrbC by a solid-phase immunoassay, we assume that the TrbC antiserum would not recognize the polypeptide after cleavage reaction. However, it was found that when mutant TraF was expressed together with another mutant (traG-traM or trbB-trbM), the TrbC-specific polypeptide was not detectable (Fig. 2, lanes b to d). This clearly demonstrates that the TraF peptidase activity is involved in the maturation of the major 8.5-kDa polypeptide.

**TABLE 3. Bacteriophage PRD1 propagation and pilus synthesis of traF mutants**

<table>
<thead>
<tr>
<th>Plasmids in strain JE2571</th>
<th>Plaque formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PRD1 adsorption&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pili synthesis&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>pML123, pWP471</td>
<td>+</td>
<td>58.0</td>
<td>+</td>
</tr>
<tr>
<td>pML123, pWP471S37A</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>pML123, pWP471K89Q</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>pML123, pWP471Δ1</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experiments were carried out as described previously (9, 22, 30).
<sup>b</sup> -, no plaques formed; +, plaques formed.
<sup>c</sup> Adsorption is expressed as percentage of the bound phage particles.
<sup>d</sup> -, no pili synthesized; +, pili synthesized.
by TraF. The antiserum was raised against a purified fusion protein consisting of the N-terminal part of the RP4 TraI gene product TraL and amino acid residues 46 to 145 of TrbC (Fig. 2, lane i) (see also Fig. 4) (22). Analyzing the reactivity of the antiserum against C-terminus-truncated trbC gene products, we found that a polypeptide lacking 29 C-terminal amino acid residues of TrbC was no longer recognized by TrbC antiserum (15). Thus, the antiserum seems to contain immunoglobulin species only against the C-terminal part of TrbC. This finding led us to the speculation that the TraF-dependent processing reaction occurs at the C terminus of TrbC.

TrbC-derived polypeptides are only very poorly stained with Coomassie blue (22). However, by using silver staining, a band corresponding to a polypeptide of 7.5 kDa (TrbC*) appeared when protein extracts of trbC- and traf-containing cells were separated in a tricine-SDS-polyacrylamide gel (Fig. 3, lane d). Simultaneously, the 8.5-kDa polypeptide (TrbC) is obtained only in the absence but not in the presence of TraF (Fig. 3, lanes c and d), indicating that the smaller polypeptide (TrbC*) is indeed the TraF-dependent cleavage product of TrbC. The bands below the 7.5-kDa TrbC* polypeptide turned out not to be trbC specific but instead were present in variable amounts in the different extracts of SCS1 cells independent of the presence or absence of trbC.

**FIG. 3. Identification of the TrbC product derived from the TraF-dependent processing reaction. IPTG-induced SCS1 cells carrying the indicated plasmids were treated with lysis buffer (50 mM Tris-HCl [pH 7.6], 5% [wt/vol] sucrose, 10 mM spermidine hydrochloride, 100 mM NaCl, 2 mg of lysozyme per ml, 1 mM EDTA, 0.25% [wt/vol] Brij 58) and broken by sequential freezing and thawing. The suspension was then centrifuged at 100,000 × g for 45 min. Protein extracts from the resulting sediment in NaCl buffer (1 M NaCl, 20 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 1 mM EDTA, 10% [wt/vol] glycerin) were separated on a tricine-SDS-polyacrylamide gel (17% acrylamide) and subsequently silver stained. Lane a, broad-range protein marker with the sizes of several reference bands below the 7.5-kDa TrbC* polypeptide turned out not to be the TraF-dependent cleavage product of TrbC. The lane b, pMS119EH (vector control); lane c, stained. Lane a, broad-range protein marker with the sizes of several reference bands.**

**DISCUSSION**

In this study, we have shown that trafa of RP4 encodes a plasmid-specific peptidase and that this enzymatic activity is essential for RP4-specific conjugative transfer, for pilus synthesis, and for the assembly of a functional receptor for the donor-specific bacteriophage PRD1. From the pattern of conserved amino acid residues in TraF and TraF analogs compared to that of the various signal peptidases and from the results obtained with defined trafa mutants, we conclude that the mechanism of the cleavage reaction and the positioning of the peptidase domain in the periplasm resemble those of host cell-encoded signal peptidases (Fig. 1).

The N termini of prokaryotic and eukaryotic signal peptidases are anchored in the cytoplasmic membrane by one or two hydrophobic transmembrane helices (12, 13, 40, 66). These segments are not directly involved in catalysis but are important for the correct localization of the catalytic domain at the periplasmic side of the cytoplasmic membrane (for reviews, see references 12 and 13). We have shown here that the deletion of the hydrophobic domain of RP4 TraF (amino acid residues 10 to 28 [Fig. 1]) resulted in a complete loss of activity. The mutant protein probably remains in the cytoplasm and thus cannot fulfill its function in the assembly of the conjugative transfer apparatus.

Signal peptidases have been shown to belong to a new class of serine proteases (7, 43, 59). Similar to LexA-like proteases, their catalytic activity depends on a serine-lysin dyad (6, 62). In analogy to chromosome-encoded signal peptidases of E. coli and B. subtilis, site-directed mutagenesis of the proposed active-site residues serine-37 and lysine-89 of TraF leads to functionally inactive gene products which did not support the synthesis of conjugative pili, the assembly of a PRD1 receptor at the cell surface, and the cleavage of the target protein TrbC. The conjugative transfer activity obtained with these mutants is dramatically reduced. However, residual transfer activity is still observed, i.e., approximately 1 in 1,000 donor cells containing the TraF mutant proteins is able to transfer the conjugative plasmid into the recipient cell. This could be explained by the very low proteolytic cleavage activity of mutant TraF which is not detectable by any other method but is sufficient for transfer at low frequency. We cannot rule out the possibility that as a result of the proposed low activity of TraF, very short or extremely few pili were produced which would support the establishment of the cell-to-cell contact between donor and recipient cells in some cases.

For B. subtilis signal peptidases, three additional amino acid residues were identified to be important for function (62). An aspartate residue, Asp-153 (Asp-155 in RP4 TraF) (Fig. 1) was thought to be catalytic (62) and is also conserved in other chromosome-encoded signal peptidases and in each of the TraF analogs. Residues Arg-84 and Asp-145 of B. subtilis (Fig. 1) are important for conformation (62). These amino acid residues are also present in other host-encoded signal peptidases and in traF gene products of RP4 and R751. Interestingly, in each of the Ti plasmid-derived TraF analogs, both residues are replaced by serine or threonine. Assuming that these two residues interact with each other and facilitate proper protein folding, one could speculate that at some time in the evolution of the transfer machineries the spontaneous mutation of one of the two residues in the Ti traF genes caused a suppressor mutation in the other residue.

Despite the similarities to host-encoded signal peptidases, the substrate specificity of TraF differs from that of host cell signal peptidases. First of all, TraF is an essential transfer gene product (22, 33, 65). Thus, the E. coli signal peptidase cannot replace TraF or carry out the TraF-specific processing reaction. Accordingly, TraF cannot complement the temperature-sensitive lepB mutant at the nonpermissive temperature (26). From our results, we conclude that RP4 traF and also the analogous functions of plasmid R751 and the Ti plasmids encode a novel class of peptidases, which catalyze a specific processing reaction using a catalytic mechanism similar to that of chromosomal signal peptidases.

We clearly demonstrated that a target protein of TraF is the trbC gene product. The results of previous and current studies...
on TrbC by our group indicate that there are also at least two host cell-mediated processing steps involved in the maturation of TrbC which are independent of TraF. The translation product of trbC is a polypeptide of 15 kDa containing a potential signal peptidase cleavage site (Pre-Pro-TrbC [Fig. 4]) (32). In this study, we reported that the predicted signal peptide of TrbC is indeed cleaved off, most likely upon action of the E. coli signal peptidase Lep (Fig. 4). The predicted size of the resulting polypeptide deduced from the amino acid sequence is 11 kDa (32), which coincides well with the weak band observed in solid-phase immunoassays (Pro-TrbC [Fig. 2 and 4]). However, the major polypeptide which is detectable with TrbC-specific antiserum or silver staining in extracts of cells containing trbC alone, together with trbB or the entire RP4 Tra2 region corresponds to a protein of about 8.5 kDa (TrbC [Fig. 2, 3 and 4]) (22). The N terminus of this polypeptide was determined to coincide with the predicted host cell signal peptidase cleavage site. Thus, we speculate that a second processing step occurs at the C-terminal part of TrbC mediated by an as yet unknown host cell function (Fig. 4). This assumption is supported by the finding that different C-terminus-truncated TrbC derivatives result in the same 8.5-kDa polypeptide and that a hexahistidine tag fused to the C terminus of TrbC is cleaved off from the full length but not from certain truncated polypeptides (15). The nature and the significance of this proteolytic cleavage reaction in the TrbC maturation process are still unclear. C-terminal processing and proteolytic degradation have also been described elsewhere for penicillin-binding protein 3 (24, 25) and certain mutant λ repressor proteins with hydrophobic C termini (52, 53) by the E. coli prerc (tps) gene product (for recent reviews, see also references 19 and 38). Interestingly, like TraF, Tsp seems to belong to the same class of serine proteases which use a hydroxyl/amine dyad catalytic mechanism (29, 43). Future studies will show whether this or other host cell functions (53) are involved in the carboxy-terminal truncation of TrbC and whether this cleavage reaction by a chromosome-encoded protease is important for pilus synthesis and conjugation. The third step of TrbC maturation is then catalyzed by TraF, resulting in a product of 7.5 kDa (TrbC* [Fig. 3 and 4]). Further experiments by our group strongly indicate that this processing reaction occurs at the C terminus of TrbC (15). Thus, maturation of TrbC involves three steps. Two reactions occur independently of any other RP4 gene product, and the third one depends on TraF (Fig. 4). Whether a certain order of these reactions is important during TrbC maturation in vivo remains to be shown. The Lep-dependent signal peptide cleavage probably enables the export of TrbC into the periplasm and might be a prerequisite for the TraF-dependent processing step. It is also not yet clear whether the host cell-mediated C-terminal truncation of TrbC is essential for the TraF-catalyzed reaction.

The determination of the substrate recognition site and the exact cleavage site for TraF still requires additional experiments. Although TraF does not seem to resemble a classical signal peptidase, the sequence similarities to the various bacterial and eukaryotic signal peptidases might reflect not only a similar catalytic mechanism but also analogies in the substrate recognition. This could include the correct positioning to the substrate at the periplasmic surface of the cytoplasmic membrane or the preference for small residues at the −1 and +1 positions of the cleavage site (64; for a recent review, see reference 43). One could also speculate that the TraF substrate TrbC is oriented toward TraF like secretory proteins toward Lep and that the cleavage of TrbC occurs shortly after one of the helical transmembrane segments, which were predicted for TrbC (20).

Since the processing reaction catalyzed by TraF is essential for pilus assembly, DNA transfer, and phage receptor formation, it is obviously the critical step in the maturation process toward a functional trbC gene product. In addition, our analysis of TraF mutant proteins reported in this study (Tables 2 and 3 and Fig. 2) revealed that processing of TrbC by TraF and pilus synthesis are directly correlated. This clearly leads to the conclusion that TrbC encodes the RP4 preplin, as has been proposed previously (2, 51). Nevertheless, we have no direct evidence that RP4-encoded pili are assembled from TrbC-derived subunits. Further experiments will require antisera against purified pili and against central parts of TrbC. However, so far we cannot rule out the possibility that proteolytic cleavage of TrbC by TraF indirectly influences pilus synthesis and as a consequence of this also affects DNA transfer and phage propagation. In principle, it is also possible that other RP4-encoded gene products are cleaved by TraF as well, although this is very unlikely, since we could not observe any influence on other transfer proteins upon coexpression with TraF (data not shown). Assuming that trbC encodes the preplin, RP4 TraF and hence the analogous functions of R751 and Ti plasmids should be regarded as novel preplin peptidases. The previously unknown maturation process is undoubtedly of great importance for the regulation of the assembly of the conjugative pilus or other structures of the transfer apparatus.

The TraF homolog of the Ti plasmid virulence system, ORF2 in the virF region, is not essential for the T-DNA transfer from Agrobacterium tumefaciens to plant cells (36). The function of ORF2 is yet unknown. However, it can be assumed that the protein is also a plasmid-specific protease which might have a different function for virulence. Fullner et al. (17) showed that the synthesis of vir-encoded pili requires the functions of the VirB region and VirD4, which are analogous to the RP4 Tra2 region and TraG, respectively. This was surprising, because TraG is dispensable for synthesis of RP4 pili (22). Thus, either a specific TraF-like proteolytic cleavage is not involved in the maturation of VirB-encoded pilin or this function is carried out by another plasmid- or host-encoded function. Since VirD4 is needed for pilus synthesis, instead of a TraF analog, one could speculate that VirD4 contains a TraF-like peptidase activity. Searching for similarities between TraF and VirD4, we found potential homologs to the three catalytic domains of TraF-like proteins and signal peptidases in the C-terminal part of VirD4 (see Fig. 5 and 1). These domains are not conserved in RP4 TraG or other analogous proteins of VirD4. The proposed peptidase domain I of VirD4 is preceded by a potential membrane-spanning helical segment, which is amphiphilic, rather than hydrophobic, like the corresponding
FIG. 5. Comparison of proposed catalytic domains from TraF ana
gologs, E. coli signal peptide, and VirD4 of T1 plasmid. Catalytic do
main I, II, and III of RP4 TraF and ORF2 of the Ti virF region and E. coli signal peptide (Sp Ec) (see also Fig. 1) were compared to potential analogous domains of the Ti VirD4 C-terminal half. Identical amino acid residues are shown on a black background, while structural or functional similar residues are shown on a gray background. TMH marks the potential transmembrane helical segment preceding domain I. Numbers indicate amino acid residues of the corresponding proteins. Gaps introduced to maximize alignment are indicated by dots.

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resolution of the btDNA transfer apparatus encoded by plasmid RP4: localization of essential components in cell fractions of Escherichia coli. Submitted for publication.


