Conjugative Pili of IncP Plasmids, and the Ti Plasmid T Pilus Are Composed of Cyclic Subunits*

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TrbC propilin is the precursor of the pilin subunit TrbC of IncP conjugative pili in Escherichia coli. Likewise, its homologue, VirB2 propilin, is processed into T pilin of the Ti plasmid T pilus in Agrobacterium tumefaciens. Trbc and VirB2 propilin are truncated post-translationally at the N terminus by the removal of a 36/47-residue leader peptide, respectively. TrbC propilin undergoes a second processing step by the removal of 27 residues at the C terminus by host-encoded functions followed by the excision of four additional C-terminal residues by a plasmid-borne serine protease. The final product TrbC of 78 residues is cyclized via an intramolecular covalent head-to-tail peptide bond. The T pilin does not undergo additional truncation but is likewise cyclized. The circular structures of these pilins, as verified by mass spectrometry, represent novel primary configurations that conform and assemble into the conjugative apparatus.

Horizontal gene transfer mediated by plasmid-borne conjugation from donor to recipient cells is initiated by a cell-to-cell bridging arrangement. These cellular interactions, whose complex is named mating pair formation (Mpf), are initiated by conjugative pili encoded by broad-host range plasmids in Gram-negative bacteria. In the case of Agrobacterium-mediated T-DNA transfer to plant cells, the VirB complex is the Mpf structure (1, 2).

Pili are usually long thin filaments extending from the surface of donor cells and upon close examination appear as tube-like structures with an outer diameter of about 8–10 nm and a central, hydrophilic lumen of 2 nm (3, 4). With both the broad-host range plasmids of the IncP group and the Ti plasmids of Gram-negative bacteria, the sex pilus will provide valuable clues directed at answering this highly interesting and perplexing question. With both the IncP and Ti plasmid pilus, the pilin subunit represents the critical monomer of the pilus structure. Insights on the physical features of pilin provide the initial step leading to our understanding of the biological role of the sex pilus.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**The Escherichia coli K-12 strain used in this study was JE2571 (leu, thr, fla, pil, str), defective in the production of pilin and flagella (14). Bacteria were grown according to (15). Agrobacterium tumefaciens strains C58, A348 (16), and NTIREB (17) were grown according to Lai and Kado (10).

**Plasmids—**Plasmids RP4 (18), R751 (19), R388 (20), F (21), pTiC58 (16), pT11955 (22), pT1A6 (23), pML123 (24), pML123mstrbc45 (5), and pWP471 (25) and the vectors pMS119EH (26) and pGZ119EH/EH (27) have been described previously. A DNA fragment generated by PCR using the synthetic oligodeoxyribonucleotides CAGTACGGTACCTAGAAAATAATTTGTTTAC and CCGTACGAATTCAGCGATTACAAGGGAATTACCCAACTAGTACGCTAGCTGTTCC were used to introduce the virB gene into the Mpf complex (1, 2). In this regard, the bacterial reliance of the pilus for DNA transfer raises a key question on whether or not the conjugative pilus is directly involved not only in bridging the donor to the recipient cell but also serving as a molecular conduit in DNA transmission. This resurrects an earlier key question raised on the possibility that the sex pilus, which appears to be tubular in structure, is used by the donor cell to transfer F-DNA rather than by direct cell-to-cell contact (3, 13). In this regard, the analyses of the structure as well as insights on the mechanism of assembly of the sex pilus will provide valuable clues directed at answering this highly interesting and perplexing question. With both the IncP and Ti plasmid pilus, the pilin subunit represents the critical monomer of the pilus structure. Insights on the physical features of pilin provide the initial step leading to our understanding of the biological role of the sex pilus.
Novel Cyclic Subunits of Conjugative Pili

6,131, GenBank accession number J03320) using synthetic oligodeoxyribonucleotides GGGTTACCCCAAGAGGAGGCCAATATG and GGGTACCCCTTACCCCTTACTCAAGGGC, creating a KpnI site at the 5′- and the 3′-end of the gene. The resulting plasmid pUCD4805 containing P2 and U2 was further digested by HindIII and ligated to a HindIII 7.6-kb fragment of pUCD1002 (29) to transform A. tumefaciens (30) directly, obtaining the plasmid pUCD4813, which can replicate in both E. coli and A. tumefaciens. Plasmid pUCD4811 is based on the vector pT7Q18 ligated to the HindIII 7.6-kb fragment of pUCD1002 and served as a control. The PCR-amplified regions were verified by nucleotide sequencing (italics in oligodeoxyribonucleotides represent RP4/ R751/Ti sequence).

Preparation of Pili—F pili were purified according to Helmuth and Achtman (31). T pili were purified according to Lai and Kado (10), followed by CsCl density gradient centrifugation. Therefore 8 ml of sucrose gradient-purified T pili were layered on a 6-step CsCl gradient (1.1–1.5 g/ml) and centrifuged at 120,000 × g for 18 h at 5 °C. Fractions containing pili were pooled, dialyzed 3 × 5 h against 1 liter of double-distilled water, concentrated by ultrafiltration (Centriprep-10, Amicon Inc.), and stored at 4 °C. For the purification of RP4 pili, JE2571 cells containing the plasmids pML123 and pWP471 were grown on 80 agar plates (23 × 23 cm) for 16 h. Production of pili was induced by 5 μM isopropyl β-D-thiogalactopyranoside, included in the medium. Cells were scraped off the plates (60 g of wet cells) using 2 ml of buffer A (10 mM Tris/HCl, pH 7.6) per plate. All the following steps were done at 4 °C, unless stated otherwise. After gentle stirring of the cells for 3 h in buffer A containing 30% sucrose, the suspension was centrifuged (30,000 × g, 20 min) two times. The combined supernatants were poured through glass wool. After dialysis (3 × 3 h) against 1 liter of buffer A each and centrifugation of pili and contaminating cells (100,000 × g, 1 h), the pellet was resuspended in buffer B (10 mM Tris/HCl, pH 8.7, 100 mM NaCl) containing 20% sucrose. The sample was layered on top of a continuous sucrose gradient (25–70%, buffer B) and centrifuged for 4.5 h (130,000 × g). Pili, still contaminated by cell debris, were visible as an opal band at a concentration of 52% sucrose. Fractions containing pili were pooled, and the latter four steps (dialysis against buffer A, centrifugation, dilution of the pellet, and sucrose gradient centrifugation) were repeated. The pooled fractions of the second sucrose gradient were pelleted again (100,000 × g, 1 h) and resuspended in buffer A containing 1.39 g/ml CsCl. The sample was placed between two layers of buffer B, the lower containing 1.43 g/ml CsCl and the upper containing 1.15 g/ml. After centrifugation (150,000 × g, 20 h), a band containing pili was visible at a density of 1.33 g/ml. The CsCl gradient centrifugation was repeated a second time. The pili were isolated at the same density of 1.33 g/ml. Fractions were pooled and dialyzed 3 × 5 h against 1 liter of buffer A each. For tryptic purification, equal volumes of the pilus preparation and 0.1 M (NH4)HCO3, were mixed with trypsin (1 mg/ml; sequencing grade, modified, Roche Molecular Biochemicals) in 10 mM HCl. After 1 h of rigorous shaking at 37 °C the pili were centrifuged by sedimentation (15,000 × g). The pellet was washed two times with 150 μl of double-distilled water. After centrifugation (150,000 × g, 20 h) fractions containing pili were dialyzed 3 × 5 h against 1 liter of double-distilled water. The pilus preparation was stored at 4 °C for longer than 1 year without significant degradation of pili.

Electron Microscopy—The negative stain for electron microscopy of pilin was carried out as described in Haase et al. (5).

Protein Expression and Analysis—Cell extracts of E. coli JE2571 cells and pilus preparation were electrophoresed on tricine-SDS-polyacrylamide gels (17%), electroblotted onto nitrocellulose (BA85, Schleicher & Schuell) or polyvinylidene difluoride (Immobilon-CD, Millipore) membranes, and incubated with the IgG fraction of purified anti-RP4 pilus serum (dilution 1:750) as described previously (32). The IgG fraction of rabbit anti-pilus immune serum was prepared using standard procedures (33).

MALDI TOF Mass Spectrometry—Analyses were performed using a Bruker Reflex II time-of-flight spectrometer (Bruker-Franzen, Bremen, Germany) equipped with an UV-nitrogen laser (337.5 nm) and delayed extraction technology. All procedures were carried out at room temperature. Small amounts of cells (A600 = 2) were harvested and washed with 1 ml of 0.1% trifuoroacetic acid (34). After centrifuging at 15,000 × g for 4 min, cell pellets were resuspended in 250 μl of 0.1% trifuoroacetic acid. Matrix-crystal layers were prepared on a stainless steel MALDI-MS target by depositing 0.3 μl of matrix solution/sample. 0.3 μl of either cell suspension or pilus preparation were transferred onto the matrix layers, followed by addition of an equal volume of matrix solution. Either trans-4-indoleacrylic acid (40 mg/ml), 4-hydroxy-2-oxo-1-cyanoaminoc acid (40 mg/ml), or 2-(4-hydroxyphenylazo)benzeneacetic acid (20 mg/ml) in acetonitrile/isopropanol (1:1 v/v) were used as matrices. The dried samples were washed by covering the crystals with 5 μl of ice-cold 5% trifluoroacetic acid solution for a few seconds. 120 single-shot spectra were accumulated for improved signal-to-noise ratio in reflector mode.

Proteolytic On-target Digestion—To prevent the loss of highly hydrophobic pilin-derived peptides by adsorption to the walls of reaction tubes or pipette tips, proteolytic cleavage of pilin was performed directly on the surface of the sample plate. 0.5 μl of the trypsinized pilus preparation were transferred onto the MS target and mixed with 0.5 μl of freshly prepared trypsin or chymotrypsin (0.25 mg/ml) in 0.1 M (NH4)HCO3. The target and a small strip of wet filter paper (~1 cm2) were enclosed into a plastic box without touching the droplet of the digestion mixture to avoid evaporation of the sample. During the digest a 60-W light bulb was adjusted about 40–50 cm above the target, keeping the temperature on the target at 37 °C. After 1–6 h of digestion the cover was removed, and 0.5 μl of the working matrix solution were added to the liquid sample. The vacuum-dried sample spot was washed shortly with 5% ice-cold trifluoroacetic acid just before MALDI-MS analyses.

Sequencing and Amino Acid Composition Analysis of RP4 and T pilus—C-terminal (35) and N-terminal sequencing was performed using an ABI 490 series protein sequencer system. For amino acid composition, pili were analyzed using a Beckman 6300 amino acid analyzer utilizing sodium citrate buffer system.

RESULTS

The RP4 Pilus Subunit Originates from trbC—For identification of the RP4 pilus subunit, two complementing strategies were followed, immunological detection of the pilin and mass spectrometric analysis. A purified pilus preparation was a prerequisite for both of these approaches. The bald E. coli strain JE2571, defective for the production of extracellular, chromosomally encoded filaments, facilitated the purification of conjugative RP4 pili. Pili were detectable only when cells were grown on semi-solid surfaces like agar plates but not in liquid media. Low concentrations (5 μM) of isopropyl β-D-thiogalactopyranoside were used to keep an acceptable balance between cell growth inhibition due to expression of the Mpf system and pilus overproduction. A five-step procedure for obtaining purified pili was followed (see “Experimental Procedures”). The purification was based on the detachment of pili from cells in high concentration of sucrose and a combination of two types of gradient centrifugation, sedimentation and equilibrium. The progress of the elimination of contaminants was monitored by electron microscopy and gel electrophoresis. We found that the protein content of purified denatured pili can be stained with silver but not with Coomassie Brilliant Blue or other protein dyes.

Electron microscopy showed that filaments of the purified RP4 pilus preparation tend to aggregate, indicated by the formation of bundles (Fig. 1A). The same observation was made with pili obtained from the IncPβ plasmid R751 (data not shown). In contrast to F and T pili, RP4 and R751 pili appeared to be rigid and inflexible.

FIG. 1. Pilus morphology. Electron micrographs of purified RP4 (A), T (B), and F pili (C) are shown. Preparations were negatively stained with 1% uranyl acetate.
The RP4 and T Pilins Contain a Highly Conserved Core Region—The sequence alignment of several related potential precursors for pilins indicates the subdivision into three apparent groups: the IncP TrbC proteins (Fig. 3, dark yellow background), the TrbC proteins of the Ti plasmid conjugal transfer system (Fig. 3, yellow background), and the Ti plasmids VirB2 proteins of the tDNA transfer system (Fig. 3, light yellow background). Within each subgroup the primary structure is highly conserved. However, the significance of this alignment becomes evident by the conserved residues in all sequences (Fig. 3, red background).

Verification of the conserved TrbC core peptide was achieved by examining the N and C termini of the RP4 propilin itself and its intermediates. The N-terminal sequence of the 15-kDa Pro-TrbC was MTTAVPFRL as predicted for unprocessed RP4 propilin. However, the 11-kDa Pro-TrbC revealed the same N-terminal sequence (Fig. 1 B), the TrbC proteins of the Ti plasmid conjugal transfer system (Fig. 3, yellow background), and the Ti plasmids VirB2 proteins of the tDNA transfer system (Fig. 3, light yellow background). Within each subgroup the primary structure is highly conserved. However, the significance of this alignment becomes evident by the conserved residues in all sequences (Fig. 3, red background).

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nal-to-noise ratio and a higher sensitivity in the detection of mature and premature pilins than those that were recorded using 2-(4-hydroxyphenylazo)benzoic acid or 4-hydroxy-α-cya-nocinnamic acid. To evaluate the reliability of the system, we first analyzed F pili with well defined characteristics. The \[\text{m/z} \text{determined for } [M+H]^+ \] ions of F pilin coincides with the calculated value of 7228.1 Da (40). The molecular mass corresponds to the 70 C-terminal amino acid residues of the F-factor TraA precursor protein, acetylated at the N terminus. The peak at 3614.6 indicated the \[\text{m/z} \text{of the double-charged, double-protonated molecular ion (Fig. 4). The data demonstrate that mass spectrometry is an extremely useful tool for pilus characterization.}

To unequivocally identify TrbC as a pilus subunit, we took advantage of the slight sequence difference between the R751 and RP4 pilin. The core regions of both TrbC proteins differ in four amino acids (Fig. 3), which results in an overall molecular mass difference of a single sulfur atom (32 Da). The analysis of both pilins, RP4 and R751, showed R751 pilin to be 32 Da heavier than that of RP4, indicating that the signals recorded indeed originate from TrbC (Fig. 4, inset).

The interpretation of the RP4 and R751 pilus spectra is based on mass calculations of TrbC molecules beginning at position Ser-37 at the N terminus and lacking residues at the C terminus. However, no calculated molecular masses of TrbC \[[M+H]^+\] corresponding to the detected \[m/z\] values of 8119.2 (RP4) and 8151.6 (R751) can be assigned (Fig. 4). For RP4/R751 TrbC, the calculated molecular masses of \[[M+H]^+\] closest to \[m/z\] of 8119.2/8151.6 were those of the molecules ending at position Gly-114, i.e. values of 8137.6/8169.6 Da.
Compositional analysis of the purified pilus preparation was in good correspondence with that of the proposed mature T pilin of 74 C-terminal amino acids when a cleavage reaction takes place between residue Ala-47 and Gln-48 of the VirB2 propilin (37, 41). Previous N-terminal sequencing attempts of the mature pilin failed to release the terminal residue, suggesting that the N-terminal residue Gln-48 is recalcitrant to Edman degradation. MALDI-TOF-MS analysis of the mature pilin produced an m/z value of 7184.3. This value differs from the mass of 7202.3 Da calculated from the primary structure of the protonated T pilin. Like the discrepancies observed for TrbC of RP4 and R751, the difference between the molecular mass derived from mass spectrometry and from the primary sequence of the T pilin was always 18 Da. This difference in mass clearly represents the loss of one molecule of water. Thus, a loss of one molecule of water within the mature pilin TrbC and the T pilin would result from an intramolecular amide linkage or the formation of an ester bond.

Intramolecular Covalent Head to Tail Linkage of TrbC and VirB2—The RP4 pilus itself generates an m/z signal of 8119.2. Interestingly, the pilus resists protease digestion. The protease-treated pilus appeared indistinguishable from untreated pilus preparations as judged by electron microscopy, and no signals representing pilin fragments were seen by mass spectrometry (data not shown). An on-target digestion procedure employing trypsin and chymotrypsin (42) was therefore used to generate peptides that were subsequently analyzed by MALDI-TOF-MS (Table I and Fig. 5). Residues Ser-37 and Gly-114 are apparently next to each other in peptides T1, C1, C2, and C4, suggesting that a head-to-tail linkage of these amino acid residues had taken place to generate a circular peptide structure. In addition to the expected [M+H]⁺ masses representing the peptides smaller than the precursor TrbC molecule, a signal at m/z 8136.7 corresponding to a linear TrbC peptide comprised of 78 residues was observed (Fig. 5). This signal increases in intensity after prolonged digestion with trypsin (data not shown). Digestion with chymotrypsin gradually reduced the intensity of this signal with the concomitant increase in the signals of the resultant peptides derived from the linearized TrbC (Table I).

The T pilin was likewise digested by the same on-target protocol in trans-3-indoleacrylic acid using trypsin digestion followed by chymotrypsin treatment. Two peptides that were generated showed one began at Thr-69 at the N terminus and ended with Phe-P24 at its C terminus, whereas the other began with residue Gly-62 and ended with Arg-44. In the T pilin, an internal residue Asp-55 and the C-terminal residue Gly-121 are potential residues bearing carboxyl groups that may interact with the amino group of residue Gln-48 located at the N terminus of the T pilin to form a peptide linkage. The resulting chymotryptic peptides ruled out residue Asp-55 and implicated residue Gly-121 as interacting with Gln-Q48 to form the potential peptide linkage. Hence, both TrbC and T pilin appear to be cyclic peptides.

Table I

| On-target digest | m/z measured | Δ | Amino acid positions | Peptide No. |
|------------------|-------------|---|----------------------|------------|
| 1–6-h 6.0-h tryptic | 2023.9 | −35.6 | 78 | 18 | T1 |
| 6131.3 | −21.6 | 19 | 77 | T2 |
| 8136.7 | −110.4 | Linearized peptide L |
| 2053.1 | −10.5 | 57 | 75 | C3 |
| 5618.5 | 15.5 | 75 | 51 | C4 |
| 5770.9 | −20.4 | 19 | 74 | TC |
| 6131.3 | −17.7 | 19 | 77 | T2 |
| 6770.8 | −44.1 | 11 | 74 | C5 |
| 8136.7 | −110.4 | Linearized peptide L |
| 1237.5 | −66.7 | 76 | 10 | C1 |
| 1384.6 | −59.1 | 76 | 10 | C2 |
| 6771.1 | 9.4 | 11 | 74 | C5 |

Methods and Results

Novel Cyclic Subunits of Conjugative Pili

To determine whether a cyclic peptide corresponding to the T pilin was indeed generated in vivo, the T pilus was digested by the same on-target procedure as used for TrbC (42). MALDI-TOF-MS analysis of the resultant peptides derived from the linearized TrbC indicated monoisotopic masses that were diagnostic of cyclization. The T pilin is a 7202.3-Da cyclic peptide. The cyclic structure is evident by the presence of 7184.3 as a diagnostic mass (Fig. 6). This cyclic peptide is not a contaminant, since MALDI-TOF-MS analysis of a second test plasmid, pTiC58, harboring either the octopine Ti plasmids TiA6 or Ti15955, demonstrated a mass of 7215.2 (Fig. 6). This value is clearly smaller than 7202.3 (a loss of one water molecule due to cyclization) and different from the mass of 7184.3 produced when VirB2 alone was analyzed by MALDI-TOF-MS (23). The signal is absent in A. tumefaciens cells containing the virB2 cloning vector pTTQ18 only in uninduced cells containing pTiC58 (Fig. 7) and in cells containing no Ti plasmid. Hence, it is apparent that the biogenesis of the T pilin through specific cleavage and cyclization does not require the Ti plasmid. Evidently, Agrobacterium host factors may play a specific role in cyclization since VirB2 undergoes processing but not cyclization in E. coli (2).

Whole Bacterial Cells Are Suitable for Pilin Detection by Mass Spectrometry—Bald E. coli strain JE2571 and bald A. tumefaciens strain NT1REB with and without their respective test plasmids were directly subjected to mass spectrometry. As demonstrated in Fig. 6, m/z signals were clearly recognizable for the protonated pilins of RP4, R751, and F in E. coli. The IncW plasmid R388 was also tested, and its pilin generated an m/z signal at 7173.5 (Fig. 6). Likewise, an m/z signal of 7184.2 representing the T pilin [M+H]⁺ in A. tumefaciens NT1REB(pTiC58) was plainly observed (Fig. 7). A. tumefaciens harboring either the octopine Ti plasmids TiA6 or Ti15955 were analyzed by the intact cell procedure (see “Experimental Procedures”). The predicted amino acid sequences of VirB2 of these plasmids are highly homologous (Fig. 3); thus, an m/z signal equal to that of VirB2 from the nopaline Ti plasmid pTiC58 is expected. Analysis of the strain harboring pTi15955 yielded an m/z peak of 7214.4 (Fig. 7), a value corresponding to the [M+H]⁺ mass of processed pT15955 VirB2 that has been cyclized by the loss of a water molecule during peptide bond formation. Analysis of pTiA6 produced an m/z peak at 7215.2 (Fig. 7), a value that does not correspond to the [M+H]⁺ mass of processed VirB2 based on published amino acid sequence data, which showed an arginine residue rather than the conserved alanine observed in all other VirB2 polypeptides (Fig. 3). We re-sequenced virB2 of pTiA6 and found the codon was indeed GCT (encoding Ala) rather than the published codon CGT (encoding Arg) (23). Correction of residue 92 into alanine provided uniform agreement that processed VirB2 has a mass lacking the mass of a single water molecule.

Upon further analysis, we found that bald strains are not required since the noise level is relatively low. Moreover, it was demonstrated recently that the mass of various proteins from whole bacterial cells can be measured by mass spectrometry (34, 43, 44).

E. Lai, R. Eisenbrandt, M. Kalkum, E. Lanka, and C. I. Kado, unpublished information.
DISCUSSION

We demonstrated that mass spectrometry accurately measures the molecular mass of pilin encoded by IncW/IncF (both acetylated) and IncP/IncRh1 plasmids (both of circular structure). These findings for the first time explain the discrepancy between the measured and the calculated masses of IncP and T pilins. For thin pili of IncI1 plasmids, a mass discrepancy of 259 Da between calculated and measured values was reported (45). These findings might correspond with modifications in analogy to the recently shown covalent addition of phosphate, glycerophosphate, as well as glycosylation for type IV pili in Neisseria (46). Although none of these modifications was found in our investigations, a post-translational modification seems to be a common motif for pilins.

For IncP and T pilins, the water molecule would be lost upon the generation of a peptide bond or more remotely by an ester bond. Pilus subunits resist degradation by acid treatment, including exposure to organic acid matrices, conditions used herein that would destroy ester bonds readily. The formation of a peptide linkage would argue that the mature pilin is likely
Novel Cyclic Subunits of Conjugative Pili

cylized. This argument is supported by the mass analyses of the peptides generated from these pilins. In every instance, an intramolecular head-to-tail association is found by the pairing of the N-terminal residue with its C-terminal residue within the mature pilin.

The mechanism of pilin cyclization remains to be elucidated. We have explored several potential mechanisms of peptide bond formation in situ. For example, such bonds can form by peptidyltransferase on ribosomes (47), by nonribosomal catalysis as in the formation of the circular antibiotic gramicidin S (48), by the generation of extein (49), by a reverse reaction of proteolysis, catalyzed by trypsin/chymotrypsin (50, 51), and by a mechanism yet to be described. Whichever mechanism operates, the biogenesis of the mature pilin appears to be a highly efficient reaction involving cleavage and ligation into a circular peptide. The absence of linear peptides supports this notion. Moreover, genetic evidence adds further credence to this idea, whereby analysis of a TrbC bearing a deletion abutted into position Gly-114, the target residue of TraF peptidase, revealed the absence of cyclization either in the presence or absence of TraF. In the absence of TraF, TrbC remains uncylized, suggesting that the removal of the four amino acid residues (Ala-115—Ala-118) by TraF peptidase is an intrinsic step in the cyclization of the pilin by the formation of the peptide bond between residues Ser-37 and Gly-114. At present, however, our data do not exclude the possibility that an enzyme other than the peptidase might be involved in forming the peptide linkage. Strategies to elucidate this mechanism are being explored, including genetic approaches directed against the trbC and trcF genes.

The results of parallel studies on the cyclization of the T pilin appear to support the notion that another enzyme is involved in peptide bond formation. We have recently found that the T pilin becomes circularized in A. tumefaciens strains but not in E. coli. 2 In addition, the present work reported herein shows that VirB2 propin is processed and is cyclized in the absence of other Ti plasmid genes. Since there is no TraF functional homologue present on the Ti plasmid except for a weak homology between TraF and the Ti plasmid-coded VirD4 (32), the pilin cyclization enzyme appears to be of chromosomal origin. Identification of an Agrobacterium chromosome-encoded specific cyclization enzyme is one of the key aims of the current research.

The recognition target of the putative peptidyl cyclase on the pilin also remains to be explored. Membrane topology studies using TnPhoA fusions have predicted that the N and C termini of VirB2 protein protrude into the periplasm with the remaining two hydrophobic regions or transmembrane helices (Fig. 3), trans-membranal helix (TMH) anchored to the inner membrane (52). Two trans-membranal helix regions are also present in TrbC propin (Fig. 3). The location of these propilins may be a prerequisite for the processing and cyclization reactions. Following specific processing reactions, the resultant N- and C-terminal residues (Ser-37/Gly-114 for TrbC and Gin-48/Gly, Ser-121 for VirB2) might be brought into close molecular contact that facilitates peptide bonding leading to the cyclic pilin product.

Clearly, the formation of pilin subunits bearing a cyclic configuration in the biogenesis of the sex pilus is novel for bacterial appendages and represents an evolutionary significant architecture required by bacteria and presumably other higher microorganisms such as Chlamydomonas-bearing appendages similar to pili. We are not arguing that cyclic peptides are absent in microorganisms but present the case for a morphologically visible structure composed of a cyclic monomeric peptide. Biologically active cyclic peptides are known as for exam-
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