Processing and Maturation of the Pilin of the Type IV Secretion System Encoded within the Gonococcal Genetic Island

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Background: Circularization of a pilin of a type IV secretion system.
Results: The TraA pilin encoded within the gonococcal genetic island is circularized by the signal peptidase LepB and the TrbI peptidase.
Conclusion: The circularization reaction shows limited flexibility in the length of the N and C termini and occurs via a covalent intermediate.
Significance: This is the first demonstration of a covalent intermediate in pilin circularization.

The type IV secretion system (T4SS) encoded within the gonococcal genetic island (GGI) of Neisseria gonorrhoeae has homology to the T4SS encoded on the F plasmid. The GGI encodes the putative pilin protein TraA and a serine protease TrbI, which is homologous to the TraF protein of the RP4 plasmid involved in circularization of pilin subunits of P-type pili. TraA was processed to a 68-amino acid long circular peptide by leader peptidase and TrbI. Processing occurred after co-translational membrane insertion and was independent of other proteins. Circularization occurred after removal of three C-terminal amino acids. Mutational analysis of TraA revealed limited flexibility at the cleavage and joining sites. Mutagenesis of TrbI showed that the conserved Lys-93 and Asp-155 are essential, whereas mutagenesis of Ser-52, the putative catalytic serine did not influence circularization. Further mutagenesis of other serine residues did not identify a catalytic serine, indicating that TrbI either contains redundant catalytic serine residues or does not function via a serine-lysine dyad mechanism. In vitro studies revealed that circularization occurs via a covalent intermediate between the C terminus of TraA and TrbI. The intermediate is processed to the circular form after cleavage of the N-terminal signal sequence. This is the first demonstration of a covalent intermediate in the circularization mechanism of conjugative pili.
Thus, the VirB4 ATPase might assemble on the cytoplasmic membrane and seems to be closed from the periplasm (18, 30). Therefore most likely the pilus subunits enter the core complex in an ATP-dependent manner from the membrane (29). The core structure opens to the cytoplasm and to the outside of the outer membrane and requires the MPF proteins (25–28) and recently it was shown that the VirB4 ATPase can release the mature VirB2 pilin in an ATP-dependent manner from the membrane (29). The core formation of a peptide bond between the N and C termini of the signal peptide cleavage mechanism, the acyl-enzyme intermediate to mediate circularization of the pilin substrate (10, 20) has been proposed that TraF uses a modification of this dyad mechanism to mediate circularization of P-type pili. The mechanism by which the pilin subunits are ready to be assembled into the pilus, they form P-type pili, which are short rigid filaments used only during mating on solid surfaces (13).

The TraF protease involved in the process of TrbC circularization belongs to the family of serine proteases, similar to the Ser-24, Ser-26 LexA/signal peptidase superfamily that includes LexA-related and type I signal peptidase families (22). These proteins use conserved serine and lysine residues in a dyad mechanism leading to cleavage of a specific site on their substrate (23). The serine residue acts as a nucleophile that attacks the sessile bond and the lysine acts as a general base (24). It had been proposed that TraF uses a modification of this dyad mechanism to mediate circularization of the pilin substrate (10, 20) in which the catalytic lysine first activates the serine by a dyad-like deprotonation, followed by nucleophilic attack of the activated hydroxyl group of the serine on the peptide bond before the leaving tetrapeptide. This should result in the formation of a TraF-acyl intermediate between the catalytic serine and the carboxyl-terminal hydroxyl group of the cleaved pilin. In the signal peptide cleavage mechanism, the acyl-enzyme intermediate is attacked by a water molecule, however, the TraF-acyl bond is instead attacked by the N-terminal amino group of the pilin. This results in hydrolysis of the TraF-acyl bond and formation of a peptide bond between the N and C termini of the pilin leading to circularization of the TrbC pilin protein.

After maturation, the pilin subunits in the inner membrane are ready to be assembled into the pilus. Assembly of the pilus requires the MPF proteins (25–28) and recently it was shown that the VirB4 ATPase can release the mature VirB2 pilin in an ATP-dependent manner from the membrane (29). The core complex of the T4SS encoded on the pKM101 conjugative plasmid spans the entire periplasm and encloses a large cavity. The structure opens to the cytoplasm and to the outside of the outer membrane and seems to be closed from the periplasm (18, 30). Therefore most likely the pilus subunits enter the core complex either from the cytoplasm or via the inner membrane (30). Thus, the VirB4 ATPase might assemble on the cytoplasmic side of the core complex to transfer the pilus subunits inside the core complex for the subsequent assembly of the pilus. The exact mechanism of pilus assembly, however, still needs to be determined.

Recently, a 57-kb horizontally acquired genetic island called the gonococcal genetic island (GGI) was discovered in the human pathogen Neisseria gonorrhoeae (31). The GGI is present in 78% of N. gonorrhoeae clinical isolates and is integrated into the chromosome at the dif site (32). The GGI encodes many genes with homology to genes involved in Type IV secretion and resembles the F plasmid T4SS (33). Interestingly, the MS11 strain in which the GGI was first identified was shown to secrete DNA into the medium (33). The released DNA is taken up by natural transformation by other gonococci. Although most T4SS-related proteins encoded within the GGI show homology to F plasmid T4SS, the GGI encodes a relaxase that belongs to a novel family of relaxases (34). The GGI encodes a putative TraF-like protein called TrbI previously only found in T4SS expressing a P-type pilus. The GGI also encodes a putative pilin TraA that has a C-terminal amino acid with homology to genes involved in Type IV secretion, respectively. E. coli cultures were grown aerobically at 37 °C in Luria-Bertani (LB) broth in a shaking incubator or on LB agar plates containing 1.5% agar with the appropriate antibiotics: ampicillin (100 µg/ml) or kanamycin (40 µg/ml). N. gonorrhoeae strains were grown at 37 °C in 5% CO2 on GCB plates containing the supplement from Kellogg (36) or GCB liquid medium containing 0.042% NaHCO3 and Kellogg’s supplements (GCB was acquired from Difco and contains per liter: 15.0 g of protease peptone number 3, 1.0 g of corn starch, 4.0 g of K2HPO4, 1.0 g of KH2PO4, 5.0 g of NaCl, and 10.0 g of agar). When necessary, chloramphenicol was used at a concentration of 10 µg/ml. Strains used in this study are listed in Table 1.

**Construction of Plasmids**—The plasmids and primers used in this study are listed in supplemental Tables S1 and S2, respectively. The full-length traA gene was obtained by performing

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth**—Escherichia coli DH5α (35) and Tuner DE3 (Novagen) strains were used for cloning and overexpression, respectively. E. coli cultures were grown aerobically at 37 °C in Luria-Bertani (LB) broth in a shaking incubator or on LB agar plates containing 1.5% agar with the appropriate antibiotics: ampicillin (100 µg/ml) or kanamycin (40 µg/ml). N. gonorrhoeae strains were grown at 37 °C in 5% CO2 on GCB plates containing the supplement from Kellogg (36) or GCB liquid medium containing 0.042% NaHCO3 and Kellogg’s supplements (GCB was acquired from Difco and contains per liter: 15.0 g of protease peptone number 3, 1.0 g of corn starch, 4.0 g of K2HPO4, 1.0 g of KH2PO4, 5.0 g of NaCl, and 10.0 g of agar). When necessary, chloramphenicol was used at a concentration of 10 µg/ml. Strains used in this study are listed in Table 1.

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**TABLE 1**

| Strains | Description | Reference |
|---------|-------------|-----------|
| DH5α    | *E. coli* strain (F−endA1 glvV44 thi-1 recA1 relA1 gyrA96 deoR supG46 lacYZA-argF)U169, hsdR17(k−, mK−), λ− | Invitrogen |
| Tuner (DE3) | *E. coli* strain (F−ompT hsdSB(rB− mB−) gal dcm lacY1 (DE3) | Novagen |
| MS11    | *N. gonorrhoeae* strain | This study |
| ND500   | *N. gonorrhoeae* strain MS11ΔGGI | This study |
| EP019   | *N. gonorrhoeae* strain MS11 with full-length traA in the native site | This study |
| S8015-MS | *N. gonorrhoeae* strain MS11 with pS8015, full-length traA behind the lac promoter inserted between lacIP and aspC | This study |
| S8015-ND | *N. gonorrhoeae* strain ND500 with pS8015, full-length traA behind the lac promoter inserted between lacIP and aspC | This study |
| S8036-ND | *N. gonorrhoeae* strain ND500 with pS8036, full-length traA and trbI behind the lac promoter inserted between lacIP and aspC | This study |
PCR on \textit{N. gonorrhoeae} genomic DNA of isolate number 4465 (37). The \textit{traA} gene has two possible start codons. All constructs described in this manuscript start at the first putative start codon and thus contain 4 extra amino acids (MRFI) compared with proteins that would start at the second start codon. Site-directed mutagenesis was performed by circular PCR using primers with the required mutations as described in supplemental Table S2. Ligation independent cloning was performed as described previously (38).

\textbf{Membrane Preparation}—Inner membranes vesicles (IMVs) of \textit{E. coli} were isolated essentially as described before (39). To isolate membranes of \textit{N. gonorrhoeae}, the strain was plated on GCB plates with the appropriate antibiotic and cells were scraped from the plate and transferred to 3 ml of GCB liquid medium. 250 ml or 1 liter of cells were grown to an A$_{660}$ of 1.0 and centrifuged at 8,000 \times g in a JLA-16.25 rotor and resuspended in 50 nm Tris-HCl, pH 7.5. Cells were broken by three passes through a French press at 15 k.p.s.i. Cell debris was removed by centrifugation at 6,000 \times g in a SS34 rotor for 10 min. The membranes were pelleted at 40,000 rpm in a Ti-45 rotor for 1 h and resuspended in 1 ml of 50 mm Tris-HCl, pH 7.5.

\textbf{Silver Staining and Western Blotting}—For all experiments 17\% polyacrylamide SDS-PAGE gels were used. To stain gels, the mass spectrometry compatible Fire silver staining kit was used (Proteome Factory Co.). TraA affinity-purified polyclonal antibody was raised against the antigen sequence TGAEFK-GLADMVTGC (Genscript Corp.). For immunodetection of TraA, proteins were transferred via wet electrotransfer from the SDS-PAGE gels to PVDF membranes. PVDF membranes were incubated overnight in a 1:1,000 dilution of the TraA polyclonal peptide antibody followed by 3 washes with 0.1\% I-Block (Roche Applied Science) in TBS buffer (50 mM Tris-HCl, pH 7.4, and 150 mm NaCl). This was followed by incubation with a 1:20,000 dilution of anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma) and three washes with 0.1\% I-Block in TBS buffer. Finally, the blot was incubated with the CDP-star substrate (Roche Applied Science) and imaged using a Roche Lumi-imager.

\textbf{In Vitro Transcription/Translation and Transport Assay}—The \textit{in vitro} transcription and translation reaction was performed as described previously (40). Shortly, the RiboMax \textit{in vitro} transcription kit (Promega) was used with the required plasmids to generate $^{35}$S-labeled proteins. To study post-translational transport, $^{35}$S-labeled TraA was synthesized \textit{in vitro}, dissolved in 6 \textmu l urea, and used in post-translational translocation reactions as described (41). To study co-translational transport, the \textit{in vitro} translation reactions were carried out for 30 min at 37 $^\circ$C in the presence or absence of IMVs (4 \textmu g/12.5-$\mu$l reaction volume) (42). Reactions were started by the addition of the $^{35}$S-labeled methionine. After 30 min at 37 $^\circ$C the reactions were stopped by addition of sample buffer, samples were incubated for 2 min at 92 $^\circ$C and analyzed by 17\% SDS-PAGE. The gels were analyzed by autoradiography.

\textbf{In Vitro Assay with Solubilized Membranes}—To study the activity of TrbI in a solubilized state, TraA was synthesized co-translationally (as described above) in the presence of LepB overexpressing IMVs. After incubation at 37 $^\circ$C for 30 min, the reaction was stopped by centrifugation in an airfuge (Beckmann). The supernatant was discarded and the pellet was resuspended in the same volume of 20 \textmu m Tris-HCl, pH 7.5, and incubated with TrbI overexpressing IMVs at 37 $^\circ$C for 30 min with or without the addition of 1% Triton X-100. The reaction was stopped by addition of sample buffer; samples were boiled for 2 min at 92 $^\circ$C, separated by 17\% SDS-PAGE, and analyzed using the phosphorimager and the LumiAnalyst software from Roche Applied Science.

\textbf{His-tag Purification of the TraA-TrbI Intermediate}—TraA was synthesized \textit{in vitro} in a total volume of 100 \textmu l (see above) in the presence of His-tagged or untagged TrbI overexpressing IMVs. After synthesis, 100 \textmu l of solubilization buffer (50 mm sodium phosphate buffer, pH 8.0, 2\% Triton X-100, 300 mm NaCl, and 10 mm imidazole) was added to the reaction mixture and the reaction was incubated 30 min at room temperature while shaking. The reaction mixture was centrifuged in an airfuge. The supernatant was removed, mixed with 50 \textmu l of nickel-nitritolriacetic acid beads (Sigma) pre-equilibrated with buffer (50 mm sodium phosphate buffer, pH 8.0, 0.2\% Triton X-100, 300 mm NaCl), and incubated while shaking for 30 min at room temperature. The beads were washed 2 times with 500 \textmu l of wash buffer (50 mm sodium phosphate buffer, pH 8.0, 0.2\% Triton X-100, 300 mm NaCl, and 60 mm imidazole). The protein was then eluted from the beads in 25 \textmu l of 5 \times sample buffer (10\% (w/v) SDS, 10 mm DTT, 20\% (w/v) glycerol, 0.2 \textmu m Tris-HCl, pH 6.8, and 0.05\% (w/v) bromophenol blue) by boiling for 5 min at 92 $^\circ$C.

\textbf{Mass Spectrometry}—TraA and TrbI bands were excised from the gel and in-gel digestion was performed using trypsin (Promega-sequencing grade modified) or chymotrypsin $^{N^-p}$-tosyl-l-lysine chloromethyl ketone (Sigma), respectively. Briefly, the gel slices were destained with 50\% acetonitrile (v/v) containing 20 mm NH$_4$HCO$_3$, dehydrated with 100\% acetonitrile, and dried. Gel pieces were rehydrated in 5 mm NH$_4$HCO$_3$ in 10\% acetonitrile (v/v) containing 15 mg/liter of sequencing-grade modified trypsin (Promega) and incubated for 10 h at 22 $^\circ$C. Tryptic peptides were extracted with 0.1\% (v/v) trifluoroacetic acid in water. The extracted peptides were concentrated under vacuum but not to dryness. The resulting peptide mixture was injected onto a PepMap100 C-18 RP nano-Colum ( Dionex, Idstein, Germany) and separated on an UltiMate 3000 liquid chromatography system (Dionex, Idstein, Germany) in a continuous acetonitrile gradient consisting of the following steps: 0–20\% B in 5 min, 20–60\% B in 40 min, 60–100\% B in 10 min (B: 80\% (v/v) acetonitrile, 0.04\% (v/v) TFA). Peptides were eluted at a flow rate of 300 nl/min. A Probot microfraction collector (Dionex, Idstein, Germany) was used to spot liquid chromatography-separated peptides on a MALDI target with a rate of 8 s/spot. The eluate was mixed with matrix consisting of 4 mg/ml of \textalpha-cyano-4-hydroxycinnamic acid in 80\% (v/v) acetonitrile and 0.1\% (v/v) TFA. MALDI-TOF/TOF analysis was carried out on a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems/MDS Sciex, Forster City, CA) in positive-ion reflector mode. For one main spectrum, 30 subspectra with 90 spots per subspectrum were averaged. Close external calibration was performed. TraB was identified with a mascot score of 2776 and sequence coverage of
Processing of the T4SS Pilin Subunit of the GGI

FIGURE 1. TrA is processed by TrBI. A, silver-stained 17% SDS-PAGE gels of IMVs of E. coli overexpressing TraA alone (lane 1) or TraA and TrBI (lane 2). B, immunoblot using TraA antibody on total membranes of N. gonorrhoeae strains MS11 (lane 1), FA19 (lane 2), SJ015-MS (MS11 overexpressing TraA, lane 3), SJ036-ND (NDS00 overexpressing TraA and TrBI, lane 4), E. coli inner membrane vesicles overexpressing TraA signal sequence mutant (pelB1–22traAT32P–102, lane 5), TraA alone (lane 6), or TraA and TrBI (lane 7). Triangles indicate the positions of nonprocessed (black), leader peptidase-cleaved (gray) and circularized (white) forms of TraA. The asterisk indicates the position of TrBI.

79%. For identification of the N-terminal to C-terminal linkage; MS/MS data (with signal to noise ratio above 200) were searched against all possible TraA bond sequences ranging from the 29th to 32nd amino acid at the N terminus with the 99th to 102nd amino acid at the C terminus.

RESULTS

Detection of TraA in N. gonorrhoeae—The F plasmid-like type IV secretion system encoded within the GGI encodes a putative pilin homolog, TraA, and a TraF-like protein named TrBI. Sequence comparison of our laboratory strain MS11 with sequences derived from other N. gonorrhoeae strains that contain a GGI, like NCCP11945, FA19, DG12, PID18, PID1, PID332, and SK-93–1035, showed that the sequence of the trbI gene was conserved in all these strains. However, the traA gene of MS11 contained a frameshift mutation resulting in a truncation of the last 14 amino acids.3 Sequence alignment of the previously studied pilins TrbC of the RP4 plasmid and VirB2 of the Ti plasmid with TraA of N. gonorrhoeae and close homologs thereof further suggested that N. gonorrhoeae strain MS11 contains a truncated TraA and that all other N. gonorrhoeae strains containing the GGI encode a full-length TraA (see supplemental Fig. S1). To test whether N. gonorrhoeae TraA is processed by TrBI, full-length TraA was overexpressed in E. coli in the presence and absence of TrBI.

TraA is Processed by TrBI in E. coli—Overexpression of TraA was observed in isolated E. coli IMVs after silver staining of the isolated membranes (Fig. 1A), but not after Coomassie staining (data not shown). Overexpression of TrBI resulted in high levels of TrBI in IMVs (data not shown). Co-overexpression of TraA and TrBI showed similarly high levels of TrBI (Fig. 1A). Moreover, TraA was processed to a smaller product (Fig. 1A). Indeed the two putative TraA bands reacted with a peptide antibody raised against TraA (See Fig. 1B, lanes 6 and 7). To determine whether these bands were processed by the E. coli signal peptidase, a construct was generated in which the TraA signal sequence was replaced by the signal sequence of the Erwinia carotovora pectate lyase B (PelB) and the amino acid at the first position after the LepB signal peptidase cleavage site was mutated to a proline. The signal sequence of PelB has been studied in detail and is often fused to other proteins to target them across the inner membrane of E. coli (43). PelB with a proline after the LepB signal peptidase cleavage site is defective in processing by signal peptidase (44). Indeed the PelB1–22–TraA32–102:T32P migrated slower on the gel than the full-length TraA (compare Fig. 1B, lanes 5 and 6) indicating that TraA is processed by the leader peptidase of E. coli. Furthermore, TraA is processed to an even smaller product in the presence of TrBI (compare Fig. 1B, lanes 6 and 7), indicating that TraA is further processed by TrBI. To identify the nature of this processing step, the band corresponding to the processed TraA was excised from a silver-stained gel, digested with trypsin, and the resulting peptides were identified by MALDI-TOF MS/MS. This showed the presence of the GMFTATTGAEFK peptide, which can only be found when the pilin is circularized between residues Ala-31 and Thr-99. This peptide was not detected when similar experiments were performed in the absence of TrBI. These data suggest that the T4SS pilin homolog TraA encoded within the GGI of N. gonorrhoeae can be processed by TrBI, independent of other proteins encoded within the GGI resulting in a 68-amino acid long circular peptide. Circularization occurs between the residues immediately after the signal sequence cleavage site and the residue before the removed LII tripeptide at the C terminus. Thus the processing mechanism of TraA resembles the circularization mechanism of TrbC of the RP4 and R751 plasmids, which also involves cleavage within the C terminus of TrbC by TraF and removal of the AIEA tetrapeptide (13). The 68 amino acid circular pilin is smaller than the circular pilins observed for both TrbC of the RP4 and R751 plasmids (76 amino acids) of the IncP1-β family and VirB2 of the A. tumefaciens Ti plasmid (72 amino acids). Sequence alignment shows that the length differences occur mainly within the periplasmic domains of the pilins (supplemental Fig. S1), but also that the cytoplasmic loop of the TraA-like pilins is 3 residues shorter that the cytoplasmic loops observed in the VirB2 and TrbC pilins. Remarkably this results in a very short predicted cytoplasmic loop for TraA and its homologs.

TraA Is Processed in N. gonorrhoeae by TrBI Independent of Other T4SS Components—To study whether TraA is also circularized in N. gonorrhoeae, membrane fractions were obtained from N. gonorrhoeae strains MS11 and FA19 (containing a nontruncated version of TraA) and analyzed by Western blotting using the peptide antibody raised against TraA. TraA could, however, not be detected within these strains (Fig. 1B, lanes 1 and 2). In a similar experiment using a strain derived from strain MS11 that overexpressed the full-length TraA (SJ015-MS), TraA could be detected (Fig. 1A, lane 3) by Western blotting and migrated at a similar height as the circularized TraA observed in E. coli. This demonstrated that TraA is also circularized in N. gonorrhoeae. The overexpression in N. gonorrhoeae was, however, lower than in E. coli, and the band was not

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observed in *N. gonorrhoeae* after Coomassie or silver staining (data not shown). To confirm that circularization depends only on TrbI and not on the other proteins encoded with the GGI, TraA and TrbI were overexpressed in ND500, a strain derived from MS11 in which the GGI has been deleted (SJ036-ND). In this strain the band representing circularized TraA was again detected (Fig. 1B, lane 4). When TraA was expressed in ND500 in the absence of TrbI, only the linear form processed by leader peptidase was observed (supplemental Fig. S3). This demonstrated that also in *N. gonorrhoeae* the circularization of TraA is independent of the other proteins encoded within the GGI, but dependent on the presence of TrbI. Because identical processing of TraA was observed in *N. gonorrhoeae* and *E. coli*, further characterization of TraA processing was performed in *E. coli*.

Analysis of the C Terminus of TraA—Processing of TrbC involves the removal of the AEIA tetrapeptide but deletion analysis of the C-terminal residues of TrbC demonstrated that TrbC can be similarly processed if only a short tail of three residues (AEI) is present (20). To determine which C-terminal residues of TraA were important for circularization, a C-terminal truncation of TraA was created. Removal of Ile-102 completely abolished TraA circularization (Fig. 2A) indicating also that for the GGI-encoded TraA at least three residues are required after the C-terminal processing site. Mutagenesis of Ile-102 to glycine, leucine, glutamine, glutamate, or cysteine did not influence circularization (data not shown). This suggests that only the presence but not the nature of the third residue is important. To test whether elongation of the C terminus influences processing, the C terminus was extended with one (Ile) or two (Ala) residues. Extension of the C terminus with one residue did not influence the processing and a band was still detected at the position of circularization. An additional band of slightly lower mobility was also observed. This band is possibly derived from a differently circularized peptide, but using mass spectrometry only a peptide demonstrating that the pilin was again circularized between Ala-31 and Thr-99 could be detected. Extension of the C terminus with two residues completely inhibited circularization (Fig. 2B).

Analysis of the N Terminus of TraA—During the circularization reaction, the first residue after the signal sequence cleavage site is linked to the residue before the C-terminal removed tripeptide. Both sequence alignment and mutagenesis of the TrbC pilin (20) indicated that the identity of this residue is not important for the circularization reaction. To study the flexibility of the N-terminal processing reaction, deletions and insertions were generated directly after the signal sequence cleavage site of TraA. To exclude any ambiguity about the length and processing of the signal sequence, the signal sequence of TraA was replaced by the signal sequence of *E. carotovora* pectate lyase B (PelB). The signal sequence of PelB has been studied in detail and it often fused to other proteins to target them across the inner membrane of *E. coli* (43). The PelB signal sequence was fused to TraA at different positions from residues 30 to 33. Co-expression of these fusion proteins and detection of circularized TraA on gel or by Western blotting showed that all the proteins were processed by signal peptidase but that only the fusions of the PelB signal sequence to positions 31 and 32 of TraA could be circularized (Fig. 3). Mass spectrometry of the processed pilin derived from the fusion with Ala-31 again identified the peptide fragment GMFTATGGAEFK indicating circularization between Ala-31 and Thr-99, as was observed for
TraA with its native signal sequence. Mass spectrometry of the processed pilus derived from the fusion at Thr-32 showed the peptide fragment GMFTTTGAEFK demonstrating that this fusion protein is circularized between Thr-32 and Thr-99, resulting in a circular peptide of 67 amino acids. In TrbC, the N-terminal serine residue located immediately after the signal sequence cleavage site could be mutated to alanine, cysteine, glycine, and threonine but not to proline without affecting circularization (20). Similarly mutation of Thr-32, directly following the PelB signal sequence cleavage site, to glycine or serine did not affect circularization, but mutation to proline again fully inhibited circularization (data not shown). These data suggest that there is a limited flexibility in the length of the N terminus and further confirm that circularization is mostly unaffected by the identity of the N-terminal amino acid. The limited flexibility further suggests that the N and C termini have to be within a certain distance of the lipid membrane to allow circularization.

**Mutation of Putative Catalytic Serine Residues Does Not Affect the Circularization**—Based on sequence and structural similarities with the bacterial type I signal peptidases it was proposed that the mechanism of pilin circularization by TraF of the RP4 plasmid resembles the catalytic serine-lysine dyad mechanism of the Ser-24, Ser-26 LexA/signal peptidase superfamily (45). For TraF of the RP4 plasmid, a nucleophilic attack of the hydroxyl group of Ser-37 on the peptide bond of the pilin was proposed to be activated by deprotonation via Lys-89 and Asp-155. Indeed mutagenesis of Asp-155 and Lys-89 abolished pilin circularization, whereas only some residual circularization remained after mutagenesis of Ser-37 (20). To study whether TrbI functions via a similar mechanism, residues Ser-52, Lys-93, and Asp-155, which align (supplemental Fig. S2) with Ser-37, Lys-89, and Asp-155 of TraF, were mutated to alanine, glutamine, and isoleucine, respectively, and tested for TrbI-dependent circularization of TraA. Expression levels of all mutants, except for the D155I mutant were comparable with the levels observed for wild type as seen by silver-stained SDS-PAGE (Fig. 4). Although D155I was expressed at a lower level, significant levels of expression were still observed. As was observed for the corresponding residues of TraF, TrbI mutants in Lys-93 (K93Q) and Asp-155 (D155I) completely abolished circularization of TraA but surprisingly, mutagenesis of Ser-52 (S52A) did not have an effect on circularization (Fig. 4). This implies that either the circularization does not involve a serine-lysine catalytic dyad mechanism or that Ser-52 is not part of the serine-lysine catalytic dyad or that the function of Ser-52 can be replaced by another serine residue after mutagenesis of Ser-52. TrbI contains another 10 serine residues. Two serines (Ser-5 and Ser-9) are located in the cytoplasm and of the other serines, only Ser-153 seems to be conserved in most TraF homologues. Ser-156 is not conserved in TraF, but shows some conservation in homologues of TrbI (supplemental Fig. S2). Neither mutagenesis of Ser-37, Ser-50, or Ser-68 located in the primary sequence close to Ser-52 (Fig. 4), nor mutagenesis of Ser-153 or Ser-156 (Fig. 4) had any influence on TraA circularization. The remaining serines (Ser-124, Ser-161, and Ser-166) were not conserved or indicated to be not in the proximity of the putative peptide cleavage position after modeling of TrbI on the LepB structure. Therefore, it is very unlikely that they function as catalytic serines. We conclude that either TrbI contains redundant catalytic serine residues or does not function via a serine-lysine catalytic dyad mechanism.

**Membrane Insertion of TraA and Interaction with TrbI**—It has been established that N-terminal cleavage by signal peptidase is essential for circularization but it is unknown how TraA is inserted into the membrane. Current models for the maturation of the TrbC pilus protein propose that TrbC is cleaved by an unknown protease in the cytoplasm and is then inserted post-translationally into the inner membrane (20). However, direct evidence for this is missing. The mechanism of insertion of proteins into the membrane is often studied by testing the protease sensitivity of an *in vitro* synthesized protein inserted into either isolated inverted IMVs or into proteoliposomes (46). These assays are, however, often difficult to perform for small membrane proteins like TraA, which have most of their amino acids located in the hydrophobic membrane, inaccessible to the added protease. Indeed our attempts to set up a conventional membrane insertion assay for TraA were unsuccessful (data not shown). Because circularization of TraA by TrbI only occurs after membrane insertion, it was tested whether the circularization of TraA by TrbI could be used as an indicator for membrane insertion of TraA. Both post-translational (Fig. 5A) and co-translational insertion (Fig. 5B) of *in vitro* 35S-labeled TraA into IMVs overexpressing TrbI were tested. Using the novel assay under conditions suitable for post-translation membrane insertion in the presence of SecA, SecB, ATP, and inner membrane vesicles no processing of TraA could be detected.
Remarkably, if TraA is synthesized co-translationally in the presence of IMVs overexpressing TrbI, again no circularized or TraA processed by leader peptidase was observed but a stable heat and SDS-resistant band was seen with a molecular mass of about 30 kDa. Because the combined molecular mass of TraA and TrbI is 30.4 kDa, it was likely that the observed band is a covalent TraA-TrbI intermediate of the cyclization reaction. This would demonstrate that TraA is inserted in a co-translational manner.

Identification of the 30-kDa Band as a Covalent TraA-TrbI Intermediate—To confirm the identity of the observed band at 30 kDa, formation of this band was tested in the TrbI K93Q and D155I mutants, both of which are deficient in formation of a circular TraA. Indeed both mutants failed to form the observed band at 30 kDa, further suggesting that the band represents a covalent TraA-TrbI intermediate (Fig. 5C). For further confirmation, a construct with TrbI fused to an N-terminal His-tag was constructed. When this construct was used in the in vitro transcription/translation/membrane insertion assay, the 30-kDa band was also observed. After purification using a His-tag affinity column indeed a band was observed at 30 kDa (Fig. 5D), whereas a similar approach using non-His-tagged TrbI only purified low levels of non-tagged TrbI (Fig. 5D). The small shift in migration of the intermediate observed after fusion to the N-terminal His-tag further confirms the identity of the 30-kDa band as a TraA-TrbI intermediate. Because the band is stable in 2% SDS after heating to 100 °C, the intermediate is very likely formed via a covalent bond. This is the first demonstration that the circularization reaction proceeds via a covalently bound intermediate.

In Vitro Analysis of Formation of the TrbI-TraA Intermediate in TraA Proteins with C-terminal Mutations—To compare the in vitro assay with the results of in vivo TraA circularization, several mutants tested above in the in vitro TraA circularization assay were also tested in the in vitro assay. As expected, the TraAΔI102 mutant, which did not show any circularization, also did not show the TraA-TrbI intermediate. Five C-terminal mutants, which showed similar circularization efficiency in vivo, I102G, I102E, I102Q, I102N, and TraA +1, also showed the TraA-TrbI intermediate, but with different efficiencies (Fig. 6). Especially the I102E mutation showed a lower efficiency of formation of the TraA-TrbI intermediate. This demonstrated that the in vitro assay is more sensitive than the in vivo assay and that mutations in the C terminus of TraA can influence the rate of formation of the TraA-TrbI intermediate.

In Vitro Circularization of TraA Requires Overexpression of LepB—In the in vitro transcription/translation/membrane insertion assay no TraA was observed that was circularized or processed by signal peptidase. This indicated that TrbI alone cannot fully process TraA and that cleavage of the signal sequence is a limiting factor. Although there are wild type levels of LepB present in the IMVs, these levels could be insufficient to process all synthesized TraA. Hence, a strain was created that overexpressed both LepB and TrbI. In vitro transcription/translation/membrane insertion assays using IMVs of this strain showed processing of TraA by leader peptidase and circularization (Fig. 7A). When similar experiments were performed with the TraAΔI102 mutant, only processing by leader peptidase was observed (Fig. 7B). This demonstrated that processing by leader peptidase and TrbI are the two steps leading to TraA circularization and that both steps can occur independently.

When this experiment was performed with fusion of the PelB signal sequence to position 33 of TraA, similar to the in vivo result, no circularization could be detected but the intermediate, although at lower levels, was still observed (Fig. 7C). This demonstrates that, when the TraA-TrbI intermediate is formed, the exact positioning of the N terminus is important for further circularization.
synthetases have been identified (50) but post-translational circularization of ribosomal proteins is not very common. However, several post-translational circularized proteins have now been identified. Among these proteins are cyclotides from the plant families Violaceae and Rubiaceae (51, 52), trypsin inhibitors from the Asteraceae and Cucurbitaceae plant families (53, 54), θ-defensins from primates (55), bacteriocins (56), and cyclic conjugative pili from bacteria (10). A possible benefit for circular proteins might be their higher stability and resistance to thermal, chemical, and enzymatic treatments (57, 58). Indeed purified pili from A. tumefaciens form structures resistant against detergents, pH variations, urea, and glycerol (59). Currently circular pili have only been identified in P-type conjugation systems, whereas to our knowledge no circular pilins have been identified in other pili systems. T4SSs expressing P- and F-type pili not only differ in their pilin processing mechanism and morphology, but F-type pili can expand and retract their pili as well as require only two instead of three ATPases for function (60). In this study, the pilin encoded by the T4SS of N. gonorrhoeae is analyzed. This pilin has no significant homology to the previously well characterized pili like TraA and TrbC pili of the T4SS of the F and RP4 plasmids, respectively. TraA has an N-terminal signal sequence. Proteins with a signal sequence are generally dependent on the SecYEG and/or YidC pathway for membrane insertion. The type I pilin subunits of the chaperone/usher assembly system are inserted via Sec translocon (61). Similarly, the pseudopilins of the type II secretion system that share similarity to the pilins of the type IV pili system are inserted co-translationally via the Sec translocon (62, 63). These pilin subunits possess special signal sequence motifs that are cleaved and methylated by the bifunctional peptidase PilD (47). Membrane insertion of the TraA pilin of the F-plasmid was proposed to be sec-independent but depended on the F plasmid-encoded membrane protein TraQ (64). It is demonstrated here that TraA of the GGI is inserted into the membrane via a co-translational pathway. Whether membrane insertion specifically depends on SecYEG, YidC, or both was not determined.

The T4SS within the GGI encodes the TrbI protease, which has homology to TraF of the RP4 plasmid, involved in the circularization of the TrbC pilin. Our study demonstrates that the TraA protein encoded within the GGI can be circularized by TrbI. This is the first demonstration of an F plasmid-like system, with the ability to process pilin subunits in a manner similar to systems encoding P-type pili. There are a few examples of hybrids of P- and F-type systems, like the IncH plasmid R27, which has a P-like relaxase and pili and an F-like T4SS (65), and IncW plasmid R388, which has an F-like relaxase and P-like T4SS (66). The circularization of TraA showed similarity to the circularization of the TrbC pilin of the RP4 plasmid (20). TrbC processing contains an extra step of C-terminal processing by an unknown protease, but both TrbC and TraA are, after insertion into the membrane, processed by the host leader peptidase and a TraF/Trlb protease. In this step, TraF removes a C-terminal tetrapeptide, whereas TrbI removes a tripeptide. TraF and TrbI are both fully functional when either a tri- or tetrapeptide is present (20) and inactive when only a dipeptide is present (20). They differ from the VirB2 pilin of the Ti plasmid.
of A. tumefaciens, which is processed without removal of a C-terminal peptide. The identity of residues in the C terminus influenced the rate at which the intermediate is formed, but their identity is not critical for the circularization reaction because no effect is observed when the mutants are tested in vivo. Truncation of the N terminus with one residue still results in a circular pilin, demonstrating also that the length of the circular pilin allows some flexibility.

Like the TraF protease, the TrbI protease encoded by the GGI seemed to belong to the Ser-24, Ser-26 LexA/signal peptidase superfamily (22), which acts via a conserved serine/lysine catalytic dyad mechanism (67). Indeed mutagenesis of Lys-93 of TrbI, showed that this residue is involved in the circularization reaction. However, no candidate for the catalytic serine could be identified. Ser-37, the putative catalytic serine of TraF, showed a strongly reduced circularization of TrbC, but mutagenesis of the corresponding conserved Ser-52 of TrbI shows no effect on circularization in vivo. Mutagenesis of several other serine residues (Ser-37, Ser-50 or Ser-68, Ser-153, and Ser-156) also did not have any influence on the in vivo circularization. The remaining serines are either located in the cytoplasm (Ser-5 and Ser-9), or are neither conserved nor indicated to be in the proximity of the putative peptide cleavage position (after modeling of TrbI on the LepB structure) making it very unlikely that they function as catalytic serines. Therefore, it is concluded that either TrbI contains redundant catalytic serine residues or does not function via a serine-lysine dyad. Remarkably, a reaction intermediate, consisting of a TrA bound with its C-terminal residue to TrbI is identified. This is the first demonstration that the circularization reaction of the pilin proceeds via a covalently linked intermediate as was proposed by Eisenbrandt et al. (20). Most likely this intermediate is formed after cleavage of the C-terminal tripeptide thus conserving the energy of the cleaved peptide bond in the newly formed acyl-intermediate as proposed before. We show here that the formation of the intermediate and the circularization reaction are two consecutive steps that can be uncoupled. The circularization reaction can only occur after the N-terminal residues become available for cleavage by leader peptidase. Because the circularization can occur in the absence of a proton-motive force (the reaction can also take place in detergent), the energy required for linking the N- and C-terminal residues might be conserved by formation of the enzyme substrate intermediate. The intermediate is most likely similar to the acyl-enzyme intermediate seen in the crystal structure of E. coli signal peptidase with the bound β-lactam (68).

The pilin TraA encoded by the GGI of N. gonorrhoeae is processed into a circular peptide by the combined action of two proteases, TrbI encoded by the GGI and LepB encoded by the host. The reaction takes place via a covalent enzyme-substrate intermediate, which is formed after removal of the three C-terminal amino acids. Formation of the intermediate is independent of leader peptidase, but circularization can only occur after cleavage by leader peptidase (see supplemental Fig. S4). Because leader peptidase is most likely present in excess to TrbI under normal conditions, TraA is most likely first processed by leader peptidase and then by TrbI. This study on the mechanism of pilin processing helps to further understand the assembly and biogenesis of the pili by T4SSs.

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