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Protein-Protein Interactions within Type III Secretion System-Dependent Pili of *Rhizobium* sp. Strain NGR234[▽]

Maged M. Saad,† Christian Staehelin,‡ William J. Broughton,* and William J. Deakin

LBMPS, Université de Genève, Sciences III, 30 quai Ernest-Ansermet, 1211 Genève 4, Switzerland

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Pili synthesized by the type III secretion system of *Rhizobium* species strain NGR234 are essential for protein secretion and thus for efficient symbiosis with many legumes. Isolation and partial purification of these pili showed that they are composed of at least three proteins, NopA, NopB, and NopX. Using biochemical assays, we show here that these proteins interact directly with one another.

Many gram-negative bacteria employ type III secretion systems (T3SS) to deliver virulence "effector" proteins directly into eukaryotic cells, which then modulate cellular functions in the host. A common feature of these secretion systems is the T3SS-dependent extracellular appendages that link bacteria to their hosts, where they probably act as conduits for the effector proteins. The appendages of animal pathogens, also called needle complexes, are mostly composed of many copies of a single, small (<10-kDa) protein. When the needle comes in contact with a host cell, synthesis of a translocation pore composed of different bacterial proteins (termed translocators) occurs in the plasma membrane of the target cell. Formation of the pore is essential for delivery of the effector proteins. Extracellular appendages of the animal pathogen *Yersinia* sp. have been well characterized (7); the needle is composed of YscF, and three translocators have been identified (LcrV, YopB, and YopD). YopB and YopD contain hydrophobic domains and can insert into erythrocyte membranes; LcrV does not possess such domains, but it is absolutely required for the assembly of functional pores (15). LcrV forms a distinct structure at the tip of the YscF needle, upon which the YopBD translocation pore is assembled (16). YscF and LcrV can interact. LcrV is probably distal to YscF since YscF needles still form if there is a mutation in *lcrV*, although the translocation of effectors is blocked. Phytopathogens produce much longer pilus-like structures, called Hrp pili, and their assembly is dependent upon the hypersensitive response and pathogenicity (Hrp) gene cluster. Hrp pili are also composed of multiple copies of a single, small protein (9, 17). T3SS of phytopathogens are also thought to form translocation pores in the plant plasma membrane. The translocating protein HrpF of *Xanthomonas campestris* pv. vesicatoria has hydrophobic domains and has been shown to insert into lipid bilayers (6). Furthermore, mutation of *hrpF* yields a phenotype characteristic of translocators; protein secretion can occur in vitro from cell cultures but not in vivo into host cells (18). Purification of Hrp pili from *Ralstonia solanacearum* yielded a second protein (PopF1) that copurified with the major subunit, HrpY. PopF1 is homologous to HrpF and functions as a translocator in *R. solanacearum*. Although this situation seems to be analogous to the situation in animal pathogens, it should be noted that HrpY and PopF1 did not interact in a yeast two-hybrid binding assay (14).

Nitrogen-fixing symbiotic bacteria, collectively called rhizobia, utilize a variety of signal molecules that govern the range of legumes with which they interact (3). Certain rhizobia also possess functional T3SS, and proteins secreted by these systems (nodulation outer proteins [Nops]) are another determinant of host range (12). For example, in *Rhizobium* species strain NGR234 at least six T3SS-secreted Nops have been identified: NopA, NopB, NopC, NopL, NopP, and NopX (1, 8, 13, 19–21). Depending upon the legume host, abolition of Nop secretion by NGR234 can improve or block symbiotic interactions.

Electron microscopic methods showed that T3SS-dependent pili of NGR234 are mostly composed of NopA but also contain NopB and NopX (8, 19). T3SS-dependent pili have also been observed in *Rhizobium fredii* USDA257, where they are also composed of several proteins. Characterization of pili using antibodies against Nops identified two of these proteins as NopB and NopX (10, 11). Based upon its relative abundance and because it has secondary structural features similar to those of other pilus subunits, NopA is the major component of NGR234 T3SS pili (none of the major pilus subunits from different bacterial species share any amino acid similarity). As NopX has significant homology to HrpF and PopF, it could also be a translocating protein, and its copurification with NGR234 T3SS pili mirrors that of PopF1 in *R. solanacearum*. The presence of NopB in pilus extracts is intriguing, however. Although BLAST searches with NopB revealed significant homology only to other rhizobial (NopB-like) proteins, the carboxy terminus of NopB has some homology to FlgK, a linker of two distinct flagellum proteins, the hook and filament (which are synthesized by related T3SS). Perhaps the role of NopB might be as a coupling protein within the NGR234 pili. We propose three simple models (Fig. 1) to explain the positions of NopA, NopB, and NopX within the pili. NopX, as a potential

* Corresponding author. Mailing address: LBMPS, Université de Genève, Sciences III, 30 quai Ernest-Ansermet, 1211 Genève 4, Switzerland. Phone: 41 22 379 3108. Fax: 41 22 379 3009. E-mail: william.broughton@bioveg.unige.ch.

† Present address: LIPM, UMR CNRS-INRA 2594/441, BP 52627, 31326 Castanet Tolosan Cedex, France.

‡ Present address: School of Life Sciences, SunYat-Sen (Zhongshan) University, 135 Xingangxi Road, Guangzhou, 510275, China.

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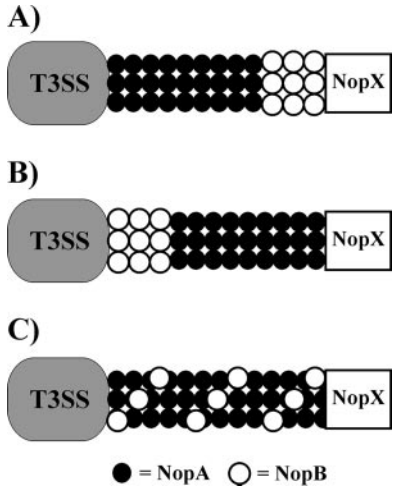

FIG. 1. Models of potential NopA and NopB interactions. Three potential arrangements of NopA, NopB, and NopX in the T3SS pili of NGR234 are shown. In all cases, the T3SS is indicated by a gray oval which spans the bacterial cell membranes, and the putative translocon (composed of NopX) is indicated by an open square which would be found in the plasma membrane of the plant cell. Between these would be the plant cell wall and perhaps an extracellular space, which the T3SS pili would bridge to allow effector release into the cytoplasm of the plant cell. Molecules of NopA are indicated by filled circles, and molecules of NopB are indicated by larger open circles. Experimental evidence for each of the three models is discussed in the text.

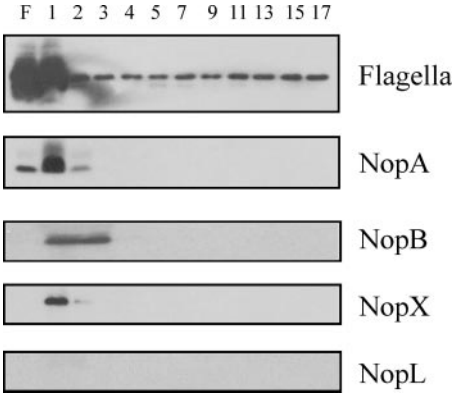

FIG. 2. Size exclusion chromatography of NGR234 surface structures. Proteins present in each fraction were identified using antibodies specific to flagella, NopA, NopB, NopL, and NopX. The monomeric sizes of the proteins are as follows: NopA, 7 kDa; NopB, 19 kDa; NopL, 38 kDa; and NopX, 63 kDa. In the case of the flagella, the major band at approximately 35 kDa corresponds to the major component flagellin. The identities of the fractions are indicated at the top, where "F" indicates the flowthrough fraction (void volume) and the numbers indicate subsequent fractions. Not all fractions are shown.

translocator, has been placed at the distal end of the pili, which would presumably be within the plasma membrane of the plant cell in vivo. We assume that in all three models there would be a continuous channel that permits direct effector secretion into the plant cell, but this is not necessarily the case; some Nops could be released into the extracellular medium before the NopX pore forms, in a manner analogous to that described for the harpins of phytopathogens (5). NopB could thus link NopA and NopX in a manner analogous to that described for LcrV in *Yersinia* (Fig. 1A) or perhaps even join the NopA filament to the basal T3SS apparatus (Fig. 1B). A specific linkage role might imply that NopB has a discrete location in the pili, but immunolocalization of NopB revealed labeling throughout the pilus structure (19); thus, NopB could interact with both NopA and NopX (Fig. 1C). In order to discriminate between these three models, we studied protein-protein interactions between NopA, NopB, and NopX of NGR234.

Size exclusion chromatography showed that NopA, NopB, and NopX belong to a high-molecular-mass structure. NGR234 surface structures were isolated as previously described (8) and then subjected to size exclusion chromatography through Sephadex G-75 columns. After the flowthrough was collected, 10 ml of 5 mM Tris-HCl (pH 8.0) was applied to a column, and 20 500-μl fractions were collected. Each fraction was concentrated and electrophoresed on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel before it was electroblotted onto polyvinylidene difluoride (PVDF) membranes. Proteins present in each fraction were identified using antibodies specific to flagella, NopA, NopB, NopL, and NopX (Fig. 2). The specificity of the anti-Nop antibodies for their respective antigens has been demonstrated previously (13, 19). Flagellar proteins represent a well-characterized multimeric surface structure that normally elutes in the void volume of the column or in the first fraction. NopA, the major pilus subunit of NGR234, did this. The other pilus subunits, NopB and NopX, cofractionated with NopA. Thus, NopA, NopB, and NopX coeluted in the high-molecular-mass fractions with the flagella, whereas NopL, an NGR234 effector protein (see below), was not detected.

Coimmunoprecipitation assays demonstrate that NopA and NopB interact. Cultures of NGR234 and NGRΩ*rhcN* were grown in rhizobial minimal medium (4) containing the flavonoid apigenin at a concentration of 10^{-6} M at 27°C for 40 h. The total extracellular proteins were purified using established techniques (13). Concentrations of supernatant proteins were determined using the Bradford assay, and 25 μg of NGR234 total extracellular protein and an equivalent volume of the NGRΩ*rhcN* extracellular protein extract were incubated (overnight at 4°C) with anti-NopA, anti-NopB, or anti-NopL antibodies (all at a 1:100 dilution). Then the antibody-antigen complexes were precipitated using a mixture of protein A and protein G agarose beads, and the precipitate was analyzed by immunoblotting (Fig. 3A and 3B). When anti-NopA antibodies were used, NopB coimmunoprecipitated with NopA (Fig. 3A), while in the reciprocal experiment (using anti-NopB antibodies), NopA coimmunoprecipitated with NopB (Fig. 3B). Preimmune sera for both antibodies did not immunoprecipitate any Nop. Furthermore, NopL was not detected in the antigen-antibody complexes when either anti-NopA or anti-NopB antibodies were used, nor were anti-NopL antibodies able to coimmunoprecipitate NopA and NopB (Fig. 3A and 3B). NopL was used as a negative control as it is an effector protein that probably functions within the host cell (2). As an effector protein, NopL is not expected to bind directly to the external T3SS machinery. Thus, although it appears that NopA and NopB are associated and might interact, it is still unclear whether direct binding between these proteins occurs or another as-yet-unidentified rhizobial protein links NopA and

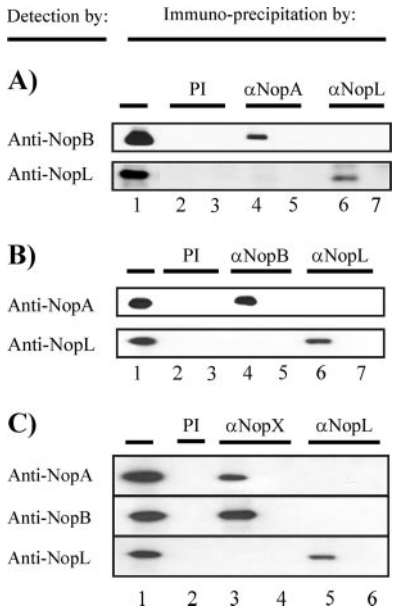

FIG. 3. Interaction between Nops detected by immunoprecipitation. (A and B) Immunoprecipitation of total extracellular proteins isolated from induced cultures of NGR234 (lanes 2, 4, and 6) or NGRΩ*rhcN* (lanes 3, 5, and 7). Nonimmunoprecipitated extracellular proteins of NGR234 were used as a positive control (lane 1). Immunoprecipitation was performed as follows: in panel A, preimmune serum (PI) (lanes 2 and 3), antibodies to NopA (lanes 4 and 5), and antibodies to NopL (lanes 6 and 7); and in panel B, preimmune serum (lanes 2 and 3), antibodies to NopB (lanes 4 and 5), and antibodies to NopL (lanes 6 and 7). (C) Immunoprecipitation from total extracellular proteins isolated from induced cultures of NGR234 (lanes 2, 3, and 5) and NGRΩ*rhcN* (lanes 4 and 6) using preimmune serum (lane 2), anti-NopX antibodies (lanes 3 and 4), and anti-NopL antibodies (lanes 5 and 6). Extracellular proteins of NGR234 were used as a positive control for the presence of Nops (lane 1). The immunoprecipitated complexes were separated by 15% SDS-PAGE and then transferred to PVDF membranes. The membranes were probed with NopA, NopB, or NopL antibodies (all at a 1:1,000 working dilution). Protein-primary antibody complexes were revealed using anti-rabbit antibodies labeled with horseradish peroxidase and ECL detection reagents (GE Healthcare).

NopB. To distinguish between these two possibilities, in vitro binding analyses were performed using NopA and NopB purified from *Escherichia coli* cells.

Overexpression of *nopA* and *nopB*. PCR was used to amplify *nopA* and *nopB*, the PCR products were cloned into a standard cloning vector, and the fidelity of the PCR was confirmed by DNA sequencing. A fragment of DNA containing *nopA* was then ligated into the overexpression vector pMal-c2, fusing *nopA* in frame to the *malE* gene encoding the maltose-binding protein (MBP). The recombinant plasmid was transformed into *E. coli* DH5α. Overexpression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C, and two proteins, one with a molecular mass of approximately 40 kDa (MBP) and one with a molecular mass of approximately 50 kDa (MBP-NopA), were observed (Fig. 4A, lanes 1 and 2), which corresponded to the predicted sizes. MBP-NopA was purified from total cell extracts using amylose resin, and then its identity was verified by SDS-PAGE (Fig. 4A, lane 3). To overexpress *nopB*, the PCR product generated as described above was subcloned into the expression vector pGEX-3x, fusing *nopB* in frame to the gene encoding glutathione *S*-transferase (GST). The recombinant plasmid was transformed into *E. coli* DH5α. Following induction with 1 mM IPTG, GST and GST-NopB were purified using glutathione agarose beads. After 30 min of incubation, the beads were washed three times with 1 ml of phosphate-buffered saline and then resuspended directly in 100 μl of SDS sample buffer for analysis by SDS-PAGE (Fig. 4B). Plasmid pGEX-3x produced a protein with a molecular mass of ~30 kDa, corresponding to the molecular mass of GST, whereas the *nopB*-containing derivative produced a protein with a molecular mass of approximately 43 kDa, corresponding to the expected molecular mass of the GST-NopB fusion protein (Fig. 4B). Two smaller proteins were coisolated with GST-NopB; both were probably degradation products.

Solid-phase interaction of NopA and NopB. The principle of the solid-phase interaction method (also termed the overlay assay or far-Western blotting) is that the first protein is fixed to a membrane and then a second, potentially interacting protein is applied and binding is allowed to occur. Washing should remove any of the second protein that is not associated. The interacting protein can then be detected with a specific antibody. The overlay assay was performed by electrophoresing 1 μg of purified GST and GST-NopB on a 12% SDS-PAGE gel and then transferring the proteins to PVDF membranes. As a control, one membrane was probed with anti-GST antibodies. A single band at a molecular mass of approximately 30 kDa was observed in the lane containing purified GST alone, while the antibodies revealed different proteins in the lanes containing extracts from GST-NopB fusions. A major band at a molecular mass of 43 kDa (the expected size of the GST-NopB fusion) was observed, as were several smaller, fainter bands that were probably degradation products of the fusion protein (Fig. 4C). Antibodies to NopB also cross-reacted with the GST-NopB fusion protein to give a similar pattern, but they did not bind to GST alone (data not shown). An identical membrane was prepared and incubated overnight at 4°C with purified MBP-NopA (at a concentration of 10 μg/ml). After extensive washing, the membrane was probed with anti-MBP antibodies (dilution, 1:10,000), and then protein-primary antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit antiserum. Only one band, at a molecular mass of ~43 kDa, was observed in the lane containing the GST-NopB fusion protein (Fig. 4D, lane 2). This band could have resulted only from MBP-NopA binding to the GST-NopB fusion protein that had been immobilized on the PVDF membrane. No bands were present in the lane containing GST alone (Fig. 4D, lane 1). Additional control experiments were performed, and identical GST- and GST-NopB-containing membranes were either incubated with MBP alone or directly probed with anti-MBP antisera; both experiments revealed no significant binding (data not shown). These results demonstrated the potential for NopA and NopB to associate but also crucially showed that these two proteins can interact directly without the need for a third, intermediary protein.

NopX, a putative translocator, also interacts with NopA and NopB. A third protein, NopX, which copurifies with T3SS pili of NGR234, is thought to be a translocator. It was demonstrated previously that NopX also cofractionates with NopA

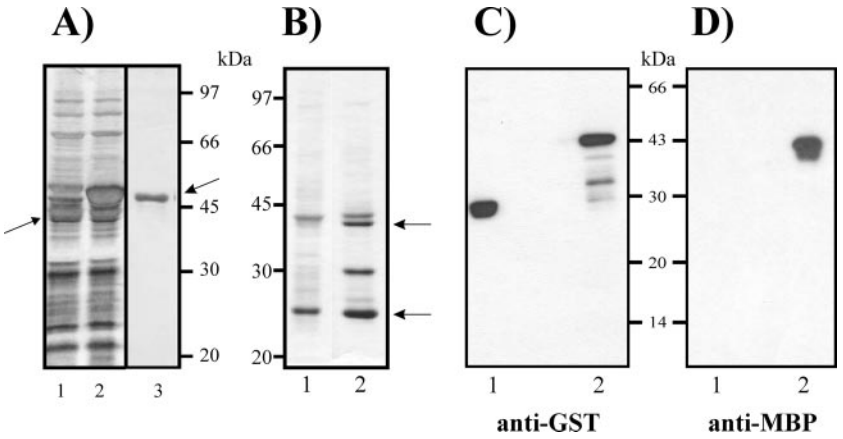

FIG. 4. Purification of NopA and NopB fusion proteins and solid-phase interaction of NopA and NopB. (A) Analysis of IPTG-induced cultures of *E. coli* overexpressing *nopA* fused to the gene encoding the MBP (lane 2). For comparison, proteins extracted from strains harboring the empty vector were also used (lane 1). The induced proteins discussed in the text are indicated by arrows. The results of purification of NopA-MBP using amylose resin are shown in lane 3. Proteins were separated on a 12% SDS-PAGE gel and stained with silver nitrate. Approximate molecular masses are indicated on the right. (B) Results of purification of the NopB-GST fusion proteins from *E. coli* cells (lane 2). Proteins were separated on a 10% SDS-PAGE gel and stained with silver nitrate. For comparison, proteins extracted from strains harboring the empty vectors were also used (lane 1). The smaller bands below GST-NopB are probably degradation products of the fusion protein, which include GST itself. Approximate molecular masses are indicated on the left. (C and D) Equal quantities of purified GST (lane 1) and GST-NopB (lane 2) were separated by 12% SDS-PAGE and blotted onto PVDF membranes. One membrane (C) was probed with anti-GST primary antibody using standard techniques. A second membrane (D) was exposed to purified MBP-NopA, washed, and then incubated with anti-MBP antiserum, followed by more washes. Protein-primary antibody complexes on both membranes were revealed using anti-rabbit antibodies labeled with horseradish peroxidase and ECL detection reagents.

and NopB after size exclusion chromatography. We thus tested whether NopX could also interact with NopA and/or NopB using immunoprecipitation assays with an antibody raised against NopX (13). NopA and NopB, but not NopL, were coimmunoprecipitated using NopX antibodies (Fig. 3C). Unfortunately, further characterization of NopX interactions was not possible, as overexpression and purification of NopX are limited by its toxicity to *E. coli* cells. Nevertheless, since a *nopX* mutant can still secrete NopA and NopB (13), whereas secretion of all Nops is blocked in *nopA* and *nopB* mutants (8, 19), we suggest that NopX is part of the distal end of a contiguous structure largely made up of NopA and NopB. If this is the case, then the order of secretion should be NopA and NopB, followed by NopX.

In terms of the pilus models presented in Fig. 1, we currently favor the model shown in Fig. 1C, as it fits best with the protein-protein interaction data, the phenotypes of all three mutants examined, and the electron microscopic immunolocalization of NopB. We would not expect NopA and NopX to interact directly, as shown in Fig. 1A, and the *nopB* mutant might still be able to secrete NopA, which is not the case. Similarly, as shown in Fig. 1B, we would not expect NopB and NopX to interact directly, and perhaps the *nopA* mutant should still be able to secrete NopB, which is also not the case. It remains to be determined exactly how these Nops interact, but more intriguing is the role of NopB in the T3SS pili. Potentially, this protein could strengthen the structure by reinforcing the NopA-NopA interactions, or perhaps its role is to camouflage the NopA subunits so that they can avoid recognition by plant receptors. Regardless of this uncertainty, the T3SS pili of NGR234 are unusual for plant-interacting bacteria in that they consist of several proteins.

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