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Characterization of Nops, Nodulation Outer Proteins, Secreted Via the Type III Secretion System of NGR234

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The nitrogen-fixing symbiotic bacterium *Rhizobium* species NGR234 secretes, via a type III secretion system (TTSS), proteins called Nops (nodulation outer proteins). Abolition of TTSS-dependent protein secretion has either no effect or leads to a change in the number of nodules on selected plants. More dramatically, Nops impair nodule development on *Crotalaria juncea* roots, resulting in the formation of nonfixing pseudonodules. A double mutation of *nopX* and *nopL*, which code for two previously identified secreted proteins, leads to a phenotype on *Pachyrhizus tuberosus* differing from that of a mutant in which the TTSS is not functional. Use of antibodies and a modification of the purification protocol revealed that NGR234 secretes additional proteins in a TTSS-dependent manner. One of them was identified as NopA, a small 7-kDa protein. Single mutations in *nopX* and *nopL* were also generated to assess the involvement of each Nop in protein secretion and nodule formation. Mutation of *nopX* had little effect on NopL and NopA secretion but greatly affected the interaction of NGR234 with many plant hosts tested. NopL was not necessary for the secretion of any Nops but was required for efficient nodulation of some plant species. NopL may thus act as an effector protein whose recognition is dependent upon the hosts' genetic background.

Additional keywords: legumes, pathogenicity, symbiosis, translocator.

Soil bacteria of the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (collectively called rhizobia) are gram-negative symbionts of legumes that induce the formation of nitrogen-fixing nodules on roots. *Rhizobium* species NGR234 (Freiberg et al. 1997; Viprey et al. 1998), *R. fredii* strains USDA257 (Krishnan and Pueppke 1993) (NCBI database, accession number AF229441) and HH103 (de Lyra et al. 2000), *Bradyrhizobium japonicum* 110*spc*4 (Göttfert et al. 2001; Krause et al. 2002), as well as *Mesorhizobium loti* MAFF303099 (Kaneko et al. 2000) are known to possess a type III secretion system (TTSS). TTSSs are common molecular machines used by symbiotic and pathogenic bacteria to deliver "messages" to eukaryotic interlocutors. TTSS machines are usually composed of about 20 proteins that form a complex across the inner and outer bacterial membranes through which proteins transit, without proteolytic cleavage, to the external environment. Secreted proteins of plant and animal pathogens are classified into four groups: effector, translocator, and regulator proteins (Hueck 1998), as well as external components of the secretion apparatus. Those from the last group mediate the transfer of the other secreted proteins out of the bacterial cells. Effector proteins subvert host metabolism. Some of them appear to be secreted into the extra-cellular compartment, whereas others are targeted into host cells (Cornelis and Van Gijsegem 2000). Translocator proteins do not exhibit direct anti-host functions but, rather, are required for protein transit through the eukaryotic plasma membrane into the cytosol of the targeted cells (Büttner and Bonas 2002). Regulatory proteins mediate the cell contact-dependent induction of genes encoding secreted proteins in some TTSS-possessing bacteria (He 1998; Hueck 1998).

Symbiotic nodule development involves the exchange of a series of molecular signals between plants and bacteria. Association of plant-produced flavonoids with the *Rhizobium* activator NodD proteins leads to the synthesis of lipo-chito-oligosaccharide signal molecules (Nod-factors) that initiate early symbiotic events, such as root-hair deformation, initiation of cortical-cell division, and entry of the bacteria into the root-hair. Bacteria trapped in the curled root-hair then penetrate the root and multiply within a tubular structure, the infection thread, that conducts rhizobia to the dividing cortical cells. At this site, a process that resembles endocytosis mediates the release of bacteria into the cytoplasm of the host cells, where they differentiate into bacteroids and fix nitrogen (Broughton et al. 2000; Perret et al. 2000).

Interestingly, Nod-factor synthesis and TTSS-dependent protein secretion by NGR234 share common regulatory networks; they both require flavonoids and NodD1 (Viprey et al. 1998). In NGR234, TTSS genes are grouped within a 30-kb region of the symbiotic plasmid, pNGR234*a* (Freiberg et al. 1997). This TTSS cluster contains all nine genes conserved between different plant and animal pathogens. These conserved genes are thought to encode proteins that constitute the core secretion machinery (Bogdanove et al. 1996). In symbiotic bacteria, they are called *rhc* (for rhizobia conserved) and are followed by the letter of the homologous *ysc* gene of *Yersinia* species (Viprey et al. 1998). Other proteins shown to play a role in protein se-

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cretion are called tts. For NGR234, the last letter of the initial nomenclature will be conserved. Thus, y4xI, a transcriptional activator belonging to the two-component regulatory family and shown to be required for TTSS gene induction has been renamed *ttsI*. NodD1 is thought to bind to the *ttsI nod*-box, a *cis*-acting regulatory element present upstream of most flavonoid-induced genes. Inducers activate transcription of *ttsI* (H. Kobayashi, X. Perret, and W. J. Broughton, *unpublished data*), subsequently modulating the expression of other TTSS genes (Viprey et al. 1998).

Functionality of the NGR234 TTSS was demonstrated by mutating the *rhcN* gene, which encodes a protein with characteristics of an ATPase that is thought to energize the secretion machinery (Viprey et al. 1998). Abolition of TTSS-dependent protein secretion was previously shown to govern the interaction of NGR234 with two plant species, *Tephrosia vogelii* and *Pachyrhizus tuberosus*. Here, we report that the secreted proteins not only modulate nodule number on other legumes but also impair the symbiotic process on *Crotalaria juncea* roots, leading to the formation of nonfixing pseudonodules. Rhizobial proteins secreted via the TTSS apparatus are now called Nops (nodulation outer proteins) (Marie et al. 2001). As a consequence, known TTSS-dependent secreted proteins, such as NolX and y4xL, have been renamed NopX and NopL, respectively. In this study, we describe the effect of single mutations in *nopX* and *nopL* on nodule formation and protein secretion. A double mutant *nopX-nopL* was also generated and shown to produce a phenotype that differs from that of NGRΩ*rhcN* when inoculated onto *P. tuberosus*. This suggested that other Nops modulate the interaction of NGR234 with this host. We found that NGR234 secretes additional proteins via the Rhc complex. The smallest of these, with a molecular mass of 7 kDa, has been identified and designated NopA.

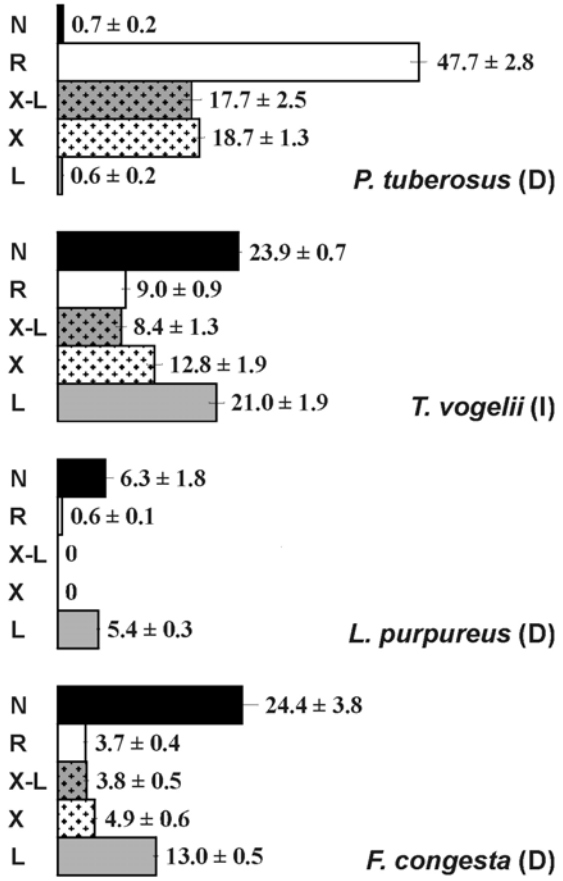


Fig. 1. Proteins secreted via the NGR234 type III secretion system modulate nodule number. Each bar represents the number of nodules obtained on roots of four different legume species after inoculation with NGR234 (N), NGRΩ*rhcN* (R), NGRΔ*nopX*Ω*nopL* (X-L), NGRΔ*nopX* (X), and NGRΩ*nopL* (L). Leguminous plants can form either determinate (D) or indeterminate (I) nodules that result from division of cells located in the outer or the inner cortex, respectively. Abolition of protein secretion led to a reduction in nodule number on plants forming either type of nodule (e.g., D for *Flemingia congesta* and I for *Tephrosia vogelii*). Similarly, Nops can have a deleterious effect on both plants that form determinate nodules (for *Pachyrhizus tuberosus*) and those that form indeterminate nodules (for *Crotalaria juncea*). On *P. tuberosus* roots, the number of nodules obtained with the *rhcN* mutant differed greatly from that observed with NGRΔ*nopX*Ω*nopL*, suggesting that other Nops govern the interaction of NGR234 with this plant species. Mutation of *nopL* moderately affected nodulation of most plant species, with the exception of *F. congesta* that formed fewer nodules than the plants inoculated with the wild-type strain. In contrast, deletion within *nopX* significantly affected nodule number on all plant species tested. Average numbers of nodules and standard errors of the means are indicated adjacent to the bars. Independent experiments were performed three times, using 10 to 15 plants in each case.

RESULTS

Abolition of Nop secretion affects the symbiotic process.

Rhizobium species NGR234 has a broad host range and is known to nodulate more than 112 genera of leguminous plants as well as the nonlegume *Parasponia andersonii* (Pueppke and Broughton 1999). Abolition of TTSS-dependent protein secretion produces one of three effects on nodulation: i) no change, ii) an increase, or iii) a decrease in nodule number, depending upon the plant tested (Viprey et al. 1998). To further study the function of Nops during the interaction, the phenotype of the wild-type NGR234 strain and the TTSS mutant (NGRΩ*rhcN*) was assessed on additional plant species. As observed with *T. vogelii*, the *rhcN* mutant formed fewer nodules than the wild-type strain on *Lablab purpureus* and *Flemingia congesta* roots (Fig. 1). On *C. juncea* roots, however, Nops appeared to have a drastic, deleterious effect on the symbiotic process. Whereas plants inoculated with the wild-type strain appeared nitrogen-starved, those grown in the presence of the TTSS mutant grew well. With NGR234, the nodulation process aborted after a few cell divisions, leading to the formation of nonfixing pseudonodules as well as rare clusters of nodules (1 to 2 per plant) (Fig. 2a). Plant roots inoculated with water were free of nodules or pseudonodules. In marked contrast, plants inoculated with the *rhcN* mutant formed many nitrogen-fixing nodules that were cylindrical in shape, a characteristic of indeterminate nodules (Fig. 2e). The persistent meristematic activity of this type of nodule allows the study of the different stages of infection to be followed. Electron micrographs of longitudinal sections of nodules obtained following inoculation with the *rhcN* mutant revealed normal nodule development from the meristematic and infection zones to the nitrogen-fixing cells (Fig. 2f). In this latter zone, the plant cytoplasm was filled with a large number of bacteroids, each individually enclosed in a peribacteroid membrane (Fig. 2g and h). In comparison, pseudo-nodules obtained with the wild-type strain were much smaller, were round in shape, and lacked the meristematic zone (Fig. 2b). Pseudonodules did, however, contain bacteria that seemed to have lysed and caused degradation of the plant cytoplasm (Fig. 2c and d). Thus, not only do Nops modulate nodule number either positively or negatively (on e.g., *F. congesta* and *P. tuberosus*, respectively), but they also impair nodule development on *C. juncea* plant roots.

Effect of mutations in *nopX* and *nopL* on nodule formation.

NopX and NopL were the first proteins shown to be secreted in an Rhc-dependent manner (Viprey et al. 1998). Genes lo-

cated within the TTSS cluster encode both proteins. Mutations were made in each gene to determine the role of NopX and NopL during symbiosis on a variety of plant species. The *nopL* mutant (NGRΩ*nopL*) had a phenotype similar to that of NGR234, when inoculated onto *P. tuberosus* and *C. juncea* roots (Fig. 1 and data not shown). On *T. vogelii* and *L. purpureus* plants, mutation of *nopL* led to a slight reduction in nodule number. This effect was more pronounced after inoculation of *F. congesta* plants. Thus, NopL seems to play a role in the symbiotic process in a restricted number of plant species.

To assess the function of NopX in symbiosis, a strain harboring a deletion in the coding sequence (NGRΔ*nopX*) was created. On *L. purpureus*, *T. vogelii*, and *F. congesta* (Fig. 1), the *nopX* mutant had a phenotype similar to that of NGRΩ*rhcN*. On *P. tuberosus* roots, however, the number of nodules obtained with NGRΔ*nopX* was intermediate between the wild-type strain and the *rhcN* mutant. In a similar manner, on *C. juncea*, the other plant that responds negatively to Nops, both nodules and pseudo-nodules were observed.

To determine whether NopX and NopL might act in synergy or if other secreted proteins could be involved in the interaction between NGR234 with its hosts, or both, a strain containing a mutation in both *nopX* and *nopL* was constructed. On all plants tested, the phenotype of NGRΔ*nopX*Ω*nopL* and NGRΔ*nopX* did not differ significantly, suggesting that these two Nops function independently. Interestingly however, the double mutant induces the formation of fewer nodules than the secretion mutant NGRΩ*rhcN* on *P. tuberosus* roots (Fig. 1). This result indicated that NGR234 secretes additional Nops that play an important role when NGR234 interacts with this plant species.

NGR234 secretes additional proteins via the TTSS.

To identify these unknown Nops, the extraction procedure described by Viprey and associates (1998) was modified to allow the identification of small proteins. After ammonium sulphate precipitation, proteins were desalted on Sephadex G25-containing columns (in place of the Microcon-10 cartridges that have a molecular mass cutoff of 10 kDa). To improve the resolution of low molecular mass proteins, samples were loaded onto 16% SDS–polyacrylamide gels (SDS-PAGE), while tricine replaced glycine as the trailing ion in the running buffer. Comparison of silver-stained proteins extracted from supernatants of the flavonoid-induced wild-type strain with those of the *rhcN* mutant revealed that at least five proteins were not secreted in the absence of a functional TTSS machine (Fig. 3A).

To detect low-abundance Nops, proteins were transferred to polyvinylidene difluoride (PVDF) membranes and were probed using a polyclonal antibody called SR-T. This antiserum was obtained by immunizing a rabbit with all of the proteins isolated from supernatants of induced cultures of *R. fredii* USDA257 (H. B. Krishnan and S. G. Pueppke, *unpublished data*), a rhizobial strain that is closely related to NGR234. This antibody revealed that both NGR234 and USDA257 secrete, in a flavonoid-dependent manner, proteins

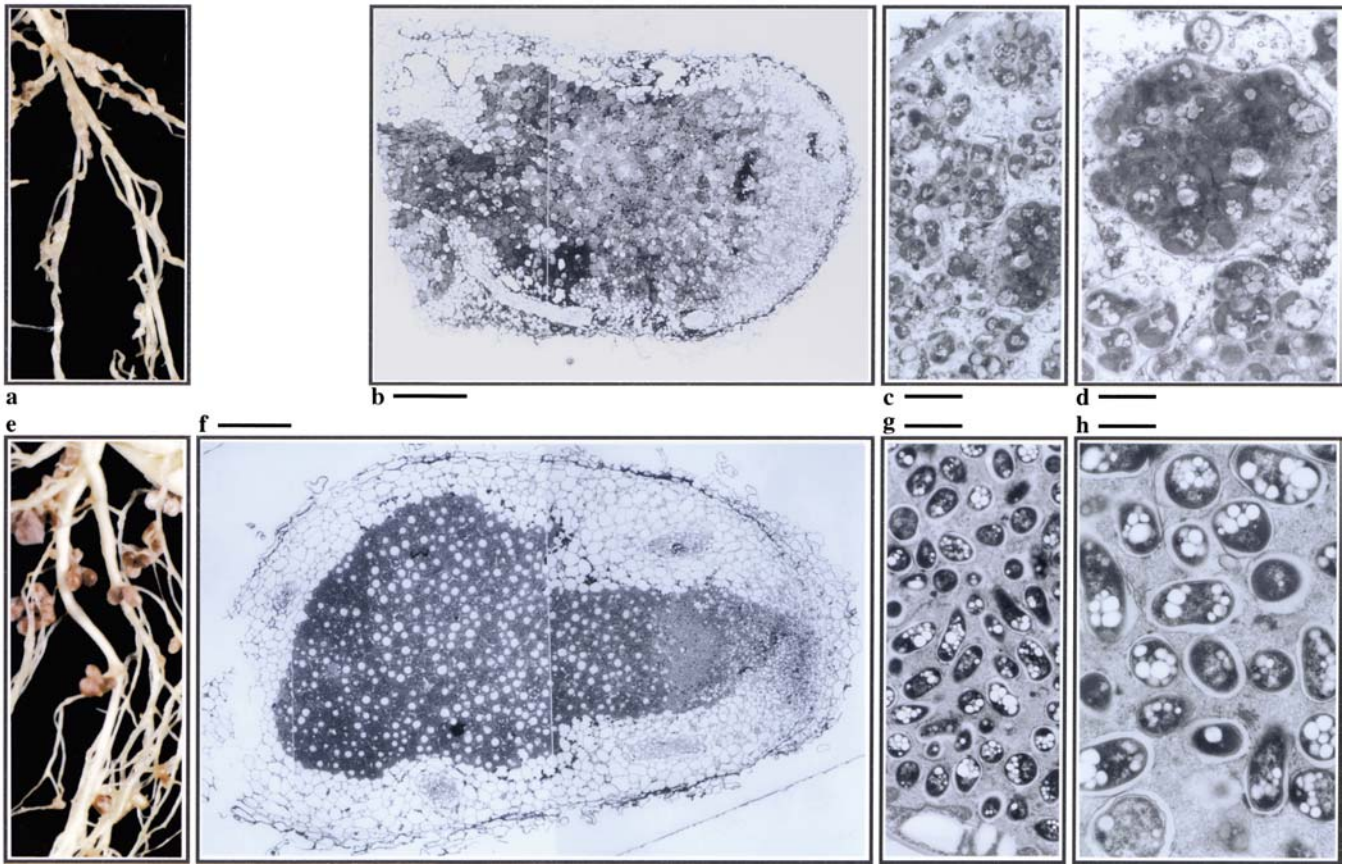


Fig. 2. Electron micrographs of longitudinal sections of pseudonodules and nodules induced by the wild-type NGR234 and the secretion mutant (NGRΩ*rhcN*) on *Crotalaria juncea* plant roots. Roots inoculated with the wild-type strain formed **a,** small nonfixing nodules that seemed to be devoid of **b,** meristematic cells. **c** and **d,** Cells of the infected zone seemed to be degraded and contained bacteroids that were dark and osmiophilic. **e,** In marked contrast, plants inoculated with NGRΩrhcN formed nitrogen-fixing indeterminate nodules. **f,** Longitudinal sections revealed that nodule development occurred normally from the meristematic and infection zones to the area in which bacteria differentiate into bacteroids. **g** and **h,** In this latter zone, symbiosomes appear to be healthy and functional. Scale bars: 250 μm for b and f; 2 μm for c and g; and 1 μm for d and h. Panel f is a composite of two pictures of the same nodule.

of similar size, with the exception of an approximately 36-kDa protein only found in cultures of the *R. fredii* strain (Fig. 3B). At least five proteins recognized by the SR-T antibody were not found in the growth medium of the *rhcN* mutant, indicating they are secreted via the TTSS. One of them is NopX, as deduced from the molecular mass of the protein and the absence of the corresponding band in extracts obtained from the *nopX* mutant (Fig. 4 and below).

Secretion of all of the Nops required the same regulatory elements, flavonoids, NodD1, and TtsI, whereas NodD2 was dispensable (Fig. 3A and B). In supernatants of the *nodD*2 mutant, the levels of Nops appeared to be slightly higher, however. Like NodD1, NodD2 belongs to the LysR family of transcriptional activators and was shown to repress the transcription of some nodulation genes after the initial flavonoid induction (Fellay et al. 1998). Surprisingly, introduction of *nodD*2 on a multiple copy-number plasmid (pRAF130) into the wild-type strain totally abolished Nop secretion (data not shown), suggesting that NodD2 may also repress the expression of TTSS genes.

Identification of NopA, a new 7-kDa TTSS-dependent secreted protein.

The amino acid sequence of Nop7 was analyzed by mass spectrometry after an in-gel trypsin digest of the protein. The determined sequence "AAVSAEATAR" is 100% identical to a stretch of amino acid residues found within a putative protein that has a molecular mass of 7 kDa (Fig. 5). The start codon of the open reading frame (ORF) is located 322 nucleotides upstream of y4yQ, which lies at one border of the NGR234 TTSS cluster. This small ORF was not annotated when pNGR234*a* was sequenced (Freiberg et al. 1997) and has now been designated NopA. To demonstrate that NopA is secreted via the Rhc complex, an antibody was raised against a synthesized peptide that matches the C-terminal end of the protein (Fig. 5). The anti-NopA antiserum detected a small protein in supernatants of induced cultures of NGR234 that was missing in extracts prepared from the *rhcN* mutant (Fig. 4), thus confirming the requirement of a functional TTSS for NopA to be secreted (Fig. 4).

BLAST searches of both amino acid and nucleotide databases revealed that NopA homologues are only present in the TTSS-containing rhizobial strains *R. fredii* USDA257, *M. loti* MAFF303099, and *B. japonicum* 110*spc*4. They are 99, 65, and 42% identical to the NGR234 NopA protein, respectively. The putative NopA protein of *B. japonicum* appears to be eight amino acids shorter, however (Fig. 5). In all three strains, *nopA* is located upstream of a gene homologous to y4yQ of NGR234. NopA counterparts were not found in either the completely sequenced genome of *R. meliloti* 1021 (Galibert et al. 2001) or the 502-kb symbiosis island of R7A, another strain of *M. loti* (Sullivan et al. 2002). Neither *R. meliloti* 1021 nor (the symbiosis island of) *M. loti* R7A have *rhc* homologues. It therefore seems as if the presence of genes encoding NopA is restricted to rhizobial strains possessing a TTSS.

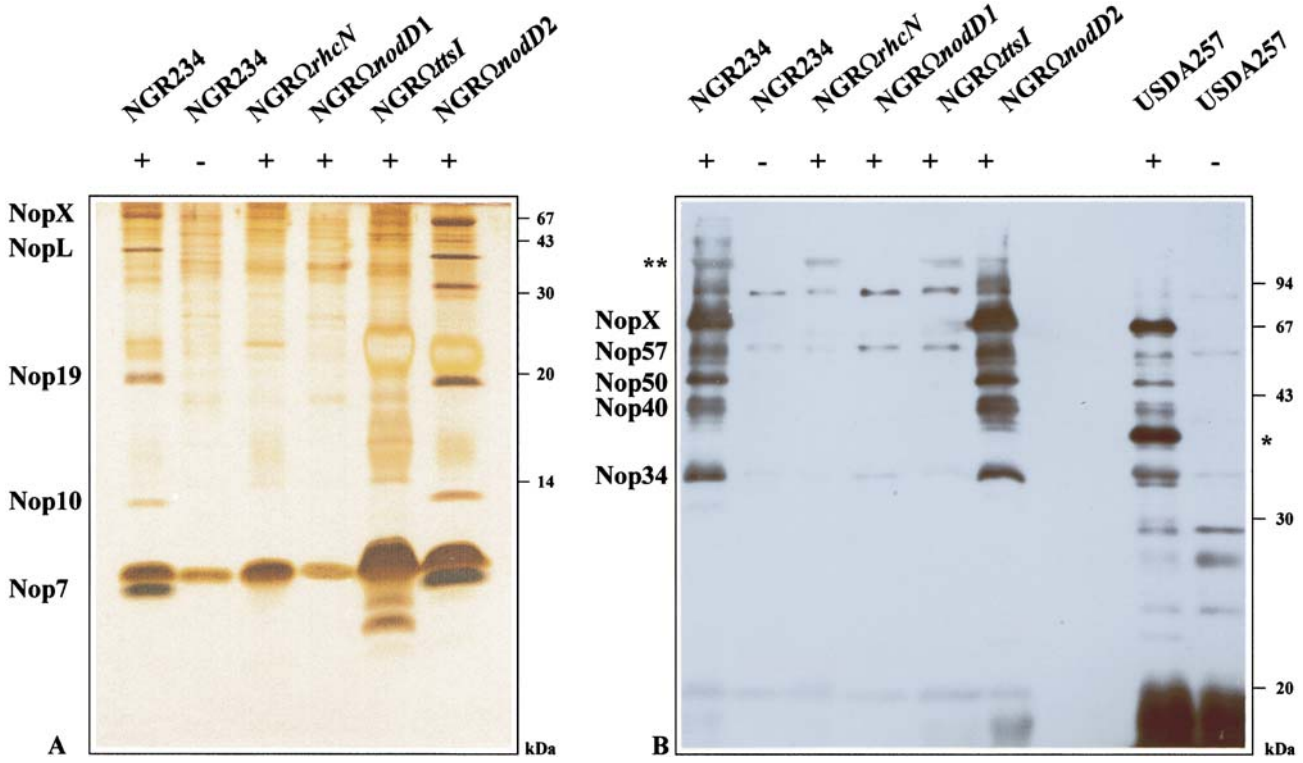


Fig. 3. Protein secretion by various *Rhizobium* strains. Proteins secreted by USDA257 and various derivatives of NGR234 grown in the presence (+) or the absence (–) of apigenin were loaded onto **A,** Tris-Tricine 16% sodium dodecyl sulfate-polyacrylamide gels (SDS-PA) or **B,** Tris-Glycine 12% SDS-PA gels. Silver staining revealed that NGR234 secretes at least five proteins in a flavonoid, NodD1-, TtsI-, and RhcN-dependent manner (low molecular weight bands observed with the *ttsI* mutant are not related to NopA; no signals were detected with the anti-NopA antiserum [data not shown]). Nop secretion appeared to be NodD2-independent, however. From their molecular masses, two bands were identified as NopX and NopL. **B,** To detect Nops produced in low amounts, proteins were separated by electrophoresis, electro-blotted onto PVDF membranes and immuno-stained using the polyclonal antibody SR-T. This serum recognized at least five proteins that require a functional type III secretion system to be secreted: NopX, Nop57, Nop50, Nop40, and Nop34. This antibody also revealed that NGR234 and USDA257 grown in the presence of apigenin, secrete, in a flavonoid-dependent manner, the same range of proteins, with the marked exception of P36 (*), which appeared only to be present in supernatants of the *R. fredii* strain. Interestingly, secretion of a high molecular mass protein (**) seems to be flavonoid- and NodD1-dependent but Rhc-independent, suggesting that another inducible secretion machine is functional in NGR234.

Are NopL and NopX required for protein secretion?

To classify NopX and NopL as effector, translocator, or external components of the secretion machine, we tested the requirement of these Nops for protein secretion, using four different polyclonal antibodies: SR-T, which recognizes many secreted proteins (as discussed above), as well as three Nop-specific antisera, anti-NopX, anti-NopL, and anti-NopA (discussed above and below). The latter three antibodies detected bands with molecular mass of 64, 37, and 7 kDa, respectively, in the growth medium of apigenin-induced NGR234 cells (Fig. 4). This is in good agreement with the predicted size of NopX, NopL, and NopA. No signals were observed with proteins isolated from supernatants of the *rhcN* mutant.

The *nopL* mutant secreted NopX and NopA, as well as all the Nops recognized by the SR-T antibody (Fig. 4). The anti-NopL antibody failed to detect a protein of 37 kDa in supernatants of NGRΩ*nopL*. Protein secretion similar to that seen with the wild-type strain was restored after introduction into the mutant of a plasmid carrying the coding sequence and upstream region of *nopL*, indicating that *nopL* is mono-cistronic. Thus, NopL does not appear to play any role in secretion of other Nops.

Similar experiments were carried out with NGRΔ*nopX*. Mutation of *nopX* abolished the secretion of the 64-kDa protein recognized by the anti-NopX antiserum but did not affect secretion of NopL and NopA. The SR-T antibody, however, failed to detect several proteins in supernatants of this strain. Downstream of *nopX* are several genes that are transcribed in the same orientation and are thought to be part of the same operon. Some of the proteins encoded by these genes (y4yB, 17 kDa; y4yA, 50 kDa; y4xP, 35 kDa; y4xO, 41 kDa; y4xN, 71 kDa; and y4xM, 42 kDa) have predicted molecular masses in the same range as that of Nop57, Nop50, or Nop40. To exclude the possibility that the lack of secretion of these three proteins by NGRΔ*nopX* was due to a polar effect of the omega interposon, the strain was complemented. Introduction of the *nopX* gene (expressed from its own promoter) into the mutant restored secretion of all the Nops recognized by the SR-T antibody (Fig. 4). It thus appears that expression of NopX is required for the secretion of a subset of Nops.

DISCUSSION

NGR234 nodulates at least 232 plant species belonging to 112 (Pueppke and Broughton 1999). Nodulation does not necessarily imply an efficient symbiosis, however, as nitrogen-fixing nodules are formed on only 59% of the NGR234 hosts. Proteins secreted via the TTSS appear to be one of the factors mediating efficient nodulation. On some plant species, such as *T. vogelii* or *F. congesta,* Nops are required for optimal nodulation. On other legumes, such as *P. tuberosus* and *C. juncea*, Nops appear to be deleterious. Similar negative effects of TTSS-dependent secreted proteins were also observed during the interaction of *R. fredii* USDA257 with some plant hosts. Whereas the wild-type strain induces the formation of nitrogen-fixing nodules exclusively on roots of primitive cultivars of soybean, inactivation of TTSS genes extends the host range of USDA257 to include advanced cultivars (Meinhardt et al. 1993). Thus, TTSS-dependent secreted proteins can both modulate nodule number and play a role in nodule development. Although USDA257 is closely related to NGR234, it has a narrower host range and nodulates an exact subset of plants that respond to NGR234 (Pueppke and Broughton 1999). This suggests that the two bacteria must produce some different symbiotic factors that govern their host specificity. The 36-kDa protein found exclusively in supernatants of induced cultures of USDA257 might be one of them.

Is NopX part of a type III translocon?

To assess the function of one of the TTSS-dependent secreted proteins, a *nopX* mutant was generated, and the effects of the mutation on protein secretion and nodule formation were tested. A mutation in *nopX* did not affect the secretion of NopL, NopA, and Nop34. The SR-T antibody, however, failed to detect a subset of Nops in the supernatant of NGRΔ*nopX.* The proteins missing in supernatants of NGRΔ*nopX* could therefore be secreted in a NopX-dependent manner or might be degradation products from NopX. Since the anti-NopX antibody, which was raised using peptides from the amino-terminus of the protein, does not detect Nop57, Nop50, or Nop40, the degradation would have to have occurred at the N-terminus after secretion. A stepwise loss of amino acids has been reported for PopA, a protein secreted in a TTSS-dependent manner by the plant pathogen *Ralstonia solanacearum.* In supernatants of the wild-type strain, two proteins, PopA2 and PopA3, appear to be truncated forms of PopA lacking the first 9 and 93 amino acids, respectively (Arlat et al. 1994). Determination of the amino acid sequence of the Nops missing in supernatants of the *nopX* mutant will allow this hypothesis to be tested.

NopX homologues were identified in *R. fredii* strains and *M. loti* MAFF303099 but were not found within the fully sequenced genome of *B. japonicum* 110*spc*4 (Kaneko et al. 2002). Interestingly, sequences similar to *nopX* are also present in plant pathogens, *hrpF* from *Xanthomonas* species (Huguet and Bonas 1997) as well as *popF1* and *popF2* in *R. solanacearum* (Salanoubat et al. 2002). The function of HrpF from *Xan-*

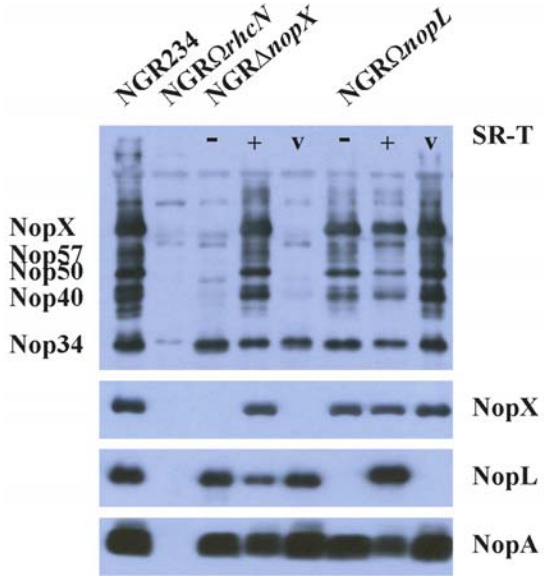


Fig. 4. Involvement of NopX and NopL in protein secretion. To determine whether NopX and NopL are required for the transit of Nops into the extracellular medium, secreted proteins were separated by electrophoresis on 12 or 16% (for NopA) sodium dodecyl sulfate-polyacrylamide gels, were transferred onto polyvinylidene difluoride membranes, and were immuno-detected, using four different antibodies: SR-T, anti-NopX, anti-NopL, and anti-NopA. All strains were grown in the presence of apigenin. Both p*nopX* or p*nopL* (+) were introduced into NGRΔ*nopX* and NGRΩ*nopL*, respectively. – indicates the strains contain no additional plasmids, and v indicates the vector with no insert. Supernatants of NGRΔ*nopX* contained NopL, Nop34, and NopA but lacked NopX and a subset of Nops. Introduction of *nopX* expressed from its own promoter into the mutant restored secretion of all proteins. In supernatants of NGRΩ*nopL*, only NopL was not detected. This suggests that the protein is not part of the secretion apparatus. Introduction of p*nopL,* which contains the promoter and coding sequence of *nopL*, into the mutant restored secretion of the protein.

thomonas campestris pv. *vesicatoria* has been characterized. It is a TTSS-dependent secreted protein, dispensable for protein secretion but required for pathogenicity of the bacterium (Rossier et al. 2000). The C-terminus of HrpF contains two hydrophobic domains that were shown by deletion analysis to be essential for function in planta (Büttner et al. 2002). HrpF is thought to be part of the translocon, a protein complex that mediates effector protein delivery across the host cell membrane. Hydrophobic domains were also identified in the NGR234 NopX amino acid sequence. If *Rhizobium* NopX homologues have a similar function to that of HrpF, the *nopX* mutant should have the same symbiotic phenotype as the *rhcN* mutant. This appears to be the case on most plant species tested. On *P. tuberosus* roots, however, NGRΔ*nopX* induces only half the number of nodules compared with that by the TTSS mutant. Thus, NopX is either not part of a translocon complex or, if it is, not all Nops are injected into the host-cell cytoplasm in a NopX-dependent manner. In *B. japonicum*, although the TTSS is required for optimal nodulation of *Macroptilium atropurpureum*, NopX homologues have not been identified, suggesting it is dispensable in this strain (Krause et al. 2002). Nevertheless, it is still possible that a protein with no obvious homology to NopX could play a similar role.

NopL—a putative effector protein required for optimal nodulation of *F. congesta*.

NopL is a hydrophilic protein that does not seem to be required for TTSS-dependent protein secretion. During symbiosis, mutation of *nopL* did not affect the interaction of NGR234 with *P. tuberosus* but significantly reduced the number of nodules on *F. congesta* roots. These results suggest that NopL could be an effector protein that interferes with a component of the signal transduction pathways mediating the nodulation process on some plant species. Database searches showed that NopL homologues were only present in two (*R. fredii* USDA257 and *B. japonicum* 110*spc*4) of the rhizobial strains that are known to contain a TTSS. Recent submission of the sequence of the *R. fredii* USDA257 TTSS cluster (NCBI database, accession number AF229441) revealed that this strain contains a predicted NopL homologue that differs from the NGR234 protein by only 15 of the 338 aa. The symbiosis island of *B. japonicum* 110*spc*4 contains a truncated gene (Göttfert et al. 2001) that encodes a protein lacking two long stretches of 91 and 86 aa located between amino acids 72 and 163 and at the C-terminus of the NGR234 sequence, respectively. None of the NopL homologues have a known biochemical function. NopL counterparts were not found in the fully sequenced genome of *M. loti* MAFF303099. These data are reminiscent of those found in plant pathogenic bacteria that share the same components of the secretion machinery but contain specific effector proteins involved in plant-bacterial recognition.

Function of NopA.

NopA is a small 7-kDa Rhc-dependent secreted protein that is present in all TTSS-containing rhizobia. This ubiquity suggests that NopA is an essential component of the secretion machinery or a key effector protein. Preliminary results indicate that NopA is required for the formation of pilus-like structures



Fig. 5. Alignments of NopA amino acid sequences from rhizobial strains. NopA homologues were only identified in rhizobial strains and were restricted to those containing *rhc* homologues: *Rhizobium* strain NGR234, *R. fredii* USDA257, *M. loti* MAFF303099, and *B. japonicum* 110*spc*4. "Seq" designates the amino acid sequence identified by mass spectrometry. "Ab" shows the part of the protein from which the synthetic peptide used to generate the anti-NopA antiserum was designed. Proteins were aligned using the ClustalW program and were manipulated with Boxshade at EMBnet. Dark and gray boxes indicate identical and similar amino acids, respectively.

Table 1. Bacterial strains and plasmids used in this study

Strains/plasmids	Characteristics	Reference / source
E. coli		
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	BRL, Bethesda, MD, U.S.A.
Rhizobium strains		
NGR234	Broad host-range bacterium isolated from nodules of *Lablab purpureus*, Rif^r	Stanley et al. 1988
NGRΩ*nodD*1	NGR234 derivative containing an Ω cassette inserted into the *Bam*HI site of *nodD*1, Rif^r, Sp^r	Relić et al. 1993
NGRΩ*nodD*2	NGR234 derivative containing an Ω cassette inserted into the *Bam*HI site of *nodD*2, Rif^r, Km^r	Fellay et al. 1998
NGRΩ*nopL*	NGR234 derivative containing an Ω cassette inserted into the *Eco*RV site of *nopL*, Rif^r, Km^r	This work
NGRΔ*nopX*	NGR234 derivative in which the 552-bp *Bam*HI internal fragment of *nopX* was replaced by an Ω cassette, Rif^r, Sp^r	This work
NGRΔ*nopX*Ω*nopL*	NGRΔ*nopX* derivative containing an Ω cassette inserted into the *Eco*RV site of *nopL*, Rif^r, Sp^r, Km^r	This work
NGRΩ*rhcN*	NGR234 derivative containing an Ω cassette inserted into the *Eco*RI site of *rhcN*, Rif^r, Sp^r	Viprey et al. 1998
NGRΩ*ttsI*	NGR234 derivative containing an Ω cassette inserted into the *Apa*I site of *ttsI*, Rif^r, Sp^r	Viprey et al. 1998
R. fredii USDA257	Broad host range strain isolated from *Glycine soja*, Km^r	Keyser et al. 1982
Plasmids		
pBluescript II KS+	High copy number ColE1-based phagemid, Ap^r	Stratagene, La Jolla, CA, U.S.A.
pKS-*nopX*	pBluescript KS+ derivative carrying a 3.1-kb *Hpa*I-*Not*I fragment containing the full-length *nopX* gene, Ap^r	This work
pLAFR-6	Broad host vector containing transcriptional terminators flanking cloning sites, Tc^r	D. Dahlbeck and B. Staskawicz, *unpublished data*
pnopL	pLAFR-6 derivative containing the full-length *nopL* gene, Tc^r	This work
pnopX	pLAFR-6 derivative containing the full-length *nopX* gene, Tc^r	This work
pRAF130	pBBR1MCS-5 derivative containing the full-length *nodD*2 gene, Ge^r	Fellay et al. 1998
pXB110	Lorist 2 derivative containing the y4xA to y4yP region of pNGR234*a*, Km^r	Perret et al. 1991

on the surface of induced NGR234 cells (W. J. Deakin and H. Krishnan, *unpublished data*). In an elegant experiment using pulsed expression of proteins combined with electron microscopy, pilus appendages were shown in *Pseudomonas syringae* to elongate from the tip by the addition of pilin subunits and to act as conduits for the long-distance translocation of effector proteins (Li et al. 2002). In *R. solanacearum* and *P. syringae*, pilin subunits are small proteins (HrpY, 7 kDa and HrpA, 10 kDa; respectively) that are secreted in a TTSS-dependent manner (Roine et al. 1997; Van Gijsegem et al. 2000). Although they do not exhibit any obvious sequence homology, they have similar functions. Given the small size of NopA, its ubiquity in all TTSS-containing symbiotic bacteria, and its possible role in pilus formation, it seems likely that, upon secretion, NopA could polymerize to form a conduit to make the junction between cells of the two symbiotic partners.

Conclusions.

First thought to be restricted to plant and animal pathogens, ORFs encoding components of TTSSs have now been identified in five rhizobial strains and have proven to be functional in NGR234, two *R. fredii* strains, and *B. japonicum* 110*spc*4 (de Lyra et al. 2000; Krause et al. 2002; Krishnan and Pueppke 1993; Viprey et al. 1998). Unlike TTSSs from plant pathogenic bacteria, the rhizobial secretion apparatus does not appear to be an absolute prerequisite for the microsymbionts to interact with their plant hosts. Many *Rhizobium* strains lack a TTSS and are still able to nodulate plant roots. Nevertheless, Nop secretion appears to govern nodule formation on some plant species. This study provides the first step in elucidating the role of individual Nops. Our current working hypothesis is summarized in Figure 6. NopX could be a component of the translocon, functioning primarily to transfer effector proteins into the plant cell cytoplasm. It is the effector Nops, of which NopL is the first to be characterized, that actually determine the plant response to the rhizobial TTSS. As more effector proteins are identified, we will need to determine whether they actually are injected into plant cells and, if so, into which cellular compartment. Our final goal is to understand how the Nops are perceived by various plant species and how they mediate symbiotic interactions.

MATERIALS AND METHODS

Molecular and microbiological techniques.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (Sambrook et al. 1989). *Rhizobium* strains were grown, at 27°C, in either complete (TY) (Beringer 1974) or minimal media (RMS, using succinate as a carbon source at a final concentration of 12 mM) (Broughton et al. 1986). Antibiotics were added to the media at the following final concentrations: ampicillin (Ap), 50μg ml^{-1}; gentamycin (Ge), 15 μg ml^{-1}; kanamycin (Km), 100 μg ml^{-1}; rifampicin (Rif), 100 μg ml^{-1}; spectinomycin (Sp), 50 μg ml^{-1}; and tetracycline (Tc), 10 μg ml^{-1}.

Standard molecular cloning techniques were carried out as described by Sambrook and associates (1989). To clone the *nopX* gene into the broad host range vector pLAFR-6, a 3.1-kb *Not*I-*Hpa*I fragment from pXB110 was first cloned into pBluescript KS+ (Stratagene, La Jolla, CA, U.S.A.) digested with *Not*I and *Sma*I. The resulting plasmid, called pKS-*nopX*, contains the 3′-end of *nolW, nopX*, and the 5′-end of y4yB. Then, the insert was released from pKS-*nopX*, using *Kpn*I and *Sac*I, and was ligated into pLAFR-6, digested with the same enzymes, to give p*nopX*. To obtain p*nopL*, the gene was first amplified by polymerase chain reaction (PCR) using Vent polymerase (Biolabs, Frankfurt am Main, Germany) and two primers with the following sequences: 5′-CGGGATCCGG-CCTGTGGCTGAAGGC-3′ (which binds 16 nucleotides upstream of the stop codon of y4xM) and 5′-TATCTAGATCA-AATGTCAAAATCCACCGA-3′ (which binds at the 3′ end of *nopL*). The PCR product was then cloned into the *Sma*I site of pBluescriptKS+ and was released by digesting the plasmid with *Bam*HI (site underlined in the primer sequence) and *Hin*dIII (from the multiple cloning site of the cloning vector). The resulting 1.3-kb fragment was ligated into pLAFR-6 digested with *Hin*dIII and *Bam*HI to give p*nopL*. Plasmids were mobilized from DH5α to *Rhizobium* strains by triparental matings using the helper plasmid pRK2013 (Figurski and Helinski 1979).

Mutagenesis of *nopX* and *nopL*.

To obtain NGRΩ*nopL*, a 2.5-kb *Xho*I-*Eco*RI fragment of pXB110 containing *nopL* was cloned into pBluescript KS+. An Ω interposon conferring resistance to kanamycin (Fellay et al. 1987) was inserted into the internal *Eco*RV site located immediately after the first ATG of *nopL*. The mutated gene was then

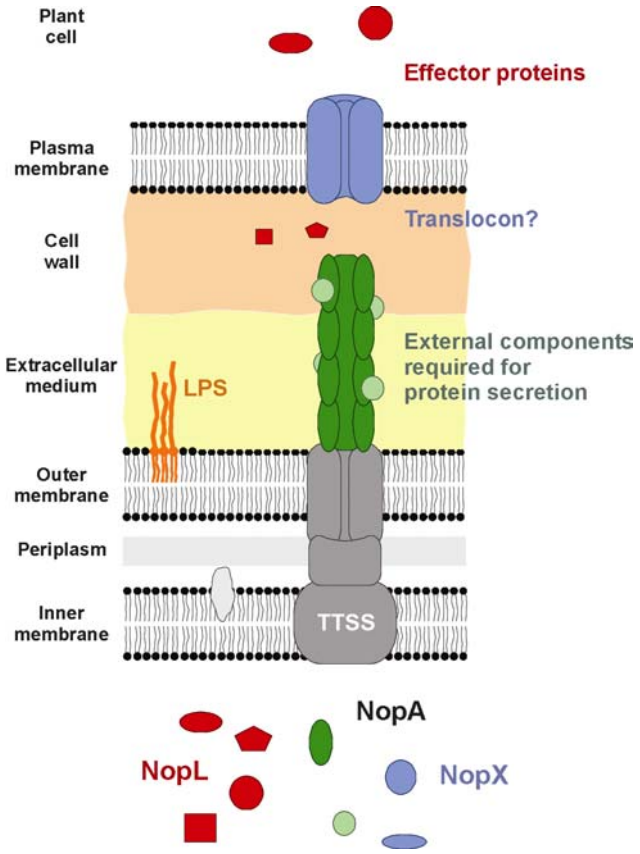


Fig. 6. Proposed model describing the putative function of NopX and NopL. Upon secretion through the type III secretion system (TTSS), proteins are directed toward different locations. Those that associate with components of the TTSS are required for the delivery of other secreted proteins into the extracellular environment. Others integrate into the host cell plasma membrane to form a type III translocon through which effector proteins transit into the host cells. During the interaction of NGR234 with its plant hosts, NopL does not appear to be required for secretion of other Nops; it is recognized by only a few plant species. We propose to classify NopL as an effector protein. We currently favor the hypothesis that Nop57, Nop50, and Nop40 are degradation products of NopX and that NopX is a component of the translocon. In the absence of NopX, the phenotype of the *rhcN* mutant differs from that of NGRΔ*nopX* (on *Pachyrhizus tuberosus*, for example). The absence of NopX probably reflects the effect of the external component of the TTSS or the nontranslocated Nops, or both, on the symbiotic process (Büttner and Bonas 2002).

subcloned into the suicide vector pJQ200SK (Quandt and Hynes 1993). Triparental matings were used to transfer the resulting plasmid into NGR234. Double recombination was selected by plating bacteria onto RMS plates containing 5% sucrose and the appropriate antibiotics. Putative mutants were confirmed by probing Southern blots of restricted genomic DNA using standard procedures (Chen and Kuo 1993; Sambrook et al. 1989). The mutant NGRΔ*nopX* was generated using a similar approach. To mutate the *nopX* gene, a 2.5-kb *Xba*I-*Sma*I fragment of pXB110 was first subcloned into pBluescript KS+. The 552-bp *Bam*HI internal fragment was then deleted and was replaced by an interposon conferring resistance to spectinomycin (Fellay et al. 1987). The double mutant NGRΔ*nopX*Ω*nopL* was obtained by introducing the mutated *nopL* gene into NGRΔ*nopX*.

Purification and analysis of secreted proteins.

Secreted proteins were prepared essentially as described by Viprey and associates (1998). Briefly, *Rhizobium* strains were grown in RMS for 40 h in the presence of the inducer apigenin (10^{-6} M final concentration) to a final optical density at 600 nm of approximately 0.8. Cell lysis did not occur under these conditions. NopX and NopL were detected in intracellular fractions of the *rhcN* mutant but not in supernatants of this strain. Cells were removed by two successive centrifugations at 5,000 × *g* for 30 min. To precipitate proteins, ammonium sulphate (60% final concentration) was added to the supernatants cleared of bacteria. Samples were desalted using Sephadex G-25-containing columns (Amersham Biosciences, Uppsala, Sweden). Proteins were separated by electrophoresis on SDS-PAGE and were stained with silver (Ausubel et al. 1991). To improve resolution of low molecular mass proteins, samples were loaded onto 16% Tricine-SDS-PAGE, prepared as described by Schägger and von Jagow (1987). For immunostaining, proteins were transferred from SDS-PAGE onto Millipore immobilon PVDF membranes by electroblotting, as described by Ausubel and associates (1991). Protein–primary antibody complexes were visualized using horseradish peroxidase-labeled anti-rabbit antibodies and ECL detection reagents (Amersham). Low range molecular mass markers from Amersham (94, 67, 43, 30, 20.1, and 14.4 kDa) and broad range prestained markers from BioRad (Hercules, CA, U.S.A.) (208, 115, 79.5, 49.5, 34.8, 28.3 20.4, and 7.2 kDa) were used as protein standards.

Antibodies.

The SR-T polyclonal antibody was obtained by injecting into a rabbit all proteins found in supernatants of induced cultures of *R. fredii* USDA257. To raise anti-NopL antiserum, NopL was expressed in *E. coli* as a fusion protein containing a 6x His-tag at the amino-terminal end. The approximately 1-kb *nopL* DNA sequence was amplified by PCR, using the primer pair 5′-GACTGGCGCCATGGATATCAATTCAACCAGC-3′ and 5′-TATCTAGATCAAATGTCAAAATCCACCGA-3′ and Vent polymerase (Biolabs). Then the PCR product was cloned into the expression vector pPROEX-1 (BRL, Bethesda, MD, U.S.A.) as an *Ehe*I-*Xba*I fragment (restriction sites underlined in the primer sequence). After purification by affinity chromatography, the 6xHis tag was removed by proteoloytic cleavage and the pure protein injected into two rabbits. Anti-NopX antiserum was generated by immunizing rabbits with two peptides (Eurogentec, Herstal, Belgium), N-LASREDLPPPDAEST and N-LEAKANDPSTPPDLK. The first amino acid of each peptide corresponds to amino acids 55 and 149 of NopX, respectively. To obtain the anti-NopA antiserum, a rabbit was immunized with the synthesized peptide (N-RSMLLRTVTTELQTTKKAADERVQ) that corresponds to the C-terminus of NopA (Sigma-Genosys, Cambridge, U.K.). Peptides were coupled to carrier proteins prior to immunization, according to the manufacturer's protocols. The anti-NopA and anti-NopL antisera detected each respective Nop monospecifically. As well as detecting NopX, the anti-NopX antibodies also cross-reacted with two smaller nonspecific bands in secreted protein extracts, which served as a useful internal loading control.

Plant material and assays.

Seed sources are listed in Pueppke and Broughton (1999). Nodulation tests were performed in Magenta jars as described by Viprey and associates (1998). Nodules were prepared for electron microscopy as detailed by Golinowski and associates (1987).

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