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Analysis of the C-terminal Secretion Signal of the *Rhizobium leguminosarum* Nodulation Protein NodO; a Potential System for the Secretion of Heterologous Proteins During Nodule Invasion

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We used deletions to analyze the domains required for secretion of the *Rhizobium leguminosarum* bv. *viciae* nodulation protein, NodO, by the *sec*-independent pathway. Deletion of the C-terminal 24 amino-acids (residues 261 to 284) reduced secretion by at least 95%. A monoclonal antibody that recognizes the C-terminal domain of NodO was used to identify four nested deletions that retained the C-terminal 24 residues of NodO but had lost up to 133 residues (amino acids 128 to 259); all four proteins were secreted into the growth medium with an efficiency between 50 and 90% of normal. A deleted derivative of NodO that retained residues 1 to 21 and 167 to 284 (and therefore lacked most of the N-terminal Ca^{2+}-binding domain) was secreted at around 80% of normal efficiency. Taken together, these observations indicate that the C-terminal 24 amino acids are sufficient for NodO secretion although the region adjacent to this domain appears to affect secretion efficiency. A derivative of the *Escherichia coli* alkaline phosphatase (*phoA*) gene was cloned into two derivatives of *nodO* such that PhoA (lacking the N-terminal transit peptide) was in-frame at both ends, with the C terminus fused to either the last 24 or 50 amino acids of NodO. These fusion proteins were secreted at 40 and 80% of the wild-type level, respectively, and the larger of the two retained alkaline phosphatase activity. A hybrid protein, containing *E. coli* β-glucuronidase (GUS) fused to the N terminus of NodO, was not secreted, and it reduced the levels of wild-type NodO secreted by *R. leguminosarum* bv. *viciae*. The nature of the NodO C-terminal secretion signal is discussed with regard to its use as a delivery system for heterologous proteins useful for investigating the Rhizobium-legume interaction.

Additional keywords: infection, symbiosis.

Significant progress has been made toward understanding signal transduction in the *Rhizobium*-legume symbiosis. A novel class of secreted plant morphogens, lipo-oligosaccharide signaling molecules, has been isolated from several rhizobia. These are oligomers of N-acetyl glucosamine carrying an N-linked acyl group on the terminal nonreducing sugar. Several substitutions, such as acyl and sulfate, determine the specificity of these signals (Dénarié and Cullimore 1993; Downie 1994; Spaink 1995). The proteins responsible for synthesis of these Nod factors are the products of the bacterial nodulation (*nod*) genes, which are induced by flavonoids released from legume roots.

NodO, a Ca^{2+}-binding protein produced by *Rhizobium leguminosarum* bv. *viciae* in response to flavonoids, is secreted and plays a role in nodulation (Economou et al. 1990, 1994). In the absence of the *nodFE* genes, which are required for the host-specific C18:4 acyl group (rather than a C18:1 group) to be present on the lipo-oligosaccharide Nod factor (Spaink et al. 1991), *nodO* is essential for nodulation of pea and vetch (Downie and Surin 1990). Introduction of the cloned *nodO* gene from *R. leguminosarum* bv. *viciae* into a *nodE* mutant of *R. leguminosarum* bv. *trifolii* enabled that strain to nodulate vetch, which is not normally a host for *R. leguminosarum* bv. *trifolii* (Economou et al. 1994). Purified NodO forms large, cation-selective channels in artificial bilayers, and it was proposed that its role in nodulation signaling may involve the formation of transmembrane channels in plant membranes (Sutton et al. 1994).

The sequence of NodO (Economou et al. 1990) suggested that it is related to the RTX-protein family (Welch 1990), which includes the following: α-hemolysins from *Escherichia coli* and other bacteria; cyclolysin from *Bordetella pertussis*; leukotoxin from *Pasteurella haemolytica*; proteases from *Erwinia chrysanthemi*, *Serratia marcescens*, and *Pseudomonas aeruginosa;* and lipases from *Pseudomonas fluorescens* and *Serratia marcescens*. All of these proteins possess a conserved domain consisting of a series of aspartate-rich nonapeptide repeats (Akatsuka et al. 1994; Delepelaire and Wandersman 1989; Duong et al. 1992, 1994; Ghigo and Wandersman 1992, 1994; Glaser et al. 1988; Gygi et al. 1990; Johnson et al. 1992; Nakahama et al. 1986) that are involved in Ca^{2+}-binding (Baumann 1994). Each of these proteins is secreted across both bacterial membranes into the culture medium by a mechanism that does not involve an N-terminal signal peptide and the *sec*-dependent secretion system. Sequencing of the N terminus of secreted NodO confirmed that no N-terminal proteolytic pro-

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cessing had occurred (Economou et al. 1990). NodO secretion can be mediated by genes (*hylBD* and *tolC* or *prtDEF*) responsible for the secretion of the other members of the RTX-protein family (Scheu et al. 1992). In addition, NodO could be secreted by all species of the *Rhizobiaceae* tested, indicating that this secretion system is highly conserved (Scheu et al. 1992).

The secretion signal of the known members of the RTX-protein family is located at the C terminus. The minimum length of the HlyA secretion signal has been described as 37, 46, 53, 60, and 62 amino acids by different authors, on the basis of deletion analysis (Jarchau et al. 1994; Kenny et al. 1991, 1992; Koronakis et al. 1989; Stanley et al. 1991) and on the secretion of chimeric proteins (Hess et al. 1990; Mackman et al. 1987). Four structures near the C termini have been proposed to play a role in secretion of RTX proteins: (i) a cluster of hydroxylated residues; (ii) a putative amphipathic helix; (iii) a cluster of charged residues; and (iv) a hydrophobic tail preceded by a negative charge (Ghigo and Wandersman 1994; Kenny et al. 1992; Koronakis et al. 1989). No single RTX protein, however, contains all of these components. The distribution of acidic residues near the C terminus of HlyA and cyclolysin is thought to play a crucial role in secretion (Kenny et al. 1994; Sebo and Ladant 1993). Three acidic motifs in cyclolysin (E/D X_{11} D X_{3-5} E/D X_{14} D) appear to operate as surrogate secretion signals if placed close to the C terminus. The distinctive Ca^{2+}-binding repeat domain does not appear to play a role in the secretion of RTX proteins, as its removal does not reduce secretion (Létoffé and Wandersman 1992), but it is required for the secretion of large heterologous protein domains by the C-terminal secretion signal (Kenny et al. 1991; Létoffé and Wandersman 1992).

It is clear that at sublytic levels, pore-forming proteins such as hemolysis can play a signaling role in animal cells (Konig et al. 1994). However, such a role for bacterial pore-forming proteins in plants has not yet been established. The ability of *nodO* genes to extend the nodulation range of different rhizobia (Economou et al. 1994; van Rhijn et al. 1996) together with the pore-forming capacity of NodO (Sutton et al. 1994) suggest a signaling role for NodO at the plant plasma membrane, possibly during infection thread growth. However, the plant cell wall might be anticipated to form a barrier limiting access of NodO to the membrane. To try to understand the interaction of NodO with plant cells, we have analyzed its secretion signal and the potential for secreting hybrid marker proteins with NodO as a carrier. Such an approach may enable us to target specific proteins to the infection thread matrix, a compartment that is normally very difficult to access.

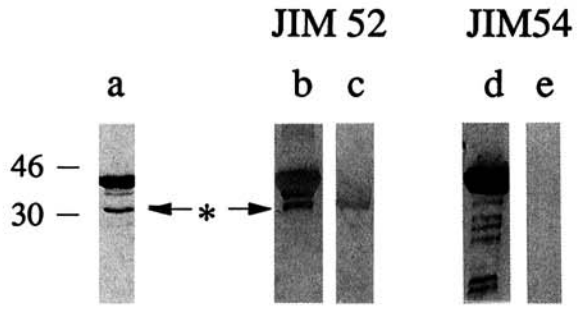

Fig. 1. Identification of JIM54, a monoclonal antibody that recognizes the C-terminal end of NodO. Partially purified NodO protein (lanes a, b, d) and cytoplasmic proteins from *Escherichia coli* DH5α/pIJ7093 (lanes c and e) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue (a), or transferred to nitrocellulose (b, c, d, e) and probed with supernatants from rat monoclonal cell lines. Antibody binding to Western blots (immunoblots) was detected with a secondary antibody conjugated to alkaline phosphatase, and stained with appropriate substrates. Two different monoclonal antibodies were identified: JIM52, which stained intact NodO, a degraded form of NodO lacking the C-terminal domain (asterisk), and the prematurely terminated NodO encoded by pIJ7093 (lane c); and JIM54, which recognized the intact form of NodO but not the protein encoded by pIJ7093.

RESULTS

Identification of a monoclonal antibody recognizing the C terminus of NodO.

During purification of NodO, three forms of the protein are often observed, with major bands at 42 kDa and 32 kDa, and a minor band of 38 kDa (Fig. 1, lane a). The N-terminal sequences of the first 15 residues of the 42- and 32-kDa proteins are identical, and correspond to the predicted sequence of NodO. Therefore, the smaller protein probably arose by loss of a C-terminal domain following proteolysis. To facilitate analysis of NodO secretion, monoclonal antibodies were screened for their ability to recognize the full-length NodO but not the 32-kDa protein. One monoclonal antibody (JIM52) recognized both forms of NodO (Fig. 1, lane b), whereas another (JIM54) recognized the full-length protein plus a series of smaller, degraded forms (Fig. 1, lane d). It appeared that none of the proteins recognized by JIM54 precisely corresponded to the 32-kDa species, indicating that JIM54 might recognize an epitope that includes the C-terminal region of NodO. The region recognized by JIM54 was further localized by inserting a translation terminating oligonucleotide into the unique *Sph*I site in *nodO* (at 1022 to 1027; Fig. 2) on pIJ1814. The resulting plasmid, pIJ7093, carries a derivative of *nodO* that encodes a protein lacking the C-terminal 24 amino acids. Immunostaining of *E. coli* extracts revealed that JIM54 did not recognize the mutant protein (Fig. 1, lane e) whereas JIM52 did (Fig. 1, lane c). Subsequent analysis with deletion and fusion derivatives of NodO, including the protein fusion encoded by pIJ7491 containing only the C-terminal 24 residues of NodO (see below), confirmed that the JIM54 epitope is wholly contained within these 24 residues.

Construction of deleted derivatives of NodO.

A series of internal deletions, all of which retained the 3′ end of the *nodO* gene, was generated with Exonuclease III (ExoIII) in conjunction with unique *Sac*II and *Sph*I sites (Fig. 2). Clones were screened for the production of "in-frame" deletions that retained the C terminus of NodO, by staining colony blots and Western blots of *E. coli* (containing deleted plasmids) with JIM54. Plasmids encoding deleted proteins of 28 to 38 kDa were selected and their DNA sequences determined (Fig. 2). The smallest deletion removed only the DNA (encoding amino acids 225 to 259) between the *Sph*I and *Sac*II sites. Further plasmids lacked the DNA encoding amino acids 189 to 259, 166 to 259, and 128 to 259, respectively (Fig. 2). All the deletions retained the first 127 amino acids encoding the Ca^{2+}-binding domain, and no stable deleted protein of smaller size was identified by this procedure. We constructed

a further deletion that encodes a derivative of NodO that lacks amino acids 22 to 166 (Fig. 2). A blot of cytoplasmic proteins from *E. coli* carrying this plasmid showed only a very faint band at around 20 kDa when stained with JIM54 (data not shown), suggesting that the protein is not stable in *E. coli*.

Analysis of NodO secretion in *Rhizobium*.

*Hin*dIII-*Xba*I fragments carrying the deleted *nodO* derivatives (Fig. 2) were subcloned into a broad host-range vector. These pML123 derivatives (pIJ7401, pIJ7465, pIJ7463, pIJ7467, pIJ7405, and pIJ7402; Fig. 2) were introduced into *R. leguminosarum* bv. *viciae* A169, which lacks a symbiotic plasmid (and hence the native *nodO* gene). Coomassie-blue staining of the concentrated culture supernatants revealed proteins of the appropriate sizes for all strains except that carrying pIJ7405. However, it was difficult to distinguish some of the proteins from background (Fig. 3A). Clearer results were obtained with immunostaining; all of the proteins containing internal deletions (pIJ7401, pIJ7402, pIJ7463, pIJ7465, and pIJ7467) could clearly be detected with JIM54, confirming that these proteins are secreted. When whole-cell extracts were similarly immunostained we did not detect any signal, indicating that these proteins were not cell associated (data not shown). The protein lacking the C-terminal 24 amino acids (pIJ7405) was almost undetectable in the concentrated culture supernatant (Fig. 3B, lane g). When a cell extract of A169/pIJ7405 was immunostained with JIM52, a weakly staining protein of the appropriate size was detected (data not shown). However, this protein did not appear to accumulate to high levels in the cells and in fact was present at levels very close to the detection limits of the immunostaining system. The simplest interpretation of these observations is that the C-terminal 24 residues (but not residues 22 to 259) of NodO are essential for its secretion, but when NodO is retained intracellularly in *R. leguminosarum* bv. *viciae* it is rapidly degraded. We have recently identified a *R. leguminosarum* bv. *viciae* mutant defective for NodO secretion. Even in a cell extract of this mutant, very little native NodO is found, suggesting that even the native protein is rapidly degraded if it is not secreted. (C. Finnie and J. A. Downie, personal communication.)

Attempts to quantitate the relative levels of different extracellular derivatives of NodO with immunoblotting of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-

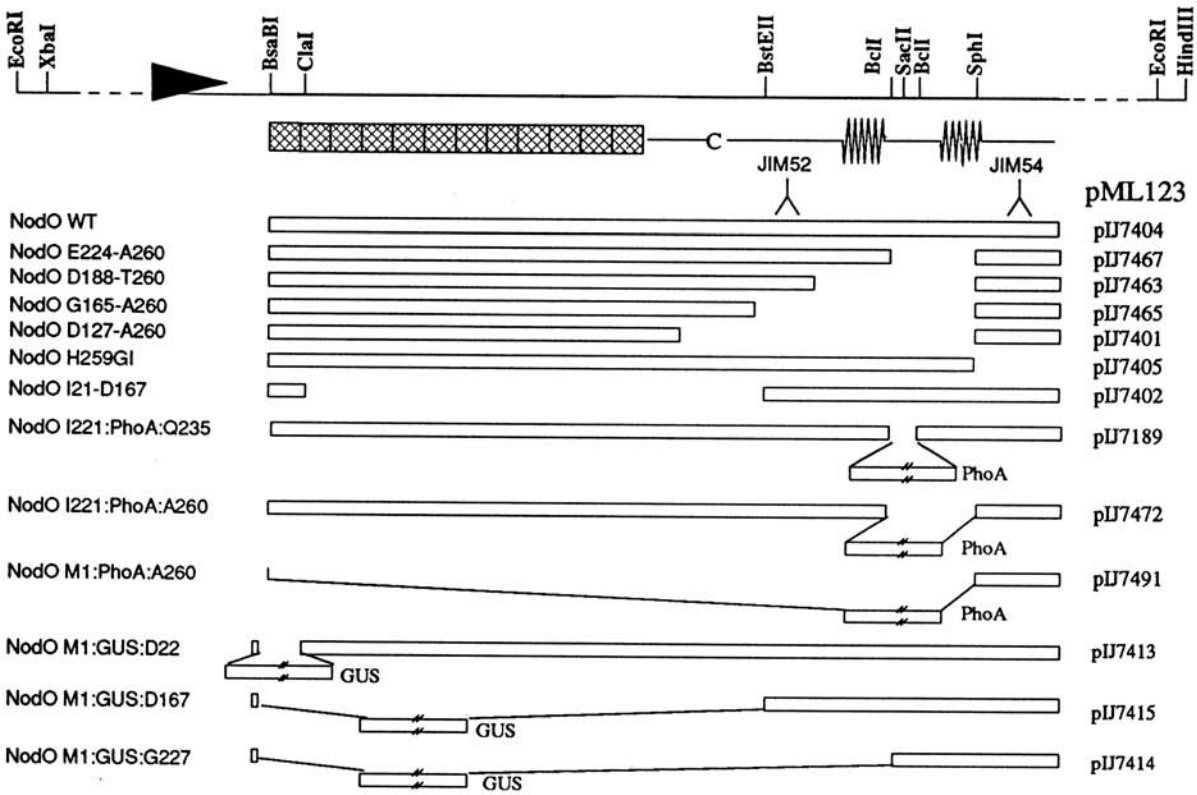

Fig. 2. Diagrammatic representation of deleted and fusion derivatives of NodO and plasmids encoding them. NodO protein is 284 amino acids long, contains a Ca^{2+}-binding domain of 12 nonapeptide repeats (indicated as hatched boxes) and is predicted to have two short α-helical regions (indicated by jagged lines), one hydrophobic and one amphipathic (nearer the C terminus). Restriction enzyme sites used in construction of NodO deletions and fusion proteins are shown; arrowhead indicates direction of transcription. Regions of proteins retained are shown as bars, and protein names define amino acid residues that form the protein fusion joint. "GI" in NodO H259GI refers to two amino acids added by the linker used to construct pIJ7093 in pUC18 (subcloned into pML123 to give pIJ7405). PhoA refers to alkaline phosphatase domain (lacking its N-terminal signal peptide) (Ehrmann et al. 1990) present in the sandwich fusions encoded by pIJ7189, pIJ7472, and pIJ7491, and GUS refers to the *uidA* gene product encoded by pIJ7413, pIJ7414, and pIJ7415. The various constructs were made in pUC18 and recloned as either *Eco*RI or *Hin*dIII-*Xba*I fragments into pML123 (as indicated) for experiments in *Rhizobium leguminosarum*. Approximate locations of epitopes recognized by monoclonal antibodies JIM52 and JIM54 are indicated, and were deduced from the observation that JIM54 recognizes all the proteins except NodOΔH259GI (Fig. 1) and JIM52 recognizes all proteins except NodOΔG165-A260, NodOΔD127-A260, and NodOMI:GusG227 (data not shown).

PAGE) gels were compromised by experimental variability, possibly due to differences in the relative recovery and/or stability of the deleted proteins. A simplified procedure in which culture supernatant was blotted directly onto nitrocellulose and quantified directly by immunostaining gave more reproducible results. When stained with JIM54, samples from A169 carrying pIJ7401 (NodOΔD127-A260), pIJ7465 (NodOΔG 165-A260), pIJ7463 (NodOΔD188-T260), pIJ7467 (NodOΔ E224-A260), or pIJ7402 (NodOΔI21-D167) showed signals that varied in intensity between 50 and 90% of the native NodO protein encoded by pIJ7404 (Fig. 4A). (JIM54 did not recognize the protein encoded by pIJ7405, as the JIM54 epitope contained within the last 24 amino acids is absent.) When protein from A169/pIJ7405 was stained with JIM52, the NodOΔH259GI protein was present at <3% of the wild-type NodO level. For comparison, data with pIJ7463 (NodOΔ D188-T260), pIJ7467 (NodOΔE224-A260), or pIJ7402 (Nod OΔI21-D167) stained with JIM52 are also shown. The amounts of protein present are similar to those detected with JIM54. Proteins from pIJ7401 (NodOΔD127-A260) and pIJ7465 (NodOΔG165-A260) do not stain with JIM52 because they lack the epitope.

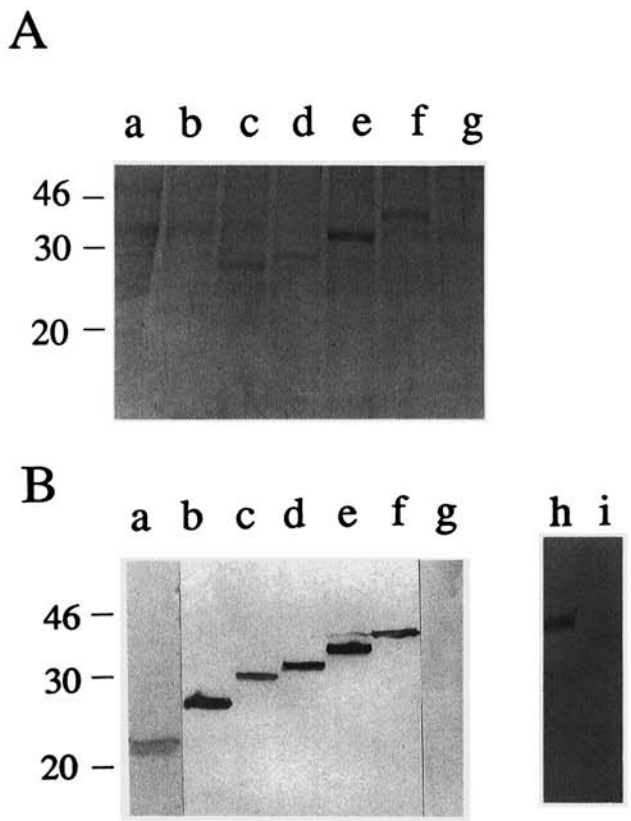

Fig. 3. C-terminal 24 residues of NodO are essential for its secretion by *Rhizobium leguminosarum*. Proteins present in growth-medium supernatant of *R. leguminosarum* A169 carrying (a) pIJ7402, (b) pIJ7401, (c) pIJ7465, (d) pIJ7463, (e) pIJ7467, (f) pIJ7404, and (g) pIJ7405 were concentrated and separated by sodium dodecyl sulfate. **A,** Gel stained with Coomassie blue. **B,** Proteins from a gel similar to **A** were transferred to nitrocellulose and visualized by immunostaining with JIM54, which recognizes C-terminal domain of NodO. Since this does not stain the protein encoded by pIJ7405, lanes h and i show results obtained following JIM52-dependent immunostaining of growth-medium supernatant of A169/pIJ7404 (wild-type NodO; h) and A169/pIJ7405 (NodOΔH259GI).

It was shown previously (Scheu et al. 1992) that NodO can be secreted from an *E. coli* strain carrying the hemolysin secretion genes *hlyBD,* and this was used to confirm secretion data in a different system. The various pUC18-based clones encoding deleted derivatives of NodO were transferred to *E. coli* MC4100 carrying the *hlyBD* genes. When the growth medium was analyzed for the deleted derivatives of NodO, results similar to those described for *R. leguminosarum* bv. *viciae* were seen. In all cases the secretion was dependent on *hlyBD* (data not shown). Analysis of cell extracts of the *E. coli* strains revealed NodO or the deletion derivatives to be present, even for those strains that secreted the protein at relatively high levels. We presume that accumulation of NodO occurs in the *E. coli* strains due to higher levels of expression from the pUC18 vector used. The observation that the relative levels of secretion by *E. coli* are similar to those seen for *R. leguminosarum* bv. *viciae* indicates that the lack of secretion of NodOΔH259GI (pIJ7405) and the lower levels of secretion of some of the other deleted NodO proteins are unlikely to be due to reduced translational efficiencies.

An active NodO-PhoA sandwich fusion secreted by *Rhizobium*.

A derivative of the *E. coli phoA* gene lacking translational start and stop codons and the DNA encoding the N-terminal

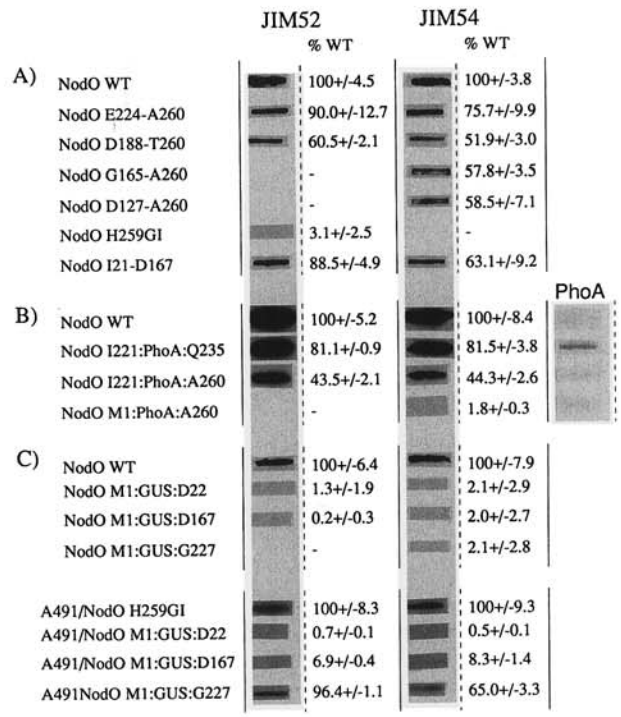

Fig. 4. Secretion of deleted proteins and protein fusions in *Rhizobium leguminosarum*. Culture supernatants equivalent to 1 ml of $OD_{600} = 0.25$ from exponentially growing cultures of *R. leguminosarum*, carrying plasmids encoding proteins indicated, were blotted directly onto nitrocellulose. Amount of NodO present in culture supernatants was calculated as a percentage of wild-type level of protein, with a scanner and an image analysis program, to determine levels of immunostaining detected with enhanced chemiluminescence. Values shown: averages of five separate experiments. In each case levels are expressed as a percentage relative to that seen with the wild-type *nodO*. Immuno blots in **B** were developed for a longer time than those in **A** or **C,** resulting in more intensive bands.

transit peptide was cloned in-frame into the region of *nodO* that was shown above to be nonessential for secretion. The *phoA* open reading frame was cloned as a 1.3-kb *Bam*HI fragment into a pair of *Bcl*I sites in pIJ1814 to make the NodO-PhoA-NodO sandwich fusion NodOΔI221:PhoA:Q235 (Fig. 2). This was confirmed to be in-frame by DNA sequencing and was recognized by the JIM54 antibody, which stained a protein of 100 kDa (the expected size of the fusion) in an extract of *E. coli* (data not shown). The *Hin*dIII-*Xba*I fragment was then recloned into pML123 to form pIJ7191, which was transferred into *R. leguminosarum* bv. *viciae*. Immunostaining with JIM52 and JIM54 revealed that the sandwich fusion protein is secreted at approximately 80% of the level of normal NodO (Fig. 4B). An identical blot was stained with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). This demonstrated that the culture supernatant contained alkaline phosphatase activity that was not present in the culture supernatant of *R. leguminosarum* bv. *viciae* lacking pIJ7191 (Fig. 4B). A Western blot of an 8% SDS-PAGE gel stained with BCIP/NBT confirmed that the major stained band was a protein of about 100 kDa, corresponding in size to the predicted fusion protein (data not shown). When *R. leguminosarum* bv. *viciae* carrying pIJ7191 was grown on plates containing BCIP a weak blue color was seen. However, this coloration was only seen in the agar around the colonies and not in the colony itself, suggesting the active form of the enzyme is only found outside the cells.

To generate a NodO-PhoA-NodO fusion protein comparable to the deleted derivatives of NodO retaining the last 24 amino acids, an internal deletion was constructed between an *Sph*I site near the end of *phoA* (equivalent to amino acid 426 of mature PhoA) and the *Sph*I site at the 3′ end of *nodO* to form pIJ7472, which encodes NodOΔI221:PhoA:A260 (Fig. 2). This fusion lacks alkaline phosphatase activity, as the C-terminal domain of PhoA is required for its activity (Kavanagh et al. 1988). The fusion protein is secreted; staining with JIM52 and JIM54 showed that the protein is present at around 40% of the level of normal NodO (Fig. 4B).

The role of the N-terminal Ca^{2+}-binding domain in the secretion of passenger proteins was addressed by constructing a further deletion of pIJ7472 between a *Bsa*BI site overlapping the translation start of *nodO* and an *Sma*I site at the start of *phoA* to form pIJ7491, which encodes NodOΔM1:PhoA:A260. This fusion protein containing only the C-terminal 24 residues of NodO could be detected in an *E. coli* cell extract with JIM54, confirming that the protein was expressed and that the JIM54 epitope is wholly contained within these 24 residues. However, JIM54 did not detect the fusion protein in culture supernatants from *R. leguminosarum* bv. *viciae* or *E. coli* (carrying *hlyBD*). This, taken together with the secretion of NodOΔI221:PhoA:A260, suggests that the N-terminal domain of NodO is required for the secretion of the PhoA passenger protein (Fig. 4B).

GUS fusions within *NodO* are not secreted by *Rhizobium*.

The *uidA* gene from *E. coli* was chosen to make fusions for two reasons: (i) to try to make a product that could be readily detected in plants if secreted; and (ii) to test if a protein that is folded in the cytoplasm could be exported. An *Nco*I site was introduced at the start of *nodO* on pIJ1814 by cloning a linker into the *Bsa*BI site that overlaps the translation start of *nodO* (pIJ7267). The *uidA* gene was then cut out as an *Nco*I to blunt fragment, with the blunt end created by S1 nuclease treatment of an *Xba*I site. This fragment was cloned into *Nco*I and either S1-nuclease-treated (blunt) *Cla*I (NodOΔM1:GUS:D22), *Bst*EII (NodOΔM1:GUS:D167), or *Sac*II (NodOΔM1:GUS:G227) sites in *nodO* on pIJ7267 (Fig. 2). Positive clones were confirmed by staining cell extracts with JIM54. The gene fusions were subcloned as *Eco*RI fragments into pML123 (pIJ7413, pIJ7415, and pIJ7414; Fig. 2) and expressed in *R. leguminosarum* bv. *viciae*. Colonies carrying the fusions showed β-glucuronidase (GUS) activity on plates supplemented with the substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc), indicating that the fusions are expressed and are relatively stable. No GUS activity was seen in the agar surrounding the colonies. Further, none of the fusion proteins was detectable in the supernatant by JIM52 or JIM54 (<2% of wild type; Fig. 4C). A NodO-GUS-NodO fusion analogous to the secreted NodO-PhoA-NodO fusion on pIJ7189 was created by subcloning the 5′ end of *nodO* on an 807-bp fragment immediately upstream of the *uidA* gene on pIJ7414. Colonies carrying the fusion protein retained β-glucuronidase activity on X-Gluc plates but no secreted proteins could be detected with the JIM52 or JIM54 antibodies (data not shown).

The lack of secretion of the GUS fusion proteins might be due to folding of the GUS proteins so that they cannot pass through the translocation apparatus, or to unusual folding that occludes the C-terminal secretion signal of NodO. If the latter, the fusions should have no effect on secretion of normal NodO from the same cell. However, in the former case it might be anticipated that the fusion protein would block export by jamming the secretion complex. To analyze inhibition of secretion, the fusion constructs were introduced into *R. leguminosarum* bv. *viciae* strain A491(*recA*), which carries a wild-type copy of *nodO* on the symbiotic plasmid.

Such competitive inhibition of NodO secretion is made complicated because the introduction of the *nodO* fusion genes on multicopy plasmids reduces the expression of the wild-type *nodO* gene on the symbiotic plasmid. This presumably occurs as a result of the *nodO* promoter (*nod*-box) on the multicopy plasmids titrating out the regulatory NodD protein. Hence, a reduction in extracellular wild-type NodO might be due to decreased levels of available NodD. Therefore, an appropriate control plasmid must be chosen that (i) does not interfere with NodO secretion and (ii) contains the *nodO* promoter on the multicopy plasmid. We found that pIJ7405 encoding the nonsecreted protein NodOΔH259GI was an appropriate control. As shown (Fig. 4) the NodOΔM1:GUS:D22 and NodOΔM1:GUS:D167 fusions inhibited secretion of the wild-type NodO protein to levels less that 10% of that observed with the NodOΔH259GI control. This indicates that these two GUS fusion proteins are sufficiently stable within the cells to block the secretion of the native NodO protein. However, expression of NodOΔM1:GUS:G227 was not as effective at inhibiting secretion of native NodO (Fig. 4). This suggests that the integrity of the secretion signal is more effectively maintained by a larger NodO domain (as in NodOΔM1:GUS:D22 and NodOΔM1:GUS:D167), and that the secretion signal is still accessible to the secretion machinery.

DISCUSSION

The lack of primary sequence similarities between members of the RTX-protein family has made the identification of the C-terminal secretion signal difficult. At 24 amino acids, the secretion signal of NodO is significantly shorter than that defined for HlyA (Hess et al. 1990; Kenny et al. 1992; Koronakis et al. 1989; Stanley et al. 1991), and similar to that defined for PrtG (27 amino acids; Ghigo and Wandersman 1994). Deletions within NodO have shown that this domain is essential and sufficient for secretion of NodO, although the adjacent domain appears to influence efficiency of secretion.

In the absence of primary sequence similarity within the C termini of RTX proteins, it is probable that secretion is determined by similar secondary structures. The structures of several members of the family have been determined (AprA, PrtSM, and PrtG), and all include a β-sheet at the C terminus of the protein in aqueous solution (Baumann 1994; Baumann et al. 1993; Wolff et al. 1994, Yin et al. 1995). In the presence of detergent micelles or trifluorethane, the PrtG C terminus undergoes a transition to an α-helical structure (Wolff et al. 1994), while the isolated C termini of both hemolysin and leukotoxin form a loop consisting of two short α-helical structures (Yin et al. 1995). A structural transition associated with secretion is observed for N-terminal signal peptides under similar conditions (Gierasch 1989; Reddy and Nagaraj 1989), suggesting that the role of the two secretion signals may be analogous in some regards. The predicted putative amphipathic α-helix within the C-terminal domain of NodO probably forms part of the NodO secretion signal, analogous to the α-helical domains of the other proteins, and this is shown, compared with the other secretion signals for which a structure has been proposed (Fig. 5).

Fig. 5. Alignment of C-terminal secretion signals of NodO constructs with HlyA and CyaA. Sequence of C-terminal 45 amino acids of NodO, internal deletions of NodO, and NodO:PhoA fusion are aligned with region of HlyA, CyaA, LktA, and PrtG proposed to form part of secretion signal (Sebo and Ladant 1993; Ghigo and Wandersman 1994; Yin et al. 1995). Predicted protein structure, based on algorithms of Chou and Fasman (1978) and Garnier et al. (1978), is shown beneath each protein: α represents α-helix, β shows β-sheet, and T represents turns. Underlining indicates where structure of these domains has been predicted by nuclear magnetic resonance (Yin et al. 1995) or circular dichroism measurements (Zhang et al. 1995). Acidic residues proposed to be important for HlyA/CyaA secretion are in underlined bold type, while their possible counterparts in NodO are in bold. Consensus 1 shows distribution of negatively charged residues based on NodO constructs, which can be fitted to the other RTX proteins shown. Consensus 2 shows distribution of acidic residues proposed by Sebo and Ladant (1993), which is not conserved in either wild-type NodO or in the NodO:PhoA sandwich fusion.

Sebo and Ladant (1993) proposed that the distribution of acidic residues at the C terminus of CyaA plays an important role in secretion. The consensus sequence E/D X_{11} D X_{3-5} E/D X_{14} D is conserved at the C terminus of HlyA, and the distribution of negative charges has been shown to be important in this case (Kenny et al. 1994). However, this motif is not strictly conserved in native NodO, or in the deleted derivatives. It is particularly badly conserved in the case of NodOΔ I221:PhoA:A260, which is still secreted at around 40% of the wild-type level. A similar motif (E/D $_{X21-23}$ E/D X_{13} E/D) can be fitted to all the secreted proteins, including the PhoA sandwich fusion, although the predicted structure outside the last 24 amino acids is variable (Fig. 5). This may be reflected by the 20 to 30% reduction in secretion caused by the deletion of an essentially α-helical domain (between amino acids 201 and 224) placed close to the C-terminal 24 amino acids in the deletion NodOΔE224-A260. The remaining fusions do not contain a predicted α-helical domain at the fusion junction and this may explain their lower secretion. The residual level of secretion (≈50%) may reflect a latitude within the RTX-protein secretion system, perhaps related to the small size of NodO compared with other members of the family.

The C terminus of NodO carries a hydrophobic tail preceded by a negative charge (EFGFI) similar to that described for proteases of the RTX family (Ghigo and Wandersman 1994). This hydrophobic domain may be involved in an early recognition event with the inner membrane secretion protein, perhaps following an initial insertion into the membrane. Such an event has been suggested for N-terminal signal peptides during *sec*-dependent secretion (Pugsley 1993). However, a similar motif is not found at the C terminus of HlyA.

The construction of PhoA and GUS protein fusions with NodO indicated that other constraints influence the secretion of RTX proteins. Others have described the secretion of chimeric proteins and the involvement of the repeat domain in secretion. Létoffé and Wandersman (1992) showed that the repeat domain is not required for secretion of short PrtB peptides, an observation in agreement with the results described here. However, the nonapeptide repeats were required for the secretion of large heterologous proteins by both the PrtB and HlyA signal sequence (Kenny et al. 1991; Létoffé and Wandersman 1992). The efficient secretion of PhoA by NodO in the case of NodOΔI221:PhoA:Q235 and NodOΔI221:PhoA:A260 probably reflects the lack of secondary structure within the PhoA domain, which is normally disulfide bridged by specific proteins in the periplasm (Akiyama and Ito 1993). The observation that alkaline phosphatase activity was found extracellularly was surprising in view of the observation that correct folding of PhoA normally requires a periplasmic enzyme, whereas secretion of proteins such as hemolysin and NodO is proposed to occur without a periplasmic intermediate

(Hughes et al. 1992). Possibly the alkaline phosphatase activity observed reflects only a somewhat low level of activity relative to the amount of fusion protein secreted; indeed, some alkaline phosphatase activity is observed with cytoplasmically located PhoA fusions (Bardwell et al. 1991) that are not exposed to the periplasmic enzyme. The failure of NodO to secrete the larger GUS fusions probably reflects the irreversible folding of the GUS protein in the cytoplasm, as judged by the GUS activity observed in the *R. leguminosarum* bv. *viciae* cells. The ability of the larger fusion proteins to compete with the wild-type NodO protein for a finite number of secretion complexes (as judged by the reduction in the secretion of wild-type NodO) indicates that the GUS domain does not simply occlude the secretion signal, but rather adopts a conformation that cannot be translocated across the bacterial membrane. This is in contrast to the apparent ability of GUS to cross membranes when coupled to other secretion signals (Kavanagh et al. 1988), and may be peculiar to this mode of secretion. The proposed involvement of the Ca^{2+}-binding domain in the maintenance of a secretion-competent state can perhaps be inferred from the loss of secretion blocking by the smaller GUS fusions that lack this domain.

The ability of the NodO to secrete PhoA suggests that the system might be used to investigate the interface between *Rhizobium* species and their host legumes, by coupling simple enzymatic domains or antibody epitopes to NodO. The existence of other RTX proteins that carry enzymatic functions (lipase, protease, and adenylate cyclase) demonstrates the potential for the secretion of carefully chosen fusion proteins by NodO, and such an approach might allow the analysis of an as yet poorly characterized environment that is inaccessible by other means. Further analysis of the secretion signal, constraints on the export of heterologous proteins, and the isolation of the genes involved in NodO secretion will facilitate this approach.

MATERIALS AND METHODS

Bacterial strains.

E. coli strain DH5α (Hanahan 1983) was used for routine manipulation, and was grown in Luria-Bertani (LB) broth (Miller 1972) supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) for the preparation of cytoplasmic proteins. Most experiments on export by *R. leguminosarum* bv. *viciae* were carried out with an exopolysaccharide-deficient strain, A169 (which lacks a wild-type copy of *nodO;* Scheu et al. 1992).

For competitive export experiments with two different *nodO* genes, a *recA*-deficient derivative of *R. leguminosarum* bv. *viciae* was made (A491). A kanamycin resistance cassette was cloned into the *Pst*I sites of the *R. leguminosarum* bv. *viciae recA* gene (from VF39; Selbitschka et al. 1991) carried on the suicide plasmid pSUP102 (Simon et al. 1983). The plasmid (pIJ7186) was introduced into a derivative of *R. leguminosarum* bv. *viciae* strain 8401(pRL1JI) (Downie et al. 1983), which carries a wild-type copy of *nodO* on the *sym* plasmid pRL1JI. Recombinants were selected for kanamycin resistance and screened for UV sensitivity. One such recombinant, strain A491, was confirmed to be recombination deficient by transduction with phage RL38.

For export experiments in *E. coli* the strain MC4100 was used, with or without pLG575 carrying *hlyBD* (Mackman et al. 1985) to supply the export functions.

Preparation of monoclonal antibodies.

Female Lou/c rats were inoculated with 200 μg of purified NodO protein (Sutton et al. 1994) at 30-day intervals over a period of approximately 120 days. Antibody titers were checked after the third injection and showed high levels of NodO-specific antibodies. A final injection was given intravenously 3 days before slaughter. The spleen of the inoculated rat was removed and spleen cells were mixed with myeloma cells (IR983F; Bazin 1982). Cell membranes were fused by the addition of polyethyleneglycol (PEG) (Galfre and Milstein 1981; Köhler and Milstein 1975), and aliquots of fused cell suspension plated into microtiter plates containing MRC5 feeder cells (Jacobs et al. 1970). Fused hybridomas were selected by growth in 2 HAT medium (100 μM hypoxanthine, 400 nM aminopterin, 16 μM thymidine; Sigma Chemical Co., St. Louis, MO) for 24 to 48 h, and surviving hybridomas cultured in 1 HAT medium.

Antibodies were screened for their ability to recognize NodO dot blot assay with 1 μg of purified NodO protein. Putative positive hybridomas were subcloned twice by limiting dilution into fresh medium to ensure that the antibodies were monoclonal.

Construction of deleted NodO lacking the last 24 amino acids.

A derivative of *nodO* was constructed by cloning an oligonucleotide (5′ GTATTTAATTAAATACCATG 3′) into the unique *Sph*I site of pIJ1814 (Scheu et al. 1992). The oligonucleotide was heated to 90°C, and cooled at a rate of 1°C/min to allow it to anneal to itself. The double-stranded linker, carrying *Sph*I adhesive ends that do not recreate an *Sph*I site after ligation, was cloned directly into pIJ1814. The ligation mixture was redigested with *Sph*I to linearize plasmids lacking this insert and then used to transform *E. coli* DH5α. The insertion and correct positioning of the linker were determined by sequencing the construct with an internal primer within *nodO*. This confirmed that the encoded protein would end at amino acid 259 of NodO with the additional amino acids GI added by the linker.

Construction of internal deletions within *nodO*.

Internal deletions within *nodO* were constructed with ExoIII essentially as described by Sambrook et al. (1989) with a Nested Deletion Kit (Promega, Madison, WI). DNA prepared from pIJ1814 was digested with *Sph*I (resistant to ExoIII), and *Sac*II (ExoIII-sensitive) for 2 h at 37°C. The linearized plasmid was digested with ExoIII over a period of 20 min with samples removed into stop solution at 2-min intervals. The deleted plasmids were treated with S1 nuclease for 30 min at room temperature and ligated overnight at 12°C with T4 DNA ligase. Religated plasmids from time points that showed appropriate sized deletions on an agarose gel were used to transform DH5α. Deletion of the DNA encoding amino acids 22 to 166 was carried out with the restriction enzymes *Bst*EII and *Cla*I followed by treatment with S1 nuclease and T4 DNA ligase as described above. All plasmids were sequenced to confirm the position of the deletions and are shown in Figure 2.

Construction of PhoA and GUS fusions.

The *phoA* sandwich vector pSWFII (Ehrmann et al. 1990) was modified to allow the cloning of *phoA* into *nodO* on pIJ1814. Two oligonucleotides (5′ GTTACGAGGATCCAG CT 3′ and 5′ GGATCCTC 3′) were annealed together and ligated into the *Bst*EII and *Sac*I sites at the 3′ end of *phoA*, thus creating a *Bam*HI site. The *phoA* gene was then cloned as a single *Bam*HI fragment into a pair of *Bcl*I sites within *nodO* on pIJ1814 such that both the 5′ and 3′ ends of *phoA* were in-frame with the *nodO* gene. An *Sph*I deletion between a site equivalent to amino acid 426 of mature PhoA and the *Sph*I site in *nodO* (equivalent to amino acid 260) was constructed by simple enzyme digest. Similarly, a deletion between a *Bsa*BI site overlapping the start codon of *nodO* and an *Sma*I site at the start of *phoA* was constructed. The correct construction of all these *phoA* fusions was confirmed by antibody staining and DNA sequencing. The gene fusions are shown schematically in Figure 2.

An *Nco*I linker (5′ CCATGG 3′) was annealed together and cloned into a *Bsa*BI site that overlaps the translation start of NodO on pIJ1814, to give the plasmid pIJ7267. The *uidA* gene from *E. coli* was then cut out as a fragment containing an *Nco*I site at its 5′ end and an S1-nuclease-treated *Xba*I site (blunt end) at its 3′ end. This was subcloned into pIJ7267, between the introduced *Nco*I site of *nodO* and S1-nuclease-treated *Cla*I (NodOΔM1:GUS:D22), *Bst*EII (NodOΔM1:GUS:D167), or *Sac*II (NodOΔM1:GUS:G227). The correct insertion of the *uidA* gene was confirmed by DNA sequencing (Fig. 2), and JIM54 antibody was used to confirm the production and correct size of the fusion proteins.

Colony blot analysis of deleted clones.

E. coli transformants carrying either putative deletions of *nodO* or fusion constructs were picked onto nitrocellulose filters and grown overnight on LB agar (Miller 1972) supplemented with carbenicillin and IPTG. Colonies were lysed with chloroform and incubated in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin and lysozyme at 40 mg/ml. In-frame fusion proteins were detected with the monoclonal antibody JIM54 (1% vol/vol) and anti-rat peroxidase secondary antibody (0.1% vol/vol, Sigma), to identify constructs that expressed the last 24 amino acids, containing the JIM54 epitope.

Preparation of cytoplasmic proteins.

During the construction of internally deleted derivatives of *nodO*, cytoplasmic proteins were prepared from DH5α carrying the deleted *nodO* genes in pUC18. Cells were grown to stationary phase in LB broth containing carbenicillin, and were subcultured (1:20) into fresh medium supplemented with IPTG. After 4 h growth at 37°C the cells were collected by centrifugation and lysed with an MSE Soniprep sonicator. Proteins were separated by discontinuous SDS-PAGE, and transferred to nitrocellulose with Tris-glycine-methanol transfer buffer (Towbin et al. 1979). Staining was carried out with monoclonal antibodies at a final concentration of 1% (vol/vol) and alkaline-phosphatase conjugated anti-rat secondary antibody (Sigma) at 0.1% (vol/vol) essentially as described by Bradley et al. (1986).

Assay of NodO secretion in *Rhizobium*.

A derivative of pML123 (Labes et al. 1990) carrying the blue-white cloning system from pUC18 was constructed to allow the cloning of the *nodO* constructs into a broad host-range vector. The *lac* region containing the polylinker from pUC18 was cut out as an *Hae*II fragment, which was blunt-ended with S1 nuclease. The polylinker from pML123 was removed as an *Eco*RI fragment, the *Eco*RI ends of the vector were blunted with S1 nuclease, and the *lac* region ligated into pML123 vector. The resultant plasmid (pIJ7395), which showed β-galactosidase activity on LB agar plates supplemented with IPTG (20 μg/ml) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal, 40 μg/ml), was checked by restriction enzyme digests.

The genes encoding NodOΔM1:GUS:D22, NodOΔM1:GUS:D167, and NodOΔM1:GUS:G227 were subcloned as *Eco*RI fragments into pIJ7395, while the remaining genes were subcloned as *Xba*I-*Hin*dIII fragments to give the plasmids shown in Figure 2. The plasmids were introduced into *R. leguminosarum* bv. *viciae* strains by triparental mating (Figurski and Helinski 1979). The strains were grown in 10 ml of TY complete medium (Beringer 1974) plus gentamicin (10 mg/ml) to stationary phase and then subcultured (1:200) into 100 ml of TY. At $OD_{600} \approx 0.5$, the cells were harvested by centrifugation, and the supernatant was passed through a nitrocellulose filter (0.2 μm Whatman). Bound proteins were released by boiling the filters in SDS-PAGE loading buffer, and a sample equivalent to material isolated from 1 ml of a culture at $OD_{600} = 0.25$ was loaded onto an SDS-PAGE gel. Secreted NodO proteins were detected on a Western blot as described above.

In the simplified protocol, culture supernatant equivalent to 1 ml of $OD_{600} = 0.25$ was passed through a 0.22-μm nitrocellulose filter in a slot blot apparatus (BioRad, Hercules, CA). The blots were immunostained with a 1% solution of each monoclonal antibody supernatant in 5% skimmed milk made up in TBS. A 0.1% solution of anti-rat peroxidase and an enhanced chemiluminescent substrate (ECL; Amersham, Little Chalfont Bucks, UK) were used to detect bound antibody. The relative intensity of the bands was determined by scanning the film with a Mirror flat bed scanner and quantitated with the Fuji MacBas Bio-Imaging analysis program (Fuji Photo Film, London, UK). Five separate experiments were carried out for each construct and the average values are presented (± standard error).

Alkaline phosphatase activity was detected by direct staining of slot or Western blots with 0.15 M Tris buffer, pH 9.6, containing 5-bromo 4-chloro indolyl phosphate and nitroblue tetrazolium at 50 and 100 μg/ml, respectively.

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