

# The *Rhizobium* nodulation gene *nodO* encodes a $\text{Ca}^{2+}$ -binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins

A.Economou, W.D.O.Hamilton<sup>1</sup>,  
A.W.B.Johnston<sup>2</sup> and J.A.Downie

John Innes Institute, Colney Lane, Norwich NR4 7UH and  
<sup>1</sup>Agricultural Genetics Company, c/o Cambridge Laboratory, AFRC  
Institute of Plant Science Research, Maris Lane, Cambridge CB2 2JB,  
UK

<sup>2</sup>Present address: School of Biological Sciences, University of East  
Anglia, Norwich NR4 7TJ, UK

Communicated by D.Hopwood

**Nodulation and host-specific recognition of legumes such as peas and *Vicia* spp. are encoded by the nodulation (*nod*) genes of *Rhizobium leguminosarum* biovar *viciae*. One of these genes, *nodO*, has been shown to encode an exported protein that contains a multiple tandem repeat of a nine amino acid domain. This domain was found to be homologous to repeated sequences in a group of bacterial exported proteins that includes haemolysin, cyclolysin, leukotoxin and two proteases. These proteins are secreted by a mechanism that does not involve an N-terminal signal peptide. The NodO protein is present in the growth medium of *Rhizobium* bacteria induced for *nod* gene expression, and partial protein sequencing of the purified protein showed that there is no N-terminal cleavage of the exported protein. It has been suggested that the internally repeated domain of haemolysin may be involved in  $\text{Ca}^{2+}$ -mediated binding to erythrocytes and we show that the NodO protein can bind  $^{45}\text{Ca}^{2+}$ . It is proposed that the NodO protein may interact directly with plant root cells in a  $\text{Ca}^{2+}$ -dependent way, thereby mediating an early stage in the recognition that occurs between *Rhizobium* and its host legume.**

**Key words:**  $\text{Ca}^{2+}$  binding/haemolysin/nodulation/*Rhizobium*

## Introduction

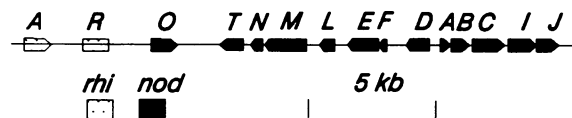
In soil bacteria comprising the 'rhizobia', which include the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, many nodulation (*nod*) genes are needed for infection of legumes, and in the genus *Rhizobium*, such genes are on 'symbiotic' (Sym) plasmids (Downie and Johnston, 1988; Long, 1989). In *Rhizobium leguminosarum* biovar *viciae* (referred to here as *R. leguminosarum*), which nodulates peas, vetches and lentils, several *nod* genes are involved in forming a series of signals that are required to initiate the infection events that lead to nodule formation. The first observed plant response in the interaction is the induction of curled root hairs caused by a soluble *Rhizobium*-made molecule (Bhuvaneshwari and Solheim, 1985; Zaat *et al.*, 1987). If normal recognition occurs, infection threads develop within root hairs and the rhizobia grow along this plant-made structure into the developing nodule meristem in the root cortex. We have identified 13 *nod* genes, arranged in five operons, *nodABCIIJ*,

*nodD*, *nodFEL*, *nodMNT* and *nolR* (*nodO*; see below) on the Sym plasmid pRL1JI (Figure 1), and *nod* genes similar in sequence and function to many of those on pRL1JI exist in other rhizobia (Long, 1989). Mutations in some *R. leguminosarum* *nod* genes (*nodA*, *B*, *C* and *D*) abolish nodulation and the induction of the early steps in the infection process, such as root hair curling. To a greater or lesser degree, mutations in the other *nod* genes delay or reduce nodulation on at least some species of the legume hosts of *R. leguminosarum*.

Expression of the nodulation genes is under the control of the *nodD* gene product which acts as a transcriptional activator in *Rhizobium* bacteria grown in the presence of flavonoids, which are secreted from legume roots. Part of the promoter region of the flavonoid-inducible *nod* genes consists of a highly conserved DNA sequence (*nod*-box) that is involved in *nod* gene expression (Rostas *et al.*, 1986; Shearman *et al.*, 1986. Spaink *et al.*, 1987). Oligonucleotides homologous to this sequence have been used to identify *nod* operons by DNA hybridization, and in *R. leguminosarum*, four *nod*-box sequences have been identified (Rostas *et al.*, 1986) which precede the four inducible *nod* operons shown in Figure 1.

The nodulation genes to the left of *nodD* in Figure 1 are involved in the highly specific recognition that occurs between *R. leguminosarum* and its host legumes (Surin and Downie, 1989), but, somewhat surprisingly, insertional mutations in those genes do not totally block nodulation. This indicates that host recognition is not mediated by a single event but is probably composed of at least two events. In the absence of one (set of) gene(s) nodulation can proceed via the other pathway, sometimes at a reduced efficiency.

In view of the intimate cell-to-cell interactions that occur between *Rhizobium* and legume root cells during infection, it has seemed likely that some *nod* gene products may be exported proteins. Indeed, the *nodC* gene product has been shown to span the inner and outer membranes of *Rhizobium* (John *et al.*, 1988). de Maagd *et al.* (1988, 1989) identified a flavonoid-inducible protein of  $M_r$  50 000 secreted by *R. leguminosarum* and showed that in some genetic backgrounds, mutations affecting the formation of this



**Fig. 1.** Map of the nodulation region of *R. leguminosarum*. The black arrows represent nodulation (*nod*) genes in the operons *nodD*, *nodABCIIJ*, *nodFEL*, *nodMNT* and *nodO* present on the symbiotic plasmid pRL1JI from biovar *viciae*. The map is based on the work of Shearman *et al.* (1986), Rossen *et al.* (1984), Evans and Downie (1986), Surin and Downie (1988), Surin *et al.* (1990) and work described here. The rhizosphere-expressed genes (*rhi*) shown as open arrows are based on the work of Dibb *et al.* (1984) and Economou *et al.* (1989).

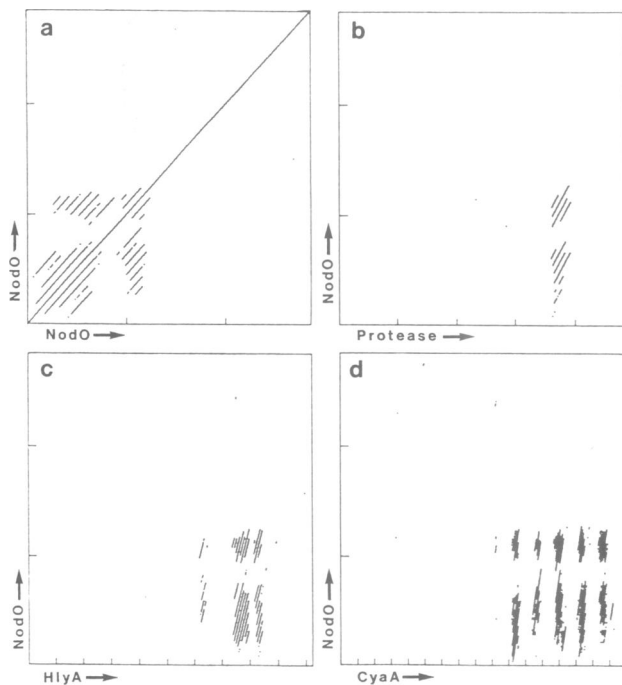


characteristic of bacterial secreted proteins described below, since they contain no cysteine residues.

### The NodO protein is homologous to haemolysin and other exported proteins

The NodO protein sequence was used to search a translation of the EMBL DNA sequence database using the protein homology search program TFASTA (Pearson and Lipman, 1988). Five significant matches were found: haemolysin, encoded by the *hlyA* gene in *E. coli*; cyclolysin, a multi-functional protein carrying adenylate cyclase and haemolytic activity, encoded by the *cyaA* gene in *Bordetella pertussis*; leukotoxin, encoded by the *lktA* gene in *Pasteurella haemolytica*; and two metalloproteases, one found in a *Serratia* species and the other in *Erwinia chrysanthemi*. These proteins are all secreted into the growth medium by a mechanism that does not require a cleaved N-terminal leader peptide, but does require other bacterial genes for their export (see Discussion). Figure 3 shows a DIAGON (Staden, 1982) comparison of the NodO protein sequence with itself and with the protein sequences of three of the homologous proteins. The N-terminal region of NodO consists of two major domains of internal repeats (Figures 2 and 3a).

It is this region that is homologous to haemolysin, cyclolysin and the *Serratia* protease (Figure 3b–d). In the *Serratia* protease sequence, one domain of repeats is seen (Figure 3b) and a similar pattern was observed with the *Erwinia* protease. With haemolysin and cyclolysin, two and five domains respectively can be seen (Figure 3c and d). The homologous domains are all in glycine/aspartic acid-



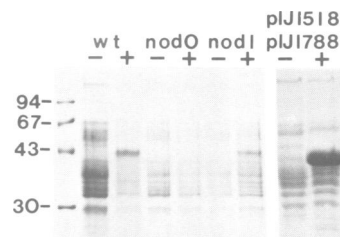
**Fig. 3.** DIAGON comparison of NodO with related proteins. The NodO protein was compared with: (a) NodO; (b) an exported protease from *Serratia* (Nakahama *et al.*, 1986); (c) haemolysin (HlyA) from *E. coli* (Felmlee *et al.*, 1985); (d) cyclolysin (CyaA) from *Bordetella pertussis* (Glaser *et al.*, 1988b), using the DIAGON protein comparison program (Staden, 1982) with a span length of 29 and a proportional score of 325. The marker bars on the axes indicate 100 amino acid residues. Since the other proteins are considerably larger than NodO, the lines of homology are not on the diagonal.

rich regions; in one stretch of 32 amino acids in NodO, 17 are glycine or aspartic acid. Ludwig *et al.* (1988) have suggested that these regions may be involved in  $\text{Ca}^{2+}$  binding by haemolysin (see Discussion). From the characteristics of the proteins homologous to NodO a number of predictions can be made about NodO: (i) it may be exported; (ii) by analogy with haemolysin, other proteins would be required to mediate its export; (iii) the exported protein would not be processed at the N-terminal region; (iv) it may bind  $\text{Ca}^{2+}$ . Each of these predictions was tested as described below.

### Analysis of exported proteins

The growth medium supernatant of *R. leguminosarum* was analysed for bacterial proteins that were induced by hesperetin, a flavanone that induces *nod*-gene expression. Figure 4 shows that few trichloroacetic acid-precipitable proteins could be detected in the spent growth-medium supernatant of the wild-type strain (WT), but after growth in the presence of hesperetin the major component is a protein of apparent  $M_r$  42 000. This protein was not induced by a strain mutated in *nodO* (Figure 4) or in a strain lacking *nodD* and thus unable to induce *nod* genes (not shown). The  $M_r$  of NodO predicted from the sequence is 30 002, somewhat different from the apparent  $M_r$  of the induced exported protein but this apparent discrepancy could be due to aberrant migration of the NodO protein on polyacrylamide gels, a characteristic of highly acidic proteins (Kaufman *et al.*, 1984).

In order to confirm if the induced protein is the *nodO* gene product, the protein of  $M_r$  42 000 was purified. A rapid purification technique was devised based on an unusually high binding affinity of the protein for nitrocellulose. It had been noted that filtration of the growth medium supernatant through a nitrocellulose filter significantly reduced the amount of the exported TCA-precipitable protein compared with that seen in Figure 4. Since filtration of the growth medium supernatant of wild-type *Rhizobium* is difficult because of the large amounts of exopolysaccharide present, a mutant strain lacking exopolysaccharide was used in the purification. After growth of this strain in medium contain-



**Fig. 4.** Analysis of NodO protein in growth medium supernatants. Proteins were precipitated from growth medium supernatants with trichloroacetic acid, separated by SDS-PAGE and stained with Coomassie blue. The growth medium supernatants were from strain 8401pRL1JI (WT) or its isogenic derivatives carrying mutations in '*nodO*' or '*nodI*'. In addition, exported NodO protein is found in a strain (8401) lacking the symbiotic plasmid but carrying the *nodO* and *nodD* genes on plasmids pJ1788 and pJ1518 respectively; the higher amount of protein in this strain is probably due to a plasmid copy-number effect. The bacteria were grown in the absence (-) or presence (+) of  $1 \mu\text{M}$  hesperetin. The position of the NodO protein is arrowed. The protein standards and their mol. wts are: phosphorylase b (94 000); bovine serum albumin (67 000); ovalbumin (43 000); carbonic anhydrase (30 000).

ing hesperetin, the centrifuged growth medium supernatant was filtered through a nitrocellulose filter. The filter was then crushed and boiled in loading buffer and the solubilized proteins were run on an acrylamide gel. Figure 5 (lane b) shows that the major protein present in such a fraction was the one of apparent  $M_r$  42 000. The proteins on the acrylamide gel were blotted onto an Immobilon membrane and, after staining, the major protein band of  $M_r$  42 000 was excised and its amino-terminal sequence was determined. The sequence obtained, Met-Asn-Ile-Lys-Gly-Ser-Asp-Asn correlates exactly with that predicted from the DNA sequence of *NodO* (Figure 2). Therefore we conclude that the exported protein is encoded by *nodO* and that the N-terminal region is not cleaved during its export.

#### Other genes required for the export of the *NodO* protein are not on pRL1JI

The export of haemolysin requires the *hlyB* gene, which is homologous to the *nodI* gene from *R. leguminosarum* (Higgins *et al.*, 1986). Therefore it seemed possible that the export of NodO protein would require NodI. However, as shown in Figure 4, the flavonoid-inducible NodO protein was found in the growth medium supernatant from a strain carrying the *nodI82::Tn5* allele. The flavonoid-inducible NodO protein was also found in the growth medium supernatants from a series of strains carrying mutations in the *nodB*, *nodC*, *nodE*, *nodL*, *nodM* or *nodN* genes. These observations were confirmed using a derivative of strain 8401 which lacks a symbiotic plasmid but carries the cloned *nodO* gene (on pIJ1788) and the cloned *nodD* gene (on pIJ1518). As shown in Figure 4, NodO protein is found in the supernatant of this strain grown in the presence of hesperetin. Therefore we conclude that other genes that may be required for NodO export are not on the symbiotic plasmid.

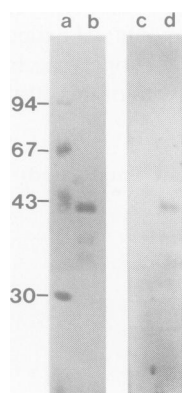


Fig. 5. NodO protein binds  $^{45}\text{Ca}^{2+}$ . The NodO protein eluted from nitrocellulose (lanes b and d), and protein standards (lanes a and c) were separated by SDS-PAGE and electroblotted onto an Immobilon membrane or nitrocellulose. The Immobilon membrane was stained with Coomassie blue (lanes a and b). The nitrocellulose membrane was used to determine  $^{45}\text{Ca}^{2+}$  binding; lanes c and d are an autoradiograph of the membrane showing a radioactive signal that corresponds to the position of the NodO protein. It is estimated that  $\sim 5 \mu\text{g}$  of NodO protein was present on the membrane. The amino acid sequence of NodO was determined from a lane similar to lane b but containing 10 times more protein.

#### *NodO* protein binds calcium

Ludwig *et al.* (1988) suggested that the aspartate/glycine-rich nonapeptide repeat region of haemolysin may be involved in binding calcium, thus facilitating the binding of haemolysin to erythrocyte membranes. The NodO protein (purified as described above) was tested for calcium binding as described by Maruyama *et al.* (1984). After electrophoresis, the protein was blotted to a nitrocellulose filter which was then incubated in a solution containing  $^{45}\text{Ca}^{2+}$  and washed. An autoradiograph of the filter (Figure 5) shows a single radioactive band corresponding with the position of the NodO protein, showing that the NodO protein can bind calcium.

#### Discussion

The observations described here show that the *nodO* gene encoded on the *R. leguminosarum* plasmid pRL1JI encodes an exported protein that is not cleaved at its amino terminus during or after its secretion into the growth medium. This protein is similar to that noted by de Maagd *et al.* (1989) who identified an exported protein whose synthesis was blocked by a mutation in the *nodO* region described here. Proteins exported by Gram-negative bacteria fall into two broad classes: the major group are translocated across the inner membrane via a system, that requires an amino-terminal 'transit' peptide that is cleaved off during or immediately after translocation; in general, these proteins are destined for the periplasmic space or the outer membrane (Pugsley and Schwartz, 1985). A second group of proteins is secreted by a mechanism that does not require an N-terminal transit sequence: these proteins are secreted through both the inner and outer membrane into the growth medium. They include the haemolysins (Felmlee *et al.*, 1985; Koronakis *et al.*, 1987; Mackman *et al.*, 1987), leukotoxin (Strathdee and Lo 1987), cyclolysin (Glaser *et al.*, 1988a) and proteases from *Serratia* (Nakahama *et al.*, 1986) and *Erwinia chrysanthemi* (Delepelaire and Wandersman 1989). Although there is cleavage of an N-terminal peptide from the proteases, this is required for activation of the proteolytic activity of the secreted enzymes and not for their export (Delepelaire and Wandersman, 1989).

One fascinating feature of this group of secreted proteins is the presence of the repeated domains illustrated in Figure 3. The periodicity of the repeat is nine amino acids and in NodO the 12 nonapeptide repeats fall into two domains separated by 18 residues (Figure 2), while the consensus repeat is  $\text{X}_L^1\text{X}_G^A\text{GXGXD}$ . Since a similar consensus is found in the other exported proteins described above, it would seem likely that this repeat may be involved in some way in their secretion. However, deletions of the *hlyA* gene have been constructed and removal of most (but not all of) the nonapeptide repeats does not block the secretion of haemolysin (Mackman *et al.*, 1987; Felmlee and Welch, 1988; Ludwig *et al.*, 1988; Koronakis *et al.*, 1989). The repeat domain of haemolysin has been shown to be responsible for its  $\text{Ca}^{2+}$ -dependent binding to erythrocytes (Ludwig *et al.*, 1988). Their structural model for  $\text{Ca}^{2+}$  binding by haemolysin involved an octahedral  $\text{Ca}^{2+}$ -binding site formed by adjacent  $\text{Asn}^{\text{Asp}}$ -Asp residues from three contiguous repeats, a structure related to but somewhat

different from the Ca<sup>2+</sup>-binding domains of other Ca<sup>2+</sup>-binding proteins (Vyas *et al.*, 1987).

The number of nonapeptide repeat units varies in the different exported proteins; the proteases each have 4, haemolysin 15, leukotoxin has 10, cyclolysin 40 and NodO 12. The last four nonapeptide repeats of these proteins show particularly strong homology and in Figure 6 they are aligned to highlight the similarities. We note that after the last nonapeptide repeat in each protein there are two highly conserved positions containing the aromatic residues tyrosine or phenylalanine. The sequences shown in Figure 6 can be folded into a tetralobate structure with glycine residues providing turns at the extremities of the lobes and with central aspartate/asparagine residues or residues with free oxygen atoms which could chelate Ca<sup>2+</sup>. This structure could possibly hold the Ca<sup>2+</sup> in a pocket protected at one end by a hydrophobic domain consisting of the large aromatic residues. Clearly X-ray crystallography would be required to test this suggestion, and since the NodO protein is the smallest of the group of proteins and has been shown to bind Ca<sup>2+</sup> it may be a useful protein to try to understand the structure common to this group of proteins.

What is the mechanism of export of the NodO protein? One characteristic of the related secreted proteins is that other gene products are specifically involved in their export (Wagner *et al.*, 1983; Welch *et al.*, 1983; Mackman and Holland, 1984; Glaser *et al.*, 1988a; Chang *et al.*, 1989). Two of these gene products, HlyB and CyaB, required for haemolysin and cyclolysin secretion respectively, are homologous to the nodulation protein NodI (Higgins *et al.*, 1986; Glaser *et al.*, 1988a). Mutation of the *nodI* gene did not prevent secretion of the NodO protein, suggesting that the NodI protein is not involved in the secretion. Since the NodO protein is exported in a strain lacking its symbiotic plasmid and carrying only the cloned *nodO* and *nodD* genes, it is likely that if, as expected, other genes are required for the export of NodO protein, then these genes are encoded elsewhere on the *R. leguminosarum* genome.

The C-terminal 100 amino acids of haemolysin are required for its export (Mackman *et al.*, 1987; Koronakis *et al.*, 1989). One feature of this region, also present in leukotoxin and cyclolysin, is characterized as a hydrophobic sequence flanked by glutamic acid or aspartic acid residues (the 'aspartic acid box' described by Holland, 1989). For example, in cyclolysin this sequence is DPGAAAAP-PAARVPD, which is similar to the sequence DRGFAS-AAAAATAID in NodO (Figure 2). Koronakis *et al.* (1989) noted that this conserved region in HlyA is preceded by a potential amphiphilic helix, and there is also a potential

amphiphilic sequence at the corresponding position in NodO (Figure 2). However, the position of the putative aspartic acid box in NodO is ~40–50 amino acids farther from the C terminus of the protein than the equivalent regions in haemolysin or cyclolysin, and data on protein export will be important to define the region(s) of NodO required for its secretion.

Although mutations in *nodO* normally have only a minor effect on nodulation of peas and *V. hirsuta* (de Maagd *et al.*, 1989; Economou *et al.*, 1989), this gene is nevertheless an important determinant of host specificity since in some genetic backgrounds it is important for nodulation of *V. sativa* (de Maagd *et al.*, 1989). Furthermore, in strains that are mutant in *NodE*, mutations in *nodO* have a severe effect on nodule formation (B.P. Surin, A. Economou and J.A. Downie, unpublished). These observations show that the *nodO* gene is a determinant of host specificity, and that the effects of mutations in *nodO* may be masked by one or more of the other host-specific *nod* genes, even though there is no similarity in their sequence. Significantly, it appears that the NodO protein is one of a class of exported proteins that are widespread in Gram-negative bacteria. Since these proteins interact with animal or plant cells, it is reasonable to conclude that the NodO protein may play a role in nodule development by direct interaction with the root hair cells or some other plant surface in a Ca<sup>2+</sup>-dependent manner. In this regard it is significant that Smit *et al.* (1989) have identified a role for Ca<sup>2+</sup> in *Rhizobium* attachment to root hair cells.

## Materials and methods

### Bacterial strains, plasmids and growth conditions

*Rhizobium leguminosarum* biovar *viciae* strain 8401pRL1JI has been described previously (Downie *et al.*, 1983) and all other strains used here are isogenic derivatives of that strain carrying the following mutations: *nodB87::Tn5*, *nodC128::Tn5*, *nodD57::Tn5*, *nodE68::Tn5*, *nodI82::Tn5* (Downie *et al.*, 1985), *nodL146::kan*, *nodM150::kan*, *nodN186::kan* (Surin and Downie, 1988), *nodO93::Tn3HoHol*, *rhiA5::Tn3HoHol* (Economou *et al.*, 1989), *pssA1::Tn5* (Borthakur *et al.*, 1986). The *nodO93::Tn3HoHol* and *rhiA5::Tn3HoHol* alleles were transferred from derivatives of pIJ1089 (Economou *et al.*, 1989) to pRL1JI by homologous recombination as described previously (Downie *et al.*, 1985), and confirmed to be correct by DNA hybridizations using pIJ1089 as a radioactive probe. Plasmid pIJ1518, carrying *nodD* was described by Rossen *et al.* (1985). pIM236 was made by subcloning a 1.5 kb *PstI*–*HindIII* fragment from pIJ1089 into the *PstI*–*HindIII* sites of pUC119. A 1.4 kb *EcoRI* fragment, carrying *nodO* and its promoter region, was excised from pIM236 and ligated to pLAFR1 to form pIJ1788.

*Rhizobium* bacteria were grown in the TY medium described by Beringer (1974) and genetic selections were made using antibiotics as described by Surin and Downie (1988). When added, the flavanone hesperetin was pre-

NodO	93	I P H S	G E G D D V L Y A G P G S D I L V A G D G A D V L T G G D D G D A F V F	132
HlyA	814	V L S G G K G N D K L Y G S E G A D L L D G G E G N D L L K G G Y G N D I Y R Y	1010	
LktA	749	R L F G G K G D D I L D G G N G D D F I D G G K G N D L L H G G K G D D I F V H	788	
CyaA	1553	V L S G G A G D D V L L G D E G S D L L S G D A G N D D L F G G Q G D D T Y L F	1592	
Serratia	338	L I V G N A A N N V L K G G A G N D V L F G G G A D E L W G G A G K D I F V F	377	
PrtB	346	I L I G N G A D N I L Q G G A G D D V L Y G S T G A D T L T G G A G R D I F V Y	385	

Fig. 6. Alignment of homologous putative Ca<sup>2+</sup>-binding domains. The four nonapeptide repeat regions of the secreted proteases from *Serratia* (Nakahama *et al.*, 1986) and *E. chrysanthemi* (PrtB; Deleplaire and Wandersman, 1989) are aligned with the last four repeats from NodO, haemolysin (HlyA; Felmler *et al.*, 1985), leukotoxin (LktA; Lo *et al.*, 1987), and cyclolysin (CyaA; Glaser *et al.*, 1988b). The numbers refer to the positions of the amino acids in the protein sequences. Identical residues are boxed and nonapeptide repeat units are indicated below the alignment of sequences. The conserved aromatic amino acid residues (see Discussion) are arrowed.

sent at 1  $\mu\text{M}$  in the growth media. Assays of  $\beta$ -galactosidase with rhizobia were as described by Rossen et al. (1985), using Tn3HoHo-1 mutations in the *nodB*, *nodE* and *nodO* genes on pIJ1089 in strain 8401.

#### Manipulation of DNA

Routine DNA manipulations were carried out using standard procedures (Maniatis et al., 1982). DNA was sequenced using the dideoxy chain termination method described by Bankier and Barrell (1983) on an ordered series of deletion fragments constructed using *Bal31* treatment of pIM236. The fragments were subcloned into M13mp18, and the DNA sequence was determined by sequencing each strand in its entirety at least twice.

#### Analysis of exported NodO protein

Bacterial cultures were grown until late logarithmic phase (48 h at 28°C) in 100 ml TY medium containing hesperetin and the bacteria were removed by three centrifugations of 20 min at 10 000 r.p.m. (Beckman JA-14 rotor). Proteins were precipitated from the supernatant with TCA (10% w/v) and after 2 h on ice the proteins were pelleted by centrifugation (10 000 r.p.m. for 20 min). The pellets were rinsed with 0.1 M Tris-HCl, pH 8.0, and then resuspended in 200  $\mu\text{l}$  of gel loading buffer (Laemmli, 1970). Samples (40  $\mu\text{l}$ ) were separated by SDS-PAGE (Laemmli, 1970) as described by Bradley et al. (1988) using 12% (w/v) acrylamide/bis-acrylamide (30:0.8) gels with a 6% stacking gel.

The NodO protein was purified by filtering 100 ml of the centrifuged growth medium supernatant (from the culture of the strains carrying the *psaA1::Tn5* mutation) through a 0.22  $\mu\text{m}$  Millipore nitrocellulose filter, which was then crushed and boiled in 400  $\mu\text{l}$  of gel loading buffer. After SDS-PAGE, the proteins were electroblotted onto PVDF membrane (Immobilon, Waters, UK) as described by Matsudaira (1987) or nitrocellulose (Schleicher and Schüll, FRG) membranes as described previously (Bradley et al., 1988). Before use, the Immobilon membrane was immersed in methanol, and after transfer, proteins were visualized with Coomassie brilliant blue (0.05% w/v) in methanol/water/acetic acid (50:40:10).

For protein sequencing, ~50  $\mu\text{g}$  of stained protein on the Immobilon membrane was excised and the amino acid sequence of the N-terminal region was determined from a portion of the sample using an Applied Biosystems (Warrington, UK) 470A gas-phase sequencer equipped with a 120A on-line PTH analyser, essentially as described by Matsudaira (1987).

#### <sup>45</sup>Ca<sup>2+</sup> binding

The nitrocellulose membrane carrying ~5  $\mu\text{g}$  of NodO protein and ~20  $\mu\text{g}$  of protein mol. wt standards (Pharmacia) was treated as described by Maruyama et al., (1984) to identify proteins binding <sup>45</sup>Ca<sup>2+</sup> (Amersham), using 20  $\mu\text{Ci}$  of <sup>45</sup>CaCl<sub>2</sub> in 20 ml of buffer. After washing in deionized water the filter was autoradiographed for 4 days on Fuji RX-X-ray film. High purity H<sub>2</sub>O and plastic containers were used throughout the experiment.

## Acknowledgements

We are indebted to Dr Pat Barker and Dr Andrew Northrop (AFRC Microchemical Sequencing Unit, Babraham, Cambridge) for determining the N-terminal protein sequence of NodO. We thank Drs V. Koronakis, J. Böllman and E. Kannenberg for advice and helpful discussions, F.K.L. Hawkins for the generous gift of nod mutant strains and Dr A. Dawson for help with the Ca<sup>2+</sup>-binding experiments. J. Timons, D. Harrison and A. Davies gave skilled technical assistance. We are grateful to David Hopwood for his comments on the manuscript. The work was supported by the Agricultural and Food Research Council via a grant in aid to the John Innes Institute; A.E. was supported by a Sectoral Training Grant from the European Commission and further support was from the Agricultural Genetics Company.

## References

- Bankier, A.T. and Barrell, B.G. (1983) In Flavell, R.A. (ed.), *The Life Sciences*, Elsevier, Ireland, pp. 137–144.
- Beringer, J.E. (1974) *J. Gen. Microbiol.*, **84**, 118–198.
- Bhuvaneshwari, T.V. and Solheim, B. (1985) *Physiol. Plant.*, **63**, 25–34.
- Borthakur, D., Barber, C.E., Lamb, J.W., Daniels, M.J., Downie, J.A. and Johnston, A.W.B. (1986) *Mol. Gen. Genet.*, **200**, 278–282.
- Bradley, D.J., Wood, E.A., Larkins, A.P., Galfre, G., Butcher, G.W. and Brewin, N.J. (1988) *Planta*, **173**, 149–160.
- Chang, Y.-F., Young, R., Moulds, T.L. and Struck, D.K. (1989) *FEMS Microbiol. Lett.*, **60**, 169–174.
- Deleplaire, P. and Wandersman, C. (1989) *J. Biol. Chem.*, **264**, 9083–9089.
- de Maagd, R.A., Wijffelman, C.A., Pees, E. and Lugtenberg, B.J.J. (1988)

- J. Bacteriol.*, **170**, 4424–4427.
- de Maagd, R.A., Spaink, H.P., Pees, E., Mulders, I.H.M., Wijffelman, C.A., Wijffelman, C.A., Okker, R.J.H. and Lugtenberg, B.J.J. (1989) *J. Bacteriol.*, **171**, 1151–1157.
- Dibb, N.J., Downie, J.A. and Brewin, N.J. (1984) *J. Bacteriol.*, **158**, 621–627.
- Downie, J.A. and Johnston, A.W.B. (1988) *Plant Cell Environ.*, **11**, 403–412.
- Downie, J.A., Ma, Q.-S., Knight, C.D., Hombrecher, G. and Johnston, A.W.B. (1983) *EMBO J.*, **2**, 947–952.
- Downie, J.A., Knight, C.D., Johnston, A.W.B. and Rossen, L. (1985) *Mol. Gen. Genet.*, **198**, 255–262.
- Economou, A., Hawkins, F.K.L., Downie, J.A. and Johnston, A.W.B. (1989) *Mol. Microbiol.*, **3**, 87–93.
- Evans, I. and Downie, J.A. (1986) *Gene*, **43**, 95–101.
- Felmler, T. and Welch, R.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5269–5273.
- Felmler, T., Pellett, S. and Welch, R.A. (1985) *J. Bacteriol.*, **163**, 94–105.
- Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A. and Danchin, A. (1988a) *EMBO J.*, **7**, 3997–4004.
- Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A. and Danchin, A. (1988b) *Mol. Microbiol.*, **2**, 19–30.
- Higgins, C.F., Hiles, I.D., Salmund, G.P.C., Gill, D.R., Downie, J.A., Evans, I.J., Holland, I.B., Gray, L., Bucke, S.D., Bell, A.W. and Hermondson, M.A. (1986) *Nature*, **323**, 448–450.
- Holland, I.B. (1989) *Biochem. Soc. Trans.*, **17**, 323–325.
- John, M., Schmidt, J., Wieneke, U., Krüssmann, H.D. and Schell, J. (1988) *EMBO J.*, **7**, 583–588.
- Kaufman, E., Geisler, N. and Weber, K. (1984) *FEBS Lett.*, **170**, 81–84.
- Koronakis, V., Cross, M., Senior, B., Koronakis, E. and Hughes, C. (1987) *J. Bacteriol.*, **69**, 1509–1515.
- Koronakis, V., Koronakis, E. and Hughes, C. (1989) *EMBO J.*, **8**, 595–605.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- Lo, R.Y.C., Strathdee, C.A. and Shewen, P.E. (1987) *Infect. Immunol.*, **5**, 1987–1996.
- Long, S.R. (1989) *Cell*, **56**, 203–214.
- Ludwig, A., Jarchau, T., Benz, R. and Goebel, W. (1988) *Mol. Gen. Genet.*, **214**, 553–561.
- Mackman, N. and Holland, I.B. (1984) *Mol. Gen. Genet.*, **196**, 129–134.
- Mackman, N., Baker, K., Gray, L., Haigh, R., Nicaud, J.-M. and Holland, I.B. (1987) *EMBO J.*, **6**, 2835–2841.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Maruyama, K., Mikawa, T. and Ebashi, K. (1984) *J. Biochem.*, **95**, 511–519.
- Matsudaira, P. (1987) *J. Biol. Chem.*, **262**, 1035–1038.
- Nakahama, K., Yoshimura, K., Marumoto, R., Kikuchi, M., Lee, I.-S., Hase, T. and Matsubara, H. (1986) *Nucleic Acids Res.*, **14**, 5843–5855.
- Pearson, W.R. and Lipman, D.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2444–2448.
- Pugsley, A.P. and Schwartz, M. (1985) *FEBS Microbiol. Rev.*, **32**, 3–38.
- Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A. and Kondorosi, A. (1986) *Proc. Natl. Acad. Sci. USA*, **77**, 3840–3844.
- Rossen, L., Johnston, A.W.B. and Downie, J.A. (1984) *Nucleic Acids Res.*, **12**, 9497–9508.
- Rossen, L., Shearman, C.A., Johnston, A.W.B. and Downie, J.A. (1985) *EMBO J.*, **4**, 3369–3373.
- Schofield, P.R. and Watson, J.M. (1986) *Nucleic Acids Res.*, **14**, 2891–2903.
- Shearman, C.A., Rossen, L., Johnston, A.W.B. and Downie, J.A. (1986) *EMBO J.*, **5**, 647–652.
- Smit, G., Logman, T.J.J., Boerrigter, M.E.T.I., Kijne, J.W. and Lugtenberg, B.J.J. (1989) *J. Bacteriol.*, **171**, 4054–4062.
- Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E. and Lugtenberg, B.J.J. (1987) *Plant Mol. Biol.*, **9**, 27–39.
- Staden, R. (1982) *Nucleic Acids Res.*, **10**, 2951–2961.
- Strathdee, C.A. and Lo, R.Y.C. (1987) *Infect. Immunol.*, **55**, 3233–3236.
- Surin, B.P. and Downie, J.A. (1988) *Mol. Microbiol.*, **2**, 173–183.
- Surin, B.P. and Downie, J.A. (1989) *Plant Mol. Biol.*, **12**, 19–29.
- Surin, B.P., Watson, J.M., Hamilton, W.D.O., Economou, A. and Downie, J.A. (1990) *Mol. Microbiol.*, in press.
- Vieira, J. and Messing, J. (1987) *Methods Enzymol.*, **153**, 3–11.
- Vyas, N.K., Vyas, M.N. and Quijcho, F.A. (1987) *Nature*, **327**, 635–638.
- Wagner, W., Vogel, M. and Goebel, W. (1983) *J. Bacteriol.*, **154**, 200–210.
- Welch, R.A., Hull, R. and Falkow, S. (1983) *Infect. Immunol.*, **42**, 178–186.
- Zaat, S.A.J., Van Brussel, A.A.N., Tak, T., Pees, E. and Lugtenberg, B.J.J. (1987) *J. Bacteriol.*, **169**, 3388–3391.

Received on October 3, 1989; revised on November 24, 1989