The EMBO Journal vol.7 no.3 pp.583-588, 1988

Transmembrane orientation and receptor-like structure of the *Rhizobium meliloti* common nodulation protein NodC

Michael John, Jürgen Schmidt, Ursula Wieneke, Heinz-Dieter Krüssmann and Jeff Schell

Max-Planck-Institut für Züchtungsforschung, Abteilung Schell, D-5000 Köln 30, FRG

Communicated by J.Schell

The 46.8-kd NodC protein of *Rhizobium meliloti* is a membrane protein, essential for nodule formation. Gene fusions of *nodC* to a portion of the λ *cI* repressor gene were used to define the membrane-anchor domain which is necessary for membrane insertion of the NodC protein into the membrane. The transmembrane orientation of NodC was confirmed by surface-specific radiolabelling and proteolysis experiments. A highly hydrophobic transmembrane-anchor domain was found near the carboxyl terminus, separating a large extracellular domain which contains an unusual cysteine-rich cluster from a short putative intracellular domain. Cross-linking studies showed that the NodC protein exists in the membrane probably as a dimer. The domain structure of the NodC protein shows striking similiarities with cell surface receptors. In nodules of various legumes a truncated form of the NodC protein was detected. The processed NodC was associated with the bacteroids and the amount of this protein increased during nodule development.

Key words: gene fusion/transmembrane orientation/NodC protein/nodules/*Rhizobium*

Introduction

The common nodulation genes *nodA, B* and *C* are highly conserved between different *Rhizobium* species and are required for the nodulation of legumes and non-legumes (Kondorosi *et al.*, 1984; Djordjevic *et al.*, 1985; Downie *et al.*, 1985; Fisher *et al.*, 1985; Marvel *et al.*, 1987). In *Rhizobium meliloti* the expression of the *nodABC* operon can be induced by the flavone luteolin, which is present in plant exudate (Peters *et al.*, 1986). Previously, we provided evidence that the nodA and B proteins are located in the cytoplasm of *R.meliloti* (Schmidt *et al.*, 1986, 1987), where NodC is a transmembrane protein (John *et al.*, 1985). When *Rhizobium* wild-type strains were inoculated onto their host plants in the presence of antibodies directed against the NodC protein, nodule formation was strongly inhibited (John *et al.*, 1985). These experiments suggested that the NodC protein is located on the cell surface of *Rhizobium*, so that extracellular antibodies would be able to bind to it, thereby causing a reduction of nodulation. Because of its transmembrane location, NodC may play an important role in the signal transduction from bacterial to host plant. The function of the highly conserved NodC protein is essential, because mutations within the *nodC* gene completely abolish root hair curling and nodule formation (Kondorosi *et al.*, 1984; Schmidt *et al.*, 1984).

We have recently shown that the NodC protein is also present in mature nodules induced by *R.meliloti* on *Medicago sativa*. During nodule development, the NodC protein appears to be processed to a smaller molecule (Schmidt *et al.*, 1986). In the current studies we have detected the processed NodC in the nodules of various other legumes and we have shown that the amount of this protein increases during nodule development. To learn more about the structure and possible function of the NodC protein, we have used gene fusions to define the membrane-anchor domain which is necessary for membrane insertion. We have determined the direction in which the NodC protein is oriented within the membrane by proteolysis experiments and vectorial labelling of intact cells. Finally, we have shown by cross-linking experiments that the NodC protein in the membrane is probably a dimer.

Results

Identification of a membrane-spanning region at the carboxyl terminus of the NodC protein

The amino acid sequence of the NodC transmembrane protein, which has been deduced from the nucleotide sequence, shows two conspicuous hydrophobic regions (Török *et al.*, 1984; Figure 1). A hydrophobic segment of ~20 amino acid residues at the amino terminus may correspond to a signal sequence for the initial steps of translocation (Sabatini *et al.*, 1982; Watson, 1984). To find out whether the large hydrophobic segment near the carboxyl terminus of NodC may serve as a membrane anchor, we fused the carboxy-terminal 119 amino acid residues of NodC to a 17.7-kd portion of the cytoplasmic CI repressor of phage λ (Figure 1). To construct this gene fusion we used the *tac* promoter vec-

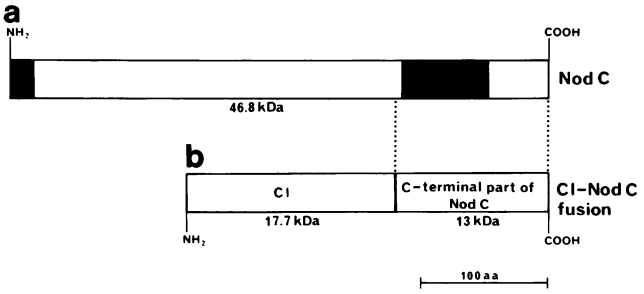

Fig. 1. Structure of the CI-NodC fusion protein. The black boxes, shown in (**a**), indicate the two large hydrophobic regions of the NodC protein. The C-terminal part of NodC, containing the major hydrophobic region, was fused to the N-terminal portion of the λ CI repressor as indicated by the dotted lines (**b**). A scale is given in amino acids (aa).

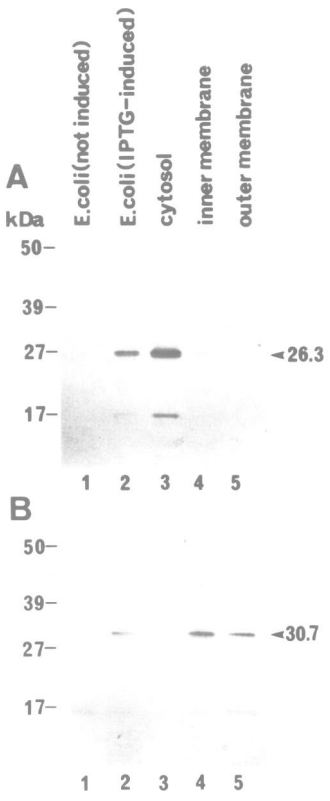

Fig. 2. Cellular location of the λ CI repressor in *E.coli* W3110 harbouring pEA305 (**A**) and the CI–NodC fusion protein synthesized from plasmid pJS1008 in the same *E.coli* strain (**B**). Cytoplasmic and membrane fractions were prepared as described in the text. Proteins were analysed by SDS–PAGE, transferred to nitrocellulose and labelled with anti-CI repressor antibodies. The position of the 26.3-kd λ CI repressor and of the 30.7-kd CI–NodC fusion protein is indicated by an arrowhead.

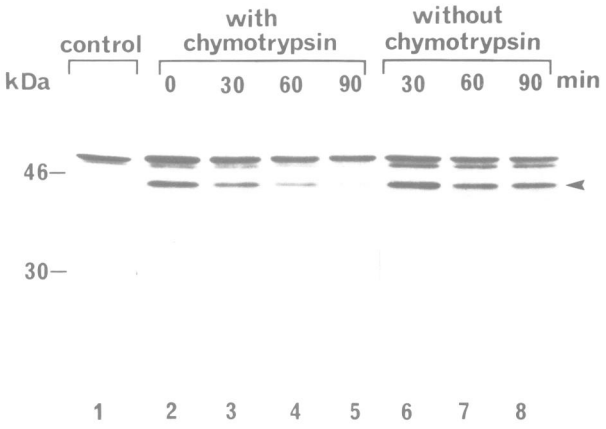

Fig. 3. Western blot analysis of NodC protein after treatment of *R.meliloti* 1021 cells with chymotrypsin. Proteolysis of bacterial cells was carried out as described in the text. Cell extracts were analysed by SDS–PAGE, immunoblotting and autoradiography. **Lane 1,** *R.meliloti* cells, not induced (control); **lanes 2–5**, induced cells after protease treatment; **lanes 6–8**, cells without protease added (control). Incubation periods (min) are indicated. Arrowhead marks the position of NodC.

tor pEA305, which directs the synthesis of the λ CI repressor upon induction with isopropyl β-D-thiogalactoside (IPTG; Amann *et al.*, 1983). The strategy was similar to that reported for the construction of other *cI-nod* gene fusions (John *et al.*, 1985; Schmidt *et al.*, 1985; see Materials and methods). The resulting plasmid pJS1008 was transformed into the *lac* repressor-overproducing strain *Escherichia coli* W3110 *lac*I^{q}L8. The expression of the fusion protein from pJS1008 had a remarkable effect on the growth rate of *E.coli*. Approximately 2 min after induction with IPTG, bacterial growth stopped completely, while the control plasmid pEA305, which synthesized the complete λ repressor without fusion, showed normal cell growth (data not shown).

E.coli strains carrying plasmids pJS1008 and pEA305 respectively, were fractionated into cytosol and membrane fractions, which were analysed by immunoblotting using anti-CI repressor antibodies. The immunoblot in Figure 2A showed that the λ CI repressor was, as expected, in the cytoplasm, while the hybrid protein was localized in the inner and outer membrane fractions of *E.coli* (Figure 2B). These data suggest that the hydrophobic segment near the carboxyl terminus of the NodC protein is responsible and sufficient for membrane insertion. In a previous experiment we fused a larger carboxy-terminal portion of NodC consisting of 287 amino acid residues to the 17.7-kd part of the λ CI repressor, and found this larger fusion protein to be localized mainly in the outer membrane of *E.coli* (John *et al.*, 1985). The presence of the shorter fusion protein both in the inner and in the outer membranes (Figure 2) may be due to the absence of sequences upstream from the large hydrophobic segment (Figure 1), which are additionally needed for proper routing of NodC to the outer membrane.

Orientation of NodC in the membrane

Since the NodC protein is present in *Rhizobium* in only tiny amounts (John *et al.*, 1985), we had to use polyclonal monospecific antibodies and radioiodination for the detection of this protein in the following experiments. Additionally, we used *R.meliloti* strain 1021, which expresses the NodC protein ~40 times more than strain AK631 used in previous studies.

To determine the direction in which the NodC protein is oriented within the membrane, we treated intact *R.meliloti* cells with chymotrypsin (Figure 3), which cleaves the NodC protein at 32 different sites. Due to its size chymotrypsin cannot penetrate biological membranes, therefore the enzyme will digest only those regions of the protein that are located at the cell surface. The treatment of intact cells with protease for 90 min leads almost to a disappearance of the NodC protein band (Figure 3, lanes 2–5). As deduced from a hydrophilicity plot using the parameters described by Hopp and Woods (1981), the possible antigenic determinants within the NodC protein are located upstream of the membrane-anchor domain (Figure 6). Apparently, protease treatment destroyed all antigenic determinants against which the polyclonal, anti-NodC antibodies were directed. This explains why no truncated forms of the NodC protein were detected. These data indicate that the large amino-terminal domain of the NodC protein (see Figure 6) is exposed to the extracellular medium and is therefore sensitive to protease digestion.

In a further experiment we labelled the surface proteins of induced and not-induced *R.meliloti* cells by lactoperoxidase-catalysed iodination. ^{125}I-Surface-labelled cells were subjected to immunoprecipitation and SDS–PAGE. As shown in Figure 4, the NodC protein, which is present

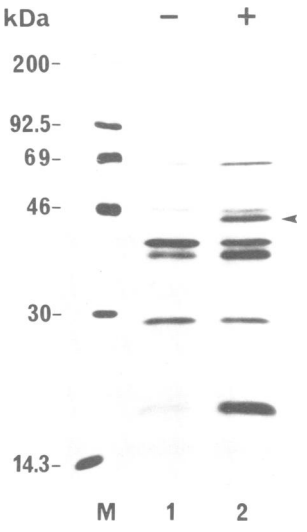

Fig. 4. Radioiodination of *R.meliloti* cells. Cell surface proteins were iodinated as described in the text and the bacteria were lysed with SDS. The ^{125}I-labelled NodC protein was immunoprecipitated, and samples were analysed by SDS−PAGE and autoradiography. **Lane 1**, *R.meliloti* grown without inducer (−, control); **lane 2**, *R.meliloti* induced with luteolin (+). **Lane M**, molecular size marker. Arrowhead marks the position of the NodC protein.

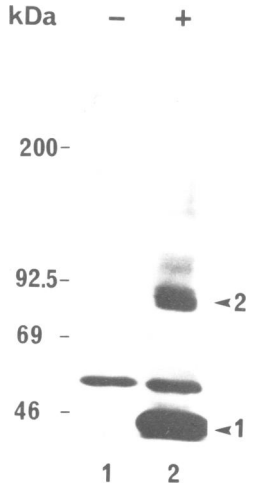

Fig. 5. Cross-linking of *R.meliloti* membranes. Membranes were cross-linked with 50 μM BS^3 (Staros, 1982) and samples were analysed in a 6% SDS−polyacrylamide gel followed by immunoblotting using anti-NodC antibodies and autoradiography. **Lane 1**, membranes from *R.meliloti* grown without inducer (−, control); **lane 2**, membranes from induced *R.meliloti* cells (+). The positions of the NodC monomer (1) and NodC dimer (2) are indicated.

in induced *R.meliloti* cells (lane 2), is reactive with extracellular lactoperoxidase. This also demonstrates that the NodC protein is indeed a cell surface protein that contains an extracellular domain.

Cross-linking of membrane proteins

To find out whether NodC is an oligomeric protein we treated *R.meliloti* membranes with BS^3, which is a hydrophilic, membrane-impermeant protein cross-linker (Staros, 1982). Cross-linking of the membrane proteins with 50 μM BS^3 yielded only one major product, identified as a dimer of the NodC protein (Figure 5, lane 2). When membranes were treated with increasing concentrations (1−2 mM) of BS^3, the NodC protein was cross-linked to very large oligomers which could not appreciably enter the 6% polyacrylamide gel. These studies suggest that the NodC protein exists in the membrane at least as a dimer.

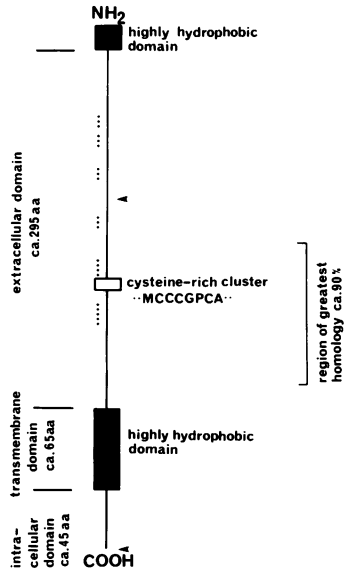

Fig. 6. Proposed domain structure of the NodC protein. Anti-NodC antibodies were raised against the major part of the NodC protein (John *et al.*, 1985). This portion is marked by the two arrowheads. The dotted lines indicate possible antigenic sites as deduced by hydrophilicity plotting (Hopp and Woods, 1981). Other characteristics are discussed in the text.

Model of NodC as a cell-surface protein

Based on these results and the predicted amino acid sequence (Török *et al.*, 1984) we propose the following structure of the NodC protein. The protein contains a large hydrophobic segment of ~65 amino acid residues near the carboxyl terminus which anchors the protein in the membrane. This anchor domain separates an ~295-residue-long extracellular domain, from a short intracellular carboxy-terminal domain (Figure 6). The polyclonal antibodies used in these studies are directed against a large portion of the extracellular domain, and this part includes a region which is highly conserved in many rhizobial species. The extracellular domain is further characterized by an unusual cysteine-rich cluster (Figure 6). As discussed later, these structural features suggest that the NodC protein may be a potential cell surface receptor.

Characterization of the NodC protein in nodules

The *R.meliloti* NodC protein is also present in mature nodules of *M.sativa* (Schmidt *et al.*, 1986). In these nodules the 46.8-kd NodC protein appears to be truncated and migrates on a gel as a 34-kd protein band (Figure 7, lanes 2 and 3). We determined the relative amounts of processed NodC in *M.sativa* nodules of different ages and found that this protein increased 2- to 3-fold during the development of the nodules (Figure 7, lanes 3−7). The same samples were analysed for the presence of NodA and NodB proteins using the appropriate antibodies. The immunoblots showed that both proteins were present in the nodules in essentially constant amounts during nodule development (data not shown).

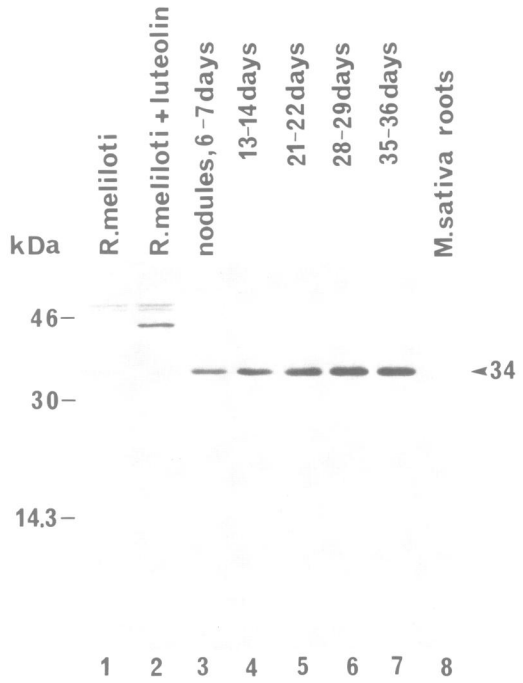

Fig. 7. Analysis of NodC during nodule development. Equal amounts of *M.sativa* nodules of different age were analysed by SDS–PAGE, immunoblotting, autoradiography and laser densitometer scanning as described by Schmidt *et al.* (1986). The arrowhead marks the position of the processed NodC protein (34 kd). **Lanes 1** and **2**, *R.meliloti* grown without and with inducer (control); **lane 8**, *M.sativa* roots (control).

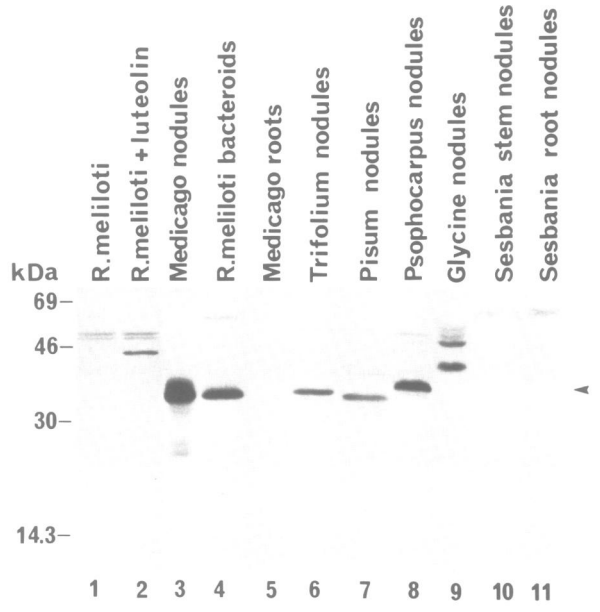

Fig. 8. Detection of the processed NodC protein in nodules of various legumes. Proteins were analysed as described in Figure 7. Arrowhead marks positions of processed NodC proteins. The controls (**lanes 1, 2** and **5**) were the same as in the previous figure.

The truncated 34-kd NodC protein was also detected in bacteroids which were isolated from *M.sativa* nodules (Figure 8, lane 4). Furthermore, truncated forms of the NodC protein were found in the nodules of other legumes infected with different rhizobia (lanes 6–9). With the exception of the processed NodC in *Glycine* nodules (lane 9), all other truncated forms were of similar size. These data suggest that the processing of NodC may play a general and important role during nodule development.

No distinct protein band was observed with *Sesbania rostrata* stem or root nodules (Figure 8, lanes 10 and 11). Apparently, there is no relevant immunologic relationship between the NodC protein of *R.meliloti* and *Azorhizobium caulinodans* since the homology between the two proteins is <50% (van den Eede *et al.*, 1987).

Discussion

Several lines of evidence suggest that the NodC protein of *R.meliloti* is a cell-surface protein. First, we treated intact *Rhizobium* cells with proteases, and found the amount of NodC to be significantly reduced (Figure 3). Since proteases cannot penetrate biological membranes, they react only with those regions of proteins that are exposed to the extracellular medium. Another independent line of evidence is provided by experiments in which cell-surface proteins of *R. meliloti* were labelled by lactoperoxidase-catalysed iodination. It was shown that NodC indeed reacts with extracellular lactoperoxidase, which labels only proteins that are exposed on the cell surface (Figure 4). This conclusion is also consistent with our previous observations, showing that antibodies were able to react with the NodC protein on the outer surface of intact *Rhizobium* cells, and cause an inhibition of nodule formation (John *et al.*, 1985).

A striking feature of the amino acid sequence of the NodC protein is a stretch of ~65 mainly hydrophobic amino acids near the carboxyl terminus. We have fused the carboxy-terminal region of the NodC protein, containing the large hydrophobic segment, to a portion of a cytoplasmic protein (λ CI), and thus created a membrane-bound fusion protein (Figure 2). This demonstrates that the carboxy-terminal hydrophobic segment is necessary and sufficient for insertion of NodC in the membrane. Other sequences upstream of the membrane-anchor domain, including the putative amino-terminal signal sequence, seem to be necessary for proper targeting of the NodC protein to the outer bacterial membrane, since previous experiments using a larger domain of the NodC protein, fused to the λ CI repressor, revealed association of the chimeric protein predominantly with the outer membrane (John *et al.*, 1985). The fact that information which determines the final location of a membrane protein is contained within the mature form of the protein has already been described by other authors (Moreno *et al.*, 1980; Hall *et al.*, 1982; Tommassen *et al.*, 1983; Benson *et al.*, 1984).

As mentioned above, antibodies directed against some antigenic determinants located upstream of the membrane-anchor domain (Figure 6) reacted with the NodC protein on the outer surface of intact *Rhizobium* cells. These recent data, together with the protease digestion experiments shown here, define the 295-residue-long amino-terminal region as the extracellular domain (Figure 6).

A computer search of the Genbank and EMBL data libraries revealed no significant homology of the NodC protein with other proteins. The structure of the NodC protein proposed here includes a membrane-anchor domain near the carboxyl terminus, a large extracellular domain and a short carboxy-terminal intracellular domain (Figure 6). Such a do-

main structure shows striking similarities with various cell-surface receptors (Pfeffer and Ullrich, 1985; Hynes, 1987). Furthermore, it is interesting to note that the extracellular domain of NodC contains an unusual cysteine-rich cluster. Extracellular cysteine-rich clusters have been found in mammalian cell-surface receptors with very diverse functions (Yamamoto *et al.*, 1984; Pfeffer and Ullrich, 1985; Ullrich *et al.*, 1985; Hynes, 1987). It is therefore tempting to speculate that the NodC protein is a potential cell-surface receptor.

There is increasing evidence that the common *nod* genes are involved in the production of a soluble factor which changes the mode of growth of the plant roots resulting in thick short roots (Tsr factor; van Brussel *et al.*, 1986; Zaat *et al.*, 1987). We have recently found that the cytoplasmic proteins NodA and B are involved in the synthesis of a low mol. wt diffusible factor which stimulates cell division of plant protoplasts (J.Schmidt *et al.*, unpublished). The signalling molecule(s) have not yet been identified. We speculate that the NodC protein, as a cell-surface receptor, may participate in transducing the diffusible growth factors from the bacterial to the plant cell.

In nodules of various legumes we detected a truncated form of the NodC protein (Figure 8). This processed NodC was associated with the bacteroids and increased during nodule development (Figure 7). The role of the truncation of this protein is unknown and needs further investigation. Since some characteristic features of NodC are now known, domain-specific anti-peptide antibodies can be prepared and used to examine the processing of this protein. We also want to find out whether NodC can bind to the growth factor provided by the NodA and B proteins or whether the peptide liberated during processing of the NodC protein itself plays a role as signal molecule.

Materials and methods

Bacterial strains and plasmids

In all experiments *R.meliloti* 1021 (Meade *et al.*, 1982) was used, with the exception of plant nodulation experiments where AK631, a compact colony morphology variant of *R.meliloti* 41 was used. *Azorhizobium caulinodans* ORS571 (Dreyfus and Dommergues, 1981), *Bradyrhizobium japonicum* USDA11 (Means *et al.*, 1964), *Rhizobium leguminosarum* T83K3 (Johnston *et al.*, 1978), *Rhizobium* sp. strain MPIK3030 (Trinick, 1980) have been described and were used for the production of nodules. *R.trifolii* Resh403 was provided by Z.Banfalvi. *E.coli* W3110*lacI*^{q}L8 (Brent and Ptashne, 1981) was used as a host for *tac* promoter-containing plasmids. Unless otherwise stated, the *E.coli* and *R.meliloti* strains were grown in M9 salts (Miller, 1972) supplemented with 0.2% casamino acids and 0.4% glycerol. Other *Rhizobium* strains were cultured in YTB medium (Orosz *et al.*, 1973) or GTS (Kiss *et al.*, 1979). Plasmid pEA305 carries the *tac* promoter and the *cI* gene of phage λ (Amann *et al.*, 1983), pJS209 contains the *nodC* gene of *R.meliloti* AK631 (John *et al.*, 1985).

Construction of cI – nodC hybrid gene

Recombinant DNA techniques were carried out essentially as described by Maniatis *et al.* (1982). The *tac* promoter vector pEA305 (Amann *et al.*, 1983), which directs the synthesis of high levels of the λ CI repressor, was digested with *Hin*dIII and protruding ends were filled-in with the Klenow fragment of DNA polymerase I. The 6-kb fragment was isolated and used as vector (pEA305Δ*Hin*dIII-1; John *et al.*, 1985).

In pJS209 the *nodC* gene is contained within a 1.8-kb *Eco*RI fragment (John *et al.*, 1985). Plasmid pJS209 was cut at its unique *Cla*I and *Tth*111I sites and the sticky ends were filled-in with the Klenow enzyme. A 0.52-kb *Cla*I – *Tth*111I fragment was isolated which encodes the major hydrophobic region of the NodC protein (Török *et al.*, 1984). Insertion of the *nodC*-containing fragment into the filled-in *Hin*dIII site of pEA305Δ*Hin*dIII-1 yielded plasmid pJS1008. Plasmid pJS1008 was stably maintained in the *lac* repressor overproducing strain *E.coli* W3110*lacI*^{q}L8.

Antibodies

Polyclonal antibodies directed against the purified CI repressor of phage λ and a previously described NodC hybrid protein (John *et al.*, 1985) were raised in rabbits, and IgG fractions of each antibody were prepared by purification on Protein A – Sepharose CL-4B. For the isolation of monospecific antibodies an affinity matrix was prepared by coupling either the λ CI repressor or the NodC hybrid protein to CNBr-activated Sepharose 4B. Antigen affinity chromatography was carried out as described by De Mey (1983).

Electrophoresis and immunoblotting

SDS – PAGE was performed in 12% polyacrylamide gels (Laemmli, 1970) unless otherwise indicated. Proteins were electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979) and the membranes were incubated with antibodies and washed as described previously (Schmidt *et al.*, 1986). Bound antibodies were localized with ^{125}I-labelled protein A (5 μCi, Amersham).

Radioiodination

R.meliloti cells were washed three times with cold phosphate-buffered saline (PBS). Cells (2 OD_{600}) were labelled in PBS containing glucose (5 mM) and $Na^{125}I$ (500 μCi/ml; Amersham). The reaction was initiated by the addition of lactoperoxidase (25 μg/ml; Calbiochem) and glucose oxidase (2.5 U/ml; Boehringer) in order to generate hydrogen peroxide (Hynes, 1973). The reaction was allowed to proceed for 20 min at room temperature and was stopped by washing the cells three times with PBS containing 10 mM NaI and 2 mM phenylmethylsulphonyl fluoride (PMSF). The bacteria were lysed with SDS and the labelled NodC protein was immunoprecipitated as described (John *et al.*, 1985).

Protein cross-linking

R.meliloti cells were fractionated into cytosol and total membranes (Schmidt *et al.*, 1986). The membranes were washed with 50 mM sodium phosphate (pH 7.4) and resuspended in the same buffer. Aliquots were treated with various concentrations of the hydrophilic, membrane-impermeant cross-linker *bis* (sulphosuccinimidyl)suberate (BS^3; Staros, 1982). After the suspensions were incubated for 30 min at room temperature, the reactions were stopped by the addition of one volume of 50 mM Tris – Cl (pH 7.4) containing 20 mM *N*-ethylmaleimide (Staros, 1982). The membranes were washed once in the same buffer, centrifuged and treated for 1 h at 37°C with electrophoresis sample buffer (pH 7.4) containing 4% SDS and 7% 2-mercaptoethanol.

Protease treatment of bacterial cells

Induced *Rhizobium* cells (1 OD_{600}) were washed three times with 50 mM Tris – Cl (pH 8.0) containing 150 mM NaCl and 2 mM $CaCl_2$. The bacteria were resuspended in 1 ml of the same buffer and incubated at 25°C in the absence or presence of chymotrypsin (9 U; Boehringer). Proteolysis was stopped by the addition of 2 mM PMSF at the times indicated in Figure 4. Cells were collected by centrifugation and the samples were analysed by SDS – PAGE and immunoblotting.

Other procedures

Seedlings of *Glycine max, Medicago sativa, Pisum sativum, Phosphocarpus tetragonolobus, Sesbania rostrata* and *Trifolium pratense* were grown on nitrogen-free medium (Kondorosi *et al.*, 1977) and were inoculated with the appropriate *Rhizobium* strain. *Glycine, Pisum* and *Sesbania* nodules were provided by Frans de Bruijn. Nodules were harvested 3 – 4 weeks after inoculation and appropriate amounts were analysed by SDS – PAGE and immunoblotting as described by Schmidt *et al.* (1986). Bacteroids from alfalfa nodules were isolated by the procedure of Corbin *et al.* (1983). *R.meliloti* 1021 was induced with 10 μM of luteolin (Roth, Karlsruhe, FRG) as described by Schmidt *et al.* (1986). Cytosol, inner and outer membrane fractions of *E.coli* were prepared as described by Ito *et al.* (1977).

Acknowledgements

We thank Frans de Bruijn (MPI, Cologne) and Adam Kondorosi (Biological Research Center, Szeged, Hungary) for discussions and critical reading of the manuscript, and Kurt Stüber for help with computer analysis. We also thank Dietrich Bock for photographic work. This work was supported by a grant from Bundesministerium für Forschung und Technologie (BCT 03652/project 8).

References

Amann,E., Brosius,J. and Ptashne,M. (1983) *Gene*, **25**, 167 – 176.
Benson,S.A., Bremer,E. and Silhavy,T.J. (1984) *Proc. Natl. Acad. Sci.*

USA, **81**, 3830–3834.
Brent,R. and Ptashne,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4204–4208.
Corbin,D., Barran,L. and Ditta,G. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3005–3009.
De Mey,J.R. (1983) In Cuello,A.C. (ed.), *Immunohistochemistry*. Wiley, Chichester, pp. 347–372.
Djordjevic,M.A., Schofield,P.R., Ridge,R.W., Morrison,N.A., Bassam,B.J., Plazinski,J., Watson,J.M. and Rolfe,B.G. (1985) *Plant Mol. Biol.*, **4**, 147–160.
Downie,J.A., Knight,C.D., Johnston,A.W.B. and Rossen,L. (1985) *Mol. Gen. Genet.*, **198**, 255–262.
Dreyfus,B. and Dommergues,Y.R. (1981) *FEMS Microbiol. Lett.*, **10**, 313–317.
Fisher,R.F, Tu,J.K. and Long,S.R. (1985) *Appl. Environ. Microbiol.*, **49**, 1432–1435.
Hall,M.N. (1982) *J. Mol. Biol.*, **156**, 93–112.
Hopp,T.P. and Woods,K.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3824–3828.
Hynes,R.O. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 3170–3174.
Hynes,R.O. (1987) *Cell*, **48**, 549–554.
Ito,K., Sato,T. and Yura,T. (1977) *Cell*, **11**, 551–559.
John,M., Schmidt,J., Wieneke,U., Kondorosi,E., Kondorosi,A. and Schell,J. (1985) *EMBO J.*, **4**, 2524–2430.
Johnston,A.W.B., Beynon,J.L., Buchanan-Wollaston,A.V., Setchell,S.M., Hirsch,P.R. and Beringer,J.E. (1978) *Nature*, **276**, 635–636.
Kiss,G.B., Vincze,E., Kalman,Z., Forrai,T. and Kondorosi,A. (1979) *J. Gen. Microbiol.*, **113**, 105–118.
Kondorosi,A., Svab,Z., Kiss,G.B. and Dixon,R.A. (1977) *Mol. Gen. Genet.*, **151**, 221–226.
Kondorosi,E., Banfalvi,Z. and Kondorosi,A. (1984) *Mol. Gen. Genet.*, **193**, 445–452.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marvel,D.J., Torrey,J.G. and Ausubel,F.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1319–1323.
Meade,H.M., Long,S.R., Ruykun,G.B., Brown,S.E. and Ausubel,F.M. (1982) *J. Bacteriol.*, **149**, 114–122.
Means,U.M., Johnson,H.W. and Date,R.A. (1984) *J. Bacteriol.*, **87**, 547–553.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Moreno,F., Fowler,A.V., Hall,M., Silhavy,T.J., Zabin,I. and Schwartz,M. (1980) *Nature*, **286**, 356–359.
Orosz,L., Svab,Z., Kondorosi,A. and Sik,T. (1973) *Mol. Gen. Genet.*, **125**, 341–350.
Peters,N.K., Frost,J.W. and Long,S.R. (1986) *Science*, **233**, 977–980.
Pfeffer,S. and Ullrich,A. (1985) *Nature*, **313**, 184.
Sabatini,D.D., Kreibich,G., Morimoto,T. and Adesdnik,M. (1982) *J. Cell Biol.*, **92**, 1–22.
Schmidt,J., John,M., Kondorosi,E., Kondorosi,A., Wieneke,U., Schröder,G., Schröder,J. and Schell,J. (1984) *EMBO J.*, **3**, 1705–1711.
Schmidt,J., John,M., Wieneke,U., Krüssmann,H.-D. and Schell,J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9581–9585.
Schmidt,J., John,M., Wieneke,U., Krussmann,H.-D. and Schell,J. (1987) Abstr. 14th International Botanical Congress, Berlin (West), 3-131-15.
Staros,J.V. (1982) *Biochemistry*, **21**, 3950–3955.
Tommassen,J., van Tool,H. and Lugtenberg,B. (1983) *EMBO J.*, **2**, 1275–1279.
Török,I., Kondorosi,E., Stepkowski,T., Posfai,J. and Kondorosi,A. (1984) *Nucleic Acids Res.*, **12**, 9509–9524.
Towbin,H., Staehelin,T. and Gordon,T. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Trinick,M.J. (1980) *J. Appl. Bacteriol.*, **49**, 39–53.
Ullrich,A., Bell,J.R., Chen,E.Y., Herrera,R., Petruzelli,L.M., Dull,T.J., Gray,A., Coussens,L., Liao,Y.-C., Rosen,O.M. and Ramachandran,J. (1985) *Nature*, **313**, 756–761.
van Brussell,A.A.N., Zatt,S.A.J., Canter Cremers,H.C.J., Wijffelman,C.A., Pees,E., Tak,T. and Lugtenberg,B.J.J. (1986) *J. Bacteriol.*, **165**, 517–522.
van den Eede,G., Dreyfus,B., Goethals,K., van Montagu,M. and Holsters,M. (1987) *Mol. Gen. Genet.*, **206**, 291–299.
Watson,M.E.E. (1984) *Nucleic Acids Res.*, **12**, 5145–5164.
Yamamoto,T., Davis,C.G., Brown,M.S., Schneider,W.J., Linett,Casey,M., Goldstein,J.L. and Russell,D.W. (1984) *Cell*, **39**, 27–38.
Zaat,S.A.J., Wijffelman,C.A., Spaink,H.P., van Brussell,A.A.N., Okker, R.H.J. and Lugtenberg,B.J.J. (1987) *J. Bacteriol.*, **169**, 198–204.

Received on December 10, 1987