Identification of the NodC Protein in the Inner but Not the Outer Membrane of *Rhizobium leguminosarum*

M. A. Barny and J. A. Downie

John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, U.K.
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An antiserum specific for the NodC protein of *Rhizobium leguminosarum* reacted with NodC in the inner, but not the outer, membrane fraction. In addition, the NodC protein in intact cells was resistant to proteolytic cleavage. Therefore, contrary to previous observations, it appears that NodC is not exposed on the outer membrane but is localized exclusively in the inner membrane.

Additional keywords: chitin synthase, membranes, nodulation.

Recognition between leguminous plants and the rhizobia that nodulate them is mediated via specific signaling molecules exchanged by the two symbiotic partners (Fisher and Long 1992). Different *Rhizobium* species that nodulate various legumes make different but closely related nodulation signaling molecules. These have a backbone of three, four, or five β1-4-linked *N*-acetyl glucosamine residues, and their specificity is determined by different substituents on this carbohydrate backbone (Dénarié *et al.* 1992). The most crucial *nod* gene products required for the synthesis of these lipo-oligosaccharides are NodA, NodB, and NodC, which in the absence of other *nod* gene products can form a "core" signal consisting of an *N*-acylated penta or tetra *N*-acetyl glucosamine oligomer (Spaink *et al.* 1991).

The NodC protein has strongly hydrophobic domains (Török *et al.* 1984; Jacobs *et al.* 1985), suggesting that it may be a transmembrane protein. John *et al.* (1985) found a NodC fusion protein to be associated with both inner and outer membranes. On the basis of a biochemical analysis of the NodC protein using surface-labeling and proteolytic cleavage, it was subsequently proposed (John *et al.* 1988) that the NodC protein spans the inner membrane and has a large N-terminal portion that is outer membrane associated and may act as a potential cell-surface receptor. The inhibition of nodulation by NodC antiserum (John *et al.* 1985) and immunolocalization studies using the same antiserum (Johnson *et al.* 1989) also supported that model. Hubac *et al.* (1992) also used the same NodC antiserum to analyze the location of NodC in fractionated *R. meliloti*. In these studies NodC was present in the inner membrane and outer membrane fractions, although a stronger reaction was seen with the inner membrane fraction.

It is now evident that NodC is significantly homologous to the polysaccharide-polymerizing enzymes chitin synthase and cellulose synthase (Bulawa and Wasco 1992; Atkinson and Long 1992; Debellé *et al.* 1992). This suggests that NodC is more likely to be involved in the synthesis of the oligo-glucosamine backbone of the nodulation factors than to function as a receptorlike protein; such a role would indicate that an outer-membrane location for NodC is unlikely. Therefore, we decided to reevaluate the location of NodC using an antiserum to identify it in subcellular fractions and to monitor its sensitivity to protease.

To raise an antibody against the *R. l.* bv. *viciae* NodC, part of NodC from *R. l.* bv. *viciae* was expressed in *E. coli* using the T7 RNA polymerase/promoter system described by Tabor (1990). The entire 3′ end of the *nodC* open reading frame (from the *SalI* site at nucleotide position 1639 equivalent to amino acid residue 86 as described by Rossen *et al.* 1984) is present in pIJ1887 on a 4.4-kb *SalI* fragment that was subcloned in both orientations into the *SalI* site of pT7-7. One of the plasmids formed (pIJ1994) carries the partial *nodC* gene cloned such that the truncated *nodC* gene was fused in frame to a linker sequence immediately downstream of the translational start site in pT7-7. pIJ1993 carrying *nodC* in the opposite orientation was used as a control. Plasmids pIJ1993, pIJ1994, and pT7-7 were introduced by transformation into strain K38 pGP1-2, and the T7 RNA polymerase on pGP1-2 was induced in the presence of rifampicin (Tabor 1990). The proteins formed were analyzed, and a major protein band having the expected size for the truncated NodC protein was observed with the strain containing pIJ1994 (Fig. 1, lane c) but not with the strains containing pIJ1993 or pT7-7 (Fig. 1, lanes a and b). About 0.2 mg of the NodC protein made by the strain carrying pIJ1994 was electroeluted from a preparative gel, and a sample was confirmed to contain the appropriate protein (Fig. 1d). The remainder was used to raise an antiserum in a rabbit. The

Corresponding author: J. A. Downie.
Present address of M. A. Barny: INRA, rue George Morel, Beaucouzé 49070, France.

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antiserum was tested for its reactivity to NodC by using it to probe proteins from *R. leguminosarum* strain 8401 carrying pIJ1216, which carries the *nodABCDEF* genes (Downie *et al.* 1985).

When the strain 8401pIJ1216 was grown in the presence of hesperetin to induce *nodC,* the antibody reacted with a protein of M_r 46,000 (Fig. 1e), corresponding with the predicted size of the intact *nodC* gene product (Rossen *et al.* 1984; Downie *et al.* 1985). In the absence of *nod* gene induction (data not shown), or with a mutant *nodC* derivative, the antiserum gave no signal (Fig. 1, lane f). The anti-NodC serum is therefore specific and could be used to detect the native NodC protein. In preliminary experiments, the antiserum to the *R. meliloti* NodC protein described by John *et al.* (1988) had been used to try to detect NodC from *R. l.* bv. *viciae*, but no significant cross-reaction was found to NodC. However, we found that there was cross-reactivity to several *R. l.* bv. *viciae* components other than NodC, a problem also seen by John *et al.* (1988) and Hubac *et al.* (1992) with *R. meliloti.*

A major part of the evidence for the outer-membrane location of NodC was its apparent sensitivity to proteases added to whole cells of *R. meliloti* (John *et al.* 1988). However, using the antibody prepared here, no degradation of NodC was detected following the addition of trypsin or chymotrypsin to whole cells of *R. l.* bv. *viciae* even after prolonged (1.5-hr) incubation (Fig. 2, lanes a, b, and e). When the cells were lysed by sonication there was complete degradation of the NodC protein by trypsin or chymotrypsin within a short (20-min) incubation period (Fig. 2, lanes d and f); in the absence of added proteases there was no significant degradation of the NodC protein in the sonicated cell extract (Fig. 2, lane c).

To localize the NodC protein, the membranes were fractionated by the procedure previously used by McEwan *et al.* (1986) for the separation of inner and outer membranes of *Rhodobacter capsulatus.* (This procedure was used because the genera *Rhizobium* and *Rhodobacter* are closely related phylogenetically, Young [1992].) Briefly, this involved growing *R. leguminosarum* in TY medium up to an OD_{600} of 0.5. The cells were harvested, washed once at 4° C in 50 mM Tris-HCl, pH8, and resuspended at 30° C in STE buffer (50 mM Tris-HCl, pH8, 1.3 mM sodium EDTA, and 0.5 M sucrose) at a concentration of 1 g wet weight of cells per 20 ml of STE buffer. Lysozyme was then added (final concentration of 600 $\mu g\ ml^{-1}$), and the cells were incubated at 30° C for 1 hr with occasional shaking. The suspension was then centrifuged (15 min, 10,000 rpm) to pellet the spheroplasts, and the supernatant containing the periplasm plus the outer membranes was

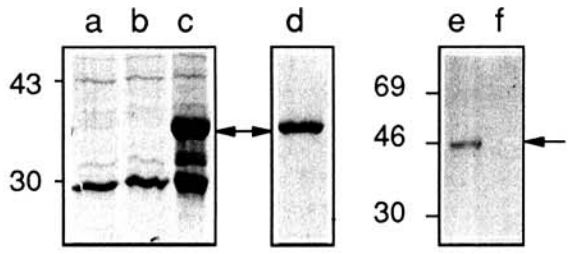

Fig. 1. Preparation of NodC antiserum: Strain K38 + pGP1-2 (Tabor 1990) plus either pT7-1 (lane a), pIJ1993 (lane b), or pIJ1994 (lane c) were grown at 30° C to an OD_{600} of 0.5 in 50 ml of L broth supplemented with kanamycin (75 $\mu g/ml^{-1}$) and ampicillin (75 $\mu g\ ml^{-1}$). At that stage the T7 polymerase was induced as described by Tabor (1990). The cells were harvested, resuspended in 1 ml of 10 mM Tris HCl (pH7.4), and sonicated (6 × 20 s) to break the cells. The envelope and membrane fractions were precipitated by centrifugation and the pellet resuspended in 1 ml of gel loading buffer (Bradley *et al.* 1988). Samples (10 μl) were run on a 10% SDS-polyacrylamide gel which was then stained with Coomassie blue. The arrowed band in lane c corresponds in size with the truncated NodC protein predicted to be made by pIJ1994. To purify this NodC protein, three preparative gels were run, each loaded with 250 μl of the protein sample from the strain carrying pIJ1994. The NodC band was then excised after staining with Coomassie blue, electroeluted from the acrylamide, freeze-dried, and resuspended in 600 μl of phosphate buffer saline. Lane d shows 10 μl of this sample after electrophoresis on an analytical SDS-polyacrylamide gel. The remainder was injected subcutaneously into a rabbit (three injections) and the resulting antiserum used to probe a Western blot of proteins from strain 8401 pIJ1216 (Downie *et al.* 1985) induced for *nodC* expression (lane e) after growth in TY medium + 1 μM hesperitin. The only band that reacted with the serum (used at a 1/2,000 dilution) corresponds with the size of the intact NodC protein which as shown in lane f was absent from 8401 pIJ1365 (pIJ1365 is a derivative of pIJ1216 carrying the *nodC*128::Tn*5*) allele. The NodC antiserum did not react with proteins from 8401 pIJ1216 that had been grown in the absence of flavonoids that induce *nod* gene expression (not shown). Antibody binding was detected with goat antirabbit immunoglobulin conjugated to alkaline phosphatase as described by Bradley *et al.* (1988).

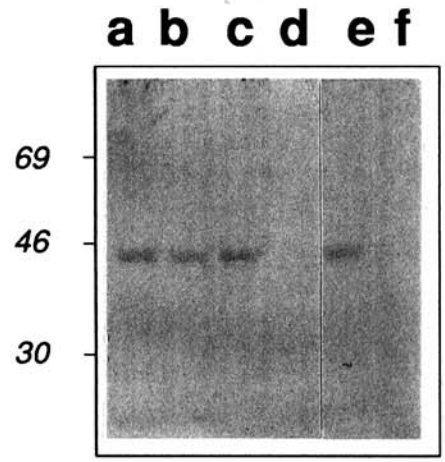

Fig. 2. NodC in cells is resistant to proteolytic degradation. Cells of 8401 pIJ1216 grown in 600 ml of TY + 1 μM hespertin were washed and resuspended in 50 mM Tris HCl buffer (pH 7.0) at a concentration of 0.5 g wet weight of cells/10 ml. Aliquots (1 ml) of the cells were then incubated for 90 min at 37° C in the absence (lane a) or presence (lane b) of trypsin (150 μg/ml final concentration). The proteins were then solubilized for electrophoresis by incubating the samples for 15 min at 100° C in sodium dodecyl sulphate loading buffer in the presence of 300 μg/ml trypsin inhibitor protein (Sigma) and 10 μM (4-amidino-phenyl) methane-sulphonyl fluoride (APMSF). After electrophoresis, the samples were transferred to nitrocellulose and probed with the NodC antiserum as described in Figure 1. A similar aliquot of the cells was first sonicated and then incubated in the presence or absence of 150 μg/ml trypsin for 20 min at 37° C. After solubilization of the sample (in the presence of trypsin inhibitor as before) complete degradation of the NodC protein was seen if trypsin was present (lane d), whereas no degradation was seen in the absence of trypsin (lane c). Under similar conditions chymotrypsin (150 μg/ml) did not degrade NodC in intact cells (lane e) although it did degrade NodC in a sonicated cell extract (lane f). The samples were incubated at 37° C following the conditions of John *et al.* (1988); similar results were seen when the samples were incubated at 5° C.

carefully removed. This supernatant was then centrifuged (20 min, 18,000 rpm) to recover a pellet of outer membranes. The spheroplast pellet was resuspended in about 4 ml of STE, sonicated (three pulses of 20 s each with a 20-s interval), and unbroken cells were removed by centrifugation (20 min 12,000 rpm). An inner membrane fraction was pelleted from the supernatant by high-speed centrifugation (2 hr, 55,000 rpm). The proteins present in the outer and inner membrane fractions derived from this procedure are shown in Fig. 3A (lanes a and b, respectively), and it is clear that there is a different pattern of protein bands. As shown in the histogram (Fig. 3D), the outer membrane fraction (lane a) has essentially no NADH oxidase activity, indicating that it is relatively free from inner membrane contamination, whereas the inner membrane fraction (lane b) had high NADH oxidase activity. The outer membrane fraction was rich in KDO (data not shown), and the lipopolysaccharide-specific monoclonal antibody JIM21 (Sindhu *et al.* 1990) was used to confirm that the outer membrane fraction was rich in lipopolysaccharide (Fig. 3C, lane a); when tested on the inner membrane preparation (Fig. 3C, lane b), this antibody revealed that, as was expected from this preparation method, the inner membrane preparation contained some outer membrane contamination. However, in terms of relative protein concentration it is evident that the inner membrane preparation is significantly enriched in inner membrane proteins compared with outer membrane components. When the two fractions were probed with the NodC antiserum (Fig. 3B), there was a clear reaction with the inner membrane fraction (lane b) but no significant cross reactivity to the outer membrane fraction (lane a). Therefore, since the outer membrane fraction as prepared here is essentially free from inner membranes (as judged by the lack of NADH oxidase activity), it is evident that, contrary to the observations made with *R. meliloti* (John *et al.* 1985, 1988), in *R. l.* bv. *vicae*, NodC appears not to be associated with the outer membrane but is found exclusively in the inner membrane fraction.

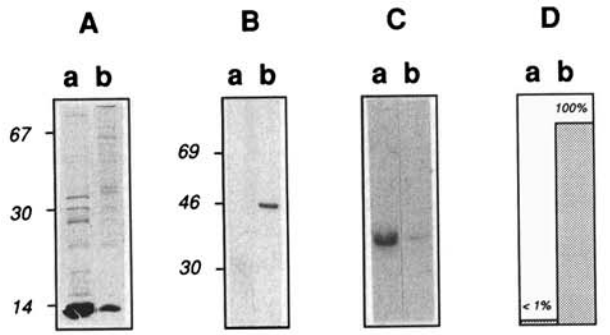

Fig. 3. NodC is in the inner membrane fraction. A: Proteins from outer (a) and inner (b) membrane fractions (about 50 μg of protein) were separated on an SDS-polyacrylamide (10%) gel stained with Coomassie blue. B: Equivalent tracks to those from A (but run on a 15% polyacrylamide gel) were electroblotted to nitrocellulose and stained with NodC antiserum showing that NodC is present in the inner (b) but not the outer (a) membrane fraction. C: Equivalent tracks to those from B were reacted with the lipopolysaccharide-specific monoclonal antibody JIM21 which reacted strongly with the outer membrane fraction (a) but only very weakly with the inner membrane fraction (b). D: A histogram showing the NADH oxidase activities of the outer (a) and inner (b) membrane fractions. Measurements were made, as described by Osborn *et al.* (1972) and 100% activity is equivalent to an NADH oxidase activity of 340 nmol min^{-1} per milligram of added protein. The molecular weights (in kilodaltons) shown in A were estimated using albumin (67 kDa) carbonic anhydrase (30 kDa) and α lactalbumin (14 kDa). Those in B and C were estimated on the Western blot using the "Rainbow" markers supplied by Amersham.

The reasons for the differences between our conclusions using *R. l.* bv. *viciae* and those of John *et al.* (1985, 1988) using *R. meliloti* are not immediately obvious and would not be expected since the two NodC proteins are functionally homologous. It should be noted that John *et al.* (1985 1988) did not carry out fractionation of *Rhizobium* inner and outer membranes. Those fractionations done were carried out with *E. coli* cells that expressed very high levels of a NodC fusion protein. The apparent location of NodC in the *E. coli* outer membranes as well as the inner membrane (John *et al.* 1985, 1988) may have been due to expression of NodC fusion proteins at too high a level (up to 19% of total cellular proteins) causing, e.g., the formation of inclusion bodies that copurified with outer membrane components. Hubac *et al.* (1992) found some NodC protein in the outer membrane preparation made from *R. meliloti* cells, although at lower levels than in the inner membrane fraction. There are significant differences with regard to the methods of membrane fractionation used by Hubac *et al.* (1992) and those used here. It is possible that the outer membranes prepared by Hubac *et al.* (1992) had higher levels of inner membrane contamination than those used here. Inner membrane contamination was monitored by Hubac *et al.* (1992) and ourselves by measuring NADH oxidase activity and, whereas Hubac *et al.* (1992) did observe low levels of NADH oxidase in the outer membrane fractions, we did not detect any activity. A low level of inner membrane contamination of outer membrane fractions could be consistent with some NodC found in the outer membrane fractions and indeed, Hubac *et al.* (1992) concluded that the exact location of NodC remained an open question.

The cross-reactivity of the *R. meliloti* NodC antiserum to components other than NodC (John *et al.* 1988 and Hubac *et al.* 1992) could have led to incorrect interpretations in the immunolocalization studies of Johnson *et al.* (1989), who described immunogold localization of NodC on the cell surface of *R. meliloti*. The same antiserum was used in the studies by John *et al.* (1988), Johnson *et al.* (1989), and Hubac *et al.* (1992). In the absence of the appropriate control (the same serum used with a strain lacking the NodC protein), the validity of the conclusion of Johnson *et al.* (1989), based on the use of an antiserum that reacts to components other than NodC (John *et al.* 1988), is clearly open to question.

John *et al.* (1988) presented two other lines of evidence for NodC being an outer membrane protein, namely its protease sensitivity and its labeling by I^{125} with lactoperoxidase. With regard to the protease sensitivity of NodC, our observations indicate that although the *R. l.* bv. *viciae* NodC protein is very susceptible to proteolytic degradation in broken cells, in intact cells it is resistant to degradation even under the same conditions of protease treatment used by John *et al.* (1988). These experiments depend on the

outer membrane remaining intact; one explanation of the contradictory results might be that the integrity of the outer membrane was lost in *R. meliloti*, but not in *R. leguminosarum*, as a result of the protease treatment. The specificity of I^{125}-labeling also depends on the outer membrane remaining intact. The NodC antiserum used to precipitate the I^{125}-labeled NodC protein also precipitated significant amounts of two I^{125}-labeled flavonoid-induced proteins which had very different molecular weights from NodC (John *et al.* 1988). If these proteins are not located in the outer membrane then this may raise doubts about the validity of the conclusions drawn about the location of NodC. However, there is no strong reason to believe that the outer membrane should have been disrupted during the I^{125}-labeling studies.

Therefore, the reasons for the differences between our conclusions and those of John *et al.* (1988) remain unresolved. We conclude that NodC is not in the outer membrane and the inner membrane location of NodC proposed here is more consistent with its proposed role in the synthesis of the *N*-actyl glucosamine oligomers that form the backbone of the nodulation factors.

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