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

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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, noduleation.

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 noduleation 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-acetylated 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 noduleation 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 chinin synthase and cellulose synthase (Bulawa and Wasco 1992; Atkinson and Long 1992; Debelle et al. 1992). This suggests that NodC is more likely to be involved in the synthesis of the oligo-glucosamine backbone of the noduleation factors than to function as a receptor-like 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. vicieae* NodC, part of NodC from *R. l. bv. vicieae* 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 plJ1887 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 (plJ1994) 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. plJ1993 carrying nodC in the opposite orientation was used as a control. Plasmids plJ1993, plJ1994, 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 plJ1994 (Fig. 1, lane c) but not with the strains containing plJ1993 or pT7-7 (Fig. 1, lanes a and b). About 0.2 mg of the NodC protein made by the strain carrying plJ1994 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
antiserum was tested for its reactivity to NodC by using it to probe proteins from *R. leguminosarum* strain 8401 carrying pJJ1216, which carries the nod*ABCDEF* genes (Downie et al. 1985).

When the strain 8401pJJ1216 was grown in the presence of hesperetin to induce *nodC*, the antibody reacted with a protein of $M_r$ 46,000 (Fig. 1c), corresponding with the predicted size of the intact nod*C* gene product (Rossen et al. 1984; Downie et al. 1985). In the absence of nod gene induction (data not shown), or with a mutant nod*C* 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, pH 8, and resuspended at 30°C in STE buffer (50 mM Tris-HCl, pH 8, 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 μg 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 + pGPI1-2 (Tabor 1990) plus either pT7-1 (lane a), pJJ1993 (lane b), or pJJ1994 (lane c) were grown at 30°C to an OD$_{600}$ of 0.5 in 50 ml of L broth supplemented with kanamycin (75 μg ml$^{-1}$) and ampicillin (75 μg 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 (pH 7.4), and sonicated (6 x 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 pJJ1994. To purify this NodC protein, three preparative gels were run, each loaded with 250 μl of the protein sample from the strain carrying pJJ1994. 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 pJJ1216 (Downie et al. 1985) induced for nodC expression (lane e) after growth in TY medium + 1 μM hesperetin. 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 pJJ1365 (pJJ1365 is a derivative of pJJ1216 carrying the nodC-128 Tn5) allele. The NodC antiserum did not react with proteins from 8401 pJJ1216 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 pJJ1216 grown in 600 ml of TY + 1 μM hesperetin 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$^{-1}$ 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$^{-1}$ trypsin inhibitor protein (Sigma) and 10 μM (4-amino-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$^{-1}$ 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$^{-1}$) 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. viciae, nodC appears not to be associated with the outer membrane but is found exclusively in the inner membrane fraction.

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 the outer membrane contamination of nodC found in the 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 antisera 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 antisera 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 conclusions of Johnson et al. (1989), based on the use of an antisera 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 $1^{125}$ 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. melliloti, but not in R. leguminosarum, as a result of the protease treatment. The specificity of $^{125}$I-labeling also depends on the outer membrane remaining intact. The NodC antisera used to precipitate the $^{125}$I-labeled NodC protein also precipitated significant amounts of two $^{125}$I-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 $^{125}$I-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-acyl glucosamine oligomers that form the backbone of the nodulation factors.

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LITERATURE CITED


