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Research Notes

The *Rhizobium, Bradyrhizobium,* and *Azorhizobium* NodC Proteins Are Homologous to Yeast Chitin Synthases

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The *nodABC* genes of rhizobia are essential for the synthesis of lipo-oligosaccharidic (*N*-acylated chitin oligomers) nodulation signals. *nodC* gene products from *Rhizobium, Bradyrhizobium,* and *Azorhizobium* exhibit extensive homology with chitin synthases, suggesting that the NodC proteins are involved in the synthesis of the chitin oligomer backbone by catalyzing the β-1,4-linkage between *N*-acetyl-D-glucosamine residues.

Additional keywords: chitinase, lipo-oligosaccharide, nodulation.

The *nodABC* genes, referred to as common *nod* genes, are structurally and functionally conserved in all *Rhizobium, Bradyrhizobium,* and *Azorhizobium* species studied so far (Dénarié and Roche 1991; Long 1989; Martinez *et al.* 1990). There is at least 40% identity in the amino acid sequences of the various NodABC proteins. The *nodABC* genes play a crucial role in infection and nodulation because a mutation in these genes results in a complete loss of the ability to elicit any detectable plant responses whatever the host, the type of infection (crack-in entry or infection thread formation), the type of nodules (determinate or indeterminate), or the location of nodules (stem or root) (Dénarié and Roche 1991; Long 1989; Nap and Bisseling 1990). The species-specific *nod* genes such as *nodFEG, nodH, nodPQ,* and *nodSU* are involved in defining the rhizobial host range (Barbour *et al.* 1991; Dénarié and Roche 1991; Martinez *et al.* 1990). Bacterial strains carrying mutations in these genes display altered infection and nodulation functions including changes in the host range.

The major biochemical function of the common and host-specific *nod* genes is to specify the synthesis of extracellular lipo-oligosaccharides, the nodulation (Nod) factors. The Nod factors from *R. meliloti* and *R. leguminosarum* bv. *viciae* share a common basic structure (Fig. 1). They are β,1-4-linked tetra or pentamers of D-glucosamine, *N*-acylated on the terminal nonreducing residue and *N*-acetylated on the other residues: In other words, Nod factors are *N*-acylated chitin oligomers (Lerouge *et al.* 1990; Roche *et al.* 1991a; Roche *et al.* 1991b; Spaink *et al.* 1991b). Purified Nod factors from *R. meliloti* elicit root hair deformations and nodule formation specifically on alfalfa at very low concentrations (Lerouge *et al.* 1990; Truchet *et al.* 1991). The Nod factors from different rhizobial species differ by the substituents linked to the chitin oligomer backbone (see Fig. 1). For example, in *R. meliloti* the molecules are *O*-sulfated on the carbon 6 of the reducing amino sugar and may be *O*-acetylated on the carbon 6 of the terminal nonreducing end (Lerouge *et al.* 1990; Roche *et al.* 1991a; Roche *et al.* 1991b). The major *N*-acyl group is a C16 chain with two double bonds in positions 2 and 9 (Lerouge *et al.* 1990; Roche *et al.* 1991a). In *R. l.* bv. *viciae* the Nod factors that elicit nodule meristem formation on vetch are *N*-acylated by a highly unsaturated C18 chain, and they are not sulfated (Spaink *et al.* 1991b).

It has been proposed that the common *nodABC* genes determine the synthesis of a Nod factor precursor(s) and that the function of the host specific *nod* genes is to mediate the decoration of this precursor(s) to generate plant-specific signals (Faucher *et al.* 1988; Banfalvi and Kondorosi 1989; Faucher *et al.* 1989). The role that individual *nod* genes play in the synthesis of the Nod factors is now subject to much attention. The *R. meliloti nodH* and *nodPQ* host range genes have been shown to control the sulfation of the NodRm factors (Roche *et al.* 1991b). The *nodP* and *nodQ* genes are homologous to *E. coli cysD, cysN,* and *cysC* genes and they encode ATP sulfurylase and APS kinase (Schwedock and Long 1990; Schwedock 1991; Leyh *et al.* 1992). They are responsible for the production of an activated form of sulfate (PAPS). The *nodH* product, homologous to sulfotransferases, is likely to transfer sulfate

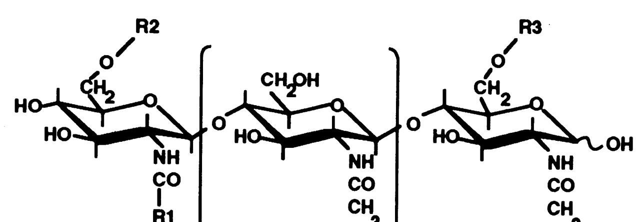

Fig. 1. Structures of *Rhizobium* lipo-oligosaccharidic Nod factors. The Nod factors from various rhizobial species share a common chitin oligomer backbone with four (n = 2) or five (n = 3) glucosamine residues. This backbone is diversely substituted in the different species. For example in *R. meliloti* the substituents are the following (Lerouge *et al.* 1990; Roche *et al.* 1991a): R1 = C16:2 (2,9), R2 = CH_3CO or H, and R3 = SO_3H; and in *R. leguminosarum* bv. *viciae* (Spaink *et al.* 1991b): R1 = C18:4 (2, 4, 6, 11) or C18:1 (11), R2 = CH_3CO, and R3 = H.

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from PAPS to the NodRm lipo-oligosaccharides (Roche *et al.* 1991b). In *R. leguminosarum* the *nodFE* host range gene products, homologous, respectively, to *E. coli* acyl carrier protein and β-ketoacyl synthase, control the synthesis of the specific highly unsaturated fatty acid moiety of the Nod factors (Spaink *et al.* 1991b). The *nodL* gene is involved in the *O*-acetylation of the terminal nonreducing glucosamine residue (Spaink *et al.* 1991b) (Fig. 1). In contrast, the biochemical role of the common *nodABC* gene products in the synthesis of the Nod factor "core" is unknown. An *R. l.* bv. *viciae* strain cured of the pSym plasmid and carrying only the regulatory *nodD* gene and the *nodABC* genes secretes lipo-oligosaccharide Nod factors similar to those produced by the wild-type *R. leguminosarum* strain with only two differences: The terminal nonreducing glucosamine residue is not substituted by an *O*-acetate group and the fatty acid chain is mono-unsaturated (Spaink *et al.* 1991b).

A search of the GenBank database, using the FASTA program for protein sequence comparisons (Pearson 1990), did not reveal any protein of known function significantly homologous to NodA and NodB. In contrast, a significant homology was found between NodC and yeast chitin synthases. This homology was independently detected by two other groups (Bulawa 1992; Atkinson and Long 1992). Using the Multalin program (Corpet 1988), we aligned the amino acid sequences of NodC proteins from *Rhizobium meliloti, R. l.* bv. *viciae, R. l.* bv. *phaseoli, R. fredii, Bradyrhizobium* sp. *Parasponia,* and *Azorhizobium caulinodans* and the four chitin synthase sequences presently available, three from *Saccharomyces cerevisiae* (Chs1, Chs2, and Cal1) and one from *Candida albicans* (canChs1). The three chitin synthases of *S. cerevisiae* play different roles in the yeast cell cycle (Bulawa and Osmond 1990; Shaw *et al.* 1991). Whereas the two Chs1 and Chs2 enzymes are activated by proteolytic cleavage, the Cal1 protein is not (Valdivieso *et al.* 1991). Figures 2 and 3 illustrate the alignments of the various proteins. The NodC proteins are highly conserved (53–70% amino acid identity between *R. meliloti* NodC and the NodC proteins from *R. l.* bv. *viciae, R. l.* bv. *phaseoli, R. fredii, B.* sp. (*Parasponia*), and *A. caulinodans*) (Rossen *et al.* 1984; Vasquez *et al.* 1991; Krishnan and Pueppke 1991; Scott 1986; Goethals *et al.* 1989). Chs1 and Chs2 are very homologous to each other (42% identity in the 650 amino acids of the

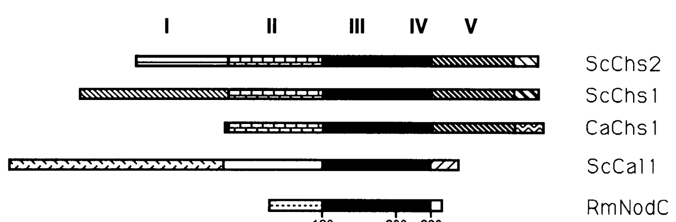

Fig. 2. Scheme of the overall sequence alignment of the NodC protein of *R. meliloti* (RmNodC) (Jacobs *et al.* 1985) with yeast chitin synthases. ScChs1 (Bulawa *et al.* 1986), ScChs2 (Silverman 1989), and ScCal1 (Valdivieso *et al.* 1991) are chitin synthases from *Saccharomyces cerevisiae* and CaChs1 (Au-Young and Robbins 1990) from *Candida albicans.* Roman numbers represent protein domains. The black boxes represent the membrane-spanning domains (domain IV). Homologous domains are represented by identical patterns.

C-terminal region) (Silverman 1989) and to the *C. albicans* chitin synthase canChs1 (37% amino acid identity between Chs1 and canChs1) (Au-Young and Robbins 1990) (Fig. 3). Chs1, Chs2, and canChs1 are homologous to Cal1, but the degree of homology between Chs2 and Chs1 or canChs1 and Chs1 is higher than that observed among any of the three and Cal1 (no more than 25% identity over 190 amino acids between Cal1 and Chs2) (Valdivieso *et al.* 1991). Cal1 is the chitin synthase most homologous to NodC proteins (30% identity over 200 amino acids between *R. meliloti* NodC and Cal1). The region of strongest homology between Cal1 and NodC is also the most conserved between the different yeast chitin synthases and between the different NodC proteins (Fig. 3).

Figure 2 shows a comparison of the various domains of the proteins. Domain I corresponds to the N-terminal part of the chitin synthases. It is not conserved in the different proteins and is absent in canChs1. In the case of Chs1 and Chs2 it has been shown that this region is not required for chitin synthase activity (Bulawa *et al.* 1986; Silverman 1989). Domain II is conserved between Chs1, Chs2, and canChs1 but shows only a very weak homology to Cal1 sequence and no homology to NodC proteins. Domain III is strongly conserved in all chitin synthases and NodC proteins. Domain IV corresponds to the highly hydrophobic transmembrane region present in all the studied proteins. Domain V, which represents the C-terminal part of the proteins, is partly conserved between Chs1, Chs2, and canChs1. The Cal1 and NodC gene products are not homologous in this domain. The alignment shown in Figure 3 highlights the fact that the sequences of domain III, which are highly conserved between the different chitin synthases, are also conserved in the NodC proteins. These results suggest that the conserved domains are involved in the creation of the β-1,4 linkage between sugar residues. This is also supported by the observation that NodC proteins also share homology with the catalytic subunit of another β-1,4-ligase, cellulose synthase from the Gram-negative bacterium *Acetobacter xylinum* (E. M. Atkinson and S. R. Long 1992). In contrast, sequence comparisons of the NodC products with chitinases did not reveal extensive homologies. In Table 1 FASTA alignment scores show that the *R. meliloti* NodC protein is clearly more closely related to chitin synthases (of eucaryotic origin) than to chitinases (of both procaryotic or eucaryotic origin). These findings suggest that NodC specifies the production of the chitin oligomers by β-1,4 synthesis from *N*-acetyl-D-glucosamine monomers rather than by degradation of chitin polymers. This is in agreement with the observation that chitin polymers have not yet been found in bacteria (Watanabe *et al.* 1990). Recently, the product of the *nodM* gene, which is present in *R. leguminosarum* and *R. meliloti,* was found to be homologous to the *E. coli* glucosamine synthetase and to exhibit a glucosamine synthetase activity (Baev *et al.* 1991; Downie *et al.* 1991). It could provide precursors for the synthesis of chitin oligomers.

NodC and chitin synthases are transmembrane proteins. Protease treatment and immunolocalization experiments indicate that NodC is located in the outer membrane (John *et al.* 1988). In contrast, other Nod proteins that are involved in Nod factor synthesis, such as NodE and NodL,

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ScChs2    435  .......TVVTLVDVG TRLNNTAIYR LWKVFDMDSN VAGAAGQIKT MKGKWGLKLF NPLVASQNFE YKISNILDKP
CaChs1    233  .......NVIVLLDVG TKPDNHAIYN LWKAFDRDSN VAGAAGEIKA MKGKGWINLT NPLVASQNFE YKLSNILDKP
ScChs1    596  .......NIVTLLDAG TMPGKDSIYQ LWREF.RNPN VGGACGEIRT DLGKRFVKLL NPLVASQNFE YKMSNILDKT
ScCal1    763  .......ETVLMVDAD TKVFPDALTH MVAEMVKDPL IMGLCGETKI ..ANKAQSWV TAIQ...VFE YYISHHQAKA
RlvNodC   133  .......DLILNVDSD TTIAPDVVSK LAHKM.RDPA VGAAMGQMKA ..SNQADTWL TRLI...DME YWLACNEERA
RfNodC    134  .......DLVLNVDSD TTIAPDVVTK LALKM.YSPA VGAAMGQLTA ..SNRSDTWL TRLI...DME YWLACNEERA
RmNodC    134  .......DLVLNVDSD STIAFDVVSK LASKM.RDPE VGAVMGQLTA ..SNSGDTWL TKLI...DME YWLACNEERA
AcNodC    132  .......DLILNVDSD TVIDKDVVTK LASSM.RAPN VGGVMGQLVA ..KNRERSWL TRLI...DME YWLACNEERI
                            *                              *                         * *

ScChs2         LESVFGYISV LPGALSAYRY RALKNHEDGT GPLRSYFLGE ........TQ EGRDHDVFTA NMYLAEDRIL CWELVAKRDA
CaChs1         LESLFGYISV LPGALSAYRY IALKNHDDGT GPLASYFKGE DLLCSHDKDK ENTKANFFEA NMYLAEDRIL CWELVSKRND
ScChs1         TESNFGFITV LPGAFSAYRF EAVRGQ.... .PLQKYFYGE IM........ ENEGFHFFSS NMYLAEDRIL CFEVVTKKNC
ScCal1         FESVFGSVTC LPGCFSMYRI KSPKGSDGYW VPVLA..... NPDIVERYSD NVTNTLHKKN LLLLGEDRFL SS.LMLKTFP
RlvNodC        AQARFGAVMC CCGPCAMYRR SA........ .......... MLSLLDQYET Q....LYRGK PSDFGEDRHL TI.LMLS..A
RfNodC         AQARFGAVMC CCGPCAMYRR SA........ .......... LLLLLDKYET Q....LFRGR PSDFGEDRHL TI.LMLN..A
RmNodC         AQSRFGAVMC CCGPCAMYRR SA........ .......... LASLLDQYET Q....LFRGK PSDFGEDRHL TI.LMLK..A
AcNodC         AQSRFGSVMC CCGPCAMYRR SA........ .......... ITPLLAEYEH Q....TFLGR PSNFGEDRHL TI.LMLK..A
                   **       *    **                                                   *** *

ScChs2         KWVLKYVKEA TGETDVPEDV SEFISQRRRW LNGAMFAAIY AQLHFYQIWK TKHSVVRKFF LHV.EFLYQF......   644
CaChs1         NWVLKFVKLA TGETDVPETI AEFLSQRRRW INGAFFAALY SLYHFRKIWT TDHSYARKFW LHVEEFIYQL......   451
ScChs1         NWILKYCRSS YASTDVPERV PEFILQRRRW LNGSFFASVY SFCHFYRVWS SGHNIGRKLL LTV.EFFYLF......   799
ScCal1         KRKQVFVPKA ACKTIAPDKF KVLLSQRRRW INSTVHN.LF ELVLIRDL.C GTFCFSMQFV IGIELIGTMV......   968
RlvNodC        GFRTEYVPSA IAATVVPDTM GVYLRQQLRW ARSTFRDTLL ALPVLPGL.. ......DRY. LTLDAIGQNV......   311
RfNodC         GFRTEYEPDA IAATVVPNSM GAYLRQQLRW ARSTFRDTLL ALRLLPGL.. ......DRY. LTLDVIGQNL......   312
RmNodC         GFRTEYVPDA IVATVVPDTL KPYLRQQLRW ARSTFRDTFL ALPLLRGL.. ......SPF. LAFDAVGQNI......   312
AcNodC         GFRTGYVPSA VARTLVPDG. SPYLRQQLRW ARSTYRDTAL ALRIKKNL.. ......SKY. ITFEICAQNL......   309
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Fig. 3. Partial sequence alignment (domain III) of rhizobial NodC proteins with yeast chitin synthases. NodC conceptual proteins from *Rhizobium leguminosarum* bv. *viciae* (Rlv) (Rossen *et al.* 1984), *R. fredii* (Rf) (Krishnan and Pueppke 1991), *R. meliloti* (Rm) (Jacobs *et al.* 1985), and *A. caulinodans* (Ac) (Goethals *et al.* 1989). Symbols and sources of chitin synthases are as in Figure 2. The multialignment was computed by MULTALIN (Corpet 1988). Amino acids were grouped as follows: (VILM) (FYWH) (DENQ) (AST) (KR) (PG) (C). The amino acids that are conserved between NodC and chitin synthases are indicated by black boxes. Amino acids identical in all compared sequences are indicated by stars.

Table 1. Sequence comparison of *Rhizobium meliloti* NodC with chitin synthases and chitinases

Enzymes	Source	Scores[a] init1	initn	Opt	References
Chitin synthases					
Chs1	*Saccharomyces cerevisiae*	43	62	78	Bulawa 1986
Chs2	*S. cerevisiae*	66	89	74	Silverman 1989
Cal1	*S. cerevisiae*	65	185	144	Valdivieso 1991
canChs1	*Candida albicans*	54	70	78	Au-Young 1990
Chitinases	*Serratia marcescens*	27	27	28	Jones 1986
	Streptomyces erythreus	25	25	42	Kamei 1989
	Cucumber	34	34	41	Metraux 1989
	Potato	35	35	36	Gaynor 1989

[a] The FASTA program was used to compare the homology of *R. meliloti* NodC with chitin synthases and chitinases. *init1*: initial score; *initn*: initial similarity score; Opt: optimized score (Pearson 1990).

are thought to be located into cytoplasmic membranes (Spaink *et al.* 1991a; Spaink *et al.* 1991b), and the NodAB proteins are predicted to be cytosolic (Schmidt *et al.* 1986). Clearly more biochemical and physiological work is required to understand the significance of the cellular compartmentalization of the various Nod enzymes. Another question concerns the control of the length of the oligosaccharide chain. The rhizobium *nodABC* genes determine the synthesis of tetra- and penta-oligomers of chitin, whereas the yeast chitin synthases are responsible for the synthesis of long chitin polymers. Does NodC itself control the length of the oligomers or are the *nodAB* gene products involved in this process? Alternatively, the NodAB proteins could be involved in the *N*-acylation of the chitin oligomer, for example, by specifying the replacement of a *N*-acetyl group by a *N*-acyl chain on the terminal nonreducing glucosamine residue. The homology found between NodC and chitin synthases allows working hypotheses for the functions of NodC and NodAB to be proposed. Further experiments are in progress to test these hypotheses.

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