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*Research Note*

# Carbamoylation of Azorhizobial Nod Factors Is Mediated by NodU

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**Lipochitooligosaccharides (LCOs) synthesized by *Azorhizobium caulinodans* ORS571 are substituted at the nonreducing-terminal residue with a 6-*O*-carbamoyl group. LCO biosynthesis in *A. caulinodans* is dependent on the *nodABCSUIJZnoeC* operon. Until now, the role of the nodulation protein NodU in the synthesis of azorhizobial LCOs remained unclear. Based on sequence similarities and structural analysis of LCOs produced by a *nodU* mutant, a complemented *nodU* mutant, and *Escherichia coli* DH5α expressing the *nodABCSU* genes, NodU was shown to be involved in the carbamoylation step.**

Nodulation of leguminous plants by *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*, collectively called rhizobia, is controlled by bacterial signals called Nod factors (reviewed by Heidstra and Bisseling 1996; Long 1996; Spaink 1996; Mergaert et al. 1997c). The synthesis of Nod factors is dependent on nodulation (*nod*, *nol*, *noe*) genes that are induced by flavonoids, phenolic compounds exuded by the plant, and a bacterial transcription activator, NodD (reviewed by Schultze et al. 1994). Nod factors are lipochitooligosaccharides (LCOs) consisting of a chitin backbone that is N-acylated at the nonreducing-terminal residue. The two terminal residues may contain strain-specific substitutions, some of which contribute to host range specificity (reviewed by Kamst et al. 1998). Methyl, carbamoyl, and acetyl residues are often found at the nonreducing-terminal residue, and sulfate and acetylated or methylated fucosyl groups belong to the major modifications at the reducing-terminal residue. The LCO backbone is synthesized by the cooperative action of the nodulation proteins NodA, NodB, and NodC. NodA is an acyl transferase that transfers an acyl group to chitooligosaccharides synthesized by NodC and deacetylated at the nonreducing-terminal residue by NodB (reviewed by Mergaert et al. 1997c; Kamst et al. 1998). Modifications at the nonreducing- and reducing-terminal residues are mediated by other nodulation proteins. For example, Bloemberg et al. (1995) proved that NodL of *Rhizobium leguminosarum* is an acetyl transferase that prefers chitooligosaccharide substrates, which are de-N-acetylated at the nonreducing-terminal residue.

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LCOs synthesized by *Azorhizobium caulinodans* ORS571, a microsymbiont of *Sesbania rostrata*, are mainly pentamers carrying vaccenic, palmitic, or stearic acid (Mergaert et al. 1997a). The nonreducing-terminal residue of the LCOs is N-methylated and 6-O-carbamoylated (Mergaert et al. 1993). The reducing *N*-acetylglucosamine can be D-arabinosylated, L-fucosylated, or D-arabinosylated and L-fucosylated (Mergaert et al. 1997a). The synthesis of azorhizobial LCOs is dependent on the *nodABCSUIJZnoeC* operon and the *nolK* operon. NodS is a methyl transferase involved in the methylation of the nonreducing-terminal residue (Geelen et al. 1995). NolK is implicated in the synthesis of GDP-fucose, utilized by the fucosyl transferase NodZ as a fucosyl donor (Mergaert et al. 1996, 1997b). Here, we extend the characterization of azorhizobial *nod* genes and show that the presence of a carbamoyl group at the nonreducing-terminal residue of LCOs produced by *A. caulinodans* is correlated with the presence of the *nodU* gene.

Previously, NodU of *A. caulinodans* (Fig. 1) has been shown to have a high similarity with NodU proteins of *Rhizobium fredii*, *R. tropici*, and *Bradyrhizobium japonicum* (Waelkens et al. 1995) and with NolO of *B. japonicum* (Luka et al. 1993). Upon re-examination of the azorhizobial NodU sequence, we confirmed these results, but, in addition, a significant similarity was found with NodU (54%) and NolO (25%) of *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997) and with CmcH (34%) of *Nocardia lactamdurans* (Coque et al. 1995; Fig. 2). CmcH is involved in the conversion of deacetylcephalosporin C into cephamicin C, a carbamoylated β-lactam antibiotic. By an in vitro assay, CmcH was shown to be a carbamoyl transferase (Coque et al. 1995). Because of this similarity, NodU of *A. caulinodans* seemed to be a good candidate to carry out the carbamoylation of azorhizobial Nod factors. Moreover, analysis of Nod factors produced by mutant and complemented mutant strains of *Rhizobium* sp. strain NGR234 showed that *nodU* of this strain was involved in the 6-O-carbamoylation (Jabbouri et al. 1995) and that *nolO* was required for the 3- or 4-O-carbamoylation of the nonreducing-terminal residue (Jabbouri et al. 1998).

*nodU* belongs to the *nodABCSUIJZnoeC* operon (Fig. 1) and its transcription is controlled by the flavonoid-inducible *nodA* promoter (Geelen et al. 1993). To obtain evidence for the proposed function of NodU, a chromosomal Tn*5* insertion mutant, ORS571-1.31U, was constructed (Geelen et al. 1993). To ensure that an effect on the LCO synthesis is caused by the mutation in *nodU* rather than by a polar influence of the Tn*5* insertion on the downstream-located genes, ORS571-1.31U was complemented with *nodU*. *nodU* was cloned behind the *lacZ* promoter in the plasmid pBBR1MCS (Kovach et al. 1994) by ligation of a 1,850-bp *Xho*I-*Psh*AI fragment (Fig. 1) in a vector digested with *Xho*I-*Sma*I. This plasmid, pBBRNU, was introduced into ORS571-1.31U by triparental mating (Ditta et al. 1980) resulting in the strain ORS571-1.31U (pBBRNU). Nod factors of both strains were prepared and purified as described by Mergaert et al. (1997a). The vaccenoylated (C18:1) and palmitoylated (C16:0) LCOs were separated from the stearoylated (C18:0) LCOs and analyzed by fast atom bombardment-mass spectrometry (FAB-MS).

A FAB-MS spectrum of the first fraction produced by ORS571-1.31U and ORS571-1.31U(pBBRNU) is shown in Figure 3A and B, respectively. The stearoylated fraction contained the same Nod factor population but the masses were two units higher (data not shown). The spectrum of LCOs synthesized by ORS571-1.31U(pBBRNU) showed three molecular ions with *m/z* 1287, 1313, and 1459 (Fig. 3B) corresponding also to LCOs produced by the wild-type strain (Mergaert et al. 1997a). The LCOs all carry a methyl and a carbamoyl group at the nonreducing-terminal residue and they are palmitoylated (*m/z* = 1287) or vaccenoylated (*m/z* = 1313 and 1459). The reducing-terminal residue contained no substitutions (*m/z* = 1287 and 1313) or an L-fucosyl residue (*m/z* = 1459) (Mergaert et al. 1997a). Ions at *m/z* 1092, 889, 686, and 483 are characteristic fragments originating from the cleavage of glycosidic bonds. The first fragmentation resulting in the ion with *m/z* = 1092 is caused by the loss of GlcNAc-OH and three other consecutive losses of GlcNAc led to the peaks with *m/z* 889, 686, and 483 (Fig. 3B). The mass of the latter molecule corresponded to the nonreducing-terminal residue that carries an *N*-methyl, a 6-*O*-carbamoyl, and a vaccenoyl group (Mergaert et al. 1993, 1997a). The FAB-MS spectrum of LCOs produced by ORS571-1.31U (Fig. 3A) is similar to that of ORS571-1.31U(pBBRNU) (Fig. 3B). Three major molecular ions were observed with *m/z* 1244, 1270, and 1416 (Fig. 3A). However, the *m/z* values of these three molecular ions were 43 mass units lower than those of the corresponding ions of ORS571-1.31U(pBBRNU). This mass difference is due to the absence of a carbamoyl residue, which suggested that ORS571-1.31U cannot synthesize carbamoylated LCOs. Cleavage of glycosidic bonds of molecular ions with *m/z* 1270 and 1416 led to the fragments with *m/z* 1049, 846, 643, and 440 (Fig. 3A). As the mass of the nonreducing-terminal residue is 43 units lower than the mass of a similar nonreducing-terminal residue, but substituted with a 6-*O*-carbamoyl residue, it is obvious that LCOs synthesized by ORS571-1.31U lack the carbamoyl group. Introduction of pBBRNU into the *nodU* mutant restored the carbamoylation completely, indicating that *nodU* rather than other downstream-located genes are involved in the carbamoylation. The results regarding the role of NodU in LCO synthesis in *A. caulinodans* confirmed the results reported for a *nodU* mutant of *Rhizobium* sp. strain NGR234 (Jabbouri et al. 1995).

ORS571-1.31U as well as ORS571-1.31U(pBBRNU) could not produce arabinosylated LCOs (*m/z* = 1445 and 1591) (Fig. 3A and B) and the amount of fucosylated LCOs was significantly lower than in the wild-type strain (Fig. 3A and B; Mergaert et al. 1997a). This is due to a polar effect of the Tn*5* insertion on the downstream-located genes, which are involved in the fucosylation and/or arabinosylation (Mergaert et al. 1996). However, a minor fraction was still fucosylated, which may be explained by a household fucosyl transferase activity (Mergaert et al. 1996).

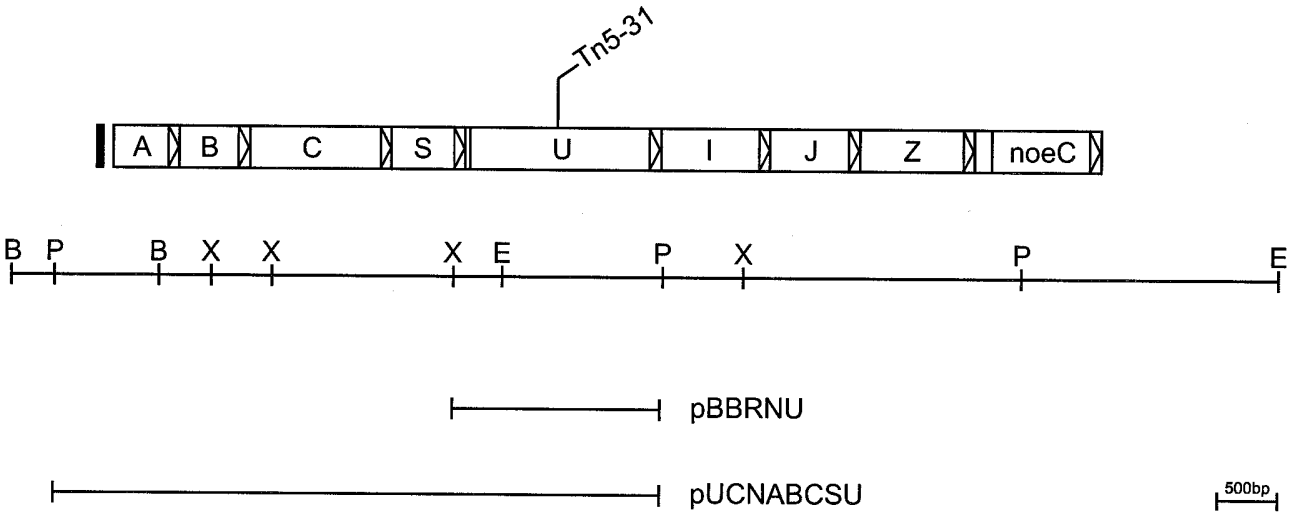


**Fig. 1.** Physical map of the *nodABCSUIJZnoeC* operon of *Azorhizobium caulinodans*. Genes *nodABCSUIJZ* and *noeC* are presented as boxes with arrowheads showing transcription direction. Black vertical box, upstream of the *nodA* gene, corresponds to the *nod* box promoter region. DNA regions present in plasmids constructed during this study are indicated. Position of the Tn*5* insertion introduced into *nodU* is designated by Tn*5*-31. B, *Bam*HI; E, *Eco*RI; P, *Psh*AI; X, *Xho*I.

As CmcH had been shown to be a carbamoyl transferase that uses carbamoyl phosphate as a carbamoyl donor, we tried to complement ORS571-1.31U with the *cmcH* gene of *N. lactamdurans*. A 2,600-bp *Pst*I fragment was cloned in a *Pst*I-digested pBBR1MCS vector, resulting in the plasmid pBBRCMCH. This plasmid was introduced into ORS571-1.31U by triparental mating. The FAB-MS spectra of LCOs produced by ORS571-1.31U(pBBRCMCH) and ORS571- 1.31U were identical (data not shown), indicating that *cmcH* cannot complement an azorhizobial *nodU* mutant. This observation suggests that carbamoylation in *A. caulinodans* demands a specialized carbamoyl transferase with a high substrate specificity. Such a substrate specificity has been described, for example, for NodL, an acetyl transferase (Bloemberg et al. 1995), and for NodS, a methyl transferase (Geelen et al. 1995), that both preferentially use end-de-N-acetylated chitooligosaccharides as a substrate (Bloemberg et al. 1995; Geelen et al. 1995).

Additional evidence was obtained from the analysis of LCOs produced by derivatives of *Escherichia coli* DH5α that carry different combinations of azorhizobial *nod* genes. Previously, DH5α(pUCNABCS), which expresses the *nodABCS* genes of ORS571, has been shown to produce methylated LCOs that carry acyl chains varying in length between C14 and C18

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ACNodU                          MKRCGL KLTHDGGVAV L.DGRDLVAC IEMEKLTNNE RYRRIEHTDE IALALHRS.G FQPSDIDEYI IDGWDGEVDA 100
BJNodU                          MRICGI KLTHDGAIAV VEDGR.RLFC VEQEKRGNGP RYQSVDNLDA VVFALAEH.G LNPRDIDQFV IDGWDGENES
RNNodU                          MRVCGI KLTHDGAIAV VEDGR.LVFC TEQEKRNNNS RYQEINNLDA VVAALAEN.G VNARDVDQFV IDGWDGEAES
RFNodU  MKTACFPVFR NRLPDLDRDL DRDTKRVCGI KLTHDGAIAV VEDGR.LVFC TEQEKRNNNS RYQEINNLDA VVAALAEN.G VNARDVDQFV IDGWDGEAES
RTNodU                          MRICGI KLTHDGAIAL IEDGR.LVFC IEQEKQDNNR RYQTIDNLDA IVTALAEH.G LNPSDVDQFV IDGWDGEIES
NLCmcH                          MLIVAF KPGHDGAVAA IGD.RRLLYS LESEK.DSRP RYSPI..LAT TVLDLAERLG EVP.DV..VA LGGWSDLRPN
BJNolO
RNNolO          ML CLGLSGGLSK IHENSLDLPN TFMHDGAAVL VRDGQ.VIAA VEEERLNRIK HSNKLPRRS. .IQYCLEYAG VQLSDIDCIA YYATEAFCNA
Cons.                           M  CG  KLTHDGA A    DGR L  C  E EK  N   RY  I  LD   V ALAE  G     D D    IDGWDGE

ACNodU  WVELLGAAGR VQLKVAPY.. VEKEPDRANE FT..QGFGLN ILGRDYTYKS APHVMGHIAS VYCTSPFAIF KQKA.LCLVW DGSIWPRLYE ISDGGIRFIN 200
BJNodU  QFQLLSGAVP VALKGAPY.. VERHAEGLLD SV..DGYGLL LGGEEFPYKS YPHVTGHVAS AYSTSPFASA GKPA.LCLVW DGCIFPRLYY VEPQGARLIG
RNNodU  QFKVLSGETP VILRGAPY.. VERHAEGLLD WI..GGSGLT LGDRVFSYRS YPHVTSHVAS AYCTSPFAKS GDPA.LCLVW DGCIFPRLYH VEGKRASFVK
RFNodU  RFKVLSGETP VILRGAPY.. VERHAEGLLD WI..GGSGLT LGDRVFSYRS YPHVTSHVAS AYCTSPFAKS GDPA.LCLVW DGCIFPRLYH VEGKRASFVK
RTNodU  RFQVLSGAAP VTLTGAPY.. VERHPDGLLD SL..DGSGLI LDDRVLSYKS YPHVTGHVAS AYCTSPFAKA GEAA.FCLVW GGCIFPRLYH VDGHGARFLE
NLCmcH  R......... ISYTGAGYSG IE...EPTVT TS..RFFG.. ...KEVKFFS STHERSHIYM ALGMAP..RD DSPVQTVLVW EGDV.GAFYV IDGHQ.RITR
BJNolO                                                                  MS AFAMSGFEQS ...LVFAIDG GGDFLSGLMA V.GSGTV.IA
RNNolO  MLERL..... ..LVSQSHMS IPLDAKLLLR GLLAQEFGTE VDPSRISFVS ..HHLSHAWS AFSMSGFEQS ...LILTIDG GGDFASGLLA V.GSGTE.VK
Cons.       L      V L GAPY   VE     LL      G GL         Y S  PHV  H AS AY TSPFA      A LCLVW  G I P LY  V G G

ACNodU  TL..FP...M IGHAYACAGH H..FG...PY KNADRTSWKL DLAGKLMSYM STG...TVDS RITAAIQTSY QNNLAGHSPN ALSYRRMSAN TSVALIQTHR 300
BJNodU  SL..FP...M IGHAYAAAGL H..FG...PY RQPNRSSWDL GIAGKLMAYI ELG...SVDE SIVEVFQGLY ETRSAADTEQ ARRYRENINN AEASLAVIHD
RNNodU  SL..FP...V TGQAYAAAGH Y..FG...PY KQTSRGGWDL GVAGKLMAFI ALG...SVHE RIVAVFQKLY QEHFAGDTAL ACAFRANINN SESSLAAVHD
RFNodU  SL..FP...V TGQAYAAAGH Y..FG...PY KQTSRGGWDL GVAGKLMAFV ALG...SVHV RIVAVFQKLY QEHFAGDTAL ACAFRANINN SESSLAAVHD
RTNodU  SL..FP...M IGQAYAAAGH Y..FG...PY KQPSRAGWDL GVAGKLMAYI ALS...SIDE DIVAVFEELY EEHFSGDAER ACRYRANIND AESSLIAVHD
NLCmcH  KV..QV...M SGPG.ARYSF L..FGLADPT FPTTGGKPRL NDAGKLMALA AFGDSADADA DITHVVERIL KQDSMYPAPK G.EYRDSV.. ..........
BJNolO  QLVSFPEHNS LGLFYLETIR YLGCGMFDEY .......... ....KVMGLA PYGDPDRYRE ....LFAQFY ELLDSG.... ..GYRVHLDR IGPALLRNIQ
RNNolO  PLATFPESDS LGLLYLETIK YLGYGMFDEY .......... ....KVMGLA PYGDPAPHRD ....LFEQFY ELLDNG.... ..GYRIYLDR IGPTLLRSIE
Cons.    L  FP      G AYA AG  Y  FG   PY     R  W L   AGKLMA     G         I  VF   Y      G     A  YR          L   H

ACNodU  FFEEIGV.LV AGAPEHDILA TFHYFVERLL IETLRHELA. .RAGRNMS.. ...RNLCISG GCGLNIKWNS ALRSSGLFRD VWVSPFPNDS GSAIGAACSA 400
BJNodU  FFESSALRLK AKRAE.DVLA SFHVFLERLL ....VKEIAM V.LLRHSS.L PGARNLCIAG GCGLNIKWNS ALRATGLFDD VWVPPFPNDS GSAIGAACGA
RNNodU  FFAASALQLG AEAPE.DVLA SSHFFLERLL ....VDEMA. .NALQHHP.L PGARNLCIAG GCGLNIKWNS ALRETGLFDS VWVPPFPNDS GSAIGAACCE
RFNodU  FFAASALQLG QRRPK.TCL. ..H....RLI FSSNVSSLTK WRTLQHHP.L PGARNLCIAG GCGLNIKWNS ALRQTGLFDS VWVPPFPNDS GSAIGAACCE
RTNodU  FFDASVVRLE DKAPE.NVLA SFHSFLELLL ....VREMAL ..AMQRHS.L PGPRNLCIAG GCGLNIKWNS ALRETGLFDA VWVPPFPNDS GSAIGAACSA
NLCmcH  LYNA.....G VESPECKIAA AL..LTERL. FETFA.EVA. .....RQE.M PEGSPLYISG GCGLNCDWNS LWAQLGHFSS VFVAPCTNDS GSALGTAIDA
BJNolO  V.RRKGMPFT QQ..HRDVSA SLQEALERIV F......... .HTLRHHRKA TGMTRLCLAG GVAHNCTLNG KLLYSGLFDD IFVQPAAHDA GCALGAAL..
RNNolO  V.RRKGMPFT QQ..HKDLSA SLQEALERIV F......... .HVLRHHSEI TGIKRLSLAG GVAHNCTLNG KLLRSGIFQD IFVQPAAHDA GCALGAAL..
Cons.   FF      L     PE D LA S H  LERL        E A     L HH    PG RNLCIAG GCGLNIKWNS ALR  GLFD  VWV PFPNDS GSAIGAAC

ACNodU  LVAND.G.LV PIN..WSVFS G.PHLVKSTP .DAN...WRG SACEL...SE LAAL....LA DGEPVVFLAG RAELGPRALG ARSILAPASD RSMKDRLNAA 500
BJNodU  MAAQD.G.FE PLE..WSVYS G.PALQESEV .PPD...WEA APCSL...PE LASI....LA DNKPVIFLSG CAGLGPRALG GRSILAAPTS PEMKDHLNDI
RNNodU  IVAQQ.G.FV PLD..WSVYS G.PSLQAGKV .PAG...WHA SPCSI...SE VAAI....LA SNKPVVFLSG RSELGPRALG GRSILAAATS PEMKDHLNEI
RFNodU  IVAQQ.G.FV PLD..WSVYS G.PSLQDRQV .PAG...WHA SPCSI...SE VAAI....LA SNKPVVFLSG RTELGPRALG GRSILAAATS PEMKDHLNEI
RTNodU  MAAHE.G.FV PLE..WSVYS G.PALKNGDA .PPG...WEA APCSI...LE LATI....LA SNKPVVFLAG RAELGPRALG GRSILAAATS PQMKDYLNEV
NLCmcH  LTTFT.G.DP HVD..WSVYS GLEFVTDTQP DPAR...WTS RPLEH...DE LSGA....LA GGRVVAWVQG RWEIGPRALC NRSLLAEPFG AVTRDRLNEI
BJNolO  MVSSELGRPA PRERLPDVYW G.PDLGNEQA TELELNAW.S GHLDIQRSDD IASSAADWMA NGAVIGWVQG RSEFGPRALG NRSILADPRP AENKDRINAV
RNNolO  MMSNELGQSA PRERLQEVYW G.PDLGSDRA VEQELIAW.G GHIEIERCDD VASRAAEWIA DGAVIGWVQG RSEFGPRALG NRSILADPRP ATNKDRINAI
Cons.     A   G    P    WSVYS G P L       P     W    PC I    E  A      LA    PV FL G R ELGPRALG  RSILA       MKD LN I
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**Fig. 2.** Alignment between NodU and NolO proteins of different rhizobia and CmcH of *Nocardia lactamdurans*. The NodU sequences from *Azorhizobium caulinodans* (ACNodU), *Bradyrhizobium japonicum* (BJNodU), *Rhizobium* sp. strain NGR234 (RNNodU), *R. fredii* (RFNodU), and *R. trifolii* (RTNodU), of NolO from *B. japonicum* (BJNolO) and *Rhizobium* sp. strain NGR234 (RNNolO), and the sequence of CmcH of *N. lactamdurans* (NLCmcH) were aligned with the Wisconsin Package version 9.1 (Genetics Computer Group [GCG], Madison, Wisc.). Below the protein sequence alignment, a consensus line (>50%) is integrated (Cons.). Dots indicate gaps introduced to allow a better alignment.

*Continued on next page.*

(Mergaert et al. 1995). A plasmid, pUCNABCSU, was constructed by cloning of a 5,300-bp *Psh*AI fragment, covering the *nodABCSU* genes (Fig. 1), into pUC18 digested with *Sma*I (Fernández-López et al. 1996). LCOs produced by DH5α(pUCNABCSU) were prepared, purified, and analyzed by FAB-MS (Fig. 3C) and collision-induced dissociation (CID)-MS (Fig. 3D) as described by Mergaert et al. (1995). The fraction, the FAB-MS spectrum of which is presented in Figure 3C, contained LCOs with *m/z* 1244, 1270, 1287, and 1313. The former two molecular ions, corresponding to products of ORS571- 1.31U (Fig. 3A), are methylated LCOs carrying a palmitoyl or a vaccenoyl chain, respectively, and were also produced by DH5α(pUCNABCS) (Mergaert et al. 1995). However, the latter two molecular ions corresponded to products of ORS571-1.31U(pBBRNU) and were 43 mass units higher, which is indicative for the presence of a carbamoyl group. Characteristic fragments originating from the molecular ions with *m/z* = 1244 are indicated with *m/z* 1023, 820, 617, and 414 and show the Nod factor nature of these products (Fig. 3C). That only part of the total amount of the LCOs is carbamoylated could be due to a deficiency of carbamoyl donor in *E. coli*.

The CID-MS spectrum of the molecular ion with *m/z* = 1287 of LCOs produced by DH5α(pUCNABCSU) showed fragments with *m/z* 1066, 863, 660, and 457 (Fig. 3D). The fragment with *m/z* = 1066 was caused by the loss of GlcNAc-OH and the other fragments formed a series separated by 203 mass units ending at *m/z* 457. This *m/z* corresponded to the mass of the nonreducing-terminal residue of the chitooligosaccharide that is N-methylated, N-acylated by palmitic acid, and 6-O-carbamoylated (Mergaert et al. 1997a). So, indeed, DH5α(pUCNABCSU) is able to synthesize carbamoylated LCOs in contrast to DH5α(pUCNABCS) (Mergaert et al. 1995), which supports the previously mentioned predictions about the function of NodU.

The NodU protein is 61.8 kDa in size and has three potentially transmembrane α-helices as predicted by the TMpred program (Hofmann and Stoffel 1993; Fig. 4). With the PSORT software (Nakai and Kanehisa 1991), it was predicted that NodU would lack a leader peptide. Together., these observations suggest that the protein may appear in the cytoplasm, probably associated with the inner membrane. These characteristics were also found for the azorhizobial NodZ protein (Mergaert et al. 1996) and support the hypothesis that LCOs might be synthesized in a "Nod factor factory" consisting of a multienzyme complex anchored in the inner membrane, as postulated by Mergaert et al. (1997c).

In an attempt to show in vitro carbamoyl transferase activity, *nodU* was cloned in pGEX-3X (Pharmacia, Uppsala, Sweden), resulting in pGEX-3XU. In this plasmid, the *nodU* open reading frame is fused to the upstream-located glutathione-*S*-transferase (GST) open reading frame. Protein extracts of *E. coli* cultures were prepared as described by Geelen et al. (1995). The NodU-GST fusion protein remained in the membrane fraction (data not shown), in agreement with the putative membranal localization. In vitro assays were carried out with crude extracts of the membrane fraction and with partially purified fusion protein as enzyme source, and carbamoyl phosphate as a carbamoyl donor. Analogous to the substrate of the acetyl transferase NodL and the methyl transferase NodS, deacetylated chitopentaoses were used, but also chitopentaose and noncarbamoylated LCOs were included in the assay as substrates. In none of the assays that were carried out in different conditions could any in vitro carbamoyl transferase activity be detected (data not shown). This result may indicate that NodU functions only in very specific conditions, or that the NodU protein is very unstable or not correctly folded, or that it only functions in a "Nod factor factory" protein complex. In conclusion, sequence similarities, mutant and

**Fig. 2.** *Continued from previous page.*

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ACNodU  .KQREYFRPV APIC.LEDRA PEIFEPGSND .....RYMLY DHKVREGWRD RVPAIMHLDG SARVQTIART SAHP.VAKLL VEYEKLTNIP LLCNTSANAL 600
BJNodU  .KRREHFRPV VPIC.LEDRA PEIFSPGTPD .....PYMLF DHQTRANWRD KIPAVVHLDG SARLQTISRN SPHK.IAALL IEFEQLTGIP LLCTTSANLH
RNNodU  .KFREHFRPV APIC.LEDRA PDIFSPGTPD .....PYMLF DHQTKMPWQD KVPAVVHLDG SARLQTISRN SQHK.VAEVL VEYEKLTGIP LLCNTSANYH
RFNodU  .KFREHFRPV APIC.LEDRA PDIFSPGTPD .....PYMLF DHQTKMPWQD KVPAVVHLDG SARLQTISRN SQHK.VAEVL VEYEKLTGIP LLCNTSANYH
RTNodU  .KFREHFRPV APIC.LEDLA PDIFSPGTPD .....PYMLF DHQTRPEWKD KIPAVVHLDG SARLQTISRS SEHA.VTELL IEYEKLTGIP LLCNTSANLH
NLCmcH  .KQREDYRPI APVCRVEDLG .KVFHEDFED .....PYMLY FRRVRES..S GLRAVTHVDG SARVQTV.RD SGNPQMHRLL SAFAAQRGVG VLCNTSLNFN
BJNolO  VKKRETYRPF AP.SALEEDA SEFFE..LPD GTRQLPFMNF VVRVREAKGN VLGAITHVDG TARLQ*
RNNolO  VKKREGYRPF AP.SVLEEDA NEFFE..LPD SRQEFPFMNF VVPVRESKRN LLGAVTHVDG TARLQTVSRN I.NQAYWEVI NAFRKRTGVP ILLNTSFNNN
Cons.    K RE FRPV APIC LED A P IF PG PD      PYMLF DH  R  W D   PAV HLDG SARLQTISR  S H      L  E EKLTGIP LLCNTSAN

ACNodU  GRGF...FPD VASACTWGR. .....IAKVW ...AENVLWS NDVDARI.P*                                                          700
BJNodU  GRGF...FPD AAAACQWGR. .....VEHVW ...CEGMLWS KTVIKKSSPT ERLLSA*
RNNodU  GRGF...FPS AAAACEWGR. .....VEHVW ...CDGMLYR K.......PS ATA*
RFNodU  GRGF...FPS AAAACEWGR. .....VEHVW ...CDGMLYR K.......PS ATA*
RTNodU  GRGF...FPD AAAACEWGR. .....IDHVW ...CNGVLFT KERVAELAPV GVADNMKMST CPR*
NLCmcH  GEGFINRMSD LVLYCE.SRG ISDMVVGDTW YQRAEG*
BJNolO
RNNolO  VEPIVDSVAD AVTTFLTTDL DGLVVGSYLI KKRTASPEDW SRLALSLPPY SSLHQVRAFT ALDRQETVCE IRTGPSSREA VRISSELFEL LMRIDGEAPL
Cons.   GRGF   FPD AA AC WGR          VW      G L           P

ACNodU                                                    750
BJNodU
RNNodU
RFNodU
RTNodU
NLCmcH
BJNolO
RNNolO  GDILDLIAPN QNQREALLNE LRGLWEQRSV RLHPMRADSA AEPLSSPINL*
Cons.
```

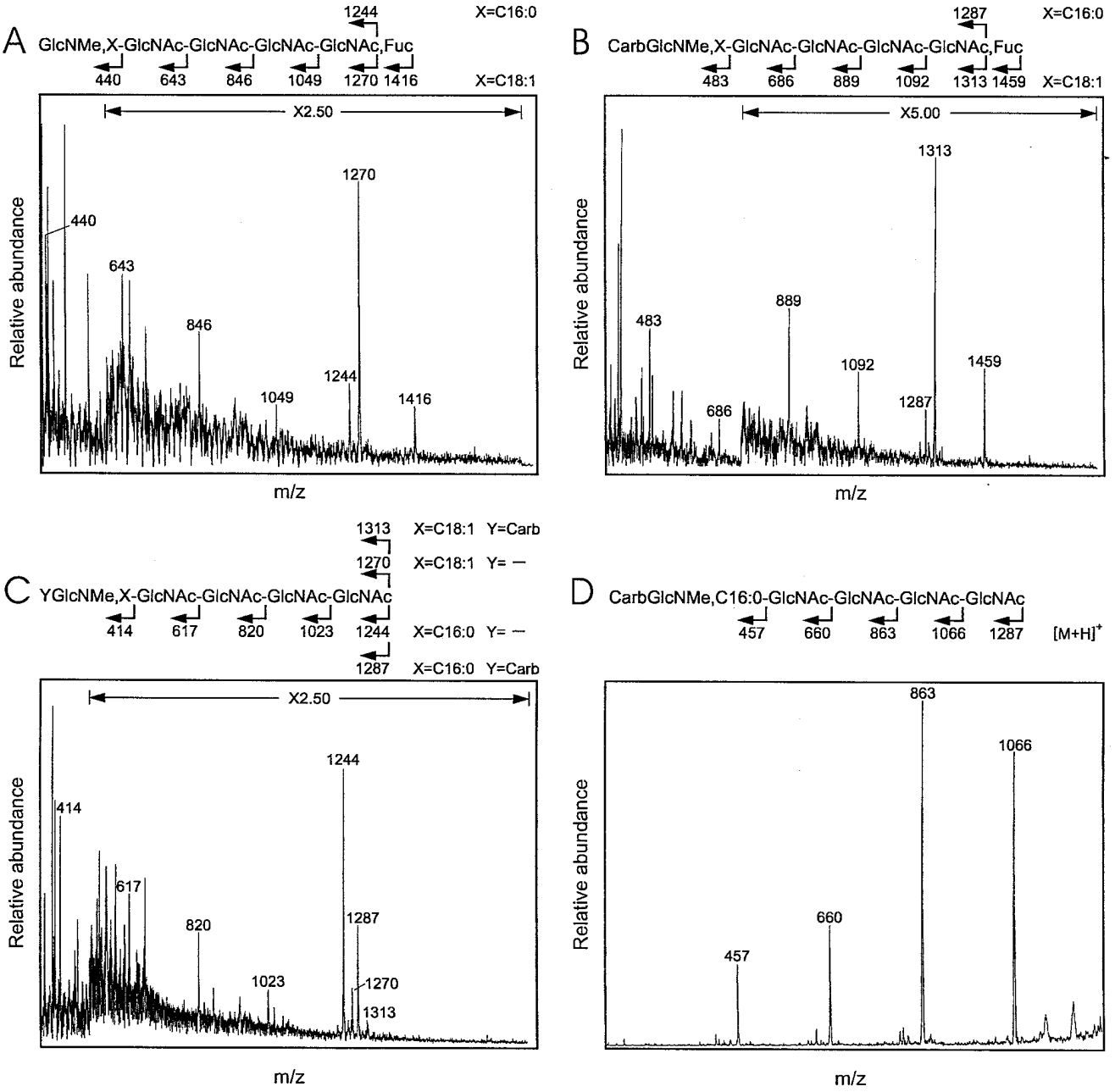


**Fig. 3.** Fast atom bombardment-mass spectrometry (FAB-MS) spectrum, collision-induced dissociation (CID)-MS spectrum, and fragmentation schemes of lipochitooligosaccharides (LCOs). FAB-MS spectrum of palmitoylated or vaccenoylated LCOs produced by **(A)** ORS571-1.31U, **(B)** ORS571-1.31U(pBBRNU), and **(C)** *Escherichia coli* DH5α(pUCNABCSU). CID-MS spectrum of a parental molecular ion with *m/z* = 1287 from **(D)** DH5α(pUCNABCSU). Abbreviations: Carb, 6-*O*-carbamoyl; Fuc, L-fucosyl; GlcNAc, *N*-acetyl-glucosamine; Me, *N*-methyl.

complementation analysis in *A. caulinodans*, and LCO production in *E. coli* provided strong support for a role of NodU in Nod factor carbamoylation.

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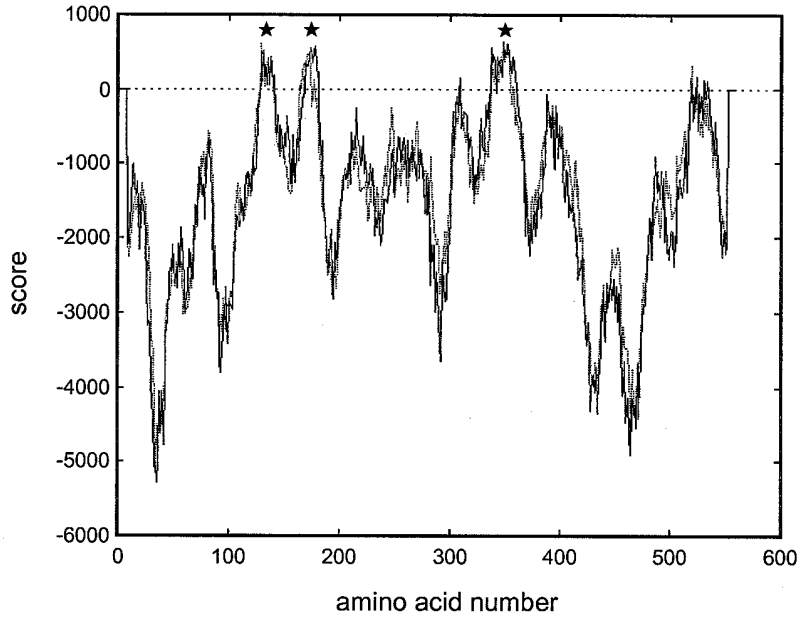


**Fig. 4.** Hydropathy profile of the predicted NodU protein from *Azorhizobium caulinodans* (according to the TMpred program). Stars indicate the three putative transmembrane α-helices.


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