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The nodulation gene *nolK* of *Azorhizobium caulinodans* is involved in the formation of GDP-fucose from GDP-mannose

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Abstract The *nolK* gene of *Azorhizobium caulinodans* is essential for the incorporation of a fucosyl group in Nod factors. A NAD(P)-binding site is present in the NolK amino acid sequence and the gene is homologous to *Escherichia coli* genes, presumably involved in GDP-fucose synthesis. Protein extracts of *A. caulinodans*, overexpressing *nolK*, have an enzyme activity that synthesizes GDP-fucose from GDP-mannose. *nolK* most probably encodes a 4-reductase performing the last step in GDP-fucose synthesis. Wild-type *A. caulinodans* produces a population of fucosylated and non-fucosylated molecules but the *nolK*-overexpressing strain produces only fucosylated Nod factors. Thus, the production of activated fucosyl donors is a rate-limiting step in Nod factor fucosylation.

Key words: Fucosylation; Nod factor; *nod* gene; *Rhizobium*

1. Introduction

Nodules are symbiotic organs formed on roots and in some cases on stems of leguminous plants. Inside the nodules, bacteria belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, or *Sinorhizobium* (collectively called rhizobia), reduce atmospheric nitrogen to ammonia. Nodule organogenesis is triggered by secreted bacterial signals, the Nod factors. Nod factors are lipo-chitooligosaccharides (LCOs) with strain-specific substitutions. The Nod factor structure plays a role in host range determination (for a recent review, see [1]).

The bacterial nodulation genes (*nod*, *nol*, and *noe*), involved in production and secretion of Nod factors, are symbiotically regulated by plant-derived flavonoids via the transcriptional activator NodD. The *nodABC* genes, common to all rhizobia, encode the enzymes for biosynthesis of the LCO backbone. Other specific nodulation genes are involved in the modification of this backbone [1].

One substitution that is often found on LCOs of tropical symbionts is a 6-O-fucosyl branch on the reducing end of the oligosaccharide [2–9]. This modification is important for nodulation on some host plants [10]. In bioassays using purified Nod factors, this substitution has also been found to be important for biological activity [9–11].

Recently, the *nodZ* gene of *Bradyrhizobium japonicum* and the *nodZ* and *nolK* genes of *Azorhizobium caulinodans* have been shown to be involved in the introduction of the fucosyl branch on the Nod factors [10,12,13]. *NodZ* encodes a fucosyl transferase that uses GDP-fucose as donor and transfers the fucosyl group to LCO precursors [12,13]. The precise function of the NolK protein is not yet determined, but inactivation of the *nolK* gene results in a complete loss of the fucosyl modification on the Nod factors of the mutant bacteria [13]. The protein shows homology with *E. coli* genes, presumably involved in the synthesis of GDP-fucose and carries a NAD(P)-binding domain. Based on these observations, the *nolK* gene is probably involved in the synthesis of the fucosyl precursor [13]. We present biochemical evidence that strongly supports this proposition.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. caulinodans and *E. coli* strains were grown at 37°C in YEB medium [14] and Luria-Bertani (LB) medium [15], respectively. Expression of *nod* genes was induced with 20 μM naringenin in *A. caulinodans* and with 1 mM isopropyl-β-D-thiogalactoside in *E. coli*. Antibiotics were used in the following concentrations for both *A. caulinodans* and *E. coli*: 100 μg/ml ampicillin (Amp), 50 μg/ml kanamycin (Km), and 50 μg/ml spectinomycin (Sp). Strains and plasmids are listed in Table 1.

2.2. Molecular and genetic methods

Cloning techniques were carried out using standard procedures [15]. A 2.3 kb *Sal*I fragment from pRG9011 (Table 1) and a 4.3 kb *Sal*I fragment from pRG9011-ΩB (Table 1) were ligated in the *Sal*I site of the cloning vector pBBR1MCS-2 (Table 1) and electroporated to DH5α. Constructs with the *nolK* gene downstream of the *lac* promoter of the vector were retained. The plasmid pBBNK carries the intact *nolK* gene (Table 1) and pBBNKΩB has an Ω-cassette inserted into the *nolK* gene (Table 1). The constructs were mobilized from DH5α to ORS571 by triparental mating [16].

2.3. In vitro assays

Protein extracts of ORS571, ORS571-4.2K, ORS571(pBBNK), ORS571(pBBNKΩB), DH5α(pBBNK), DH5α(pBBNKΩB) and ORS571(pUCNZ) were prepared as follows. Overnight cultures were diluted 10-fold in fresh medium in a total volume of 20 ml. 1 h later, *nod* gene inducer was added and 4 h after induction, the cultures were centrifuged. The bacterial pellet was washed once with 1 ml 20 mM Tris-HCl (pH 8) and then resuspended in 1 ml of the same buffer. Cells were broken by sonication.

Assays for the in vitro synthesis of GDP-fucose from GDP-mannose were carried out at 30°C for 2 h in 50 μl reaction mixtures containing 20 mM Tris-HCl (pH 8), 50 μM GDP-mannose, 50 nCi GDP-[U-^{14}C]-mannose (281 mCi/mmol; Amersham, Aylesbury, UK), and protein extract (approximately 400 μg protein). When NADH or NADPH (0.1 mM or 1 mM) was included, their addition was repeated twice during the incubation. Reactions were stopped by boiling and the precipitate was centrifuged off. The supernatant was lyophilized and the residue resuspended in 5 μl water. Nucleotide sugars were analyzed by PEI-cellulose-TLC (see below). Fucosyl transferase assays were as described [13]. In brief, 5 μl of a 1 mM Nod factor solution (containing unfucosylated Nod factors) was added and 20 μl of a protein extract of DH5α(pUCNZ). The mixture was incubated at

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Abbreviations: RP, reverse phase; TLC, thin-layer chromatography

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30°C during 15 min after which Nod factors were extracted with *n*-butanol and analyzed by RP-C18-TLC (see below).

GDP-mannose oxidoreductase activity was measured using the spectrophotometric assay of Kornfeld and Ginsburg [17].

2.4. In vivo labelling of Nod factors

Nod factors were labelled in vivo and extracted using a procedure previously described [18].

2.5. TLC analysis of radioactive compounds

Nucleotide sugars were analyzed with PEI-cellulose-TLC plates (CEL 300 PEI, Macherey-Nagel, Dürren, Germany). Prior to use, the plates were washed by migration in 10% NaCl until the solvent was 5 cm from the bottom, followed by developing twice in water. The samples (0.5 μl) were chromatographed first in 1 N acetic acid until the solvent front was 2 cm above the sample application and then in 1 N acetic acid/3 M LiCl (9/1). The migration was stopped when the solvent was approximately 15 cm high. The TLC plate was not dried between the two runs.

Nod factors were chromatographed on reverse phase octadecyl (RP-C18) TLC (Merck, Darmstadt, Germany) using water/acetonitrile (1/1) as the solvent. After chromatography, the plates were dried and radioactive compounds were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. NolK is involved in the conversion of GDP-mannose to GDP-fucose

Protein extracts of *A. caulinodans* strains were incubated with ^{14}C-labelled GDP-mannose and the formation of GDP-fucose was monitored in two ways. Firstly, reaction products were separated on PEI-cellulose-TLC. Secondly, reaction products were incubated in the presence of both unfucosylated Nod factors and extracts of *E. coli* expressing the azorhizobial *nodZ* gene. GDP-fucose, formed during the first incubation, can be transferred by NodZ to Nod factors. This transfer was detected by Nod factor extraction with *n*-butanol and RP-C18-TLC analysis.

Protein extracts of the ORS571 wild-type strain converted GDP-mannose to several new compounds amongst which compounds 'X' and 'Y' that migrated close to GDP-mannose and GDP-fucose (Fig. 1, lane 1). However, the formation of these compounds was not dependent on induction of *nod* genes (data not shown) or on NolK, as they were also formed by the *nolK* mutant ORS571-4.2K (Fig. 1, lane 2). Moreover, in the fucosyltransferase reactions, no radioactivity was transferred to the Nod factor substrate (Fig. 2, lanes 1 and 2). This indicated that no detectable amounts of GDP-fucose were formed.

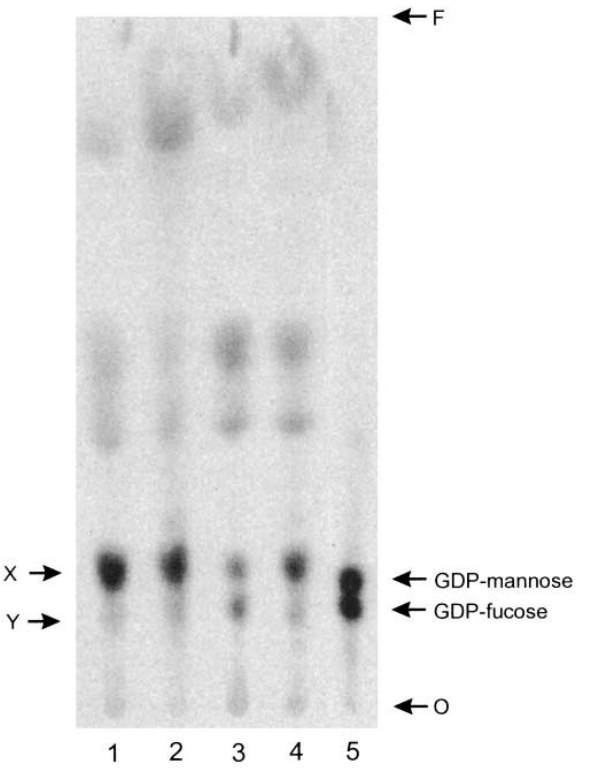

Fig. 1. PEI-cellulose-TLC analysis of nucleotide sugars. ^{14}C-labelled GDP-mannose was incubated with protein extracts of *A. caulinodans* and derivatives. After incubation, one tenth of the mixture was analyzed by PEI-cellulose TLC. The protein extracts were prepared from the strains ORS571 (lane 1), ORS571-4.2K (lane 2), ORS571(pBBNK) (lane 3) and ORS571(pBBNKΩB) (lane 4). On lane 5, GDP-[^{14}C]-mannose and GDP-[^{14}C]-fucose standard was spotted. Rf values are for GDP-mannose 0.183, for GDP-fucose 0.143, for compound X 0.197 and for compound Y 0.128. O is de origin and F the solvent front of the TLC.

Therefore, a *nolK*-overexpressing strain, ORS571(pBBNK), was constructed, carrying the *nolK* gene on the broad-host-range vector pBBR1MCS-2. The control strain ORS571(pBBNKΩB), carried an Ω-cassette in the plasmid-borne *nolK* gene.

Protein extracts of the strain ORS571(pBBNK) were capable of transforming GDP-mannose to GDP-fucose. PEI-cellulose-TLC analysis showed a clear spot comigrating with a GDP-fucose standard (Fig. 1, lane 3). The transfer of radioactivity from reaction products to Nod factors (Fig. 2, lane 3)

Table 1
Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Origin
Strain		
DH5α	*E. coli* strain used for cloning purposes	[23]
ORS571	*A. caulinodans* wild-type strain, Amp^R	[24]
ORS571-4.2K	ORS571 derivative carrying a Tn*5* insertion in *nolK*, Amp^{R}Km^R	[13]
Plasmid		
pBBR1MCS-2	broad-host-range cloning vector, Km^R	[25]
pBBNK	pBBR1MCS-2 containing *nolK*, cloned downstream from the *lac* promoter of the vector, Km^R	This work
pBBNKΩB	pBBR1MCS-2 containing *nolK* with an Ω-insertion cloned downstream from the *lac* promoter of the vector, Km^{R}Sp^R	This work
pRG9011	pBR325 plasmid carrying *nolK* on a 2.3 kb *Sal*I fragment	[22]
pRG9011-ΩB	pRG9011 with an Ω-cassette (Sp^R) cloned in the *nolK* gene	[22]
pRK2013	helper plasmid used in triparental matings	[16]
pUCNZ	pUC18 plasmid containing *nodZ* cloned downstream from the *lac* promoter	[13]

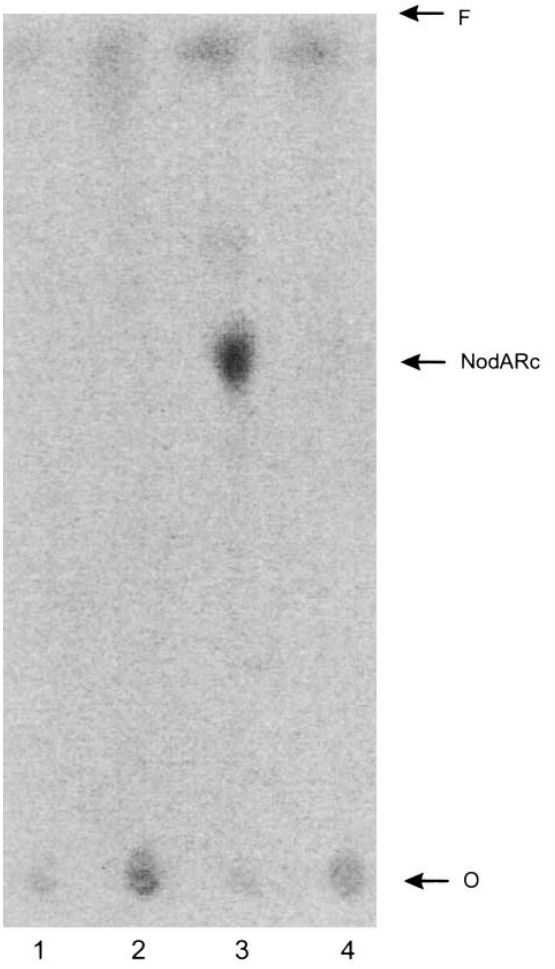

Fig. 2. RP-C18-TLC analysis of fucosyltransferase reaction products. The products of the enzymatic reactions presented in Fig. 1 were incubated with unfucosylated Nod factors and protein extracts of the strain DH5α(pUCNZ). The Nod factors were analyzed by RP-C18-TLC after the incubation. Lanes are in the same order as in Fig. 1. NodARc are the ^{14}C-labelled Nod factors. O is de origin and F the solvent front of the TLC.

indicated the presence of GDP-fucose as a substrate of NodZ action. The control strain ORS571(pBBNKΩB) displayed an identical picture as ORS571 and ORS571-4.2K (Fig. 2, lanes 1, 2, and 4), proving that GDP-fucose formation was depending on the overproduction of the NolK protein.

GDP-fucose formation by extracts of ORS571(pBBNK) was not stimulated by 0.1 mM NADH, slightly inhibited by 0.1 mM NADPH, and strongly inhibited by 1 mM NADPH or 1 mM NADH (data not shown). This observation was unexpected because a stoichiometric amount of NAD(P)H is required for formation of GDP-fucose [19]. Probably, the extracts contained sufficient amount of NAD(P)H. Inhibition with high NADH concentrations was also reported for a mammalian GDP-fucose-synthesizing system [20].

Extracts of *E. coli* DH5α(pBBNK) were unable to form GDP-fucose. Mixing DH5α(pBBNK) and ORS571-4.2K extracts resulted in GDP-fucose formation (data not shown). These results demonstrated that DH5α lacks the pathway for conversion of GDP-mannose to GDP-fucose and that additional enzymes, other than NolK, are required for GDP-fucose synthesis.

3.2. *The expression level of nolK determines the degree of Nod factor fucosylation*

Since overexpression of *nolK* resulted in the production of detectable amounts of GDP-fucose in vitro, the effect of overexpression on Nod factor production in vivo was investigated. Nod factors of the strains ORS571, ORS571-4.2K, ORS571(pBBNK) and ORS571(pBBNKΩB) were in vivo radioactively labelled and analyzed by TLC (Fig. 3). In the wild-type strain, four LCO spots were present on the RP-C18-TLC. Spots 1 and 2 corresponded to the more hydrophilic vaccenoylated LCOs and spots 3 and 4 to the stearoylated LCOs. Spots 1 and 3 were Nod factors carrying an arabinosyl and/or fucosyl group and spots 2 and 4 corresponded to molecules lacking a glycosyl group. The wild-type strain produced more or less equal amounts of fucosylated and unfucosylated Nod factors (Fig. 3, lane 1). The *nolK* mutant ORS571-4.2K did not produce fucosylated Nod factors (Fig. 3, lane 2) [13].

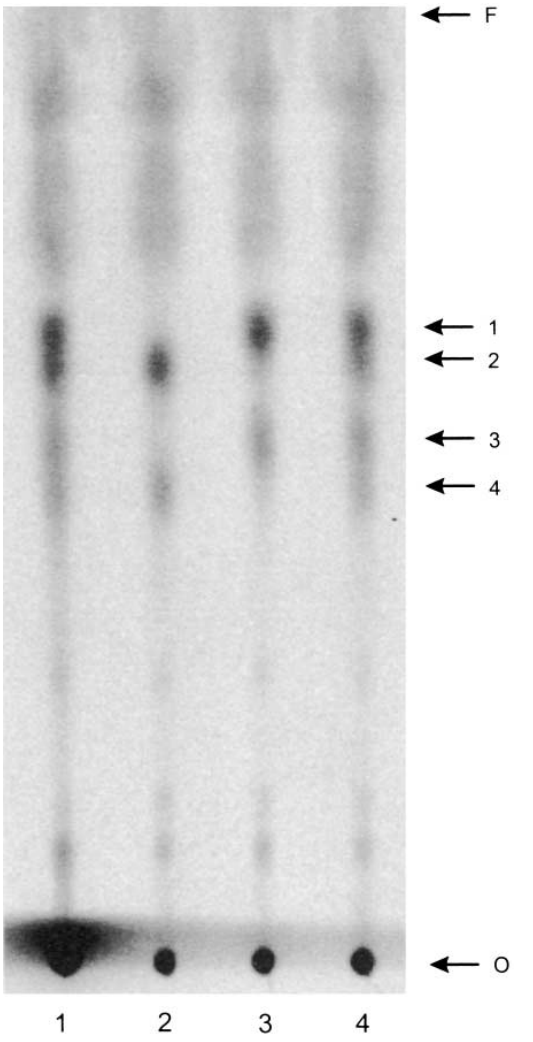

Fig. 3. RP-C18-TLC analysis of in vivo labelled Nod factors. Nod factors, in vivo labelled with [^{14}C]-acetate were extracted from cultures from the strains ORS571 (lane 1), ORS571-4.2K (lane 2), ORS571(pBBNK) (lane 3) and ORS571(pBBNKΩB) (lane 4). Spots 1 and 2 correspond to vaccenoylated Nod factors and spots 3 and 4 to stearoylated Nod factors. Spots 1 and 3 are Nod factors carrying an arabinosyl and/or fucosyl group and spots 2 and 4 correspond to molecules lacking a glycosyl group. O is de origin and F the solvent front of the TLC.

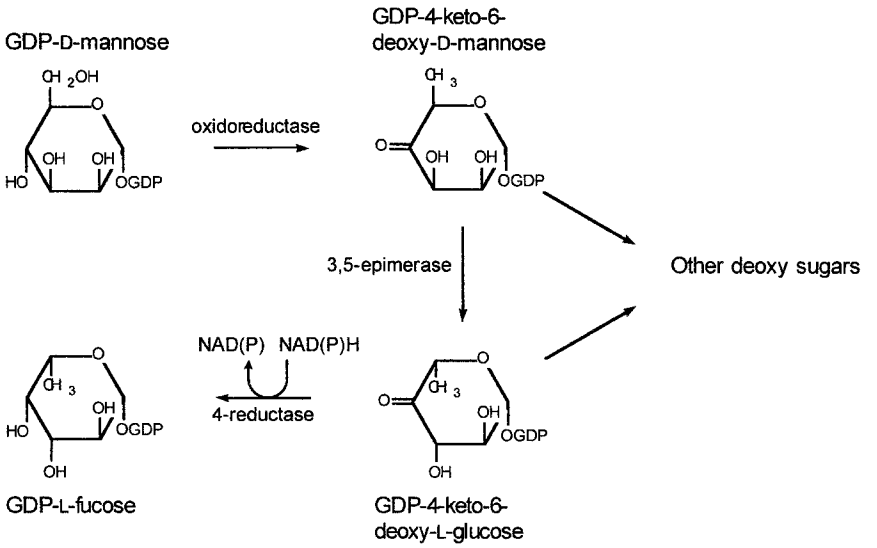

Fig. 4. Pathway for synthesis of GDP-L-fucose from GDP-D-mannose [19]. See text for some more details. The proposed function for NolK is the 4-reductase step transforming GDP-4-keto-6-deoxy-L-glucose to GDP-L-fucose.

In contrast, the *nolK*-overexpressing strain ORS571(pBBNK) produced only fucosylated Nod factors (Fig. 3, lane 3), whereas the control strain ORS571(pBBNKΩB) produced a mixture similar to that of the wild-type strain (Fig. 3, lane 4). Thus, the level of expression of the *nolK* gene determined the proportion of fucosylated Nod factors.

4. Discussion

4.1. GDP-fucose synthesis by NolK

GDP-mannose is converted to GDP-fucose in three enzymatic steps (Fig. 4; [19]). First an oxidoreductase, using NAD^+ as cofactor, forms GDP-4-keto-6-deoxy-mannose. This intermediate is at a branching point and serves as the precursor for several deoxy sugar nucleotides such as GDP-D-rhamnose, GDP-6-deoxy-D-talose, GDP-colitose (a dideoxy sugar) and GDP-L-fucose. Subsequent steps required to convert GDP-4-keto-6-deoxy-mannose to GDP-fucose are a 3,5-epimerization and a 4-reduction. The latter step consumes a stoichiometric amount of NAD(P)H. It is not yet unambiguously clear whether the epimerization and reduction step are performed by one or two distinct enzymes. In porcine liver, a single enzyme seems to be involved [20]. In contrast, in *E. coli* K-12 two enzymes are possibly involved [21].

We propose that *nolK* encodes the 4-reductase or a 3,5-epimerase-4-reductase for the following reasons. (i) NolK is essential for Nod factor fucosylation but is not the fucosyltransferase [13]; (ii) NolK is part of the biochemical pathway forming GDP-fucose from GDP-mannose because *nolK* is essential for in vitro conversion of GDP-mannose to GDP-fucose and the expression level of *nolK* is a rate-limiting step in this pathway (Figs. 1 and 2); (iii) *A. caulinodans* may possess the pathway for production of other deoxy sugar nucleotides (e.g. compounds X and Y; Fig. 1) and NolK would then form a one-step branch on that pathway leading to GDP-fucose synthesis under Nod factor-synthesizing conditions (Fig. 4); (iv) oxidoreductase activity in protein extracts of the *A. caulinodans* strains described here and of DH5α-expressing *nolK* was found to be independent of the *nolK* gene (data not shown); and (v) an NAD(P)-binding motif is present in the amino terminus of NolK [22], consistent with the fact that the 4-reduction step utilizes NAD(P)H.

4.2. Factors determining the level of Nod factor fucosylation

The *A. caulinodans* wild-type strain produces a mixture of fucosylated and unfucosylated Nod factors [11] whereas the *nolK*-overexpressing strain ORS571(pBBNK) produces mainly fucosylated Nod factors. Thus, the formation of the GDP-fucose precursor via NolK is a limiting step in Nod factor fucosylation. Also bacterial growth conditions influenced fucosylation. Higher aeration led to a higher proportion of fucosylated Nod factors [11], which could be due to the metabolic status of the cells. Alternatively, the *nolK* gene could be oxygen regulated, possibly resulting in the synthesis of different Nod factors in the rhizosphere and in infection threads. Fucosylated and unfucosylated Nod factors could have a different role in the induction of nodules and in the infection process.

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