Rhizobium leguminosarum exoB Mutants Are Deficient in the Synthesis of UDP-glucose 4'-Epimerase*

(Received for publication, February 16, 1990)

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Rhizobium leguminosarum bv. viciae exoB mutant strains RBL5523,exo7::Tn5, RBL5523,exo8::Tn5 and RBL5523,exo52::Tn5 are affected in nodulation and in the syntheses of lipopolysaccharide, capsular polysaccharide, and exocellular polysaccharide. These mutants were complemented for nodulation and for the syntheses of these polysaccharides by plasmid pMP2603. The gene in which these mutants are defective is functionally homologous to the exoB gene of Rhizobium mellitii. The repeating unit of the residual amounts of EPS still made by the exoB mutants of R. leguminosarum bv. viciae lacks galactose and the substituents attached to it. The R. leguminosarum bv. viciae and R. mellitii exoB mutants fail to synthesize active UDP-glucose 4'-epimerase.

In recent years the role of polysaccharides in nodulation by Rhizobium bacteria has once again gained interest. Several groups described the isolation of mutants of various Rhizobium strains defective in the production of particular polysaccharides. Some of these mutants are pleiotropic, e.g. Diebold and Noel (1989) recently described the isolation of two mutants of Rhizobium leguminosarum bv. phaseoli defective in the syntheses of both exopolysaccharide (EPS) and lipopolysaccharide (LPS). The best characterized pleiotropic Exo- mutants are the exoB mutants of Rhizobium mellitii (Long et al., 1988), which were shown to be defective in the syntheses of both LPS and EPS (Leigh and Lee, 1988). Like the other Exo- mutants of R. mellitii (Leigh et al., 1985), the exoB mutants form ineffective nodules. Genes functionally homologous to the R. mellitii exoB gene were shown to be present in Agrobacterium (Cangelosi et al., 1987) and Azospirillum (Michiels et al., 1988).

R. leguminosarum bv. viciae strains synthesize EPS with a different chemical structure (Roberts et al., 1981) in comparison with the succinoglycan synthesized by R. mellitii (Jansson et al., 1977). Furthermore, R. leguminosarum bv. viciae strains synthesize a neutral gelling capsular polysaccharide (CPS; Zevenhuizen and Van Neerven, 1993b).

Previously we reported the isolation of several Exo- mutants of R. leguminosarum bv. viciae strain RHL5523 (Canter Cremers et al., 1988a). In this paper we describe that three of these mutants are impaired in a gene which is functionally homologous to the exoB gene of R. mellitii and that these exoB mutants are deficient in an enzyme involved in galactose metabolism, namely UDP-glucose 4'-epimerase.

**MATERIALS AND METHODS AND RESULTS**

Isolation of Pleiotropic Polysaccharide Mutants of R. leguminosarum bv. viciae—Previously we reported the isolation of Exo- mutants of R. leguminosarum bv. viciae strain RBL5523 that failed to nodulate Vicia sativa plants (Canter Cremers et al., 1988a). In order to identify mutants not only defective in the synthesis of EPS, the LPS, CPS, and cyclic β(1-2)-glucan synthesized by these strains were analyzed. Most Exo- mutants produce a LPS profile identical to that of parental R. leguminosarum bv. viciae strain RBL5523 (Fig. 1, lane a). The LPS profile of this strain consists of two functions, LPSI and LPSII. LPSII presumably consists of lipid A and core LPS, whereas LPSI contains in addition O-antigen (De Maagd et al., 1988). Mutant strains RRL5523,exo7::Tn5, RRL5523,exo8::Tn5 and RRL5523, exo52::Tn5 produce an LPS (Fig. 1, lane b), which consists mainly of LPSII and a few intermediate bands, which may consist of LPSh to which a low number of O-antigen units are attached.

Zevenhuizen and Van Neerven (1983b) described that R. leguminosarum bv. viciae strains produce a CPS. The amount of CPS produced by strain RBL5523 and most of the Exo- mutants was about 0.15 g of CPS/g of cellular protein. However, the Exo- mutants RBL5523,exo7::Tn5, RBL5523, exo8::Tn5, and RBL5523, exo52::Tn5 produce no CPS. These latter mutants are thus affected in the syntheses of EPS, LPS, and CPS.

Zevenhuizen and Van Neerven (1983a) described that R. leguminosarum bv. viciae strains synthesize and secrete cyclic β(1-2)-glucan. We investigated the presence of β(1-2)-glucan in strain RBL5523 and in its pleiotropic mutants. Therefore culture supernatants of these strains were fractionated using an Amicon Hollow Fiber apparatus as described previously (Djordjevic et al., 1986). When the fraction containing molecules with an apparent size between 500 and 3000 Da isolated from the culture supernatant of strain RBL5523 or any of the

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* Portions of this paper (including "Materials and Methods," an explanatory part of the "Results," Figs. 1-4, and Tables 1-3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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**Notes:**

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three pleiotropic mutants was eluted from a Sephadex DEAE A-25 column, only one evident neutral hexose-containing peak was observed. After further purification and analysis by 13C NMR spectroscopy, the peaks obtained were identical to those published previously for the neutral cyclic \( \beta(1\rightarrow2) \) glucan isolated from \( \text{Rhzobium strain NGH2254} \) (Batley et al., 1987). Similarly, also the fractions containing molecules with an apparent molecular size between 3000 and 100,000 Da, isolated from the culture supernatant of these four strains were assayed for the presence of cyclic \( \beta(1\rightarrow2) \) glucans. In all these fractions cyclic \( \beta(1\rightarrow2) \) glucan was present as a neutral polysaccharide. Therefore, we concluded that both \( R. \text{leguminosarum bv. viciae} \) strain RBL5523 and the three pleiotropic mutants synthesize only neutral cyclic \( \beta(1\rightarrow2) \) glucan.

**Symbiotic Characterization of the Pleiotropic Mutants of \( R. \text{leguminosarum bv. viciae} \)—**When inoculated on \( V. \text{sativa, V. hirsuta or Pismum sativum} \) plants, the three pleiotropic mutant strains RBL5523,ex0B::Tn5, RBL5523,ex08::Tn5, and RBL5523,ex052::Tn5 failed to induce root hair curling, nodules, or nodule-like structures. The mutants induce some root hair deformation and rare abortive infection threads. Hence the three pleiotropic mutants are impaired in an early step of infection.

**Complementation of the Pleiotropic \( \text{Exo}^+ \) Mutants—**To isolate the complementing DNA fragment(s), a DNA library of a Sym plasmid-derivative of \( R. \text{leguminosarum bv. viciae} \) strain RBL5523, namely strain RBL5515, was constructed in IncP plasmid pTJS133, which carries the \( \text{Te}^+ \) gene. The DNA library was introduced into \( \text{Exo}^- \) mutant strain RBL5523, ex08::Tn5, and the resulting strains were inoculated on forty \( V. \text{sativa} \) plants. On two plants nodules appeared after 2 weeks. About 90% of the bacteria reisolated from these nodules were tetracycline-resistant. Unlike the original RBL5523,ex08::Tn5 mutant strain, these tetracycline-resistant colonies were mucoid on YMB plates. From these colonies, plasmid pTJS133, harboring five HindIII additional DNA fragments with a total size of 14.8 kb, could be isolated. When this plasmid, pMP2602, was introduced into either of the \( \text{Exo}^- \) mutant strains RBL5523,ex08::Tn5, RBL5523,ex052::Tn5, or RBL5523,ex052::Tn5, they acquired a mucoid colony morphology and induced nitrogen-fixing nodules on \( V. \text{sativa} \) and \( V. \text{hirsuta} \) as fast as parental strain RBL5523. The three mutant strains were also complemented for the synthesis of CPS and LPS (Fig. 1, lane e), as well as nodulation on \( V. \text{sativa} \) or \( V. \text{hirsuta} \) plants.

To determine which of the \( R. \text{meliloti} \) \( \text{exo} \) genes is involved in the complementation, we tested whether plasmid pMP2602 was able to functionally complement \( R. \text{meliloti} \) \( \text{exo}^- \) mutants. Plasmids pMP2602 and its smaller derivative pMP2603 were able to complement only \( R. \text{meliloti} \) \( \text{exo}^- \) mutant strain Rm7094 (exoB) (Table 2). In order to locate the complementing locus more precisely, derivatives of pMP2603 in which Tn5 was inserted at various sites (Fig. 2) were introduced into \( R. \text{meliloti} \) mutant strain Rm7094. The resulting strains were tested for fluorescence on plates containing calcifluor and for nodulation on \( \text{Medicago sativa} \). The only strain that was not complemented for both these characteristics was strain Rm7094 harboring pMP2603,ex0B::Tn5 (Table 2). A gene functionally homologous to the \( \text{exoB} \) gene of \( R. \text{meliloti} \) is thus present on plasmid pMP9803 in a locus defined by pMP2603,ex0B::Tn5 (Fig. 2). We propose to designate this gene as the \( \text{exoB} \) gene of \( R. \text{leguminosarum bv. viciae} \) strain RBL5523.

**Chemical Structure of the EPS of \( \text{exoB} \) Mutants—**Colonies of \( R. \text{leguminosarum bv. viciae} \) \( \text{exoB} \) mutants on YMB plates are not completely rough. We therefore checked whether the mutants still produce some EPS.

After growth for 6 days in \( B^- \) minimal medium, parental strain RBL5523 produces about 0.8 g of EPS/g protein present in the culture. Under these circumstances, the \( \text{exoB} \) mutants of \( R. \text{leguminosarum bv. viciae} \) produce approximately 0.07 g of EPS/g of protein. The sugar composition of this residual EPS produced by the \( \text{exoB} \) mutants was analyzed by NMR spectroscopy. To facilitate the spectroscopy, the EPS was depolymerized by phage RL38 and purified by column chromatography. In comparison to the depolymerized EPS of strain RBL5523 (Fig. 3A), that of strain RBL5523,ex08::Tn5 eluted earlier from the Sephadex DEAE A25 column (Fig. 3B), suggesting that it contained molecules with a lower anionic strength. After further purification over Bio-Gel P2 and Dowex 50WX2 columns, the structures of the depolymerized EPS from the parent strain RBL5523 and strain RBL5523,ex08::Tn5 were then determined by \( ^1H \) and \( ^13C \) NMR spectroscopy.

Apart from small differences caused by variations in the extent of esterification of acetate and 3-hydroxybutanoate,
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the $^{13}$C NMR spectrum of material from RBL5523 (Fig. 4A) was identical to that of the repeating unit of R. leguminosarum bv. trifolii strains ANU843 (Hollingsworth et al., 1988) and LPR5 (McNeil et al., 1986). The chemical structure of the repeating unit of RBL5523 is therefore identical to that of these strains (Fig. 5).

When the $^{13}$C NMR spectrum of the repeating unit of the EPS of strain RBL5523, exoB: Tn5 (Fig. 4B) is compared with that of RBL5523 (Fig. 4A), it is apparent that several peaks are absent. Carboxyethylidene signals at 26.1 and 101.8 ppm and galactose peaks for C2 to C6 at 71.3, 66.7, 71.9, 72.5, and 65.8 ppm, respectively, are missing. Furthermore, C3 of the glucose carboxyethylidene residue is no longer glycosylated, and the peak at 80.3 ppm assigned to it has moved several parts/million upfield. The remaining peaks can all be assigned as in the $^{13}$C spectrum of the repeat unit from strain RBL5523. It may therefore be concluded that R. leguminosarum bv. viciae ExoB mutant strain RBL5523, exoB: Tn5 produces small amounts of EPS, similar in structure to that of strain RBL5523, but lacking the terminal galactose carboxyethylidene from the side chain (Fig. 5). Methylation analysis confirmed these findings.

The 3-hydroxybutanoate that is usually esterified to a proportion of the galactose rings was also absent in the mutant EPS. The small peak at 23.0 ppm and others in the 60-70 ppm region were absent from the spectrum of the ExoB mutant (Fig. 4B). In the $^1$H spectrum, the 3-hydroxybutanoate peak at 1.51 ppm was also absent.

Small peaks in the $^{13}$C spectrum at 90.4 and 95.2 ppm showed the presence of some acetate on the sugar at the reducing terminus of material from strain RBL5523, as was also reported for R. leguminosarum strains 128C53 (Kuo and Mort, 1986) and LPR5035, a derivative of LPR5 (Philip-Hollingsworth et al., 1989). Mutant strain RBL5523, exoB: Tn5 had the same acetylation pattern. The $^1$H spectra also showed the additional complexity in the acetate methyl signal at 2.2 ppm that was reported by Hollingsworth et al. (1988) as being an indication of acetylation of glucose at the branch point, but the carbon spectra have the advantage that the assignment is unequivocal and do not require confirmation by methylation analysis.

Finally, the structure of the repeat unit of EPS produced by RBL5523, exoB: Tn5 harboring either pD56 or pMP2603 was determined. Hydrolyzed EPS from both strains gave spots on TLC plates indicating the presence of galactose as well as glucose and glucuronic acid. When the depolymerized EPS of these strains were purified over a Sephadex DEAE A25 column, peaks eluted at the same time as the repeating unit from strain RBL5523 (Fig. 3). The $^{13}$C NMR spectrum from the oligosaccharides obtained from these peaks were identical to that of strain RBL5523. Summarizing, 1) the three ExoB mutants synthesize residual amounts of EPS that lacks the terminal galactose residue h (Fig. 5) and the substituents attached to it and 2) the synthesis and structure of the EPS is restored to wild type after complementation of these strains by plasmids pMP2602, pMP2603, or pD56.

Analysis of the Activity of Enzymes Involved in Galactose Metabolism—Generally bacteria use UDP-galactose for the incorporation of galactose into polysaccharides (Stoddart, 1984). Therefore we analyzed the exoB mutants of R. leguminosarum bv. viciae and R. mellitii for the presence of enzymes involved in the synthesis of UDP-galactose (Fig. 6).

In cell-free extracts prepared from R. leguminosarum bv. viciae strains RBL5515 and RBL5523 galactose dehydrogenase, glucokinase, glucose dehydrogenase, glucose-6-phosphate dehydrogenase, UDP-glucose 4'-epimerase, and galactose-1-phosphate uridyltransferase activities are present, whereas galactokinase activity was absent (Table 3). UDP-glucose 4'-epimerase activity was also present in the cell-free extract of R. mellitii strain 1021.

In the extracts of the three exoB mutants of R. leguminosarum bv. viciae, as well as in the R. mellitii exoB mutant strain Rm7094, UDP-glucose 4'-epimerase activity is absent (Table 3), whereas the other tested enzymes activities were present (Table 3). In the cell-free extracts prepared from R. leguminosarum bv. viciae and R. mellitii exoB mutant strains harboring either one of the complementing plasmids pMP2602, pMP2603, or pD56, UDP-glucose 4'-epimerase activity was present (Table 3).

**DISCUSSION**

In the present paper we describe three indistinguishable pleiotropic mutants of R. leguminosarum bv. viciae with the following characteristics. 1) They are non-mucoid as a result of a strongly reduced amount of EPS. The residual EPS lacks the terminal galactose residue (Fig. 5, residue h) and the

Fig. 5. Chemical structure of the octasaccharide repeat unit of the EPS of strain RBL5523. Glc, glucose; GlcA, glucuronic acid; HB, 3-hydroxybutanoate; Gal, galactose; p, pyranose. The repeating unit of the EPS formed by mutant strain RBL5523, exoB: Tn5 lacks the galactose residue h and consequently the substituents attached to this residue.
substituents attached to it. In addition, these mutants fail to synthesize CPS, whereas their LPS profile is also affected. Furthermore, these mutants synthesize unsubstituted neutral cyclic β(1-2) glucans indistinguishable from that synthesized by the parental strain. 2) The mutants are defective in an early step of the nodulation process, since they only induce root hair deformation but not marked root hair curling and only rarely infection threads which are abortive. 3) The three mutants are affected in a DNA region only about 1 kb in size, whereas the characteristics of the mutants are indistinguishable. It is therefore likely that these mutants are affected in the same gene. 4) The same region of plasmid pMP2603 is involved in the complementation of the three *R. leguminosarum* bv. *viciae* Exo− mutants as well as a *R. meliloti* exoB mutant. A clone harboring the exoB gene of *R. meliloti* is able to complement the three pleiotropic mutant strains of *R. leguminosarum* bv. *viciae*, for nodulation and the syntheses of EPS, LPS, and CPS. We therefore designated the mutant strains as *R. leguminosarum* bv. *viciae* exoB mutants.

**Function of the exoB Gene** —The EPS of *R. leguminosarum* bv. *viciae* contains galactose as a structural element (Fig. 5). In *R. leguminosarum*, galactose is also a structural element of CPS (Zevenhuizen and Van Neerven, 1983b) and of LPS (Carlson et al., 1987; Zovenhuizen et al., 1980). The *R. leguminosarum* bv. *viciae* exoB mutants are affected in the syntheses of all three polysaccharides. In addition, both types of EPS isolated from *R. meliloti*, EPSI and EPSII, contain galactose, and it was reported that exoB mutants of *R. meliloti* fail to synthesize either one of these EPS species (Glazebrook and Walker, 1989). If the exoB gene is involved in some step in the synthesis or polymerization of UDP-galactose, the defects seen in the EPS, LPS, and CPS isolated from exoB mutants of *R. meliloti* and *R. leguminosarum* can be understood.

The exoB mutants of both *R. leguminosarum* bv. *viciae* and *R. meliloti* grow on galactose as sole carbon source. This indicates that in these mutants all the enzymes required for the uptake and conversion of galactose are functioning. Initially we were misled by this observation and the knowledge that in *Escherichia coli* galactose is lethal for galE mutants (Fukasawa and Nakaido, 1961). However the situation in *Rhizobium* differs from that in *E. coli* as it was reported previously that *R. leguminosarum* bv. *trifolii* (Ronson and Leigh, 1987) and *R. leguminosarum* (Arias and Cervenansky, 1986) make use of the De Ley-Douderoff pathway for the conversion of galactose (Fig. 6). In the strain used in the present study, namely *R. leguminosarum* bv. *viciae* strain RBL5523 and its derivatives, a crucial enzyme of the De Ley-Douderoff pathway, galactose dehydrogenase, is present (Table 3), indicating that strain RBL5523 also makes use of this metabolic pathway. Furthermore, one of the enzymes of the De Ley-Douderoff pathway, galactokinase, is absent (Table 3). In strain RBL5523, UDP-galactose can therefore only be formed by the epimerization of UDP-glucose (Fig. 6), a reaction which is catalyzed by UDP-galactose-4′-epimerase (Maxwell et al., 1961).

The fact that our *R. leguminosarum* bv. *viciae* exoB mutants still produce polysaccharides that contain glucose indicates that these mutants are still able to synthesize UDP-glucose. The exoB mutants of *R. leguminosarum* bv. *viciae* and *R. meliloti*, however, lack UDP-glucose-4′-epimerase activity (Table 3) and are thus unable to synthesize UDP-galactose (Fig. 6). The most likely explanation for our results is that the exoB genes of *R. leguminosarum* bv. *viciae* and *R. meliloti* are structural genes for UDP-glucose 4′-epimerase. We plan to sequence the *R. leguminosarum* bv. *viciae* exoB gene to see whether it shares homology with the *E. coli* galE gene.

**The Role of exoB in Nodulation** —Our results point out that there is an effect of the mutation in the exoB gene on the early steps of the nodulation process, even though the attachment of the mutants to root hairs of *V. sativum* plants is comparable with that of the parental strain RBL5523. Our data indicate that the exoB mutants are not able to synthesize any molecule which requires UDP-galactose for its synthesis. A possible explanation is that the *R. leguminosarum* bv. *viciae* sign molecule that precedes root hair curling contains galactose. However, this explanation implies that this compound is different from its *R. meliloti* counterpart which lacks galactose (Lerouge et al., 1990).

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Materials and methods

Bacterial strains

All bacterial strains and plasmids used are listed in Table 1. R. leguminosarum bv. trifolii 86-127 was cultured on medium 86-127 (2% yeast extract, 1% tryptone, 0.5% NaCl, 0.05% agar), followed by the introduction of the plasmid. The strain 86-127 (2% yeast extract, 1% tryptone, 0.5% NaCl, 0.05% agar) was cultured in M9 minimal medium with 0.5% lactose (Sigma Chemical Co., St. Louis, MO) as described by Leppel et al. (1984).

DNA isolation and hybridization

DNA from R. leguminosarum was isolated according to Kraus et al. (1983). DNA from R. leguminosarum was isolated with 1% SDS at 90°C in a total volume of 1 ml. After 15, 30 and 60 minutes of boiling, 1 ml of phenol was added to the sample. After treatment with chloroform:isoamyl alcohol (25:1) the DNA was precipitated with ethanol. DNA was isolated from E. coli strain DH5α with the Qiagen kit according to the manufacturer's instructions.

Enzyme assays

For the preparation of crude culture extracts, bacteria were cultured in M9 medium to an A600 of 0.3. Subsequently, bacterial cells were collected by centrifugation at 10,000 rpm for 10 minutes, resuspended in 0.5 M NaCl and sonicated overnight for extraction. The crude extracts were obtained by incubation at 24,000 g for 1 hour at 0°C after sonication using a Branson sonicator for 1 minute as described by Poirier (1987). Activity of glucosidase (EC 3.2.1.21) was estimated spectrophotometrically according to Anderson and Hanson (1983), galactosidase (EC 3.2.1.23) according to Anderson and Hanson (1983), glucuronicidase (EC 3.2.1.23) according to Anderson and Hanson (1983) and galactosidase (EC 3.2.1.23) according to Anderson and Hanson (1983).

Results

The three Enzyme mutants of R. leguminosarum 86-127 were isolated by Tn5 mutagenesis. Three mutations were identified that affected the three sequential steps of the enzyme activities. The three Enzyme mutants were isolated from a total of 100,000 transformants in 10 plates. The three Enzyme mutants were isolated from a total of 100,000 transformants in 10 plates. The three Enzyme mutants were isolated from a total of 100,000 transformants in 10 plates. The three Enzyme mutants were isolated from a total of 100,000 transformants in 10 plates.
### Table 1

| Relevant characteristics of bacteria and plasmids | Source/reference |
|--------------------------------------------------|------------------|
| R. leguminosarum bv. phaseoli strain RC574 | Rhee and Whitman, 1984 |
| R. leguminosarum bv. vignae strain NC3874 | Rhee and Whitman, 1984 |
| R. leguminosarum bv. trifolii strain NZP159 | Center O'Connell et al., 1984a |
| R. leguminosarum bv. trifolii strain NZP1571 | Center O'Connell et al., 1984b |
| R. leguminosarum bv. trifolii strain NZP1574 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1560 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1572 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1562 | Van der Ploeg et al., 1989 |

### Table 2

| Relevant characteristics of bacteria and plasmids | Source/reference |
|--------------------------------------------------|------------------|
| P. vulgaris strain 249 | Johnson et al., 1970 |
| P. vulgaris strain 77 | Spano et al., 1987 |
| P. vulgaris strain 249 | This paper |
| P. vulgaris strain 77 | This paper |
| P. vulgaris strain 249 | This paper |
| P. vulgaris strain 77 | This paper |
| P. vulgaris strain 249 | This paper |
| P. vulgaris strain 77 | This paper |

### Table 3

| Relevant characteristics of bacteria and plasmids | Source/reference |
|--------------------------------------------------|------------------|
| R. leguminosarum bv. phaseoli strain RC574 | Rhee and Whitman, 1984 |
| R. leguminosarum bv. vignae strain NC3874 | Rhee and Whitman, 1984 |
| R. leguminosarum bv. trifolii strain NZP159 | Center O'Connell et al., 1984a |
| R. leguminosarum bv. trifolii strain NZP1571 | Center O'Connell et al., 1984b |
| R. leguminosarum bv. trifolii strain NZP1574 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1560 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1572 | Lang et al., 1988 |
| R. leguminosarum bv. trifolii strain NZP1562 | Van der Ploeg et al., 1989 |

### Figure 1

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Rhizobium leguminosarum exoB mutants are deficient in the synthesis of UDP-glucose 4'-epimerase.
H C Canter Cremers, M Batley, J W Redmond, L Eydems, M W Breedveld, L P Zevehusizen, E Pees, C A Wijffelman and B J Lugtenberg

*J. Biol. Chem. 1990, 265:21122-21127.*