Oligo- and polysaccharide synthesis by *Rhizobium leguminosarum* and *Rhizobium meliloti*

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Oligo- and polysaccharide synthesis by *Rhizobium leguminosarum* and *Rhizobium meliloti*

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
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dr. H.C. van der Plas,
in het openbaar te verdedigen
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des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Het onderzoek beschreven in dit proefschrift werd uitgevoerd binnen het Innovatief Onderzoeksprogramma koolhydraten (IOP-k) en werd begeleid door de Programmacommissie koolhydraten van het Ministerie van Economische Zaken.
De afronding van het proefschrift kwam mede tot stand door een geldelijke ondersteuning van het LEB-Fonds.

1. De hoge trehalose-concentratie in *Rhizobium* cellen tijdens osmotische stress is verklaarbaar door de rol, die trehalose speelt als beschermer van het interne milieu van de cel.

2. De waarneming dat i.a.v 2.5 mM xanthine de specifieke aktiviteit van xanthine-oxidase van *Arthrobacter* S-2 50x hoger is dan dat van koeiemelk, is gezien de oplosbaarheid van xanthine bij pH=7.0 nogal troebel.
CA Woolfolk en JS Downard. 1978. J Bacteriol 135:422-428

3. Het uitdrukken van een hoeveelheid geaccumuleerd celgeassocieerd produkt als gewichtspercentage van het drooggewicht leidt tot een zekere mate van onderwaardering van dat produkt. Het is beter te relateren aan een onafhankelijke parameter, bijv. cultuurvolume of eiwitconcentratie.

4. Een apart kledingadvies voor de mannelijke promovendus tijdens de promotieplechtigheid is niet in overeenstemming met het m/V beleid.
(Promotiereglement Landbouwuniversiteit ingegaan op zondag 1 april (!) 1990).

5. Het besluit om *Rhizobium trifolii* op grond van de sterke mate van verwantschap met *R. phaseoli* en *R. leguminosarum* als een biovar binnen de *Rhizobium leguminosarum* groep te plaatsen maakt een proefschrift onnodig lijvig.

6. "De consument vraagt ernaar" als motief van fabrikanten van voedings- of genotsmiddelen bij de introduktie van nieuwe produkten is zondermeer een farce.

7. Het geleringsvermogen van anionische extracellulaire polysacchariden van *Rhizobium leguminosarum* is zeer gering vergeleken met de neutrale kapsulaire polysacchariden van hetzelfde organisme. Het is dan ook merkwaardig dat de aandacht voor het fysische en biologische gedrag van polysacchariden zich voornamelijk heeft beperkt tot de eerstgenoemde.
Morris VJ en anderen. 1990. Carbohydr Polym 13:165-183

8 De in een review gewekte suggestie dat osmoprotectanten accumuleren tot hoge concentraties door transport maar niet door *de novo* synthese wordt niet gestaafd door experimentele waarnemingen m.b.t. trehalose-accumulatie in de geciteerde referenties.
Czonka en Hanson. 1991. Ann Rev Microbiol 45:569-606
Elsheikh en Wood. 1990. Lett Appl Microbiol 90:127-130

9a Stellingen, die op een andere dan het eigen vakgebied betrekking hebben, veranderen in het geheel niets aan de kwaliteit van het proefschrift zelf.

9b Een reden tot handhaving van stellingen bij de verdediging van het proefschrift is mogelijk ten behoeve van publiciteit in een landelijk dagblad.

9c "Tradities zijn er om in ere gehouden te worden". Als dit het argument blijkt om een proefschrift *met stellingen* te moeten verdedigen, dan behoren de stellingen als een verplicht onderdeel van de promotie te worden geschrapt.

10. Alle kapsulaire polysacchariden (CPS) zijn extracellulaire polysacchariden (EPS); het beweren van het omgekeerde hangt af van de manier van cultiveren van de bakteriën. Het niet konsekwent hanteren van deze begrippen in de naamgeving van de *Rhizobium* polysacchariden wekt verwarring in de literatuur.

11. De in de politiek gehanteerde term "valutaire tegenwind" doet vermoeden dat je voor de juiste koersontwikkelingen naar het weerbericht moet luisteren.

12. De phylogenetische verwantschap tussen de dierpathogeen *Brucella* sp en de plantensymbiont *Rhizobium* sp biedt in potentie een oplossing voor het mestoverschot. Men moet dan wel eerst op zoek naar de eerste N_2-fixerende koe.

13. Gezien het tijdstip van de cursus oecologie van de vegetatie van de alpen (juni-juli 1992), waarbij een van de onderzoeksopdrachten luidt: "de invloed van de skisport op de vegetatie zal ter plekke worden bestudeerd", is het aan te bevelen roller-skies mee te nemen.
Oecologie Progamma AIO/OIO 1991-1992.

14. Voor rood, geel en blauw hoeft niemand meer bang te zijn.

15. De rol van exopolysacchariden in het infektie- en nodulatiegedrag van *Rhizobium* moet nog gevonden worden.

16. De excretie van cyclische glucanen door *Rhizobium leguminosarum* vanuit mannitol als substraat als gevolg van stress is te vergelijken met de rol van de huidige universiteit als onderwijsinstelling: je stopt er studenten in, je laat ze flink zweten en het levert afgestudeerden af.

17. De huidige hypothese dat voornamelijk anionische cyclische glucanen van *R. meliloti* een osmotische druk opwekken in de periplasma en daarmee een belangrijke rol spelen in de osmoregulatie houdt in dat die rol van cyclische glucanen in *R. leguminosarum* van onderschikt belang is.

Stellingen behorend bij het proefschrift "Oligo- and polysaccharide synthesis by *Rhizobium leguminosarum* and *Rhizobium meliloti*" van Michaël Breedveld

Wageningen, 10 maart 1992

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CHAPTER 1

INTRODUCTION

INTRODUCTION

PREVIEW

In this chapter a general introduction is given on the production of *Rhizobium* oligo- and polysaccharides. Both structural investigations and structure-function relationships will be discussed. After an introduction on extracellular polysaccharides (1.1) and their possible role in the bacterium-plant interaction (1.2), the knowledge on oligo- and polysaccharides of *Rhizobium* will be summarized (1.3.1). Special emphasis will be given to the cell-surface and extracellular polysaccharides (1.3.2). Their possible technical application is given in 1.4. This chapter concludes by an outline of this thesis (1.5). The cyclic (1,2)-ß-glucans will be discussed separately in Chapter 2.

ABBREVIATIONS: bv=biovar; CPS=capsular polysaccharide; EPS= extracellular polysaccharide; HM_r=high molecular weight; LM_r=low molecular weight; LPS=lipopolysaccharide.

1.1 EXTRACELLULAR POLYSACCHARIDES

Cell-surface (extracellular) polysaccharides like capsular polysaccharide (CPS) and extracellular polysaccharides (EPS) are highly hydrated molecules and form the outermost barrier between the bacterial cell and its surroundings. The polymers protect the cell against phagocytosis, phage attachment, or adverse environmental conditions like dessication. The polysaccharides can also be involved in binding and/or detoxification of metal-ions (Dudman, 1977; Sutherland, 1985). Polysaccharides can function as an adhesive compound for the attachment to inert or biological surfaces (Allison and Sutherland, 1987; Sutherland, 1985).

The presence of EPS associated with bacterial cells grown on solid media is often recognizable by the mucoid colonial morphology. However, it does not indicate whether this EPS is part of a capsule firmly attached to the bacterial cell surface or forms a loose slime layer secreted by the bacterial cells, which may easily be slough off the cells by stirring in water. The anionic EPS of *Rhizobium leguminosarum* is sometimes called CPS, because of the fact that it has been isolated from colonies on agar-plates on which the material is overlaying the cells (e.g. Sherwood *et al.*, 1984). Sutherland (1985) used the term capsule only when the polymer was positively identified by electron or light microscopy. In this thesis the term EPS is used for the viscous, water-soluble, anionic extracellular HM_r polysaccharides of *Agrobacterium* and *Rhizobium*, which can be found in the culture supernatant, and CPS is used for the gelling neutral HM_r polysaccharide, not extractable in cold-water and found firmly attached to the cells.

Polysaccharides are known to undergo transition from ordered to disordered states as the temperature of the aqueous solution is raised. In bacterial cultures grown at 25°C, the polymers are in the ordered state as the transition temperature is around 30°C or above. Physical properties like viscosity and gelation are dependent on the primary structure and the molecular weight of the polymer, the non-carbohydrate substituents and on the concentration of both cations and anions present in solution (Rees *et al.*, 1983; Sutherland, 1988). The physical properties of the EPS (succinoglycan) of *R. meliloti* and *A. tumefaciens*, the EPS of *R. leguminosarum* and the CPS of *R. leguminosarum* bv *trifolii* TA-1 are extensively discussed in "Recent developments in industrial polysaccharides" (Stivala *et al.* 1987) to which the interested reader is referred to.

1.2 *RHIZOBIUM*-PLANT INTERACTION

The association between bacteria and the tissues of both animals and plants has been a major research topic for many years. This association is a very important aspect in the control of health in humans, animals and plants, and can be both beneficial or disadvantageous for the host. For the symbiotic or pathogenic behaviour of the bacteria their cell-surfaces are of great importance in pro-

cesses like recognition, attachment, invasion and activation of the host immune system.

The so-called "cell-surface carbohydrates" of rhizobia are supposed to be of importance in the specific recognition and infection process of the bacteria at the root hair surface of leguminous plants (Long, 1989). Rhizobia are classified according to their ability to infect the roots of leguminous plants (Jordan, 1984). The *Leguminoseae*, the third largest family of higher plants, are able to enter into nitrogen-fixing symbiosis with rhizobia possessing the appropriate symbiotic genes. In the process of root hair infection of these plants, starting from recognition and attachment, followed by processes like infection thread formation, nodule formation, invasion and nitrogen fixation, it is believed that *Rhizobium* oligo- and polysaccharides play both a generic and specific role (Halverson and Stacey, 1986). Recently conclusive evidence for an important role of a novel, sulfated oligosaccharide of *R. meliloti* as signal molecule in the infection process was presented (Lerouge *et al.*, 1990).

The family of *Rhizobiaceae* consists of 3 important genera, *Rhizobium*, *Bradyrhizobium* and *Agrobacterium*. The species of *Rhizobium* (the so-called "fast-growers") which will be discussed here are *R. leguminosarum* and *R. meliloti*. *R. leguminosarum* with biovars *viciae*, *trifolii* and *phaseoli*, is able to nodulate pea, clover and bean respectively, and *R. meliloti* nodulates alfalfa.

Bradyrhizobium sp., designated as the "slow-growers," live often in symbiosis with tropical legumes and the thusfar only recognized species is *B. japonicum* which nodulates soybean. Members of the genus *Agrobacterium* are able to form crown gall tumors on plants. Like for *Rhizobium* spp., their growth on carbohydrate-containing media is usually accompanied by production of viscous exopolymers. With respect to exopolysaccharide structures the agrobacteria are related to *R. meliloti*.

1.3 OLIGO- AND POLYSACCHARIDES OF *RHIZOBIUM*

1.3.1 PRODUCTION OF OLIGO- AND POLYSACCHARIDES BY *RHIZOBIUM*

Fast-growing *Rhizobium* and *Agrobacterium* are capable of synthesizing a variety of polysaccharides. Changes in physiological and environmental conditions like pH, dissolved oxygen (Thompson and Leps, 1985), phase of growth (De Hollander *et al.*, 1979), temperature, nutrient supply and medium composition (Courtois *et al.*, 1986) may all affect the composition, physical properties, and relative amounts of oligo- and polysaccharides (for reviews, see Sutherland, 1985, 1988; Zevenhuizen, 1990). Several types of polysaccharides, clearly differing in both chemical and physical properties, can be observed during cultivation of a single strain (Figure 1). Rhizobia excrete the viscous HM_r-EPS which is water-soluble. Sometimes LM_r compounds like repeating units, the building blocks of EPS, and cyclic (1,2)-ß-glucans can be found in culture

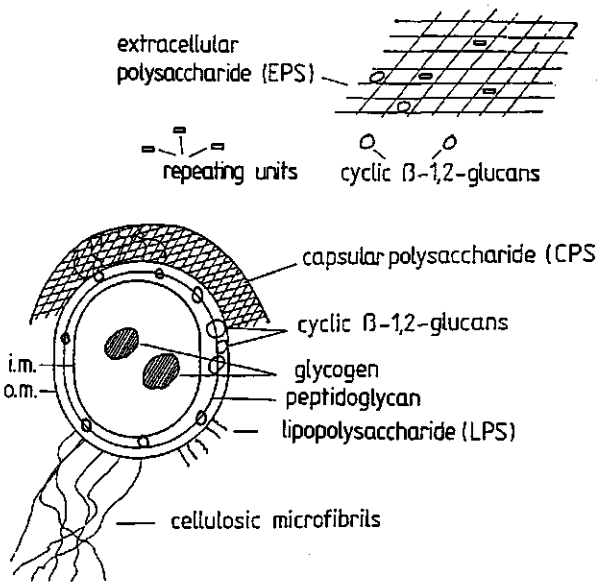

FIGURE 1. Representation of a cell of *Rhizobium* and its intra- and extracellular oligo-and polysaccharides. i.m. = inner membrane; o.m. = outer membrane

medium as well (Amemura *et al.*, 1983; Zevenhuizen and Van Neerven, 1983). Rhizobia also cover their cells with the insoluble neutral CPS (Dudman, 1968; Zevenhuizen and Van Neerven, 1983) and can form cellulosic microfibrils, which cause the cells to form flocs (Deinema and Zevenhuizen, 1971).

The cell envelope of *Rhizobium*, with the cytoplasmic or inner membrane, the peptidoglycan layer and outer membrane, contains also different polysaccharides. Lipopolysaccharide (LPS) consists of (i) lipid A, which is anchored in the outer membrane, (ii) the core, containing LPS-specific sugars like 2-keto-3-deoxyoctonate, and (iii) the O-antigen, which is highly variable in its sugar content among strains of the same species (Nikaido and Vaara, 1985). Peptidoglycan, which separates the inner and outer membranes, plays a very important role in maintaining the structural cellular integrity. Cyclic (1,2)-ß-glucans are found in the periplasm, the space between inner and outer membrane, and are present in high amounts when the osmolarity of the medium is low (Miller *et al.*, 1986).

Within the cells glycogen can be stored as a reserve material in the cytoplasm (Zevenhuizen, 1981). Oligosaccharides like trehalose can be found in all *Rhizobium* species (Streeter, 1985) and are present in high amounts when the cells are osmotically-stressed.

1.3.2 EXTRACELLULAR POLYSACCHARIDES OF *RHIZOBIUM* AND *AGROBACTERIUM*

CELLULOSIC MICROFIBRILS

Cellulose, or (1,4)-ß-glucan, has been found to occur on the cell-surface of many Gram-negative bacteria, including *Rhizobium* and *Agrobacterium* cells (Ross *et al.*, 1991). They are involved in floc formation (Deinema and Zevenhuizen, 1971) and attachment to surfaces like the root hairs of their host (Smit, 1988) or on the tumor initiation site (Matthyse, 1987). Correlation has been found between the uptake by *Rhizobium* colonies of the color dye Congo-red and the ability to form cellulosic microfibrils (Zevenhuizen *et al.*, 1986). Calcofluor-white, a fluorescent dye used as a brightener, interacts with glucose polymers containing contiguous (1,4)-ß-linked (cellulose) or (1,3)-ß-linked D-glucopyranoyl units (Haigler *et al.*, 1980). Succinoglycan of *R. meliloti* and *A. tumefaciens* contains both linkage-types. In many studies it is reported that succinoglycan-negative mutants of *R. meliloti* can be distinguished from the wild type by the failure of the mutant colonies to fluoresce on Calcofluor-white agar plates (e.g. see Leigh *et al.*, 1985; Long *et al.*, 1988). However, purified succinoglycan preparations were found to fluoresce only very weakly or not at all on Calcofluor-white agar plates (Zevenhuizen, unpublished results, 1988). It could therefore be that the succinoglycan-negative mutants described in the literature are impaired in cellulose synthesis as well.

CAPSULAR POLYSACCHARIDES

Typical insoluble capsular polysaccharides have been found in both *Agrobacterium* spp. and *R. leguminosarum*. Curdlan, a (1,3)-ß-glucan, covers the cells of *Agrobacterium* spp. as gelatinous substance (Harada *et al.*, 1968; Harada and Amemura, 1981). CPSs of *R. leguminosarum* are insoluble neutral gelling polymers which are synthesized mainly in the stationary phase. The presence and amount of capsular materials in *R. leguminosarum* is dependent on the cultural conditions and on the particular bacterial strain (Dudman, 1968). CPS was found to be present in several species of *R. leguminosarum*, but not in *R. meliloti*. It could be extracted from stationary phase grown cells cultivated in media with excess carbon source (Zevenhuizen, 1984). It had a sugar composition of glucose:galactose:mannose in a ratio of 1:4:1, without any non-sugar substituents. The structure was determined by methylation and Smith degradation (Zevenhuizen and Van Neerven, 1983); the anomeric configurations of the sugar residues were established by NMR analysis (Gidley *et al.*, 1987) as presented in Figure 2.
Both curdlan of *A. tumefaciens* and the CPS of *R. leguminosarum* can be extracted with hot water or with NaOH at room temperature. The CPS of *R. leguminosarum* does not require divalent cations for gelation and is able to form gels in water already at 0.1% $^{w}/_{v}$

which is less than needed for agar, which forms gels at 0.4% $^{w}/_{v}$ (Zevenhuizen, 1984; Creszenzi *et al.*, 1987). The sol-gel transition temperature lies between 40 and 55°C. As this capsular polysaccharide with its unique gelling properties has considerable potential applications it is desirable to select for strains which do not produce EPS or only in low amounts in order to keep the viscocity of the medium low. This strategy was used for selecting succinoglycan-negative, curdlan-producing strains of *A. tumefaciens* (Harada and Amemura, 1981). Alternatively, by choosing the right cultural conditions a selective CPS synthesis over EPS can be achieved as was shown for *R. leguminosarum* bv *trifolii* TA-1 (Zevenhuizen, 1986).

The relationship between the presence of CPS in *R. leguminosarum* and the ability to nodulate the host plant has never been studied. It is doubtful that CPS plays an important role in nodulation. During nodulation bacteria are actively growing but CPS synthesis starts only in the late logarithmic phase. CPSs from many *R. leguminosarum* strains (all biovars) have constant compositional and structural properties (Zevenhuizen, 1990) thus suggesting a more generic role for this polymer in the free-living state (Dudman, 1977).

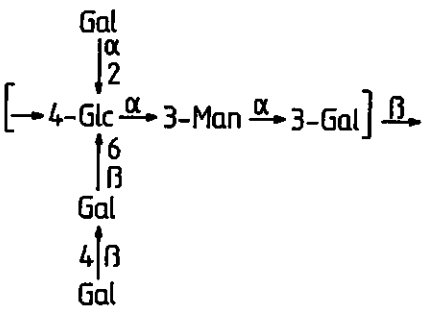

FIGURE 2. Structure for CPS of *R. leguminosarum* bv *trifolii* TA-1

ANIONIC EXTRACELLULAR POLYSACCHARIDES

In media with excess carbon source high concentrations of EPS can be found in supernatants of *Rhizobium* and *Agrobacterium* cultures, rendering the cultures viscous. The synthesis of EPS starts already in logarithmic growth phase and continues in stationary phase. These EPSs are build up of repeating-units. In some cases these repeating-units can be found in the supernatant as well. Nutrient limitations of nitrogen, phosphorus and sulphur have all a stimulating effect on polysaccharide production (Sutherland, 1985). Both the diversity of structures and the multiproductive pattern of polysaccharides produced by rhizobia have been reviewed recently (Zevenhuizen, 1987, 1990).

Methylation analysis of the EPSs derived from species of *R. leguminosarum* bv *viciae*, *trifolii* and *phaseoli* gave identical patterns of partially methylated sugars (Zevenhuizen, 1973). The general type of EPS found in *R. leguminosarum* is the K36-EPS, named after *R. leguminosarum* bv *phaseoli* 127 K36, consisting of an octasaccharide repeating unit with sugar composition of glucose:glucuronic acid:galactose in the ratio of 5:2:1 (Dudman *et al.*, 1983a; Figure 3). Per repeating unit approximately 2 pyruvate, and varying amounts of acetate and 3-hydroxybutanoate are present, apparently depending on the growth medium and/or the strain (McNeil *et al.*, 1986). The structure of this type of EPS obtained from strains of *R. leguminosarum* bv *trifolii* and *viciae* was for the first time resolved by Robertson *et al.* (1981). Using oligosaccharide fragments from a phage depolymerizing activity on the EPS of *R. trifolii* 843, the presence of non-sugar substituents on the EPS was elucidated using a combination of ^{1}H- and ^{13}C-NMR, fast-atom bombardment-mass spectrometry (FAB/MS), periodate oxydation and methylation analysis. Two pyruvate-groups acetal-linked to the O-4- and O-6-positions of the terminal glucose and galactose residues, and non-stoichometric amounts of acetate and 3-hydroxybutanoate were detected (Hollingsworth *et al.*, 1988). In all the EPSs of *R. leguminosarum* studied so far, the backbone consisted of 2 glucuronic acid and 2 glucose residues with the same linkage types. However, variations in the structure of the side chain of EPSs from *R. leguminosarum* bv *phaseoli* 127 K38, 127 K44 and 127 K87 were reported

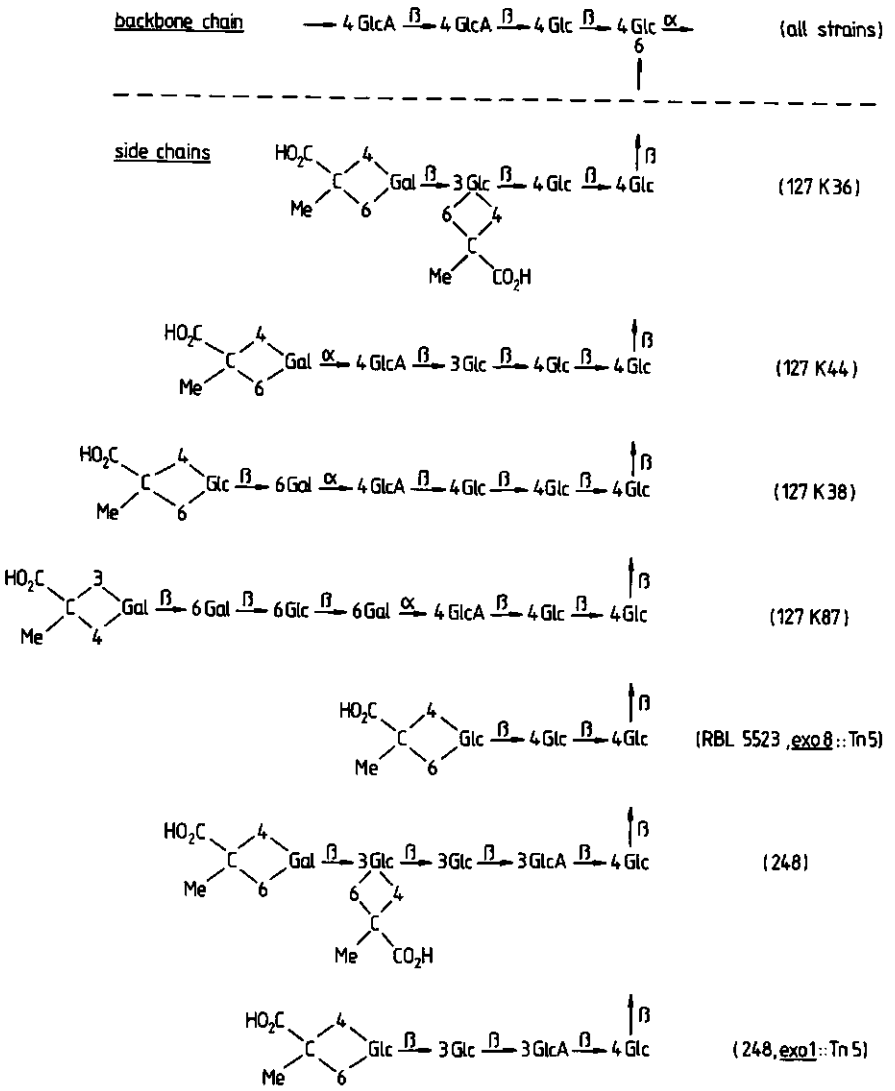

FIGURE 3. Proposed structures for the EPSs of *R. leguminosarum* spp. Abbreviations: The 127 K-numbers are EPS-types from strains of *R. leguminosarum* bv *phaseoli*; 5523,*exoB*::Tn5= mutant of *R. leguminosarum* bv *viciae* 5523 (strain 5523 has the K-36 type EPS); 248= *R. leguminosarum* bv *viciae* strain 248; 248,*exo1*::Tn5= mutant of strain 248. Non-stoichometric substituents are not shown.

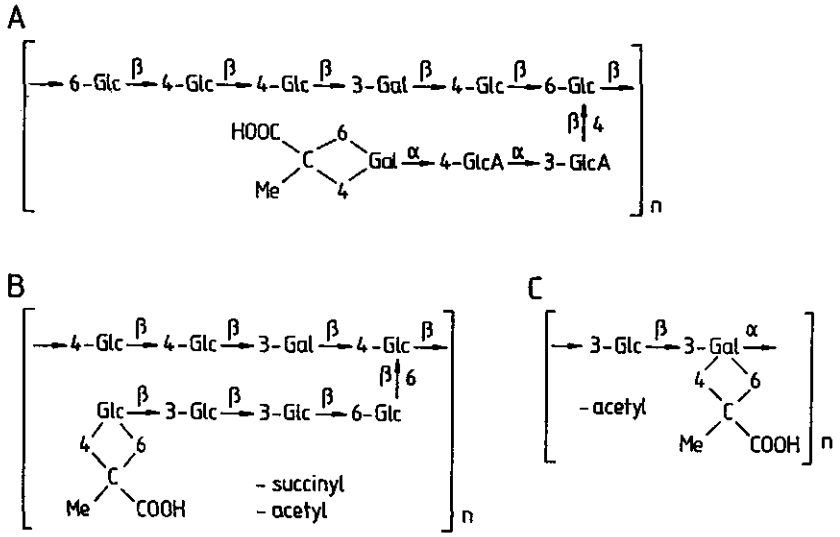

FIGURE 4. Proposed structures for the EPSs. Wide host range *Rhizobium* sp. strain NGR234 (A); succinoglycan of *Agrobacterium* spp. and *R. meliloti* (B); galactoglucan of mutants of *R. meliloti* (C)

(Aman *et al.*, 1982; Franzen *et al.*, 1983; Dudman *et al.*, 1983b). Recently, *exoB* mutants of *Rhizobium leguminosarum* bv *viciae* 5523 were shown to be defective in the enzyme galactose-4'-epimerase, necessary for incorporation of galactose into the carbohydrate polymer. While the wildtype strain 5523 synthesized the regular octasaccharide type EPS, the *exoB* mutants synthesized EPS missing the terminal galactose (Canter Cremers *et al.*, 1990; Figure 3). The glucose:glucuronic acid ratio (5:2) within these truncated EPS molecules is identical to that of *R. trifolii* 4S (Amemura *et al.*, 1983). However, strain 4S has a (1,3)-linked glucose in the side chain. The EPS of *R. leguminosarum* bv *viciae* 248 is composed of a nonasaccharide repeating unit with sugar composition of glucose: glucuronic acid: galactose ratio of 5:3:1, while its mutant strain 248, *exo1*::Tn5 missed the galactose end-group (Figure 3). The side chain of the EPS of strain 248 contained a glucuronic acid (Canter Cremers *et al.*, 1991a) which is rather exceptional. Glucuronic acid in side chains of EPS has been found in the broad host range bacterium *Rhizobium* NGR 234 (Figure 4; Djordjevic *et al.*, 1986) and some strains of *R. leguminosarum* bv *phaseoli* (Figure 3). However, the majority of EPS side-chains of *R. leguminosarum* do contain only neutral hexoses (Zevenhuizen and Bertocchi, 1989).

The EPS commonly found in *R. meliloti* and *Agrobacterium* sp. is of the succinoglycan-type. This EPS, composed of octasaccharide repeating units with sugar composition of glucose:galactose ratio of 7:1, and approximately 1 pyruvate, 1 acetate and a succinate group, was structurally identified in several laboratories (e.g. Hisamatsu *et al.*, 1978; Aman *et al.*, 1983), but the exact location of the succinyl- and acetyl groups has still not been identified (Figure 4). Recently a galactoglucan with a disaccharide repeating-unit was found in cultures of mutants of *R. meliloti* strain SU-47 (Zhan *et al.*, 1990) and its related strain 1021 (Glazebrook and Walker, 1989; Figure 4). The mutant strains of *R. meliloti* 1021 and SU-47 produced galactoglucan at the same level as succinoglycan produced by the wildtype strain. A mutant of *R. meliloti* strain YE-2 was found to excrete galactoglucan, together with the octasaccharide-repeating units of succinoglycan. By increasing the osmotic strength of the medium to

0.6 M NaCl, synthesis of galactoglucan was repressed while the succinoglycan polymer was produced. It made up 80% of the total excreted carbohydrates (Zevenhuizen and Faleschini, 1991).

Many different metabolic and regulatory enzymes are involved in the biosynthesis of exopolysaccharides, like enzymes involved in synthesis of the nucleotide precursors, transferases for each monosaccharide to the growing subunit, enzymes involved in the transfer of non-sugar substituents like pyruvate, acetate, hydroxybutanoate and/or succinate ("decorations"), enzymes involved in export of the unit over the membrane, and at least one polymerase for each type of sugar (Sutherland, 1982). Although some of these enzymes (e.g. the transferases) are unique for the synthesis of the particular polysaccharide, others (basal metabolic enzymes) are involved in the synthesis of more than one polymer. *In vitro* experiments revealed that lipid-linked intermediates are involved in the synthesis of EPS of *R. trifolii* (Bossio *et al.*, 1986), and succinoglycan of *A. tumefaciens* and *R. meliloti* (Tolmasky *et al.*, 1982; Staneloni *et al.*, 1984). In the biosynthesis of succinoglycan, galactose is the first residue in the assemblage to which glucose is bound via ß-(1,3)-linkage. After completion of the octasaccharide unit, the non-sugar subsituents are attached. Subsequently, polymerization takes place.

BIOLOGICAL ROLE OF EPS

Research on *Rhizobium* polysaccharides has mainly focused on their role during recognition and infection of root hairs of legimunous plants (for a review, see Halverson and Stacey, 1986). Most studies deal with the function of EPS in the infection- and nodulation process.

With *Rhizobium meliloti* strong correlation has been found between infection behaviour and polysaccharide synthesis. Mutants unable to synthesize succinoglycan formed ineffective pseudonodules in the host plant, alfalfa (Leigh *et al.*, 1985; Zhan *et al.*, 1989; Long *et al.*, 1988). However, the finding that both the succinoglycan producing wildtype strains and galactoglucan producing mutant strains of *R. meliloti* are able to infect their host plant properly (Glazebrook and Walker, 1989; Zhan *et al.*, 1990) suggest that the structure of EPS may have little influence on nodulation. Although both polymers have the disaccharide unit Glc-ß-(1,3)-Gal in common (Figure 4), they have different substituents and flanking linkages.

For *R. leguminosarum*, the importance of presence of EPS in the infection process or as host-range determinant is not clearly established. While exopolysaccharide-deficient mutants of *R. leguminosarum* bv *viciae* failed to nodulate their proper host-plant, such mutants of *R. leguminosarum* bv *phaseoli* were fully infective. It was concluded that the requirement for EPS in the nodulation, depended on the cross-inoculation group (Diebold and Noel, 1989). Purified EPS from *R. leguminosarum* bv *trifolii* ANU843 restored the abilitiy to fix nitrogen of EPS-deficient mutants (Djordjevic *et al.*, 1987). Purified deacetylated EPS of *R. leguminosarum* bv *viciae* added prior to inoculation with the strain did not block nodulation, while the untreated EPS inhibited (Skorupska *et al.*, 1985). The suggestion that nodulation genes on the symbiosis (Sym) plasmid of *R. leguminosarum* bv *trifolii* determined host-specificity by influencing the pattern of acetylation (Philip-Hollingworth *et al.*, 1989) was contradicted recently by two research groups who found that the acetylation pattern of the EPS is solely determined by the bacterial genome (Canter Cremers *et al.*, 1991b; O'Neill *et al.*, 1991). The identity of EPS in most *R. leguminosarum* strains but their different host-specificity (McNeil *et al.*, 1986) is a further argument against the host-specific action of EPS.

Mutations in a gene coding for basal metabolic enzymes leading to a defect in the synthesis of EPS may affect the synthesis of their repeating-units, LPS, CPS, cyclic glucans and internal carbohydrates like glycogen or trehalose as well. In studies on (exo)-polysaccharide-negative mutants the biochemical background of the mutation involved is often not well defined. Therefore, conclusions about the role of EPS in the infection process should be used carefully.

Although some EPSs show clearly differences in composition and amount of hexo-

ses, some common structures and properties are still present. It therefore may be that (i) EPS is required for successful nodulation because the backbone structure in these EPSs are the same, and/or that (ii) a certain viscosity in the infection thread is needed. In the latter case, the quantity of EPS might be of importance. Curiously, an EPS-endo-depolymerase from *B. japonicum* has been isolated which was active against the own EPS produced (Dunn and Karr, 1990). The *in vivo* function of the depolymerase might be to decrease viscosity of EPS. Alternatively, (iii) the oligosaccharides (repeating units) synthesized or produced by depolymerization of the polymer might be of importance as signalling molecule in the infection process. Furthermore, it has been shown that high concentrations of EPS and Ca^{2+} promoted the gelation of EPS of *R. leguminosarum*, suggesting that (iv) EPS might play an unspecific role in attachment to root hair cells (Morris *et al.*, 1989).

A role of EPS for rhizobia in the free-living state might also be the creation of an area of high viscosity around the cells, which could limit oxygen diffusion, resulting in the derepression of nitrogenase activity (Jain *et al.*, 1990). It is generally believed that these polysaccharides do not function as a source of carbon or energy for the organism itself.

1.4 APPLICATION

Microorganisms are capable to synthesize polysaccharides which are unique in the mode of linkage and of the sugar residues which constitute the polymeric chains. Out of a vast scala of natural compounds microbial polymers are getting more attention. Because of their interesting physico-chemical properties *Rhizobium* polysaccharides can be of importance in their application as viscous (EPS) or gelling agent (CPS) or as inclusion compounds (cyclic (1,2)-ß-glucans), e.g. in food and pharmaceutical products and for enhanced oil-recovery. They could well replace some tradionally obtained polysaccharides of plant- and/or seaweed origin, or synthetic polymers. One of the important advantages of these natural polymers over products of synthetic origin is their biodegradability.

Microbial polymers like carrageenan, xanthan, pullulan and alginate are used as food-additives. Despite the potential of rhizobia to produce various oligo- and polysaccharides only a few of them are commercially produced, e.g. curdlan (1,3)-ß-glucan (Harada and Amemura, 1981), the CPS synthesized by *Agrobacterium* sp., and succinoglycan (Linton *et al.*, 1987), the EPS produced by many *Agrobacterium* and *Rhizobium meliloti* strains (Figure 4; Kenne and Lindberg, 1983; Sandford and Baird, 1983; Sutherland, 1985).

1.5 OBJECTIVES AND OUTLINE OF THIS THESIS

Interests in the field of *Rhizobium* polysaccharides has resulted from a development in two distinct areas, (i) the role of surface molecules, amongst them oligo- and polysaccharides, in the microbe-plant interaction, and (ii) studies on the physico-chemical properties of microbial polysaccharides with a potential technical application. In both areas, research has focused mainly on the structural status of a single polysaccharide produced by a particular organism, sometimes without considering that polysaccharide synthesis is very much dependent on the environmental conditions, and/or that rhizobia are multiproductive with respect to polysaccharide production. The aim of this thesis is to extend the knowledge on the production of *Rhizobium* polysaccharides as influenced by environmental conditions taking into account their multiproductive pattern.

Because a majority of the experimental results dealt with cyclic (1,2)-ß-glucans a separate extensive overview will be given on this polysaccharide (chapter 2). *Rhizobium leguminosarum* bv *trifolii* TA-1 and *Rhizobium meliloti* SU-47 were used as a model strain to study the extent of polysaccharide-production as influenced by cultural conditions. Chapter 3 describes the observation of excessive excretion of cyclic (1,2)-ß-glucan by growing *R. leguminosarum* bv *trifolii* TA-1 at superoptimal temperature for growth, or by allowing the cells to grow to high cell densities. Because

cyclic glucans play a role in osmoregulation, the influence of the osmotic pressure of the medium on growth and synthesis of cyclic glucans and other polysaccharides was also investigated. Osmotically-induced oligo- and polysaccharide synthesis by *R. meliloti* SU-47 and *R. leguminosarum* bv *trifolii* TA-1 is discussed in the chapters 4 and 5, respectively. In chapter 6 the response of cellular carbohydrates like trehalose and cyclic (1,2)-ß-glucans in *R. leguminosarum* bv *trifolii* TA-1 and *R. meliloti* SU-47 to a NaCl-shock is investigated. Chapter 7 focuses on the biosynthesis of cyclic (1,2)-ß-glucans by strain *R. leguminosarum* bv *trifolii* TA-1. The influence of medium composition and growth rate on exopolysaccharide-production by strain TA-1 is described in chapter 8. Chapter 9 focuses on the polysaccharide-production by some *R. leguminosarum* mutants altered in polysaccharide-synthesis, and the implication of the alteration for the infection behaviour. Finally, the results described in the preceeding chapters are summarized in chapter 10.

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CHAPTER 2

Cyclic (1,2)-ß-D-glucans in *Rhizobium* and *Agrobacterium*

Cyclic (1,2)-ß-D-glucans in *Rhizobium* and *Agrobacterium*

SUMMARY

Cyclic (1,2)-ß-D-glucans are unique molecules which are almost exclusively found in the genera of *Agrobacterium* and *Rhizobium*. The molecules consist of glucose as the only hexose monomer and can be substituted with glycerol-1-phosphate groups. Their degree of polymerization (DP) ranges from DP=17-25 in *Rhizobium leguminosarum* and *Agrobacterium* species, and to over 40 in *Rhizobium meliloti*. It is established that cyclic (1,2)-ß-glucans play a role in osmoregulation in *R. meliloti* and *A. tumefaciens*. Furthermore, a role of cyclic glucans in the infection behaviour of the bacterium is claimed. This chapter summarizes the information on cyclic glucans with respect to occurrence, synthesis, application and structure-function relationships, related both to osmoregulation and to infection behaviour.

ABBREVIATIONS: bv=biovar; CPS=capsular polysaccharide; DP=degree of polymerization; EPS= extracellular polysaccharide; HPLC=high-performance liquid chromatography; HM_r=high molecular weight; LM_r=low molecular weight; LPS=lipopolysaccharide; MDO=membrane-derived oligosaccharide; π=osmotic pressure (atm); TLC=thin-layer chromatography; UDP=uridine-diphosphate.

2.1.1 ANALYIS OF CYCLIC GLUCANS

Cyclic (1,2)-ß-D-glucans were found for the first time in the culture medium of *Agrobacterium tumefaciens* (McIntire *et al.*, 1942). The so-called crown-gall polysaccharide was described as a LM_r glucose polymer of around 3600 Da. This poly-saccharide was isolated from the supernatant by precipitation with 10 volumes of ethanol, after HM_r-EPS had been removed by precipitation with 2 volumes of ethanol. Since then, (1,2)-ß-glucans were found in culture fluids of other *Agrobacterium* species (Gorin *et al.* 1961; Hisamatsu *et al.*, 1982) and *Rhizobium* species (York *et al.* 1980; Hisamatsu *et al.*, 1983).

For a first detection of cyclic glucan production a spectrofotometric assay on hexoses, like the anthrone-sulphuric acid (Trevelyan and Harrison, 1952) or phenol-sulphuric acid method (Dubois *et al.*, 1951), is often used. For that purpose, HM_r-EPS has to be separated from the LM_r glucan fraction by precipitation with alcohol or by complex formation using quarternary ammonium salts (Scott, 1965). Except for the cyclic glucans, repeating units of the HM_r-EPS may be present in the LM_r extracellular carbohydrate fraction (Amemura *et al.*, 1983; Zevenhuizen and Van Neerven, 1983) and can be separated by anion-exchange chromatography followed by gel-permeation chromatography.

Cellular cyclic (1,2)-ß-glucans of *Rhizobium* and *Agrobacterium* species have been overlooked for a long time since attention was directed to extracellular secretion of cylic (1,2)-ß-glucans together with production of copious amounts of HM_r-EPS. By extraction of *Rhizobium* cells with hot phenol-water a cyclic glucan fraction next to a HM_r-LPS-fraction could be obtained (Zevenhuizen and Scholten-Koerselman, 1979). With the saccharose-osmotic shock method it could be shown that the glucans are localized in the periplasmic space (Abe *et al.*, 1982). It is widely accepted that cyclic glucans are indeed in the periplasmic space, despite the fact that most isolations of cyclic glucans were performed with whole cell pellets. Cellular glucans can also be obtained by extracting the cell pellet with hot 75% alcohol (Zevenhuizen and Van Neerven, 1983), or with trichloroacetic acid (Miller *et al.*, 1986), followed by anion-exchange and/or gel-permeation chromatography. With these methods the glucans are well separated from LPS, CPS, glycogen and smaller molecules like trehalose and other oligosaccharides.

2.1.2 STRUCTURE AND RING-SIZE DISTRIBUTION OF (1,2)-ß-CYCLIC GLUCANS

The (1,2)-linked D-glucosyl structure was established by methylation analysis of the glucans followed by hydrolysis, which yielded

3,4,6-tri-O-methyl-D-glucose as the only methylated product (Putman *et al.*, 1950; Gorin *et al.*, 1961; Zevenhuizen and Scholten-Koerselman, 1979; York *et al.*, 1980; Da Costa Castro *et al.*, 1983), and by periodate oxydation followed by Smith degradation (Madsen, 1962; Zevenhuizen and Scholten-Koelselman, 1979). The macrocyclic, unbranched form was postulated because of the absence of reducing and non-reducing end-groups in the molecules (Zevenhuizen and Scholten-Koelselman, 1979; York *et al.*, 1980). The cyclic character of the glucans of *Agrobacterium* sp. was unequivocally established by ^{13}C-NMR spectroscopy (Dell *et al.*, 1983). The ß-anomeric configuration at the C_1-carbon atoms was suggested by optical rotation and confirmed by ^{1}H-NMR (Hisamatsu *et al.*, 1982; Amemura *et al.*, 1983).

Hisamatsu *et al.* (1983) found that the glucan fraction eluted as a single peak on gel-permeation chromatography, but with HPLC the mixture could be separated into fractions consisting of rings with different DPs. Koizumi *et al.* (1983) reported that the retention of the glucan rings was related to the molecular mass of the different rings in case of amino-bonded HPLC columns (elution buffer acetonitril:water), but with reverse phase columns (elution buffer methanol:water) the retention correlated with the solubility of the glucans in water. Higashiura *et al.* (1985) described an EPS-negative mutant of *R. phaseoli* which excreted cyclic (1,2)-ß-D-glucan with only one ring-size, DP=17.

The (1,2)-ß-glucans of *Rhizobium leguminosarum* and *Agrobacterium tumefaciens* consist of a mixture of rings with different DPs between 17 and 25 as determined by fast atom bombardment-mass spectrometry (FAB-MS; Dell *et al.*, 1983) and by HPLC and TLC analysis of partial acid hydrolysates of the separated components (Koizumi *et al.*, 1984; Hisamatsu *et al.* 1987; Benincasa *et al.*, 1987; Rizzo *et al.*, 1989; Figure 1). *Rhizobium meliloti* contains ring sizes of up to 40 (Koizumi *et al.*, 1984). The glucans of *Rhizobium* and *Agrobacterium* spp. could be classified into four classes according to the degrees of polymerization and the relative abundances of the different rings (Table 1; Hisamatsu *et al.*, 1983). Cyclic (1,2)-ß-D-glucans with DP below 17 have never been found. It has been calculated that (1,2)-ß-glucan rings with lower DP are energetically impermissible (Palleschi and Creszenzi, 1985). A representation of a cyclic (1,2)-ß-glucan ring with DP=18 is shown in Figure 2.

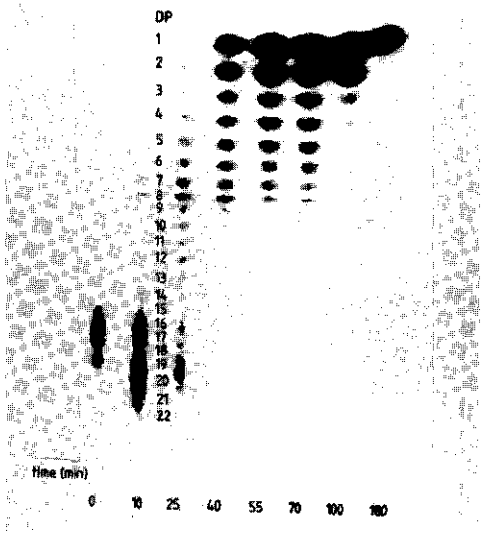

FIGURE 1. TLC chromatogram of the cyclic (1,2)-ß-glucan fraction of *R. leguminosarum* bv *trifolii* TA-1, partially hydrolyzed with 1 M trifluoroacetic acid. Incubation times and DPs of the partially hydrolyzed glucans are indicated.

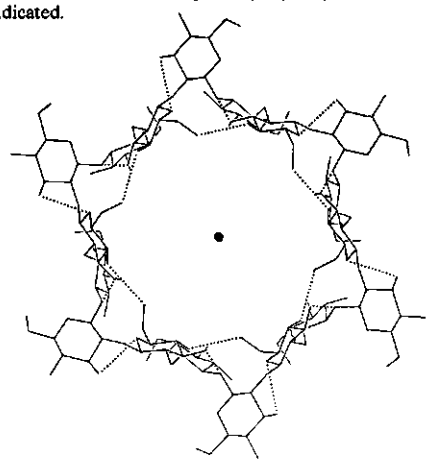

FIGURE 2. Minimum-energy, maximum-symmetry conformation of cyclic (1,2)-ß-glucan with DP=18 (from: Palleschi and Creszenzi, 1985).

TABLE 1 Classification of cyclic (1,2)-ß-D-glucans according to their DPs (Hisamatsu *et al.* (1983)

Glucan type	I	II	III	IV
Main DPs	17	19-20	21-22	19-22
Highest DP	19-20	24-25	40	25
Lowest DP	17	17	17	17
Species	*R. phaseoli* AHU 1133	*R. leguminosarum* sp.	*R. meliloti* sp.	*Agrobacterium* sp.
% Charged[a]	Not reported	< 10%	10-90%	30-90%

a= charged with phosphoglycerol-groups

2.1.3 SUBSTITUTION OF CYCLIC GLUCANS

In *R. meliloti* and *A. tumefaciens* a large part of the glucans are glycerophosphorylated (Batley *et al.*, 1987; Miller *et al.*, 1986; Miller *et al.*, 1987). This was determined by analyzing a charged fraction consisting of glucans with anionic properties. Glucan fractions eluting from anionic exchange columns were obtained containing 0, 1, 2, 3 and 4 glycerol-1-phosphate groups per ring cycle connected to the O-6 of the glucose residue. The substituents were derived from phosphatidylglycerol moieties from the membrane-phospholipids in *R. meliloti* (Miller *et al.*, 1988). It was noticed earlier that the (1,2)-ß-glucan structure was present in the anionic LM_r fraction and they were thought to be bound to the acidic repeating units of the EPS (Hisamatsu *et al.*, 1982; Barreto-Bergter *et al.*, 1980). In fact the glucans themselves were acidic. Hisamatsu *et al.* (1987) reported methylmalonic acid and succinic acid substituents in the glucans of strains of *Rhizobium* and *A. radiobacter*. In *R. leguminosarum* glucans were found to be mostly low-substituted or neutral molecules (Zevenhuizen *et al.*, 1990).

2.1.4 ß-LINKED LM_r GLUCANS IN OTHER GENERA

(1,2)-ß-D-glucans are not widely distributed in genera other than *Rhizobiaceae*. From the cell-envelope of *Escherichia coli* MDOs have been isolated with DP=6 to 12 and consisting of ß-(1,2)- and ß-(1,6)-linkages (Schneider *et al.*, 1979; Schulman and Kennedy, 1979) substituted with phosphoglycerol, phosphoethanolamine and succinyl groups (for a review on MDOs, see Kennedy, 1987). Amemura *et al.* (1985b) found linear (1,2)-ß-D-glucosacharides in the supernatant of strains of *Acetobacter* with DP=6 to 42. In biosynthetic studies using membrane fractions of *A. xylinum* linear HM_r (1,2)-ß-D-glucans were produced (Sandermann and Dekker, 1979). Tropical strains of *Rhizobium* have been described which produced linear (1,2)-ß-D glucans with DP=15 to 19 (Amemura *et al.*, 1985a). *Xanthomonas* sp. produced linear unbranched (1,2)-ß-glucans with DP=8-20 and a cyclic (1,2)-ß-D-glucan with DP=16 containing one (1,6)-linkage and one α-linkage (Amemura and Cabrera-Crespo, 1986). Cyclic (1,2)-ß-glucan, described as polysaccharide B, was also detected in the mammal pathogen *Brucella* sp. (Bundle *et al.*, 1988). On the basis of rRNA 16S sequence comparison and lipid A composition, a close phylogenetic relationship was found between brucellae and other members of the alpha-2 subdivision of the class *Proteobacteria*, amongst which rickettsia, agrobacteria and rhizobia (Moreno *et al.*, 1990). Interestingly, these organisms all live in close association with eukaryotic cells. Therefore, it would be interesting to know more about the role for cyclic glucans of *B. abortus* in the infection process.

Bradyrhizobium sp., a third genus of the family *Rhizobiaceae*, synthesizes a different kind of glucan with ß-(1,3) and ß-(1,6) linkages (Dudman and Jones, 1980), which appeared to be ß-1,3,6-branched, forming cyclic structures with DP=10-13 (Miller *et al.*, 1990).

Cyclic glucans do not function as carbon-

or energy source (Zevenhuizen, 1981), and no (1,2)-ß-D-glucanase activity in rhizobia has been reported. The Glc-(1,2)-ß-Glc linkage is not widespread in nature, and only a few (1,2)-glucanases are known. Reese *et al.* (1961) discovered (1,2)-ß-D-glucanases in fungi. Mendoza and Amemura (1983) isolated an endo-(1,2)-glucanase and a ß-glucosidase activity in the soil-bacterium *Cytophaga arvensicola*, which was able to grow on (1,2)-ß-D-glucans as the sole source of carbon.

SUMMARY

Cyclic (1,2)-ß-D-glucans have been found almost exclusively in *Agrobacterium* and *Rhizobium* species. The molecules consist of glucose as the only hexose, and are build up in a linear, unbranched macrocyclic form. Their degree of polymerization (DP) ranges from DP=17-25 in *Rhizobium leguminosarum* and *Agrobacterium* species, and to over 40 in *Rhizobium meliloti*. Cyclic glucans in *Agrobacterium* sp and *R. meliloti* can be substituted with glycerol-1-phosphate groups, while in *R. leguminosarum* the molecules are neutral.

2.2 BIOSYNTHESIS OF CYCLIC (1,2)-ß-D-GLUCANS

The *in vitro* (1,2)-ß-D-glucan synthesis was studied for the first time in *R. japonicum* (Dedonder and Hassid, 1964). In a cell-free extract label derived from UDP-[^{14}C]-glucose was incorporated into a ß-(1,2)-glucan fraction. Cell-free particulate fractions obtained from *A. radiobacter*, *R. phaseoli*, *A. tumefaciens*, *R. leguminosarum*, *R. meliloti* were also found to catalyze the formation of cyclic (1,2)-ß-D-glucan.

Membrane-extracts of *R. phaseoli* AHU1133 (Amemura, 1984) and *R. japonicum* (DeDonder and Hassid, 1964) strains needed NAD or ATP for optimal activity, while extracts of *R. meliloti* R41, *A. tumefaciens* LBA 4011 (Zorreguieta *et al.*, 1985a; Zorreguieta, 1985b) and *A. radiobacter* IFO 12665b1 (Amemura, 1984) did not require these factors. The requirement for ATP or NAD was explained by the fact that these compounds protected the substrate UDP-glucose against the action of contaminating pyrophosphatases (Dedonder and Hassid, 1964). The metal ion Mn^{2+} was necessary for activity and could be replaced by Mg^{2+}, except in *R. phaseoli* fractions. Recently, a novel ß-(1,3)-ß(1,6)-glucosyltransferase activity was detected in *B. japonicum* (Cohen and Miller, 1991) which was apparently responsible for the biosynthesis of cyclic ß-(1,3)-ß-(1,6)-glucan (Miller *et al.*, 1990). The authors discussed that the *R. japonicum* strain (nowadays called *B. japonicum*) showing (1,2)-ß-D-glucosyltransferase activity (Dedonder and Hassid, 1964) in fact represented a fast-growing *Rhizobium* species.

Mutants in two chromosomal virulence regions of *Agrobacterium* called *chvA* and *chvB* were found to be homologous to and could be functionally complemented by the chromosomal *ndvA* and *ndvB* genes of *R. meliloti* involved in nodule development (Dylan *et al.*, 1986). The *ndvA* gene product, a 67 kDa protein presumably situated in the cytoplasmic membrane, is involved in translocation of cyclic glucans and has structural homology with an ATP-binding transport protein in *E. coli* (Stanfield *et al.*, 1988). A similar locus *chvA* exists in *A. tumefaciens* (Inon de Ianino and Ugalde, 1989; Cangelosi *et al.* 1989). The *ndvB*/*chvB*-gene encodes for a cytoplasmic membrane protein ("235 kDa protein") which incorporates UDP-glucose into (1,2)-ß-glucan (Geremia *et al.*, 1987; Zorreguieta *et al.*, 1986). Recently the *ndvB* gene product was described as a 319 kDa protein, due to the fact that the apparent molecular mass of this membrane protein was smaller than its true molecular mass (Ielpi *et al.*, 1990). This inner membrane protein forms an intermediate with the glucan chain starting from the activated form of glucose, UDP-glucose, until circularization is complete. Only the amino-terminal part of the *ndv*B and *chvB* protein was required for biosynthesis of the (1,2)-ß-D-glucans (Zorreguieta *et al.*, 1988; Ielpi *et al.*, 1990). It could therefore be that the mutation in the gene caused synthesis of linear versus circular glucan. However, linear glucans show different gel-chromatographic behaviour and the authors should have noted this. To our knowledge, the circularization reaction of a linear ß-1,2-glucan has never been studied.

Linear glucans as possible intermediates have never been detected, nor with TLC (Amemura, 1984), HPLC (Zorreguieta *et al.*, 1985) nor with antibodies directed against partially hydrolyzed (1,2)-ß-D-glucan (Geiger *et al.*, 1991). Lipid intermediates, involved in the biosynthesis of many polysaccharides (Sutherland, 1985), are not involved in the biosynthesis of cyclic glucans (Zorreguieta *et al.*, 1986).

Cyclic (1,2)-ß-D-glucans of *R. loti* were found with DPs higher than for *A. tumefaciens*, as deduced from the chromatographic behaviour of the glucans. By integrating the *Agrobacterium chvA* and *chvB* genes into *R. loti* the produced glucans were indistinguable from those of *A. tumefaciens*. The authors concluded that the *chvB* gene product had the enzymatic activity for synthesis and determined the degree of polymerization (Lepek *et al.*, 1990). Similar experiments were performed with *chvB* genes which were incorporated into *Azospirillum* (Altabe *et al.*, 1990).

Miller *et al.* (1987) reported that up to 90% of the glucans in *R. meliloti* strain 1021, a streptomycin resistent derivative of strain SU-47, were anionic in character. On the contrary, Dylan *et al.* (1990a) found 96% neutral glucans in *R. meliloti* 102F34. *In vitro* studies revealed that neutral glucans are the precursors of the anionic glucans in *R. meliloti* 1021, and that excretion of glucans *in vivo* was strongly enhanced in yeast extract-mannitol-salts medium. It was suggested that glucans in excess of the amount needed for periplasmic functions were excreted (Geiger *et al.*, 1990). Therefore differences between strains of *R. meliloti* with respect to glucan substitution and excretion may be ascribed to different cultural conditions.

SUMMARY

In cell-free particulate fractions of rhizobia, label derived from UDP-[^{14}C]-glucose was incorporated into a (1,2)-ß-glucan fraction. At least two genetic loci are involved in the biosynthesis of cyclic glucan from UDP-glucose. The *ndvA/chvA* gene product, a 67 kDa protein, is involved in translocation of cyclic glucans over the cytoplasmic membrane. The *ndvB/chvB*-gene encodes for a cytoplasmic membrane protein which incorporates UDP-glucose into cyclic (1,2)-ß-glucan. Linear glucans as intermediates have never been detected. Substitution degrees of anionic glucans differ significantly between similar strains and could be the result of different cultural conditions.

2.3 BIOLOGICAL FUNCTION OF CYCLIC (1,2)-ß-GLUCANS

Cyclic (1,2)-ß-glucans of the *Rhizobiaceae* are believed to play a role in both osmoregulation and infection behaviour. In the following both the osmoregulatory aspects of the cyclic glucans (2.3.1) and their role in the microbe-plant interaction (2.3.2) will be discussed.

2.3.1 OSMOREGULATION

At hypo-osmotic conditions the medium has an osmotic pressure (π) below 0.5 atm; for a medium with low osmotic strength π is between 1 and 2.5 atm. A medium with high osmotic strength (or a medium with elevated osmotic pressure) contains at least 100 mM NaCl or a comparable equivalent of osmolyte concentration. The osmotic pressure π in these media is above 4.8 atm.

At low osmotic values of the medium a net flow of water into the cytosol takes place leading to an increase of turgor pressure. In Gram-negative but especially in Gram-positive bacteria the peptidoglycan-layer plays a very important role in maintaining the structural cellular integrity against this pressure. It is supposed that the periplasm, the compartment between the cytoplasmic membrane and the cell wall, remains isoosmotic with the cytoplasm over a wide range of environmental osmotic pressures (Stock *et al.*, 1977). The osmotic pressure of the periplasm in *E.coli*, at low osmotic pressure of the medium, is mainly achieved by the presence of the MDOs (Kennedy 1982). Their accumulation results in a Donnan potential across the outer membrane (Stock *et al.*, 1977). It seems that the presence of MDO is only one of the requirements for osmo-adaptation since MDO-negative mutants have been found to grow

almost as well as the wild type in a low osmolarity environment (Kennedy 1982). In *Rhizobium* and *Agrobacterium* spp. the neutral and especially the anionic periplasmic cyclic glucans may be very well suited as a fixed osmolyte for increasing the turgor pressure in the periplasm against the cytoplasmic membrane (Miller *et al.*, 1986; Miller *et al.*, 1987). Alternatively, anionic glucans of *A. tumefaciens*, *R. meliloti* or *E. coli* might well function as a source of phosphoglycerol as suggested by Batley *et al.* (1987), but conversion of anionic into neutral glucans has never been reported.

Rhizobium meliloti mutants which were defective in the synthesis of (1,2)-ß-D-glucans were strongly impaired in their ability to grow under hypoosmotic conditions (π<0.5 atm) and were altered in motility, phage-sensitivity and antibioticum resistance. Their growth was restored by raising the osmolarity of the medium by the addition of 100 mM NaCl, after which these properties were like in the wild type (Dylan *et al.*, 1990a). With mutants of *A. tumefaciens* defective in glucan synthesis growth was also relieved when the osmotic pressure of the medium was elevated (Cangelosi *et al.*, 1990). These two examples give strong arguments for the involvement of cyclic (1,2)-ß-D-glucans in osmo-regulation of *A. tumefaciens* and *R. meliloti* at hypo-osmolar conditions. However, cyclic glucans may in addition have a function that is unrelated to its role in osmo-adaptation, since *Agrobacterium chvA* and *chvB* mutants impaired in glucan synthesis were avirulent and showed reduced motility regardless of the osmolarity of the medium (Cangelosi *et al.*, 1990).

At high osmotic strength of the medium the synthesis of the periplasmic MDOs in *E. coli* was repressed (Kennedy 1982). The amount of both the neutral and the anionic cyclic glucans of *A. tumefaciens* C58 was strikingly reduced in media supplemented with 0.4 M NaCl compared to a NaCl-free medium (Miller *et al.*, 1986). The cyclic glucans show not only structural resemblance with the MDOs of *E. coli* (Schulman and Kennedy, 1979), the regulation of the biosynthesis of cyclic glucans and MDOs is also comparable. The synthesis of cyclic ß-(1,3)-(1,6)-glucans from *Bradyrhizobium japonicum* was found to be osmo-regulated as well (Tully *et al.*, 1990; Cohen and Miller, 1991). Zorreguieta *et al.* (1990) showed that glucan accumulation by osmotically-stressed cells of *R. meliloti* was strongly inhibited *in vivo* as compared to non-stressed cells, while inner membrane preparations of both cell types showed no differences in biosynthetic activity. This *in vitro* reaction was inhibited in the presence of ionic osmolytes like NaCl and KCl but not with neutral osmolytes like glucose or mannitol.

Care should be taken to consider all LM_r carbohydrates as glucans. Clark (1985) took the anthrone value of this fraction as a measure of the MDO content of *E. coli*. Later it was established that this fraction consisted mainly of a disaccharide, apparently trehalose (Kennedy and Rumley, 1988). In media of high osmolarity *E. coli* increases its intracellular concentration of potassium and glutamic acid, followed by accumulation of trehalose (Welsh *et al.*, 1991). The so-obtained higher level of the osmolarity of the cytoplasm balances that of the medium and therefore the decrease of cell volume is avoided. Like other compatible solutes such as glycine-betaine and glycine-proline, trehalose can function as osmo-protectant when cells are exposed to a high osmotic strength (Czonka, 1989). In a low osmolarity environment, trelalose is present in several species of *Rhizobium* in low amounts between 0-40 μg/mg protein (Streeter, 1985). Therefore, at both low and high osmotic pressures of the medium, LM_r-carbohydrates play an important role in osmo-adaptation.

2.3.2 INVOLVEMENT IN BACTERIUM-PLANT INTERACTION

Experimental evidence has been presented that (1,2)-ß-D-glucans are of importance during the process of infection of root hairs of their hosts plants by rhizobia or in tumorformation by agrobacteria. The relation between the presence of oligo- and/or polysaccharides and the ability to infect plants (*Rhizobium* sp.) or to form tumors (*Agrobacterium* sp.) has been an important topic in the research area of the microbe-plant interactions. In

Rhizobium not only cyclic glucans, but other cell-surface carbohydrates like EPS, CPS, and LPS (for reviews see Halverson and Stacey, 1986; Long, 1989; Gray and Rolfe, 1990) are thought to be of importance in the complex process from recognition and attachment to invasion and nitrogen fixation. Recently, a sulfated tetrasaccharide in cultures of *R. meliloti* was found which elicited root hair deformation on the host in nanomolar concentrations (Lerouge *et al.* 1990).

The leguminous plants contribute to host specificity by synthesis of flavonoids and lectins (for a recent review, see Long 1989). When studying the lectin-polysaccharide binding capacity with LPS fractions of *R. leguminosarum* an unidentified glucan fraction interfered with pea lectins while EPS and the polysaccharide part of LPS did not (Planqué and Kijne, 1977). In other studies on LPS purification, similar glucan fractions were described (Wolpert and Albersheim, 1975; Humphrey and Vincent, 1975). This cell-wall associated glucan was identified as cyclic (1,2)-ß-D-glucan and the cellular concentration depended on the cultural conditions, while the LPS remained constant (Zevenhuizen and Scholten-Koerselman, 1979). Enzyme preparations from *Trifolium repens* were able to degrade polysaccharides like LPS, CPS, EPS and and a LM_7-glucan fraction, apparently (1,2)-ß-glucan, from its symbiont *R. leguminosarum* bv *trifolii* better than those from bv *viciae* (Solheim and Fjellheim, 1984). However, since no qualitative differences exist in the structures of either glucans, CPS or EPS between the two biovars no conclusive explanation can be given for these observations.

Addition of purified cyclic glucans increased the number of nodules and enhanced the formation of infection threads in the *R. leguminosarum* bv *trifolii*-clover symbiosis. Addition of γ-cyclodextrin showed a comparable effect, while the effects with anionic EPS were less pronounced (Abe *et al.*, 1982). The beneficial influence on nodulation by two comparable but distinct cyclic structures suggests that the glucans might function as a carrier of signalling molecules during infection behaviour. In wildtype *R. meliloti* cyclic glucan addition in the micromolar range stimulated nodule formation as well (Dylan *et al.*, 1990b).

Mutants of *Rhizobium* and *Agrobacterium* affected in glucan synthesis are often impaired in their infection or virulence behaviour as well, but it often required quite a bit of effort to fully understand the implication of a single mutation on both glucan synthesis and infection behaviour. For instance, *A. tumefaciens* (Thomashow *et al.*, 1987) and *R. meliloti* (Cangelosi *et al.*, 1987) mutants in the *exoC* gene were found to be non-infective and appeared to be defective in EPS, cyclic glucan synthesis and in motility. It was established that the *exoC* gene encoded for phosphoglucomutase, an enzyme involved in the biosynhesis of UDP-glucose which is substrate in the biosynthesis of several polysaccharides (Uttaro *et al.*, 1989).

***ChvA* and *chvB* mutants of *A. tumefaciens*.** *ChvA* mutants of *A. tumefaciens* A348 were avirulent and defective in a 75 kDa membrane-protein, did not contain glucans in the periplasmic space or extracellular matrix but accumulated glucans in the cytoplasm (Cangelosi *et al.*, 1989). These glucans were nonsubstituted (Inon de Iannino and Ugalde, 1989). Cyclic glucan deficient mutants of *A. tumefaciens* A348 were avirulent and did not attach to plant cells, suggesting that cyclic (1,2)-ß-D-glucans were involved in the attachment of these bacteria to plant cells (Puvanaserah *et al.*, 1985). However, after addition of purified cyclic glucans in virulence tests *chvA* mutants were still avirulent. It was suggested that extracellular glucans were not involved in virulence (O'Connell and Handelsman, 1988). This suggestion was strenghtened by the fact that *chvB* mutants of *A. tumefaciens* also failed to synthesize rhicadhesin, a calcium-binding protein involved in attachment to the host cell-surface (Smit, 1988). Addition of rhicadhesin restored, although not to 100%, the attachment ability and virulence of the mutants indicating that rhicadhesin and not (1,2)-ß-glucan is essential for virulence (Smit, 1988). The *chvA* and *chvB* genes of *A. tumefaciens*, which are involved in synthesis of cyclic glucans, were not only essential for tumor formation but also for formation of root nodules. *ChvA* or *chvB* mutants of *A.*

tumefaciens containing the Sym plasmid of *R. leguminosarum* bv *phaseoli* failed to form nodules while the wild type did (Van Veen *et al.*, 1987).

***NdvA* and *ndvB* mutants of *R. meliloti*.**
In studies with *ndvA* mutants of *R. meliloti* 104F34 defective in nodulation, the oligosaccharide containing glucose and galactose, apparently the repeating unit of succinoglycan, was absent in the medium while with the wild type both cyclic glucans and the oligosaccharide were present. It was suggested that *ndvA* is involved in transporting next to cyclic glucans other compounds like the oligosaccharides as well (Stanfield *et al.*, 1988). Geremia *et al.* (1987) reported that *ndvB* mutants of *R. meliloti*, lacking the 235kDa protein and therefore did not synthesize glucans, induced only pseudonodules in alfalfa. The mutants produced twice the amount of succinoglycan. It was hypothezised that the overproduction of EPS resulted from increased availability of endogenous UDP-glucose being the precursor for both glucans and EPS (Geremia *et al.*, 1987).

CONCLUSION

Mutations in genes coding for proteins involved in basal metabolic enzymes or export-proteins, and leading to a defect in the synthesis of cyclic glucans, may affect levels of other polysaccharides as well. Therefore, firm conclusions about the role of cyclic glucans in osmoregulation or in the infection process have to be taken with care, when results were obtained with such pleiotropic mutants. It might well be that mutants of *Rhizobium* and *Agrobacterium* defective in (1,2)-ß-glucan synthesis are not able to infect properly because their osmotic adjustments are impaired, but such a supposed role can not explain all the results. Therefore, (i) the *chv/ndv* genes might be involved in other processes except for the synthesis and translocation of glucans, or (ii) cyclic glucans may in addition have a function that is unrelated to its role in osmoadaptation. This parallels the findings in *E. coli*, where the MDO synthesis is not only osmotically regulated, but genetically linked to outer membrane composition and chemotactic behaviour (Fiedler and Rotering, 1988).

Most of the studies concerning the biological role of cyclic glucans are performed with *R. meliloti* and *A. tumefaciens*. The role of cyclic glucans in osmo-adaptation of *R. leguminosarum* at low osmolarity has not been investigated so far. Studies with glucan-negative mutants might be of use in assessing the role of glucans in *R. leguminosarum*.

2.4 PRODUCTION AND TECHNICAL APPLICATION OF CYCLIC GLUCANS

2.4.1 PRODUCTION OF CYCLIC GLUCANS

Cellular concentrations of (1,2)-ß-D-glucans follow a distinct pattern depending on the species, medium composition and the growth phase. In a 0.5% mannitol-0.1% glutamic acid-salts medium (osmotic pressure π=1.3 atm) cells of *R. leguminosarum* build up concentrations of (1,2)-ß-D-glucans to a maximum of 100 mg/g dry cell weight at the end of the logarithmic phase. These concentrations were reduced by more than 50 % during the stationary phase, during which CPS was deposited around the cells, while the glucans were found to be excreted into the medium. In contrast, *R. meliloti*, which forms no capsules, produces up to 200 mg (1,2)-ß-D-glucans/g dry cell weight. During the stationary phase, this concentration remained constant (Zevenhuizen, 1981; Zevenhuizen and Van Neerven, 1983).

Attempts to optimize glucan production in *Rhizobium* spp. were made by Higashiura *et al.* (1985). They selected a *R. phaseoli* mutant strain AHU 1133, obtained by chemical mutagenesis, which did not produce EPS but excreted a single glucan ring of DP=17. This mutant strain excreted 1.2 g of (1,2)-ß-D-glucan per liter (estimated as 0.8 g glucan/g cell protein) which was about 4.5 times more than the wildtype strain. In media with high concentrations of nutrients which promote growth to high cell densities, *R. trifolii* TA-1 excreted 4 g of glucan per liter into the medium (calculated as 2.2 g glucans/g cell protein; Zevenhuizen, 1986). Comparable results were achieved with EPS-negative mutants of *R. leguminosa-*

num 8401 pRL1JI pss1::Tn5 with yields between 2.6-3.2 g cyclic glucans/l (calculated as 1.3-1.6 g glucans/g protein; Harris *et al*, 1991). For production of (extracellular) cyclic glucans, it is not necessary to separate cells from supernatant. By treating whole cultures with excess ethanol cells were both extracted and precipitated together with HM_r-EPS from the supernatant. The cyclic glucans can then be isolated from the alcoholic supernatant (Hisamatsu *et al.*, 1983).

In these studies which were aimed towards the optimization of cyclic glucan production, the cyclic glucans were excreted under nitrogen limiting growth conditons in stationary phase in the presence of excess carbon-source, and at incubation times of at least 10 days. In most studies concerning the biological role of glucans, harvesting of cells occurs in mid-logarithmic phase, and often various kinds of media are used. Therefore, comparisons between reported production capacities are difficult to make.

2.4.2 TECHNICAL APPLICATION

Cyclic (1,2)-ß-D-glucans are potentially useful molecules for inclusion of agents used for pharmaceutical purposes, because of their relatively hydrophobic internal space. Their internal space makes the glucans suitable as inclusion agents, comparable to the cyclodextrins. The cyclodextrins are a mixture of rings consisting of 6 to 8 glucose residues arranged in a linear manner with α-(1,4)-bonds. Their synthesis occurs by conversion of linear maltooligosaccharides into cyclomalto-oligosaccharides catalysed by cyclodextrin glycosyltransferase (Sjejtli 1990). Geometric parameters for maximum-energy-minimum symmetry conformations of cyclic glucans were calculated for rings with DP=15-24 (Palleschi and Crescenzi, 1985). The cavities of the cyclodextrin ring with DP=8 and cyclic (1,2)-ß-D-glucan of DP=18 (88 nm) were calculated to be nearly equal (Pallaschi and Creszenzi, 1985), but cyclic (1,2)-ß-D-glucan are better soluble in water, up to 250 g/l (Koizumi *et al.*, 1984). The application of cyclodextrins as inclusion agents of lipophilic compounds has been described for processes like the enzyme-catalyzed transformation of steroids in aqueous media. Cyclodextrins enhance the solubility of complexed substrates in aqueous media, but do not damage the microbial cells or the enzymes involved. Furthermore, cyclodextrins can form complexes with toxic compounds which may be of use for bacterial populations in waste waters of polluting factories (for a review, see Szejtli 1990). The naringenin-complexing capacity of cyclic glucans of *R. leguminosarum* bv *viciae* 8401 has been recently reported. Naringenin, a flavonoid essential insoluble in water, is known to induce nodulation genes, suggesting a role of cyclic glucan as complexing agent of naringenin *in vivo* (Morris *et al.*, 1991). Aqueous solutions of cyclic (1,2)-ß-D-glucan (DP=17) of *R. phaseoli* AHU 1133 were shown to be a carrier of slightly soluble guest molecules, supplied as a solution in acetone. Vitamin K_1, which is essentially insoluble in water, was solubilized in this manner to 0.2 mg/ml (Koizumi *et al.*, 1984). No other applications on cyclic glucan inclusion complexes have been reported.

SUMMARY

Depending on cultural conditions, cyclic (1,2)-ß-glucans are excreted in high amounts in culture supernatants of *R. leguminosarum* sp., and can be isolated in a relatively easy manner. Cyclic glucans and cyclodextrins both have a relatively hydrophobic internal space and can function as carrier of hydrophobic molecules. While the advantage of cyclic glucans over cyclodextrins is the greater solubility in water, only a few reports have appeared on the inclusion capacity of cyclic glucans.

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CHAPTER 3

Excessive excretion of cyclic ß-(1,2)-glucan by *Rhizobium trifolii* TA-1

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Excessive Excretion of Cyclic β-(1,2)-Glucan by *Rhizobium trifolii* TA-1

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At 25°C, the optimal temperature for growth of *Rhizobium trifolii* TA-1, extracellular and capsular polysaccharide (EPS and CPS) were the main carbohydrate products synthesized in mannitol-rich medium (10 g of mannitol and 1 g of glutamic acid per liter). In the same medium at 33°C, EPS and CPS production was inhibited, and up to 3.9 g of cyclic β-(1,2)-glucan was produced during an incubation period of 20 days with a total biomass of 0.55 g of protein. In a medium containing 50 g of mannitol and 10 g of glutamic acid per liter, high cell densities (3.95 g of protein) were obtained at 25°C. This biomass excreted 10.9 g of cyclic β-(1,2)-glucan within 10 days. Concomitantly, 4.8 g of EPS was synthesized, while CPS production was strongly suppressed. The excreted cyclic β-(1,2)-glucans were neutral and had degrees of polymerization ranging from 17 to 25, with a degree of polymerization of 19 as the major glucan cycle.

Fast-growing rhizobia are capable of synthesizing a variety of polysaccharides. Depending on the growth phase and cultural conditions, their production patterns may vary considerably (23). These organisms excrete high-molecular-weight anionic extracellular polysaccharide (EPS) into the medium, cover their cells with the insoluble neutral capsular polysaccharide, and can form cellulosic microfibrils (24). Sometimes, low-molecular-weight compounds like repeating units of EPS and cyclic β-(1,2)-glucans can be found in the culture medium as well (8, 25).

Bacteria of the genera *Rhizobium* and *Agrobacterium* contain cyclic β-(1,2)-glucans in their cells. These molecules accumulate preferentially in the periplasmic space (1). It is believed that they play a role, like the membrane-derived oligosaccharides of *Escherichia coli*, during osmotic adaptation (10, 15, 19). It is also suggested that β-(1,2)-glucans may be of importance during the process of root hair infection by these bacteria. Mutants of *R. meliloti* unable to form β-(1,2)-glucan induced only pseudonodules in alfalfa (5). For *Agrobacterium tumefaciens*, it is thought that cyclic β-(1,2)-glucans are involved in the attachment of these bacteria to plant cells (17).

In media with high concentrations of substrates and nutrients which promote growth to high cell densities, *R. trifolii* TA-1 excretes up to 4 g of glucan per liter into the medium (23). Attempts to optimize glucan production in *Rhizobium* spp. were made by Higashiura et al. (7). These authors used EPS-negative mutants by selecting nonslimy colonies from solid medium. One mutant strain excreted 1 g of β-(1,2)-glucan per liter, which was about 4.5 times more than the wild type.

In this report, we show that *R. trifolii* TA-1 can be forced to excrete large amounts of cyclic β-(1,2)-glucan as the main polymer product. The greatly increased production of glucans was obtained by growing the organism either at greater-than-optimal temperatures or in media which promoted the formation of high cell densities.

MATERIALS AND METHODS

Microorganisms and cultivation. The following organisms were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands: *R. trifolii* TA-1, 0403, and Coryn and *R. leguminosarum* 1044 and PF-2. These bacteria were maintained on agar slants containing a medium of yeast extract, mannitol, and mineral salts (22). Precultures were prepared by inoculating the organism into standard medium with the following composition (in grams per liter of distilled water): mannitol, 5; glutamic acid, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; $FeCl_3 \cdot 6H_2O$, 0.0025; $MnCl_2 \cdot 4H_2O$, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.00001; $ZnSO_4 \cdot 7H_2O$, 0.00001; $CuSO_4 \cdot 5H_2O$, 0.00001; H_3BO_3, 0.00001; $CoCl_2 \cdot 6H_2O$, 0.00001; biotin, 0.00001; and thiamine, 0.0001. The pH was adjusted to 7.0 with NaOH. All media were autoclaved at 121°C for 25 min. Most batch culture experiments were done in a production medium which contained 10 g of mannitol per liter. Because of the higher carbon/nitrogen ratio in this medium, higher concentrations of polysaccharides were obtained. To obtain high cell densities, extra mannitol plus glutamic acid and/or vitamins and mineral salts were added. The exact amounts for each experiment are given in Results.

Cells from the late logarithmic phase of a culture were used as an inoculum (1% [vol/vol]) for 50 ml of medium in a 300-ml Erlenmeyer flask. The flasks were incubated on a rotary shaker at 200 rpm. Alternatively, cells were grown in a 2-liter batch fermentor (Applikon, Meyvis, Schiedam, The Netherlands) with 1 liter of medium. This fermentor was used because it allowed control of aeration and agitation. The cultures were aerated at the rate of 1 liter of air per min and stirred at 500 rpm. Oxygen measurements were done with a Clark-type electrode.

The purity of the culture was routinely checked by plating appropriate dilutions on yeast extract-mannitol-Congo red agar (24). On these plates, cellulosic microfibrils color deep red. Cell shape and uniformity of the culture were also checked by phase-contrast microscopy at a magnification of ×1,500.

Measurement of growth and polysaccharide production. Cells and culture supernatant were separated by centrifuga-

tion at 35,000 × *g* for 30 min. Cultures that were too viscous were diluted with distilled water before centrifugation. The growth rate was calculated from the optical density measured at 660 nm at time intervals. In the stationary phase, accumulation of polysaccharides and poly-β-hydroxybutyric acid interfered with optical density measurements. Total cellular protein was routinely used for quantifying the biomass (see below).

Excreted glucan and extracellular polysaccharide were determined in the supernatant by the anthrone-sulfuric acid method for hexoses (21) and the 3-hydroxydiphenyl-sulfuric acid method for hexuronic acids (3). The determination of EPS was based on the uronic acid content of the supernatant and the known hexose/hexuronic acid ratio of 3:1 for anionic EPS produced by *R. trifolii* (23) and was expressed as the sum of glucose and glucuronic acid equivalents (in milligrams per liter of culture). The β-(1,2)-glucan content was derived from the amount of glucose which was not accounted for in the calculation of the EPS concentration.

Mannitol was determined by periodate oxidation followed by the chromotropic acid colorimetric method (4). The relative viscosity of the culture supernatant was measured with an Ubbelohde viscometer (Tamson, Zoetermeer, The Netherlands) at 25°C.

Cell pellets were suspended in 10 ml of water and briefly sonicated to obtain a homogeneous suspension. Capsular polysaccharide (CPS) was extracted from the pellet with 1 N NaOH at 70°C for 15 min. The mixture was centrifuged, the clear supernatant was decanted, and the CPS was precipitated by 1 volume of alcohol and centrifuged. The precipitate was redissolved in NaOH. CPS, which had a sugar composition of 4:1:1 galactose:glucose:mannose (23), was quantified by the anthrone-sulfuric acid method and given as milligrams of galactose equivalents per liter of culture. The carbohydrate fraction of the supernatant after alcohol precipitation consisted mainly of cellular cyclic β-(1,2)-glucan. This compound was quantified by the anthrone-sulfuric acid method and expressed as milligrams of glucose equivalents per liter of culture.

Total cellular protein was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard.

Isolation and purification of β-(1,2)-glucan. After cells were removed from the culture by centrifugation, the EPS remaining in the culture supernatant fluid was precipitated by the addition of 3 volumes of ethanol. After subsequent centrifugation (5,000 × *g* for 10 min.), the 75% alcoholic supernatant fluid was concentrated by rotation evaporation under vacuum to about 1/10 of the original volume. The remaining EPS was removed by the addition of 3 volumes of ethanol, and the precipitate was collected by centrifugation. Up to 10 volumes of ethanol was added to the remaining clear solution. A white precipitate of β-(1,2)-glucan formed after this solution stood overnight at 4°C and was collected by centrifugation. This precipitate was checked for contamination by uronic acid-positive components and was purified further by filtration through an Ultrogel AcA 202 column (2.5 by 30 cm; fractionation range, 1,000 to 15,000 daltons; Pharmacia-LKB, Woerden, The Netherlands) with water as the eluent. Fractions of 5 ml were collected, and the major peak containing anthrone-positive material was collected, freeze-dried, and used for further analysis.

Characterization of the cyclic β-(1,2)-glucan fraction. To check for the neutral sugar composition, 3 mg of the freeze-dried material was hydrolyzed for 8 h in 1 N trifluoroacetic acid. The sugar composition was determined with gas-liquid chromatography by converting the liberated sugars into their alditolacetates (25). Glycosyl linkage type was determined by methylation of the free hydroxyl groups followed by acid hydrolysis. Determination of the partially methylated sugars was done with gas-liquid chromatography (6) by using a capillary CP Sil 43-CB column (Chrompack, Middelburg, The Netherlands) connected to a flame ionization detector in a Kipp Analytica 8200 gas-liquid chromatograph (Kipp & Zonen, Delft, The Netherlands) at 200°C.

To obtain an approximation of the ring size distribution, 1-mg samples of β-(1,2)-glucan were hydrolyzed at time intervals (10, 20, 40, 60, 120, and 240 min) at 100°C in 1 N trifluoroacetic acid. After evaporation of trifluoroacetic acid at 40°C under vacuum, the partial hydrolysates were put on thin-layer chromatography plates of Silica gel 60 (Merck, Amsterdam, The Netherlands) and chromatographed with butanol-ethanol-water (5:5:4 [vol/vol/vol]) as the eluent in the ascending way (two times). After drying, spraying with 5% ethanolic H_2SO_4, and heating the plates for 15 min at 105°C, black spots developed corresponding to linear fragments with various degrees of polymerization.

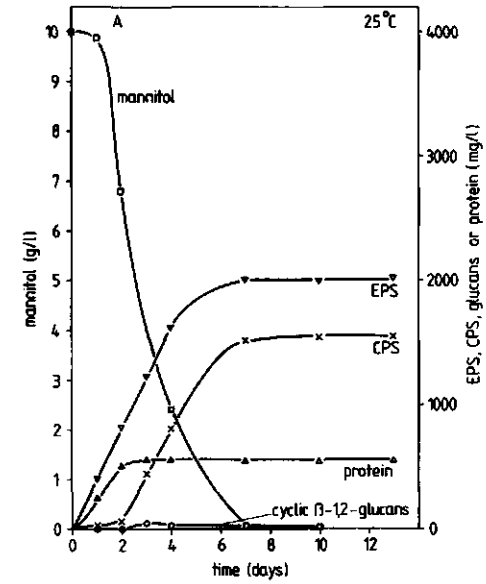
Ring size distribution was accurately determined by high-performance liquid chromatography (HPLC) (2). Five-microliter samples (5 mg of glucan per ml) were analyzed by using a Chrompack Hypersyl APS column (Chrompack, Middelburg, The Netherlands) connected to a refractive index detector (LKB 2142; Pharmacia-LKB, Woerden, The Netherlands). The mobile phase was chromatography-grade acetonitrile:water (Milli-Q; Millipore, Etten-Leur, The Netherlands) (2:1) at a flow rate of 0.5 ml/min. Using these conditions, degrees of polymerization (DP) up to 29 could easily be recognized. Pure β-(1,2)-glucan with a ring size of 21 residues (a gift from R. H. Fokkens, University of Amsterdam) was used as a standard. This had been purified by preparative HPLC, and its molecular mass was determined by FAB-mass spectroscopy as described in reference 18. By adding the standard to the glucan fraction, the ring with 21 residues could be positively identified.

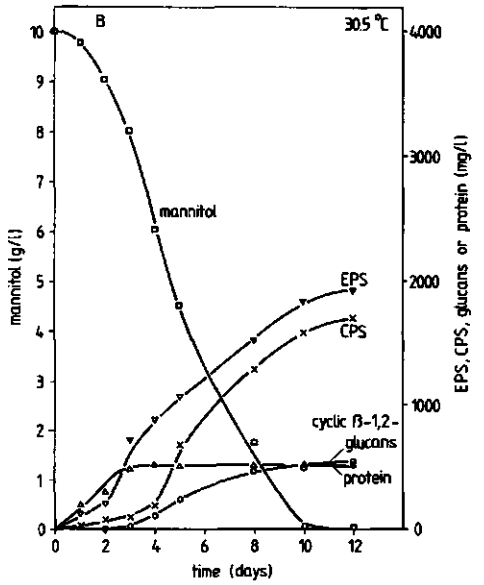
Chemicals. All chemicals were at least of analytical grade and were obtained from Merck if not indicated otherwise.

RESULTS

Temperature effects on growth and polysaccharide production. To compare temperature effects on growth and production of polysaccharides by *R. trifolii* TA-1, cultures were incubated at 25, 30.5, or 33°C in production medium and analyzed at various times (Fig. 1).

At 25°C, the generation time for *R. trifolii* TA-1 was 5.5 h and the cells were in stationary phase after 48 h. At this point, CPS synthesis started and EPS synthesis progressed because of the surplus mannitol. Rod-shaped cells were embedded in a polysaccharide matrix as the culture aged. Growth characteristics and polysaccharide production by *R. trifolii* TA-1 remained the same between 25 and 30°C (data not shown). At 25°C, a very small amount of cyclic β-(1,2)-glucan (40 mg/liter of medium) was excreted into the medium during the stationary phase (Fig. 1A). The viscosity of the medium was 32 cP because of the extended production of high-molecular-weight EPS. At 30.5 to 31°C, a shift took place from the EPS and CPS production to cyclic glucan excretion (Fig. 1B). At 33°C the growth rate was diminished (generation time, 12 to 13 h), and growth was nonexponential. The cells were of irregular shape and much larger than at 25°C. Glucan release started in early stationary phase and reached 3,900 mg/liter, while production of EPS (400 mg/liter) and CPS (100 mg/liter) was considerably reduced (Fig.





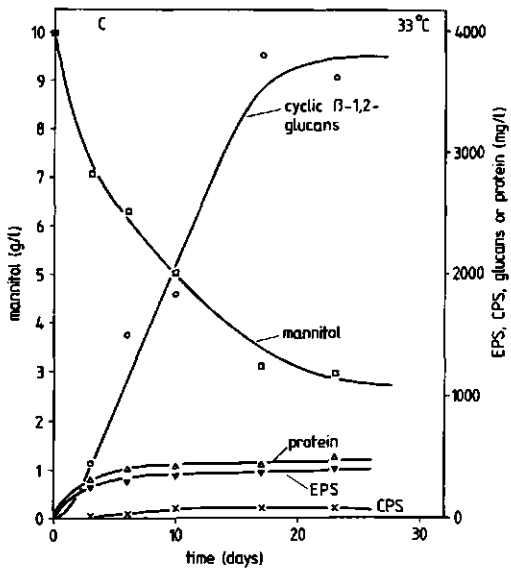


FIG. 1. Growth and polysaccharide production by *Rhizobium trifolii* TA-1 at 25 (A), 30.5 (B), and 33°C (C). Cells were incubated in 300-ml Erlenmeyer flasks on a rotating platform (300 rpm). These data are representative for at least three independent experiments done at each temperature. The final polysaccharide concentrations varied by not more than 5% of the data shown.

1C). The viscosity of the medium was only 1.2 cP. Cellular glucan content at all temperatures was about 30 to 50 mg/g of protein. *R. trifolii* TA-1 did not grow above 37°C.

To check whether oxygen limitation could have occurred during incubation at 33°C and triggered overproduction of cyclic β-(1,2)-glucan, experiments in the fermentor were done with a constant air supply at both 80 and 10% air saturation. The results from these studies (data not shown) did not differ from the polysaccharide production pattern at 33°C given in Fig. 1C.

Mannitol-dependent β-(1,2)-glucan excretion at 33°C. To determine the influence of the mannitol concentration on the excretion of cyclic glucan at 33°C, various mannitol concentrations ranging from 0 to 10 g/liter were tested in standard medium (Fig. 2). With no mannitol added, cell protein reached 325 mg/liter of medium. In all other incubations, cell protein content was the same (490 to 540 mg/liter). Increased initial mannitol concentrations were paralleled with increased cyclic β-(1,2)-glucan release. Incubations with initial mannitol concentrations over 10 g/liter did not lead to a higher production of glucans (data not shown).

Ring size distribution. At 33°C, more than 4,000 mg of glucose equivalents per liter were found in the supernatant. The low glucuronic acid content indicated only a small EPS fraction. The 75 vol% alcohol-soluble portion of the culture supernatant contained 90% of the anthrone-positive material. Purification of this portion over an Ultrogel AcA 202 column gave one peak of anthrone-positive material, consisting exclusively of glucose (85% of the total sugar portion was recovered in this peak). Methylation resulted solely in 3,4,6-trimethylglucose, indicating exclusively 1-2 glucosyl bonds in an unbranched molecule of cyclic nature. On thin-layer chromatography plates, the black spots were characteristic for cyclic β-(1,2)-glucans of *R. trifolii* (11). Only neutral cyclic β-(1,2)-glucans could be detected. HPLC analysis of the intact glucan revealed a degree of polymerization (DP) pattern from 17 to 25, with a DP of 19 as the major peak (Fig. 3).

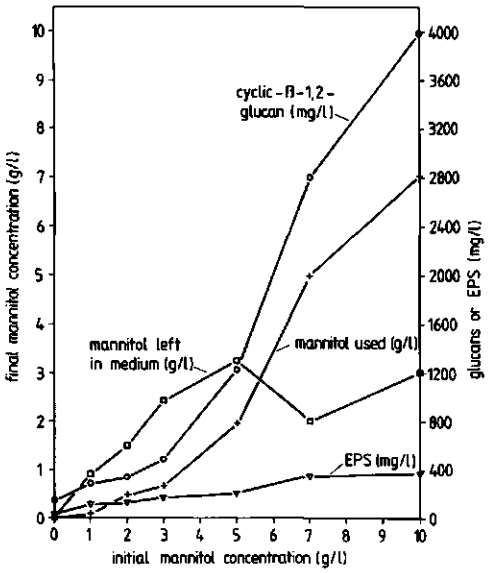


FIG. 2. Influence of the initial mannitol concentration on polysaccharide production by *R. trifolii* TA-1 incubated at 33°C for 17 days. The mannitol consumed was determined by subtracting the final concentration of mannitol measured in the supernatant after 17 days from the initial concentration of mannitol. Cells were incubated in 300-ml Erlenmeyer flasks on a rotating platform (300 rpm). These data are representative for two experiments, in which values varied within 5% of the data presented.

Since the anthrone-positive fraction in the alcoholic supernatant consisted solely of cyclic β-(1,2)-glucan and the glucan fraction made up almost 90% of the total hexose content of the supernatant, it appeared safe to assume that the anthrone-positive fraction detected in the supernatant, which could not be accounted for as EPS, was indeed cyclic β-(1,2)-glucan.

Temperature-dependent excretion of β-(1,2)-glucan by other *Rhizobium* strains. Temperature-dependent glucan excretion was not restricted to *R. trifolii* TA-1. *R. leguminosarum* and other *R. trifolii* strains also produced an excess of glucans at 33°C (Table 1). Except for *R. trifolii* Coryn, all strains showed reduced EPS and CPS synthesis at 33°C, and the major part of the anthrone-positive fraction in the supernatant consisted of cyclic β-(1,2)-glucan, as calculated from the glucuronic acid and glucose content of the supernatant.

Response of *R. trifolii* TA-1 to high growth substrate concentrations. It has been reported that in media which promote growth to high cell densities, TA-1 excreted up to 4 g of cyclic glucan per liter of medium (23). Therefore, standard medium was supplemented with additional amounts of mannitol and glutamic acid in an attempt to produce higher concentrations of cyclic β-(1,2)-glucan (Table 2). Biomass increased over the whole range tested, though above 20 g of mannitol and 4 g of glutamic acid per liter the increase in biomass was no longer proportional to the substrate concentration. Proportionally lower protein synthesis was paralleled by a slight decrease in EPS and by a dramatic decrease in CPS production. The excretion of cyclic glucan, however,

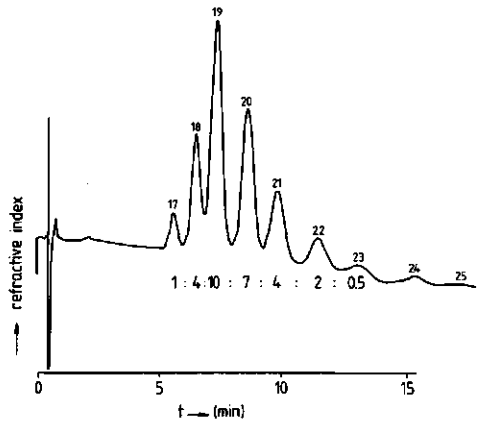


FIG. 3. Elution profile of the purified glucan fraction on an amino-bonded silica column as determined by HPLC. Eluent acetonitril:water ratio was 2:1. The ring size distribution pattern is indicated in the figure. The glucan fraction was obtained from the supernatant of a 1-liter *R. trifolii* TA-1 culture grown at 33°C in a 2-liter fermentor and was purified by gel filtration as described in Materials and Methods.

increased considerably with higher mannitol and glutamic acid concentrations, reaching 10.9 g/liter at the highest substrate concentrations. This yield of cyclic glucan represented 21.8% of the mannitol initially added.

The relative reduction of protein content in the incubations with high amounts of mannitol and glutamic acid compared with those using standard medium could have been the result of a lack of vitamins and inorganic nutrients. Therefore, not only the amounts of mannitol and glutamic acid but also the concentrations of vitamins and mineral salts were increased. Extra mineral salts and vitamins (Table 3, 10 ×GMS medium) did not result in a proportionally higher protein production compared with 10×GM medium. Compared with the 10×GM or 10×GMS medium, 10 times the concentrations of either mineral salts plus vitamins, glutamic acid, or mannitol did not drastically affect the production of cyclic glucan. It is clear from these data that growth in

TABLE 1. Polysaccharide-production at 25 and 33°C by various *Rhizobium* strains in production medium

Organism	Temperature (°C)[a]	Production (mg/liter) of:		
		EPS[b]	Glucans[c]	CPS[d]
R. trifolii TA-1	25	1,540	50	1,370
	33	500	1,950	400
R. trifolii 0403	25	650	15	260
	33	150	260	0
R. trifolii Coryn	25	370	0	160
	33	360	110	35
R. leguminosarum 1044	25	2,200	20	975
	33	400	2,250	90
R. leguminosarum PF-2	25	890	25	65
	33	335	550	15

[a] Incubation times were 10 days (25°C) and 14 days (33°C).
[b] Expressed as glucose plus glucuronic acid equivalents.
[c] Expressed as glucose equivalents.
[d] Expressed as galactose equivalents.

TABLE 2. Polysaccharide production by *R. trifolii* TA-1 at 25°C at different concentrations of mannitol and glutamic acid

Medium composition[a]		Incubation time (days)[b]	Production (mg/liter) of:			
Mannitol (g/liter)	Glutamic acid (g/liter)		Cellular protein	Glucans[c]	EPS[d]	CPS[e]
2.5	0.5	7	320	0	835	210
5	1	7	550	0	1,500	325
10	2	10	950	10	2,200	790
15	3	10	1,260	380	2,665	870
20	4	15	1,750	2,150	2,695	1,110
25	5	15	1,800	5,040	2,060	545
50	10	15	2,350	10,900	2,085	295

[a] Experiments were done in 300-ml Erlenmeyer flasks containing 100 ml of standard medium with mannitol and glutamic acid as indicated. These data were obtained in one experiment but are representative for a number of replicates. Polysaccharide concentrations in the different experiments varied maximally by 8% from the numbers shown here.
[b] All mannitol was consumed except in the 50 g of mannitol per liter condition, in which 44 g of mannitol was consumed per liter.
[c] Expressed as glucose equivalents.
[d] Expressed as glucose plus glucuronic acid equivalents.
[e] Expressed as galactose equivalents.

production medium was nitrogen limited because the addition of glutamic acid resulted in an increase of biomass. Although glutamic acid can serve as a carbon source, the cultures were certainly not carbon limited.

At the very high biomass concentrations (2.8 g of protein per liter with 10×GMS), oxygen supply to the cells might have limited growth efficiency and the yields of various polysaccharides. Therefore, an experiment was performed in a continuously aerated (2 liters of air per min) and agitated (1,200 rpm) 2-liter fermentor containing 1 liter of the 10× GMS medium. During logarithmic growth, the dissolved oxygen concentration never fell below 25% air saturation. After exponential growth ceased, the medium contained 90% air. Growth, mannitol consumption, and polysaccharide production from this experiment are shown in Fig. 4. A comparison of the results in Fig. 4 and Table 3 suggested that the organisms grown in the Erlenmeyer flasks were probably oxygen limited for a certain period, since less biomass, cyclic glucan, and EPS were formed. The ratio between cyclic glucans released and the total biomass (estimated on the basis of protein content), however, was much higher in the Erlenmeyer flasks than in the well-aerated fermentor, namely 3.2 versus 2.3 g/g. Because of these data, we do not assume that massive lysis can be the reason for the high glucan content found in the supernatant, although some protein was measured in the supernatant as well.

TABLE 3. Growth and polysaccharide production of *R. trifolii* TA-1 in different media[a]

Supplements to production medium[b]	Production (mg/liter) of:			
	Cellular protein	Glucan[c]	EPS[d]	CPS[e]
Control	530	40	2,340	1,680
10×S	510	300	1,450	1,320
10×M	540	80	2,600	1,260
10×G	2,800	560	930	140
10×GMS	2,800	9,400	2,100	180
10×GM	2,500	9,000	2,500	120

[a] Cells were incubated for 12 days at 25°C. Data from replicates did not vary by more than 5% from the numbers shown here.
[b] Control, No supplement; 10×S, 10× concentrated mineral salts and vitamins; 10×M, 10× concentrated mannitol (50 g/liter); 10×G, 10× concentrated glutamic acid (10 g/liter); 10×GMS, 10× concentrated mineral salts, vitamins, mannitol, and glutamic acid; 10×GM, 10× concentrated glutamic acid and mannitol.
[c] Expressed as glucose equivalents.
[d] Expressed as glucose plus glucuronic acid equivalents.
[e] Expressed as galactose equivalents.

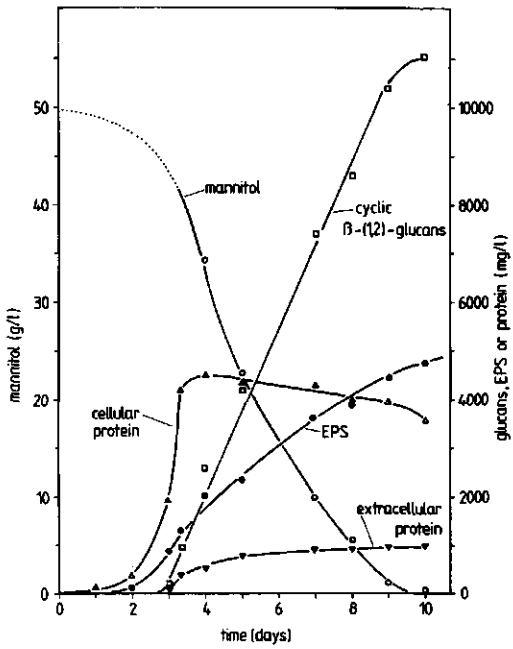


FIG. 4. Growth and polysaccharide production by *R. trifolii* TA-1 in the 10-fold-concentrated standard medium (10×GMS; see Table 3) at 25°C in a 2-liter batch fermentor with strong aeration (2 liters of air per min) and high agitation (impeller speed, 1,200 rpm). These data are representative for three independent experiments. The final concentrations varied by not more than 5%.

The distribution patterns of the ring sizes of the cyclic glucans obtained in the experiments reported in Table 3 and Fig. 4 did not differ markedly from those given in Fig. 3.

Effect of high growth substrate concentrations at 33°C. When *R. trifolii* TA-1 was incubated at 33°C in 10×GMS medium, biomass reached only 0.84 g of protein within 7 days and remained constant up to 17 days. At this point, EPS and CPS syntheses were greatly reduced (185 and 120 mg/liter, respectively), while cyclic glucan production reached 3,200 mg/liter of medium. In this experiment, the ratio between glucan and biomass produced was 3.8. This value was slightly higher than the ratio of 3.2 obtained with the 10×GMS medium incubated at 25°C but lower than the 7.1 obtained with cells incubated at 33°C in production medium.

DISCUSSION

Cyclic β-(1,2)-glucans are present in the periplasmic space of *Agrobacterium* and *Rhizobium* spp. (1). Their sizes range between 3,000 and 4,000 daltons. In stationary phase, small quantities are found in the culture medium of *R. trifolii* TA-1 (23), possibly because the outer membrane is partially per-

meable for cyclic glucans. However, active transport cannot be excluded, for in *R. meliloti* a protein involved in the export of β-(1,2)-glucan has been found (20). Colorimetric detection of low concentrations of glucan in the medium is often biased by the high concentration of EPS. Cyclic glucans were found for the first time in the culture medium of *Agrobacterium tumefaciens* (14). This crown-gall polysaccharide was described as a low-molecular-weight glucose polymer (3,600 daltons). It was isolated by precipitation with 10 to 20 volumes of ethanol after high-molecular-weight EPS was removed (14).

By increasing the incubation temperature from 25 to 33°C, an enormous shift in polysaccharide production by *R. trifolii* TA-1 and other rhizobia was observed. By promoting *R. trifolii* TA-1 to grow to high cell densities, the polysaccharide production pattern changed drastically as well. *R. leguminosarum* and *R. trifolii* are closely related to each other and are presented as biovars of the same species, *R. leguminosarum* (9). Furthermore, both biovars synthesize anionic EPS with the same sugar composition and structure of the repeat unit and, as is shown here, the investigated strains show the same temperature effect. At 33°C, the higher incubation temperature rather than the high mannitol concentration was the trigger for the observed glucan excretion. Whereas at 33°C glucans were produced independently of the mannitol concentration under all circumstances tested, at 25°C excessive excretion was observed only at high concentrations of mannitol and glutamic acid. It is known that polysaccharide production in *Rhizobium* is strongly dependent on the cultural conditions (23). Cyclic β-(1,2)-glucan excretion by *R. trifolii* TA-1 under extreme conditions of temperature and high cell densities might be an indication for a deregulation of its synthetic capacity.

The ring size distribution patterns of the extracellular cyclic β-(1,2)-glucans in cultures of *R. trifolii* TA-1 were the same in all experiments. They corresponded well to that of the type II cyclosophoraoses [cyclic β-(1,2)-glucans] with DPs of 17 to 25 and a main cycle with a DP of 19 (11). Higashiura et al. (7) succeeded in isolating a *R. phaseoli* mutant strain, ANU 1133, which did not produce EPS but excreted a single glucan ring with a DP of 17. Cyclic β-(1,2)-glucans may be substituted with glycerol-1-phosphates (15, 16). However, *R. trifolii* TA-1 makes only neutral glucan. Charged glucans were not found; these would run faster on thin-layer chromatography plates and would have been excluded from the neutral glucans by gel filtration.

Cyclic β-(1,2)-glucans are potentially useful molecules because of their relatively hydrophobic internal space. This makes them suitable as an inclusion agent, comparable to the cyclodextrins, which contain 6 to 8 glucose residues with α-(1,4) bonds. The internal spaces of both molecules are about the same, but the solubility of cyclic β-(1,2)-glucan is higher. Although much has been published about inclusion of compounds in cyclodextrins, little is known about cyclic β-(1,2)-glucan as a carrier (12). With *R. trifolii* TA-1, cyclic β-(1,2)-glucans are now easily accessible. This might help to stimulate more application-oriented research on this class of compounds.

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CHAPTER 4

Osmotically-induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47

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Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47

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In standard liquid medium containing 5 g mannitol l^{-1} and 1 g glutamic acid l^{-1}, *Rhizobium meliloti* SU-47 cells accumulated 350 mg cyclic 1,2-β-glucans (g protein)$^{-1}$. The cyclic glucans were 36% glycerol-1-phosphate-substituted and 64% were uncharged. In the same medium with 10 g mannitol l^{-1}, repeating units of succinoglycan (1110 mg l^{-1}) were found as extracellular carbohydrates, and only low amounts of the succinoglycan polymer (up to 300 mg l^{-1}) were excreted. By raising the osmotic pressure of the medium by the addition of NaCl or other ionic and non-ionic osmolytes, succinoglycan production could be stimulated: up to 2·4 g l^{-1} at 0·2 M-NaCl was produced at the expense of the repeating units. Above 0·2 M-NaCl growth was slowed down, and succinoglycan excretion diminished. At 1 M-NaCl growth stopped completely. In standard medium containing 0·6 M-NaCl the amount of cellular cyclic 1,2-β-glucans was lowered to 150 mg (g protein)$^{-1}$ out of which the glycerol-1-phosphate-substituted glucan fraction was reduced to 15%. Instead, high amounts of oligosaccharides were synthesized as osmoprotectants, with trehalose as the major component [up to 200 mg (g protein)$^{-1}$]. Glycogen synthesis was completely suppressed at this salt concentration, while poly β-hydroxybutyric acid synthesis was unaffected.

Introduction

Cyclic 1,2-β-glucans of rhizobia are considered to function as osmoregulants in order to equilibrate the osmotic balance between the cytoplasm and the periplasm, in particular at low osmotic values of the medium (Miller *et al.*, 1986). They are localized in the periplasmic space (Abe *et al.*, 1982). Cyclic 1,2-β-glucans were isolated for the first time from the extracellular fluid of an *Agrobacterium tumefaciens* culture (McIntire *et al.*, 1942). Since then, several authors have found 1,2-β-glucans in the cell (Zevenhuizen and Scholten-Koerselman, 1979; Miller *et al.*, 1986) or in the medium (Amemura *et al.*, 1983, 1985; Zevenhuizen, 1986) of a number of *Rhizobium* and *Agrobacterium* species.

Cellular concentrations of 1,2-β-glucans follow a distinct pattern depending on the species and the growth phase. In mannitol–glutamic acid–salts medium of low osmotic value cells of *R. leguminosarum*, biovars *viciae*, *trifolii* and *phaseoli* build up concentrations of 1,2-β-glucans to a maximum of 100 mg (g dry cell wt)$^{-1}$ at the end of the exponential phase. Their concentrations are reduced by more than 50% during the stationary phase, during which capsular polysaccharide (CPS) is deposited around the cells. In contrast *R. meliloti*, which forms no capsules, produces up to 200 mg 1,2-β-glucans (g dry cell wt)$^{-1}$. During the stationary phase, this concentration remains constant (Zevenhuizen, 1981; Zevenhuizen & Van Neerven, 1983).

At high osmotic values of the medium the synthesis of cyclic glucans is repressed in *Agrobacterium tumefaciens* (Miller *et al.*, 1986). Several authors have reported the formation of compatible solutes in the cytoplasm of cells grown under conditions of high osmotic pressure (Le Rudulier & Bernard, 1986; Czonka, 1989). At concentrations up to 1·3% NaCl growth of *R. meliloti* is not affected, while growth of *R. leguminosarum* biovar *trifolii* is impaired at 0·4% NaCl (Vincent, 1974).

In this paper we report how the osmotic values of the medium affect oligo- and polysaccharide synthesis by *Rhizobium meliloti* strain SU-47. Comparison is made with other *R. meliloti* and *A. tumefaciens* strains. A role for cellular and capsular carbohydrates in osmoregulation is also discussed.

Abbreviations: CPS, capsular polysaccharide, DP, degree of polymerization; EPS extracellular polysaccharide; PHB, poly β-hydroxybutyric acid.

Methods

Organisms and cultivation. The following strains were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands: *Rhizobium meliloti* strains SU-47, SU-255, and SU-256; *Agrobacterium tumefaciens* strains PD15, PD18 and PD51. These bacteria were maintained on agar slants of *Rhizobium* medium containing yeast extract, mannitol and mineral salts. Precultures were prepared by inoculating the organism into 'standard medium' containing 5 g mannitol and 1 g glutamic acid $litre^{-1}$, and mineral salts and vitamins according to Zevenhuizen (1986). The term 'production medium' is used when the mannitol concentration was 10 g l^{-1}. In the experiments on osmoregulation media were supplemented with NaCl, other electrolytes or non-electrolytes as indicated.

Cells from the late exponential phase were used as inoculum (1%, v/v). They were cultivated at 25 °C in 50 ml medium in 100 or 300 ml Erlenmeyer flasks at 25 °C. To obtain larger quantities of cultures, cells were grown in 300 ml medium in 2 litre Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 200 r.p.m. Experiments with controlled pH and oxygen supply were done in a 2 litre batch fermenter with 1 litre of medium. The culture was aerated at the rate of 1 l air min^{-1}, and stirred at 500 r.p.m.

Calculation of osmotic pressure. The osmotic pressure π was calculated as $\pi = \Sigma n_i \cdot M_i \cdot \mathbf{R} \cdot T$ where n = no. of moles of solute i, M = molarity of the solute i (mol l^{-1}), $\mathbf{R}$ = 0·0821 l atm K^{-1} mol^{-1}, and T = 298·15 °K. Ideal behaviour of the solutions is assumed (Chang, 1977). The osmotic pressure of the production medium was calculated as 2·0 atm (1 atm = 101 325 Pa).

Analysis of the cultures. Cells and culture supernatant were separated by centrifugation at 35000 *g* for 30 min. Highly viscous cultures were first diluted with distilled water prior to centrifugation. Cell pellets from 50 ml cultures were resuspended in 10 ml distilled water and briefly sonicated to obtain a homogeneous suspension.

Supernatant. The supernatant was analysed for total excreted carbohydrates by the anthrone–H_2SO_4 method (Trevelyan & Harrison, 1952). The carbohydrate concentration was expressed as glucose equivalents. Mannitol was measured by the periodate oxidation–chromotropic acid colorimetric method (Burton, 1957). Viscosity measurements were made with an Ubbelohde viscometer at 25 °C.

Excreted carbohydrate was fractionated into a low-M_r and a high-M_r fraction by precipitating the high-M_r fraction with 3 vols ethanol. The precipitated succinoglycan was redissolved in water, and the sugar content was measured by the anthrone–H_2SO_4 assay. Repeating units of succinoglycan which remained in the ethanolic supernatant were also quantified by the anthrone–H_2SO_4 assay. The extracellular low-M_r carbohydrates of this fraction were separated according to charge on a DEAE-Sepharose anion-exchange column (3 × 25 cm, Pharmacia–LKB), and were eluted with distilled water followed by a linear KCl gradient (0–0·5 M). Fractions (5 ml) were collected, and checked for hexose content by the anthrone method. Peak fractions were desalted by gel-filtration over a Bio-Gel P2 column. The amount of succinate found within the collected peak fractions after methanolysis was determined as methylsuccinate on a capillary Wax 57-CB column (Chrompack) connected to a flame ionization detector in a Kipp Analytica 8200 gas chromatograph at 100 °C (Zevenhuizen & Van Neerven, 1983).

Cell pellet. Fractionation into high-M_r and low-M_r cellular carbohydrates was as follows. Cell suspensions of *A. tumefaciens* were extracted with 1 vol. 2 M-NaOH for 15 min at 70 °C. After centrifugation, the NaOH extract was decanted. Ethanol (1 vol.) was added to this extract, which precipitated CPS [curdlan, a linear 1,3-β-glucan (Hisamatsu *et al.*, 1978)]. The precipitate was collected by centrifugation, and redissolved in 1 M-NaOH. Curdlan was quantified by the anthrone method and expressed as glucose equivalents. In the ethanolic extract, containing cyclic 1,2-β- glucans and oligosaccharides, total carbohydrate was measured by the anthrone assay. In the case of *R. meliloti* strains, which do not produce CPS, cell suspensions were extracted with 3 vols ethanol for 30 min at 70 °C, followed by centrifugation and resuspension of the extracted cells in water. The hexose content of this suspension was determined by the anthrone method and could be taken as a measure of the glycogen content of the cells (Zevenhuizen, 1981). In the ethanolic extract, low-M_r carbohydrates (cyclic glucans and oligosaccharides) were present. These were separated after evaporation of the ethanol on a column of Ultrogel AcA-202 (2·5 × 37 cm; M_r range 1000–15000; Pharmacia–LKB) with water as the eluent. Fractions of about 2·5 ml were collected and measured for hexose content.

TLC was done out on silica gel plates (Merck) with the solvent [n-butanol/ethanol/water (5:5:4, by vol.)] ascending the plates (Amemura *et al.*, 1985). Cell protein was measured according to the Lowry method with BSA as standard. PHB was determined after methanolysis of whole cell suspensions with GLC on a capillary Wax 57-CB column at 100 °C by the method of Braunegg *et al.* (1978).

Sugar composition and linkage types of isolated poly- and oligosaccharides. Separation of neutral sugar components was done after hydrolysis and conversion of the liberated sugars into alditol acetates by GLC (Blakeney *et al.*, 1983). Glycosidic linkages were determined after methylation analysis according to Harris *et al.* (1984). With both GLC experiments a capillary Sil 43-CB column (Chrompack) was used at 210 °C. HPLC of glucans and oligosaccharides was done according to Breedveld *et al.* (1990). ^{1}H-NMR was done with a Bruker Ac-200E apparatus with 4,4'-dimethyl-4-silapentane sulphonate (DDS) as internal standard.

Results

Influence of NaCl and other osmolytes on extracellular and cellular carbohydrate production by R. meliloti and A. tumefaciens

A difference in succinoglycan production between *A. tumefaciens* and *R. meliloti* strains was observed (Table 1). The *A. tumefaciens* strains PD15, PD18 and PD51 formed highly viscous cultures in production medium without and with added NaCl. With *R. meliloti* strains SU-47, SU-255 and SU-256 the major part (70–80%) of the extracellular carbohydrate produced in the NaCl-free medium was of low-M_r. By adding NaCl to the medium the proportion of high-M_r succinoglycan was greatly enhanced. Succinoglycan production was parallelled by an increase in viscosity of the medium (data not shown).

The low content of low-M_r cellular carbohydrates (1,2-β-glucans) and the high curdlan content of *A. tumefaciens* cells in media not supplemented with NaCl was in contrast to the higher amounts of 1,2-β-glucans of *R. meliloti* cells, which did not produce capsules. At increasing NaCl concentrations, large amounts of oligosaccharides were synthesized, functioning probably as compatible osmoregulants in the cytosol of the cells (see below for *R. meliloti* SU-47).

Table 1. *NaCl-induced osmotic effects on extracellular and cellular carbohydrates of A. tumefaciens and R. meliloti strains*

Cultures were incubated in production medium (with NaCl added as indicated) for 14 d on a rotary shaker (200 r.p.m.) at 25 °C. Carbohydrate contents are expressed as mg glucose equivalents per litre of culture. The data shown here are representative of two experiments between which values varied not more than 5%.

Strain	Supernatant		Cell pellet		
	High-M_r*	Low-M_r†	Protein (mg l^{-1})	Low-M_r‡	CPS§
(*a*) *A. tumefaciens*					
Strain PD15	2600	40	425	30	980
+0·2 M-NaCl	2100	50	435	60	880
+0·6 M-NaCl	770	340	360	140	60
Strain PD18	2400	450	450	45	760
+0·2 M-NaCl	2100	400	440	105	1400
+0·6 M-NaCl	875	240	390	180	30
Strain PD51	3000	210	430	50	340
+0·2 M-NaCl	2940	180	450	70	270
+0·6 M-NaCl	680	620	330	180	30
(*b*) *R. meliloti*					
Strain SU-47	240	1100	455	160	-
+0·2 M-NaCl	2350	450	470	190	-
+0·6 M-NaCl	750	320	455	180	-
Strain SU-255	350	800	540	240	-
+0·2 M-NaCl	1290	280	550	245	-
+0·6 M-NaCl	540	385	360	190	-
Strain SU-256	470	165	510	250	-
+0·2 M-NaCl	945	10	530	230	-
+0·6 M-NaCl	400	85	380	220	-

* High-M_r carbohydrates in supernatant (succinoglycan) quantified as mg glucose equivalents per litre of culture.

† Low-M_r carbohydrates in supernatant quantified as mg glucose equivalents per litre of culture.

‡ Low-M_r carbohydrates in pellet (cyclic glucans + oligosaccharides) quantified as mg glucose equivalents per litre of culture.

§ Curdlan quantified as mg glucose equivalents per litre of culture; absent in *R. meliloti*.

The production of succinoglycan in media supplemented with 0·6 M- as compared to 0·2 M-NaCl was lowered in all strains tested. Curdlan production in *Agrobacterium* was strongly reduced in these media. None of the strains were able to grow in the presence of 1 M-NaCl.

To distinguish between an ion-specific (Na^+, Cl^-) and a general osmotic effect on succinoglycan production by *R. meliloti* SU-47 various ions and non-ionic osmolytes were tested (Table 2). Initial experiments showed that 0·2 M-NaCl was the optimal NaCl concentration for succinoglycan production by strain SU-47. With divalent ions ($CaCl_2$, $MgCl_2$, $MgSO_4$ and Na_2SO_4) much lower concentrations (25 mM) were needed for an even higher production on succinoglycan and a higher viscosity of the cultures. Stimulation of succinoglycan production and concomitant rise in viscosity occurred also with 0·2 M-KCl and high concentrations of mannitol. Sucrose (0·5 M) also stimulated succinoglycan production as could be seen from a rise in viscosity (because sucrose interfered with the anthrone assay, results were not shown).

Osmotic effects on excretion of extracellular carbohydrates of R. meliloti SU-47 at different incubation times

R. meliloti SU-47 was cultivated in the presence or absence of 0·2 M-NaCl in a well-aerated batch-fermenter (Fig. 1). Hardly any differences in growth rate and substrate uptake were observed. In both cases, synthesis of extracellular carbohydrates occurred mainly in the stationary phase. Because of the high carbon/nitrogen ratio of the medium not all the mannitol was consumed. The total amount of carbohydrate material excreted was higher in the NaCl-supplemented medium, and, as a

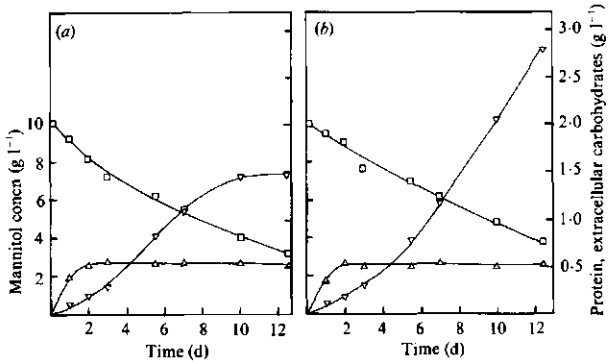

Fig. 1. Growth (as protein; △), production of exocellular carbohydrates (▽) and mannitol consumption (□) by *R. meliloti* SU-47 at 25 °C as a function of time in production medium without (*a*) and with (*b*) 0·2 M-NaCl.

Table 2. *Osmotically induced cellular and extracellular carbohydrate synthesis by R. meliloti SU-47*

Cultures were incubated for 14 d at 25 °C on a rotary shaker (200 r.p.m.) in production medium (with additions as indicated). The data shown here are representative of two experiments between which values varied not more than 10%.

Addition	Osmotic pressure (atm)*	Supernatant			Cell pellet	
		Viscosity (cP)	High-M_r†	Low-M_r‡	Low-M_r§	Protein (mg l⁻¹)
None	2·0	1·5	240	1100	160	455
200 mM-NaCl	11·8	30	2330	470	190	470
200 mM-KCl	11·8	45	2280	290	140	445
25 mM-Na_2SO_4	3·8	75	2870	660	155	420
25 mM-$MgCl_2$	3·8	92	2990	120	210	450
25 mM-$CaCl_2$	3·8	ND	2400	130	205	455
500 mM-mannitol	14·2	80	2700	700	180	395

ND, Not determined; the culture had to be diluted prior to centrifugation because of high viscosity.

* $\pi = \Sigma n_i \cdot M_i \cdot \mathbf{R} \cdot T$ with π = calculated osmotic pressure, n = no. of moles of solute, M = molarity of the solutes, $\mathbf{R}$ = gas constant and T = absolute temperature (see Methods). (1 atm = 101 325 Pa.)

† High M_r succinoglycan quantified as mg glucose equivalents per litre of culture.

‡ Low M_r extracellular carbohydrates quantified as mg glucose equivalents per litre of culture.

§ Low M_r cellular carbohydrates quantified as mg glucose equivalents per litre of culture.

consequence, the viscosity (28 cP at 0·2 M-NaCl vs 1·5 cP without added NaCl) also. Although in Erlenmeyer flasks oxygen limitation might have occurred, the distribution of both high- and low-M_r carbohydrates was very much comparable to that shown in Table 1. As the pH in NaCl-supplemented media often fell below 5·5 during incubation an experiment was set up in a batch fermenter with the pH set at 7·0. No differences from the data in Fig. 1 were observed.

Succinoglycan production was also enhanced by solely elevating the concentrations of medium components. In a medium with 40 g mannitol, 8 g glutamic acid and 8 times the concentration of salts and vitamins (8 × GMS medium), 7·8 g succinoglycan per litre of medium (95% of the total extracellular carbohydrate fraction) was produced by a biomass of 4 g protein (Fig. 2).

Identification of extracellular carbohydrates

Both the high- and low-M_r carbohydrate fractions showed glucose : galactose ratios of 6·5–7·2 : 1 as determined by component analysis. This agrees with succino-

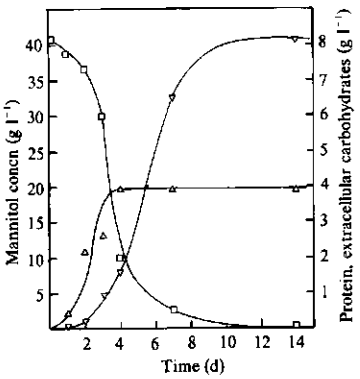

Fig. 2. Growth, succinoglycan production and mannitol consumption by *R. meliloti* SU-47 at 25 °C as a function of time in a medium containing (l^{-1}) 40 g mannitol and 8 g glutamic acid. Symbols, as Fig. 1.

glycan having a glucose:galactose ratio of 7:1. To exclude interference from excreted cyclic glucans, the low-M_r carbohydrate fraction of *R. meliloti* SU-47 cultures grown in the presence or absence of 0·2 M-NaCl was chromatographed with a DEAE-Sepharose anion-exchange column. In the experiment without added NaCl, three peaks were obtained. They corresponded to the repeat-units of succinoglycan having 0 (peak I, 5%), 1 (II, 35%) and 2 (III, 60%) succinate substituents per repeat-unit, respectively. With 0·2 M-NaCl, peak I could not be detected while peak II was predominant (70%). In neither case were cyclic glucans detected.

Influence of NaCl on cellular carbohydrates of R. meliloti strain SU-47

R. meliloti SU-47 was grown in standard medium containing 0 or 0·6 M-NaCl at 25 °C, and analysed at different time intervals (Table 3). When inoculating cells from salt-free medium into standard medium containing 0·6 M-NaCl, maximal cell yield was obtained after 4 d instead of 2 d. Most striking was the absence of glycogen at 0·6 M-NaCl. Without added NaCl, glycogen accumulation occurred within 2 d. Thereafter the glycogen disappeared gradually. PHB synthesis was unaffected by NaCl. At both 0 and 0·6 M-NaCl, a large fraction of cellular low-M_r carbohydrates was present, which remained constant during the whole incubation period (see below).

Identification of low-M_r cellular carbohydrates

The low-M_r carbohydrate fractions from cells of *R. meliloti* SU-47 grown at 0 and 0·6 M-NaCl were separated on a gel-filtration column (Fig. 3*a*, *b*). With cells grown in the absence of added NaCl almost all carbohydrates detected were glucans (Fig. 3), both charged (peak I) and uncharged (peak II). With NaCl-grown cells, a low-M_r oligosaccharide fraction became predominant (peak III), whereas the total amount of glucans was lowered, especially the charged glucans (Table 4).

Peak fractions were collected and freeze-dried. Component analysis of all peaks revealed exclusively glucose as the sugar constituent. Peak fractions I, II and III were further separated by TLC on silica gel. Peak II displayed

Table 3. *Growth (as cell protein), cellular carbohydrates and PHB formation in R. meliloti SU-47 grown in the presence or absence of 0·6 M-NaCl during the course of incubation*

Cells were incubated at 25 °C on a rotary shaker (200 r.p.m.) in standard medium. The data shown here are representative of at least three experiments.

	Time (d)	High-M_r*	Low-M_r†	Protein (mg l^{-1})	PHB (mg l^{-1})
No NaCl	2	340	125	510	75
	4	280	150	500	225
	7	250	170	475	250
	10	170	170	480	180
0·6 M-NaCl	2	10	0	240	0
	4	30	210	475	185
	7	35	215	425	215
	10	35	225	425	180

* High-M_r cellular carbohydrates (glycogen) in mg glucose equivalents per litre of culture.
† Low-M_r cellular carbohydrates (cyclic glucans and oligosaccharides) in mg glucose equivalents per litre of culture.

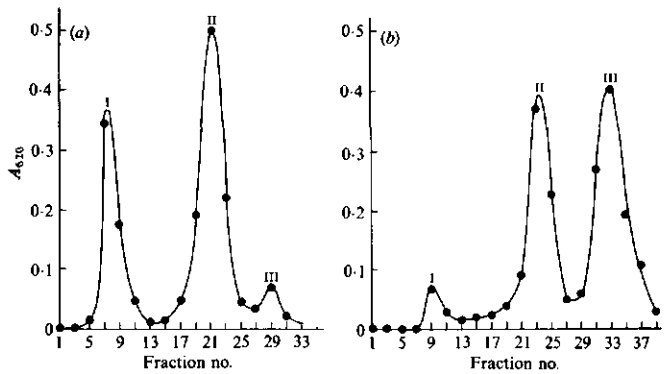

Fig. 3. Gel chromatography on Ultrogel AcA-202 of low-M_r cellular carbohydrates of *R. meliloti* SU-47. Cells were cultivated at 25 °C in standard medium without (*a*) and with (*b*) 0·6 M-NaCl.

Table 4. *Distribution of the cellular low-M_r fractions (cyclic glucans and oligosaccharides) of R. meliloti SU-47 grown at different NaCl concentrations*

Peak fractions I, II and III were obtained after gel-permeation chromatography (Fig. 3). Values are expressed relative to the total low-M_r fraction (100%).

NaCl concn (M)	Percentage of total low-M_r cellular fraction: I	II	III	Total*
0	35	61	4	160
0·2	24	45	31	190
0·4	10	39	51	155
0·6	6	34	60	160

* Total cellular low-M_r carbohydrates in mg glucose equivalents per litre of culture.

a pattern of bands of neutral cyclic 1,2-β-glucans of DPs 19–25 (Zevenhuizen *et al.*, 1990). Peak I gave a pattern of closely situated bands that moved at faster rates. These bands have been identified as glycerol-1-phosphate-substituted cyclic glucans (Batley *et al.*, 1987; Zevenhuizen *et al.*, 1990). On methylation analysis of peak I and II the principal linkage was found to be 1,2- since only 3,4,6-tri-*O*-methyl-D-glucose was detected. With HPLC, ring cycles with DPs of 17–29 could be found.

Peak III consisted of gluco-oligosaccharides with DPs of 2–6. The DP values were determined by comparing the R_F values of the gluco-oligosaccharides with those of a series of sophoro-oligosaccharides. These were obtained by partial hydrolysis of 1,2-β-glucans and analysed by HPLC and TLC (results not shown). Methylation analysis of peak III fractions from cells cultivated at various salt concentrations yielded 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose in the ratios 95:5 at 0·6 M-NaCl, 84:16 at 0·4 M-NaCl, and 62:38 at 0·2 M-NaCl. These data show that the 1,1- bond was predominant in all cases tested, indicating a trehalose structure. Some 1,2- bonds were also present in the higher oligosaccharides, with a maximum at 0·2 M-NaCl. Peak III fractions were subjected to ^{1}H-NMR with trehalose as a reference. The

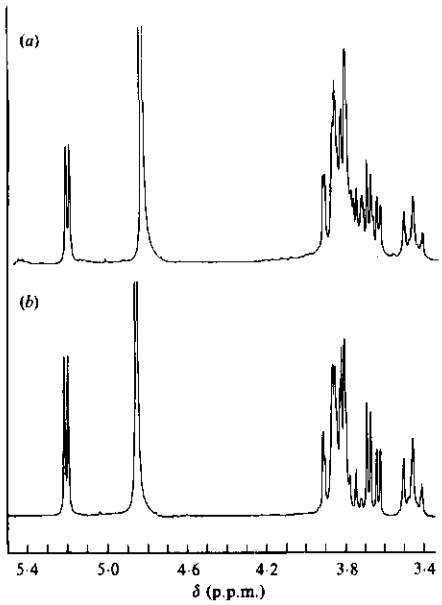

Fig. 4. ^{1}H-NMR spectra of peak fraction III (*a*) taken from the experiment in Table 4 (0·4 M-NaCl) compared with that of α,α-trehalose (*b*). Concentration 20 mg D_2O ml^{-1}.

spectra for the oligosaccharide fraction of peak III from cells grown at 0·4 M-NaCl and pure trehalose (α-D-glucopyranosyl α-D-glucopyranoside) were identical (Fig. 4). Most characteristic is the resonance at 5·20 p.p.m. (J_1–J_2 = 3·3 Hz) as a result of the α-anomeric protons (Usui *et al.*, 1974). As in α,α-trehalose the two glucose residues are identical, only one doublet being observed. Some very weak signals in the region between 5·0–5·5 could be observed as well, possibly due to anomeric protons of glucose in the 1,2-α- configuration.

Discussion

Osmoregulation at low osmolarity

Cyclic glucans of *Agrobacterium* and *Rhizobium* are localized in the periplasmic space, have an intermediate M_r of about 3500, and contain a 1,2-β-glucan backbone. Because of their structural resemblance to the so-called membrane-derived oligosaccharides (MDO) of *E. coli* (Schulman & Kennedy, 1979), the regulation of the biosynthesis of cyclic glucans is comparable (Miller *et al.*, 1986). At low osmotic values of the medium a net flow of water into the cytosol takes place leading to an increase of turgor pressure, and therefore it was supposed that the periplasm tends to remain iso-osmotic with the cytoplasm (Stock *et al.*, 1977). The neutral and especially the anionic periplasmic cyclic glucans may be very well suited as a fixed osmolyte for increasing the turgor pressure in the periplasm against the cytoplasmic membrane (Miller *et al.*, 1986, 1987).

R. leguminosarum cells (all biovars) build up considerable amounts of 1,2-β-glucans during the exponential growth phase, but during the stationary phase their glucan content falls. At this point the cells became embedded in a CPS-matrix (Zevenhuizen, 1981, 1984). It appears that strains of *R. leguminosarum* grown in standard medium combine a low 1,-2-β-glucan concentration with a high deposition of CPS as is the case with *A. tumefaciens* cells. However *R. meliloti* cells, which do not form capsules, usually have constantly high 1,2-β-glucan concentrations (Table 1). Therefore, we suggest that both properties – constant high periplasmic 1,2-β-glucan concentration and the presence of capsular material – can be mechanisms for cells of the *Rhizobiaceae* to maintain their integrity in standard medium of low osmolarity.

Capsular polysaccharides could very well function to counteract the turgor pressure of the cells against the outer membrane. Studies on isolated curdlan of *A. tumefaciens* and of CPS of *R. leguminosarum* biovar *trifolii* TA-1 have shown that these polymers form strong gels in water (gel strength about 500 g cm^{-2} of a 1% CPS solution; Zevenhuizen, 1984). These gelling matrices may act mechanically against the turgor pressure of the cells *in vivo* also.

At certain critical conditions of osmolarity and of temperature, when cells of *R. leguminosarum* biovars *viciae* and *trifolii* are unable to form CPS and can form small amounts of EPS only, continuous 1,2-β-glucan excretion into the medium takes place in amounts up to several grams per litre (Breedveld *et al.*, 1990). It thus seems that in biovars of *R. leguminosarum*, which are osmotically and temperature sensitive, 1,2-β-glucan synthesis is the first action undertaken by the cells in an effort to maintain cell integrity. *R. meliloti* cells, which are osmotically much more tolerant, are not deregulated in such a way, but can maintain a high glucan concentration within their cells at low osmolarity.

The ring size distribution of the cyclic glucans from *R. meliloti* SU-47, as determined by HPLC, is clearly different from that of *R. leguminosarum* biovar *trifolii* TA-1 (Breedveld *et al.*, 1990), namely DPs 17–29 and 17–25, respectively. The proportion of the glucans which were glycerol-phosphorylated was 40% in *R. meliloti* SU-47 while substituted glucans were not detected, or formed a minor fraction, in *R. leguminosarum* biovars (Zevenhuizen *et al.*, 1990).

Osmoregulation at high osmolarity

When *A. tumefaciens* C58 was grown in yeast extract–mannitol medium supplemented with 0·4 M-NaCl, the amount of both the neutral and the anionic cyclic glucan fraction was strikingly lower in comparison to the situation without added NaCl (Miller *et al.*, 1986). No osmoregulatory function can be ascribed for periplasmic 1,2-β-glucans in media with high NaCl concentrations. However, the neutral cyclic glucans were still synthesized by *R. meliloti* SU-47, although at a lower level.

In media containing NaCl, a considerable increase of oligosaccharides was observed, with DP = 2 [trehalose, up to 200 mg (g protein)$^{-1}$] as the principal one, and with minor amounts of oligomers of DPs' 4–6. Their accumulation at high osmotic values of the medium is most likely to function as osmoregulants for the cytoplasm of the cell. Because of the presence of 1,2- and 1,1- linkages in these latter oligosaccharides they are probably identical with the three koji-oligosaccharides (containing 1,2-α-bonds and a terminal 1,1-α-bond) of DPs 4–6 from cultures of *R. meliloti* J7017 studied by Hisamatsu *et al.* (1985). These authors detected the non-reducing oligosaccharides after ethanol extraction of whole cell cultures without reporting the origin (cellular or extracellular) of the molecules.

Trehalose was found in small quantities, varying from 1–20 mg (g dry wt)$^{-1}$ within the cytoplasm of cells of *Rhizobium* spp. grown in media of low osmolarity (Streeter, 1985). The high cellular low-M_r carbohydrate

content detected in *A. tumefaciens* cells grown at 0·4 M-NaCl by Miller *et al.* (1986) was probably due to trehalose as osmoprotectant. Recently, Dylan *et al.* (1990) showed that *R. meliloti* 102F34 mutants defective in 1,2-β-glucan accumulation were impaired in their growth rate under low-osmotic conditions as compared to the wild-type. Their growth was restored by raising the osmolarity of the medium, at which point a cellular low-M_r oligosaccharide appeared, apparently trehalose. As is shown in these examples, the cellular low-M_r carbohydrate fraction did not consist solely of cyclic glucans. We found evidence that the majority of the cellular low-M_r carbohydrate fraction in NaCl-supplemented media consisted of trehalose.

It is important to keep in mind that the nature of the medium and the growth phase can influence the amount of trehalose synthesized. In carbon-rich media as used in our study high concentrations of various carbohydrates can be obtained. In nitrogen-rich media and at other growth phases different osmoregulatory mechanisms may take place, like glycine, proline and betaine accumulation (Le Rudulier & Bernard, 1986; Czonka, 1989).

EPS and osmoregulation

Osmotically induced changes in extracellular polysaccharide production have been reported for a *Pseudomonas aeruginosa* strain (Berry *et al.*, 1989). With *A. tumefaciens* strains, and with *A. radiobacter* NCIB 11883 (Linton *et al.*, 1987), succinoglycan was produced in media of low osmotic strength. The production of 2–4 g succinoglycan (g protein)$^{-1}$ by *R. meliloti* SU-47 was comparable to the data reported for *R. meliloti* J7017 and *R. leguminosarum* biovar *trifolii* J60 by Amemura *et al.* (1983). Although these authors found acidic oligosaccharides and succinoglycan in addition to cyclic glucans in the medium, we did not find extracellular cyclic glucans for strain SU-47.

Many reports claim strong correlations between functional genes needed for succinoglycan biosynthesis and the ability of *R. meliloti* to invade root hairs (Leigh *et al.*, 1985; Zhan *et al.*, 1989) or to induce the formation of nodules (Long *et al.*, 1988). The differences in the relative production of high- and low-M_r extracellular carbohydrates depending on the environmental osmolarity, as was found for different *R. meliloti* strains, may be related to this phenomenon.

It has been shown that growth of *R. meliloti* NSI was inhibited by various salts depending on the specific ion rather than the increase in osmolarity (Botsford, 1984). Our report shows that succinoglycan production by *R. meliloti* appeared to be enhanced by increasing osmotic pressure. With increasing succinoglycan production viscosity rose, but the relatively small differences in production of EPS by *R. meliloti* SU-47 could not explain the large differences in viscosity, especially with the divalent ions tested. This last phenomenon is probably an effect of a different conformation of the polysaccharide in the presence of divalent ions. This was found for the Ca-form of succinoglycan from *Alcaligenes faecalis* var. *myxogenes* 10C3 (Harada & Amemura, 1981) and for the acidic EPSs from *R. leguminosarum* (Morris *et al.*, 1989).

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CHAPTER 5

Osmotically-regulated trehalose accumulation and cyclic ß-(1,2)-glucan excretion by *Rhizobium leguminosarum* biovar *trifolii* TA-1

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Osmotically-regulated trehalose accumulation and cyclic β-(1,2)-glucan excretion by *Rhizobium leguminosarum* biovar *trifolii* TA-1

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Abstract. *Rhizobium leguminosarum* biovar *trifolii* TA-1 produced high molecular weight extracellular (EPS) and capsular polysaccharides (CPS) as the main carbohydrate products in a medium (10 g of mannitol and 1 g of glutamic acid per liter) with low osmotic pressure of 0.20 MPa. By increasing the osmotic pressure of the medium with the addition of NaCl or other osmolytes up to 1.44 MPa, the synthesis of EPS and CPS was suppressed. Cyclic β-(1,2)-glucans were excreted instead. Concentrations of over 1500 mg of glucans/l medium were produced by a biomass of 520 mg protein at 200 mM NaCl (1.20 MPa). Intracellular cyclic β-(1,2)-glucan concentrations remained at 45 to 100 mg/g protein during the stationary phase, independent of the osmotic strength of the medium. Parallel to the increasing osmotic pressure of the medium, the disaccharide trehalose accumulated in the cells as osmo-protectant. Concentrations of up to 130 mg/g protein were reached. Strain TA-1 could tolerate 350 mM NaCl.

Key words: Osmotic pressure – Osmoregulants – Cyclic β-(1,2)-glucans – Extracellular polysaccharides – Capsular polysaccharides – Trehalose – *Rhizobium leguminosarum* biovar *trifolii*

Cellular cyclic β-(1,2)-glucans are involved in osmoregulation in members of the family of the *Rhizobiaceae*, especially at low osmotic strength of the medium. *Rhizobium meliloti* mutants with a defect in the synthesis of β-(1,2)-glucans were strongly impaired in their ability to grow under hypoosmotic conditions. Their growth was restored by raising the osmolarity of the medium (Dylan et al. 1990). At high osmotic strength of the medium the synthesis of glucans was strongly repressed in *Agrobacterium tumefaciens* (Miller et al. 1986) and *R. meliloti* (Dylan et al. 1990; Zorreguieta et al. 1990; Breedveld et al. 1990b). Instead, the disaccharide trehalose accumulated in *R. meliloti* cells functioning as osmo-protectant (Breedveld et al. 1990b).

The β-(1,2)-glucans of *R. leguminosarum* consist of a mixture of rings with different degrees of polymerization between 17 and 25 (Zevenhuizen et al. 1990). Although cyclic β-(1,2)-glucans are generally found in the periplasmic space of the cells (Abe et al. 1982) they were also found to be excreted into the culture liquid (Amemura et al. 1983, 1985), especially at superoptimal temperatures for growth or at high cell densities (Zevenhuizen 1986; Breedveld et al. 1990a). Cyclic β-(1,2)-glucans are comparable both in structure and regulation of biosynthesis with the membrane-derived oligosaccharides of *Escherichia coli* (Miller et al. 1986).

Cellular concentrations of glucans vary depending on the species and the growth phase. In media of low osmotic values *Rhizobium leguminosarum* cells (all biovars) have a β-(1,2)-glucan content of maximally 100 mg/g dry cell weight during logarithmic phase. During stationary phase the concentration decreased below 50 mg/g dry cell weight while capsular polysaccharide (CPS) is continuously deposited around the cells (Zevenhuizen 1984). *R. meliloti* forms no capsular material. Its β-(1,2)-glucan content of up to 200 mg/g dry cell weight remained constant during stationary phase (Zevenhuizen and van Neerven 1983).

The ability of rhizobia to grow under conditions of high osmolarity depends strongly on the species. *Rhizobium leguminosarum* biovar *trifolii*, e.g. was much less tolerant towards NaCl than *R. meliloti* (Vincent 1974). Strains of *R. meliloti* and *A. tumefaciens* could tolerate NaCl up to 1 M (Breedveld et al. 1990b).

As part of our study on osmotically-induced oligo- and polysaccharide synthesis by rhizobia we describe here the growth and cellular carbohydrate regulation of *Rhizobium leguminosarum* biovar *trifolii* TA-1 under conditions of enhanced osmotic pressure of the medium. We

Abbreviations. CPS, capsular polysaccharide; EPS, extracellular polysaccharide; LM_r, low molecular weight; HM_r, high molecular weight

found that under these conditions cyclic glucans were excreted in high amounts. Excessive cyclic β-(1,2)-glucan excretion by *R. leguminosarum* as a general response to various stress-conditions like growth at superoptimal temperature or at high osmotic pressure of the medium is discussed.

Methods

Organisms and cultivation

The following strains were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands: *Rhizobium leguminosarum* biovar *trifolii*, strains TA-1, Coryn; *Rhizobium leguminosarum* biovar *viciae*, strains 1044, PF-2; and *Rhizobium leguminosarum* biovar *phaseoli*, strains Blink, K44. *R. leguminosarum* biovar *viciae* VF-39 was obtained from Dr. U. Priefer, University of Bielefeld, Bielefeld, FRG. Precultures were prepared by inoculating the organism into "standard medium" with the following composition (in grams per liter of distilled water): mannitol, 5; glutamic acid, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; $FeCl_3 \cdot 6H_2O$, 0.0025; $MnCl_2 \cdot 4H_2O$, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.00001; $ZnSO_4 \cdot 7H_2O$, 0.00001; $CuSO_4 \cdot 5H_2O$, 0.00001; H_3BO_3, 0.00001; $CoCl_2 \cdot 6H_2O$, 0.00001; biotin, 0.00001 and thiamine, 0.0001. The pH was adjusted to 7.0 with NaOH. All media were autoclaved at 121°C for 25 min. The term "production medium" is used when the mannitol concentration was 10 g/l. Media were supplemented with NaCl or other osmolytes as indicated.

Cells from late logarithmic phage were used as inoculum (1%, v/v). They were cultivated at 25°C in 50 ml medium in 300-ml Erlenmeyer flasks at 25°C. The flasks were incubated on a rotary shaker at 200 rpm. For preparative purposes cellular carbohydrates were obtained from a 2 l batch fermenter (Applikon, Meyvis, Schiedam, The Netherlands) with 1 l of medium (aeration rate 1 l air/min, stirring speed 500 rpm). Polysaccharide production in time was followed in the same culture.

Analysis of the cultures

Cells and culture supernatant were separated by centrifugation at $35000 \times g$ for 30 min. Highly viscous cultures were first diluted with distilled water prior to centrifugation. Cell pellets from 50-ml cultures were resuspended in 10 ml distilled water and briefly sonicated to obtain a homogeneous suspension.

Supernatant

The supernatant was analysed for its total excreted carbohydrates for hexoses by the anthrone-sulfuric acid method (Trevelyan and Harrison 1952) and for hexuronic acid by the 3-hydroxydiphenyl-sulfuric acid method (Blumenkrantz and Asboe-Hansen 1973). Mannitol was measured with the periodate oxydation-chromotropic acid colorimetric method (Burton 1957). Viscosity measurements were made with an Ubbelohde viscometer (Tamson, Zoetermeer, NL) at 25°C.

The concentration of HM_r EPS, with hexose:hexuronic acid ratio of 3:1 (Zevenhuizen 1986), was expressed as the sum of glucose and glucuronic acid equivalents in mg/l culture. The cyclic β-(1,2)-glucan content was routinely derived from the amount of glucose which was not accounted for in the calculation of the HM_r EPS concentration and expressed as glucose equivalents in mg/l culture. This was shown to be correct for *R. leguminosarum* (Breedveld et al. 1990a).

Fractionation of the supernatant by precipitation with 3 vol ethanol resulted into a HM_r and a LM_r fraction. The precipitate, comprising the HM_r EPS, was dissolved in distilled water followed by extensive dialysis. This material was freeze-dried and used for component analysis.

The LM_r fraction in the alcoholic supernatant, which contained cyclic glucans and/or repeat-units of EPS, could be next isolated by precipitation of these carbohydrates with another 7 volumes of ethanol. The LM_r fraction so obtained was separated from coprecipitated NaCl and mannitol by filtration on a Biogel P2-column (Biorad, Veenendaal, NL), after which the carbohydrates were further purified on an Ultrogel AcA 202 gel-filtration column (2.5 × 37 cm; Pharmacia-LKB, Etten-Leur, NL) as described by Breedveld et al. (1990a). Fractions of 2.5 ml were collected and analyzed for hexose and hexuronic acid content.

Cell pellet

Cellular carbohydrates were fractionated by treating the pellet with 1 M NaOH for 15 min at 70°C. After centrifugation the extracted cells were resuspended in distilled water. The hexose content of this suspension was quantified with the anthrone method and taken as a measure of the glycogen content of the cells (Zevenhuizen 1981). CPS was precipitated from the alkaline supernatant by the addition of 1 vol ethanol, separated by centrifugation and redissolved in NaOH. CPS, with hexose composition galactose:glucose:mannose of 4:1:1, was quantified with the anthrone method and expressed as Gal equivalents in mg/l culture. The total LM_r carbohydrate fraction in the alkaline, alcoholic supernatant, containing β-(1,2) cyclic glucans and oligosaccharides, was measured with the anthrone assay. For separation of these LM_r components the whole cell pellet was extracted with 3 vol ethanol for 30 min at 70°C followed by centrifugation. The alcoholic supernatant, containing the LM_r fraction, was concentrated to 1 ml by rotation evaporation, centrifuged again to remove particulates, and separated by gelfiltration on Ultrogel AcA 202. Fractions of about 2.5 ml were collected and measured for hexose content.

Thin-layer chromatography of LM_r carbohydrates was carried out on silicagel plates (Merck, Amsterdam, NL) and chromatographed two times with n-butanol:ethanol:water (5:5:4 v/v) in the ascending way, sprayed with 5% sulfuric acid in ethanol and developed for 30 min at 100°C (Amemura et al. 1985). Cell protein was measured according to the method of Lowry et al. (1951) with bovine serum albumine as standard.

Sugar composition and linkage types of isolated poly- and oligosaccharides

Separation of neutral sugar components was done by hydrolysis and conversion of the liberated sugars into alditol-acetates followed by gaschromatography (Blakeney et al. 1983). Glycosidic linkages were determined by methylation analysis according to Harris et al. (1984). The sugar derivatives were separated on a capillary Sil 43-CB column (Chrompack, Middelburg, NL) at 210°C. Uronic acids (Blumenkrantz and Asboe-Hansen 1973), pyruvate (Katsuki et al. 1971) and acetate (Hestrin 1949) were measured spectrophotometrically. High pressure liquid chromatography of cyclic β-(1,2)-glucans was carried out according to Breedveld et al. (1990a).

Calculation of osmotic pressure

The osmotic pressure (Ψ) was calculated from $\Psi = \Sigma n_i \cdot M_i \cdot R \cdot T$ with n = number of mol of solute i, M = molarity, R = 8314 l.Pa/K.mol and T = 298.15°K (Chang 1977). The osmotic pressure Ψ of

Table 1. Influence of 150 mM NaCl on polysaccharide-production of strains of *Rhizobium leguminosarum* biovar *viciae*, *trifolii* and *phaseoli*. Incubations were done for 14 days at 25°C in the production medium

Biovar	Strain	Supernatant			Pellet	
		NaCl (mM)	EPS[a]	glucans[b]	CPS[c]	protein (mg/l)
trifolii	TA-1	0	1890	25	1580	510
		150	1200	400	390	490
	Coryn	0	910	70	210	500
		150	50	85	0	480
viciae	1044	0	1800	30	1015	520
		150	180	1190	20	490
	PF-2	0	1630	20	220	480
		150	60	90	65	490
	VF-39	0	2680	90	820	500
		150	325	1280	80	520
phaseoli	Blink	0	2160	0	380	470
		150	100	270	120	470
	K44	0	930	70	110	520
		150	170	360	15	510

[a] Expressed as the sum of glucose and glucuronic acid equivalents in mg/l culture
[b] Expressed as glucose equivalents in mg/l culture
[c] Expressed as galactose equivalents in mg/l culture

the production medium was calculated to be 2 atm or 0.2 MPa ($1 \text{ Pa} = 9.87 \times 10^{-6}$ atm).

Results

Influence of NaCl and other osmolytes on polysaccharide production by some Rhizobium leguminosarum strains

The effect of NaCl on the polysacharide production of seven strains of *Rhizobium leguminosarum* were tested. Initial experiments showed no growth at 350 mM NaCl, indicating a low salt-tolerancy of these organisms. At 150 mM NaCl, EPS and CPS synthesis was reduced in all strains tested. However, an enhanced production of cyclic β-(1,2)-glucans was observed, except for *R. leguminosarum* biovar *trifolii* strain Coryn (Table 1). Experiments were continued with *R. leguminosarum* biovar *trifolii* strain TA-1. This organism is used as a model strain in our laboratory for studying the effects of environmental conditions on poly- and oligosaccharide synthesis by *Rhizobiaceae*. As a consequence much physiological information is already available (Zevenhuizen 1986; Breedveld et al. 1990a).

An increase of NaCl concentrations in cultures of strain TA-1 was parallelled by higher cyclic β-(1,2)-glucan excretion (Table 2). Both CPS and EPS synthesis were suppressed with increasing NaCl concentration. Interestingly, the viscosity of the culture supernatants increased between 0 to 100 mM NaCl, though the EPS concentration gradually decreased (Table 2). The viscosities in pure water (2.0 g EPS/l) were 14 and 29 cP for the EPSs obtained from the 0 and 100 mM NaCl culture, respectively. The composition of EPS samples yielded in both cases glucose: glucuronic acid:galactose:pyruvate:acetate ratios of 4.9 ($\pm$0.2):2.0 ($\pm$0.1):1.0 ($\pm$0.1):2.0 ($\pm$0.2):0.9 ($\pm$0.1) which is the generally occurring composition of EPS of *Rhizobium leguminosarum* (Zevenhuizen and Bertocchi 1989). In all cases tested, more than 95% of the hexuronic acids in the culture supernatants were present in the HM_r fraction. The increase in viscosity at a moderate osmotic pressure of the medium (0.46 to 0.70 MPa) could be the result of a higher degree of polymerization of EPS.

To distinguish between an ion-specific and a general osmotic effect on carbohydrate synthesis by strain TA-1 some other osmolytes were tested as well. Growth was completely inhibited by 50 mM Na-acetate, 10 mM Na_2SO_4, and 350 mM KCl. Both KCl (150 mM) and Na_2SO_4 (50 mM) showed increased glucan excretion and reduced CPS and EPS synthesis. Mannitol (0.55 M) caused a sharp decrease in CPS synthesis while EPS was only moderately decreased (Table 3). The effect of $MgCl_2$ was concentration-dependent: at 25 to 50 mM $MgCl_2$ a much higher viscosity of the supernatant was observed as compared to 50 and 100 mM NaCl (Tables 2, 3). At 100 mM $MgCl_2$ growth was already inhibited, glucan excretion was enhanced and EPS and CPS production was strongly suppressed.

NaCl-dependent carbohydrate synthesis by R. leguminosarum biovar trifolii TA-1

Cellular LM_r carbohydrate fraction. The LM_r cellular fraction increased with increasing NaCl (Table 2). The composition of this crude fraction was measured with gel-permeation chromatography (Fig. 1). The first peak (fractions 23 to 27) corresponded in all cases with a purified sample of cyclic β-(1,2)-glucan obtained from the supernatant of strain TA-1 after incubation at 33°C (Breedveld et al. 1990a). The second peak (fractions 29 to 34) consisted of an oligosaccharide. This fraction increased with increasing NaCl. The material of this peak behaved as a single spot on thin-layer chromatography at the position of the disaccharide trehalose. Methylation yielded exclusively 2,3,4,6-tetra-O-methyl-D-glucose. This is in accordance with the 1-1 bond in trehalose. The amounts of the LM_r cellular carbohydrates were (mg glucose equivalents/l culture): 25 glucans (0 mM NaCl, Fig. 1), 25 glucans and 3 trehalose (50 mM NaCl), 44 glucans and 7 trehalose (100 mM NaCl), 47 glucans and 23 trehalose (150 mM NaCl), 37 glucans and 37 trehalose (200 mM NaCl), 40 glucans and 65 trehalose (250 mM NaCl, Fig. 1).

Extracellular LM_r carbohydrate fraction. The LM_r fraction excreted by strain TA-1 cells grown at 200 mM NaCl during 14 days, eluted in a major anthrone-positive peak from the Ultrogel AcA 202 gel-filtration column at the same position as the cellular glucans (not shown). No hexuronic acid could be detected in this peak. Both the cellular and the extracellular glucans had the same mobility and ring size distribution on thin-layer chromatography and high-performance liquid chromatography,

Table 2. NaCl-dependent polysaccharide-production by *Rhizobium leguminosarum* biovar *trifolii* TA-1. Cells were incubated for 12 days at 25°C in the production medium

Medium [NaCl] (mM)	Supernatant				Pellet		
	Ψ[a] (MPa)	EPS (mg/l)	Glucans (mg/l)	Visc[b] (cP)	CPS (mg/l)	Protein (mg/l)	LMW[c] (mg/l)
0	0.20	1710	40	14	1240	500	25
50	0.46	1560	130	20	1260	490	30
100	0.70	1390	290	26	915	475	55
150	0.94	1260	390	13	570	480	75
200	1.20	920	1050	7	180	465	80
250	1.44	440	1670	2	70	420	110

[a] $\Psi = n \cdot M \cdot R \cdot T$ with Ψ = calculated osmotic pressure, n = number of mol of solute, M = molarity of the solutes, R = gas constant, and T = absolute temperature
[b] Viscosity of the supernatant in cP (centipoise). 1 Poise = 0.1 $N \cdot s \cdot m^{-1}$
[c] Low Molecular Weight cellular carbohydrate fraction
For other abbreviations, see legend Table 1

Table 3. Osmotically-induced polysaccharide-synthesis by *Rhizobium leguminosarum* biovar *trifolii* TA-1. Cultures were incubated for 14 days at 25°C in production medium with various amounts of additions

Addition	Supernatant				Cell pellet	
	Ψ (MPa)	Visc (cP)	EPS (mg/l)	Glucans (mg/l)	CPS (mg/l)	Protein (mg/l)
None	0.20	15	1800	35	1280	510
150 mM KCl	0.94	1	270	1100	280	435
150 mM NaCl	0.94	13	1300	410	570	475
50 mM Na_2SO_4	0.58	1	240	620	30	400
25 mM $MgCl_2$	0.39	125	2200	30	970	520
50 mM $MgCl_2$	0.58	80	2060	55	710	500
100 mM $MgCl_2$	0.94	1	255	460	70	250
500 mM Mannitol	1.44	8	1060	420	280	540

For abbreviations, see legend Tables 1 and 2

with degrees of polymerization between 17 and 25 (Breedveld et al. 1990a). Component-analysis revealed glucose as the only hexose. Methylation-analysis gave exclusively 3,4,6-tri-O-methyl-D-glucose, thus confirming a molecular structure of cyclic nature with solely (1,2) bonds. As a control the same procedure was repeated with the LM_r fraction excreted by a 14-day-old culture grown in production medium without any added NaCl (50 mg LM_r carbohydrates/l). Two small peaks appeared during gel-filtration. The first (30 mg/l) consisted of repeat-units of EPS with a ratio of hexose:hexuronic acid of 2.9:1 which is about the same as the composition of EPS. The second peak contained exlusively cyclic glucans (20 mg/l). As found earlier (Zevenhuizen et al. 1990; Breedveld et al. 1990a) this material only contained neutral glucans.

Production of polysaccharides by R. leguminosarum biovar trifolii TA-1 during growth

Strain TA-1 was incubated in 1 l production medium supplemented with 200 mM NaCl in a well aerated batch-fermenter. Samples were taken at various time-intervals (Fig. 2). During this incubation intracellular glycogen (maximally 50 mg/l) and CPS production (50 mg/l) was low, while in production medium these values were 10 to 30 times higher (Zevenhuizen 1986; Breedveld et al. 1990a). Cyclic β-(1,2)-glucan excretion followed a linear course during stationary phase, reaching almost 1500 mg/l medium after 17 days (Fig. 2). In stationary phase glucan synthesis proceeded with a rate of 220 mg glucans/g protein · day. EPS production was low and the viscosity of the culture supernatant had only reached 2.4 cP after 17 days. The LM_r cellular carbohydrate fraction (100 mg/l medium at 14 days of incubation) had comparable gel chromatographic behaviour to the profile shown in Fig. 1.

Discussion

A higher osmotic pressure of the medium affected carbohydrate synthesis of *Rhizobium leguminosarum* biovar *trifolii* TA-1 in three ways: (i) excess cyclic β-(1,2)-glucans were excreted into the medium, (ii) CPS and EPS synthesis were reduced, and (iii) trehalose was produced in the cells. While in *Rhizobium meliloti* and *Agrobacterium tumefaciens* the concentration of cellular glucans, especially the glycerophosphorylated glucans, decreased drastically with increasing osmolarity (Miller et al. 1986; Dylan et al. 1990; Zorreguieta et al. 1990; Breedveld et al. 1990b), the β-(1,2)-glucan content in *R. leguminosarum*

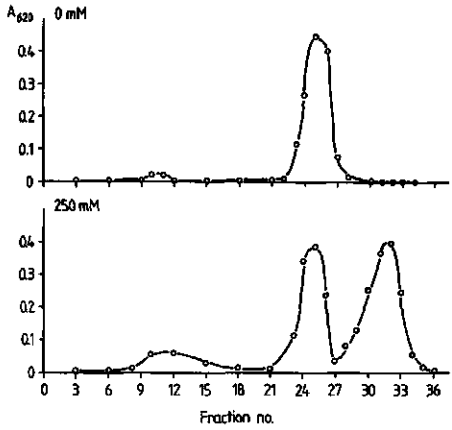

Fig. 1. Gel-filtration profiles of the LM_r cellular carbohydrate fractions of *Rhizobium leguminosarum* biovar *trifolii* TA-1 grown in the presence of 0 and 250 mM NaCl. After 14 days of incubation cells obtained from 200 ml culture were extracted with ethanol. This extract was subjected to gel-chromatography (Ultrogel AcA 202), fractions were collected and measured with the anthrone-sulfuric acid assay (A_{620}). The sample volume was 200 µl for cultures grown at 0 and 100 µl for 250 mM NaCl. Fractions 23 to 27 (first peak) contain cyclic β-(1,2)-glucans and fractions 29 to 34 (second peak) trehalose

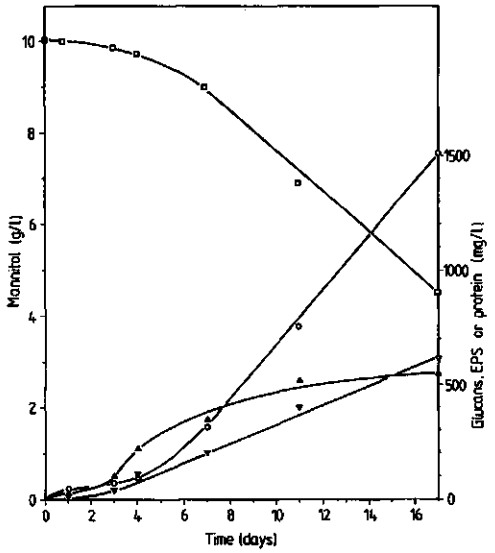

Fig. 2. Growth (as cellular protein, ▲) of *Rhizobium leguminosarum* biovar *trifolii* TA-1, EPS (▽) and cyclic glucan (○) excretion and substrate (mannitol, □) consumption in a 1 l fermenter batch culture with production medium containing 200 mM NaCl

biovar *trifolii* TA-1 was not affected by increasing osmotic pressure (this paper).

In our recent paper (Breedveld et al. 1990b) we discussed that the presence of capsular material (in *R. leguminosarum*) or high concentrations of periplasmic cyclic glucans (in *R. meliloti*) may both be mechanism to maintain cell integrity under conditions of low osmotic pressure of the medium. We showed that CPS synthesis, which occurred only in the stationary growth phase of *R. leguminosarum* in the presence of excess carbon source (Zevenhuizen 1984, 1986), was delicately regulated. Under conditions of growth at superoptimal temperatures or at high cell densities (Breedveld et al. 1990a), or at high osmotic pressure of the medium, CPS synthesis by *R. leguminosarum* was almost completely suppressed. Apparently in an effort to maintain cell integrity, osmotically and temperature sensitive *R. leguminosarum* cells continuously synthesized and excreted cyclic glucans. *R. meliloti* cells (which do not produce capsules) are osmotically much more tolerant, do not excrete glucans under these conditions, and can maintain a high cellular glucan concentration at low osmotic pressure of the medium (Breedveld et al. 1990b).

Salt tolerance of rhizobia depends strongly on the species and the type of ions involved. We showed that strains of *R. meliloti* and *A. tumefaciens* could grow well in media up to 1 M NaCl (Breedveld et al. 1990b), while *R. leguminosarum* could only tolerate up to 350 mM NaCl. Growth of *R. meliloti* strain NSI was hardly inhibited by Na^+ and K^+ ions at 100 mM, while Mg^{2+} ions inhibited severely already at 25 mM (Botsford 1984). The anions Cl^- and SO_4^{2-} had little effect, but PO_4^{3-} and acetate showed inhibition at 20 mM. Under phosphate-limiting conditions *R. leguminosarum* biovar *trifolii* TA-1 caused an increase of the viscosity of the culture to extremely high values (data not shown), comparable to the effect of 25 to 50 mM $MgCl_2$ (Table 3). This suggested that excess PO_4^{3-} or Mg^{2+} had caused precipitation of $(Mg)_3(PO_4)_2$, resulting in a limitation of Mg^{2+} and PO_4^{3-}, respectively. In a solution containing 50 mM $MgCl_2$ and 5.7 mM K_2HPO_4 the free PO_4^{3-} concentration is only 10^{-7} mM, assuming a solubility product for $(Mg)_3(PO_4)_2$ of 9.86×10^{-25} (Chang 1988). This value for free PO_4^{3-} may be somewhat higher in the production medium, due to complexation of Mg^{2+} with compounds like glutamic acid. In an autoclaved production medium supplemented with 50 mM $MgCl_2$ (pH = 7, 25°C) a white precipitate indeed can be observed. Therefore, the inhibition of growth and the enhancement of the viscosity may be explained rather by a lack than by an excess of each of the necessary nutrient ions Mg^{2+} or PO_4^{3-}.

Trehalose has been found in several species of *Rhizobium* grown in unamended culture media at varying concentrations, ranging from 0 to 40 mg/g dry cells (Streeter 1985). Trehalose concentration in *R. meliloti* SU-47 in media with 0.6 M NaCl could reach 200 mg/g cell protein and thus constituting the major cellular carbohydrate compound (Breedveld et al. 1990b). The same holds for strain TA-1 which accumulates up to 130 mg trehalose/g cell protein at 200 to 250 mM NaCl. Enhanced levels of trehalose (up to 100 mg/g protein) have also been found in *Rhizobium* cultured under low oxygen tensions (Hoelzle and Streeter 1990). In yeast

cells, trehalose is supposed to play a role in protecting against adverse conditions like desiccation, heat-shocks and frost (Wiemken 1990). In this report *R. leguminosarum* cells have been found to build up increased levels of trehalose as a result of osmotic stress, but the same could not be found in cells grown at superoptimal temperature for growth (unpublished observation). Therefore, further investigations are needed to determine whether trehalose accumulation functions as a general protectant in *R. leguminosarum* cells exposed to various conditions of stress.

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CHAPTER 6

The response of cellular carbohydrates in *Rhizobium leguminosarum* biovar *trifolii* TA-1 and *Rhizobium meliloti* SU-47 to a NaCl shock

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The response of cellular carbohydrates in *Rhizobium leguminosarum* biovar *trifolii* TA-1 and *Rhizobium meliloti* SU-47 to a NaCl shock

SUMMARY

The response to a NaCl-shock on cellular carbohydrates of *Rhizobium leguminosarum* biovar *trifolii* TA-1 (0.25 M NaCl) and *R. meliloti* SU-47 (0.4 M NaCl) grown in NaCl-free medium was investigated in non-growing cell cultures and in cell suspensions using *in vivo* NMR. After transferring NaCl-free grown cells to a glutamic acid-free medium containing mannitol and NaCl, both strains immediately responded to the increased osmotic pressure by the increase the trehalose content of the cell. Without mannitol in the medium trehalose synthesis was slower but clearly detectable. Its synthesis parallelled the breakdown of the reserve materials glycogen and poly-hydroxybutyric acid (PHB). NMR experiments with 25-fold concentrated cell-suspensions using $^{13}C_1$-mannitol as substrate revealed that 15-20% of the trehalose synthesized was derived from mannitol, but 80-85% from other sources. Trehalose was mainly formed from the internal pool of glycogen and/or PHB, whether mannitol was present or not, and reached 135 and 280 μg/mg cell protein in the strains TA-1 and SU-47, respectively. At low osmolarity intracellular trehalose was metabolized by strains TA-1 and SU-47. Intracellularly accumulated phosphoglycerol-substituted and neutral cyclic (1,2)-ß-glucans of SU-47 cells grown in the absense of NaCl were not degraded nor excreted after exposure to NaCl. On the contrary strain TA-1, which only makes neutral cyclic (1,2)-ß-glucans, continued to synthesize and excrete cyclic glucans after exposure to NaCl. With *in vivo* ^{31}P-NMR a sharp peak at 1.34 ppm was present in cell suspensions of SU-47. This peak representing glycerol-1-phosphate substituted cyclic glucans, was absent in strain TA-1.

Abbreviations: bv=biovar; HM_r=high molecular weight; LM_r=low molecular weight; PHB=poly-hydroxybutyric acid

INTRODUCTION

Rhizobia, like other non-halophilic Gram-negative bacteria, are able to adapt to differences in osmotic pressure between the cytoplasm and the environment. The processes involved in adaptation take place in different compartments of the cells, depending on the osmotic pressure of the environment. In media with low osmotic pressure ($\leq$ 3 atm) the organisms accumulate LM_r-carbohydrates in their periplasm, as e.g. the membrane-derived oligosaccharides in *Escherichia coli* or the cyclic (1,2)-ß-glucans in rhizobia and *Agrobacterium* spp. Their synthesis is repressed at high osmotic pressure of the medium (Kennedy and Rumley, 1988; Miller *et al.*, 1986, Zorreguieta *et al.*, 1990).

Cells of *E. coli* or *Rhizobium meliloti* respond to increased environmental osmotic pressure (5 to 20 atm) by import of potassium-ions and concomitant synthesis and accumulation of glutamic acid in their cytoplasm, followed in a later growth stage by the formation of trehalose as compatible solute (Le Rudulier and Bernard, 1986; Czonka, 1989; Welsh *et al.*, 1991). NaCl inhibition could be removed by adding exogenously proline-betaine, an osmo-protectant not synthesized by *Rhizobium*. It was found to accumulate in the cells. Without NaCl in the medium proline-betaine was metabolized by the cells (Gloux and Le Rudulier, 1989). Actively growing *R. meliloti* cells accumulate 0.5 μmol glutamic acid/mg protein in media containing 0.4 M NaCl (Botsford and Lewis, 1990) and 0.2 μmol/mg protein of a dipeptide N-acetylglutaminylglutamine amide (Tombras Smith and Smith 1989). Strains of *A. tumefaciens* accumulate both glutamic acid and a novel disaccharide ß-fructofuranosyl-α-mannopyranoside, or mannosucrose (Tombras Smith *et al.* 1990).

Recently it was discussed that trehalose in microorganisms has a function in the protection against various stress conditions rather than solely being a reserve material for carbon and energy (Van Laere, 1989; Wiemken, 1990; Zevenhuizen, 1991). *Rhizobium* and their bacteroids accumulate trehalose in small

amounts at low osmotic pressure of the medium (Streeter, 1985). At microaerophilic conditions (1% O_2) trehalose is increased 10 to 20 fold, reaching up to 0.25 μmol trehalose/mg protein (Hoelzle and Streeter, 1990). To compensate for the difference in osmotic pressure over the cytoplasmic membrane *R. meliloti* cells accumulate up to 0.85 μmol trehalose/mg protein in the cytoplasm when grown in media containing 200-800 mM NaCl (Breedveld *et al.*, 1990b).

We have investigated the ability of members of the *Rhizobiaceae* to grow at high osmotic pressures of the medium, and their tolerance towards several ionic and non-ionic osmolytes (Breedveld *et al.*, 1990b, 1991). Two model-organisms were used, *R. leguminosarum* bv *trifolii* TA-1 which grows in media containing up to 350 mM NaCl, and *R. meliloti* SU-47 which can grow at concentrations up to 1 M NaCl. The two strains were found to differ in their (1,2)-ß-glucan synthesis. Strain TA-1 synthesized and excreted continuously neutral cyclic (1,2)-ß-glucans in media containing 250 mM NaCl (Breedveld *et al.*, 1991). Both anionic and neutral (1,2)-ß-glucan formation was repressed in cells of strain SU-47 in media containing 400 mM NaCl (Breedveld *et al.*, 1990b). In both strains, glycogen synthesis was repressed, while trehalose was accumulated at enhanced osmotic pressure.

In this study we investigate the dynamics of cellular carbohydrate production in non-growing cells of *Rhizobium* upon exposure to NaCl.

METHODS

Organisms and cultivation. *Rhizobium leguminosarum* bv *trifolii* TA-1 and *Rhizobium meliloti* SU-47 were obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands. Precultures were prepared by inoculating the organism into standard medium with the following composition: 5 g mannitol/l, 1 g glutamic acid/l and mineral salts and vitamins, having a total osmotic pressure of 1.3 atm (Breedveld *et al.*, 1990b). The pH was adjusted to 7.0 with NaOH. All media were autoclaved at 121°C for 25 min. Cells from the late logarithmic phase were inoculated (1% $^{v}/_{v}$) into 3 l Erlenmeyer flasks containing 500 ml medium. The flasks were incubated on a rotary shaker at 200 rpm for 48 h at 25°C. Cells and culture supernatant were separated by centrifugation at 35000 x *g* for 30 min in sterile tubes. For the fermenter-experiments, cells obtained from 1 liter of a batch culture were washed and resuspended twentyfold concentrated in the washing buffer (standard medium without glutamic acid and mannitol). The entire cell suspension was transferred into a 2 l fermenter with 1 l of standard medium containing 250 mM NaCl (TA-1) or 400 mM NaCl (SU-47) but no glutamic acid. To study the influence of the mannitol concentration on cellular LM_t-carbohydrates of strain TA-1, incubations were done in 300 ml Erlenmeyer flasks with 50 ml standard medium containing different concentrations of mannitol. The flasks were incubated at 25°C on a rotary shaker at 200 rpm for 7 days.

Separation of cells and supernatant. Samples were taken at different time intervals, centrifuged for 30 min at 35000 x *g* to separate cells and supernatant. Both pellet and supernatant were used to quantify the different oligo- and polysaccharides.

Cell pellet. For determination of LM_t-carbohydrates, cell pellets were extracted with 75% ethanol at 70°C for 30 min, after which the mixture was centrifuged. In the 75% alcoholic supernatant the total LM_t-carbohydrate fraction (containing cyclic glucans and/or trehalose) was measured for hexoses. For determination of glycogen, the alcohol-extracted cells of *R. meliloti* SU-47, which do not produce capsules, were incubated in 1 M HCl at 100°C for 2 h in order to hydrolyze the glycogen fraction. The mixture was centrifuged, after which glycogen was measured in the supernatant and expressed as glucose equivalents/l culture. In case of *R. leguminosarum* bv *trifolii* TA-1, alcohol-extracted cells were suspended first in 1N NaOH for 15 min at 70°C in order to remove capsular polysaccharides, the mixture was thereafter centrifuged and glycogen determination was performed in the alcohol and alkaline extracted cell pellet after hydrolysis in HCl.

Supernatant. HM_r polysaccharides were separated from the LM_r-carbohydrates by precipitation with 3 vol. ethanol. The alcoholic supernatant was measured for cyclic glucans as described earlier (Breedveld *et al.*, 1990a).

Analytical methods. Hexoses were measured by the anthrone-sulphuric acid method (Trevelyan & Harrison, 1952). Mannitol was determined by periodate oxidation followed by the chromotropic acid-colorimetric method (Burton, 1957).

Total cellular protein was determined according to Lowry *et al.* (1952) using bovine serum albumin as the standard.

PHB determinations were performed according to the method of Braunegg *et al.* (1974), in which PHB was measured by GLC with benzoic acid as the standard and expressed as mg PHB/l culture.

Amino acids were detected in the supernatant of ethanol-extracted cells by measuring α-amino nitrogen groups with the ninhydrin-reaction, with glutamic acid as the standard (Herbert *et al.*, 1971).

Cellular LM_r-carbohydrates were separated with gel-permeation chromatography using a column of Ultrogel AcA 202 (25x2.5 cm). Fractions of 2.5 ml were collected and hexoses were measured. Charged glucans, neutral glucans and trehalose were found in the fractions 10-13, 20-24 and 27-30 respectively (Breedveld *et al.*, 1990b). Peak fractions were analyzed by methylation analysis (Harris *et al.*, 1984).

Thin-layer chromatography of the total cellular LM_r-fraction corresponding to 50 μg glucose-equivalents was performed on Silica gel-plates with the solvent butanol:ethanol:water (5:5:4 by volume). The plates were run two times in the ascending way. Compounds were made visible by spraying the plate with 5% H_2SO_4 in ethanol and heating for 15 min. at 100°C as described earlier (Breedveld *et al.*, 1990b).

***In vivo* NMR.** Cell pellets were washed and taken up in 50 mM phosphate pH 7.0 or 50 mM Tris-HCl pH 7.8. To 10 ml of a 25-fold concentrated cell suspensions (12.5 mg protein/ml buffer) of strains SU-47 and TA-1 grown in standard medium for 72 hours, 1 ml D_2O was added together with 91.8% $^{13}C_1$-labelled mannitol (Merck Sharp and Dome, Haarlem, the Netherlands) or unlabelled mannitol (^{31}P-NMR) and the suspension was continuously sparged with O_2 (1 l/h). Fourier transform NMR ^{13}C and ^{31}P spectra were obtained using a Bruker CXP-300 spectrometer equipped with a 20 mm internal diameter $^{13}C/^{31}P$ switchable probe, operating either at 75.46 MHz (^{13}C) or 121.47 MHz (^{31}P). The WALTZ pulse sequence was used to decouple protons during the recording of the ^{13}C-NMR spectra, the temperature being kept close to 25°C. Spectra (1h), covering a spectral width of 20,000 Hz, were obtained after Fourier transformation of blocks of 7200 FID's that were sequentially stored on disk in 8K data points, using a 60° pulse, a pulse delay of 0.5 sec and a line broadening of 5 Hz. The ^{31}P-NMR spectra, covering a spectral width of 10,000 Hz, were recorded using a 60° pulse, a pulse delay of 0.5 sec and a line broadening of 20 Hz. WALTZ decoupling was only used in the case of cell extracts, that were measured at a temperature kept close to 4°C. ^{13}C-NMR chemical shifts were referenced to the mannitol C_1-resoncance at 64.3 ppm, whereas ^{31}P-NMR chemical shifts were referenced to glycerophosphoryl-choline at 0.49 ppm.

RESULTS

Dynamics of carbohydrate metabolism after exposure to NaCl. The response of cellular carbohydrates in non-growing cells of *R. leguminosarum* bv *trifolii* TA-1 and *R. meliloti* SU-47 to NaCl was investigated. Cells grown in 1 l standard medium (500 mg cellular protein) were transferred to 1 liter of a glutamic acid-free standard medium containing 0.25 M (TA-1) or 0.4 M NaCl (SU-47). After separation of the LM_r-fraction of strain TA-1 by gel-chromatography, 2 peaks could be detected, which eluted at the same position as neutral cyclic (1,2)-ß-glucans and trehalose (Breedveld *et al.*, 1991; Table 1). Methylation analysis of the cellular glucan fraction, which remained constant, resulted in 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-glucitol as sole methylated product, confirming the cyclic glucan structure

with (1,2)-linkages. The second peak, which increased with time, revealed exclusively 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol, confirming the (1,1)-structure of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside).
With strain SU-47 3 peaks could be distinguished after separation of the cellular LM_r-carbohydrate fraction by gel-chromatography. The total internal cyclic (1,2)-ß-glucan pool comprising both the anionic (peak I) and neutral cyclic glucans (peak II), remained constant during the experiment (80 h). Trehalose (peak III, Table 2) synthesis occurred faster and reached a higher level than in strain TA-1. The presence of both neutral and charged cyclic glucans, and the accumulation of trehalose by strain SU-47 is illustrated by Thin-layer chromatography (Figure 1).

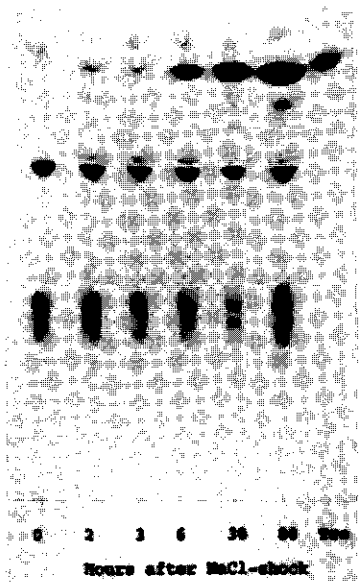

FIGURE 1 Thin-Layer chromatogram of the cellular LM_r-carbohydrates of *Rhizobium meliloti* SU-47. The numbers indicate the time (h) after exposure to NaCl.

The external carbon-source (5.0 g/l mannitol) was partially utilized, and after 120 h still 1.5 g (TA-1) or 3.1 g mannitol/l (SU-47) was present. Glycogen was degraded almost completely (Table 1,2). The total cell protein remained constant, indicating that no cell lysis had occurred after exposure to NaCl. The TA-1 cells excreted cyclic glucans, up to 450 mg/l after 120 h of incubation. Cyclic glucans of strain SU-47 were not excreted (data not shown) nor degraded by strain SU-47 after exposure to NaCl.

When no external mannitol was present, trehalose synthesis was still observed, both in SU-47 (Table 3) and TA-1 (not shown). This synthesis could only take place at the expense of the reserve materials present, since no other carbon source was available. Therefore, PHB was measured as well. In fact, PHB and glycogen decreased. This decrease parallelled the synthesis of trehalose. The onset of trehalose synthesis was much slower in the absence than in the presence of mannitol (Tables 2 and 3).

Influence of mannitol on levels of cellular LM_r carbohydrates and amino-acids in strain TA-1 grown in low and high osmolarity. Strain TA-1 was grown for 7 days in batch cultures containing standard medium with varying amounts of mannitol, in the presence or absence of 0.2 M NaCl. In a mannitol-free medium the pH of the medium rose to 8.9. In all other cases pH-values ranged between 6.8 and 8.0, which is normally observed for strain TA-1 (data not shown). Mannitol had a beneficial effect on growth, especially at 0.2 M NaCl. The concentration of cellular LM_r carbohydrates increased with increasing concentration of mannitol, both in the absence and presence of NaCl (Table 4). TA-1 cells grown in the presence of 0.2 M NaCl accumulated increasing amounts of trehalose with increasing concentrations of mannitol in the medium (Table 4). The amino acid content of the cells, expressed as glutamic acid equivalents, was inversely correlated to the mannitol concentration in NaCl-containing media. In NaCl-free media the LM_r-fraction of strain TA-1 consisted of neutral cyclic glucans only, and the cellular amino acid content (with 5 g mannitol/l) was less than 2 mg/g protein.

TABLE 1. Time course of cellular carbohydrate metabolism (μg/mg cell protein) by cells of *R. leguminosarum* bv *trifolii* TA-1 pregrown in NaCl-free medium and resuspended in a glutamic acid-free medium containing 5 g/l mannitol and 250 mM NaCl

Time (h)	LM_r[1]	Glucan[1]	Trehalose[1]	Glycogen (μg/mg protein)
0[2]	80	80	0	390
6	100	70	30	145
24	125	75	50	50
48	150	75	75	20
120	220	85	135	10

[1] low molecular weight carbohydrates in μg glucose equivalents/mg protein. This fraction was separated by gelchromatography in a cyclic glucan and a trehalose fraction.
[2] The data at t=0 are from the TA-1 cells grown for 72 h in the absence of NaCl.

TABLE 2. Time course of cellular carbohydrate metabolism (μg/mg cell protein) by cells of *R. meliloti* SU-47 pregrown in NaCl-free medium and resuspended in a glutamic acid-free medium containing 5 g/l mannitol and 400 mM NaCl.

Time (h)	LM_r[1]	Glucans[1]		Trehalose[1]	Glycogen (μg/mg protein)
		I	II		
0[2]	250	80	165	3	450
1	270	70	150	50	340
2	300	70	140	90	270
3	320	70	150	100	240
6	360	70	160	130	210
30	410	60	140	210	120
80	495	70	140	280	80

[1] low molecular weight carbohydrates in μg glucose equivalents/mg protein. This fraction was separated by gelchromatography in a charged cyclic glucan-fraction (I), a neutral cyclic glucan fraction (II) and trehalose fraction.
[2] The data at t=0 are from the SU-47 cells grown for 72 h in the absence of NaCl.

TABLE 3. Time course of cellular carbohydrate metabolism and PHB (μg/mg cell protein) by cells of *R. meliloti* SU-47 pregrown in NaCl-free medium and resuspended in a glutamic acid- and mannitol-free medium containing 400 mM NaCl.

Time (h)	LM_r[1]	Glucans[1]		Trehalose[1]	Glycogen	PHB
		I	II		-------(μg/mg protein)------	
0[1]	260	80	175	1	630	410
3	260	95	160	3	505	340
6	320	90	170	55	445	280
24	410	90	170	150	350	260
52	445	85	150	210	320	240

[1] see table 2

TABLE 4. Mannitol-dependent LM_t cellular carbohydrate content of *R. leguminosarum* bv *trifolii* TA-1 grown in the presence or absence of 0.2 M NaCl. Incubation was for 7 days in standard medium (1 g glutamic acid/l) with varying mannitol-concentrations.

Mannitol	0 M NaCl		0.2 M NaCl				
	LM_t[a]	protein	LM_t[b]	glucan	trehalose	amino-acids[c]	protein
(g/l)		(mg/l)	μg/mg protein				(mg/l)
0	4	450	1	nd[d]	nd[d]	nd[d]	110
1	19	550	20	10	10	16.5	550
2	35	540	30	20	10	8.0	560
3	40	510	55	25	30	4.6	540
4	45	550	125	60	65	3.0	560
5	60	520	140	70	70	2.0	540
10	80	540	145	70	75	2.1	550

a) total low molecular weight cellular carbohydrates expressed as glucose equivalents in μg/mg cell protein. This fraction consists of cyclic (1,2)-ß-glucans only as determined by gel-chromatography.
b) as a, but this fraction was separated by gelchromatography in a glucan and trehalose fraction
c) amino-acids are expressed as glutamic acid equivalents in μg/mg cell protein
d) not determined

***In vivo*-^{13}C-NMR.** NMR-experiments were performed to obtain more insight into the dynamics of trehalose synthesis and breakdown of reserve-materials. Cell suspensions obtained from the strains *Rhizobium meliloti* SU-47 and *R. leguminosarum* bv *trifolii* TA-1 were first incubated in the absence of NaCl and $^{13}C_1$-mannitol to measure the accumulation of natural abundance ^{13}C compounds. The intracellular concentration of cyclic (1,2)-ß-glucans in the cell suspension of strain SU-47 was 260 μg/mg cell protein or 3 mg/ml suspension. The resonances of C_1-C_6 of the cyclic (1,2)-ß-glucans at 102.9, 83.3, 76.5, 69.8, 77.2 and 61.7 ppm (Figure 2A) could be easily assigned (Zevenhuizen *et al.*, 1990). Resonances of low intensity at 63.7 and 67.7 ppm could be assigned to the C_3- and C_1-carbons of the glycerol-phosphate subsitutions being present on part of the C_6-glucoses of the anionic cyclic glucans in *R. meliloti* (Zevenhuizen *et al.*, 1990). The C_2-resonance of the glycerol-phosphate substitution at 71.9 ppm was less resolved. The cellular concentrations of the neutral cyclic glucans in the TA-1 cell suspension was 0.8 mg/ml.

The broad resonance around 20 ppm that was observed in suspensions of both SU-47 (Figure 2A) and TA-1 (not shown) after the onset of aeration, was most likely due to the CH_3-carbon atoms in a mobilized PHB fraction (Claassen *et al.*, 1986). This peak diminished upon prolonged incubation, both in the presence or absence of mannitol and/or NaCl, indicating breakdown of PHB.

The buffer capacity of 50 mM phosphate pH 7.0 was not enough to prevent fast acidification of the SU-47 suspension to 5.4 after 5 h of incubation. This could be due to excretion of a keto-acid intermediate which is sometimes observed with *R. meliloti* (Courtois *et al.*, 1979). In the presence of 10 g/l $CaCO_3$ in the SU-47 suspensions, the pH-drop after 20 h was only 0.8 units for the phosphate buffer and 0.6 for Tris-HCl pH 7.8 buffer. The pH drop in phosphate buffered cell suspensions of strain TA-1 was only 0.4 units after 20 h, both in the absence and presence of $CaCO_3$.

Exposure to NaCl. 50 mg $^{13}C_1$-mannitol (4.5 g/l) and NaCl in a concentration of 0.4 M (SU-47) or 0.25 M (TA-1) were added to cell supsensions of cells grown in the absence of NaCl. Resonances at 94.1 and 61.6 ppm were a clear indication for C_1 and C_6 of trehalose (Figures 2B and 3). Maximum incorporation of mannitol-derived label in trehalose was already obtained after 5 to 9 h and remained more or less constant thereafter. Cyclic glucan synthesis by strain TA-1 was observed after exposure to NaCl by the enhancement of the C_1 and C_6-resonances of the glucose-residues in cyclic glucan (Figure 3).

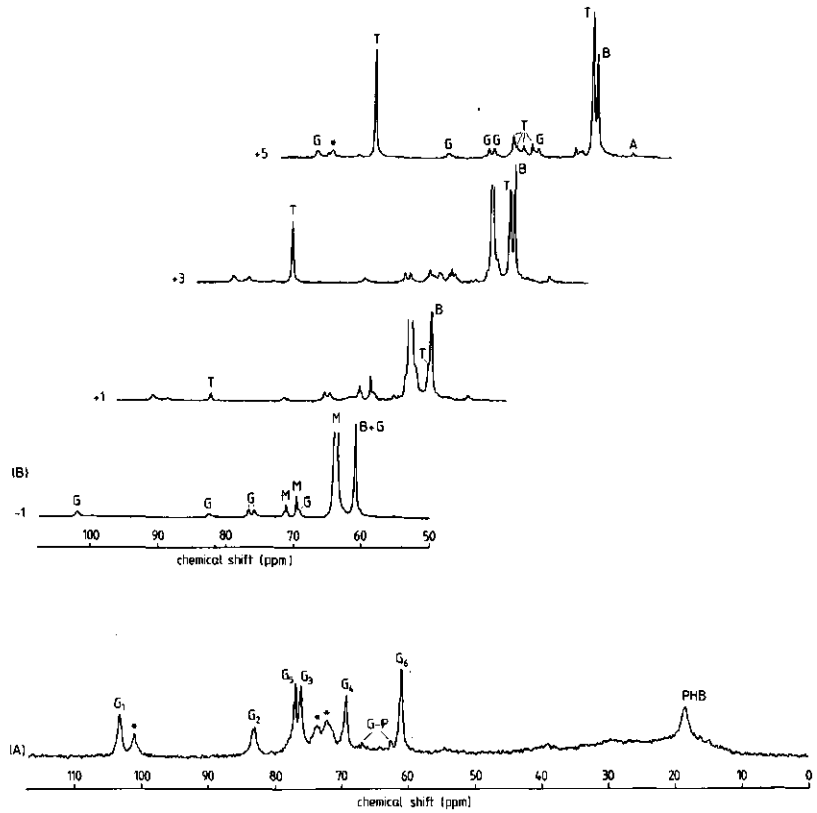

FIGURE 2 *In vivo* 13C-NMR spectra of cells of *R. meliloti* SU-47. **(A)** Incubation of cells in phosphate-buffer in the absence of mannitol, showing all 6 resonances of cyclic (1,2)-ß-glucans (G_1-G_6) and the mobile CH_3 resonances of PHB; * reflects most likely the broad resonances of endogenous high-molecular weight polysaccharides and/or PHB-compounds; **(B)** Incubation of cells in Tris-buffer (B) in the presence of $13C_1$-mannitol (M), before (t=-1h) and after (t=1 to 5 h) theexposure to NaCl, showing the synthesis of trehalose (T); A=C_2-glutamic acid. Note the overlap of the C_6 resonances of glucan and trehalose.

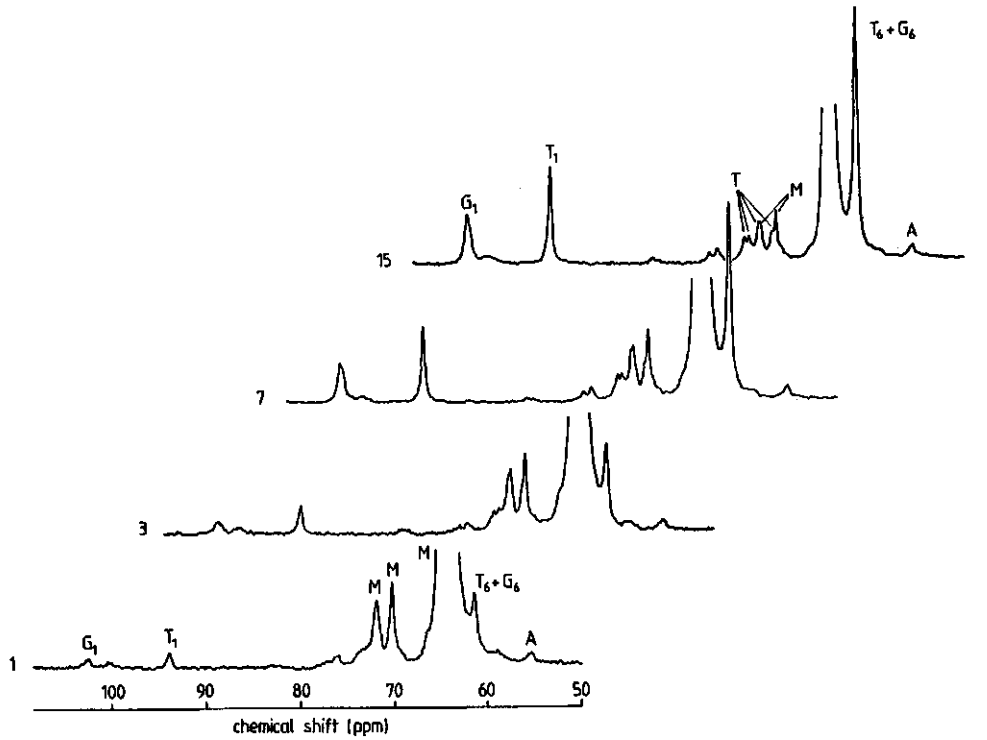

FIGURE 3 *In vivo* ^{13}C-NMR of cells of *R. leguminosarum* bv *trifolii* TA-1. Cells in phosphate-buffer were incubated in the presence of $^{13}C_1$-mannitol (M). The spectra show the appearance of trehalose (T) and cyclic glucans (G) after (t=1 to 15 h) the exposure to NaCl. For other abbreviations see Figure 2.

Low levels of ^{13}C-enriched glutamate were measured in both strains after exposure to NaCl. Its C_2 (55.6 ppm) and C_3 carbons (28.0 ppm; not shown) had half the intensity of theC_4 carbon (34.4 ppm; not shown), indicating that the carbon atoms were derived from $^{13}C_1$-mannitol after entering the Krebs-cycle (Claassen *et al.*, 1986).

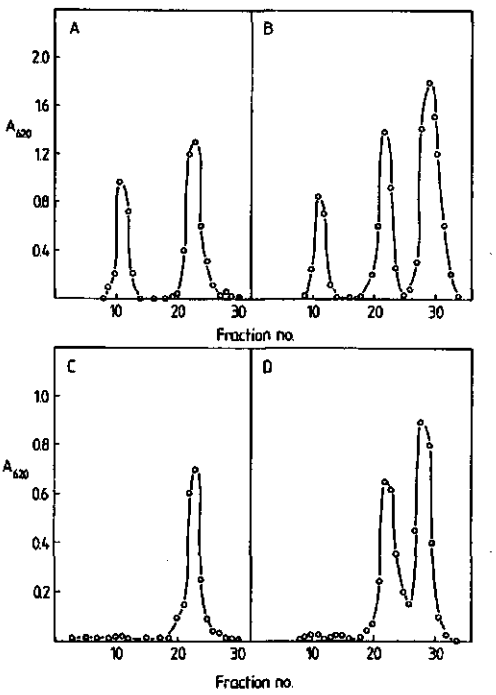

FIGURE 4 Gel-chromatogram of LM_t-cellular carbohydrates of *Rhizobium meliloti* SU-47 (A,B) and *R. leguminosarum* bv *trifolii* TA-1 (C,D) of the cell-suspensions obtained for the NMR-experiments after exposure to NaCl at t=0 (A and C) and t=20 h (B and D). LM_t-carbohydrates in the cell suspension were extracted with 3 vol. of ethanol and fractionated over an Ultrogel AcA-202 gel-filtration column. Charged glucans, neutral glucans and trehalose are found in the fractions 10-13, 20-24 and 26-31, respectively

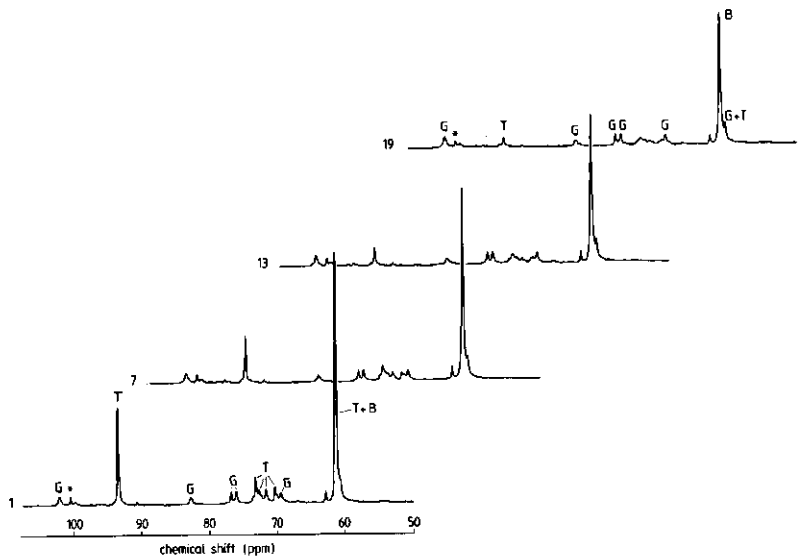

FIGURE 5 *In vivo* 13C-NMR spectra of trehalose-accumulated *R. meliloti* SU-47 cells in NaCl-free buffer. Note the overlap of the buffer (B) and C_6 resonance of trehalose at t=1 h. For other abbreviations, see Figure 2

Trehalose formed a major fraction in the LM_r-carbohydrate pool of cell suspensions of both SU-47 and TA-1 incubated for 20 h in the NaCl-containing buffer (Figure 4). Synthesis of trehalose could not be explained by sole conversion from the enriched $^{13}C_1$-mannitol. Indeed, at 72.1, 73.5, 70.5 and 73.1 ppm the four remaining natural abundant resonances of equal amplitude belonging to the C_2-C_5-carbon atoms of trehalose (Meikle *et al.*, 1991) could easily be detected in both strains (Figures 2B and 3). Since ^{13}C is only abundant in nature by 1.1%, it was calculated that about 15-20% of the trehalose was derived from the external mannitol and 80-85% from the internal carbon-reserve pool like glycogen and PHB.

SU-47 cells with trehalose incubated for 20 h in Tris-buffer in the presence of 0.4 M NaCl, were transferred to NaCl-free buffer without external carbon. Trehalose was immediately broken down. This confirms that its function was dispensable in a low osmolarity environment. The C_6-resoncances of the cyclic glucans and trehalose overlapped, and coincided with Tris-buffer carbons (Figure 5).

***In vivo* ^{31}P-NMR.** The charged glucans of strain SU-47 were investigated with ^{31}P-NMR. In ^{31}P-spectra of whole aerobic cell suspensions differences between SU-47 and TA-1 could be clearly observed, TA-1 showing much lower levels than SU-47. Since the cells were suspended in Tris buffer, no external phosphate was present. The spectra of SU-47 cells showed the resonances of alkaline cytosolic inorganic P_i (2.91 ppm) besides hexose-phosphates (around 4.9 ppm), co-factors NAD(P)/NAD(P)(H) (-10.7), ATP (-5.0, - 10.0, and - 18.6 ppm belonging to γ, α and ß ATP) and UDPG (-10.7 and -12.3 ppm). In addition, the SU-47 spectra revealed a sharp peak at 1.34 ppm, which was absent in TA-1 (Figure 6). Its resonance position, being non-titrable in the physiological region, was calibrated against added glycerophosphoryl-choline (0.49 ppm). The peak position and area remained unchan-

ged during the whole incubation period of 20 h in the presence of NaCl. Upon addition of glycerol-1-phosphate substituted cyclic glucan, obtained from SU-47 cells after separation by gel-chromatography of the LM_r-carbohydrate fraction, either to the cell suspensions or the cell extract, the amplitude of this single narrow resonance at 1.34 ppm increased, whereas its shape remained constant. This clearly indicates that the resonance at 1,34 ppm is due to the phosphate group of the glycerol-1-phosphate substituted cyclic glucans.

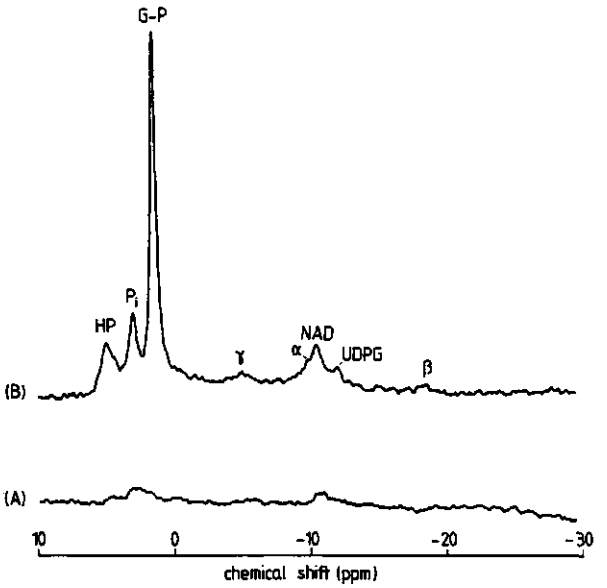

FIGURE 6 ^{31}P-NMR spectra (0.5 h) of whole cell suspensions of *R. leguminosarum* bv *trifolii* TA-1 (A) and *R. meliloti* SU-47 (B). The resonance at 1,34 ppm represents the phosphoglycerol-subsitution (G-P) in the cyclic glucans of strain SU-47. HP=hexose-phosphates; P_i=cytosolic inorganic phosphate; NAD= phosphates present in NAD(P)/NAD(P)(H); α, ß, and γ= phosphates present in ATP.

DISCUSSION

Trehalose formation from glycogen. Non-growing cultures of *R. leguminosarum* bv *trifolii* TA-1 and *R. meliloti* SU-47 which are exposed to NaCl synthesize their trehalose for at least 80 % from the internal pool of reserve materials glycogen and/or PHB, both in the presence (NMR-experiments) or absence (batch fermenter experiments) of NaCl. It can be assumed that the formation of the disaccharide trehalose (containing the α-(1,1) bond) is likely to be formed rather from glycogen (α-(1,4)-glucan) than from the structurally non-related PHB. Experimental evidence is not available, because these polymers contain hardly any mobile carbon-chains and do not show well resolved resonances in the NMR spectra, except for the PHB-CH_3 resonance. While the role of glycogen as a true reserve material is unambiguously accepted, the role of trehalose as a general protectant against adverse conditions has recently been proposed. Therefore, the hypothesized synthesis of trehalose out of glycogen may help to stimulate the discussion on the role of trehalose as a general protection against so-called stress-conditions (Van Laere, 1989, Wiemken, 1990, Zevenhuizen, 1991).

Osmo-regulants. Depending on culture conditions, growth phase and the organism involved, cells of *R. meliloti* and *E.coli* respond to high osmotic pressure of the medium by accumulation of osmo-protectants. These compounds can be rich in nitrogen, like glycine-betaine, proline-betaine, or glutamate, and carbon, like trehalose (Czonka, 1989). Apparently, depending on the availability of carbon or nitrogen, strain TA-1 growing at high osmotic pressure of the medium favoured accumulation of amino-acids in mannitol-limited medium, and trehalose in mannitol-rich medium (Table 4). At low osmotic pressures of the medium it is thought that periplasmic cyclic (1,2)-ß-glucans of *Rhizobium* play an important role in osmo-adaptation (Miller *et al.*, 1986, Dylan *et al.*, 1990). We discussed that the capsular polysaccharide (CPS) around cells of *R. leguminosarum* could withstand the turgor pressure of the cell against the outer membrane at low osmotic

pressure of the medium (Breedveld *et al.*, 1990b). CPS is only synthesized in media with excess carbon (chapter 8). The osmotic pressure of our NaCl-free media is largely dependent upon the concentration of mannitol. Since low concentrations of mannitol gave rise to concomitantly low concentrations of cyclic glucans (Table 4), other yet unknown mechanisms should be responsible for osmotical adaptation of the TA-1 cells to low osmolarity environments.

Detection of cyclic glucans with *in vivo* ^{13}C and ^{31}P NMR. With SU-47 cell suspensions the carbon-resonances of the cyclic glucans were well resolved reflecting the high intrinsic flexibility of the cyclic (1,2)-ß-glucans having an average molecular weight of 4000 Da. The cyclic glucan concentration in TA-1 cells is much lower than in SU-47 and its resonances were inherently less visible in the spectra of TA-1 cells before exposure to NaCl. However, after exposure of TA-1 cells to NaCl glucan synthesis occurred, in agreement with the osmotically-induced glucan excretion reported earlier (Breedveld *et al.*, 1991). It is safe to assume that the cyclic glucans, which were found continuously excreted in media with high osmotic pressure, are formed from the substrate mannitol, and not from the reserve materials since under these circumstances the synthesis of glycogen and PHB was strongly repressed (Breedveld *et al.*, 1991). Furthermore, the osmotically-induced glucan excretion occurring both in NaCl-adapted and in NaCl-exposed cultures may be an effect of the osmolyte on the cell wall rendering it more permeable.

Batley *et al.* (1987) observed the phosphate resonance of a purified phosphoglycerol substituted cyclic (1,2)-ß-glucan fraction of *Rhizobium* sp. NGR234 in a proton-decoupled ^{31}P spectrum as a single peak. The same was found for the phosphoglycerol-substituted glucans in whole cell suspensions of strain SU-47 (Figure 6). The synthesis of phosphoglycerol-substituted cyclic glucans of *R. meliloti* SU-47, being for 35% substituted in low osmolarity media, was strongly repressed in a medium containing 0.4 M NaCl, while the neutral cyclic glucans were only moderately repressed (Breedveld *et al.*, 1990b). However, in the experiments presented here cells were grown in standard medium (having a low osmotic pressure of 1.3 atm) and therefore cyclic glucan concentration had already reached its maximum value. Cyclic glucans were not excreted nor degraded by strain SU-47 after exposure to NaCl. Miller *et al.* (1988) found up to 90% anionic glucans in *R. meliloti* strain 1021 (a streptomycin resistent derivative of strain SU-47), while Dylan *et al.* (1990) found 96% neutral glucans in *R. meliloti* 102F34. In the last two cases, the accumulation of glucans completely suppressed in media containing 0.5 M NaCl. Differences in substitution grade, due to synthesis of anionic glucans and/or release of the substiuent from the rings, as found within strains of *R. meliloti* may be ascribed to different cultural conditions. We did not find any change in the phosphoglycerol-substituted glucan content after exposure to NaCl, suggesting that no release of the substituent from the glucans had occurred. However, the glycerolphosphate group might be released from the cyclic glucan under other yet unknown conditions.

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CHAPTER 7

NaCl-induced excretion of cyclic ß-(1,2)-glucans by *Rhizobium leguminosarum* biovar *trifolii* TA-1 is the result of enhanced permeability of the cells

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NaCl-induced excretion of cyclic ß-(1,2)-glucans by *Rhizobium leguminosarum* biovar *trifolii* TA-1 is the result of enhanced permeability of the cells

ABSTRACT

The synthesis of cyclic ß-(1,2)-glucans from UDP-[^{14}C]-glucose by a crude membrane preparation and whole cells of *Rhizobium leguminosarum* biovar *trifolii* TA-1 was investigated. The crude membrane system needed Mn^{2+}, ATP and NAD^+ for optimal activity. Hardly any difference in biosynthetic activity was observed between membrane fractions of TA-1 cells grown in the presence (200 mM) or absence of NaCl. Whole TA-1 cells grown in the presence of NaCl excreted labelled, neutral cyclic ß-(1,2)-glucan during incubation with added UDP-[^{14}C]-glucose. With NaCl-free cultured TA-1 cells no excretion was observed, however after 8 times alternately freezing and thawing these cells excreted glucans. Glucan formation *in vitro* and glucan excretion by whole cells was strongly inhibited in the presence of 50 mg/ml cyclic glucan (about 15 mM), indicating biosynthesis of cyclic ß-(1,2)-glucans in strain TA-1 to be controlled by end-product inhibition. These observations indicate that TA-1 cells become permeable for cyclic glucans at high NaCl concentrations. The constant loss of glucans from cells grown in the presence of 200 mM NaCl prevents end-product inhibition and results in glucan accumulation in the medium up to 1600 mg/l.

Abbreviations: TLC= thin-layer chromatography; UDP= uridine-diphosphate

INTRODUCTION

ß-(1,2)-glucan is a polysaccharide synthesized by members of the family of *Rhizobiaceae* and has been shown to consist exclusively of ß-(1,2)-linked D-glycosyl residues (25, 26). Dell *et al* (14) demonstrated unequivocally the cyclic character of the glucans. The ß-(1,2)-glucans of *Rhizobium leguminosarum* and *Agrobacterium tumefaciens* consist of a mixture of rings with different degrees of polymerization (DPs) between 17 and 25 (29), while *Rhizobium meliloti* contains ring sizes of up to 40 (19). In *R. meliloti* and *A. tumefaciens* a large part of the glucans are glycerophosphorylated (5,22). In *R. leguminosarum* glucans are mostly low substituted, or unsubstituted, neutral molecules (29). Although cyclic ß-(1,2)-glucans are generally supposed to be located in the periplasmic space of the cells (1) they were also found to be excreted into the culture liquid (3,4,7,27,28).

Periplasmic cyclic ß-(1,2)-glucans are involved in osmoregulation in members of the family of the *Rhizobiaceae* at low osmotic strength of the medium. The glucans are synthesized to obtain an increased osmolarity in the periplasm, in order to minimize the differences in osmotic pressure across the inner membrane (21). Mutants of *R. meliloti* (15) and *A. tumefaciens* (11) defective in the synthesis of ß-(1,2)-glucans were strongly impaired in their ability to grow at hypoosmotic conditions in diluted glutamic acid-mannitol-salts media (osmotic pressure π=0.3 atm). Their growth was restored by the addition of osmolytes to the medium. These mutants were also ineffective (*Rhizobium*) and avirulent (*Agrobacterium*), indicating some role of cyclic glucans in attachment to or during infection of their hosts.

At high osmotic strength of the medium (containing 0.5 M NaCl) the production of cyclic glucans was strongly repressed in *A. tumefaciens* (21) and *R. meliloti* (15,32). Under the same conditions, glycogen synthesis was strongly inhibited and trehalose was accumulated in *R. meliloti* cells to function as osmo-protectant (8). However, the *in vitro* synthesis of cyclic glucans by a particulate membrane fraction of *R. meliloti* was not repressed (32). The *in vitro* ß-(1,2)-glucan synthesis from UDP-[^{14}C]-glucose was studied for the first time in a particulate preparation obtained from *R. japonicum* (13). Since then, cell-free particulate fractions obtained from *A. radiobacter* and *R. phaseoli* (2) and *A. tumefaciens* and *R. meliloti* (30,31) were found to

catalyze the formation of cyclic ß-(1,2)-glucan.

Recently we showed that in a medium containing 200 mM NaCl or other osmolytes *R. leguminosarum* bv *trifolii* TA-1 and other *R. leguminosarum* cells excreted up to 1600 mg/l cyclic glucans. In NaCl-free media only low amounts of glucans were excreted (maximally 50 mg/l). The cells contained 50-100 mg glucans/g protein independently of the osmotic pressure of the medium (9). In this study we compare the *in vitro* synthesis of cyclic glucans by strain TA-1 grown in the presence or absence of NaCl. No difference in biosynthetic activity was found. Therefore, the observed excretion of glucans *in vivo* in the presence of NaCl cannot be explained by a higher biosynthetic capacity. We provide evidence that the outer membrane becomes more permeable for cyclic glucans when *R. leguminosarum* cells are exposed to NaCl.

METHODS

Organisms and cultivation. *Rhizobium leguminosarum* bv *trifolii* TA-1 was obtained from the culture collection of the Department of Microbiology, Wageningen, The Netherlands. *R. leguminosarum* bv *viciae* RBL5523 and mutant RBL5523, *exo*8::Tn5 (12) were obtained from the culture collection of the Department of Plant Molecular Biology, State University Leiden.

Precultures were prepared by inoculating the organism into "standard medium" containing 5 g/l mannitol, 1 g/l glutamic acid and mineral salts according to Breedveld *et al.* (7). In this medium 500 mg cellular protein per liter could be produced. The term "production medium" is used when the mannitol concentration was 10 g/l. Media were supplemented with EDTA, NaCl or other osmolytes as indicated. Cells from late logarithmic phase were used as inoculum (1%, v/v). They were cultivated for 32 hours (standard or production medium) or 48 hours (medium supplemented with NaCl) in 1 liter Erlenmeyer flasks containing 200 ml medium at 25°C. The flasks were incubated on a rotary shaker at 200 rpm. Cells and culture supernatant were separated by centrifugation at 35000x *g* for 30 min. Cell pellets were washed in 100 ml 50 mM Tris-HCl pH=7.8 (buffer A), centrifuged and resuspended in 20 ml buffer A. In case of whole cell experiments, cultures were grown in 300 ml Erlenmeyer flasks with 50 ml standard medium. The cultures were centrifuged, pellets were washed in 20 ml buffer A, centrifuged again and resuspended in 2 ml buffer A. For the EDTA experiments, cultures of strain TA-1 were grown in 300 ml Erlenmeyer flasks with 50 ml production medium containing 0, 0.1, 0.5 or 1 mM EDTA for 12 days. After centrifugation of the cultures, the supernatant was used for the quantification of high molecular polysaccharides and cyclic glucans as reported previously (9). The pellet was used for quantification of capsular polysaccharide and low molecular cellular carbohydrates via NaOH extraction and alcohol fractionation (7).

Preparation of the membrane fraction. To obtain a cell-free extract cell suspensions were 8 times sonicated at 0°C for 30 sec at 40W (Branson Sonifier) interrupted by intervals of 60 sec. After centrifugation at 12000x *g* for 15 min, the extract was ultracentrifuged for 1 hour at 150000x *g*. The crude membrane fraction in the pellet was washed and taken up in buffer A and used as the enzymic fraction. This fraction could be stored at -20°C for several months without significant loss in activity. Cell protein was measured according to the method of Lowry *et al.* (20) with bovine serum albumine as standard. The protein content of the membrane fractions was quantified according to the method of Bradford (6).

Enzyme assay. All solutions were prepared in buffer A. The standard assay was done in a total volume of 75µl containing 100µg protein of the crude membrane fraction, 3µl UDP-[^{14}C]-glucose (925 Bq/µl, specific activity 12.9 MBq/µmol), 5 mM ATP, 5 mM NAD^+, 12.5 mM $MnCl_2$, and 2.5 mM mercapto-ethanol. After incubation at 20°C for 2 hours the mixture was heated for 3 minutes at 100°C and centrifuged. To obtain more product the mixture with 4 mg membrane protein, 2.6 mg unlabelled and 50µl labelled UDP-[^{14}C]-glucose (46.3 kBq), 5 mM ATP, 5 mM NAD^+, 12.5 mM $MnCl_2$ and 2.5 mM mercapto-ethanol (total volume of 1.1 ml) was

incubated for 5 hours. In case of whole cell experiments the assay mixture (total volume of 75 μl) with cell suspension (containing 350μg cellular protein), 5μl UDP-[^{14}C]-glucose (4.6 kBq), 5 mM ATP, 5 mM NAD^+, 12.5 mM $MnCl_2$ and 2.5 mM mercapto-ethanol was incubated for 45 minutes after which the mixture was centrifuged. To avoid the extraction of labelled products from the cells during the inactivation at 100°C, the boiling step was omitted. To obtain permeable cells, cell suspensions were alternately 8 times frozen (-20°C in the freezer) and thawed (room temperature).

Chromatography. Reactants and products in the supernatant (50 μl) were applied to a DEAE-trisacryl column (LKB-Pharmacia, 0.7x10 cm) with H_2O as eluent. The bound fraction was eluted with 1 M KCl. Fractions of 0.4 ml were collected and 100μl was counted for radioactivity by liquid scintillation counting. In the presence of high concentrations of NaCl or KCl (0.1-0.4 M) in the assay mixture, samples were first desalted on a Biogel P2 column (0.7x10 cm). To separate the labelled compounds in the bound fraction a DEAE-trisacryl column (2x25 cm) was used with H_2O followed by a linear gradient of 0-1 M KCl as eluent. Fractions of 5 ml were collected and counted for radioactivity. Gel-chromatography of the combined neutral glucan fractions, obtained after DEAE-chromatography, was performed with Ultrogel AcA202 (2.5x30 cm) as described earlier (7). The so obtained labelled glucan fraction was used for methylation analysis.

HPLC-experiments were performed with 20μl sample applied to an NH_2-bonded silica column with acetonitril:H_2O in a ratio of 62:38 as liquid phase at a flow of 1 ml/min (7). Fractions of 0.5 ml were collected and counted for radioactivity. Thin-layer chromatography (TLC) was carried out on Silica gel-plates with the solvent butanol:ethanol:water (5:5:4 by volume). Labelled compounds were located by autoradiography. Reference compounds were made visible by spraying the plate with 5% H_2SO_4 in ethanol and heating for 15 min. at 100°C (2).

Linkage types of the isolated glucan fraction. Glycosidic linkages were determined by methylation analysis according to Harris *et al.* (17). The methylated sugars were separated by TLC and visualized as described above.

Osmotic pressure. The osmotic pressure π of a solution was calculated as described previously (8).

Chemicals. UDP-^{14}C-glucose (specific activity 12.9 MBq/μmol) was obtained from Amersham (Den Bosch, The Netherlands). Reference compounds of cyclic ß-(1,2)-glucan were isolated from the supernatant of a culture of strain TA-1 grown at 33°C. Cyclic ß-(1,2)-glucan was purified by gel-chromatography and its ring-size distribution tested by TLC and HPLC.

RESULTS

Incorporation of ^{14}C-glucose into a neutral glucan fraction by crude membrane fractions of *Rhizobium leguminosarum* bv *trifolii* TA-1. When a membrane fraction of *strain* TA-1 was incubated in the presence of Mn^{2+}, NAD^+, ATP, mercapto-ethanol and UDP-^{14}C-glucose, about 71% of the label appeared in a neutral fraction as a single peak which was not retained by DEAE-trisacryl (Table 1; Figure 1, peak I). This fraction co-migrated with purified cyclic glucans on thin-layer chromatography (data not shown). The reaction required functional protein since with a boiled fraction almost all label remained in the charged fraction (Figure 1, peak II). This fraction comigrated with UDP-glucose on TLC (Figure 2) and HPLC (Figure 3). Without Mn^{2+} and Mg^{2+} almost no activity was shown in the neutral fraction. The reaction required ATP or NAD^+ (Table 1).

Identification of the labelled compounds. For a further identification of the products the supernatant was applied to a DEAE-trisacryl column and eluted with a gradient of 0 to 1 M KCl (Table 2). Now 3 peaks were obtained, one neutral (I) and two charged peaks (II, III). The combined neutral peak fractions (I) were eluted over the Ultrogel AcA202 gel-filtration column. Only one labelled peak appeared. Following HPLC the label appeared in exactly the same fractions as a reference glucan fraction obtained from

train TA-1 (Figure 3). After methylation and hydrolysis only one labelled band appeared on TLC which showed the same mobility as the partially methylated sugar obtained from a reference cyclic ß-(1,2)-glucan (3,4,6-tri-O-methyl-D-glucose; experiment not shown). We therefore conclude that the labelled neutral fraction detected in these experiments consists of cyclic glucans only.

TABLE 1. Incorporation of ^{14}C-glucose into the glucan fraction by a membrane fraction from *Rhizobium leguminosarum* bv *trifolii* TA-1 cells grown in production medium. Various omissions or additions are indicated. Values are expressed relative to the complete mixture, in which 71% of the total label appeared in the glucan fraction (set at 100%). In all experiments 9-13% of the total label was found in the membrane pellet. The values are shown from one typical experiment. Experiments were performed 3 times in which values differed no more than 5%.

INCUBATION	% in glucan fraction
Complete mixture	100
no NAD^+	102
no ATP	98
no ATP/NAD^+	22
no mercapto-ethanol	94
boiled protein	6
no Mn^{2+}	5
12.5 mM $MgCl_2$, no Mn^{2+}	85
+200 mM glucose	98
+200 mM mannitol	95
+100 mM NaCl	63
+200 mM NaCl	40
+200 mM KCl	43
+300 mM NaCl	9
+400 mM NaCl	1

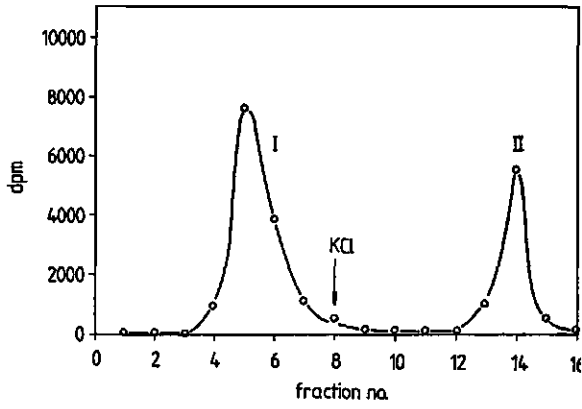

FIGURE 1. DEAE-trisacryl chromatogram of the labelled reaction mixture obtained after incubation of a membrane fraction of *Rhizobium leguminosarum* bv *trifolii* TA-1 with ^{14}C-UDP-glucose, Mn^{2+}, ATP, NAD^+ and mercapto-ethanol. Peak I represents the neutral fraction containing cyclic glucan, peak II represents the combined charged fractions with UDP-glucose and glucose-1-phosphate. KCl: eluent is switched from H_2O to 1M KCl.

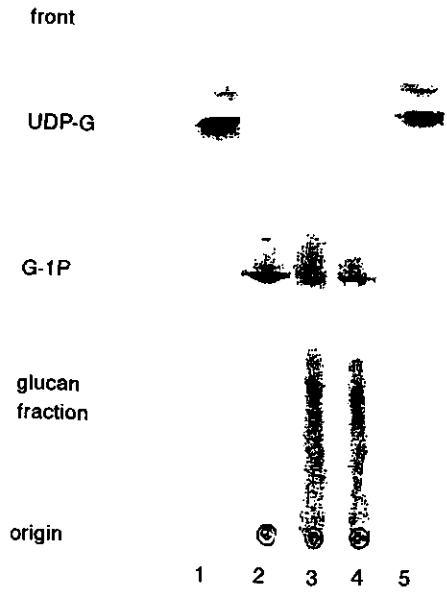

FIGURE 2. TLC chromatogram (3µl) of the reaction mixture with membrane fractions of *Rhizobium leguminosarum* bv *trifolii* TA-1. Lanes: 1= UDP-^{14}C-glucose, 2= incubation without NAD^+ and ATP; 3= complete mixture; 4= incubation with Mg^{2+} instead of Mn^{2+}; 5= incubation without Mn^{2+} and Mg^{2+}. G-1P= glucose-1-phosphate

TABLE 2. Distribution of label between neutral (I) and charged (II, III) peak fractions after incubation of membrane fraction (4 mg protein) of Rhizobium leguminosarum bv trifolii strain TA-1 with 50 µl UDP-^{14}C-glucose (925 Bq/µl) and 2.6 mg unlabelled UDP-glucose incubated for 5 h. I= cyclic glucans; II= glucose-1-phosphate; III= UDP-glucose. The peaks were obtained with a DEAE trisacryl column eluated with a gradient of 0 to 1 M KCl. The amount of label is expressed as a percentage of the total label (=100%)

Addition of NAD$^+$ and ATP	(% label) I	II	III	Pellet	Glucans[1] (mg hexose)
+	46	2	42	10	0.35
–	10	54	24	12	ND[2]

[1] The hexose content in fraction I was measured with the anthrone-sulphuric acid assay (9).
[2] not determined.

TABLE 3. Cyclic glucan excretion by whole cells of *Rhizobium leguminosarum* bv *trifolii* TA-1, *R. leguminosarum* bv *viciae* strain RBL5523 and mutant RBL5523,*exo*8::Tn5. Cells (350µg protein) were incubated in the presence of UDP-^{14}C-glucose and radioactivity was counted in the different fractions. Values are expressed as percentage of the total activity (100%). The results shown here are from one typical experiment. Experiments were performed 3 times and the obtained values differed maximally 6%.

Strain	Growth Condition	F/t[1] 8 times	Glucan Addition (50 mg/ml)	Pellet %	Soluble fraction[2] Neutral %	Charged %
TA-1	Standard[3]	-	-	9	0	91
TA-1	Standard	+	-	15	13	72
TA-1	Standard	+	+	12	0	88
TA-1	150mM NaCl	-	-	10	13	77
TA-1	200mM NaCl	-	-	16	56	28
TA-1	200mM NaCl	+	-	15	53	32
TA-1	200mM NaCl	+	+	9	4	87
TA-1	33°C	-	-	15	24	61
RBL5523	Standard	-	-	24	1	75
*exo*8::Tn5	Standard	-	-	25	23	52

[1]: Cells were either alternately frozen (at -20°C) and thawed (room temp.) 8 times (+) or not (-)
[2]: Fractions separated on DEAE-column.
[3]: Incubation of cells grown in standard medium at 25°C.

Without addition of NAD$^+$ and ATP peak I decreased and peak II formed the major fraction (Table 2). In a study on glucan synthesis by *R. japonicum* DeDonder en Hassid (13) detected glucose-1-phosphate formed from UDP-glucose by a pyrophosphatase-like activity which was apparently present in the membrane fraction. With the addition of excess NAD$^+$ or ATP the breakdown of UDP-glucose into glucose-1-phosphate could be prevented. Indeed, the major spot on TLC plates co-migrated with glucose-1-phosphate when NAD$^+$ and ATP were omitted (Figure 2). Peak III in Table 2 was unreacted UDP-glucose.

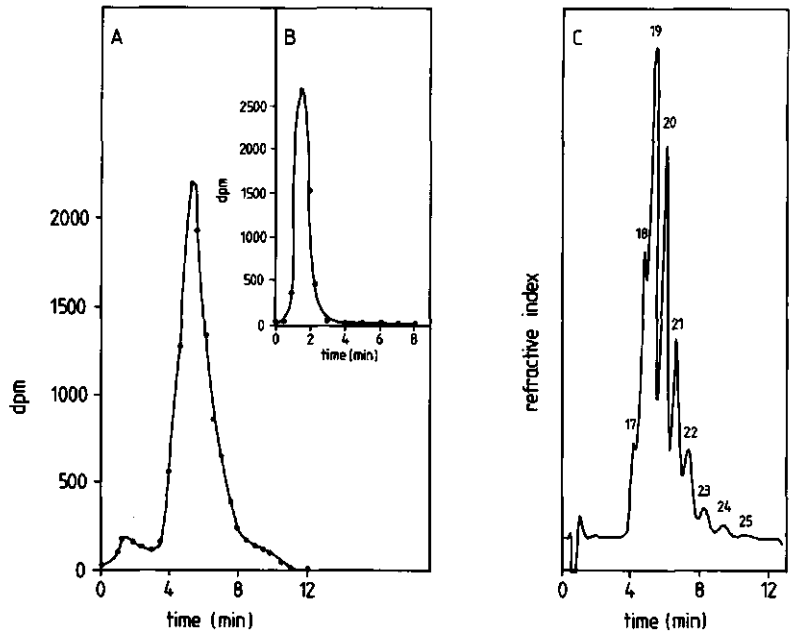

FIGURE 3. HPLC chromatogram of the labelled glucan fraction (A), labelled UDP-glucose (B) and a purified glucan fraction (C). The numbers in C refer to the degree of polymerization (number of glucose molecules) of the neutral glucans.

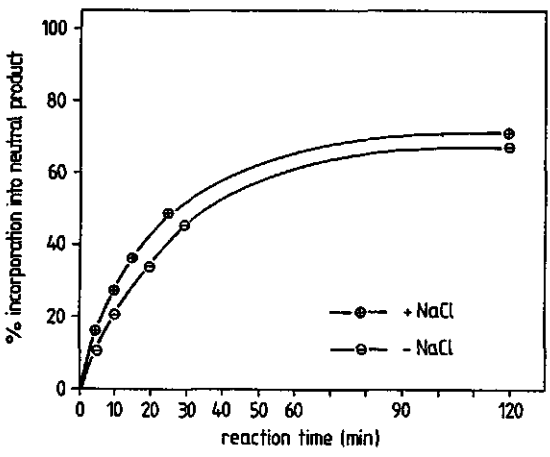

FIGURE 4. Time-dependent biosynthesis of cyclic ß-(1,2)-glucans by a membrane fraction of *Rhizobium leguminosarum* bv *trifolii* TA-1 grown in the presence (+) or absence (-) of 200 mM NaCl

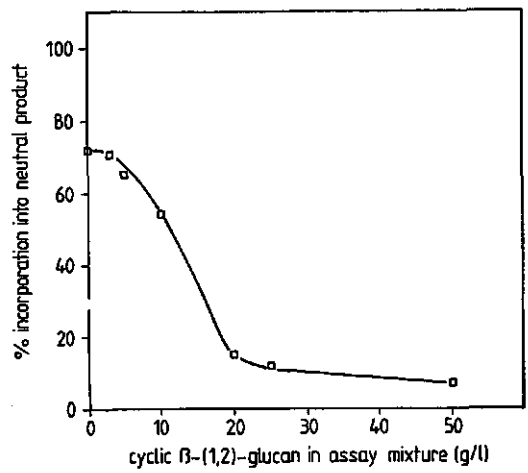

FIGURE 5. Biosynthesis of cyclic ß-(1,2)-glucans from [^{14}C]-glucose by a membrane fraction of *Rhizobium leguminosarum* bv *trifolii* TA-1 in the presence of increasing amounts of cyclic ß-(1,2)-glucans.

TABLE 4. Influence of EDTA on polysaccharide-production by *Rhizobium leguminosarum* bv *trifolii* TA-1. Cells were grown for 12 days in production medium containing various amounts of EDTA as indicated. The results shown here are from one typical experiment. Experiments were performed 2 times and the obtained values differed maximally 5%.

[EDTA] (mM)	SUPERNATANT		PELLET		
	EPS[a]	glucans[b]	CPS[c]	LMW[d]	protein[e]
0	1515	50	1500	55	580
0.1	480	350	195	60	580
0.5	265	170	0	30	300

[a] extracellular polysaccharides expressed as glucose + glucuronic acid equivalents in mg/l culture.
[b] extracellular cyclic glucans expressed as glucose equivalents in mg/l culture
[c] capsular polysaccharide expressed as galactose equivalents in mg/l culture
[d] low molecular weight cellular carbohydrates (taken as a measure for the cyclic glucan content) expressed as glucose equivalents in mg/l culture
[e] protein determined according to Lowry *et al* (20) in mg/l culture

***In vitro* glucan synthesis by membrane fractions of NaCl-grown TA-1 cells.** We have reported high temperature-induced and osmotically-induced excretion of cyclic ß-(1,2)-glucans by strain TA-1 (7,9). However, no significant differences in activity could be seen with membrane fractions from cells grown at 25°C in the presence of 0, 100 and 200 mM NaCl or from cells grown at 33°C (data not shown). In order to check the initial activities, membrane fractions (containing 100 μg protein) of both 0 and 200 mM NaCl grown cells were incubated for various time intervals (Figure 4). Hardly any difference in biosynthetic activity was observed.

End-product inhibition of the glucan synthesis *in vitro*. The synthesis of cyclic glucans showed end-product inhibition (Figure 5). Glucose, the only hexose present in cyclic ß-(1,2)-glucan, or mannitol, the carbon- and energy source used in our experiments, did not inhibit synthesis at 200 mM concentration. However, ionic osmolytes like KCl or NaCl markedly inhibited glucan synthesis (Table 1).

Glucan excretion by whole cell suspensions. Labelled neutral glucan was released into the medium when cells, grown in the presence of NaCl, were incubated in the presence of UDP-[^{14}C]-glucose (Table 3). This glucan fraction eluted at the same position as cyclic ß-(1,2)-glucans, and its behaviour on TLC and HPLC was identical to the patterns in the Figures 2 and 3. The release of glucans by the cells could have been the result of a sudden negative change in osmotic pressure between the culture medium containing 200 mM NaCl (osmotic pressure 11.8 atm) and the assay mixture (osmotic pressure 5.6 atm). However, addition of 200 mM NaCl to the assay mixture prior to the reaction (osmotic pressure 15.3 atm) did not reveal any difference in glucan release from cells grown in the presence of 200 mM NaCl (data not shown).

The labelled neutral fraction decreased by 91% in the presence of 50 mg/ml cyclic ß-(1,2)-glucans. This value corresponds to 15 mM assuming an average molecular weight of 3200 D. With NaCl-free cultured TA-1 cells hardly any labelled neutral products appeared in the supernatant. When cells of strain TA-1 were made permeable by alternately 8 times freezing and thawing a labelled glucan fraction in the supernatant appeared as well (Table 3). The glucan excretion was again strongly inhibited in the presence of cyclic ß-(1,2)-glucan. Cells of strain TA-1 grown in the absence of NaCl at 33°C excreted as well a labelled neutral glucan fraction, when incubated with UDP-[^{14}C]-glucose (Table 3).
High amounts of glucans (up to 800 mg/l) were also found in whole cultures of strain *R. leguminosarum* bv *viciae* RBL5523,*exo*8::Tn5 grown for 14 days in production medium without NaCl, while the wildtype strain RBL5523 excreted only little glucans (up to 40 mg/l), comparable to strain TA-1 (10). Therefore, we incubated both the wildtype and the mutant cells, grown for 32 h in production medium without NaCl, with UDP-[^{14}C]-glu-

cose. The mutant strain excreted a labelled glucan fraction, while the wildtype strain did not (Table 3).

Glucan excretion by whole cultures of TA-1 in the presence of EDTA. Strain TA-1 grown in the absence of NaCl was inoculated in medium containing varying concentrations of EDTA. EDTA weakens the LPS layer, amongst other effects, by complexing divalent cations. At 0.1 mM EDTA production of high molecular weight extracellular polysaccharide but especially capsular polysaccharide was repressed, while cyclic glucan excretion was increased by a factor 7 (Table 4). At 0.5 mM EDTA biomass production was already inhibited. No growth was observed in the presence of 1 mM EDTA during the incubation period of 12 days.

DISCUSSION

NaCl-grown TA-1 cells are more permeable towards cyclic glucans. Under conditions of cultivation at high temperature (30-33°C) (7) or high osmotic pressure of the medium (4-13 atm) (9) *Rhizobium leguminosarum* bv *trifolii* TA-1 excreted excessive amounts of cyclic ß-(1,2)-glucans into the culture medium. In this paper we present evidence that the enhanced excretion is due to an increased permeability of the outer membrane for cyclic glucans. This evidence is based on the following observations: (i) Incubation of repeatedly frozen and thawed TA-1 cells with UDP-^{14}C-glucose resulted in the appearance of a labelled glucan in the medium. Cells grown in the presence of NaCl and cells cultured at 33°C showed the same effect. Cells cultured at 25°C in the absence of NaCl did excrete only little cyclic glucans; (ii) the presence of 0.1 mM EDTA in cultures of strain TA-1 also induced glucan excretion. EDTA is a complexing agent of divalent cations which results in a weakened LPS layer; (iii) *R. leguminosarum* bv *viciae* RBL5523,*exo*8::Tn5 mutant cells excreted a labelled glucan fraction, while the parent strain RBL5523 did not. The mutant was shown to be defective for the enzyme galactose-4'-epimerase, which catalyses the conversion of UDP-glucose into UDP-galactose (12). This mutation resulted in a decreased production of extracellular polysaccharide, hardly any production of capsular polysaccharide, and synthesis of modified LPS deficient in O-antigen units (12). Especially the last fact might explain the greater permeability of the mutant cells towards cyclic ß-(1,2)-glucans. Kennedy and Rumley (18) described an *Escherichia coli* K-12 mutant DC2 which was selected because of increased sensitivity to antibiotics and excreted high amounts of membrane-derived oligosaccharides (MDOs). It was postulated that the mutant's outer membrane had become permeable for these periplasmic oligosaccharides (18). The MDOs contain 6-12 glucose residues with ß-(1,2) and ß-(1,6) linkages and have a similar regulation of biosynthesis as the cyclic glucans of *R. meliloti* and *A. tumefaciens* (21).

It has been found that the *ndv*A gene product of *R. meliloti*, which is involved in synthesis of cyclic glucans, has structural homology with an ATP-binding export protein in *Escherichia coli* (23). Such a protein could be present in strain TA-1 as well. However, the increased permeability of the cells towards glucans did not result from a higher transport rate, since all our observations point in the direction of a partially leaky outer membrane. An increased biosynthetic activity could be excluded as well since hardly any difference in glucan synthesis rates was found between membrane fractions derived from TA-1 cells grown at 25°C in the presence or absence of NaCl.

In vitro synthesis of cyclic glucans. By comparing the requirements for the *in vitro* synthesis of cyclic ß-(1,2)-glucans from UDP-[^{14}C]-glucose by *Rhizobium leguminosarum* bv *trifolii* TA-1 with other members of the *Rhizobiaceae*, the following resemblances and differences can be found. *R. phaseoli* AHU1133 (2) and *R. japonicum* strains (13) needed NAD^+ or ATP for optimal activity like strain TA-1, while *R. meliloti* R41, *A. tumefaciens* LBA 4011 (31) and *A. radiobacter* IFO 12665b1 (2) did not require these factors. The presence of Mn^{2+} was necessary and could be replaced by Mg^{2+} for all strains investigated, except for *R. phaseoli*. *R. leguminosarum* is able to synthesize different types of oligo- and polysacchari-

des (7,12,28), but under the conditions employed here only neutral cyclic glucans were detected.

Cyclic glucan synthesis is controlled by end-product inhibition. The internal glucan concentration can be calculated as 10-20 mg glucans/ml cell, or 25-50 mg glucans/ml periplasm using the following assumptions: (i) the volume of the periplasm is 20-40% of the cellular volume (24), (ii) the cellular glucan concentration is 50-100 mg/g protein or 25-50 mg/l medium (7), and (iii) the total cellular volume of 1 l culture is 3 ml with an estimated 2 $*10^{13}$ cells per liter. Thus, the concentration at which strong inhibition of synthesis of glucans occurs (Figure 5) is in the same range as the internal periplasmic glucan concentration and therefore of physiological importance. Consequently, we propose the regulation of cyclic ß-(1,2)-glucan synthesis by *R. leguminosarum bv trifolii* TA-1 to occur as follows. TA-1 cells synthesis cyclic glucans until the periplasmic concentration reaches about 50 mg/ml, at which point end-product inhibition occurs. TA-1 cells which have been growing in a medium with high osmotic pressure or at high temperature become more permeable towards cyclic glucans, apparently because the outer membrane becomes more leaky. The constant loss of glucans from the leaky cells prevents end-product inhibition and results in glucan accumulation in the medium. It is postulated that cyclic glucans function in the periplasm of *Rhizobiaceae* as osmotic barrier between the cytoplasm and the low osmolarity environment (21). Accordingly, their function in media with high osmotic pressure is dispensable. Therefore it is intriguing that the synthesis of cyclic glucans by *R. leguminosarum* cells is not suppressed at high osmotic strength of the medium (9, this paper).

Comparison of cyclic glucan synthesis in strain TA-1 with that in *R. meliloti*. The cellular glucan concentration of *R. meliloti* strains growing in production medium without NaCl (π=2.0 atm) can reach 350 mg/g protein, part of it being substituted with glycerol-1-phosphate (8,29). At high osmolarity (π>6.0 atm) the accumulation of cyclic glucans by *R. meliloti* was repressed (8,15,32). However, the *in vitro* synthesis by membrane fractions of osmotically-stressed *R. meliloti* cells was not repressed (32). Ionic osmolytes inhibited its enzyme system, while non-ionic osmolytes did not (32). We found the same for membrane fractions of strain TA-1. Very recently, Geiger *et al* (16) showed with *R. meliloti* 1021 that excretion of glucans is strongly influenced by the nutrient composition of the medium. Enhanced glucan excretion was observed in yeast extract-mannitol-salts medium, having a comparable osmotic pressure (π=2.0 atm) as the production medium. The authors suggested that glucans in excess of the amount needed for periplasmic functions were excreted (16). With *R. meliloti* SU-47 (closely related to strain 1021) grown in production medium, we found no glucan excretion but high amounts of the succinoglycan repeating unit in the supernatant (8). Therefore, it needs to be determined whether cyclic glucan synthesis in *R. meliloti* is regulated in a comparable way as proposed for *R. leguminosarum*.

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CHAPTER 8

Influence of medium composition and growth rate on production of capsular and extracellular polysaccharides by *Rhizobium leguminosarum* biovar *trifolii* TA-1

Influence of medium composition and growth rate on production of capsular and extracellular polysaccharides by *Rhizobium leguminosarum* biovar *trifolii* TA-1

SUMMARY

Rhizobium leguminosarum biovar *trifolii* TA-1 was used as a model organism to study the influence of growth rate and medium composition on exopolymer production. In batch cultures in production medium (10 g mannitol and 1 g glutamic acid/l medium) the maximum specific growth rate was μ_{max}=0.133 h^{-1}. Extracellular polysaccharide (EPS) was synthetized during active growth, with a rate of 75 mg EPS/g protein.h and continued in stationary phase (up to 1.6-2.1 g/l of 500 mg protein as biomass). Capsular polysaccharide (CPS)-synthesis which took place only in stationary phase in the presence of excess carbon-source, occurred with a rate of 13 mg CPS/g protein.h. Maximal CPS-yield was 2.9 g CPS/l medium, with 1 g protein as biomass during 14 days in a medium containing 20 g/l mannitol and 2 g/l glutamic acid. In a non-growing cell-medium containing 10 g/l mannitol CPS synthesis reached 2.1 g/l, but EPS-synthesis was lower (0.8 g/l). In continuous cultures in production medium with a constant biomass of 500 mg cell protein/l, EPS was at every dilution rate D (between 0.02 and 0.12 h^{-1}) the most abundant polysaccharide present. CPS synthesis occurred only at low specific growth rates. Only low amounts of cyclic glucans were excreted (10-30 mg/l) over the entire range of growth rates.

ABBREVIATIONS: bv=biovar; CPS=capsular polysaccharide; EPS= extracellular polysaccharide; HM_r=high molecular weight; LM_r=low molecular weight; YEMCR =Yeast Extract-Mannitol-Congo Red agar.

INTRODUCTION

Members of the family *Rhizobiaceae* are capable of synthesizing a variety of polysaccharides. Depending on cultural conditions several types of polysaccharides, clearly differing in both chemical and physical properties, can be observed during cultivation of a single rhizobial strain. (Sutherland, 1985, 1988). *R. leguminosarum* strains produce exopolysaccharides like high molecular weight anionic extracellular polysaccharide (EPS), insoluble neutral capsular polysaccharide (CPS) and cellulosic microfibrils, while sometimes periplasmic cyclic ß-(1,2)-glucans can be found in the medium (Zevenhuizen, 1990). The EPS type commonly found with this group of organisms is composed of an octasaccharide repeating unit with sugar composition of glucose: glucuronic acid: galactose in the ratios of 5:2:1 (McNeil *et al.*, 1986). The CPS from a number of *R. leguminosarum* strains, composed of neutral sugars without any non-sugar substituents, has a constant sugar composition of glucose: galactose: mannose in the ratios of 1:4:1 (Zevenhuizen, 1984). CPS of *R. leguminosarum* is a polymer which forms gels already at 0.2 % $^{w}/_{v}$ which is even less than the 0.4% for agar (Zevenhuizen, 1984; Gidley *et al.*, 1987; Creszenzi *et al.*, 1987). The CPS with its unique gelling properties has considerable application potentials. Therefore, it is desirable to select for strains which do not produce EPS (Chapter 9), or to choose the right cultural conditions in which a selective CPS synthesis over EPS can be achieved (Zevenhuizen, 1986). In this chapter *R. leguminosarum* bv *trifolii* TA-1 was used as a model strain to study the production of polysaccharides, and their distribution as a function of growth rate and composition of the medium.

METHODS

Cultivation. *Media.* The standard medium contained 5 g mannitol and 1 g glutamic acid per liter. Its carbon/nitrogen (C/N) ratio of 29 made it nitrogen-limited medium. Sometimes the production medium was used, which contained 10 g of mannitol, sucrose, glucose or galactose per liter as carbon and energy source, and 1 g glutamic acid or 0.36 g NH_4Cl per liter as nitrogen source. Mineral salts and vitamins (Chapter 3) were present in sufficient amounts. Reducing one of each of the mineral

salts or vitamins by 50% did not alter growth yield expressed as cellular protein.

Batch cultivation. Precultures of *Rhizobium leguminosarum* bv *trifolii* strain TA-1 were prepared by inoculating the organism into standard medium. Cells from the late logarithmic phase were inoculated (1%; $^{v}/_{v}$) into 50 ml medium in 300 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 200 rpm at 25°C for various incubation times. For non-growing cell cultures, 72 h grown cells were centrifuged, and the cell pellet transferred to glutamic acid-free production medium containing 10 g/l mannitol. The effect of different oxygen concentrations on growth and polymer production at 25°C was tested (i) in 100 ml Erlenmeyer flasks containing different volumes of production medium, or (ii) in a 2 *l* batch fermenter with 0.5 *l* culture volume which was flushed with gas-mixtures containing 40% O_2/60% N_2/0.5% CO_2, air, or 4% O_2/96% N_2/CO_2 at a rate of 1 *l* gas mixture/min and *l* medium, impeller speed was 600 rpm.

Continuous cultivation. For continuous culture experiments TA-1 cells grown for 72 h in standard medium were inoculated 1% ($^{v}/_{v}$) in 1 *l* of production medium in a 2 *l* vessel aerated at 1 *l* air/min and *l* medium, impeller speed 600 rpm. Such, the oxygen tension never fell below 25 % air saturation at 25°C. The culture was run the first 24 h in batch mode. Thereafter it was operated as a chemostat. Samples were taken at certain D-values after steady-state had been reached (routinely after 5 volume changes). The pH was kept at 7.0 with 2 N NaOH and 2 N HCl. To prevent foaming, routinely 1 drop of polypropyleneglycol was added.

Two-step continuous cultivation was performed with the fermentor containing 0.5 *l* medium and run at D=0.11 h^{-1}. The outlet was connected to a 10 *l* vessel with a working volume of 4.4 *l* run at D=0.021 h^{-1}. This vessel was aerated at 0.5 *l* air/min and *l* volume, and stirred by a magnetic stirrer.

Purity of the cultures was routinely checked by plating appropriate dilutions on Yeast Extract-Mannitol-Congo Red (YEMCR) agar (Zevenhuizen *et al.*, 1986). Cell shape and uniformity of the culture were also checked by phase-contrast microscopy.

Analytics. *Cells and culture supernatant* were separated by centrifugation at 35000 x *g* for 30 min. The cell pellet was resuspended fivefold concentrated in distilled water and briefly sonicated to obtain a homogeneous suspension.

To measure *growth* 3 methods were used: (i) From the slope of a growth curve (optical density at 660 nm versus time) during early exponential growth in batch culture the maximum specific growth rate was determined; (ii) dry weight, as measured after overnight incubation of a cell pellet at 105°C; (iii) total cellular protein content of the pellet, determined according to Lowry *et al.* (1951) after pretreatment of the cell pellet in N NaOH at 100°C for 30 min.

Extracellular carbohydrates were determined in the supernatant by the anthrone-sulphuric acid method for hexoses (Trevelyan & Harrison, 1952) and the 3-hydroxydiphenyl-sulphuric acid method for hexuronic acids (Blumenkrantz & Asboe-Hansen, 1973). The concentration of EPS and cyclic glucans were calculated as described in Chapter 3.

Cellular carbohydrates were determined in the pellet by the anthrone method. CPS was determined after alkaline-excraction of the cells followed by alcohol-precipitation and expressed as galactose equivalents, and periplasmic cyclic glucans were determined in the alcoholic extract and expressed as glucose-equivalents as described (Chapter 3). Glycogen was determined in the alkaline-extracted cells after hydrolysis in 2 N HCl and expressed as glucose-equivalents, and the hexose content in the acid-resistent, alkali-insoluble material was a measure of the cellulose content of the cells (Zevenhuizen *et al.*, 1986).

HM, EPS was precipitated from the supernatant by the addition of 3 volumes of ethanol, centrifuged, washed with alcohol:water= 3:1, centrifuged again and redissolved in distilled water. Finally, it was reprecipitated with 75% alcohol, dissolved in distilled water and freeze-dried.

CPS was isolated by extraction of a cell suspension with 1M NaOH for 1 h at room temperature, followed by centrifugation. The

supernatant was decanted and CPS was precipitated by the addition of 1 volume of ethanol. After centrifugation the CPS pellet was washed with 50 % ethanol several times until the pH reached 7.0. CPS was dissolved in hot water, freeze dried and used for further analysis.

Mannitol was determined in the supernatant by periodate oxidation followed by the chromotropic acid-colorimetric method (Burton, 1957).

The relative *viscosity* of the culture supernatant was measured with an Ubbelohde viscometer (Tamson, Zoetermeer, The Netherlands) at 25°C.

PHB was determined by GLC according to Braunegg *et al.* (1978).

The *neutral sugar composition* of freeze-dried samples was determined after hydrolysis with 2 M TFA for 8 h at 100°C, followed by conversion of the liberated sugars into alditol-acetates and separation by GLC (Blakeney *et al.*, 1983) on a capillary Sil 43-CB column (Chrompack, Middelburg, The Netherlands) at 210°C.

RESULTS

Growth. The generation time of strain TA-1 was calculated as g=5.3 h, resulting in a maximum specific growth rate μ_{max}=0.133 h^{-1}. In early logarithmic phase of growth a linear relationship existed between optical density and cell protein content, but in stationary phase the optical density still increased while the protein content remained constant at 500 mg/l.

CPS-production had a strong impact on the dry weight of the pellet in stationary phase (Figure 1). Reserve materials like PHB and glycogen contributed to the dry weight as well. Glycogen accumulated in late logarithmic phase (up to 500 mg/l) and degraded in stationary phase. PHB accumulated in early stationary phase (up to 900 mg/l) and degraded after exhaustion of the external carbon-source upon prolonged incubation. We did not further use the dry weight of the pellet as an indication of biomass, but routinely used cellular protein (Figure 1).

The higher carbon/nitrogen ratio in the production medium (C/N ratio of 53) allowed a higher yield of polysaccharides. A doubling of the mannitol concentration in the medium from 5 to 10 g/l resulted in an increase of the CPS yield in stationary phase (Figure 1) with a constant production rate of 13 mg CPS/g protein.h. EPS was produced already during logarithmic growth phase with a rate of 75 mg EPS/g protein.h (not shown). Final EPS concentrations in the standard- and production medium after 14 days of incubation were 1540 and 1850 mg/l respectively.

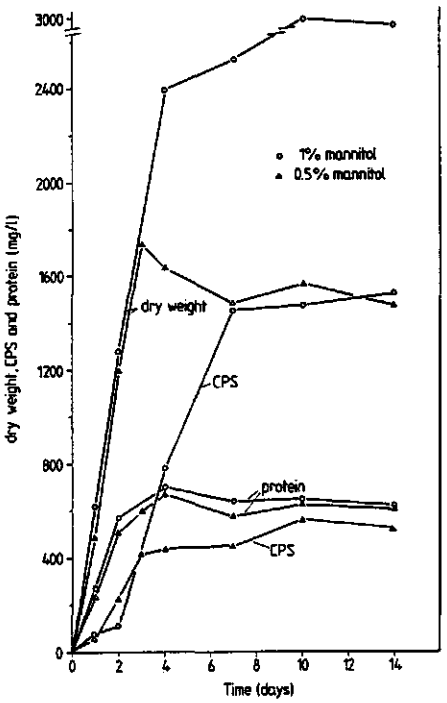

FIGURE 1 Comparison CPS-production, dry weight and cell protein in cell pellets of *Rhizobium leguminosarum* bv *trifolii* TA-1 grown in standard- or production medium (0.5 and 1.0% mannitol, respectively) in batch culture

Nitrogen source. The nitrogen-sources NH_4Cl and glutamic acid were compared on their influence on growth and polymer production, on the basis of the same nitrogen-equivalents. NH_4^+ is the preferred nitrogen source for several species of *Rhizobium* at constant pH (Poole *et al.*, 1987). Other strains prefer an

organic nitrogen source like glutamate (Bergensen, 1961). The buffer capacity of the phosphate-buffered medium (5.8 mM; pH=7.0) with NH_4Cl as nitrogen-source was not enough to prevent acidification of the medium. In the presence of $CaCO_3$ higher EPS-yields were obtained with NH_4Cl, while CPS synthesis was much higher with glutamic acid as nitrogen-source (Table 1: a-c). The generation times with NH_4Cl (in the presence of $CaCO_3$) and glutamic acid were 4.8 and 5.3 h, respectively.

Carbon source and its concentration. The influence of the amount of mannitol on the polysaccharide production was determined (Figure 2). In media with a higher concentration of mannitol than necessary for biomass production (above 1 g mannitol/l) polysaccharide-production was enhanced. High concentrations of mannitol led to viscous cultures due to the high EPS production. The highest CPS/EPS ratio was achieved with 10 g mannitol/l.

The substrates sucrose, glucose, galactose were compared with mannitol, on the basis of the same carbon equivalents. With glucose and galactose as substrate the medium quickly acidified, which could be prevented by adding $CaCO_3$. The CPS production with glucose and galactose as substrate was lower than with sucrose (Table 1: a, d-h). Sucrose is with respect to EPS and CPS production comparable to mannitol.

By trying to increase the volumetric production of CPS the concentrations of both glutamic acid and mannitol in the production medium were increased with a factor 2. CPS-production was enhanced up to 2900 mg/l medium on a basis of 1 g protein, with a rate of 10 mg CPS/g protein.h. In the medium 600-800 mg/l cyclic glucans were found. When supplying the mannitol in a fed-batch mode, comparable results for CPS were obtained, but EPS synthesis and cyclic glucan excretion was lower (Table 1: i-j).

Batch experiments were performed in which growth phase was separated from CPS-production phase. In this non-growing cells incubation, polysaccharides were measured after a total incubation time of 14 days (Table 1: k). CPS production reached 2100 mg/l, and the EPS-production was relatively low, with a concomitant low viscosity of 5.9 cP.

Influence of oxygen tension. By changing the ratio of volumes of Erlenmeyer flask (100 ml) and culture (between 15-75 ml), the availability for oxygen could be influenced and thereby the polysaccharide production. The EPS yield was slightly higher with low volumes, and CPS with intermediate volumes. Only slight differences in yields between different culture volumes after 2 (not shown) or 7 days of incubation could be seen (Table 2). Comparable results were found with batch cultures flushed with a gas-mixture containing 40% oxygen (slightly higher EPS production) or air (slightly higher CPS production). Even with the gas-mixture containing only 4% oxygen the polymer production remained the same (data not shown).

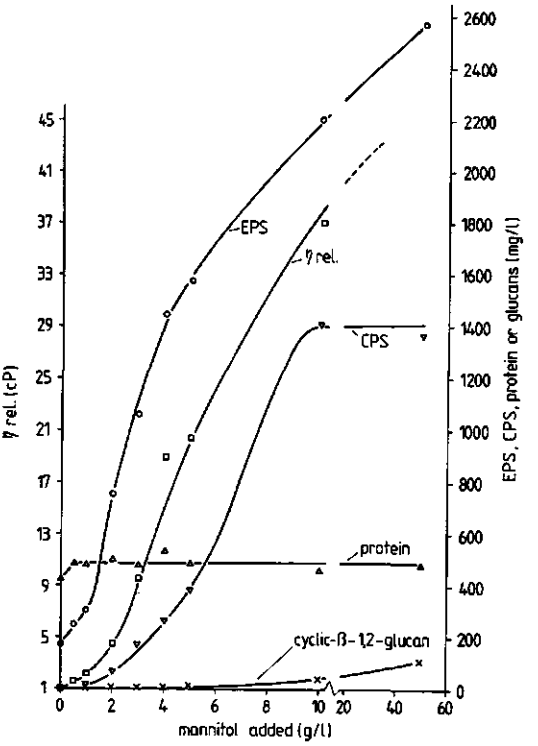

FIGURE 2 Effect of the concentration of mannitol in the medium on extracellular polysaccharide production of *Rhizobium leguminosarum* bv *trifolii* TA-1 grown in batch culture for 14 days

TABLE 1. Influence of the carbon- and nitrogen-source on polysaccharide-yields by *Rhizobium leguminosarum* bv *trifolii* TA-1 in batch cultures incubated for 14 days at 25 °C

	C-source (g/l)	N-source (5.8 mM)	$CaCO_3$ (1 g/l)	HM_1EPS (mg/l)	glucans (mg/l)	pH	CPS (mg/l)	protein (mg/l)
a	Man (10)	Glu	-	1900	30	7.2	1600	520
b	Man (10)	NH_4Cl	-	400	60	4.7	80	410
c	Man (10)	NH_4Cl	+	2100	70	6.9	600	510
d	Suc (10)	Glu	-	1680[a]	b	7.0	1470	530
e	Glc (10)	Glu	-	580[a]	b	5.7	300	490
f	Glc (10)	Glu	+	1580[a]	b	8.0	980	510
g	Gal (10)	Glu	-	560[a]	b	5.6	310	450
h	Gal (10)	Glu	+	1760[a]	b	8.1	870	520
i	Man (20)	2xGlu[c]	-	1400	800	7.6	2900	1050
j	Man (20)[d]	2xGlu[d]	-	900	650	7.4	2800	990
k	Man (10)	-[e]	-	800	40	7.4	2100	510

a) HM_1 EPS was determined after precipitation and washing with 3 vol alcohol and expressed as glucose+glucuronic acid in mg/l culture; b) glucans were not determined because of the presence of hexose-containing substrates; c) Incubation in 2 times concentrated production medium (containing 20 g/l mannitol and 2 g/l glutamic acid (11.6 mM)); d) Incubation in fed-batch system composed of 2 g/l glutamic acid and 5 g/l mannitol. After 60 h and 180 h mannitol is added, 10 g/l resp. 5 g/l; e) cells are transferred after 72 h from production medium to glutamic acid-free production medium.
Man= mannitol; Suc= sucrose; Glc= glucose; Gal= galactose; Glu= glutamic acid

TABLE 2. Influence of cultural volume on polysaccharide-production by *Rhizobium leguminosarum* bv *trifolii* TA-1 at 25°C grown in production medium in 100 ml Erlenmeyer flasks on a rotation shaker (200 rpm) for 7 days.

Volume (ml)	EPS (mg/l)	glucans (mg/l)	protein (mg/l)	CPS (mg/l)
15	2340	25	520	1000
25	2170	10	560	1170
50	2150	10	520	1240
75	2020	10	525	1010

EPS was expressed as glucose+glucuronic acid in mg/l culture; cyclic glucans were expressed as glucose equivalents in mg/l culture; CPS was expressed as galactose equivalents in mg/l culture

Continuous culture-experiments. The influence of the growth rate on polysaccharide-production was investigated in a continuous culture. After reaching steady state (at least 5 volume changes) cell protein, mannitol consumption and polysaccharide production were measured at different dilution rates D (Figure 3). EPS was synthesized at both high and low

growth rates and was the prominent polysaccharide produced, while CPS synthesis occurred only at low dilution rates.

The intracellular concentration of cyclic glucans did not differ significantly and ranged between 34-45 mg/l medium (about 70-90 mg/g protein). The supernatant contained only 10-30 mg/l of cyclic glucans. At all growth rates except around μ_{max} cell counts were $(3\pm0.2)x10^9$/ml. Upon prolonged cultivation at D=0.10 hr^{-1} variants of the TA-1 colonies could be observed on YEMCR plates at a frequency of 0.0001-0.01% at 5-25 volume changes, respectively. These colonies took up the Congo red dye intensively. These variants were not altered in their EPS and CPS production but contained up to 150 mg cellulose/g protein in their cell pellets, while usually 20-60 mg/g protein is measured (data not shown). At low growth rates below 0.02 h^{-1} EPS made the medium highly viscous. At the same time heavy wall growth occurred by the cellulose-producing variants.

Two-step continuous cultivation. Since CPS was found to be produced only at low dilution rates, polysaccharide production in a 2-step continuous culture was investigated. In the first fermentor (the continuous culture vessel) biomass was produced at a constant D=0.11 h^{-1}. In the second step (D=0.021 h^{-1}) no growth occurred, since all the glutamic acid was already used up. However, polysaccharide production could proceed since the influent still contained mannitol. Despite the non-growth-conditions in this second vessel EPS was still the main polysaccharide present (Table 3; cc1, cc2).

When the second vessel was disconnected from the first fermentor and further incubated in batch mode another 5 days (Table 3: batch), EPS and CPS production in this aerated and stirred vessel was comparable to that obtained in a conventional batch culture.

Monomer composition of EPS and CPS. EPS-samples from continuous culture at D=0.02 and 0.11 h^{-1} and from batch-culture (production medium) in stationary phase (72 and 168 h), and CPS samples from continuous culture (D=0.02 h^{-1}) and batch culture (168 h) were investigated for their neutral hexose composition. The hexose composition of CPS yielded galactose: mannose: glucose in a ratio of 4.0 (±0.2): 1.0 (±0.1): 1.0 for both samples. The hexose composition of EPS was glucose:galactose in a ratio of 5.0 (±0.3) :1.0, and the hexose: hexuronic acid ratio was 3.0 (±0.2):1.0 in all samples.

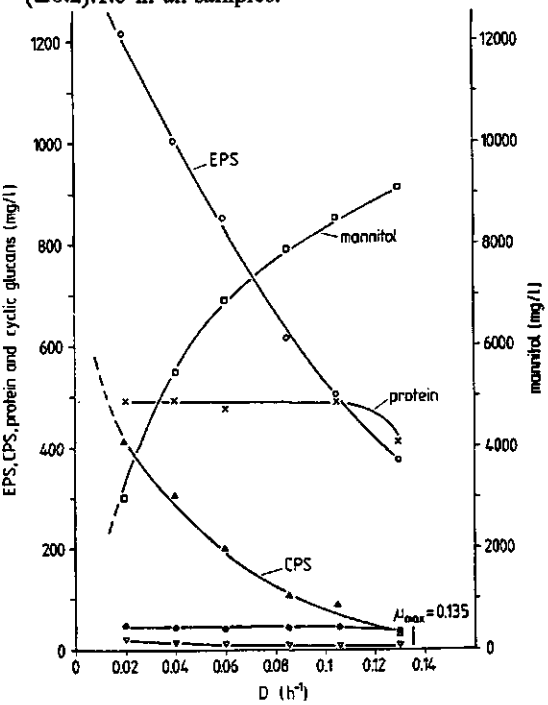

FIGURE 3 Effect of the dilution rate D on polysaccharide production of *Rhizobium leguminosarum* bv *trifolii* TA-1 in a continuous culture in production medium. □ = mannitol; o =EPS; ▵ =CPS; ● ,▽ =intracellular and extracellular cyclic glucans, respectively; x=protein

DISCUSSION

The sugar composition of the EPSs of *Rhizobium* sp. was found to be independent of cultural conditions (Courtois *et al.* 1986). Although yields of polysaccharides greatly differed between different cultural conditions, the hexose compositions of both CPS and EPS of strain TA-1 were apparently independent of growth phase and/or medium composition. Like all fast-growing rhizobia, strain TA-1 is multi-productive with respect to polysaccharide-production.

TABLE 3. Polysaccharide-production by *Rhizobium trifolii* TA-1 in the two-stage fermentor system.
cc1= first fermentor run at D=0.11 h^{-1}; cc2= second fermentor run at D=0.021 h^{-1}; batch= production vessel cultivated in batch-mode, during 5 days.

VESSEL	growth rate (h^{-1})	SUPERNATANT mannitol (g/l)	EPS (mg/l)	glucans (mg/l)	PELLET CPS (mg/l)	glucans (mg/l)
cc1	0.11	8.8	510	5	110	35
cc2	0.021	4.8	1100	10	550	45
batch	-	0.2	1650	45	1290	55

See legend Table 1 for explanation

A selective synthesis of polysaccharides can be achieved with this strain in mannitol-rich media, like (i) EPS under forced aeration, (ii) CPS in fed-batch cultures (Zevenhuizen, 1986), or (iii) cyclic glucans at incubation at 33°C or at high osmotic pressures of the medium (chapters 3 and 5).

Mannitol is a good carbon-and energy source for fast-growing rhizobia (Stowers, 1985). EPS and CPS production was found to be comparable with sucrose and mannitol. For laboratory experiments, mannitol is most convenient, since it does not interfere with the hexose measurements and can easily be measured separately. However, on large scale sucrose would be more favorable, for economical reasons.

Exopolysaccharide synthesis by *R. leguminosarum* bv *trifolii* A131 was found both in carbon-limited as well as in carbon-sufficient continuous cultures. It strongly depended on the growth rate in carbon-limited cultures (De Hollander *et al.*, 1979). In batch and continuous cultures EPS was often the most abundant polysaccharide present, while CPS-synthesis only occurred in media with excess carbon at low dilution rates or in stationary phase. Continuous cultivation aimed at polysaccharide-production is therefore only beneficial for EPS, because its synthesis is growth-associated. In the two-step fermentation process EPS synthesis was predominant since only the growth rates were separated. By separating cells from supernatant in the non-growing cell-experiment, CPS synthesis took place up to 2.1 g/l in a medium with low viscosity, which made recovery of CPS easier. The highest CPS-yield (2.9 g CPS/l) was achieved in batch culture with 1 g cell protein as biomass. Higher yields than 3.0 CPS g/l by strain TA-1 found in a fed-batch culture (Zevenhuizen, 1986) have never been reported. Higher substrate concentrations resulted in higher biomass (glutamic acid; chapter 3) or higher EPS-production (mannitol; figure 2), but decreased CPS synthesis and enhanced glucan excretion (chapter 3). Apparently the synthesis of CPS is tightly regulated, but its mechanism is not yet known. Suboptimal conditions for growth lead to a strong repression of CPS synthesis (chapters 3 and 5). Furthermore, in an acidified medium polymer production stopped. When during exponential growth the pH fell below 6, polysaccharide synthesis by *R. meliloti* stopped (Courtois *et al.*, 1979). By analogy, the drop of the pH as a result of growth of strain TA-1 on the substrates glucose, galactose, or NH_4Cl resulted in a repression of EPS, but in particular CPS synthesis. The CPS of *R. leguminosarum* is unique in its chemical composition and interesting for its physical properties. Because of these specific properties it would be interesting to find conditions optimal for high CPS yields. In this study we were unfortunately not able to identify these conditions.

Oxygen limitation affected both growth and polysaccharide yields (Sutherland, 1985). Zevenhuizen (1986) found increased EPS production and decreased CPS production by strain TA-1 with forced aeration in a Kluyver flask. Circumstances leading to a high CPS-

synthesis lead often to concomitantly high EPS-production, rendering the medium viscous, and making the process of recovery of CPS more difficult. It could be that high EPS production, resulting in a high viscosity of the medium, regulated the oxygen availability as was suggested by Jain *et al.* (1990). CPS production, being dependent on the amount of carbon-source and growth phase, could therefore depend indirectly on the previously synthesized EPS. Low dissolved oxygen (below 4%) and growth-limiting oxygen transfer rates promoted exopolymer (EPS) production in *R. trifolii* 0403 (Thompson and Leps, 1985). Thus, it is not yet clear how oxygen tensions influences the production of EPS and CPS.

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CHAPTER 9

Polysaccharide production by mutants of *Rhizobium leguminosarum* affected in carbohydrate synthesis

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Polysaccharide production by mutants of *Rhizobium leguminosarum* affected in carbohydrate synthesis

SUMMARY

The polysaccharide production by *Rhizobium leguminosarum* bv *viciae* RBL5515 pRL1JI, *R. leguminosarum* RBL5515 and some of their Tn::5 generated mutants affected in polysaccharide synthesis was studied in a medium containing 10 g mannitol and 1 g glutamic acid per liter, allowing the production of about 500 mg cellular protein/l. Wild type strain RBL5515 produced anionic extracellular polysaccharide (EPS, up to 2200 mg/l) composed of the regular octasaccharide repeating unit, and neutral capsular polysaccharide (CPS, up to 1700 mg/l) as main other polysaccharides. The presence of either pRL1JI or pSym5, the Sym plasmids of *R. leguminosarum* bv *viciae* and *trifolii*, respectively, did not influence the quantities of these polysaccharides synthesized. All mutants showed increased cyclic (1,2)-ß-glucan excretion (400-1000 mg/l) as compared to strain 5515 (50 mg/l). The mutants *R. leguminosarum* strains RBL5515,*exoB8*::Tn5 and RBL5515,*exo336*::Tn5 were defective in LPS and CPS synthesis while mutants RBL5515,*exo4*::Tn5 and RBL5515,*exo344*::Tn5 produced CPS at the wild type level. The mutants RBL5515,*exo4*::Tn5, RBL5515,*exoB8*::Tn5 and RBL5515,*exo344*::Tn5 formed 5-20% of wild type EPS-level. Both mutants RBL5515,*exoB8*Tn::5 and RBL5515,*exo344*::Tn5 synthesized a truncated EPS with a heptasaccharide-repeating unit missing the terminal galactose in the side chain. While the RBL5515,*exoB8*::Tn5 mutant was shown to be devoid of UDP-glucose 4' epimerase activity, the activity of this enzyme and of other enzymes involved in the synthesis of UDP-galactose were comparable in RBL5515,*exo344*::Tn5 and the wild type. It was concluded that RBL5515,*exo344*::Tn5 was affected in a galactose transferase activity. Since this mutant failed to nodulate plants belonging to the pea inoculation group, and the presence of the terminal galactose in the side chain of the EPS of *R. leguminosarum* bv *viciae* was not required for succesful nodulation, it it postulated that the amount of EPS produced by RBL5515,*exo344*::Tn5 is insufficient for nodulation.

ABBREVIATIONS: bv=biovar; CPS= Capsular Polysaccharide; EPS= Extracellular Polysaccharide; HM_r=High Molecular Weight; K-36-type EPS = EPS belonging to *Rhizobium leguminosarum* bv *phaseoli* strain K-36; LM_r= Low Molecular Weight; LPS=lipopolysaccharide; YEMCR =Yeast Extract-Mannitol-Congo Red agar.

INTRODUCTION

Rhizobium leguminosarum (all biovars) is capable of synthesizing a variety of polysaccharides. Depending on the growth phase and cultural conditions the production pattern may vary considerably. These organisms excrete high molecular weight anionic extracellular polysaccharide (EPS) into the medium, synthesize insoluble neutral capsular polysaccharide (CPS) and can form cellulosic microfibrils (Zevenhuizen, 1990). The EPS type commonly found with this group of organisms is composed of a K-36 type octasaccharide repeating unit with sugar composition of glucose: glucuronic acid: galactose in the ratios of 5:2:1 (Figure 1; McNeil *et al.*, 1986). However, for *Rhizobium leguminosarum* bv *phaseoli*, exceptions of these ratios are reported as reviewed by Zevenhuizen and Bertocchi (1989). The CPS from a number of *R. leguminosarum* strains with sugar composition of glucose: galactose: mannose in the ratios of 1:4:1 was found to be constant (Figure 1; Zevenhuizen, 1984). Sometimes, low molecular weight compounds like repeating units of EPS and cyclic (1,2)-ß-glucans can be found in the culture medium as well (Amemura *et al.*, 1983a, Zevenhuizen and Van Neerven, 1983).

Strong correlation between infection behaviour and polysaccharide synthesis has been found with *Rhizobium meliloti* . Mutants unable to synthesize succinoglycan, the typical EPS of *R. meliloti*, formed ineffective pseudonodules in the host plant, alfalfa (Leigh *et al.*,

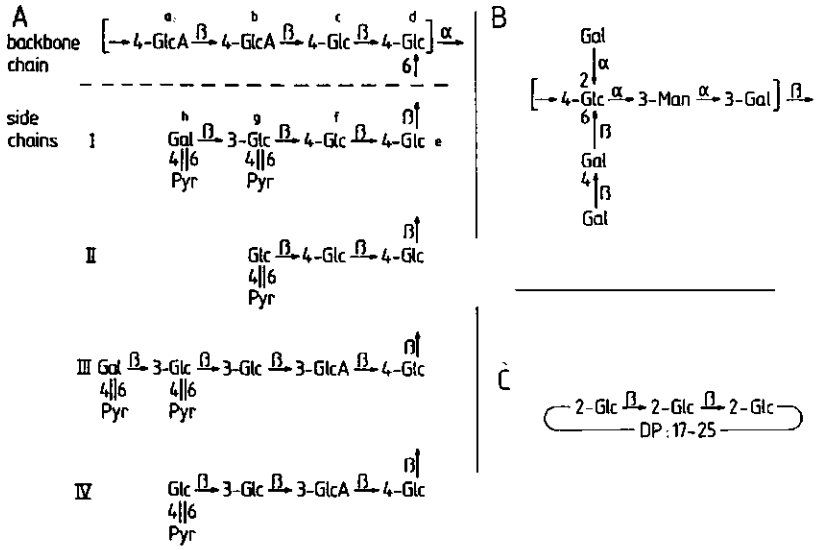

FIGURE 1. Proposed structures of EPS (A), CPS (B) and cyclic (1,2)-ß-glucans (C) of the *Rhizobium leguminosarum* strains used in this study. A-I: structure of EPS of strains TA-1, RBL5515, RBL5515,*exo4*::Tn5 and RBL5515,*exo336*.::Tn5, representing the K-36-type EPS; A-II: EPS of mutants RBL5515,*exo8*::Tn5 and RBL5515,*exo344*::Tn5; A-III: EPS of strain 248; A-IV: EPS of mutant 248,*exo1*::Tn5. Non-stochiometric substituents are not shown.

defective in EPS, CPS and LPS synthesis have been described, which were unable to fix nitrogen in peas, their host plant (Canter Cremers *et al.*, 1990). It was shown that the mutants were affected in the enzyme galactose-4'-epimerase, which is necessary for incorporation of galactose into the carbohydrate polymers. While the wild type strain synthesized the regular octasaccharide type EPS (Figure 1), the *exoB* mutants synthesized an EPS missing the terminal galactose residue. In another study, *R. leguminosarum* bv *viciae* 248, which synthesizes EPS composed of a nonasaccharide repeating unit with sugar composition of glucose: glucuronic acid: galactose in the ratios of 5:3:1 (Figure 1), nodulated pea while a mutant with a galactose-missing EPS did not (Canter Cremers *et al.*, 1991).

In this report we present studies on polysaccharide structure and production of *R. leguminosarum* bv *vicae* strain RBL5515 pRL1JI and some of its Tn::5-induced polysaccharide mutants. Furthermore, we present a novel mutant, RBL5515, *exo344*::Tn5 which is defective in a galactose transferase activity. The implications of this defect for nodulation is discussed.

METHODS

Organism and cultivation. A list of the organisms used is shown in Table 1. All strains were obtained from the Department of Plant Molecular Biology, State University, Leiden, except for strain TA-1. This strain was used as a reference strain with known polysaccharide production pattern (Chapter 3, 8) and was obtained from the culture collection of the Department of Microbiology, Wageningen Agricultural University. Precultures were prepared by inoculating the organism into standard medium with the following composition (in g/l of distilled water): mannitol, 5; glutamic acid, 1; and vitamins and mineral salts (Chapter 3). Most batch culture experiments were done in a production medium, which contained 10 g mannitol/l. Because of the higher carbon/nitrogen ratio in this medium, higher concentrations of polysaccharides were obtained. Cells from the late logarithmic phase were inoculated (1%; $^{v}/_{v}$) into 50 ml medium in 300 ml Erlenmeyer flasks. The

TABLE 1. DESCRIPTION OF ORGANISMS USED IN THIS STUDY.

Strain	Full name
RBL5515	*R. leguminosarum* bv *trifolii* LPR5[1] cured for its Sym plasmid[2]
LPR5039	Strain RBL5515 harboring *R. leguminosarum* bv *trifolii* pSym5 plasmid[3]
RBL5522	Strain RBL5515 harboring *R.leguminosarum* bv *viciae* pRL1JI Sym plasmid, Nod$^-$Fix$^-$.
RBL5515 pRL1JI	RBL5515 harboring the pRL1JI Sym plasmid of *R. leguminosarum* bv *viciae*
RBL5515*exo4*,::Tn5	*R. leguminosarum* bv *trifolii* strain 5515*exo4*::Tn5 harboring the *trifolii* pSym5 plasmid [4]
RBL5515,*exo8*::Tn5	*R. leguminosarum* bv *trifolii* strain 5515,*exo8*::Tn5 harboring the *trifolii* pSym5 plasmid[4]
RBL5515,*exo336*::Tn5	*R. leguminosarum* bv *trifolii* strain 5515*exo336*::Tn5 harboring the *trifolii* pSym5 plasmid[4]
RBL5515,*exo344*::Tn5	*R. leguminosarum* bv *trifolii* strain 5515*exo344*::Tn5 harboring the *trifolii* pSym5 plasmid
248	*R. leguminosarum* bv *viciae* strain 248[5]
248,*exo1*::Tn5	*R. leguminosarum* bv *viciae* strain 248,*exo1*::Tn5[5]
TA-1	*R. leguminosarum* bv *trifolii* strain TA-1

[1] McNeil *et al.* (1986); [2] Priem and Wijffelman (1984); [3] Hooijkaas *et al.* (1981); [4] Canter Cremers *et al.*(1988); [5] Canter Cremers *et al.*. (1991)

flasks were incubated on a rotary shaker at 200 rpm at 25°C. The purity of the culture was routinely checked by plating appropriate dilutions on Yeast Extract-Mannitol-Congo Red (YEMCR) agar (Zevenhuizen *et al.*, 1986). Cell shape and uniformity of the culture were also checked by phase-contrast microscopy.

Nodulation tests. Plant seeds, media and climate chambers were used, and the acetylene reduction assay was performed as described (Canter Cremers *et al.*, 1989).

Complementation of RBL5515,*exo344*::Tn5 mutant. DNA-isolation and complementation were performed as described (Canter Cremers *et al.*, 1990). Briefly, a DNA-library of RBL5515 was constructed in IncP plasmid pTJS133 (tetracycline^r), and introduced in the mutant RBL5515,*exo344*::Tn5 harboring the pRL1JI plasmid, the resulting strains being inoculated on *Vicia sativa* plants. Introduction of plasmids into the mutant strain was done as described (Canter Cremers *et al.*, 1990).

Measurement of growth and polysaccharide production. Cells and culture supernatant were separated by centrifugation at 35000 x *g* for 30 min. The cell pellet was resuspended fivefold concentrated in distilled water and briefly sonicated to obtain a homogeneous suspension.

a) **Supernatant.** Excreted glucan and extracellular polysaccharide were determined in the supernatant by the anthrone-sulphuric acid method for hexoses (Trevelyan and Harrison, 1952) and the 3-hydroxydiphenyl-sulphuric acid method for hexuronic acids (Blumenkrantz and Asboe-Hansen, 1973). The determination of EPS was based on the uronic acid content of the whole supernatant and the known ratio (see below) of hexose:hexuronic acid for the particular EPS and was expressed as the sum of glucose and glucuronic acid equivalents (in mg/l medium). The (1,2)-ß-glucan content was derived from the amount of glucose which was not accounted for in the calculation of the EPS concentration.

Mannitol was determined spectrofotometrically according to Burton (1957). The relative viscosity of the culture supernatant was measured with an Ubbelohde viscometer (Tamson, Zoetermeer, The Netherlands) at 25°C.

b) **Pellet.** Total cellular protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

CPS was extracted from the pellet with 1N NaOH, precipitated by 1 volume of alcohol, quantified by the anthrone-sulphuric acid method and expressed as mg galactose equivalents/l culture as described (chapter 3). The carbohydrate fraction of the 50% alcoholic supernatant consisted mainly of cyclic (1,2)-ß-glucans. This fraction was quantified by the anthrone-sulphuric acid method and expressed as mg glucose equivalents/g cell protein. The alkaline-extracted cells were treated with 1 n HCl for 2 h. The hexose content in the acid-resistent, alkali-insoluble material was a measure of the cellulose content of the cells (Zevenhuizen *et al*, 1986).

Enzyme assays. Cell-free extracts were prepared in N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), pH=7.6 containing 1 mM dithioerythritol (Canter Cremers *et al.*, 1990), and UDP-glucose 4'-epimerase, UDP-glucose pyrophosphorylase, glucose dehydrogenase, glucokinase, glucose-6-P-dehydrogenase, galactose dehydrogenase, galactokinase, and galactose-1-P-uridyl transferase were measured, as described (Canter Cremers *et al.*, 1990). Enzyme activities were expressed as nmol substrate used per minute per mg protein.

Isolation and characterization of oligo- and polysaccharides. HM_r EPS was precipitated from the supernatant by the addition of 3 volumes of ethanol as described (chapter 4). In the 75% alcoholic supernatant cyclic glucans and/or repeating-units could be present. For separation of these compounds, DEAE-sepharose anion-exchange column chromatography (25x2.5 cm) was performed and fractions of 5 ml were collected. Distilled water followed by a linear gradient of 0 to 1 M KCl were used as eluens. Both hexoses and hexuronic acids were measured (chapter 4). Isolation of the CPS was performed as described (chapter 8).

Ratios of hexose:hexuronic acids of EPS were determined by applying anthrone-sulphuric acid reagent (hexoses) and 3-hydroxydiphenyl-sulphuric acid method (hexuronic acids) respectively. The neutral sugar composition of EPS and CPS were identified after hydrolysis with 2 M trifluoroacetic acid for 8 h at 100°, followed by conversion of the liberated sugars into alditolacetates and separation by GLC (Blakeney *et al.*, 1983). Glycosidic linkages were determined by methylation analysis (Harris *et al.*, 1984) and identified by mass spectrometry using a GLC/MS combination of V.G. Micromass, type 70-70-F (capillary column DB-225, J&W; 30mx0.25mm, filmthickness 0.25μm). Resolution: 1000; ionisation energy of the electron source: 70eV at 200°C. The relative amounts of the partially methylated sugars were corrected with Effective Carbon Recovery (Sweet *et al.*, 1975).

Pyruvate was determined spectrofotometrically according to Katsuki *et al.* (1971), and HPLC of cyclic glucans was carried out as described (chapter 3).

Depolymerization of EPS. Purified EPS was depolymerized by the use of purified phage RL38 and fractionated with ethanol , followed by further purification using colums of Sephadex DEAE A25, Biogel P2, and Dowex 50W-X2 as described (Canter Cremers *et al.*, 1990)

NMR. Purified depolymerized EPS was dissolved in D_2O and ^{13}C-spectra were recorded on a Varian XL-200 spectrometer with methanol (Δ= 50.04 ppm) as reference (Canter Cremers *et al.*, 1991).

RESULTS.

Polysaccharide production by *R. leguminosarum*. Previously we described (Canter Cremers *et al.*, 1988, 1990, 1991) the isolation of transposon mutants of *R.leguminosarum* bv *viciae* affected in the synthesis of 1) EPS (RBL5515, *exo4*::Tn5, RBL5515,*exo344*::Tn5, 248, *exo1*::Tn5), 2) LPS (RBL5515, *exo336*::Tn5) or 3) EPS and LPS (RBL5515,*exoB8*::Tn5). In this paper we analyse the quantities of EPS, CPS, cellulose fibrils and (1-2)-ß-glucans synthesized after growth for 14 days and compared it with the amounts synthesized by wild type strains in order to gain insight if and how the mutations affected other polysaccharides than initially screened for. The polysaccharide-production during growth was studied as well (Figure 2).

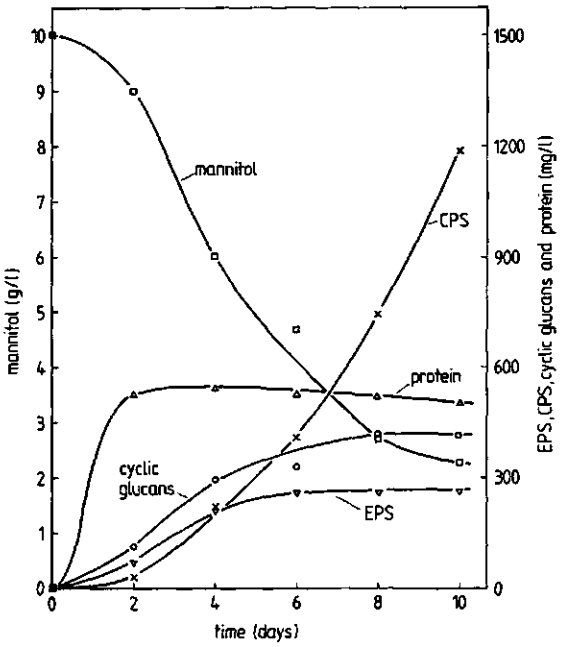

FIGURE 2. Growth, expressed as total cellular protein, polysaccharide production and mannitol consumption of *Rhizobium leguminosarum* bv *viciae* strain *RBL5515,exo344*::*Tn5* in the course of time.

(i) **EPS.** *R.leguminosarum* bv *viciae* strain RBL5515 pRL1JI, *R.leguminosarum* strain RBL5515, *R.leguminosarum* bv *trifolii* strains LPR5039 and TA-1, all produced about 2.0 g EPS/l culture, while *R.leguminosarum* bv *viciae* strain 248 produced significantly more EPS, namely 3,2 g/l culture (Table 2). Of the mutants, strain RBL5515,*exo336*::Tn5 synthesized similar amounts of EPS as the wild type strain, while the other mutants of strain RBL5515 produced significantly less EPS, namely between 90 and 350 mg/l medium (Table 2).

The hexuronic acids measured in the supernatant of strain 5515 and all strains and mutants derived from it could for more than 95% be precipitated with 3 vol of alcohol, indicating that only very small amounts of low molecular weight repeating units of this EPS were present. The high concentration of EPS measured in the supernatant of strain 248 was not parallelled by a proportionally high viscosity (Table 2). Indeed, 25% of the hexuronic acids were present in the LM, fraction of the supernatant. After separation of the LM, fraction by DEAE sepharose anion exchange chromatography, one neutral and one charged peak fraction could be obtained. The charged peak fraction with hexose:hexuronic acid ratio 2:1 contained the repeating units of the anionic EPS (830 mg/l).

(ii) **cyclic glucans.** While the pellets of all strains contained about the same quantities of (1-2)-ß-glucans, namely about 80-150 mg/g protein, considerable differences were found in the quantities of (1-2)-ß-glucans secreted into the medium. In the supernatant of cultures of strains RBL5515 pRL1JI, RBL5515, LPR5039 and TA-1 about 40 mg (1-2)-ß-glucans/l culture was present, while strain 248 excreted 300 mg/l. From the supernatant of cultures of the mutants affected only in the synthesis of EPS, strains RBL5515,*exo4*::Tn5, and RBL5515,*exo344*::Tn5, more (1,2)-ß-glucans (400 mg/l) could be isolated, than from that of strain RBL5515 itself. The highest quantities however could be isolated from the culture supernatants of LPS-deficient strains RBL5515,*exoB8*::Tn5 (790 mg/l) and RBL5515,*exo336*::Tn5 (1050 mg/l).

(iii) **CPS.** Strains RBL5515 pRL1JI, RBL5515, LPR5039 and TA-1 synthesized between 0,75 and 1,5 g CPS/l culture, while *R.leguminosarum* bv *viciae* strain 248 produced significantly less CPS, namely only 0,2 g/l culture (Table 2). Two mutants, RBL5515,*exo4*::Tn5 and RBL5515,*exo344*::Tn5 synthesized as much CPS (1.0 g/l) as the wild type strain they were derived from, while mutants RBL5515,*exoB8*::Tn5 and RBL5515,*exo336*::Tn5 formed hardly any CPS at all (0.1 g/l; Table 2).

In the nitrogen-limited production medium, strains of *R. leguminosarum* produced considerable amounts of CPS during stationary phase, together with EPS which is synthesized both in logarithmic and stationary phase (Zevenhuizen, 1984). We tried to increase the volumetric CPS production of the EPS-deficient mutant *exo4* by increasing the concentration of nutrients of the medium. As a result the amount of biomass rose, but in a medium containing twice the amount of the nutrients of the production medium the CPS production was only increased by a factor 1.2 (Table 3).

TABLE 2. Polysaccharide-production by wild type strains and mutants of *R. leguminosarum*. Bacteria were grown for 14 days at 25°C in the production medium.

STRAIN	SUPERNATANT			PELLET			
	EPS (mg/l)	glucans (mg/l)	viscosity cP	CPS (mg/l)	cellulose (mg g^{-1})	LM_r* (mg/l)	protein (mg/l)
LPR5039	2220	40	18	1305	50	95	530
RBL5515	2010	60	15	990	45	135	540
RBL5515pRL1JI	2105	55	19	750	55	115	525
RBL5515,*exo4*	90	380	1	1590	45	150	560
RBL5515,*exo8*	280	790	2	70	25	160	510
RBL5515,*exo336*	1920	1050	11	110	15	155	530
RBL5515,*exo344*	350	400	2	1190	40	150	490
248	3250	300	13	200	45	160	510
248,*exo*1	1880	400	12	180	50	115	520
TA-1	1690	30	14	1410	35	80	520

* Low molecular weight cellular carbohydrates (mainly cyclic (1,2)-ß-glucans) expressed as mg glucose equivalents per g cellular protein

TABLE 3. Influence of concentration of medium components on polysaccharide synthesis by *R. leguminosarum* mutant RBL5515,*exo4*::Tn5. Cells were incubated for 14 days at 25°C.

MEDIUM	SUPERNATANT			PELLET	
	EPS (mg/l)	glucans (mg/l)	protein (mg/l)	CPS (mg/l)	Protein (mg/l)
standard	150	280	0	480	475
production	160	430	0	1630	530
2x standard[b)]	250	650	30	570	1110
2x production[b)]	270	850	nd[a)]	1940	1050
4x standard[b)]	465	3800	375	240	1880
8x standard[b)]	470	8400	1650	200	2900
10x standard[b)]	500	11900	1800	310	3250

[a)] nd=not determined

[b)] 2x standard is a medium consisting twice the amount of substates and nutrients of the standard medium, 2x production is a medium with twice the amount of nutrients and substrates of the production medium, etc.

By further increasing the concentration of nutrients of the production medium, CPS synthesis was strongly repressed (data not shown). Under these circumstances protein was found in the supernatant, indicating that considerable cell lysis had occurred. The CPS production by mutant *exo4* in concentrated "standard medium" was lowered as well. Instead, cyclic glucan excretion was greatly enhanced to more than 10 g/l (Table 3), as was also shown for strain TA-1 (chapter 3). The viscosity of the medium remained low due to a small EPS production.

(iv) cellulose. The strains RBL5515 pRL1JI, RBL5515, LPR5039, and RBL5515, *exo336*::Tn5 grew as disperse homogeneous liquid cultures, *exo*8 formed small flocs, and the mutants *exo*4 and *exo*344 aggregated very strongly in liquid cultures. Aggregation might be the result of a high cellulose content of the cells. On YEMCR agar, the colonies of mutants affected in the synthesis of EPS (RBL5515,*exo4*::Tn5, *exo8*::Tn5 and *exo344*::Tn5) were coloured deep red, while

the colonies of strains RBL5515, RBL5515 pRL1JI, LPR5039, and RBL5515,*exo336*::Tn5 colonies were pale pink. Congo Red binds preferentially to cellulose, and this phenomenon could be used as a measure of the cellulose content of the cell pellet (Zevenhuizen *et al.*, 1986). However, there was no correlation between the cellulose content (between 10-40 mg/l, Table 2), and the colour uptake of the colonies, or the state of aggregation. The lack of large amounts of EPS around colonies of the EPS-deficient mutants might allow a better uptake of the dye.

Structural analysis of the isolated polysaccharides.

(i) **EPS**. The structural features of the EPS synthesized by strains RBL5515, RBL5515 pRL1JI, LPR5039, RBL5515,*exoB8*::Tn5 (Canter Cremers *et al.*, 1990), 248, 248,*exo1*::Tn5 (Canter Cremers *et al.*, 1991) and TA-1 (Zevenhuizen and Bertocchi, 1989) have been described previously.

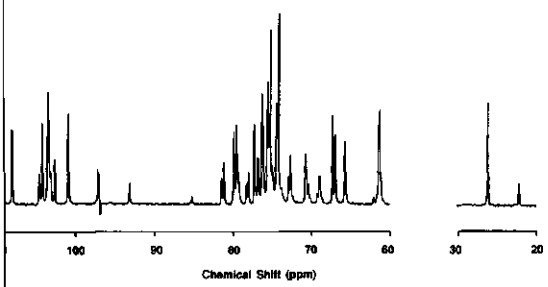

FIGURE 3. ^{13}C- NMR spectrum of the depolymerized EPS of strain RBL5515,exo344::Tn5

The structure of the EPS synthesized by the other strains, RBL5515, *exo4*::Tn5, RBL5515, *exo336*::Tn5 and RBL5515, *exo344*::Tn5, was determined by (i) component analysis, (ii) methylation analysis and (iii) ^{1}H- and ^{13}C-NMR spectroscopy.The results obtained indicated that the structures of the EPS synthesized by strains RBL5515,*exo4*::Tn5 and RBL5515,*exo336*::Tn5 were indistinguishable from that of strain RBL5515 itself (Figure 1) with almost identical sugar (Table 4) and glycosidic linkage compositions (Table 5). The EPS of strain TA-1 also belongs to this so-called K-36 type EPS (Figure 1). However component analysis showed that the EPS synthesized by mutant strain RBL5515, *exo344*::Tn5 lacked galactose, with (4,6)-di-O-carboxyethylidene (pyruvate) subsituted glucose as terminal hexose (Table 5). The spectrum obtained by ^{13}C NMR spectroscopy of the depolymerized EPS, confirmed that strain RBL5515,*exo344*::Tn5 synthesized repeating units which are comparable to that synthesized by mutant strain RBL5515,*exoB8*::Tn5 (Canter Cremers *et al.*, 1990) lacking the terminal galactose residue, as well as the hydroxybutanoyl and carboxyethylidene substituents attached to it (Figure 3). Unlike that of strain RBL5515,*exoB8*::Tn5 (Canter Cremers *et al.*, 1990) or strain RBL5515 itself (Canter Cremers *et al.*, 1991), the repeating unit of the EPS synthesized by strain RBL5515, *exo344*::Tn5 harbors O-acetyl groups attached to residue b only. Furthermore, the ^{13}C NMR spectrum of strain RBL5515,*exo344*::Tn5 contains small peaks at about 85.1 and 61.9 ppm that indicate the presence of a small amount of glucose residue g without 4,6-carboxyethylidene attached to it. The relative size of the peaks suggests that about 10% of the repeating units have a side chain that contains no carboxyethylidene at all. Similar peaks, although less pronounced can also be found in the ^{13}C NMR spectrum of EPS isolated from wild type strain RBL5515 pRL1JI. We therefore regard the presence of repeating units in which residue g has no carboxyethylidene attached to it, as imperfections in the synthesis of EPS.

In summary, we have isolated a mutant, RBL5515,*exo344*::Tn5, which synthesizes repeating units which lack galactose and the substituents attached to it and therefore resembles the *exoB* mutant strains (Canter Cremers *et al.*, 1990). But strain RBL5515, *exo344*::Tn5 differs from the *exoB* mutants, in that it is not affected in the synthesis of LPS (Canter Cremers *et al.*, 1988). We therefore analysed this latter mutant in more detail.

TABLE 4. Component analysis of EPS of the wild type *Rhizobium leguminosarum* RBL5515, RBL5515,*exo4*::Tn5, RBL5515,*exo336*::Tn5, RBL5515,*exo344*::Tn5, and *R. leguminosarum* bv *trifolii* TA-1. Cells were grown for 14 days at 25°C. Values are expressed in molar ratios.

EPS of strain:	Glc[a]	Gal[a]	Man	GlcA[a]	Pyr[b]
EPS					
RBL5515	4.8	1.0	-	2.0	1.9
RBL5515,*exo4*	4.8	0.9	-	2.0	2.0
RBL5515,*exo336*	4.5	1.0	-	2.0	1.7
RBL5515,*exo344*	5.1	-	-	2.0	0.9
TA-1	5.1	1.0	-	2.0	1.1

a) The Glc:Gal ratio was measured by GLC (Blakeney *et al.*, 1983). Total hexose was determined with the anthrone-sulphuric acid reaction and the glucuronic acid content was determined according to Blumenkrantz and Asboe-Hansen (1973). From these values the ratio glucuronic acid:hexose was determined.
b) Pyruvate was determined according to Katsuki *et al.* (1971)

TABLE 5. Methylation analysis of EPS of *Rhizobium leguminosarum* RBL5515, RBL5515,*exo336*::Tn5 and RBL5515,*exo344*::Tn5, and *R. leguminosarum* bv *trifolii* TA-1. Cells were grown for 14 days at 25°C. The molar ratios of the partially methylated sugars were calculated according to the area on their GLC-chromatogram, corrected with Effective Carbon Recovery (Sweet *et al.*, 1975) and identified with mass spectrometry.

		Bacterial strains			
		5515	*exo336*	*exo344*	TA-1
Sugar[a]	T[b]	Molar ratios of partially methylated sugars			
2,3,4,6-Glc[c]	1	0.4	0.4	0.0	0.0
3,4,6-Glc[d,e]	1.38	-	0.2	0.0	-
2,3,6-Glc	1.56	3.3	2.6	2.9	3.2
2,3-Glc	2.42	1.0	1.0	2.0	1.0
2,3-Gal	2.52	1.1	0.8	-	1.2
2-Glc	3.22	1.2	1.0	-	1.1

a) 2,3,4,6-Glc = 1,5 di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, etc.
b) Retention times. Expressed relative to 2,3,4,6-Glc.
c) Minor amounts of 2,3,4,6-Glc were due to end-groups.
d) 2,4,6-Glc and 3,4,6-Glc could be separated by applying a temperature program (140-240°C; 2°min^{-1}) using a Fused Silica Capillary Column DB-225.
e) These small amounts of 3,4,6-Glc were due to contamination of EPS preparates with cyclic (1,2)-ß-glucan.

(ii) CPS. The CPSs of strains LPR5039, RBL5515, RBL5515 pRL1JI, RBL5515,*exo4*::Tn5, RBL5515,*exo344*::Tn5, 248, and TA-1 all contained the same glucose: galactose: mannose ratios of 1:4:1. The patterns of methylated sugars investigated for the CPSs of strain TA-1 and RBL5515,*exo4*::Tn5 were identical as well (not shown).

(iii) cyclic (1,2)-ß-glucans. Upon methylation analysis of the cellular and extracellular glucan fractions of both wild type and mutant strains 3,4,6-tri-O-methyl-D-glucose was found as sole methylated sugar, confirming the 1,2-glycosidic bond and a molecule with an unbranched, cyclic character. The cyclic glucans were all neutral as deduced by Thin-Layer Chromatography and HPLC, their degrees of polymerization ranging from 17 to 25 as was shown for strain TA-1 (chapter 3).

Nodulation. After introduction of the *R.leguminosarum* bv *viciae* Sym plasmid pRL1JI, we tested the resultant strain

RBL5515,*exo344*::Tn5 pRL1JI for nodulation on *V.sativa* plants. It failed to induce nodules completely. Of the characteristics of the initial steps of nodulation, it only induced root hair deformation and rare, abortive infection threads. However, when we introduced the *R.leguminosarum* bv *trifolii* Sym plasmid pSym5 in strain RBL5515,*exo344*::Tn5, the resultant strain induced marked root hair curling, wild type infection threads and nitrogen fixing nodules on *T.repens* and *T.pratense* as fast as strain RBL5515 pSym5. We therefore concluded that the nodulation defect of strain RBL5515,*exo344*::Tn5 is dependent on the cross-inoculation group studied.

We also coinoculated mutant RBL5515,*exo344*::Tn5 pRL1JI with strain RBL5522 on *V.sativa* plants. Strain RBL5522 is a derivative of strain RBL5515 and harbors a *R.leguminosarum* bv *viciae* pRL1JI Sym plasmid which carries a deletion of about 40 kb, spanning the entire Nod and Fix region. Therefore the strain by itself cannot nodulate *V.sativa* plants. We used this strain in our coinoculation experiments, because the presence of the deleted Sym plasmid near completely inhibits the transfer of other pRL1JI Sym plasmids into this strain. After coinoculation both strains in equal proportions in equal proportions on *V.sativa*, nitrogen fixing nodules were induced with a delay of 2 to 4 days. From the thus obtained nodules, both strain RBL5522 and RBL5515,*exo344*::Tn5 could be reisolated in a proportion of 1 to 100. Random samples of the reisolated colonies of both strains failed to nodulate *V.sativa* plants, which indicates that no transfer of genetic material had occurred. Apparently strain RBL5522 helps strain RBL5515,*exo344*::Tn5 through the first stages of nodulation. Once inside in the plant, RBL5515,*exo344*::Tn5 is apparently still capable of forming nitrogen fixing bacteroids.

Since the mutant strain RBL5515,*exo344*::Tn5 induces abortive infection threads, we propose that the inability to nodulate is due to a mutation in a factor affecting stabilisation and outgrowth of the infection thread, which can be provided in trans by another live *Rhizobium* bacterium.

Complementation with previously described *exo* genes. First we tried to complement strain RBL5515,*exo344*::Tn5 for the synthesis of EPS by the introduction of a DNA library of strain RBL5515 (Canter Cremers *et al.*, 1990). Unfortunately we did not succeed in finding a complementing clone. Also the introduction in strain RBL5515,*exo344*::Tn5 of plasmid pMP2602, which carries the *R.leguminosarum* bv *viciae exoB* gene (Canter Cremers *et al.*, 1990), failed to restore EPS synthesis or nodulation ability. Furthermore neither of the plasmids pD56, pEX154 or pEX312, which carry most of the described *R.meliloti exo* genes (Leigh *et al.* 1987; Long *et al.*, 1988) could complement strain RBL5515, *exo344*::Tn5. This clearly demonstrates that RBL5515,*exo344*::Tn5 is not affected in the *ExoB* gene, nor in any other gene present on plasmid pMP2602 or any of the *exo* genes of *R.meliloti* provided by plasmids pD56, pEX154 or pEX312.

Analysis of the activity of enzymes involved in galactose metabolism. Previously we described that *R.leguminosarum* bv *viciae exoB* mutants are deficient in the synthesis of UDP-glucose 4'-epimerase (Canter Cremers *et al.*, 1990). This enzyme is involved in the synthesis of UDP-galactose, which is generally used by bacteria to incorporate galactose in polysaccharides. Just as the *ExoB* mutants, the EPS of strain RBL5515,*exo344*::Tn5 lacks galactose. Therefore we analyzed strain RBL5515,*exo344*::Tn5 and the other mutants not tested before for the presence of enzymes involved in the synthesis of UDP-galactose. In cell free extracts of *R.leguminosarum* bv *viciae* strains RBL5515, RBL5515 pRL1JI, RBL5515, *exo4*::Tn5 pRL1JI, RBL5515, *exo344*::Tn5 pRL1JI and RBL5515, *exo336*::Tn5 the activity levels of the following enzymes were comparable: UDP-glucose 4'-epimerase (90 nmol/min per mg protein), UDP-glucose pyrophosphorylase (120 nmol/min per mg protein), glucose dehydrogenase (80 nmol/min per mg protein), glucokinase (90 nmol/min per mg protein), glucose-6-P-dehydrogenase (90 nmol/min per mg protein), galactose dehydrogenase (450 nmol/min per mg protein), galactokinase (no activity), and galactose-1-P-uridyl transferase (100 nmol/min

per mg protein). In our previous paper we concluded that RBL5515 makes use of the De Ley-Douderoff pathway for the conversion of galactose, having galactose dehydrogenase as crucial enzyme, and the absence of galactokinase implied that UDP-galactose only could be formed by the epimerization of UDP-glucose as catalyzed by UDP-glucose 4'-epimerase (Canter Cremers *et al.*, 1990). We therefore concluded that in all these strains, including mutant strain RBL5515,*exo344*::Tn5, the enzyme activities required for the synthesis of UDP-galactose are present.

DISCUSSION

Polysaccharide production. *R.leguminosarum* bv *viciae* strain RBL5515 pRL1JI, *R.leguminosarum* bv *trifolii* strain RBL5515 pSym5 and *R.leguminosarum* strain RBL5515 itself, all synthesize about the same amounts of EPS, CPS, cellulose fibrils and (1-2)-ß-glucans and are representative for those of many other *R. leguminosarum* strains from our culture collections studied thus far. We therefore conclude that the presence of either the pRL1JI or pSym5 Sym plasmid does not influence the quantities of these polysaccharides synthesized.

R.leguminosarum bv *viciae* strain 248 produced EPS with a glucuronic acid in the side chain (Canter Cremers *et al.*, 1991) together with its corresponding nonasaccharide repeating unit. Excretion of relatively large amounts of repeating units of EPS has been reported mainly for *Rhizobium meliloti* strains (Amemura *et al.*, 1983a, Zevenhuizen and Van Neerven, 1983, chapter 4). The presence of glucuronic acid in the side chains of EPS of *R. leguminosarum* is rather exceptional. It has been found in the EPS of the broad host range bacterium *Rhizobium* NGR 234 (Djordjevic *et al.*, 1986) and in some strains of *R. leguminosarum* bv *phaseoli* having EPS of the K-38, K-44 and K-87 types (Zevenhuizen and Bertocchi, 1989). Therefore, great variations in EPS-structure can exist between strains of *R. leguminosarum* bv *phaseoli*. In our present study the largest variations in structure and/or quantities of polysaccharides synthesized were found between two *R.leguminosarum* bv *viciae* strains, namely strain 248 and RBL5515 pRL1JI. It is therefore unlikely that variation in the structure of a polysaccharide is a factor in the determination of host specificity in *R. leguminosarum* bv *viciae*. Rather, the variation in the production patterns of these polysaccharides is a reflection of the chromosomal background of the particular strain studied.

The production patterns of exopolysaccharides of their Tn::5 generated mutants are clearly different from most wild-type production patterns: (i) synthesis of altered EPS-structures (RBL5515,*exoB8*::Tn5, *exo344*,::Tn5), (ii) production of CPS but hardly any EPS (RBL5515,*exo4*::Tn5, *exo344*::Tn5), (iii) enhanced excretion of cyclic (1,2)-ß-glucans (all mutants), and (iv) defects in the synthesis of LPS and CPS (RBL5515,*exoB8*::Tn5, RBL5515, *exo336*::Tn5).

(i) **The synthesis of altered EPS-structures.** Both mutants RBL5515,*exo8*::Tn5 and RBL5515,*exo344*::Tn5 produced considerably lower amounts of EPS, missing the terminal galactose and the pyruvate residue attached to it, as compared to the wild type strain producting the K-36 type EPS. Linton (1990) discussed that with some transposon generated mutants of *Xanthomonas campestris* synthesizing truncated xanthan molecules the production capacity was reduced, apparently because the energetics of synthesis of truncated polymers was less favorable than for the fully acetylated and pyruvylated wild type EPS. This could well be the case with some of the mutants studied here. Howvever, the EPS of mutant 248, *exo1*::Tn5 also missed the galactose end group and therefore the pyruvate attached to it, but in contrary to RBL5515,*exo8*::Tn5 and RBL5515, *exo344*::Tn5, the EPS production was not greatly affected.

(ii) **Capsular polysaccharide.** The neutral CPS is a commonly found capsular polysaccharide within the *R. leguminosarum* group (Zevenhuizen, 1984). All the capsule producing strains presented here show identical sugar patterns upon component analysis. Until now, no alteration of the neutral CPS structure has been found within this group. CPS of *R. leguminosarum* is a polymer which forms gels already at 0.2 % $^{w}/_{v}$ (Zevenhuizen, 1984; Gid-

ley *et al.*, 1987; Crescenzi *et al.*, 1987). As this capsular polysaccharide has considerable potential applications as a gelling agent the mutants RBL5515,*exo4*::Tn5 and RBL5515, *exo344*::Tn5 which do produce wild type level CPS are advantageous as they synthesize EPS in low amounts only. As a result the viscocity of the medium remains low. However, by increasing the biomass in media with high concentration of nutrients excessive glucan excretion occurred just as was shown for strain TA-1 (chapter 3). The CPS synthesis was strongly suppressed. Therefore, by choosing the right cultural conditions, these types of mutants can produce CPS (at low biomass) or cyclic glucans (at high biomass) as the main polysaccharides. Because of the low viscosity of the medium these mutants are advantageous as production organisms of CPS and/or cyclic (1,2)-ß-glucans over all wild type *Rhizobium* strains investigated thus far.

(iii) Increased synthesis of (1-2)-ß-glucans by *exo* mutants. Cyclic (1-2)-ß-glucans are generally concentrated in the periplasmic space of rhizobia where they play a role in osmotic adaptation (Miller *et al.*, 1986, Dylan *et al.*, 1990). EPS, CPS and LPS normally form protective layers around the rhizobial cell. Defects in the synthesis of either compound, can facilitate the exchange between the periplasmic space and the environment, thus affecting the osmotic balance between the cell and the environment. The increased excretion of (1-2)-ß-glucans by the *exo* mutants described here, could therefore be a reaction of the organism in order to osmotically adjust itself to the environment (see also chapter 7).

Proposed roles for the affected gene(s) of the *exo* mutants. Mutant strain RBL5515, *exo336*::Tn5 is affected in the synthesis of both LPS and CPS. Carlson *et al.* (1987) described that LPS of *R.leguminosarum* bv *viciae* contains glucose, galactose, glucuronic acid and mannose. In addition, Zevenhuizen (1984) found that CPS of *R. leguminosarum* consists of galactose, glucose and mannose. Since mutant RBL5515,*exo336*::Tn5 still synthesizes EPS, it is still capable of synthesizing UDP-galactose, UDP-glucose and UDP-glucuronic acid (Stoddart, 1984). It is however possible that this mutant is affected in the synthesis of the other common precursor of CPS and LPS, UDP-mannose.

Mutant strain RBL5515,*exo4*::Tn5 synthesizes all polysaccharides, but only residual amounts of EPS. It is therefore difficult to determine the nature of the affected gene(s). Since the residual amounts of EPS synthesized still have the wild type structure, the mutant can still synthesize all sugar nucleotides and sugar transferases required for its assembly (Stoddart, 1984). We therefore hypothesized that this mutant is affected in a gene involved in the regulation of the synthesis of EPS. Investigations have shown that mutant strain RBL5515,*exo4*::Tn5 is affected in a gene homologous to *pss2* (Canter Cremers, personal communication), a regulatory gene involved in the synthesis of EPS by *R. leguminosarum* bv *phaseoli* (Borthakur *et al.*, 1986, 1988). Mutant strain RBL5515,*exo344*::Tn5 is also only affected in the synthesis of EPS. The residual amounts of EPS still secreted into the medium however lack the terminal galactose residue of the side chain and, as a consequence, the substitutions attached to it. Previously we described a mutant, strain RBL5515,*exoB8*::Tn5, which synthesized EPS similar in structure to that synthesized by mutant RBL5515,*exo344*::Tn5. This ExoB mutant was affected in the synthesis of UDP-galactose. Since mutant RBL5515,*exo344*::Tn5 (i) still synthesizes other polysaccharides which require the presence of UDP-galactose, (ii) harbors all the enzymes required for the synthesis of UDP-galactose, and (iii) cannot be complemented by plasmids harboring the *exoB* gene, the defect in the structure of its EPS is not due to an inability to synthesize UDP-galactose. When UDP-galactose is present, the addition of galactose to EPS only requires the presence of a specific sugar transferase (Stoddart, 1984; Sutherland, 1979). Apparently this transferase is absent in this latter mutant strain. We therefore propose that mutant strain RBL5515,*exo344*::Tn5 is affected in either a structural gene coding for the transferase or in a gene which regulates the synthesis of this specific galactose transferase.

Nodulation ability. The relationship between the exopolysaccharide production of *Rhizobi-*

um and the ability to nodulate the host-plant has been the subject of many studies, mostly done with transposon-mutants affected in the synthesis of EPS (Diebold and Noel, 1989; Leigh *et al.*,1985; Leigh and Lee, 1988; Sanders *et al.*, 1981). Most studies concerning *R. leguminosarum* are hampered by the fact that the defect in the synthesis of EPS was poorly defined. Previously we have shown that defects in the synthesis of EPS can be a result of mutations in genes coding for basal metabolic enzymes (Canter Cremers *et al.*, 1990). Similar consequences of mutations in metabolic genes have also been described for other bacterial species, like for instance for *Salmonella* (Germanier, 1970). Clearly, multiple defects - detectable and non-detectable - can result from a mutation in one of these genes. Firm conclusions on the involvement of polysaccharides in nodulation, which are based on experiments with this kind of mutant, are therefore impossible.

Sugar transferases involved in the assembly of polysaccharides are highly specific, whereby each polysaccharide has its own set of transferases, namely at least one for each addition (Stoddart, 1984; Sutherland, 1979). A mutation in a gene coding for a sugar transferase will therefore only affect the polysaccharide, in which assembly it is involved. As we described above, mutant strain RBL5515, *exo344*::Tn5 is such a mutant. Since this mutant synthesizes low amounts of EPS with a aberrant structure and fails to nodulate plants belonging to pea cross-inoculation group, we can conclude that either the structure of EPS or the amount of EPS synthesized is important for nodulation.

Amemura *et al.* (1983b) described the isolation of *R.leguminosarum* bv *trifolii* strain 4S which synthesized EPS, structurally comparable to that synthesized by strain RBL5515,*exo344*::Tn5, but strain 4S having a (1,3)-linked glucose residue instead of a (1,4)-linked glucose residue (next to the terminal glucose) in the side chain. When we introduced the *R.leguminosarum* bv *viciae* Sym plasmid pRL1JI into this strain it induced nitrogen fixing nodules on *V.sativa* plants (Canter Cremers *et al.*, 1990). This indicates that succesful nodulation on plants of the pea cross-inoculation group does not require the presence of the terminal galactose group in the side chain of the EPS, nor the substituents attached to it. We therefore have to conclude that the amount of EPS synthesized by mutant strain RBL5515,*exo344*::Tn5 is insufficient for the nodulation of plants of the pea cross-inoculation group. Since strain RBL5515, *exo344*::Tn5 harboring the *R.leguminosarum* bv *trifolii* Sym plasmid pSym5 induced nitrogen fixing nodules on plants belonging to clover cross-inoculation group, the requirement for the amount of EPS is dependent on the cross-inoculation group studied. Coinoculation experiments with help of strain RBL5522 support and extend this last conclusion. *R.leguminosarum* RBL5522, which synthesizes as much EPS as strain RBL5515 (data not shown), harbors no nitrogen fixation genes and can therefore under no circumstances fix nitrogen. Coinoculation of strain RBL5522 and RBL5515,*exo344*::Tn5 pRL1JI on *V.sativa* plants resulted in the induction of nitrogen fixing nodules. In these nodules, the nitrogen fixation has to be due to formation of bacteroids by strain RBL5515,*exo344*::Tn5. Strain RBL5515,*exo344*::Tn5 by itself induced abortive infection threads only, while the combination of strain RBL5522 and RBL5515,*exo344*::Tn5 induced wild type infection threads. It is possible that for proper nodulation a certain viscosity in the infection thread is needed. In that case, the quantity of EPS is of importance. The infective cells are considered to be metabolically active and in their phase of growth during the process of infection, and RBL5515,*exo344*::Tn5 produced very little EPS during logarithmic phase (Figure 2) while media of the wild type strains are already viscous (data not shown). Therefore we propose that the amount of EPS synthesized, and herewith connected the viscosity, is important in the stabilisation and outgrowth of infection threads.

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CHAPTER 10

SUMMARY

SAMENVATTING

SUMMARY

Rhizobium and *Agrobacterium* species are capable of synthesizing a variety of extracellular and cellular oligo- and polysaccharides. Changes in environmental conditions may all affect the composition, physical properties, and relative amounts of oligo- and polysaccharides. Interest in the field of *Rhizobium* polysaccharides has resulted from a development in two distinct areas, (i) the role of oligo- and polysaccharides in the microbe-plant interaction, and (ii) studies on the physico-chemical properties of microbial polysaccharides with a potential technical application (CHAPTER 1).

In this thesis two model strains, *Rhizobium leguminosarum* bv *trifolii* TA-1 and *Rhizobium meliloti* SU-47, were used to study the extent of polysaccharide-production as influenced by cultural conditions. Although this study was aimed at polysaccharide-production in general, most research was focused on the regulation of cyclic (1,2)-ß-glucan synthesis. Therefore, in CHAPTER 2 a summary on the chemistry, occurrence, biological function and potential applications of these compounds is given.

CHAPTER 3 describes the enhanced excretion of cyclic (1,2)-ß-glucan by *Rhizobium leguminosarum* bv *trifolii* TA-1 as the result of (i) incubation at superoptimal temperature for growth (30-33°C) and (ii) growth at high cell densities. At 33°C, EPS and CPS production was inhibited and up to 3.9 g of cyclic (1,2)-ß-glucan/l was produced in the production medium (10 g mannitol and 1 g glutamic acid/l medium) with a rate of 400 mg glucans/g protein/day by a biomass of 540 mg protein/l. At 25°C, the optimal temperature for growth of strain TA-1, EPS and CPS were the main carbohydrate products synthesized, while hardly any glucans were detected in the medium. Other *R. leguminosarum* strains showed a comparable "temperature-effect". In a medium containing 50 g mannitol and 10 g glutamic acid per liter high cell densities of strain TA-1 (3.95 g protein/l) were obtained and 10.9 g/l of cyclic (1,2)-ß-glucan within 10 days at 25°C were excreted, while CPS production was strongly suppressed. The cyclic (1,2)-ß-glucans were neutral and had degrees of polymerization (DP) ranging from 17 to 25 with DP=19 as the major glucan cycle.

In production processes, aimed at high volumetric yields, high concentrations of organic nutrients and mineral salts in liquid media may lead to a considerable high osmotic pressure. Therefore, and because synthesis of cyclic glucans were found to be osmo-regulated in some members of the family of *Rhizobiaceae*, the influence of the osmotic pressure of the medium on the growth of and polysaccharide synthesis by *R. meliloti* SU-47 and *R. leguminosarum* bv *trifolii* TA-1 was studied (CHAPTERS 4 and 5). The ability of members of the *Rhizobiaceae* to grow at high osmotic pressures of the medium, and their tolerance towards several ionic and non-ionic osmolytes, depend strongly on the species and the type of osmolyte. Strains of *R. meliloti* and *A. tumefaciens* could grow well in media up to 1 M NaCl while *R. leguminosarum* could only tolerate up to 0.35 M NaCl. In standard- or production medium with low osmotic pressure cells of strain SU-47 accumulated up to 350 mg cyclic (1,2)-ß-glucans/g protein, of which 36% were glycerol-1-phosphate substituted and 64% were neutral. By increasing the osmotic pressure of the medium by the addition of NaCl or other ionic and non-ionic osmolytes, succinoglycan production could be stimulated (up to 2.4 g/l at 0.2 M NaCl), at the expense of the repeating units. Furthermore, the amount of cellular cyclic (1,2)-ß-glucans was lowered, to 150 mg/g protein at 0.6 M NaCl, of which the glycerol-1-phosphate substituted glucan fraction was reduced to 15%. Instead, oligosaccharides up to 250 mg/g protein were synthesized with trehalose as the major component. Glycogen synthesis was fully suppressed at this salt concentration. No cyclic glucans were found in the medium (CHAPTER 4). By increasing the osmotic pressure of the medium, the synthesis of EPS and CPS by *R. leguminosarum* bv *trifolii* TA-1 was suppressed, and cyclic glucans were excreted instead (1500-2000 mg of glucans/l). This proceeded with a rate of 220 mg glucans/g protein/day by a biomass of 520 mg protein/l at 0.2 M NaCl. Intracellular cyclic (1,2)-ß-

glucan concentrations remained at 45-100 mg/g protein during the stationary phase, independent of the osmotic strength of the medium. Parallel to the increasing osmotic pressure of the medium, the disaccharide trehalose accumulated in the cells, up to 130 mg/g protein (CHAPTER 5).

The response to a NaCl-shock on cellular carbohydrates of NaCl-free grown cells of *R. leguminosarum* bv *trifolii* TA-1 and *R. meliloti* SU-47 was investigated in non-growing cultures in a batch-fermenter and in cell suspensions using *in vivo* NMR (CHAPTER 6). In a glutamic acid-free medium containing NaCl TA-1 cells but especially SU-47 cells responded immediately by synthesizing trehalose, while glycogen and the external substrate mannitol were metabolized. Without mannitol in the medium trehalose synthesis was slower and parallelled the breakdown of the reserve materials glycogen and PHB. ^{13}C-NMR experiments with 25-fold concentrated cell-suspensions using $^{13}C_1$-mannitol as substrate revealed that 20% of the trehalose synthesized was derived from the substrate, but 80% from other sources. Therefore, trehalose synthesis occurred from the internal pool of glycogen and/or PHB, whether mannitol was present or not. Cells of strains TA-1 and SU-47 that had accumulated trehalose metabolized this compound again in a low osmolarity environment. As trehalose is a general occurring compound found in cells of *Rhizobium* at much lower concentrations the higher concentrations which were measured during the conditions of osmotic stress could be explained by the role of trehalose as osmo-protectant. The cellular phosphoglycerol-substituted and neutral cyclic (1,2)-ß-glucans of NaCl-free grown SU-47 cells were not degraded nor excreted after the NaCl-shock. With *in vivo* ^{31}P-NMR the glycerol-1-phosphate substituted cyclic glucans in cell suspensions of strain SU-47 could well be observed.

In CHAPTER 7 the synthesis of cyclic (1,2)-ß-glucans from UDP-[^{14}C]-glucose by a crude membrane preparation and whole cells of *Rhizobium leguminosarum* bv *trifolii* TA-1 was investigated. The observed enhanced excretion of cyclic (1,2)-ß-glucans by strain TA-1 was due to an increased permeability of the outer membrane for cyclic glucans. This was concluded from the following observations: (i) Incubation of repeatedly frozen and thawed TA-1 cells with UDP-^{14}C-glucose resulted in the appearance of a labelled glucan in the medium. Cells grown in the presence of NaCl and cells cultured at 33°C showed the same effect, while cells cultured at 25°C in the absence of NaCl did excrete only little cyclic glucans; (ii) the presence of 0.1 mM EDTA, a complexing agent of divalent cations which results in a weakened LPS layer, also induced glucan excretion in growing cultures of strain TA-1; (iii) *R. leguminosarum* bv *viciae* RBL5515,*exoB8*::Tn*5* mutant cells excreted a labelled glucan fraction, while the parent strain RBL5515 did not. The mutant was affected in the production of EPS, CPS, and LPS (CHAPTER 9), which all might explain the greater permeability of the mutant cells towards cyclic (1,2)-ß-glucans. Hardly any difference in biosynthetic activity was observed between membrane fractions of TA-1 cells grown in the presence (0.2 M) or absence of NaCl. Glucan formation *in vitro* and glucan excretion by whole cells was strongly inhibited in the presence of 50 mg/ml cyclic glucan, indicating biosynthesis of cyclic (1,2)-ß-glucans in strain TA-1 to be controlled by end-product inhibition. Therefore, the constant loss of glucans from osmotically-stressed TA-1 cells prevented end-product inhibition and resulted in glucan accumulation in the medium of up to 1600 mg/l. Cyclic (1,2)-ß-glucans from *Rhizobium meliloti* and *Agrobacterium tumefaciens* are thought to be involved in both infection behaviour and osmo-regulation at low osmotic pressure of the medium. Combining the results obtained on the synthesis of cyclic glucans, it is doubtful that cyclic glucans of *R. leguminosarum* play an important role in osmo-adaptation at low osmolarity, for the following reasons: (i) cellular glucan concentrations are much lower than in *R. meliloti*; (ii) the great majority of the glucans in *R. leguminosarum* are neutral molecules, and (iii) no repression of glucan synthesis at enhanced osmolarity of the medium occurs.

In CHAPTER 8 *Rhizobium leguminosarum* bv *trifolii* TA-1 was used a model organism to study the influence of growth rate and me-

dium composition on exopolymer production. Circumstances leading to a high CPS-yield also lead often to concomitantly high EPS-production, rendering the medium viscous, and making the process of recovery of CPS more difficult. In continuous cultures, EPS was at every dilution rate (between D=0.02-0.12 h^{-1}) the most abundant polysaccharide present in production medium, while CPS synthesis occurred only at low specific growth rates. Only low amounts of cyclic glucans were excreted (10-30 mg/l). In production medium EPS was synthetized in the active phase of growth and continued in stationary phase (up to 1.6-2.1 g/l). CPS-synthesis which takes place only in stationary phase and in the presence of excess mannitol, was produced up to 1.8 g/l during batch incubation for 14 days at 25°C. Maximal CPS production was 2.9 g CPS/l medium, with 1 g protein as biomass in a medium containing 20 g/l mannitol and 2 g/l glutamic acid. The maximum specific growth rate was μ_{max}=0.133 h^{-1}. To washed cells at which 10 g/l mannitol was added CPS synthesis reached 2.1 g/l, but EPS-synthesis was lower (0.8 g/l).

In CHAPTER 9 the polysaccharide production by *Rhizobium leguminosarum* RBL5515 and some Tn::5 generated mutants affected in polysaccharide synthesis was studied in production medium. The EPS of the wildtype strain was composed of a K-36 type octasaccharide repeating unit with sugar composition of glucose: glucuronic acid: galactose in the ratios of 5:2:1. The CPS from the *R. leguminosarum* strains investigated had all a constant sugar composition of glucose: galactose: mannose in the ratios of 1:4:1. The presence or absence of either the pRL1JI or pSym5, the Sym plasmids of *R. leguminosarum* bv *viciae* and *trifolii*, respectively, did not influence the quantities of these polysaccharides synthesized, being comparable to strain TA-1. The production patterns of exopolysaccharides of their Tn::5 generated mutants were clearly different from most wild-type production patterns by the synthesis of altered EPS-structures, low production of CPS and/or EPS, and enhanced excretion of cyclic (1,2)-ß-glucans (all mutants; 400-1000 mg/l) as compared to strain RBL5515 (50 mg/l). The mutants RBL5515,*exo4*::Tn5, RBL5515,*exoB8*::Tn5 and RBL5515,*exo344*::Tn5 formed 5-20% of wild-type EPS-level. Both mutants RBL5515, *exoB8*Tn::5 and RBL5515,*exo344*::Tn5 synthesized a truncated EPS with a heptasaccharide-repeating unit missing the terminal galactose in the side chain. While the RBL5515,*exoB8*::Tn5 mutant was shown to be devoid of UDP-glucose 4' epimerase activity, the activity of this enzyme and of other enzymes involved in the synthesis of UDP-galactose were comparable in RBL5515, *exo344*::Tn5 and the wildtype. It was concluded that RBL5515,*exo344*::Tn5 was affected in a galactose transferase activity. Since this mutant failed to nodulate plants belonging to the pea inoculation group, and the presence of the terminal galactose in the side chain of the EPS of *R. leguminosarum* bv *viciae* was not required for succesful nodulation, it was postulated that the amount of EPS produced by RBL5515*exo344*::Tn5 and the herewith related viscosity is insufficient for nodulation.

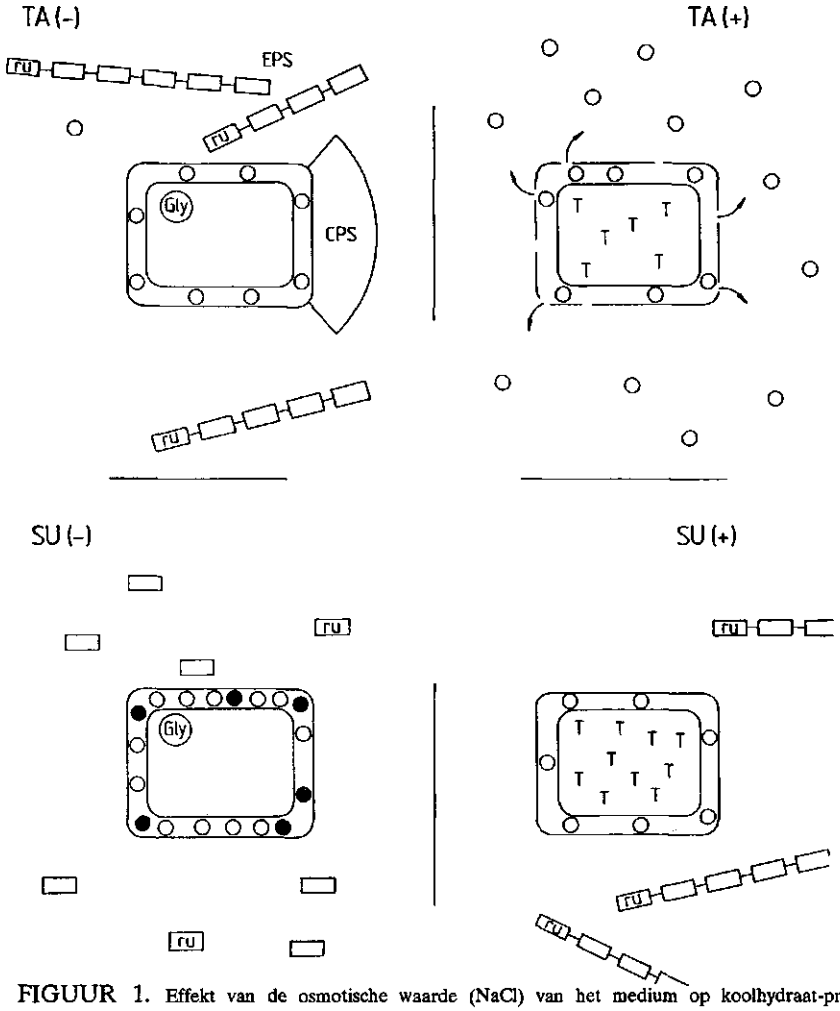
Finally, CPS of *R. leguminosarum* is a polymer which forms gels already at 0.2 % $^{w}/_{v}$, which is even lower than for agar and therefore has considerable potential applications. Cyclic (1,2)-ß-glucans are potentially useful molecules because of their relatively hydrophobic internal space, which make them suitable as an inclusion agent. By choosing the adequate cultural conditions and/or strains, *R. leguminosarum* was able to produce CPS (CHAPTERS 8, 9) or cyclic glucans (CHAPTERS 3,5) as the main polysaccharides. The aim of this thesis was to extend the knowledge on the production of *Rhizobium* polysaccharides as influenced by cultural conditions. The results described in this thesis work can help to stimulate the application-oriented research on hydrocolloïds (CPS, EPS) and inclusion agents (cyclic glucans), and may lead to a better understanding of the biological role of these compounds.

SAMENVATTING PROEFSCHRIFT "OLIGO- EN POLYSACCHARIDE SYNTHESE DOOR *RHIZOBIUM LEGUMINOSARUM* EN *RHIZOBIUM MELILOTI*"

Bakteriën van de geslachten *Rhizobium* en *Agrobacterium* maken een aantal verschillende extracellulaire en cellulaire oligo- en polysacchariden. Veranderingen in cultuurcondities kunnen een effekt hebben op zowel samenstelling, fysische eigenschappen, als hoeveelheid van de geproduceerde oligo- en polysacchariden. De interesse op het gebied van de *Rhizobium* polysacchariden komt voort uit de ontwikkelingen binnen twee onderzoeksvelden: (i) de bakterie-plant interakties en de rol die oligo- en polysacchariden daarin spelen, en (ii) de fysisch-chemische eigenschappen van microbiële polysacchariden met een mogelijke technische applikatie (HOOFDSTUK 1). Dit proefschrift beschrijft 2 model-organismen, *Rhizobium leguminosarum* bv *trifolii* TA-1 en *Rhizobium meliloti* SU-47, waarmee de invloed van cultuur-condities op de polysaccharide-produktie werd bestudeerd. Hoewel deze studie gericht was op polysacchariden in het algemeen, werd de meeste aandacht besteed aan de synthese van cyclische glucanen. In HOOFDSTUK 2 wordt daarom een samenvatting van de struktuuropheldering, het voorkomen, de biologische funktie en de mogelijke toepassing van deze unieke verbindingen gegeven.

HOOFDSTUK 3 beschrijft de verhoogde uitscheiding van cyclische (1,2)-ß-glucanen door *Rhizobium leguminosarum* bv *trifolii* TA-1 als het resultaat van (i) incubatie bij hogerdan-optimale temperatuur voor groei (30-33°C) en (ii) groei bij hoge celdichtheden. Incubatie van stam TA-1 bij 33°C leidde tot verlaagde EPS en CPS produktie, terwijl 3.9 g cyclische (1,2)-ß-glucanen/l werden uitgescheiden in het produktiemedium (10 g mannitol en 1 g glutaminezuur/l medium), met een produktiesnelheid van 400 mg glucanen/g eiwit/dag door een biomassa van 550 mg eiwit/l. Bij 25°C, de optimale temperatuur voor groei, vormden EPS en CPS de voornaamste koolhydraat-produkten, terwijl slechts lage glucaanconcentraties werden waargenomen. Andere *R. leguminosarum* stammen vertoonden een vergelijkbaar temperatuur-effekt. In een medium met 50 g mannitol en 10 g glutaminezuur per liter werden hoge celdichtheden bereikt van stam TA-1 (3.95 g eiwit/l). Deze biomassa scheidde 10.9 g/l cyclische (1,2)-ß-glucanen uit gedurende 10 dagen bij 25°C, en CPS-produktie werd sterk geremd. De cellulaire en extracellulaire cyclische (1,2)-ß-glucanen waren neutrale verbindingen en hadden een polymerisatiegraad (DP) van 17 to 25 glucose-eenheden met DP=19 als hoofdcomponent.

Bij produktieprocessen, gericht op hoge volumetrische opbrengsten, kunnen hoge concentraties van nutrienten en zouten aanleiding geven tot een aanzienlijke osmotische druk van het medium. De synthese van cyclische glucanen in sommige leden van de *Rhizobium*-familie is osmotisch gereguleerd. Het onderzoek richtte zich daarom op de invloed van de osmotische druk van het medium op de groei van en de polysaccharide-produktie door *R. meliloti* SU-47 en *R. leguminosarum* bv *trifolii* TA-1 (Figuur 1). De mogelijkheid van *Rhizobium* bakteriën te groeien bij hoge osmotische druk van het medium, en de tolerantie voor ionische en niet-ionische osmolieten, hangt sterk af van de soort en het type osmoticum. Zo bleek dat *R. meliloti* en *A. tumefaciens* soorten tolerant waren tot 1 M NaCl, terwijl *R. leguminosarum* tolerant was tot 0.35 M NaCl. In het standaard- of produktiemedium (met een lage osmotische druk) hoopten SU-47 cellen 350 mg cyclische (1,2)-ß-glucanen/g eiwit op, waarvan 36% glycerol-1-fosfaat gesubsitueerd en 64% neutraal waren. Door de osmotische druk te verhogen, door toevoeging van NaCl of andere osmolieten, werd de produktie van succinoglycaan gestimuleerd (tot 2.4 g/l bij 0.2 M NaCl), ten koste van de zgn. repeating units. Tevens werd de accumulatie van de cellulaire cyclische (1,2)-ß-glucanen bij 0.6 M NaCl verlaagd tot 150 mg/g eiwit, waarvan het aandeel van de gesubsitueerde glucanen was gereduceerd tot 15%. Hoge concentraties cellulaire oligosacchariden werden aangetroffen tot 250 mg/g eiwit, waarvan trehalose de hoofdcomponent vormde. De synthese van glycogeen was volledig gerepresseerd in aanwezigheid



FIGUUR 1. Effekt van de osmotische waarde (NaCl) van het medium op koolhydraat-produktie door *Rhizobium leguminosarum* bv *trifolii* TA-1 (0.2M NaCl) en *R. meliloti* SU-47 (0.4 M NaCl). (-), (+)= incubatie in afwezigheid (-) en aanwezigheid (+) van NaCl. Gly= glycogeen; ru=repeat-unit of repeterende eenheid; T=trehalose. ○, ● = geladen en ongeladen cyclische (1,2)-ß-glucanen.

van 0.6 M NaCl. Er werden geen cyclische glucanen aangetroffen in het medium (HOOFDSTUK 4). EPS- en CPS-synthese door *R. leguminosarum* bv *trifolii* TA-1 was geremd in een medium met verhoogde osmotische druk. In een medium met 0.2 M NaCl werden 1500-2000 mg glucanen/l uitgescheiden, met een produktiesnelheid van 220 mg glucanen/g eiwit/dag door een biomassa van 520 mg eiwit/l. De concentratie van cellulaire cyclische (1,2)-ß-glucanen bedroeg 45-100 mg/g eiwit gedurende de stationaire fase, en was onafhankelijk van de osmotische druk van het medium. Parallel aan de verhoogde osmotische druk van het medium werd trehalose in de cel opgehoopt, tot 130 mg/g eiwit (HOOFDSTUK 5). De reaktie van een NaCl-shock op de cellulaire koolhydraten van *R. leguminosarum* bv *trifolii* TA-1 en *R. meliloti* SU-47, vooraf gegroeid in een NaCl-vrij medium, werd bestudeerd in een batch-fermenter, en in celsuspensies met behulp van de *in vivo* NMR-techniek (HOOFDSTUK 6). In een gluta-

minezuur-vrij medium met NaCl reageerden TA-1 cellen (0.25 M NaCl), maar vooral SU-47 cellen (0.4 M NaCl) onmiddellijk door synthese van trehalose, terwijl glycogeen en het externe substraat mannitol werden verbruikt. In afwezigheid van mannitol vond bij een verlaagde snelheid trehalosevorming plaats, die parallel liep aan de afbraak van de koolstofreservebronnen glycogeen en PHB. ^{13}C-NMR experimenten met 25-maal geconcentreerde celsuspensies met $^{13}C_1$-mannitol als C-bron toonden aan dat 20% van het gesynthetiseerde trehalose afkomstig was van mannitol, maar 80% van andere bronnen. Het bleek dat trehalose synthese plaats vond vanuit glycogen en/of PHB, zowel in de aan- als afwezigheid van mannitol. Trehalose, aanwezig in NaCl-gestresste cellen, werd gemetaboliseerd in een medium met lage osmotische druk. Lage concentraties trehalose komen algemeen voor in cellen van *Rhizobium*. De sterk verhoogde concentraties gemeten tijdens osmotische stress kunnen daarom verklaard worden door de rol van trehalose als beschermer van het interne milieu van de cel. De cellulaire fosfoglycerol-gesubstitueerde en neutrale cyclische (1,2)-ß-glucanen die al aanwezig waren in de SU-47 cellen werden na de shock niet afgebroken noch uitgescheiden. Verder werden de glycerol-1-fosfaat gesubstitueerde cyclische glucanen in celsuspensies van stam SU-47 met *in vivo* ^{31}P-NMR waargenomen.

In HOOFDSTUK 7 werd de synthese van cyclische (1,2)-ß-glucanen vanuit UDP-[^{14}C]-glucose door een ruw membraanpreparaat en hele cellen van stam TA-1 bestudeerd. De waargenomen verhoogde uitscheiding van cyclische (1,2)-ß-glucanen door stam TA-1 was het resultaat van een verhoogde permeabiliteit van de buitenmembraan. Deze conclusie werd getrokken op grond van de volgende waarnemingen: (i) na incubatie van TA-1 cellen, die afwisselend bevroren en ontdooid waren, met UDP-^{14}C-glucose verscheen een gelabeld glucan in de supernatant. Hetzelfde gold voor cellen gegroeid bij hoge NaCl-concentraties, en cellen die bij 33°C gegroeid waren. Echter, cellen gegroeid bij 25°C in afwezigheid van NaCl vertoonden maar weinig uitscheiding; (ii) de aanwezigheid van 0.1 mM EDTA in cultures van TA-1, een complexant van o.a Ca^{2+} wat kan resulteren in een verzwakt LPS, leidde ook tot excretie; (iii) cellen van de mutant *R. leguminosarum* bv *viciae* RBL5515, *exoB8*::Tn5 vertoonden glucaan-uitscheiding, maar het wildtype RBL5515 niet. De mutant had een defekt in de produktie van zowel EPS, CPS, als LPS (HOOFDSTUK 9). Er was nauwelijks verschil in biosynthetische aktiviteit tussen membraanfrakties van TA-1 cellen die in aan- (0.2 M NaCl) en afwezigheid van NaCl waren gegroeid. Glucaanvorming *in vitro* en -excretie door hele cellen werd sterk geremd in aanwezigheid van 50 mg/ml cyclisch glucaan, waaruit bleek dat de biosynthese hiervan in stam TA-1 werd gecontroleerd door eindproduktremming. Het weglekken van glucanen vanuit osmotisch-gestresste TA-1 cellen verhinderde de controle via eindproduktremming, waardoor accumulatie van glucanen in het medium tot 1600 mg/l mogelijk werd. Cyclische (1,2)-ß-glucanen van *Rhizobium meliloti* en *Agrobacterium tumefaciens* zijn betrokken bij zowel het infektiegedrag als bij osmo-regulering, bij lage osmotische druk van het medium. De volgende observaties brengen de potentiële rol van cyclische glucanen in osmo-adaptatie in *R. leguminosarum* in twijfel: (i) de cellulaire glucaanconcentraties zijn veel lager dan in *R. meliloti*; (ii) de overgrote meerderheid van de glucanen zijn neutraal in *R. leguminosarum*, en (iii) repressie van de glucaansynthese bij verhoogde osmolariteit van het medium treedt niet op.

In HOOFDSTUK 8 wordt *Rhizobium leguminosarum* bv *trifolii* TA-1 gebruikt als modelorganisme om de invloed van de groeisnelheid en medium-compositie op produktie van exopolymeren te bestuderen. Omstandigheden die leiden tot een hoge CPS-opbrengst gaan vaak vergezeld met een hoge produktie van EPS, wat het medium visceus maakt en waardoor isolatie en opwerking van CPS bemoeilijkt wordt. In continue cultures was EPS bij elke verdunningssnelheid (tussen D=0.02 en 0.12 h^{-1}) het meest voorkomende polysaccharide, terwijl CPS synthese plaatsvond alleen bij lage specifieke groeisnelheden. Er werden slechts 10-30 mg glucanen/l aangetroffen in het medium. De maximum specifieke groeisnelheid bedroeg μ_{max}=0.133 h^{-1}. In batch-cultures in

het produktie-medium (10 g en 1 g glutaminezuur per *l*) werd EPS zowel in de aktieve groeifase als in de stationaire fase gesynthetiseerd en bedroeg 1.6-2.1 g/l. CPS-synthese vond alleen in de stationaire fase plaats en in aanwezigheid van overmaat koolstof, en bereikte 1.8 g/l gedurende een batch-incubatie van 14 dagen. De gevonden maximale CPS-produktie bedroeg 2.9 g CPS/l medium, vanuit 1 g eiwit als biomassa in een medium met 20 g/l mannitol en 2 g/l glutaminezuur. Gewassen cellen waaraan 10 g/l mannitol was toegevoegd, produceerden 2.1 g/l CPS, en 0.8 g/l EPS.

HOOFDSTUK 9 beschrijft de polysaccharide-produktie door *Rhizobium leguminosarum* RBL5515 en enkele met Tn::5 gegenereerde mutanten, gestoord in de synthese van polysacchariden synthese, in het produktie-medium. Het EPS van de ouderstam was opgebouwd uit octasaccharide repeterende eenheden van het K-36 type, met suikersamenstelling glucose: glucuronzuur: galactose in the verhoudingen 5:2:1. Het CPS vertoonde een konstante samenstelling bestaande uit de hexoses glucose: galactose: mannose in de verhoudingen 1:4:1. De aan/of afwezigheid van pRL1JI of pSym5, de symbiontische plasmiden van *R. leguminosarum* bv *viciae* resp. *trifolii*, beinvloedde de hoeveelheden van deze polysacchariden nauwelijks, en waren te vergelijken met die van stam TA-1. Echter, de produktiepatronen van de exopolysacchariden van de Tn::5-mutanten waren duidelijk te onderscheiden van die van de meeste wildtype stammen, zoals bijv. de synthese van EPS of LPS met gewijzigde strukturen, of een lage produktie van EPS en/of CPS. Verder vertoonden alle mutanten een verhoogde uitscheiding van cyclische ß-glucanen (400-1000 mg/l) vergeleken met stam RBL5515 (50 mg/l). De mutanten RBL5515,*exo4*::Tn5, RBL5515, *exoB3*::Tn5 en RBL5515,*exo344*::Tn5 produceerden 5-20% van het wildtype EPS-niveau. RBL5515,*exoB8*Tn::5 en RBL5515,*exo344*::Tn5 maakten beiden een gewijzigd EPS met een heptasaccharide-repeterende eenheid zonder de terminale galactose in de zijketen. Hoewel RBL5515,*exoB8*::Tn5 geen UDP-glucose 4' epimerase aktiviteit vertoonde, was er geen aantoonbaar verschil in aktiviteit van dit enzym en andere enzymen die betrokken zijn in de synthese van UDP-galactose tussen RBL5515,*exo344*::Tn5 en RBL5515. Het is daarom waarschijnlijk dat RBL5515, *exo344*::Tn5 een defekt had in een galactose transferase aktiviteit. Doordat deze mutant niet in staat was erwteplanten te noduleren, en de aanwezigheid van galactose in de zijketen van EPS van *R. leguminosarum* bv *viciae* niet vereist was voor nodulatie, werd geconcludeerd dat de hoeveelheid EPS die door RBL5515*exo344*::Tn5 wordt gesynthetiseerd (en de hiermee gerelateerde viscositeit) niet voldoende is voor nodulatie van de erwt.

Tot besluit, CPS van *R. leguminosarum* is een uniek polymeer dat in staat is te geleren bij 0.2 % $^{w}/_{v}$ zonder aanwezigheid van divalente kationen en heeft mogelijk interessante toepassingen. Cyclische (1,2)-ß-glucanen zijn interessante verbindingen vanwege de relatief hydrophobe interne ruimte, wat de molekulen potentieel geschikt maakt als een gastheer-molecuul voor insluitverbindingen. Door de juiste cultuuromstandigheden voor *R. leguminosarum* te kiezen, was een selektieve synthese te bereiken van CPS (HOOFDSTUKKEN 8, 9) of cyclische glucanen (HOOFDSTUKKEN 3, 5). Het doel van dit proefschrift was meer kennis te verzamelen over de invloed van cultuurcondities op polysaccharide-produktie door *Rhizobium*. De resultaten die in dit proefschrift zijn beschreven kunnen het toepassings-gerichte onderzoek aan hydrocolloïden (EPS, CPS) en gastheer-molekulen voor insluitcomplexen (cyclische glucanen) steunen, maar kunnen tevens leiden tot een beter begrip van de biologische rol van deze verbindingen.

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NAWOORD

Op een bepaald moment krijg je dan een gevoel van euforie. Het proefschrift is eindelijk klaar, het heeft een kop en een staart. Na al die jaren staren naar die cirkelstruktuur die nooit zal verjaren kan ik nu verklaren: de zaak is rond. Het laatste waarop de wereld dan zit te wachten is een nawoord. Echter, de ervaring leert dat de meeste lezers binnen en buiten het vakgebied het nawoord, de stellingen en het curriculum het meest kritisch lezen. Daarom wil ik een ieder bedanken die op-wat-voor-wijze-dan-ook een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Deze zin lees je overal. Nu wil ik sommigen speciaal (met name) noemen.

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-Pa en Ma, voor alle mogelijkheden
-Huisgenoten, voor het samen leven in een lekker huisje

Levensloop

Op een koude herfstdag, 19 november 1962, werd ik in Den Haag geboren, en kreeg de namen Michaël Willem Breedveld mee. Op de lagere school, Willem de Zwijgerschool te Scheveningen, wist ik al wat afkijken was. De onderwijzeres dacht aan een oogafwijking en daarvoor ben ik haar nog eeuwig dankbaar. In juni 1980 verkreeg ik het diploma VWO aan de Prot. Chr. Sch. Gem. "Zandvliet" te Den Haag.

In september 1980 begon ik aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Het kandidaatsexamen werd in januari 1984 behaald. Het doktoraalexamen in de Landbouwwetenschappen, studierichting Moleculaire Wetensachappen, omvatte de volgende vakken: Erfelijkheidsleer (bijvak; Bos/van der Veen), Biochemie (hoofdvak; Müller), Chemische Microbiologie (hoofdvak; Brons/Zehnder), en Pedagogiek en Algemene Didactiek (extra vak, ter verkrijging van de onderwijsbevoegdheid scheikunde 1e-graads). Gedurende de maanden juni 1986-mei 1987 voerde ik een boeiende stage uit aan de vakgroep Pathology, Faculty of Medicine, University of British Columbia, Vancouver, Canada (Moore/Autor). Het was een leerzame periode: ik leerde dat bij een vliegreis de maaltijden bij de vliegprijs waren inbegrepen. Het ingenieursdiploma behaalde ik in juni 1987.

Gedurende augustus 1987-december 1991 verrichtte ik een promotie-onderzoek bij de vakgroep Microbiologie, Landbouwuniversiteit Wageningen, aan de oligo-en polysaccharide synthese van *Rhizobium* bakteriën, waarvan dit proefschrift de afsluiting vormt.