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Succinoglycan and galactoglucan

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Succinoglycan and galactoglucan, also known as EPS-I and EPS-II, respectively, have much been studied in *Rhizobium meliloti* isolated from the root nodules of lucerne. These polysaccharides have also been obtained from a number of other bacteria, which have been isolated from diverse sources of soil and water communities.

In 1965 Harada *et al.* isolated from soil a bacterium named *Alcaligenes faecalis* var. *myxogenes* which produces a polysaccharide that for the first time was characterized and named as 'succinoglycan'. Succinoglycans were found to be synthesized normally by strains of *Agrobacterium tumefaciens*, *A. radiobacter* and *Rhizobium meliloti* and were also produced by many strains of *Pseudomonas* spp. isolated from activated sludge and from other sources. They had component compositions of D-glucose, D-galactose, pyruvate, succinate and acetyl in the ratios 7:1:1:1:1. Because of their useful applications as an emulsion-stabilizing, suspending and thickening agent, succinoglycans were produced industrially by Shell.

Galactoglucan-producing bacteria were first encountered in activated sludge in 1974 and could reproducibly be isolated from water sediments and from biofilms in water streams and were identified as *Agrobacterium radiobacter* and *Pseudomonas* spp. On isolation of these bacteria, colonies were recognized by their very viscous appearance on agar plates. Polymers were found to have very simple component compositions of D-glucose, D-galactose and pyruvate in the ratios 1:1:1. These highly pyruvylated polymers, which were named 'galactoglucans', were structurally identified as having a disaccharide repeating unit of alternatively (1→3)-linked D-glucose and D-galactose residues. Surprisingly, *Rhizobium meliloti* mutant strains were found, which overproduced galactoglucans instead of normal succinoglycan.

The osmotic conditions of the growth media, in which the bacteria were cultivated, were found to have a profound effect on the direction of synthesis of the succinoglycan/galactoglucan oligomeric and polymeric products and it was proposed that these products are related by a common biosynthetic pathway (presented in part at the Beijerinck Centennial Congress in The Hague, The Netherlands, 10–14 December 1995). © 1997 Elsevier Science Ltd

INTRODUCTION

Succinoglycan and galactoglucan, secreted by *Rhizobium meliloti*, have been extensively studied to understand their roles in symbiosis. Much work has been done to unravel the fine structure, biosynthesis and genetic analysis of these polysaccharides and to relate their chemical structures to the infective properties of this bacterium in its host plant *Medicago sativa* (lucerne) (Her *et al.*, 1990; Glucksmann *et al.*, 1993; Reinhold *et al.*, 1994). *R. meliloti* mutant strains synthesizing principally either succinoglycan or galactoglucan were found to be successful in nodule induction and development leading to nitrogen fixation (Zhan *et al.*, 1989).

Apart from the above-mentioned bacteria belonging to the family *Rhizobiaceae* many other bacteria have been isolated from diverse sources which produce succinoglycan and/or galactoglucan (for a recent review on curdlan and succinoglycan see Harada and Harada, 1995). These bacteria which have been isolated from soil and water communities have no proven relationships with plants to form nodules or galls and will be described in this review.

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SUCCINOGLYCAN AND GALACTOGLUCAN PRODUCING BACTERIA BELONGING TO THE FAMILY OF RHIZOBIACEAE ISOLATED FROM PLANTS

Succinoglycan was found to be synthesized generally by many strains of *Agrobacterium tumefaciens* and by *Rhizobium meliloti* (Zevenhuizen, 1971, 1973). The polysaccharide was found to be composed of octasaccharide repeat units (see Fig. 1(a)) (Jansson *et al.*, 1977). In the medium generally used for cultivation of *Rhizobium* and containing (per litre) 10 g mannitol and 1 g glutamic acid and salts, phytopathogenic *Agrobacterium tumefaciens* strains produced high amounts of succinoglycan (3.0 g) and variable amounts of insoluble curdlan, a linear β-(1→3)-linked glucan, whereas *R. meliloti* produced only low amounts (300 mg/litre) of succinoglycan and in addition was found to excrete high amounts of monomeric repeat units of succinoglycan (Zevenhuizen and van Neerven, 1983). Addition of 0.2 M NaCl to the medium stimulated succinoglycan production by *R. meliloti* strain SU-47 at the expense of the repeating units. Increasing the concentration of medium components in the growth medium to 40 g mannitol and 8 g glutamic acid yielded exclusively HMW succinoglycan (8 g/litre; Table 1) (Breedveld *et al.*, 1990).

Mutants of *R. meliloti* SU-47 have been isolated that are unable to produce (normal) succinoglycan, but do induce and invade nodules in lucerne roots. These mutants were found to overproduce galactoglucan composed of a disaccharide repeating unit -β-D-Glc*p*-(1→3)-α-D-Gal*p*(1→3)- having a 6-*O*-acetyl group attached to most of the D-glucose residues and a 4,6-*O*-(1-carboxyethylidene)-group attached to all D-galactose residues (Fig. 1(b)) (Her *et al.*, 1990; Levery *et al.*, 1991).

Rhizobium meliloti, strain YE-2, was found to produce galactoglucan instead of normal succinoglycan (Zevenhuizen, 1989). The population of the bacteria was clonally heterogeneous in polymer production and mucoid character. On plating YE-2 on agar the population segregated into small white colonies (YE-2w) producing succinoglycan and heavy slimy colonies (YE-2SL) producing galactoglucan. The latter colonies were not stable and on subculture they separated again into small and viscous colony forms on agar plates. In liquid glutamic-acid–mannitol medium YE-2SL was found to excrete comparable quantities of succinoglycan repeating units and galactoglucan. As NaCl was added progressively to the growth medium, the repeating units nearly completely disappeared and the galactoglucan was gradually replaced by a succinoglycan (Table 1) (Zevenhuizen, 1990; Zevenhuizen and Faleschini, 1991).

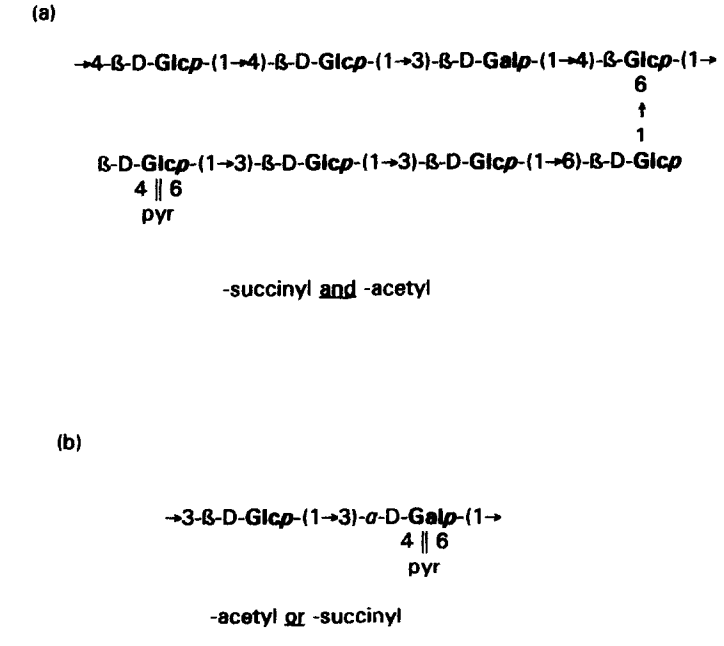

Fig. 1. Repeating unit structures and substituent groups of: (a) succinoglycan (from a number of succinoglycans of different microbial sources NMR data showed that succinate is located on the O-6 of either one or two side chain 3-linked β-D-Glc residues, whereas the acetate (when it is present) is located on one of the O-6 of backbone 4-linked β-D-Glc units (Matulová *et al.*, 1994); (b) galactoglucan (the *Rhizobium meliloti* polymer was found to be substituted by *O*-acetyl at the 6-position of most of the glucose units (Her *et al.*, 1990)); on re-examination by NMR analysis the polymer of *Pseudomonas marginalis* was found to be substituted by succinate at the O-2 of the D-galactose residues (Matulová *et al.*, 1996).

Table 1. Production of HM EPS (succinoglycan and galactoglucan) and LM EPS (octasaccharide repeat units of succinoglycan) by *Rhizobium meliloti* at different concentrations of sodium chloride in the medium. Bacteria were cultivated in glutamic acid–mannitol–salts (50 ml) in Erlenmeyer flasks (100 ml) on a shaker at 25°C (Breedveld *et al.*, 1990; Zevenhuizen, 1990)

Bacterial strain	Cultural conditions			Cell yields and polymer production			
	Glu (g/litre)	Man (g/litre)	Incubation time (days)	Cell protein (μg/ml)	Excreted products		
					LM EPS (μg/ml)	HM EPS (μg/ml)	
Rhizobium meliloti strain SU-47						Succinoglycan	
without added salt	1	5	7	500	664	114	
+200 mM NaCl	1	5	7	500	228	744	
+600 mM NaCl	1	5	7	500	105	440	
without added salt	1	10	14	455	1100	240	
+200 mM NaCl	1	10	14	470	470	2330	
without added salt	8	40	12	4000	400	7800	
Rhizobium meliloti strain YE-2SL						Succinoglycan + galactoglucan	
						Total	Ratios
without added salt	1	5	10	500	755	715	25:75
+200 mM NaCl	1	5	10	500	450	1000	50:50
+600 mM NaCl	1	5	10	500	129	1100	85:15

Procedure. After cultivation of the bacteria at the indicated cultural conditions cells were removed by centrifugation. They were resuspended in water and measured for their cell protein content according to Lowry. Culture supernatants were mixed under stirring with three volumes of ethanol in order to precipitate HM EPS. Precipitated polymers were dissolved in water and measured with anthrone–sulphuric-acid for carbohydrates and expressed as μg glucose equivalents per ml of culture. Ratios of succinoglycan:galactoglucan in the HM EPS preparations were determined by component and glycosidic linkage analysis of isolated dry specimens. LMW carbohydrates in the alcoholic supernatants and containing principally succinoglycan repeat units were also measured with anthrone–sulphuric-acid and expressed as glucose equivalents per ml of culture (Zevenhuizen and Faleschini, 1991).

PRODUCTION OF SUCCINOGLYCAN AND GALACTOGLUCAN BY BACTERIA ISOLATED FROM SOIL AND WATER WITH NO PROVEN ABILITIES TO FORM NODULES OR GALLS

In 1965 Harada *et al.* isolated from soil a bacterium named *Alcaligenes faecalis* var. *myxogenes* 10C3, which produced a polysaccharide that for the first time was characterized and named as a succinoglycan (Misaki *et al.*, 1969; Harada *et al.*, 1979). It produced 2 g succinoglycan in 100 ml medium containing 4 g glucose and 150 mg $(NH_4)_2HPO_4$. A spontaneous mutant obtained on subculture of this bacterium had lost the capacity to produce succinoglycan but instead produced curdlan (reviewed by Harada and Harada, 1995).

During research on floculation of bacteria isolated from activated sludge from water purification plants, sometimes heavy slime-forming bacteria were isolated which produced a polysaccharide identified as a succinoglycan. One such bacterium, strain SL, was identified as a species of *Pseudomonas* and its polymeric product was characterized by component analysis to contain D-glucose:D-galactose:pyruvate:acetyl in the ratios 7:1:1:0.5 (Ebbink and Zevenhuizen, 1971). From a polydiskfilter in a paper factory a bacterium, strain 201-25, was isolated and identified as *Agrobacterium radiobacter*. On testing its polysaccharide synthesis in a standard 0.1% glutamic acid–0.5% mannitol–salts medium, it was found to produce 2.3 mg/ml (46% yield) of a polysaccharide which on methylation analysis afforded a pattern of methylated sugars identical with those obtained with succinoglycan products of *Rhizobium meliloti* bacteria (van der Worp and Zevenhuizen, 1985).

Because of their useful applications as an emulsion-

stabilizing, suspending or thickening agent, succinoglycan was produced industrially by Shell and marketed as Alphaflo (International Bio-Synthetics, 1988).

Galactoglucan-producing bacteria could reproducibly be isolated from municipal and dairy waste activated sludge as follows. Cells were loosened by mechanical treatment from the flocs and after serial 1:10 dilution of the suspension in sterile water they were streaked onto 0.1% casitone–0.2% glycerol–0.035% yeast extract–agar. After incubation for 3 days at 25°C large (diameter 1 cm), clear polysaccharide-overproducing colonies were obtained, which were cultivated to purity. Polysaccharide production did not appear to be a constant property, as, during storage on agar, repeated checks gave mixtures of large, viscous colonies and small, less viscous colony types. They were identified as *Achromobacter* spp. (Zevenhuizen and Ebbink, 1974) and later, when they were again isolated from water sediments, identified as *Agrobacterium radiobacter* (Table 2) (Zevenhuizen, 1989). The polymers had component compositions of D-glucose, D-galactose and pyruvate in a 1:1:1 ratio, were devoid of succinate and variable in acetyl substituents. They were structurally identified by methylation analysis, periodate oxidation, NMR and partial hydrolysis to have a disaccharide backbone of -3-β-D-Glc*p*-(1→3)-α-D-Gal*p*-1- which was 4,6-*O*-(1-carboxyethylidene) (pyruvate) substituted at the D-galactose units. It was also noted that the solabiosyl -β-D-Glc*p*-(1→3)-D-Gal*p*- structural element is common to both succinoglycan and galactoglucan repeating units (see Fig. 1(a) and (b)). During this research a polymer of *Bacillus megaterium* was obtained, which on structural investigation was found to be composed of a trisaccharide repeating unit -4-D-Glc*p*-(1→4)-D-Glc*p*-(1→3)-D-Gal*p*-1- and likewise was highly pyruvylated at the D-galactose residues (unpublished data 1973; re-examined by Segond *et al.*, 1983).

Galactoglucan-type polymers are produced by bacterial species from diverse sources. From cobble-associated biofilms in water streams large numbers of polysaccharide-overproducing organisms were isolated

Table 2. Galactogluco-polysaccharides (molar compositions)

Source	Glucose	Galactose	Pyruvate	Succinate	Acetate
Succinoglycans					
Agrobacterium tumefaciens[a]	7	1	1	n.d.	+
Pseudomonas strain SL[b]	7	1	1	n.d.	tr.
Agrobacterium radiobacter[c]	6	1	1	n.d.	n.d.
Alcaligenes faecalis[d]	7	1	n.d.	1.5	n.d.
Rhizobium meliloti[e]	7	1	1	1	1
Galactoglucans					
Achromobacter spp.[f]	1	1	1	—	+
Agrobacterium radiobacter[g,h]	1	1	1	—	—
Rhizobium meliloti[h,i]	1	1	1	—	+
Pseudomonas putida[j]	1	1	1	?	?
Pseudomonas fluorescens[j]	1	1	1	?	?
Pseudomonas marginalis[k]	1	1	1	1	—

+, Present; —, absent; tr, trace; n.d., not determined.
[a] Isolated from diseased plants (Zevenhuizen, 1973).
[b] Isolated from activated sludge (Ebbink and Zevenhuizen, 1971).
[c] From a polydiskfilter in a paper factory (van der Worp and Zevenhuizen, 1985).
[d] Misaki *et al.* (1969).
[e] From root nodules of lucerne (Zevenhuizen, 1973; Zevenhuizen and van Neerven, 1983).
[f] From activated sludge (Zevenhuizen and Ebbink, 1974).
[g] Isolated from water sediments.
[h] Zevenhuizen (1989).
[i] Zevenhuizen (1990), Zevenhuizen and Faleschini (1991).
[j] From biofilms in water streams (Read and Costerton, 1987); associated with commercial mushroom production (Fett *et al.*, 1995).
[k] Spoilage of fruits and vegetables (Osman and Fett, 1989).

in numbers of 10^5 cells per cm^2 of *Pseudomonas* which were very mucoid in nature. *P. fluorescens* strain MC2 produced an exopolysaccharide composed of D-glucose, D-galactose and pyruvate in the ratios of 1:1:1 and *P. putida* strain BR7 produced an exopolysaccharide composed of D-glucose, D-galactose and pyruvate in the ratios of 1:1:0.5. Polymers from both strains were acetylated to a variable degree but their structures were not determined (Read and Costerton, 1987).

An acidic exopolysaccharide termed marginalan was extracted from cultures of phytopathogenic *Pseudomonas marginalis* and characterized as a (1→3)-linked galactoglucan substituted with Gal(4,6-pyruvylated) and Glc (presumably succinylated at either position 2 or position 4) (Osman and Fett, 1989). *Pseudomonas putida* and *P. fluorescens*, which are associated with commercial mushroom production, likewise produced galactoglucan (Fett *et al.*, 1995).

PROPERTIES OF SUCCINOGLYCAN AND GALACTOGLUCAN IN SOLUTION AND IN THE SOLID STATE

Solution properties indicated that the conformation of galactoglucan from *Rhizobium meliloti* is not ordered (entanglement network) in dilute solution. Rheological studies of succinoglycan produced results typical of a weak gel system which can be attributed to the ordered conformation adopted by the succinoglycan in solution (Navarini *et al.*, 1992; Cesàro *et al.*, 1992). Three-dimensional molecular structure has been determined by X-ray diffraction of polycrystalline and well oriented specimens of the potassium salt of galactoglucan from *R. meliloti* (Chandrasekaran *et al.*, 1994a, b).

CONCLUDING REMARKS

The fact that *Rhizobium meliloti* strains synthesizing principally either succinoglycan (*R. meliloti* SU-47) or galactoglucan (*R. meliloti* YE-2SL), and both cases simultananeously excreting LMW succinoglycan, are equally effective in promoting nodule invasion in alfalfa begs the question of whether there is a common role for these oligosaccharide forms during infection processes. It was indeed found that specific oligosaccharide forms of succinoglycan promoted nodule invasion in alfalfa (Pühler *et al.*, 1991; Battisti *et al.*, 1992).

In bacterial ecosystems the production and functioning of succinoglycan/galactoglucan should not be restricted to a mere specific role during symbiosis of *Rhizobium* bacteria with their appropriate host plant; they point to a much more general functioning in bacterial systems, such as water-holding capacity, adhesive properties in bacterial flocs and biofilms of water organisms, and in aiding soil aggregation and stability. In literature studies on bacterium–plant relations — apart from soluble acidic heteropolysaccharides — no consideration was given to the roles of another major group of insoluble neutral polymers produced by Rhizobiaceae, namely the gel-forming curdlan of *Agrobacterium tumefaciens* and the gel-forming capsular polysaccharide (CPS) of *Rhizobium leguminosarum* (Zevenhuizen, 1990). Only by systematically measuring all carbohydrate fractions, cellular and extracellular, during the development of the bacteria will their occurrences, their identities and clues to their functioning be discovered.

Therefore, the use of Calcofluor-white (Leigh *et al.*, 1985) in agar plates for screening is too simple a selection criterium to distingish between succinoglycan/galactoglucan producing bacteria (succinoglycan is assumed to be the Calcofluor-binding polymer) because it is a well known fact that many strains of *Rhizobium* bacteria in addition produce cellulosic microfibrils (Zevenhuizen *et al.*, 1986) as extracellular polysaccharide and give even stronger fluorescence on Calcofluor-containing agar plates; the same holds also in the case of curdlan when present in pellets of *Agrobacterium* bacteria (unpublished data).

The osmotic pressure of the media, in which bacteria in this study were grown and from which they were isolated, determines a selection mechanism for the organism to produce either galactoglucan or succinoglycan. Low osmolarity, which often prevails in freshwater communities, gives rise to organisms among which galactoglucan-producing bacteria are predominant, while bacteria from soil with variable salinity often give rise to succinoglycan-producing organisms. Bacteria synthesizing both types of polysaccharides, such as *R. meliloti*, excrete at low osmotic pressure of the growth medium the galactoglucan-type polymer and low molecular weight succinoglycan repeat units, while at high osmotic pressure (> 10 atm) of the growth medium succinoglycan production was stimulated at the expense of the repeating units.

Bacteria producing galactoglucan were not found to excrete particular repeat units connected with this type of polymer, but instead produce as a low molecular weight fraction pyruvylated and succinylated repeat units of succinoglycan (*R. meliloti* strain YE-2) and sometimes cyclic β-1,2-glucans (*A. radiobacter*, Is.II) (Zevenhuizen and van Veldhuizen, 1988). Because of the common structural motif -β-D-Glc*p*-(1→3)-D-Gal*p*- in succinoglycan/galactoglucan it was tempting to assume a common biosynthetic route for both types of polysaccharides. Pathways for the biosynthesis of succinoglycan by *Rhizobium meliloti* (Tolmasky *et al.*, 1982) and by *Agrobacterium tumefaciens* (Staneloni *et*

al., 1984) have been proposed starting with UDPG and by the transfer of sugar residues to lipid-bound intermediates as follows: D-galactose, disaccharide solabiosyl -β-D-Glc*p*-(1→3)-D-Gal*p*-, tetrasaccharide, octasaccharide, pyruvylated octasaccharide followed by polymerization. Highly productive succinoglycan-producing organisms will follow this route leading exclusively to final succinoglycan polymer (Linton *et al.*, 1987). Others, like many strains of *R.meliloti*, seem to be partially blocked — at low osmolarity of the growth medium — at the polymerization step and excrete besides succinoglycan considerable quantities of octasaccharide repeat units. Others again, like *R. meliloti* YE-2SL, although still excreting octasaccharide repeat units, seem to be totally blocked at the polymerization step to succinoglycan, but have found an alternative pathway for polymerization taking place after the first two sugar tranfer steps of the pathway, giving rise to galactoglucan polymer. Still other bacteria (*A. radiobacter*, Is.II) do not excrete octasaccharide repeat units either and will follow only the short route leading to galactoglucan (Zevenhuizen, 1989).

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