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Effect of the concentration of sodium chloride in the medium on the relative proportions of poly- and oligo-saccharides excreted by *Rhizobium meliloti* strain YE-2SL

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ABSTRACT

Rhizobium meliloti mutant strains have been found which, in the presence of low concentrations of NaCl, produce a galactoglucan instead of the usual succinoglycan. When grown in a mannitol–glutamic acid–salts medium, the principal products secreted by *R. meliloti* YE-2Sl were comparable quantities of succinoglycan repeating-units and galactoglucan. As NaCl was added progressively to the culture medium, the repeating units nearly completely disappeared and the galactoglucan was gradually replaced by a succinoglycan.

INTRODUCTION

Fast-growing strains of *Rhizobium* and *Agrobacterium* (*Rhizobiaceae*) are well known producers of a broad range of oligo- and poly-saccharides. It is common to find[1,2] exopolysaccharides and capsular polysaccharides in the slime that surrounds the cells, in addition to glycogen, (1→2)-β-D-glucans, and oligosaccharides which are contained within the cells. *R. meliloti* SU-47, in addition to producing a succinoglycan (**1**, EPS-I), excretes considerable amounts of its octasaccharide repeating-unit. This octasaccharide, as well as a galactoglucan (**2**, EPS-II), are excreted by *R. meliloti* YE-2.

A common pathway has been proposed for the biosynthesis of the exopolysaccharides[3], and those for *R. meliloti* strains YE-2 and SU-47 can be modified by the stimulation or inhibition of a particular enzymic system. Studies[4] of the regulation of the biosynthesis by *R. meliloti* SU-47 have suggested that different osmotic conditions modify not only the production of exopolysaccharides, but also the low-molecular-weight carbohydrates in the cells.

We now report on the effects of different concentrations of NaCl in the culture medium on the production of exopolysaccharides by *R. meliloti* YE-2.

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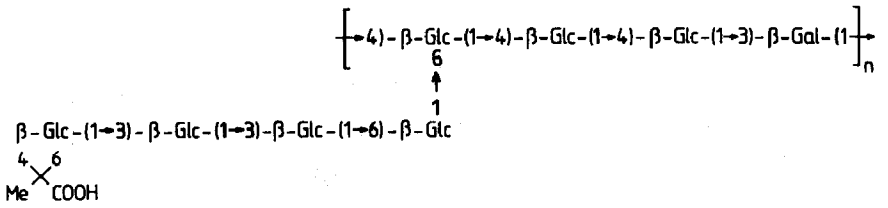
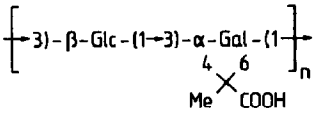


1 (EPS-I)



2 (EPS-II)

EXPERIMENTAL

Bacterial strains and their cultivation. — *R. meliloti* YE-2 was obtained from the bacterial collection of the Agricultural University in Wageningen. The bacteria were isolated from the root nodules of lucerne grown in acid (pH 4.5) peat and inoculated with soil of Yemen. The bacteria, which originally had been purified to homogeneity, displayed dimorphy with large, viscous (YE-2Sl) and small, white colony types (YE-2w). The slimy variant, which produced EPS-II (**2**), was selected from the plates and used as an inoculum. The cultures were grown on a rotary shaker at 25° in 50 mL of GMS medium contained in 100-mL conical flasks. The osmotic conditions tested were 0, 0.2, 0.4, and 0.6M NaCl. The GMS medium contained glutamic acid, 1; mannitol, 5; K_2HPO_4, 1; $MgSO_4 \cdot 6H_2O$, 0.2; and $CaCl_2$, 0.04 g/L; together with $FeCl_3 \cdot 6H_2O$, 2.5; H_3BO_3, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $MnCl_2$, 1.0; and $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg/L; biotin, 10; and thiamine, 100 μg/L; pH 7.0.

The cultures were removed from the shaker after 1, 2, 4, 7, and 10 days, and centrifuged at 40 000*g* for 30 min. The sedimented cells were resuspended in water (10 mL) and assayed[5] for protein (expressed as μg of serum albumin/mL of culture).

The total concentration of exopolysaccharides was measured by the anthrone–sulphuric acid reaction[6] in the supernatant solutions and expressed as μg of glucose equiv./mL of culture. The same samples were used to measure the concentration of mannitol, using the periodate–chromotropic acid method[7].

The fractionation of products of low (octasaccharide) and high molecular weight (EPS-I and EPS-II) was performed by adding 3 vol. of ethanol to each supernatant solution. The precipitate was collected by centrifugation and redissolved in the original volume of water. Both the ethanolic supernatant solutions and the aqueous solutions were assayed with the anthrone–sulphuric acid reagent.

Analysis of the exopolysaccharides. — Colorimetric determinations of pyruvate[8] and acetyl[9] were performed on aqueous solutions of the freeze-dried exopolysaccharides. Succinate was determined by g.l.c. of the methyl ester after hydrolysis of the

exopolysaccharide in 2M trifluoroacetic acid[2]. The neutral sugars in the hydrolysates were converted into the alditol acetates and analysed by g.l.c. on a Sil 43CB column (Chrompack, Middelburg, The Netherlands) at 210°. Methylation analysis was carried out according to the procedure of Harris *et al.*[11]. Viscosimetry on the culture supernatant solutions involved an Ubbelohde capillary viscometer at 25°.

RESULTS

Production of exocellular saccharides by R. meliloti *YE-2SL during batch cultivation, as a function of added salt.* — The growth of the bacteria was followed by determination of the protein content of the biomass as a function of time at different conditions of added salt (Fig. 1). In the presence of 0.2, 0.4, and 0.6M NaCl, the growth showed a longer lag period compared to that in the absence of salt. There was an induction period also in the biosynthesis and secretion of the exopolysaccharides (Fig. 2). Nevertheless, the *total* amount of such compounds (expressed as glucose equiv.)

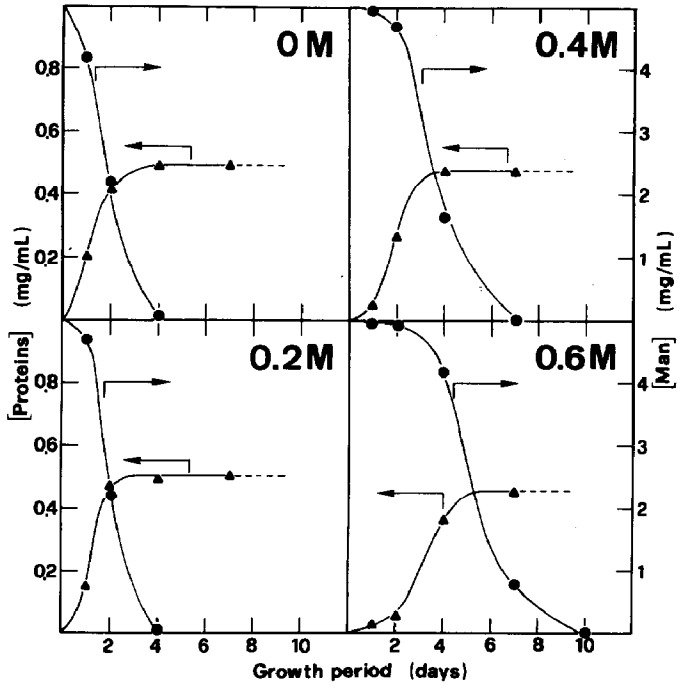


Fig. 1. Production of protein (▲) and consumption of mannitol (●) during batch cultivation of *R. meliloti* YE-2Sl at different concentrations of NaCl.

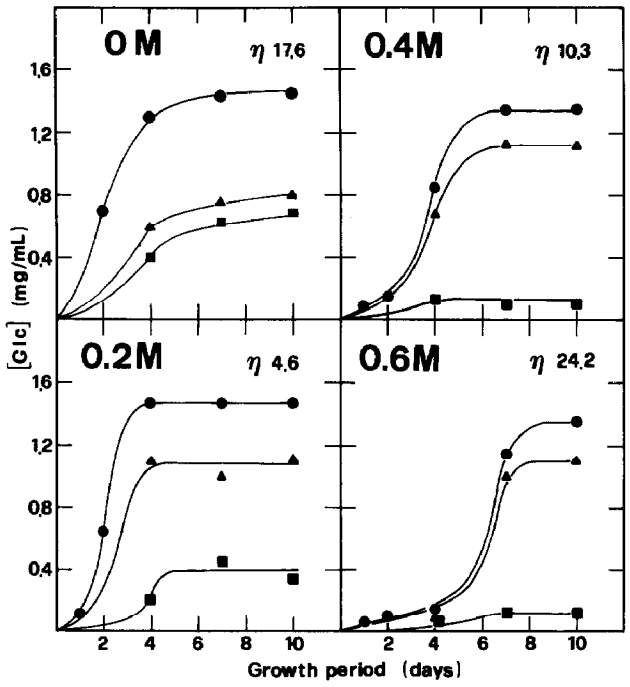


Fig. 2. Carbohydrates excreted from *R. meliloti* YE-2Sl during batch cultivation in the presence of different concentrations of NaCl: total carbohydrate production (●), EPS-I + EPS-II (▲), EPS-I repeating units (■); η, end viscosities (cPoises) of the cultures.

released into the medium at the end of the exponential phase of growth did not change significantly with increase in the amount of salt added.

EPS-I (**1**) and EPS-II (**2**) were found[3] to be present only as separate products in the culture media of different YE strains. In the present work, there was a significant change in the pattern of production on changing the concentration of the added salt (Fig. 2). In the absence of NaCl, there was a comparable production of EPS-I repeating units and EPS-II. On adding NaCl to the culture medium, the synthesis of the repeating units was depressed. This behaviour could reflect stimulation of the polymerisation of the repeating units to give EPS-I. In this situation, EPS-I cannot be separated from EPS-II by precipitation with ethanol and it was necessary to analyse the mixture of freeze-dried materials obtained by precipitation with ethanol. The proportions of EPS-I (**1**) and EPS-II (**2**) after incubation for 10 days were calculated from the Glc:Gal ratios (EPS-I contains Glc:Gal:pyruvyl:succinyl:acetyl in the ratios 7:1:1:1:1 and EPS-II contains Glc:Gal:pyruvyl:acetyl in the ratios 1:1:1:1). Analyses of the pyruvyl, acetyl and succinyl groups also showed the change in the polysaccharide compositions of the mixtures. The results are shown in Table I.

That there were no repeating units other than those of EPS-I and EPS-II was evident from the results of the methylation analyses (Table II), which showed that only those two repeating units were present, namely, for EPS-II with (1→3)-linked Glc and

TABLE I

Analysis of high-molecular-weight exopolysaccharide obtained from cultures of *R. meliloti* YE-2Sl after incubation for 10 days in the presence of various concentrations of salt

NaCl (M)	*Glc*	*Gal*	*Pyruvate*	*Acetyl*	*Succinate*	*EPS-I (%)*	*EPS-II (%)*
	(molar ratio)						
0	1.47	1	0.94	0.89	0.08	25	75
0.2	2.18	1	0.87	0.80	0.25	50	50
0.4	3.90	1	1.05	1.23	0.54	78	22
0.6	4.60	1	1.07	1.40	0.71	85	15

TABLE II

Methylation analysis (weight %) of high-molecular-weight exopolysaccharides excreted by *R. meliloti* YE-2Sl in the presence of various concentrations of salt

Methylated sugar[a]	T[b]	*NaCl (M)*			
		0	*0.2*	*0.4*	*0.6*
2,4,6-Glc	1.43	43.8	38.0	27.2	25.4
2,4,6-Gal	1.52	5.7	8.5	11.5	12.3
2,3,4-Glc / 2,3,6-Glc	1.62	7.8	16.0	25.8	27.0
2,3-Glc	2.50	7.9	14.5	26.6	29.8
2-Gal	3.30	34.8	22.9	8.9	5.5

[a] 2,4,6-Glc connotes 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol *etc.* [b] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a column of Sil-43 CB at 210°. The products were identified by comparison of their retention times with those of methylated sugars obtained from reference compounds[2,12].

Gal (4,6-pyruvylated), and for EPS-I with (1→3), (1→4)-, and (1→6)-linked Glc, (1→3)-linked Gal, branching (1→4,6)-linked Glc, and terminal Glc (4,6-pyruvylated). The Glc:Gal ratios could be determined also from the results of the methylation analyses. High-molecular-weight products, formed in the absence of NaCl, appeared to be composed mainly of EPS-II together with 25% of EPS-I. On increasing the concentration of NaCl in the medium to 0.6M, the proportions of EPS-I increased to 85%, and the viscosity of the culture medium increased after an initial drop of viscosity (Fig. 2). Aqueous solutions of EPS-I and EPS-II exhibit high viscosities. However, EPS-II had a much reduced viscosity in 0.2M NaCl, whereas that of EPS-I was insensitive to added NaCl. Consequently, there was a sharp drop in viscosity in the culture medium at 0.2M NaCl when the ratio of EPS-I and EPS-II was 1:1.

DISCUSSION

R. meliloti YE-2 shows variability with respect to its synthesis of exopolysaccha-

rides. During prolonged storage of the bacterium on agar slants, subsequent growth on agar plates always leads to different forms of colonies of a non-viscous white form (YE-2w) and a heavy slimy form (YE-2Sl). On introduction of the YE-2w variant into an MGS medium (see Experimental), there was low production of EPS-I, which was stimulated by the addition of salt at the expense of the EPS-I repeating units also present in the supernatant solution (results not shown). The YE-2w variant was stable and did not tend to convert into the slimy YE-2Sl variant. The slimy form, in the presence of low concentrations of NaCl, produced mainly EPS-II together with EPS-I repeating units. As the concentration of NaCl was increased, EPS-I became almost the sole product.

Galactoglucan-producing bacteria with an unstable character have been found as isolates from water sediments (*Achromobacter* spp.)[12] and showed dimorphy with small compact colonies and large viscous colony forms. Such bacteria with a more stable character have been isolated and identified as *Agrobacterium radiobacter*[3], which did not excrete EPS-I repeating units, but excreted cyclic (1→2)-β-D-glucans highly substituted with glycerol 1-phosphate moieties[13].

Galactoglucan-type polymers are produced by diverse bacterial species, including mutant strains of *R. meliloti*, and by many bacteria isolated from water sediments. Recently, an acidic exopolysaccharide (marginalan) isolated from a phytopathogenic pseudomonas *P. marginalis*[14] was characterised as a (1→3)-linked galactoglucan substituted with Gal (4,6-pyruvylated) and Glc (succinylated at either positions 2 or 4).

Apart from the naturally occurring bacterial isolates that synthesise galactoglucans, several mutant strains of *R. meliloti* SU-47 have been described during genetic experiments aimed at the production of exo-mutants, that failed to produce (normal) EPS-I and were defective in nodulation and nitrogen fixation[15,16]. Their polymers were identical in structure to that proposed for EPS-II, to be devoid of succinate, and to contain one acetyl group per repeating unit[16]. This EPS-II replaced EPS-I in nodule invasion[15] and suppressed the symbiotic defects of EPS-I-deficient strains on lucerne. Likewise, the mutant forms of *R. meliloti* YE-2w and YE-2Sl were each equally effective in nodulation and nitrogen-fixation experiments on lucerne seedlings[17].

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