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Manganese elicits the synthesis of a novel exopolysaccharide in an arctic *Rhizobium*

Vasu D. Appanna and Caroline M. Preston*

Lady Davis Institute for Medical Research of Sir Mortimer B. Davis, Jewish General Hospital, Montreal, Quebec H3T 1E2 and **Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada*

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Under the influence of manganese (400 μM), the arctic *Rhizobium* N_{11} [(1986) Arch. Microbiol. 146, 12–18] appeared to elaborate an exopolysaccharide that showed marked diversity with that synthesized in a medium deficient in added manganese. Both total carbohydrate and the uronic acid contents of these biomolecules appear to differ substantially. ^{13}C-NMR spectroscopy revealed three distinct signals in the anomeric region of the spectrum derived from the biopolymer secreted in the manganese rich medium, while that from the other exopolysaccharide exhibited numerous peaks. The resonance lines indicative of the non-carbohydrate moieties were also found to be dissimilar. Further evidence for the unidentical nature of these biopolymers was obtained from viscosity and optical rotation measurements.

Manganese; Exopolysaccharide; Uronic Acid; Non-carbohydrate moiety; ^{13}C-NMR; (*Rhizobium*)

1. INTRODUCTION

Numerous microbes are known to produce copious amounts of exopolysaccharides. These biomolecules possess diverse chemical structures and appear to be involved in a wide array of biological processes [2]. While polysaccharide antigens confer pathogenic properties to *E. coli* [3], immunomodulatory characteristics have been shown to be associated with exopolysaccharides from other bacteria [4]. Many plant diseases have been attributed to microbes that have the ability to produce these biopolymers [5]. The possible involvement of these complex carbohydrates in microenvironmental regulation has also been documented [6].

Bacteria belonging to the genus *Rhizobium*, when residing in the root nodules of leguminous plants, contribute immensely to the global nitrogen budget [7]. Secretion of exopolysaccharides appears to be a common feature of these microorganisms [8]. Although these complex compounds may be associated with unknown function(s), the notion that they have a determinant role in establishing an effective symbiosis between the *Rhizobium* and the plant host has been subject of active investigation [9]. In this paper we demonstrate that the exopolysaccharide elaborated by the arctic *Rhizobium* N_{11} [1] under the influence of manganese appears to be different from that obtained in a normal growth culture (unamended with manganese). The possible biological significance of such structural variations is also discussed.

Correspondence (present) address: V.D. Appanna, Dept of Chemistry, McGill University, 3420 University St, Montreal, Quebec H3A 2A7, Canada

2. MATERIALS AND METHODS

m-Hydroxydiphenyl and D-glucuronic acid were obtained from Sigma while Folin Ciocalteu's phenol reagent was from Anachemia. D-Glucose assay kit was a product of Sclava Diagnostics.

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2.1. *Organism and culture conditions*

The arctic *Rhizobium* N_{11} [1], was isolated from the root nodules of the leguminous plant, *Oxytropis maydelliana*, found in the Canadian northwest territories. The *Rhizobium* was maintained at 4°C on yeast extract and mannitol [10], solidified by the inclusion of 2% agar, and subcultured monthly. The control liquid medium contained yeast extract ($0.5\ g \cdot l^{-1}$), mannitol ($10\ g \cdot l^{-1}$), K_2HPO_4 ($0.18\ g \cdot l^{-1}$), NaCl ($0.1\ g \cdot l^{-1}$), $MgSO_4$ ($0.15\ g \cdot l^{-1}$) and was amended with an appropriate amount ($400\ \mu M\ Mn^{2+}$) of filter-sterilized aqueous solutions of $MnCl_2 \cdot H_2O$ in test experiments. The cultures were incubated at 23°C and aerated on a rotary shaker at 250 rpm.

2.2. *Cell growth determination*

At various time intervals samples of culture (10 ml) were harvested by centrifugation ($5000 \times g$) and cellular growth was monitored by the Lowry method [11]. Serum albumin was used as standard.

2.3. *Isolation and purification of exopolysaccharides*

At the stationary phase of growth, cells were removed by centrifugation ($20000 \times g$), for 30 min at 4°C and the exopolysaccharides were liberated from the supernatant by 3 vols of cold ethanol. Purification was effected by reprecipitation and extensive dialysis (72 h) against distilled water.

2.4. *Analytical methods*

Total carbohydrate content of the purified exopolysaccharides was determined in triplicate by the phenol-sulfuric acid assay with D-glucose as standard [12]. The amount of uronic acid in the biopolymers was analysed colorimetrically [13]. D-Glucuronic acid served as the standard. Following the hydrolysis of the complex carbohydrates in an evacuated tube at 110°C for 2 h in 2 M trifluoroacetic acid and the subsequent removal of the acid by repeated evaporation, D-glucose was estimated enzymatically by the D-glucose oxidase method.

^{13}C-NMR spectroscopy was performed with a Bruker WM 250 spectrometer in the Fourier transform mode. All spectra were obtained at 80°C at sample concentration of ~30 mg/ml in D_2O. Chemical shifts are given relative to tetramethylsilane (TMS, 0.0 ppm) that was used as the external reference. Viscosities of aqueous solutions (0.2 mg/ml) were measured at 25°C with a capillary tube having an efflux time of 178 s for H_2O. Experimental variations were less than 12%. Optical rotation was monitored with a digital polarimeter model Jasco Dip-140.

3. RESULTS

Exopolysaccharides isolated from the two aforementioned media at a stationary phase of growth were purified and analysed for their chemical composition. The total carbohydrate content accounted for 86.2% of the biopolymer elaborated in the manganese-rich medium while that from the culture without added manganese amounted to only 72.6%. The exopolysaccharide from the latter medium was found to contain approximately twice as much uronic acid than the polymer produced by the arctic *Rhizobium* N_{11} in the medium supplemented with manganese. D-Glucose was found to constitute 18% of the carbohydrate in the complex biomolecule secreted by the microbe in the normal culture, while the biopolymer released in the manganese-enriched medium comprised of 31.4% of this same monosaccharide (table 1).

The dissimilarity in the sugar residues and the non-carbohydrate moieties constituting these two biopolymers was further revealed by ^{13}C-NMR spectroscopy (fig.1). Anomeric carbons in sugar residues are known to resonate between 112 and 90 ppm [14,15]. This region of the spectrum obtained from the complex carbohydrate isolated from the manganese-enriched medium contained three fine peaks (100.5, 100.0 and 98.2 ppm) with relative intensity of approx. 1:1:1, while the spectrum derived from the other exopolysaccharide revealed numerous signals with chemical shifts ranging from 104 to 98 ppm. Further variations were also observed in the spectra, especially in the 66–60 ppm region that is commonly attributed to carbon of the CH_2OH moiety of monosaccharides (fig.1) [14,15]. Such differences in chemical shifts and their relative intensities may be indicative of the unidentical nature of the sugar residues constituting these two biopolymers. Substituent peaks in complex carbohydrates characteristic of acyl and alkoxyl functions having moieties like CH,

Table 1

Chemical and physical properties	Exopolysaccharide elaborated by arctic *Rhizobium* N_{11} in normal medium (unamended with manganese)	Exopolysaccharide elaborated by arctic *Rhizobium* N_{11} in medium with added manganese (400 μM)
1. Total carbohydrate content[a]	72.6%	86.2%
2. Uronic acid content[a]	40.8%	20%
3. D-Glucose content[a]	18%	31.4%
4. Viscosity (0.2 mg/ml) (in cP)	2.36 ± 0.26	1.42 ± 0.15
5. Specific rotation $[\alpha]_D^{22}$ (0.1% w/v in H_2O)	+112.6°	+71.9°

[a] Mean values from experiments performed on exopolysaccharides (1–3 mg/ml) by procedures described in section 2. Carbohydrate expressed as D-glucose equivalent and uronic acid as D-glucuronic acid equivalent

CH_2 and CH_3 usually resonate upfield of 59 ppm [14,15].

This region of the spectra exhibited numerous distinctive features. Notably, the spectrum of the exopolysaccharide elaborated in the normal medium had signals at 26.1 and 21.0 ppm that were absent in the spectrum derived from the complex carbohydrate isolated when the microbe was subjected to manganese. However, this latter spectrum had sharp fine peaks at 55.6, 53.4, 47.8 and 8.2 ppm in relative proportion of 1.0:1.1:2.3:1.8 respectively. On the other hand, the spectrum derived from the exopolysaccharide isolated from the medium deficient in added manganese revealed signals at 56.0, 54.0, 48.6 and 8.7 ppm with relative intensity of 1.4:1.0:2.0:1.1, respectively. Thus the complete absence of some signals and marked differences in the relative abundance of others may indeed be reflective of the diverse nature of the substituents in the exopolysaccharides elaborated in media with and without added manganese. Values obtained from viscosity and optical rotation measurements further revealed the disparate nature of these two biopolymers (table 1).

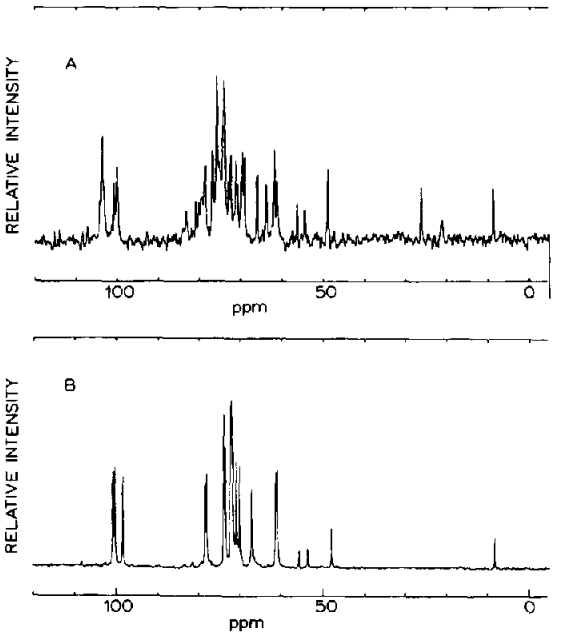

Fig.1. ^{13}C-NMR spectra of exopolysaccharides in D_2O at 80°C. (A) Spectrum derived from exopolysaccharide elaborated in the absence of added manganese. (B) Spectrum derived from exopolysaccharide produced in manganese rich medium.

4. DISCUSSION

The data presented in this paper clearly demonstrate that the arctic *Rhizobium* N_{11} elaborates a different exopolysaccharide under the influence of manganese. Both the quantity and quality of these complex carbohydrates synthesized by microorganisms are known to be influenced by a variety of factors. Sulfate limitation in *Rhizobium trifolii* has been shown to trigger an increased production of exopolysaccharide [12]. Pyruvate and acetate moieties in these biopolymers have been found to vary with the age of the culture [16]. Other factors like oxygen tension, antibiotics and root exudate of leguminous plants have also been reported to be effectors of exopolysaccharide biosynthesis [17–19].

In this study both the components involved in the glycosidic linkages and the non-carbohydrate moieties in the extracellular polysaccharide elaborated by the arctic *Rhizobium* N_{11} appear to be affected by the inclusion of manganese in the medium. Since the production and discharge of this biomolecule necessitate the expenditure of considerable amounts of energy by the microbe, it is conceivable that the exopolysaccharide contributes to a some significant biological function(s). Thus it is possible that this novel exopolysaccharide secreted during manganese assault, may be more apt to trap this metal and hence may be playing a role in manganese homeostasis. Both a scarcity and an excess of metallic elements are known to trigger the production of unique biochelators [20,21]. These variations reflected in the novel biopolymer may also be due to the influence of manganese on the biomolecules involved in the synthesis of this complex carbohydrate. Manganese does participate in saccharide biosynthesis [22] and is also known to be involved in transcriptional control mechanisms [23]. Thus whether this exopolysaccharide with its novel moieties plays the dual role of sequestering manganese and helping mediate the adaptation of the microbe to this non-ideal situation has to await further investigation.

Although the exact structural features of the biopolymers elaborated by this *Rhizobium* under the aforementioned conditions have to be determined and their possible biological role(s) delineated, the present report shows that the nature of the exopolysaccharide production in this nitrogen fixing microbe is sensitive to manganese.

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