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Osmoregulated trehalose-derived oligosaccharides in *Sinorhizobium meliloti*

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ABSTRACT

***Sinorhizobium meliloti* is a soil bacterium accumulating glutamate, *N*-acetylglutaminyl glutamine amide and trehalose in hyperosmolarity. Besides these compatible solutes, we highlighted several compounds in *S. meliloti* Rm1021 wild-type strain. The purification and the structural characterization based on liquid chromatography evaporative light scattering detector, electrospray ionization high resolution mass spectrometry and nuclear magnetic resonance techniques showed they were four linear oligosaccharides composed of 3, 4, 5 and 6 glucose units all linked by α-(1 → 2) linkages except a terminal α-(1 ↔ 1) linkage. These oligosaccharides were cytoplasmic and were observed in several wild-type strains suggesting they were common features in *S. meliloti* strains grown in hyperosmolarity.**

1. Introduction

Sinorhizobium meliloti is a Gram-negative soil bacterium which ecological niche is continuously submitted to osmotic variations depending on weather conditions. To survive in the rhizosphere *S. meliloti* develops active adaptative mechanisms quite different in hypoosmolarity (humidity) and hyperosmolarity (aridity) [1]. In hypoosmolarity, this bacterium accumulates in the periplasm cyclic β-glucans composed of 17–40 glucose residues linked by β-(1 → 2) glycosidic bonds and eventually substituted by phosphoglycerol, succinyl or methylmalonyl groups [2,3]. Conversely, the hyperosmoadaptation proceeds with the accumulation of intracellular uncharged or zwitterionic compounds, called compatible solutes [1,4]. In *S. meliloti*, the endogenous compatible solutes are the amino acid glutamate, the dipeptide *N*-acetylglutaminyl glutamine amide (NAGGN) and the disaccharide trehalose (αGlucose-(1 ↔ 1)-αGlucose) [5–7].

Besides these classical compatible solutes, we discovered new molecules accumulated in hyperosmolarity by *S. meliloti*. The purification and the structural characterization of these compounds showed they were oligosaccharides composed by 3–6 glucose residues all linked by α-(1 → 2) linkages except an α-(1 ↔ 1) terminal bond, corresponding to a trehalose-terminal structure.

2. Materials and methods

2.1. Bacterial strains, growth media and ethanolic extraction

The *S. meliloti* strains used were the wild-type Rm1021 and the mutant Rm10004 (Rm1021, *exoA*::Tn5, *ndvB*::Tn5), kindly provided by Prof. G.C. Walker (MIT, Massachusetts), and five other wild-type strains: Rm2011, Rm102F34Sm^R, Su47, Rm5000 and M5N1 [8–12]. Cultures were grown at 30 °C and 130 rpm in a minimal growth medium (Tris: 25 mM; NH_4Cl: 10 mM; KH_2PO_4: 2.5 mM; K_2HPO_4: 2.5 mM; $MgSO_4{\cdot}7H_2O$: 0.4 mM; $CaCl_2{\cdot}2H_2O$: 0.3 mM; H_3BO_3: 0.2 mM; Biotin: $4.1.10^{-2}$ mM; $FeCl_3{\cdot}6H_2O$: $3.7.10^{-3}$ mM; $ZnSO_4{\cdot}7H_2O$: $3.5.10^{-3}$ mM; $CuSO_4{\cdot}5H_2O$: $2.0.10^{-3}$ mM; $Na_2MoO_4{\cdot}2H_2O$: $4.1.10^{-4}$ mM; pH 7.2) with glucose at 6 g/L and streptomycin at 200 μg/mL. This medium osmolarity was low (103 mosM) and was eventually increased by NaCl at 0.1 M (278 mosM or isoosmolarity) or 0.4 M (961 mosM or hyperosmolarity) [3,4,13]. The RC rich medium [10] was also used and its osmolarity (87 mosM) was eventually increased by NaCl at 0.1 M (255 mosM) or 0.4 M (761 mosM). The medium osmolarity was measured with an automatic osmometer (Roebling). Bacterial growth was determined spectrophotometrically at 600 nm. Intracellular content extractions were all realized in stationary growth phase as previously described [11]. Specific extraction of the periplasm and the

Abbreviations: DP, Degree of polymerization; ESI-HRMS, Electrospray ionization high resolution mass spectrometry; HMBC, Heteronuclear multiple bond correlation; LC-ELSD, Liquid chromatography evaporative light scattering detector; NAGGN, *N*-acetylglutaminyl glutamine amide; NMR, nuclear magnetic resonance

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cytoplasm was adapted from Cogez et al. [14]. All experiments were done at least twice.

2.2. HPLC experiments

Waters 600 and Waters LC4000 (Milford, MA, USA) systems were used for analytical and semi-preparative HPLC, respectively. The Prevail Carbohydrate ES analytical (250 mm × 4.6 mm i.d., 5 μm particle size) and semi-preparative (250 mm × 10 mm i.d., 5 μm particle size) columns were purchased from Alltech (Deerfield, IL, USA). This polymeric-based NH_2 stationary phase was chosen for its capacity to separate oligosaccharide isomers with different inter-glycosidic linkages [15]. The analytical column was kept at room temperature. The sample injection volume was 20 μL. A gradient elution was applied at 1 mL/min with a ratio acetonitrile/water from 80/20 to 51/49 in 70 min, followed by 10 min at 80/20. For the semi-preparative column a 200 μL injection volume and a 7.6 mL/min flow rate were used. Molecules were detected by a PL-ELS 1000 (Polymer Labs, Amherst, MA, USA) ELSD with a 1 mL/min nitrogen gas flow, a 40 °C nebulization and a 70 °C evaporation temperatures. Data acquisition was performed using EMPOWER software.

2.3. MS experiments

High-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF *Ultima Global* hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound (0.1% orthophosphoric acid solution). Extracts were directly infused (5 μL/min) through an integrated syringe pump into the electrospray source. For the purified oligosaccharides 0.1 g/L solutions in water were used. The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 L/h, respectively. The capillary voltage was 3.5 kV, the cone voltage 100 V and the RF lens1 energy was optimized for each sample (50–150 V). The mass range was 50–1550 Da and spectra were recorded at 1.5 s/scan in the profile mode at a resolution of 9000 (FWHM). For MS/MS experiments argon was used as collision gas at an indicated analyzer pressure of 5.10^{-5} Torr and the collision energy was set to 40 V. Data acquisition and processing were performed with MassLynx 4.0 software.

2.4. Nuclear magnetic resonance (NMR) experiments

^{1}H and ^{13}C-NMR spectra were done as previously described [11]. 2D-Heteronuclear multiple bond correlation (2D-HMBC) spectra were recorded on a Bruker DRX 500 NMR spectrometer (Bruker BioSpin) equipped with a 5 mm $^{1}H/^{13}C/^{15}N$ z-gradient triple resonance probe and operated at 500.13 and 125.89 MHz (^{1}H and ^{13}C, respectively).

3. Results

3.1. S. meliloti intracellular content in hypo-, iso- and hyperosmolarity

The *S. meliloti* Rm1021 wild-type strain was grown in a minimal medium which osmolarity was low and eventually modulated by NaCl at 0.1 M (isoosmolarity) or 0.4 M (hyperosmolarity). At stationary growth phase, intracellular contents were extracted and analyzed by ^{13}C-NMR (Fig. 1). In hypoosmolarity (Fig. 1A), the only signals detected were those of the glucose units constituting β-glucans (C1: 103.2 ppm, C2: 83.3 ppm, C3: 76.5 ppm, C4: 70.0 ppm, C5: 77.4 ppm, C6: 61.7 ppm). No β-glucan substituent was detected. In isoosmolarity (Fig. 1B), the β-glucan peaks were observed and weak signals corresponding to trehalose (C1: 94.2 ppm, C2: 72.0 ppm, C3: 73.5 ppm, C4: 70.7 ppm, C5: 73.1 ppm, C6: 61.5 ppm). In hyperosmolarity (Fig. 1C), β-glucans were still detected, trehalose peaks increased, and supplementary signals appeared (101.5 ppm, 100.6 ppm, 98.6 ppm, 97.4 ppm, 96.7 ppm and 91.4 ppm). These peaks did not correspond to the compatible solutes usually accumulated in *S. meliloti*, like NAGGN or glutamate, and the chemical shifts of these signals suggested molecule(s) of the carbohydrate family different from trehalose and β-glucans.

To characterize the structure of these unassigned signals, we used a *S. meliloti* Rm10004 mutant strain (Rm1021, *exoA*::Tn5, *ndvB*::Tn5) to eliminate β-glucans from the extracts with the inactivation of the *ndvB* gene [16] and simplify the NMR spectra. Then, we analyzed the extracts of the Rm10004 mutant grown in the same way than previously with the parent strain to verify the presence of the unassigned signals (Fig. 1). No signal were detected in hypoosmolarity (Fig. 1D), only trehalose was present in isoosmolarity (Fig. 1E) and trehalose with the same unassigned signals were observed in hyperosmolarity (Fig. 1F). Therefore results were identical for the Rm10004 mutant and the Rm1021 wild-type strains except β-glucans and only results concerning the Rm10004 mutant will be further shown to get rid of the β-glucans and to get clearer results on the unknown molecule structures.

3.2. Identification of the molecules

The Rm10004 hyperosmotic extract was analyzed by electrospray ionization high resolution mass spectrometry (ESI-HRMS) and the spectrum (Fig. 2) revealed the presence of ions corresponding to the sodium salt of the compatible solute NAGGN (*m*/*z* 338.14), in agreement with the literature [5,17] but its concentration was too low to be detected by ^{13}C-NMR. Trehalose was also observed in a $[M+Na]^+$ (*m*/*z* 365.11) and a $[M+K]^+$ (*m*/*z* 381.08) forms. The spectrum showed other peaks corresponding to $[M+Na]^+$ ion of four oligosaccharides (*m*/*z* 527.16, 689.21, 851.26, 1013.32) and to their $[M+K]^+$ adducts (*m*/*z* 543.13, 705.19, 867.24, 1029.29). To specify the elemental compositions of these molecules, accurate mass measurements were performed on their $[M+Na]^+$ ions (Table 1). The results confirmed the presence of four oligosaccharides constituted of hexose units ($C_6H_{10}O_5$) with degree of polymerization (DP) from three to six without any substituent. The bacterial extract was then hydrolysed with concentrated trifluoroacetic acid to identify the nature of the hexose monomer and analyzed by ^{13}C-NMR [11]. The spectrum clearly showed glucose as the unique constituent of these oligosaccharides.

3.3. Oligosaccharide purification and structural characterization

Further structural characterization needed a prior purification of each oligosaccharide by liquid chromatography evaporative light scattering detector (LC-ELSD). The chromatogram of the Rm10004 hyperosmotic extract (Fig. 3) showed five peaks with retention times of 20.6, 27.3, 32.1, 37.1 and 40.7 min. The first one was assigned to trehalose after comparison with a commercial standard. The four other peaks (noted Peak 1–4) were collected and corresponded, respectively, to DP3, DP4, DP5 and DP6 oligosaccharides, as confirmed by ESI-HRMS.

A scale-up to the preparative mode was achieved to afford at least 5 mg of each oligosaccharide. The purity of the final batches was verified by LC-ELSD and ESI-HRMS and only one oligosaccharide was present in each collected fractions, showing the great efficiency of the preparative method.

The DP3 to DP6 oligosaccharide structure was then determined by NMR experiments. The following part will only detail results for

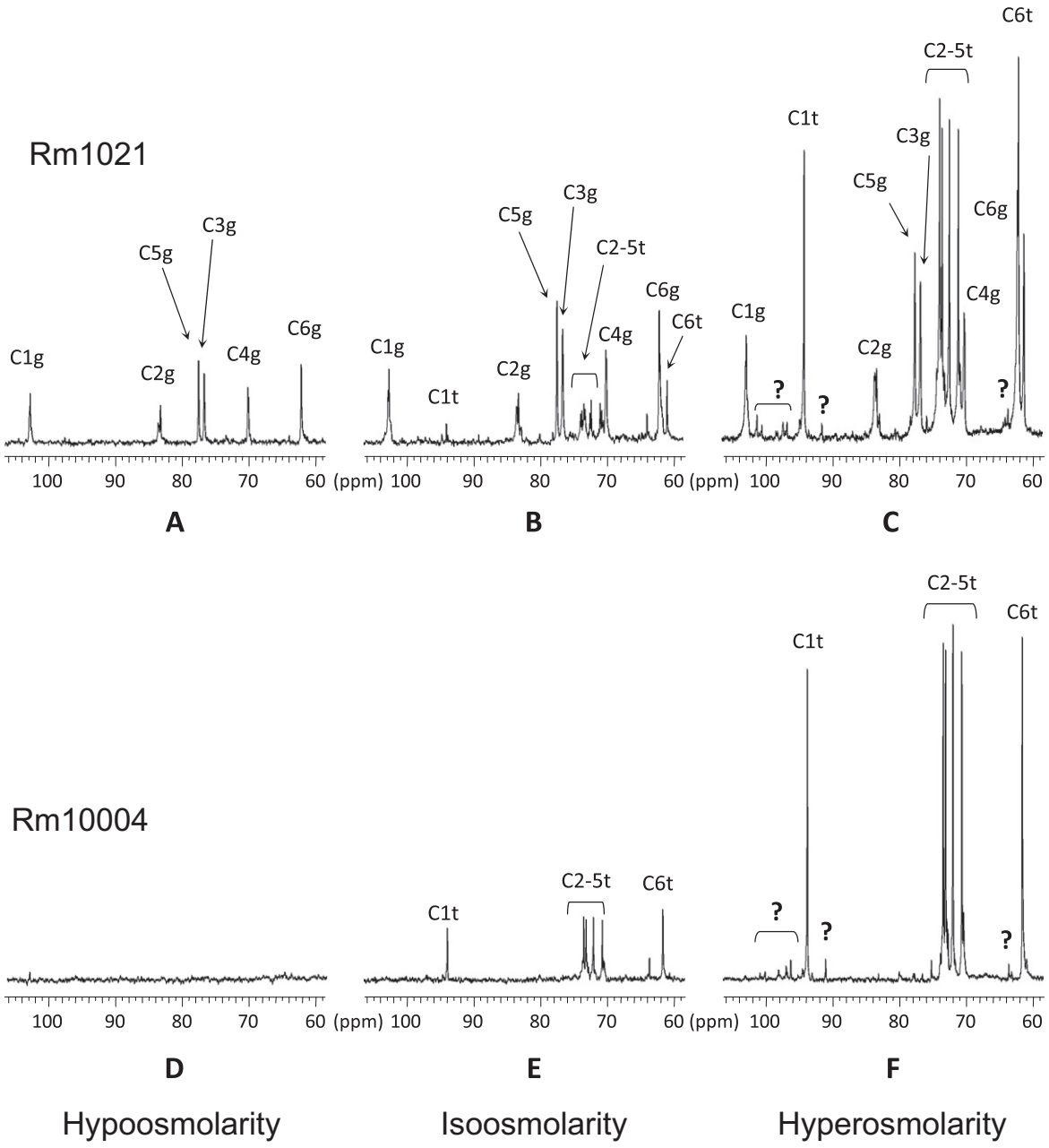

Fig. 1. Ethanolic extract ^{13}C-NMR analysis of *S. meliloti* Rm1021 wild-type strain (A, B and C) and Rm10004 mutant strain (D, E and F) in stationary growth phase in hypo-, iso- and hyperosmolarity, respectively, (g: β-glucans, t: trehalose, ?: unassigned signals).

the DP5 oligosaccharide, but similar results were obtained with the other DPs. The anomeric region of ^{1}H-NMR spectrum showed five doublet signals at 5.25, 5.24, 5.09, 5.06 and 4.94 ppm (not shown), indicating that 5 glucose units (noted A, B, C, D and E) were detected corresponding to a single DP5 isomer. All the glucose units were in α-anomeric configuration because all the chemical shifts were between 4.8 and 5.3 ppm and no β-signal was detected between 4.4 and 4.8 ppm [18]. The J_{H1-H2} coupling constants were 3.5, 3.7, 3.0, 3.6 and 3.6 Hz, respectively, confirming the α-anomeric configuration of all the glucose units [18].

The glucosidic linkages were determined by HMBC (Fig. 4). This 2D NMR experiment showed the cross-peaks between protons and carbons that are three bonds away (indicated by the arrows). The cross-peak between H1 of glucose unit A (5.06 ppm) and C1 of unit B (93.24 ppm) demonstrated a (1 ↔ 1) linkage between these two units, confirmed by the reverse cross-peak between H1 of unit B (5.25 ppm) and C1 of glucosyl A (94.25 ppm). Cross-peaks were also observed between C2 of unit B (79.39 ppm), unit C (79.22 ppm) and unit D (77.27 ppm), respectively, with H1 of unit C (5.24 ppm), unit D (5.09 ppm) and unit E (4.94 ppm), revealing (1 → 2) inter-glucosidic linkages between the glucosyl units B, C, D and E, confirmed by the correlation between C1 of unit C (97.64 ppm), unit D (96.75 ppm) and unit E (97.88 ppm), respectively, with H2 of unit B (3.49 ppm), unit C (3.49 ppm) and unit D (3.48 ppm). The NMR results demonstrated that the DP5 structure was αD-Glc-(1 → 2)-αD-Glc-(1 → 2)-αD-Glc-(1 → 2)-αD-Glc-(1 ↔ 1)-αD-Glc. Similar results were obtained with the DP3, DP4 and DP6 oligosaccharides and all the glucosidic units were linked with α-(1 → 2) linkages except an α-(1 ↔ 1) terminal linkage, corresponding to a trehalose-terminal structure.

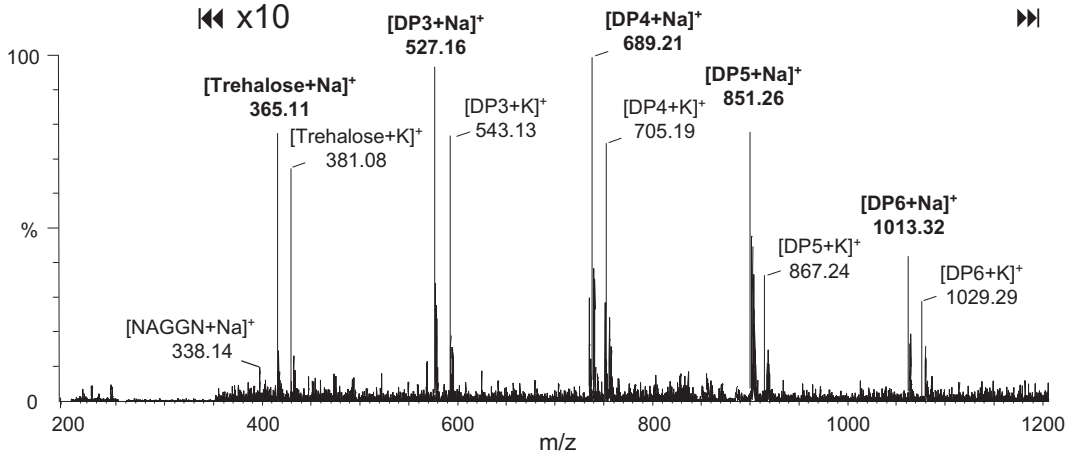

Fig. 2. ESI-HRMS spectrum of the stationary growth phase Rm10004 hyperosmotic extract.

Table 1
Accurate mass measurements of the $[M+Na]^+$ ions of the oligosaccharides from DP3 to DP6 using the Q-TOF *Ultima Global* instrument equipped with a LockMass sprayer.

Compound	Mass	Calcd mass	ppm	Formula
DP3	527.1570	527.1588	−1.3	$C_{18}H_{32}O_{16}Na$
DP4	689.2109	689.2116	−1.1	$C_{24}H_{42}O_{21}Na$
DP5	851.2647	851.2645	0.3	$C_{30}H_{52}O_{26}Na$
DP6	1013.3161	1013.3173	−1.2	$C_{36}H_{62}O_{31}Na$

The NMR results and the structure of each oligosaccharide were confirmed by ESI-MS/MS experiments [19–21] performed on the $[M+Na]^+$ ions of DP3, DP4, DP5 and DP6 (see Appendix) .

3.4. Other wild-type strains, growth media and cellular localization

Five other *S. meliloti* wild-type strains were tested in stationary growth phase: Rm2011, Rm102F34Sm^R, Su47, Rm5000 and M5N1. LC-ELSD analysis of the hyperosmotic extracts showed the DP3 to DP6 oligosaccharides in all the strains tested, while they were absent in hypoosmolarity. Same experiments in the RC rich medium more commonly used to cultivate *S. meliloti* species [10] gave the same results.

ESI-HRMS analysis of the extracellular medium, periplasmic and cytoplasmic extracts showed the four oligosaccharides only in the cytoplasm, neither in the periplasm nor outside the bacteria.

4. Discussion

Oligosaccharides with close structures have already been observed in *Rhizobiaceae*. In 1982, Watanabe et al. [22] found an oligosaccharide with 6 glucose units linked by α-(1 → 2) bonds, excreted by *Rhizobium japonicum* strain 516. In *S. meliloti* J7017, Hisamatsu et al. [23] detected three oligosaccharides composed of 4, 5 and 6 glucose units linked with α-(1 → 2) linkages and an α-(1 ↔ 1) terminal linkage, but these compounds were extracellular. Breedveld et al. [7] observed in *S. meliloti* Su47 the presence of intracellular DP4 to DP6 oligosaccharides accumulated in hyperosmolarity. The authors noticed the presence of (1 → 2) and (1 ↔ 1) glycosidic linkages possibly in α-configuration and without any further structural characterization suggested they were identical to those described by Hisamatsu et al. [23]. To our knowledge, these are the only three references dealing with DP4-6 α-oligosaccharides in *Rhizobiaceae*. In our work, the oligosaccharides are clearly cytoplasmic, not periplasmic nor extracellular, composed of 3–6 glucose residues linked with α-(1 → 2) linkages and an α-(1 ↔ 1) terminal linkage, accumulated in hyperosmotic conditions by all the *S. meliloti* strains tested.

The oligosaccharide osmoregulation and the trehalose-terminal structure suggest there is a metabolic link between oligosaccharides and trehalose. *S. meliloti* possesses three pathways to synthesize trehalose: OtsAB generating trehalose from glucose-

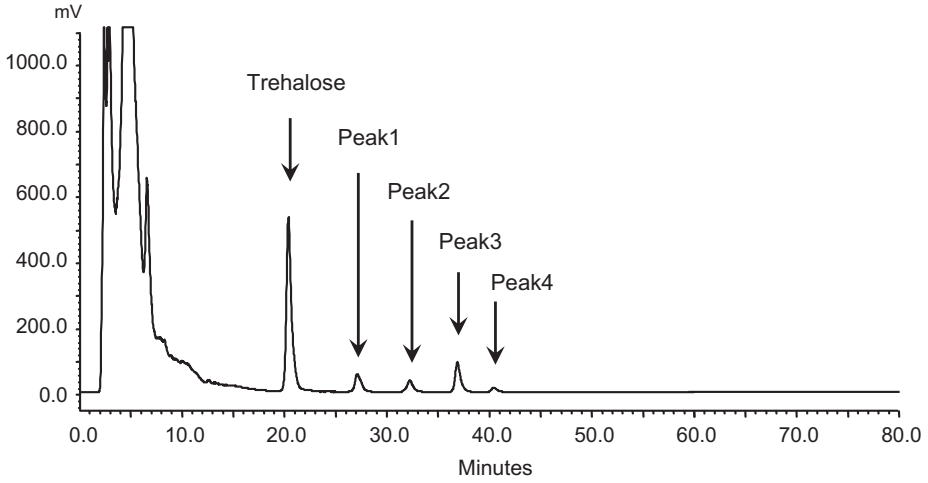

Fig. 3. LC-ELSD analytical chromatogram of the stationary growth phase hyperosmotic Rm10004 extract.

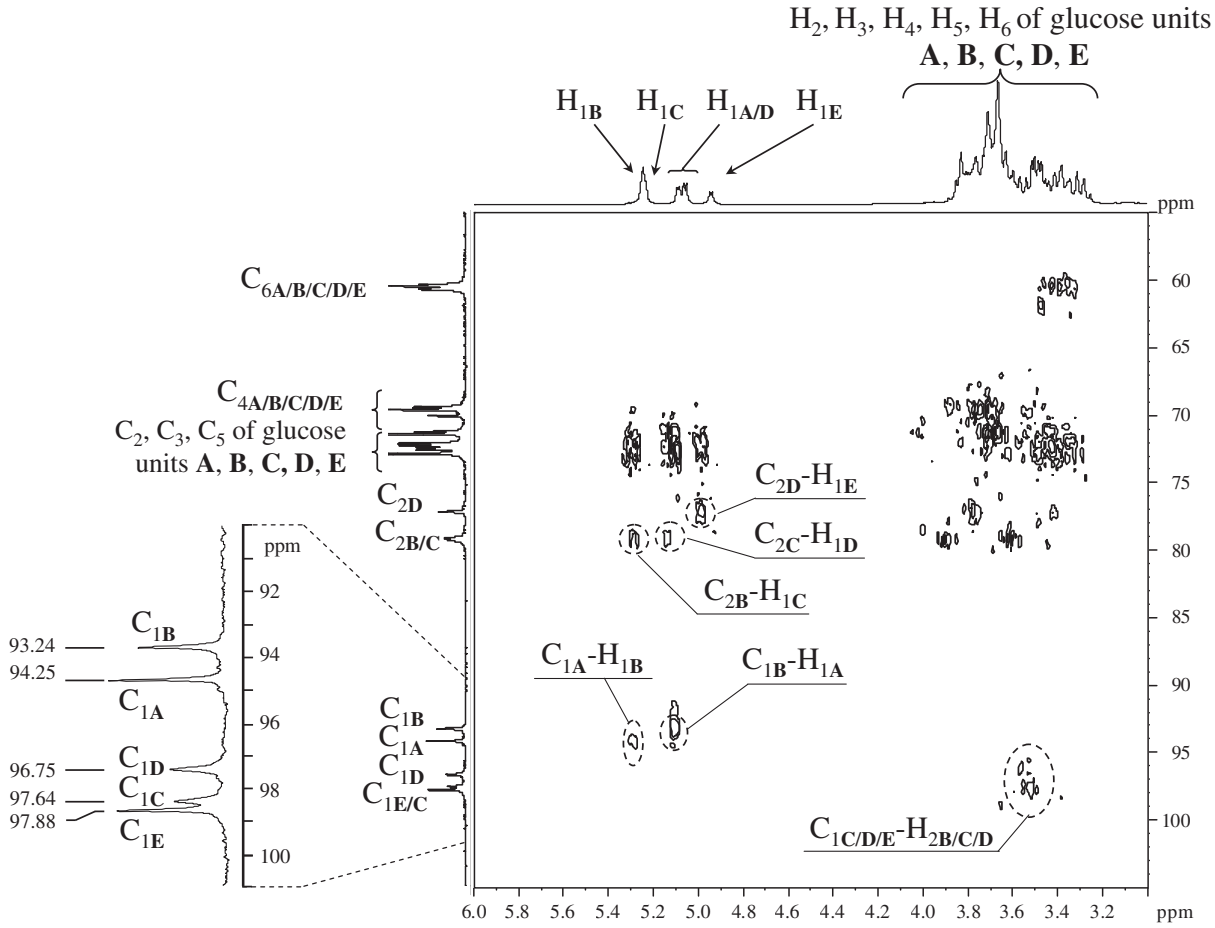

Fig. 4. 2D-HMBC NMR spectrum of DP5 at 20 g/L in D_2O at 25 °C. The significant correlations ^3J (^{13}C–^1H) corresponding at the different inter-glycosidic linkages are circled.

6-phosphate and UDP-glucose, TreYZ isomerizing maltooligosaccharides (α-(1 → 4) linked glucans) in maltooligosyl-trehalose and cutting the terminal trehalose feature and TreS consisting in the reversible conversion of maltose (αGlc-(1 → 4)-αGlc) in trehalose (αGlc-(1 ↔ 1)-αGlc) [24,25]. The OtsAB pathway has been described as the major one involved in osmoregulated trehalose biosynthesis [25], if not the sole [26], but a surprising point is the genome sequence of *S. meliloti* Rm1021 lacking the *otsB* gene [27] and no open-reading frame encoding an otsB-like product was detected [25]. So even though the activity of this biosynthetic pathway has been reported, the OtsAB pathway in *S. meliloti* remains unclear. The TreYZ pathway has been shown to be active in several *S. meliloti* strains in vitro from crude protein preparations [24]. In 2006, Domínguez-Ferreras et al. [28] found in *S. meliloti* Rm1021 a salt stress induction of a putative maltooligosyl-trehalose synthase gene coding for a TreY activity. Contradictory the TreYZ pathway has been shown in the same strain to be active only when OtsA is absent [25] or completely inactive [26]. Therefore the implication of TreY and TreZ activities in trehalose biosynthesis is uncertain and could be linked to the growth medium composition [26]. And the last pathway, TreS, has recently been shown not to be involved in osmoregulated trehalose accumulation in *S. meliloti* [25,26].

Finally, the question of the osmoregulated trehalose biosynthesis in *S. meliloti* is still raising and is currently source of debate in the literature. The trehalose-derived oligosaccharides could be involved, maybe in a way similar to the TreYZ pathway, and could act as metabolic precursors for a trehalose-hydrolase which could cut the final trehalose feature of the oligosaccharides. This speculation could explain their osmoregulation, but further work is necessary to understand the synthesis mechanisms and the physiological role of these new molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.047.

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