Structural Analysis of a Novel Polysaccharide of the Lipopolysaccharide-Deficient Extremophile Gram-Negative Bacterium *Thermus thermophilus* HB8

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A novel polysaccharide was isolated from the extremophile Gram-negative bacterium *Thermus thermophilus* HB8. Its structural characterization employed compositional and methylation analyses, and 2D ^{1}H and ^{13}C NMR spectroscopy. Various chemical degradations (methanolysis and Smith degradation) were also carried out in order to depolymerise this complex macromolecule, and subsequent chemical and spectroscopic analyses were also carried out on the obtained fragments. All the data suggested a novel polymeric structure that consists of a disaccharide repeating unit to which a branching trisaccharide chain is linked, although not stoichiometrically.

Introduction

Thermophilic bacteria of the genus *Thermus* have been isolated worldwide from hot neutral or alkaline springs, and from hot water or natural waters subjected to thermal pollution.[1,2] These organisms form one of the major aerobic heterotrophic populations of those sites with temperatures between 55 and 85 °C. However, only a few strains, all closely related to *Thermus thermophilus* HB8, are capable of growth at 80 °C or above.[3,4] *Thermus thermophilus* strain HB-8 is a Gram-negative bacterium that was first isolated from marine hot springs in Japan. It is halotolerant and able to grow at temperatures as high as 80−82 °C.

The outer membrane of Gram-negative bacteria performs a great variety of functions and is the source of a great number of compounds, such as lipopolysaccharides, glycoproteins, etc.[5] Among these, lipopolysaccharides play a key role in bacterial survival and are the object of our research study. Lipopolysaccharides (LPSs) are complex macromolecules that are unique, vital, and exclusive components of Gram-negative bacteria; they are part of the external leaf of the outer membrane. Their structure, biosynthesis and function are of crucial importance and, because of their location, they mediate the contact between the cell and the surrounding environment; the carbohydrate portion, in particular, is directly involved in this interaction process.

Previous studies on the basis of fatty-acid composition have suggested the absence of lipopolysaccharides in the outer membrane of this bacterium[6] and, thus, the outer-membrane polysaccharide portion of *Thermus thermophilus* was studied within this frame. The structure of a novel polysaccharide associated with the outer membrane of the bacterium is described and, the complete absence of lipopolysaccharide components is definitely established.

Results

Isolation and Characterization of the PS

Dried cells of *Thermus thermophilus* HB8 were extracted with hot aqueous phenol. This mixture was separated into two phases by centrifugation, both of which were treated with nucleases and protease to get rid of protein and nucleic acid contaminants. After enzymatic hydrolysis, the water phase was found to contain a significant amount of carbohydrate material, while the phenol phase contained no carbohydrate material at all; further studies were therefore conducted on the water phase. The fatty-acid composition of the water phase revealed the presence of *iso* and *ante* C15:0 and *iso* and *ante* C17:0. No traces of the typical 3-hydroxy fatty acids of lipopolysaccharides were detected. In addition, Kdo colorimetric assay was negative and it was not detected in compositional analysis. SDS-PAGE electrophoresis showed neither the characteristic ladder migration of LPSs nor a band to the bottom of the gel, which is the typical migration pattern of lipooligosaccharides.

The polysaccharide fraction contained in the water phase was further purified by successive precipitation with organic solvents and then by gel-permeation chromatography to

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yield a purified polymer (PS). At this stage, further analyses showed the complete absence of any fatty-acid residue and Kdo.

A monosaccharide compositional analysis showed the presence of D-Man, D-Gal, D-Glc, D-ManN, and D-GalN, and methylation analysis showed the presence of terminal Glc, 4-substituted Man, 3-substituted GalN, 6-substituted-ManN, 3,4 disubstituted Gal, and a small amount of 3-substituted Gal (*t*-Glc: 3.7; 4-Man: 3.4; 3-GalN: 3.0; 6-ManN: 3.0; 3,4-Gal: 4.0; 3-Gal: 1.0)

Despite the presence of six monosaccharide derivatives in the methylation analysis, the ^{1}H (Figure 1) and ^{13}C NMR spectra of PS suggested the presence of a heterogeneous polysaccharide fraction consisting of a major component and a minor one and/or a polysaccharide possessing a non-repetitive structure. Various anomeric proton signals of different intensity are present in the ^{1}H NMR spectrum in the region $\delta = 5.11-4.73$ ppm, which also has a crowded ring-signals region and two acetyl methyl signals occurring at $\delta = 2.10$ ppm (not shown in Figure 1). Similarly, the ^{13}C NMR spectrum contains several signals of different intensity in the anomeric region ($\delta = 104.0-96.6$ ppm), numerous carbon ring signals in the range $\delta = 79.8-60.0$ ppm, two nitrogen-bearing carbon signals at $\delta = 54.3$ and 52.0 ppm, and acetyl carbonyl and methyl signals ($\delta = 175.6$ and 22.6 ppm, respectively).

Given the heterogeneity of the sample, the polysaccharide fraction was subjected, once more, to several kinds of gel-permeation chromatography in order to achieve a separation of the putative components. Unfortunately, though, all attempts failed, therefore a full 2D NMR analysis was carried out on the sample in order to understand its main structural features. The use of DQF-COSY, TOCSY, NOESY, HSQC and HMBC techniques allowed the identification of ^{1}H and ^{13}C resonances of six major spin-systems (Table 1 and Figure 1), which corresponded to the monosaccharide residues detected by methylation analysis. Five of these residues (**A**, **A′**, **B**−**D**) possess an α-configuration, as shown by a coupled HSQC spectrum ($^1J_{C,H}$ in the range of 173 Hz). In particular, the **A** and **A′** residues possess small $J_{H,H}$ values for H-3/H-4 and H-4/H-5, and, in addition, residue **A** possesses C-3 and C-4 carbon signals that are shifted downfield due to glycosylation;[7] it was thus identified as a 3,4-di-substituted α-Gal and, likewise, residue **A′** was identified as a 3-substituted α-Gal since its C-3 carbon signal resonates at low field. Residue **B** possesses small $^3J_{H1,H2}$ and $^3J_{H2,H3}$ values that are diagnostic of an H-2 equatorial orientation, and, in the TOCSY spectrum it was possible to assign all the other cross-peaks within the spin system, from H-2, thus allowing us to identify it as a mannose residue. The chemical shift of C-4 ($\delta = 76.1$ ppm) is diagnostic of glycosylation. Spin system **C** was identified as a terminal α-glucose residue since all its $^3J_{H,H}$ ring values are large and it possesses the expected ^{13}C chemical shifts for a non-substituted residue.[7] Spin system **D** was identified as a 6-substituted α-ManN as its anomeric proton signal correlates to the H-2 proton at $\delta = 4.55$ ppm, which, in turn, is correlated to a carbon signal at $\delta = 54.3$ ppm in the

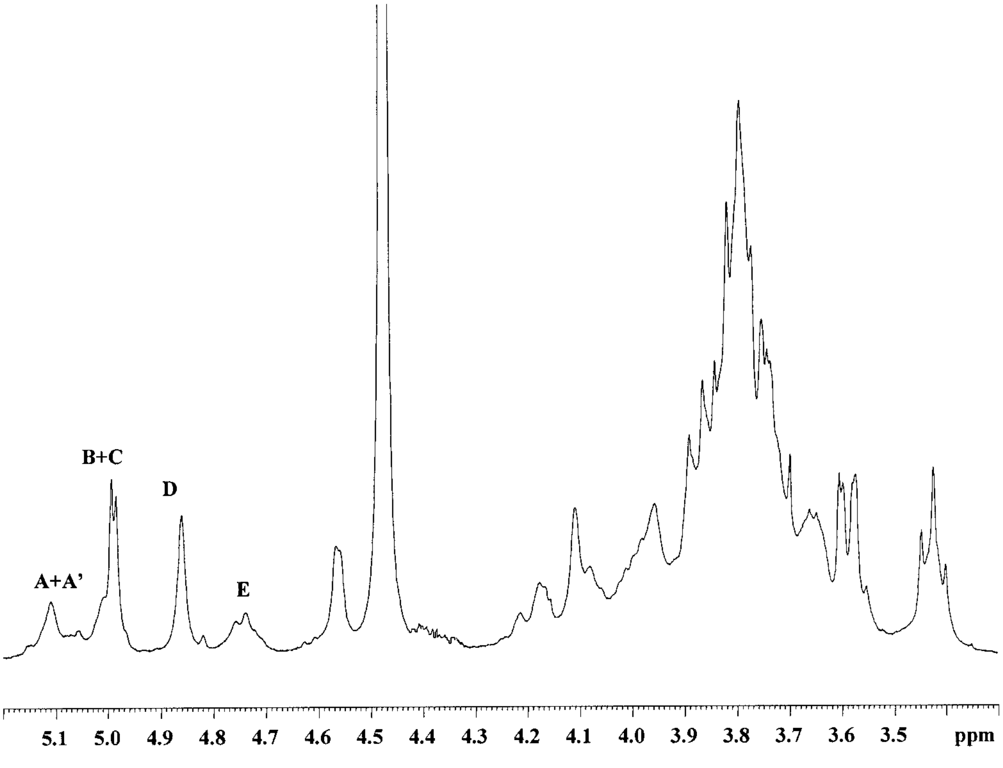


Figure 1. Section of the ^{1}H NMR spectrum of the PS from *Thermus thermophilus* HB8; the letters refer to the carbohydrate residues shown in Table 1

HSQC spectrum. From this H-2 signal it was possible to assign all other resonances within the spin system up to the H-6 proton signals. In the same HSQC spectrum, the deshielded C-6 signal at δ = 66.9 ppm because of glycosylation was also visible.

Table 1. ^{1}H and ^{13}C (in italics) NMR chemical shifts of sugar residues of the PS from *Thermus thermophilus* HB8 relative to acetone (^{1}H: δ = 2.225 ppm; ^{13}C: δ = 31.45 ppm, both at 35 °C); the resonances for the acetyl group appear at δ = 2.10 and δ = 22.6 and 175.6 ppm, respectively

A	5.11	3.94	3.79	4.12	3.98	3.78/3.71
3,4-Gal	*96.6*	*68.2*	*76.7*	*78.2*	*68.1*	*62.1*
A′	5.11	3.90	3.81	4.22	3.93	3.83
3-Gal	*96.6*	*70.0*	*76.8*	*70.2*	*68.4*	*62.0*
B	5.06	4.17	3.99	3.64	3.91	3.69/3.71
4-Man	*102.1*	*71.2*	*70.1*	*76.1*	*70.8*	*61.0*
C	4.98	3.58	3.81	3.42	3.74	3.88/3.76
t-Glc	*99.5*	*74.2*	*72.0*	*70.0*	*71.4*	*61.7*
D	4.85	4.55	3.84	3.58	3.65	3.80/3.97
6-ManNAc	*100.2*	*54.3*	*74.2*	*72.5*	*70.5*	*66.9*
E	4.73	4.07	3.81	4.17	3.88	3.70
3-GalNAc	*104.0*	*52.0*	*79.8*	*65.8*	*75.9*	*61.7*

Spin-system **E** was characterized from a $^{1}J_{C,H}$ value of 163 Hz and a $^{3}J_{H1,H2}$ value of 7.8 Hz, both of which point to a β-anomeric orientation. This assumption was further confirmed by the NOESY spectrum, where intra-residue NOE connectivity is present between H-1, H-3 and H-5. The *galacto* configuration was confirmed by the low $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ values, whereas C-2 nitrogen substitution was evident from the C-2 chemical shift at δ = 52.0 ppm.

The presence of acetamido groups at C-2 of the **D** and **E** residues was verified by a HMBC spectrum, where both H-2 **D** and H-2 **E** and the methyl signals at δ = 2.10 ppm correlate to a carbonyl signal at δ = 175.6 ppm.

In the NOESY spectrum (Figure 2) H-1 **A** is correlated to H-3 **E**, while H-1 **E** is correlated to H-3 **A**; similarly, H-1 **A′** is correlated to H-3 **E**, while H-1 **E** is correlated to H-3 **A′**. These data are explicable by considering a simple disaccharide backbone built up of **A** and **E** residues in which **A** can be further substituted at its C-4 position. Actually, H-4 **A** has an NOE with H-1 **B**, and H-4 **B** has an NOE with H-1 **D**; moreover, H-6$_{a,b}$ **D** has an NOE with the anomeric signal of the terminal glucose **C**. The HMBC spectrum confirms both the attachment points of the residues, as deduced from the glycosylation shifts, and the sequence proposed for the oligosaccharide, as determined from the NOE data, as it contains all the significant long-range scalar correlations. The most determinant for the sequence are those between H-1/C-1 **A-A′** and C-3/H-3 **E**, H-1/C-1 **E** and C-3/H-3 **A-A′**, H-4/C-4 **A** and C-1/H-1 **B**, H-4/C-4 **B** and C-1/H-1 **D**, and H-6/C-6 **D** and C-1/H-1 **C**. Thus, taking into consideration all the above data, two

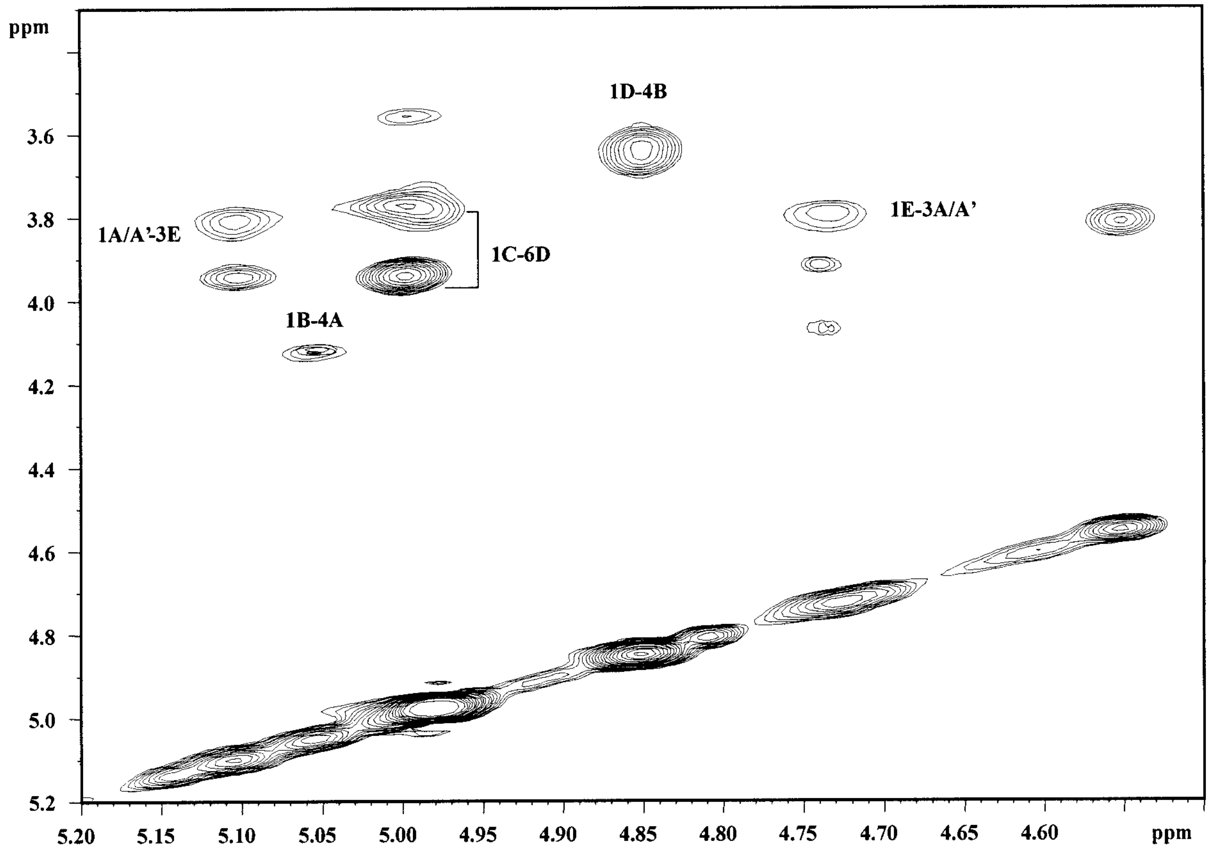


Figure 2. The anomeric region of the 2D NOESY spectrum of the PS from *Thermus thermophilus* HB8; the spectrum was recorded in D$_2$O at 500 MHz and 35 °C with a mixing time of 200 ms; the relevant interresidual correlations are shown; the letters refer to the carbohydrate residues shown in Table 1

oligosaccharide sequences **1** and **2** were proposed to belong to the membrane polysaccharide (PS) from the Gram-negative bacterium *Thermus thermophilus.*

→3)-α-Gal-(1→3)-β-GalNAc-(1→
1

and

→3)-α-Gal-(1→3)-β-GalNAc-(1→
α-Glc-(1→6)-α-ManNAc-(1→4)-α-Man-(1→4)┘
2

In order to confirm the presence of the oligosaccharide structure depicted above, and to gain further structural information, the polysaccharide was submitted to two different chemical degradations, from which two useful oligosaccharides were obtained. The first oligosaccharide was unintentionally obtained by incomplete methanolysis during the course of the monosaccharide analysis procedure. It is well-known that the glycosidic bond of 2-acetamido-2-deoxy monosaccharides is fairly resistant to cleavage and, in fact, after methanolysis of the intact polysaccharide, a pure disaccharide *O*-methyl glycoside (compound **3**) was isolated, along with monosaccharides residues. After workup of the reaction, *N*-acetylation, de-*O*-acetylation, and HPLC purification this was submitted to chemical analysis and 2D NMR characterization (Figure 3). Methylation analysis revealed the presence of 4-substituted Man (**W**) and terminal ManNAc (**X**) and, in fact, NMR spectroscopic data were in full agreement with the presence of the methyl glycoside of α-ManNAc-(1→4)-α-Man disaccharide (Table 2, Figure 3).

Table 2. ^{1}H and ^{13}C (italic) NMR chemical shifts of sugar residues of the disaccharide (compound **1**) obtained by methanolysis of the PS from *Thermus thermophilus* HB8 relative to acetone (^{1}H: δ = 2.225 ppm; ^{13}C: δ = 31.45 ppm; both at 25 °C); the resonances for the acetyl group appear at δ = 2.07 and δ = 22.0 and 175.6 ppm, respectively, and those for the *O*-methyl group at δ = 3.4 and 54.1 ppm, respectively.

W	4.94	4.05	3.94	3.55	3.73	3.90/4.01
4-Man	*98.3*	*68.9*	*68.1*	*77.1*	*71.2*	*60.8*
Y	4.84	4.61	3.90	3.57	3.95	3.91
t-ManNAc	*100.2*	*52.3*	*71.2*	*68.0*	*72.1*	*60.8*

An alternative and more selective degradation was carried out on the polymer by treatment with periodic acid, which oxidises the *vic*-diol groups (a Smith degradation). After the reaction a single component was obtained which retained polymeric behavior — it eluted in the void volume of a gel-permeation chromatogram (Sephadex G-50). Its methylation data showed the presence of 3-substituted Gal and 3-substituted GalN, as expected, as the Smith degradation does not affect 3-substituted residues since they do not possess any *vic*-diol function. In fact, in the ^{1}H NMR spectrum (Figure 4) only two anomeric signals are present at δ = 5.12 (**X**) and 4.72 (**Z**) ppm, and both spin-system signals were fully assigned from the COSY, TOCSY, and HSQC spectra. The spin-system **X** was identified as 3-substituted α-Gal since it possesses an anomeric coupling con-

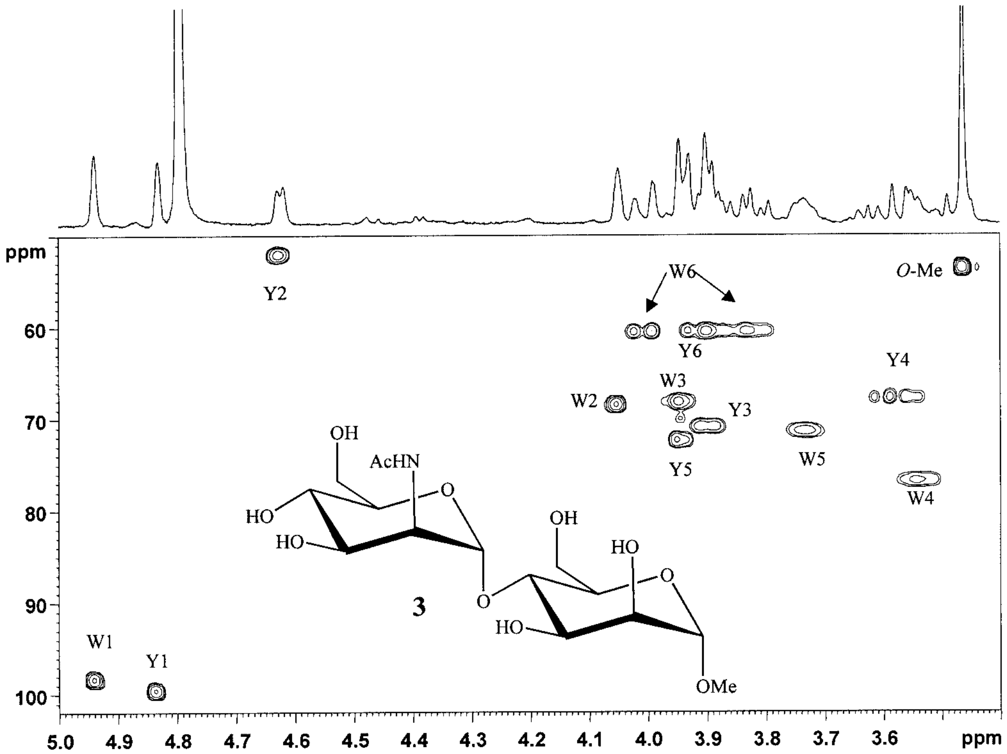
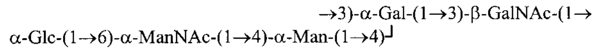


Figure 3. A close-up of the ^{1}H and HSQC spectra of compound **3** (sketched inside the HSQC spectrum) obtained by methanolysis of the polymer; the letters refer to the carbohydrate residues shown in Table 2

stant value of 3.4 Hz and small $J_{H,H}$ values for H-3/H-4 and H-4/H-5, and, furthermore, its C-3 carbon signal (δ = 76.9 ppm) is deshielded, thus indicating that C-3 is glycosylated. Likewise, spin-system **Z** shows small $J_{H,H}$ values for H-3/H-4 and H-4/H-5, an anomeric proton *J*-value of 8.0 Hz, and its C-3 carbon signal is found at δ = 80.5 ppm, in agreement with the presence of a 3-substituted β-GalNAc. The sequence of the two sugar residues in the polymer was inferred from the HMBC spectrum (Figure 4), which shows interresidual, long-range scalar interactions between H-1/C-1 **X** and C-3/H-3 **Z** and H-1/C-1 **Z** and C-3/H-3 **X**. Thus, the polymer resulting from Smith degradation is built up of the repeating unit **1**.

The results of the two degradations described above are in agreement with the presence of the two oligosaccharide structures **1** and **2** in the polysaccharide fraction of *Thermus thermophilus* HB8. As can be seen in the ^{1}H NMR spectrum (Figure 1), the anomeric signals of 3-substituted Gal*p* and of 3,4-disubstituted Gal*p* overlap, and so it is not possible to establish whether oligosaccharide structures **1** and **2** are present as a mixture of two regular polysaccharides or whether only one polysaccharide, built up of a disaccharide backbone **1** to which a linear side-chain trisaccharide is attached in a not fully stoichiometric fashion, is present. Moreover, the 1:4 ratio between 3-substituted Gal*p* and 3,4-disubstituted Gal*p* residues obtained from the methylation data reveals a minor content of the disaccharide polymer with respect to the pentasaccharide one. This is in conflict with the hypothesis of two different polysaccharides, which, in this case, would have been distinguishable by gel-permeation chromatography because of their quite different hydrodynamic volumes and therefore, probably, their molecular weights.

Thus, we suggest a polysaccharide with a nonregular pentasaccharide repeating unit where the trisaccharide side is not stoichiometric.



$$\begin{array}{r}\rightarrow 3)\text{-}\alpha\text{-Gal-}(1\rightarrow 3)\text{-}\beta\text{-GalNAc-}(1\rightarrow\\ \alpha\text{-Glc-}(1\rightarrow 6)\text{-}\alpha\text{-ManNAc-}(1\rightarrow 4)\text{-}\alpha\text{-Man-}(1\rightarrow 4)\lrcorner\qquad\qquad\qquad\quad\end{array}$$

In this regard, the minor signals present in the ^{1}H NMR spectrum are probably due to heterogeneity arising from the absence of the trisaccharide branching chain — the spin systems **X** and **Z** were also identified as **A′** and **E** in the 2D NMR spectra of the polymer (PS). In addition, it is worth noting the different width and shape of the proton anomeric signals of the *galacto* residues, which is greater than that of the side-chain residues, in agreement with their expected higher mobility with respect to that of the sugar backbone. Finally, the lack of terminal mannose or terminal ManNAc in the methylation analysis indicated that it was not possible that a shorter (one or two residue) side-chain was present.

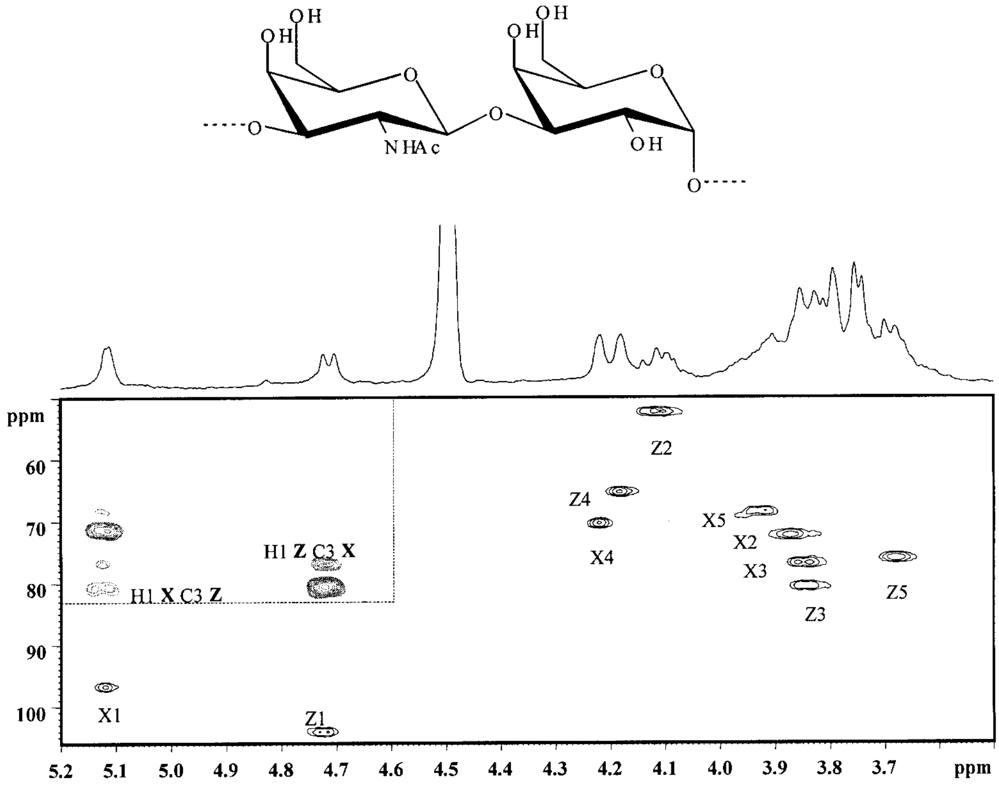


Figure 4. The ^{1}H NMR, HSQC and HMQC spectra of the disaccharide repeating unit of the polymer (compound **2**) obtained by Smith degradation of the PS from *Thermus thermophilus* HB8; all the HSQC correlations and the relevant interresidual correlations are visible; the letters refer to the carbohydrate residues shown in Table 3

Discussion

Lipopolysaccharides are characteristic glycoconjugates present in the outer leaflet of the external membrane of almost all Gram-negative bacteria. They provide permeability of the barrier, the adhesive properties, and the peculiar physical architecture of the membrane through their chemical elements. The most important information obtained from this work is that *Thermus thermophilus* HB8 does not possess any lipopolysaccharides in its external membrane and this is, in our opinion, a very interesting scientific point.

Only a very few of the outer membranes of Gram-negative bacteria studied so far have been found to be lacking lipopolysaccharides, namely those from *Sphingomonas capsulata*, *Treponema denticola* and *Chloroflexus aurantiacus*, *Borrelia burgdorferi*, and *Fibrobacter succinogenes*.[8−12] A few preliminary data were available before now[6] on the outer-membrane composition of the bacterium *Thermus* — these suggested the absence of lipopolysaccharide in its external membrane — but no complete information was available. This work is the definitive confirmation of these proposals as a novel outer-membrane polysaccharide has been structurally characterised, and, furthermore, the fatty acid composition of the glycolipid fraction ascertained. These latter, in some way, could substitute the lipopolysaccharides in the structure of the external membrane.[8−12] In our opinion, these findings could also be relevant in the understanding of the adaptive biochemical alterations of the outer membrane of *T. thermophilus* HB8 as a consequence of its exposure to harsh environmental conditions.

The existence of polymers assembled from a nonrepeating unit in the outer membrane of Gram-negative bacteria is now well accepted, although mainly for O-chain polysaccharides. Several O-polysaccharide chains from the LPS of *Xanthomonas* possess this attribute,[13−17] and a few other examples from *Pseudomonas* also possess the same feature.[18] More often, however, the nonrepetitiveness is caused, as in the present case, by a substituent attached in a nonstoichiometric fashion. In several examples the substituent masking the repetitiveness of the polymer is a noncarbohydrate group (i.e., an acetyl group),[19] but, recently, carbohydrate residues have been found to be unsystematically attached to a carbohydrate chain, and, in two cases, a saccharide chain with a completely unsystematic collection of carbohydrate residues was reported.[14,20]

Even in the polysaccharide derived from the outer membrane of the Gram-negative bacterium *Thermus thermophilus* HB8, there is a repeating unit masked by a nonstoichiometric carbohydrate substituent. Its peculiarity resides in the fact that the "substituent" linked to the always present oligosaccharide unit is a trisaccharide chain.

Experimental Section

Growth of Bacteria and Isolation of PS: *Thermus thermophilus* HB-8 (ATCC 27634) was grown at 75 °C for 3 d, in TH medium: peptone (Oxoid) 8.0 g/L, yeast extract (Oxoid) 4.0 g/L, NaCl 2.0 g/L at pH 7.0. The flask was inoculated to give an initial turbidity of 0.05 O.D. at 540 nm. The strain (inocula 1:10 v/v) was grown in a 1-L Erlenmeyer flask containing 200 mL of TH medium. The culture was grown to the late exponential phase (turbidity 1.7 O.D. at 540 nm) and was then transferred into 3 L of fresh TH medium contained in a 5-L flask and grown again to the late exponential phase. Active cultures (3 L) were inoculated in a 95 L fermenter (Terzano, Milano, Italy) with low mechanical agitation and an aeration flux of 30 mL/min/L of broth. The cells were harvested in the early stationary phase of growth by continuous-flow on an Alfa-Laval model Lab 102 B-20 centrifuge. The dried cells (5.1 g) were washed twice with $CHCl_3$/MeOH (2:1), twice with $CHCl_3$/MeOH (1:3), and then extracted with hot phenol and wate.[21] The two phases obtained were further purified of nucleic and protein material by enzymatic digestion with nuclease and protease to obtain a carbohydrate-containing product (yield: 240 mg, 4.7% of bacterial dry mass) only in the water phase. This was further purified by gel-permeation chromatography using columns (100 × 3 cm, eluent 50 mM NH_4CO_3) of Sephacryll S-300, S-400 and S-100 (Pharmacia); the resulting fraction represented the polysaccharide fraction (PS).

General and Analytical Methods: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) was stained with silver nitrate for detection of LPSs/LOSs, as described previously.[22] The monosaccharides were identified by GLC and GLC-MS as acetylated *O*-methyl glycosides derivatives: briefly, samples were treated with 2 M HCl/MeOH at 85 °C for 24 h, then dried under a stream of air and acetylated with acetic anhydride in pyridine at 80 °C for 30 min. The absolute configuration of the residues was determined by the published method, by GLC of the acetylated (*S*)-2-octyl glycosides;[23] temperature profile: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C and 6 °C min^{-1} from 200 to 260 °C, and 260 °C for 5 min.

Methylation analysis was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide.[24] The hydrolysis of the methylated *O*-polysaccharide was carried out with 2 M TFA (120 °C, 2 h) and the partially methylated monosaccharides, after reduction with $NaBD_4$, were converted into alditol acetates by treatment with acetic anhydride in pyridine at 80 °C for 30 min, and then analyzed by GLC-MS.

The total fatty-acid content was determined as described previously.[25] Briefly, the hydrolysis step was performed in 4 M HCl at 100 °C for 4 h. Then, the pH was adjusted to slight acidity, and the fatty acids were extracted with chloroform, esterified by treatment with diazomethane, and analyzed by GLC-MS.

All GLC analyses were performed on a Hewlett−Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco). For sugar-methylation analysis the temperature program was: 150 °C for 2 min, then 2 °C min^{-1} to 200 °C and 10 °C min^{-1} to 260 °C for 11 min, then 8 °C min^{-1} to 300 °C for 20 min; for absolute configuration analysis: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C and 6 °C min^{-1} to 260 °C for 5 min. For fatty-acids analysis the temperature program was: 80 °C for 2 min, then 8 °C min^{-1} to 300 °C for 15 min.

NMR Spectroscopy: For structural assignments of polysaccharide (PS) and compounds **1** and **2**, 1D and 2D ^1H NMR spectra were recorded of a solution at pD 7 (uncorrected value) of 5 mg of product in 0.6 mL of D_2O, at 35 °C (PS), 25 °C (compound **3**), and at

52 °C (compound deriving from Smith degradation). ^{1}H and ^{13}C experiments were carried out using a Varian Inova 500 spectrometer. Spectra were calibrated against internal acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm).
NOESY spectra were measured using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with a 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 80 ms, using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase-sensitive double-quantum-filtered correlation spectroscopy (DQF-COSY).[26,27]

HSQC and HMBC experiments were measured in the ^{1}H-detected mode by single-quantum coherence, with proton decoupling in the ^{13}C domain, using data sets of 2048 × 512 points, and 64 scans were acquired for each t_1 value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[28] A 60 ms delay was used for the evolution of long-range connectivities in the HMBC experiment.

All NMR analyses were carried out following literature procedures[29] and the spectra were assigned using the computer program Pronto,[30] which allows the simultaneous display of different two-dimensional spectra and the individual labelling of cross peaks.

Selective Chemical Degradations: An aliquot of PS (15 mg) was cleaved by methanolysis with 1 M HCl in MeOH at 80 °C for 16 h. The reaction mixture was then dried in a stream of nitrogen and *N*-acetylated with acetic anhydride in 0.5 M NaHCO$_3$. After workup of the reaction, the product was de-*O*-acetylated of any possible *O*-acetyl groups by treatment with 30% ammonium hydroxide at room temperature overnight. The mixture of *N*-acetylated products was purified by HPLC on a reverse-phase column (Nucleosil C-18 Nautilus, eluent water), from which five products were obtained: Man (1 mg), Gal (4 mg), Glc (2 mg), ManNAc (1 mg), and GalNAc (3 mg) as their *O*-methyl glycosides, along with compound **3** (1 mg), as its α-ManNAc-(1→4)-α-Man-*O*-methyl glycoside derivative.

An aliquot of PS (20 mg) was degraded by Smith degradation:[31] briefly, it was treated with 50 mM NaIO$_4$ at 4 °C for 7 d, followed by addition of ethane-1,2-diol, reduction (NaBH$_4$), acidification (2 M acetic acid), dialysis, and freeze-drying. Then, the oxidized polymer was hydrolyzed with 1% HOAc at 100 °C for 1.5 h, and the acid was removed by freeze-drying. The product obtained was purified by Bio-Gel P2 (2 × 100 cm), eluted in the void volume with 50 mM ammonium hydrogen carbonate buffer (pH 5), monitored with a Waters differential refractometer, and dried (compound **2**, 10 mg).

Table 3. ^{1}H and ^{13}C (italic) NMR chemical shifts of the sugar residues of the disaccharide repeating unit of the polymer (compound **2**) obtained by Smith degradation of the PS from *Thermus thermophilus* HB8 relative to acetone (^{1}H: δ = 2.225 ppm; ^{13}C: δ = 31.45 ppm; both at 52 °C); the resonances for the acetyl group appear at δ = 2.11 and δ = 23.0 and 175.8 ppm, respectively

X	5.12	3.87	3.85	4.22	3.93	3.78
3-Gal	*96.6*	*72.0*	*76.9*	*70.2*	*68.4*	*62.1*
Z	4.72	4.11	3.84	4.18	3.67	3.67
3-GalNAc	*104.1*	*52.2*	*80.5*	*65.2*	*75.9*	*62.2*

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