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The structure of the O-specific polysaccharide from *Thiobacillus ferrooxidans* IFO 14262

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Abstract

Lipopolysaccharide (LPS) was isolated from *Thiobacillus ferrooxidans* IFO 14262 by the hot phenol–water extraction procedure. The O-specific polysaccharide, liberated from LPS by mild acetic acid hydrolysis, had a branched pentasaccharide repeating-unit composed of D-glucose, L-rhamnose, D-rhamnose, and 3-*O*-methyl-L-rhamnose in approximate molar ratios of 2:1:1:1. On the basis of methylation analysis, ^{1}H and ^{13}C NMR spectroscopy, including 2D shift-correlated (COSY) and 1D NOE spectroscopy, the structure for the repeating unit of the O-specific polysaccharide was established, and the assumed biological repeating unit indicated.

1. Introduction

Thiobacillus ferrooxidans is a mesophilic chemolithotroph characterized by its ability to obtain energy from the oxidation of elemental sulfur and ferrous iron at low pH. It is phylogenetically placed into the β-branch of the *Proteobacteria* [1] and is commercially applied in bacterial leaching, especially for dumps around old and already exploited copper and iron mines [2].

The direct attachment of these bacteria to distinct copper and iron sulfide ores, e.g., to pyrite, has been described as being due to hydrophobic interactions [3] between surface structures of the bacterial cell and the ore. In a previous

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communication the chemical composition of the cell-wall lipopolysaccharide (LPS) of *T. ferrooxidans* IFO 14262 was reported [4], as well as the sodium deoxycholate–polyacrylamide gel electrophoretic (DOC-PAGE) pattern of the isolated LPS, showing a very regular banding profile indicating long O-chains (with up to 20 repeating units). The phosphate-free lipid A moiety was shown to possess a mixed lipid A backbone, being composed of GlcN and 2,3-diamino-2,3-dideoxy-D-glucose [4]. Rhamnose, 3-*O*-methylrhamnose, and glucose were reported as the main sugars in the O-specific chain and smaller amounts of mannose, L-*glycero*-D-*manno*-heptose, Kdo, and D-glucosamine originating most probably from the R-core oligosaccharide [4]. Herein, we now report on the detailed chemical structure of the O-specific polysaccharide of *Thiobacillus ferrooxidans* IFO 14262.

2. Results and discussion

The LPS was isolated in 1–2% yield (based on bacterial dry weight) by the hot phenol–water procedure as described previously [4] and was characterized by its DOC-PAGE profile [5]. The O-specific polysaccharide (PS) was liberated by mild acid hydrolysis of the lipopolysaccharide. The pentasaccharide repeating-unit contained D-glucose, rhamnose, and 3-*O*-methylrhamnose in approximate ratios of 2:2:1. The determination of the absolute configuration of the monosaccharides by GLC of their glycosides with (*S*)-2-octanol [6] revealed the D configuration of the Glc and the simultaneous presence of D- and L-rhamnose. Methylation with trideuteriomethyl iodide according to Ciucanu and Kerek [7], hydrolysis with 4 M

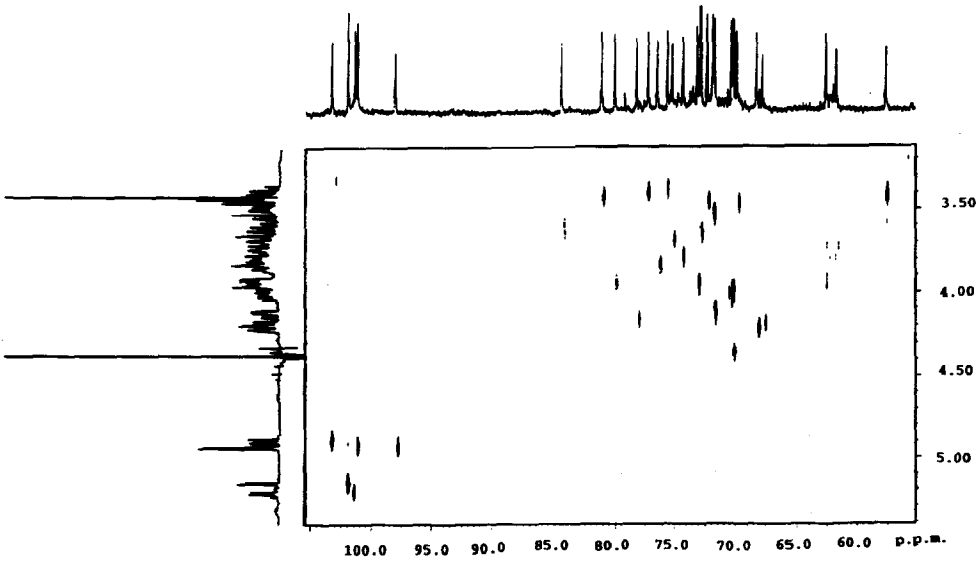


Fig. 1. 2D ^{13}C–^{1}H correlation spectrum for the isolated O-chain from *Thiobacillus ferrooxidans* IFO 14262 and the corresponding one-dimensional spectra.

CF_3CO_2H (100°C, 2 h) and GC–MS analysis of the methylated constituents after reduction with NaB^2H_4 and acetylation revealed the presence of 3-*O*-methyl-rhamnose as a terminal unit and two 3-substituted rhamnoses, 3-, and 3,4-substituted glucose, as well as a small amount of 4-substituted glucose and the following disaccharide product: 2,6-di-*O*-trideuteriomethyl-glucopyranosyl-(1 → 3)-2,4-di-*O*-trideuteriomethyl-rhamnitol-1-*d*. The presence of this disaccharide indicated that the residue of the disubstituted glucose was attached to O-3 of one of the rhamnose residues. The ^{13}C NMR spectrum of the PS (shown in the upper part of the COSY spectrum, Fig. 1) contained one major series of signals corresponding to a pentasaccharide repeating-unit with three 6-deoxyhexoses (C-6 signals around 17.6 ppm), one methylated sugar (Me group at 57.2 ppm), and two hexoses having unsubstituted C-6 (signals at 61.3 and 62.1 ppm). A series of minor signals, which correspond very probably to the ends of the polymeric chains, and not to core-derived sugars, was also present. The major series of signals in the ^1H NMR spectrum of the PS was completely assigned using COSY, COSY RCT, and COSY RCT2 methods (Table 1). From the $J_{1,2}$ coupling constants (3.5 Hz for α-Glc and 8.0 Hz for β-Glc) it may be concluded that glucose **D** has the α configuration, whereas glucose **C** is β-linked. To determine the sugar sequence, NOE measurements with pre-irradiation of all anomeric protons were performed (Fig. 2 and Table 2). Pre-irradiation of proton **D1** (α-linked Glc) provides NOE for **D2** (strong), **A3** (strong), and **A2** (weak), indicating that residue **D** is attached to O-3 of the rhamnose residue **A**. Pre-irradiation of proton **B1** (rhamnose) gives

Table 1
Chemical shifts (^1H and ^{13}C) for the repeating unit of *Thiobacillus ferrooxidans* IFO 14262 [a]

Residue	H-1	4.92	C-1	97.6	(+2.4)	Residue	H-1	5.15	C-1	101.6	
A	H-2	4.11	C-2	71.2	(−0.4)	**B**	H-2	4.21	C-2	67.7	(−3.8)
D-Rha	H-3	3.93	C-3	79.6	(+8.3)	L-Rha	H-3	3.83	C-3	75.9	(+4.6)
	H-4	3.65	C-4	72.3			H-4	3.52	C-4	71.4	
	H-5	3.97	C-5	69.7			H-5	4.00	C-5	69.9	
	H-6	1.25	C-6	17.6			H-6	1.25	C-6	17.6	
Residue	H-1	4.88	C-1	102.9		Residue	H-1	5.21	C-1	101.0	(+7.7)
C	H-2	3.37	C-2	75.1		**D**	H-2	3.78	C-2	73.8	
D-Glc	H-3	3.59	C-3	84.0		D-Glc	H-3	4.16	C-3	77.7	
	H-4	3.45	C-4	69.4	(−1.5)		H-4	3.68	C-4	74.7	
	H-5	3.39	C-5	76.7			H-5	3.93	C-5	72.6	
	H-6	3.92	C-6	62.1			H-6	3.84	C-6	61.3	
	H-6′	3.77					H-6′	3.71			
Residue	H-1	4.92	C-1	100.8		Residue	H-1	5.23			
E	H-2	4.18	C-2	67.3		**F**	H-2	3.56			
3-*O*-Me-	H-3	3.42	C-3	80.6		D-Glc	H-3	3.85			
L-Rha	H-4	3.46	C-4	71.8		Residue	H-1	4.89			
	H-5	4.36	C-5	69.6		**G**	H-2	4.17			
	H-6	1.22	C-6	17.6		3-*O*-Me-	H-3	3.42			
						L-Rha					

[a] The calculated glycosylation effects are given in parentheses.

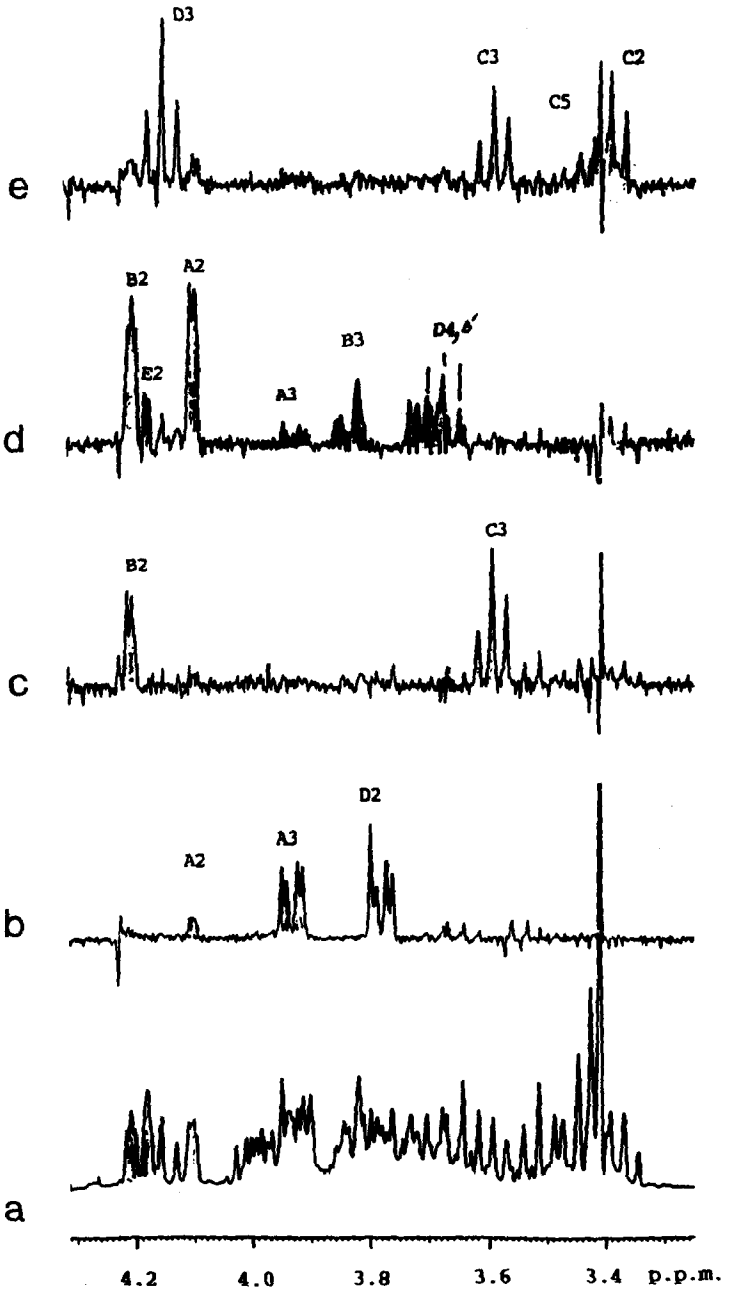


Fig. 2. ^{1}H NMR spectrum of *T. ferrooxidans* polysaccharide (trace a) and NOE spectra with pre-irradiation of H-1 protons of the residues: **D** (b); **B** (c); **A**, **E** (d); and **C** (e).

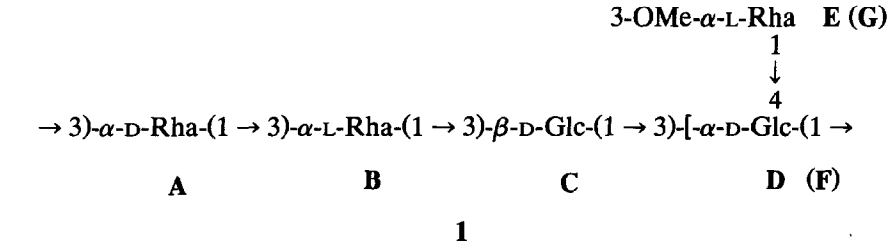
NOE for **B2** (strong) and **C3** (strong), thus residue **B** is attached to O-3 of the β-linked glucose. Similarly, on pre-irradiation of **C1**, NOEs were observed on **C2**, **C3**, **C5**, and on **D3** (strong), indicating the attachment of the β-linked glucose **C** to

C-3 of the α-linked glucose (**D**). A more-complex situation was observed on pre-irradiation of the H-1 protons of the two residues **A** and **E**, which showed exactly the same chemical shifts (Fig. 2). As unit **A** is substituted by unit **D** then all NOEs at unit **D** (**D4** and **D6′**) result not from an interaction between **A** and **E** but from an interaction between **E** and **D**. A substitution at position 6 is excluded according to the results of the methylation and the ^{13}C NMR analyses. Consequently, unit **E** (3-*O*-methylrhamnose) is attached to **D4** and the α-linked glucose is the branching point. Strong NOEs at **B2** and weak ones at **B3** are the result of the attachment of unit **A** to C-3 of unit **B**. Substitution at O-2 of unit **B** is not in agreement with the methylation data. Studies on a synthetic model showed that pre-irradiation of H-1 of mannose in a methyl α-D-mannopyranosyl-(1 → 3)-α-L-rhamnopyranoside results in a twice-stronger NOE at H-2 than on H-3 of the rhamnose residue, as a result of the specific conformation of such a fragment [8]. Assignments of the ^{13}C NMR spectrum were made on the basis of a C–H correlation spectrum (Fig. 1). The high-field position of all C-5 signals of the rhamnose residues at 69.6–69.9 ppm (Table 1) confirmed the α configuration for all rhamnose units. The low-field signal of C-3 at 80.6 ppm of unit **E** not being substituted by sugar unit indicated that unit **E** is the 3-*O*-methylrhamnose. Analysis of the chemical shifts of residue **A**, substituted by the α-linked D-glucose, is in good agreement (the largest difference being 0.8 ppm) with the calculated values for such a disaccharide [9], whereas, assuming that unit **A** is L-rhamnose, almost 3 ppm differences from the calculated C-2 and C-3 signals are observed. Thus, unit **A** has the D configuration. Analogous considerations concerning the spectra of the disaccharide partial structures **A–B** and **B–C** clearly show that rhamnose **B** has the L configuration. A relatively large difference from the observed value was found for the chemical shift of C-2 of the β-linked glucose (unit **C**), namely 75.1 ppm instead of the expected 73.3 ppm. This may be explained as a result of the attachment of the unit **C** to the branched unit **D**, which usually gives deviations of this order when compared with the spectra of the nonbranched model substances. The L configuration of 3-*O*-methylrhamnose was confirmed by the presence of a significant NOE for H-6′ of the residue **D** (see earlier and [10]) and by the small negative glycosylation effect for C-6 of the same residue (−0.6 ppm) in the ^{13}C NMR spectrum [8] (Table 1).

Table 2
NOEs observed at pre-irradiation of the anomeric protons of the polysaccharide from *Thiobacillus ferrooxidans*

Irradiated H-1 proton or residue	A+E	B	C	D
Response at proton number	Of residue			
2	**A, B, E**	**B**	**C**	**A, D**
3	**A, B**	**C**	**C, D**	**A**
4	**D**			
5			**C**	
6′	**D**			

On the basis of these data the structure of the branched repeating-unit of the O-polysaccharide from *T. ferrooxidans* IFO 14262 could be completely assigned (**1**).



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1 [repeating unit of the O-chain of strain IFO 14262; square brackets indicate the assumed end of the polymeric chain and the units in parentheses (**F**, **G**) the units forming the end of the chain]

The COSY and COSY RCT spectra of the polysaccharide contained well evident cross-peaks, corresponding to low-intensity signals in the one-dimensional ^{1}H NMR spectrum. From the observed couplings and the positions of these signals (units **F** and **G** in **1** and in Table 1) it may be suggested that unit **F** is the α-linked glucose without a substituent at O-3, which correlates with the presence of small amounts of derivatives of 4-substituted glucose amongst the methylated sugars (see earlier). Unit F has H-1, H-2, and H-3 very close to the signals of 3-*O*-methylrhamnose, and thus this monosaccharide is probably attached to O-4 of glucose **F** (which consequently is unit **D** without a substituent at C-3) and which therefore constitutes a terminal sugar of the polymeric chain of PS (**1**).

The overall structure of the O-specific chain of *T. ferrooxidans* with the hydrophobic 3-*O*-methylrhamnose occupying the terminal positions in the branched repeating-units is of interest for the hydrophobic interaction of this species with the surface of distinct ores resulting in a rather firm attachment to the surface of sulfide ores and in the generation of microbial corrosion pits. Whether extracellular substances are also involved in the corrosion process is not unequivocally proven [11].

3. Experimental

General methods.—The isolation and purification of LPS has been described, as well as the component analysis by GLC and GLC–MS [4]. Determination of the absolute configuration of the monosaccharides by GLC of their glycosides with optically pure (*S*)-2-octanol was performed as described [6].

Methylation.—The polysaccharide (1 mg) was methylated according to Hakomori with CH_3I or according to Ciucanu and Kerek [7] using CD_3I. The methylated polymer was isolated by $CHCl_3$–water extraction, hydrolyzed with 4 M CF_3CO_2H (100°C, 2 h), reduced with NaB^2H_4, acetylated, and analysed by

GLC–MS. The NMR measurements were carried out on a Bruker WM-360 spectrometer at 323°K. Bruker software was applied for 2D COSY, single- and two-step relayed coherence transfer COSY (RCT-COSY) and heteronuclear ^{13}C–^{1}H-COSY spectra.

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References

[1] C.R. Woese, *Microbiol. Rev.*, 51 (1987) 221–271.
[2] A. Brunesteyn, *J. Biotechnol.*, 11 (1989) 1–10.
[3] P. Harrison, Jr., *Arch. Microbiol.*, 131 (1982) 68–72.
[4] A. Yokota, M. Rodriguez, Y. Yamada, K. Imai, D. Borowiak, and H. Mayer, *Arch. Microbiol.*, 149 (1987) 106–111.
[5] T. Komuro and C. Galanos, *J. Chromatogr.*, 450 (1988) 381–387.
[6] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, (1978) 359–362.
[7] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[8] N.K. Kochetkov, G.M. Lipkind, A.S. Shashkov, and N.E. Nifant'ev, *Carbohydr. Res.*, 221 (1991) 145–168.
[9] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
[10] G.M. Lipkind, A.S. Shashkov, S.S. Mamyan, and N.K. Kochetkov, *Carbohydr. Res.*, 181 (1988) 1–12.
[11] M. Ostrowski and A. Sklodowska, *World J. Microbiol. Biotechnol.*, 9 (1993) 328–331.